BACTERIAL PATHOGENICITY

Adherence of *Aeromonas caviae* to human cell lines Hep-2 and Caco-2

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Adherence of *Aeromonas caviae* to HEp-2 and Caco-2 cell monolayers was investigated with 24 clinical isolates. Growth phase, temperature, multiplicity of infection and length of incubation affected adherence. Treatment of the bacteria with trypsin, sodium metaperiodate, mechanical shearing and the addition of cytochalasin B and cycloheximide to the monolayer significantly reduced the adherence capabilities of the strains investigated. The use of chloramphenicol to inhibit protein synthesis reduced the adhesive capabilities of bacteria grown in liquid medium and those subjected to mechanical shearing. Light microscopy, scanning and transmission electron microscopy were employed in the investigation of bacteria–bacteria and bacteria–monolayer interactions and indicated similarities with the aggregative adherence patterns of the Enterobacteriaceae. The presence of extracellular bacterial appendages and their correlation with increased adhesive capacity may indicate a role in the process of adherence.

Introduction

Aeromonas species are important pathogens in paediatric, geriatric and immunocompromised patients, causing sporadic diarrhoea, dysentery and extra-intestinal infections and may be life-threatening [1–5]. It is not known if all strains are pathogenic, or if, like *Escherichia coli*, there are sub-populations of pathogenic strains. The quality of conventional antibiotic therapy for the treatment of motile aeromonad infections is still debatable as antibiotic resistance is of growing concern [6, 7]. *A. hydrophila* and *A. sobria* were thought to be the main causes of aeromonas-related diarrhoea and aeromonas-associated colitis; however, recent investigations implicate *A. caviae* as a significant enteropathogen [8–10]. Difficulties in the correlation of toxin production, invasive properties, haemagglutination and adherence with disease symptoms, and the problems with speciation of the aeromonads may account for much of the confusion surrounding the role of these organisms in disease. The role of these virulence factors in the pathogenicity of

Aeromonas spp. remains, for the most part, vague [11–13]. Studies on other enteropathogens indicate that a pathogen must be able to attach to host target cells to cause gastrointestinal disease, via either toxin production or host cell invasion, or both [14, 15].

Adherence has been shown to be a major determinant of pathogenicity for other enteropathogenic bacteria, including the aeromonads, but few studies on factors affecting colonisation by *A. caviae* have been undertaken [16–19]. Adhesion to intestinal cell models has been investigated previously and, as with *Escherichia coli* and many other pathogenic species, differences in adherence level and patterns exist [20–23]. The work reported here was conducted as a preliminary investigation into the interaction between epithelial and bacterial cells with 24 clinical isolates of *A. caviae*.

Materials and methods

Bacterial strains

Twenty-four clinical isolates of *A. caviae* from patients with diarrhoea were studied. They were isolated [10] in the Bacteriology Departments at the Sheffield Children's and Royal Hallamshire Hospitals, identified as

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described previously [24] and stored in the vapour phase of liquid nitrogen until required.

Cell culture

Two cell lines were used throughout this study – HEp-2 and Caco-2. The HEp-2 cells (ATCC CCL23, derived from human epidermoid larynx carcinoma) were grown in Basal Medium Eagles (BME; Gibco, Paisley) containing new-born calf serum. The Caco-2 cells (ATCC HTB37, derived from human colon carcinoma) were grown in Eagle's Minimal Essential Medium (EMEM; Gibco) supplemented with fetal calf serum 10%, sodium pyruvate 1%, 2 mM glutamine and amphotericin B 250 mg/L. The antibiotic was removed from the cell layer by washing 3 days before experimental use and the cells were incubated further in maintenance medium. Both cell lines were grown until confluent at 37°C in air with CO₂ 5%, diluted to 50–100 cells/mm² and seeded in 1-ml amounts into 24-multiwell tissue-culture plates (Costar, High Wycombe). The cell lines were used at confluence in duplicate for each separate experiment with monolayer age being kept as constant as possible. The HEp-2 cells were 24 h old after the final seeding, while the Caco-2 cells were at least 14 days old to allow for full cellular differentiation.

Growth and preparation of bacteria

The test strains were inoculated into Brain Heart Infusion Broth (BHIB; Oxoid) from the frozen stocks and incubated statically in air for 16 h at 37°C [5]. Subcultures were then made by *c.* 1 in 100 dilution into fresh BHIB and incubation at 4, 12, 26 or 37°C statically for a further 16–24 h as required. Culture density was estimated by serial dilution and plating on to Columbia Aga (Oxoid) with horse blood 5%, for viable counting, and by the measurement of optical density at 600 nm.

Adherence assay

This was conducted by a modification of the method of Carello *et al.* [26]. Briefly, bacteria were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS, pH 7.4; Oxoid) and resuspended in an appropriate volume of cell monolayer culture medium. One ml of this bacterial suspension (containing *c.* 5×10^6 cfu/ml with an OD₆₀₀ of 0.07) was inoculated on to the cell monolayer and incubated for 90 min at 37°C in air with CO₂ 5%. For multiplicity of infection (moi) experiments, bacteria were added at varying concentrations from 3×10^3 to 3×10^9 cfu/ml. Control wells without bacteria or monolayer cells were also prepared, as were wells with chloramphenicol 25 mg/L (MIC₉₀ for *A. caviae* 8 mg/L) to stop bacterial protein synthesis [27]. Chloramphenicol was also used to stop protein synthesis for adherence assays when bacteria grown at 4, 12, 26 and 37°C were employed to

investigate the effect of altering growth temperature. After incubation, the medium and non-adherent bacteria were removed by washing three times with PBS. The cell layer and remaining adherent bacteria were then fixed with a methanol (Fisons, Loughborough): acetic acid (BDH Chemicals, Poole) 3:1 solution for 5 min. After washing with distilled water, the remaining cells were stained with Giemsa stain (BDH Chemicals) 10% for 20 min, mounted and viewed by light microscopy.

Treatment with cytochalasin B and cycloheximide

A modification of the method described by Favre-Bonte *et al.* [20] was used. The adhesion assays were conducted as above with the addition of either cytochalasin B (Sigma) 2.5 or 5 mg/L, or cycloheximide (Sigma) 200 mg/L 1 h before the addition of bacteria. Wells with no cycloheximide or cytochalasin B served as controls, and pretreated bacteria added to untreated cells served as controls for any unwanted effects that cytochalasin B or cycloheximide may have upon the bacteria. All parts of the experiment were repeated in triplicate.

Treatment with trypsin, sodium metaperiodate and mechanical shearing

The bacteria were prepared as before in PBS. Positive control samples were taken before any bacterial treatment. Trypsin (Sigma) 1 g/L was added for 30 min at room temperature [28]. Sodium metaperiodate treatment was achieved by resuspending the bacteria in 0.01 M sodium metaperiodate (Sigma) in 0.2 M acetate buffer (pH 4.5) at room temperature for 15 min in the dark as described by Naess *et al.* [29]. Mechanical shearing was performed with a 'whirring' type blender (Kinematica, Lucerne, Switzerland) for 15 min on ice. After treatment, all bacterial preparations were washed twice in PBS, resuspended in cell culture medium to an OD₆₀₀ of 0.07 spectrophotometrically, and the adherence assay was conducted as above, with and without the addition of chloramphenicol.

Transmission electron microscopy

Bacterial suspensions were placed on to formvar-coated copper grids (A.G.A.R., Stanstead) and negatively stained with a solution of potassium phospho-tungstate (KPT; A.G.A.R.) 2%. Bacteria were then viewed on a Philips EM 400 transmission electron microscope. Monolayers grown on Thermanox coverslips (Gibco) and adherent bacteria were prepared for sectioning by being fixed in glutaraldehyde 3% for 1 h, washed in cacodylate buffer (pH 7.4), post-fixed in osmium tetroxide (A.G.A.R.) 1% in cacodylate buffer at 4°C for 1 h and dehydrated in a series of graded ethanol solutions, before being embedded in a 3-mm epon coating and allowed to polymerise. Ultra-thin sections

were cut and stained in aqueous uranyl and lead salts and viewed as above.

Scanning electron microscopy

Monolayer cells grown on glass coverslips and inoculated with bacteria to demonstrate adherence, were fixed in glutaraldehyde 3% and washed as above in cacodylate buffer. They were then post-fixed in osmium tetroxide 1% before being dehydrated through a series of graded ethanol solutions, critical point dried and coated with a 20-nm layer of gold. They were viewed with a Philips 401 scanning electron microscope.

Results

Determination of experimental parameters

This was necessary as variables such as growth phase, toxin production and moi are known to have profound effects on bacterial adherence. Maximum adherence was observed to occur early in the exponential phase, falling to a constant level after the start of the stationary phase at about 4 h and noted to be consistent for 24 h post-inoculation (Fig. 1). This consistent level of adherence was noted to be proportionate to the maximum levels reached during the early exponential phase for all strains investigated. Strains expressing very high levels of adherence had consistently high levels during the stationary phase of growth, and *vice versa* for the poorly adherent strains. Light microscopy showed monolayer destruction commonly associated with toxin production, increasing with the adherence ability of the bacteria towards 1.6 h; however, little monolayer damage was noted when stationary phase

bacteria were applied. A similar toxic effect was noted if monolayers were incubated with bacteria for prolonged periods. For this reason a 90-min incubation with 16-h stationary phase bacteria was used, to demonstrate bacterial adherence to monolayers without toxin-mediated tissue damage.

Adhesion to HEp-2 cells, with a strain of medium adherence by previous studies, was investigated by the use of different moi increasing from 3×10^3 cfu/ml to 3×10^9 cfu/ml [25] to determine the moi required to give the optimal number of bacteria in relation to monolayer cells before pathogenic effects were noted. Excessive bacterial adhesion to the monolayer cells was noted when $> 3 \times 10^7$ cfu/ml were used, resulting in tissue damage and too large a number of bacteria to count. When 3×10^6 cfu/ml were added, each monolayer cell was shown to have *c.* 10 adherent bacteria, making it feasible to count and minimising any associated damage. The addition of fewer bacteria would make adherence assays with low adherent strains difficult as few adherent bacteria would be observed.

Variation of adhesion patterns with growth phase

Following investigations with strains known to express aggregative adherence patterns when grown at 37°C [25], the patterns of adhesion to the monolayers were seen to vary with growth phase. Small numbers of grouped (localised) bacteria were seen during the lag phase, while larger numbers of bacteria demonstrated aggregative adherence to the cell monolayer during the early exponential growth phase, when most adhesion was taking place. A more diffuse adhesion pattern was seen concurrently in the stationary phase, with aggregative adhesion becoming less frequent (Figs 1 and 2).

Variation of adhesion with growth temperature

The adhesion assay was conducted as described above, with the bacteria grown at 4, 12, 26 or 37°C and protein synthesis arrested by chloramphenicol before the addition of bacteria to the monolayer. Maximum adhesion occurred at 12°C with all strains investigated. Patterns of adhesion differed for all strains with changes in growth temperature (Table 1). Diffuse patterns of adhesion were predominant at 4°C; increasing the growth temperatures favoured bacterial grouping to form microcolonies on the monolayers. Aggregative adhesion patterns, similar to those of the stacked brick appearance noted for members of the Enterobacteriaceae, were seen with nine of the 24 strains investigated at 37°C [25].

Fig. 1. Effect of growth phase on adhesion of *A. caviae* to HEp-2 cells. Samples were taken at different times of growth. The optical density at 600 nm (—◇—) was measured, and bacterial adherence (—○—) was determined.

Adhesion to HEp-2 and Caco-2 cells

Investigations with the 24 *A. caviae* strains showed varying degrees of attachment to the cell line

Fig. 2. Light micrographs of Giemsa-stained *A. caviae* (strain Sch3) adhering to HEP-2 cell monolayers after bacterial growth at 37°C in BHIB for: **a**, 1 h; **b**, 2 h; **c**, 24 h (magnification $\times 6$).

monolayer as observed by light and electron microscopy. All strains tested were classified according to the number of adherent bacteria retained on the cell line after washing (Figs 2 and 3). The greatest mean number of adherent bacteria per tissue culture cell observed was 50.5 for HEP-2 cells and 51.6 for Caco-2

Table 1. Changing adhesive patterns and degrees of adhesion for *A. caviae* strain Sch3 grown at different temperatures in BHIB

Temperature (°C)	Average adherent bacteria monolayer cell (SD)	Predominant adhesive pattern
4	12.8 (4.7)	Diffuse
12	54.6 (9.2)	Aggregative
26	21.8 (6.5)	Diffuse/aggregative
37	49.4 (7.3)	Aggregative

cells. Strains that expressed a high degree of adherence to HEP-2 cells also showed a high degree of adherence to Caco-2 cells. A similar correlation was observed with medium and low adherent strains. Few bacteria of any strain adhered to either glass or plastic coverslips without a monolayer.

Inhibition of adhesion

Chemical and mechanical alterations to the monolayers and bacteria were investigated to determine specific roles in the adhesion process (Fig. 4).

Cytochalasin B. Adhesion to HEP-2 cell monolayers was inhibited by up to 93% when the bacterial strains were exposed to cytochalasin B, an inhibitor of microfilament production.

Cycloheximide. Treatment of the monolayer with cycloheximide 200 mg/L produced an 85% reduction in adhesion.

Trypsin. Addition of trypsin to the bacteria produced 78% inhibition, presumably by the proteolytic removal of extracellular protein structures.

Sodium metaperiodate. Exposure of the bacteria to sodium metaperiodate produced a 68% reduction in adhesion, possibly due to the re-arrangement and blockage of carbohydrate receptors on the bacterial cell surface.

Mechanical shearing. Removal of extracellular bacterial appendages by mechanical shearing reduced adhesion to the cell monolayers by up to 72%.

Addition of chloramphenicol to the adherence assay

When chloramphenicol was added to the bacterial suspension prior to the adherence assay, a fall in adherence was noted when compared to the same assay without the antibiotic. The reduction was greater when the bacteria had been grown in a broth medium (BHIB) than when they were harvested from a solid medium, i.e., blood agar. However, this medium-dependent difference was removed when the bacteria were exposed to a shearing force before the addition of the antibiotic (Fig. 5).

Fig. 3. Scanning electron micrograph showing adherence of *A. caviae* (strain Sch3) during stationary phase to HEp-2 cells after growth in BHIB at 37°C (bars 1 μ m).

Fig. 4. Graph showing the effect of treating the monolayer or bacteria with agents to reduce adhesion prior to the adherence assay. Column 1, 100% adhesion without any prior change to the monolayer or bacteria; 2 and 3, the reduction caused by pretreatment of the monolayer with cytochalasin B 2.5 and 5 mg/L, respectively; 4, the effect of cycloheximide 200 mg/L pretreatment on the monolayer; 5 and 6, the reduced adhesive effect of trypsin 1 g/L and 0.01 M sodium metaperiodate on the bacteria; 7 and 8, the effect of a mild shearing force for 15 and 30 min, respectively, upon the bacteria.

Fig. 5. The adherence of *A. caviae* to monolayer cells after growth in BHIB (columns 1–4) and on BA (5–8), without any treatment (⊞), and following treatment of the bacteria by shearing (▨), with chloramphenicol (□) and both shearing and chloramphenicol (▤).

Discussion

This investigation was undertaken to examine the interactions leading up to and involving the adherence process of *A. caviae*, now commonly considered the primary step bacteria make in colonising, invading or causing disease. A previous study showed that the

majority of the strains demonstrated adherence potential. However, variables such as growth phase, moi, toxin production and extracellular appendage expression alter the interpretation of bacterial adhesion to a cell-line model [25].

Two well recognised cell culture monolayers, HEp-2 and Caco-2, were used in the present study. They were chosen because of their universal availability and their similarity to epithelial cells of the gastrointestinal tract, which are considered to be the target cells in the majority of aeromonas infections. Control strains of *A. caviae*, *A. hydrophila* and *A. sobria* were included in a parallel study, and the *A. caviae* ATCC15468 results were included. The results from this investigation show a high degree of similarity with previous studies [13, 19], with minor exceptions probably due to strain divergence, species differences and geographical variations.

The change in aggregative adherence over the first phase of exponential growth was seen in the majority of the strains in the study, and coincides with the time of maximum adherence. Microscopic examination of the adhesive process indicated that these adhesion patterns were similar to those of other species of Enterobacteriaceae [14, 15]. The correlation of these adhesive patterns suggests similarities between the pathogenic mechanisms of these strains and those of other recognised pathogens [22, 29]. The fact that this study showed these patterns changing with time and temperature indicates that the bacteria were expressing different adhesins and surface structures dependent upon their surrounding conditions. An increase in expression of extracellular filamentous structures was also seen at these temperatures, along with a change to ordered aggregative adherence patterns. The environmental dependent adherence factor variation was also reflected in the reduction in adherence seen when protein synthesis was inhibited by the addition of chloramphenicol before the bacterial interaction with the cell monolayer. From these results it appears that the structure responsible for the majority of adhesion is expressed on a solid growth medium but not in a liquid environment. The numbers of extracellular filamentous appendages seen by transmission electron microscopy have been noted to alter under these conditions, with most present after growth in solid surface. The reduction of adhesion of bacteria grown in solid medium to levels comparable with those of liquid-grown cultures by shearing and the addition of chloramphenicol also indicates their previous expression upon the solid medium and their absence from bacteria grown in liquid media.

The dramatic effect on adherence of mechanical shearing and treatment with trypsin suggests the possible role of protein structures capable of being removed by the shearing force, while the effect of sodium metaperiodate on adhesion indicates a carbo-

hydrate interaction. Transmission electron microscopy revealed several different filamentous structures that appear capable of removal by shearing. Examination of post-sheared bacteria showed the absence of these structures, while an adhesion assay in the presence of chloramphenicol confirmed the inability of cells to adhere when protein synthesis was inhibited. The failure to inhibit adhesion completely by any one method, and the ability to competitively inhibit adhesion with sheared structure preparations (unpublished data) suggest that there are multiple adhesion mechanisms, while the effects of the arrest of protein synthesis before shearing indicate that there may be rapid re-synthesis of removed structures.

The considerable reduction in adhesion with cytochalasin B and cycloheximide shows that bacterium-cell line adhesion is a two way process. The inhibitory effect of cytochalasin B indicates that adhesion is microfilament-dependent, although it is not known whether it is the bacterial or the eukaryotic cell that controls the re-arrangement of the cytoskeletal proteins, as has been demonstrated for *E. coli* [20, 30, 31]. The inhibiting effect of cycloheximide indicates the necessity for metabolic activity of the eukaryotic cell if adhesion is to occur.

This study has shown the variation of adherence pattern and capacity with growth phase and temperature. The adhesion of *A. caviae* strains to eukaryotic cells requires the active participation of those cells. Initial indications point to an extracellular filamentous structure of protein and carbohydrate composition as a putative adhesin. The regulation of the expression of these extracellular structures and their individual roles in the adhesion process remains to be determined for *A. caviae*. As with other *Aeromonas* spp., filamentous structure expression is noted to increase at *c.* 12°C and on specific types of medium, where adhesion is greatest [7, 32, 33]. From attachment of a single bacterium to the formation of bacterial aggregates, it seems reasonable to assume that a foothold has been established which the bacteria may utilise to further colonise and cause disease.

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