BACTERIAL PATHOGENESIS

Flow cytometric analysis of *Clostridium difficile* adherence to human intestinal epithelial cells

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Clostridium difficile is the most common cause of diarrhoea in hospitalised patients. Bacterial adherence to gut epithelial cells is a likely prerequisite to infection and toxin production. A novel flow cytometric method was developed for detecting adherence of *C. difficile* to human colonic and small intestinal epithelial cells (EC) and human intestinal cell lines. Small intestinal and colonic EC were isolated from biopsy specimens with mucolytic and chelating agents. Adherence of fluorochrome-labelled *C. difficile* to EC was measured by flow cytometry and was calculated as increase in median fluorescent intensity (Δ MFI). Cells with bacteria attached could be distinguished easily from cells alone or cells with unlabelled bacteria attached. Toxin-positive *C. difficile* adhered to colonic and small intestinal EC (Δ MFI mean 21.2 SD 16.7, $n=33$ and 16.5 SD 20.7, $n=19$ respectively). The toxin-negative strain also adhered to both epithelial cell types (Δ MFI 26.1 SD 32.5, $n=17$ and 18.3 SD 31.3, $n=16$). Adherence of toxin-positive *C. difficile* to the intestinal cell lines Caco-2 (Δ MFI 9.4 SD 4.4, $n=14$) and HT29 (Δ MFI 8.1 SD 3.1, $n=12$) was quantifiable, although at a significantly lower level than with primary colonic epithelial cells. Adherence of the toxin-negative strain was slightly lower, Δ MFI 6.5 SD 1.8, $n=9$ with Caco-2 cells and Δ MFI 6.0 SD 2.0, $n=10$ with HT29 cells. Adherence of *C. difficile* to epithelial cell lines was blocked with *C. difficile* antiserum, confirming specificity of adherence. In conclusion, flow cytometry is a useful approach to quantifying adherence of *C. difficile* to human colonic and small intestinal epithelial cells. Binding of toxin-negative as well as toxin-positive bacteria was detectable by this approach. Analysis of *C. difficile* adherence to target cells may have important implications for the understanding of the pathogenesis of *C. difficile*-related disease.

Introduction

Clostridium difficile, a gram-positive spore-forming anaerobic bacillus, is the most common cause of infectious diarrhoea in hospitalised patients [1]. Optimal conditions for colonisation and infection develop when antibiotic treatment leads to disruption of the normal colonic flora [2, 3]. Infection results in a wide spectrum of disease ranging from mild self-limiting diarrhoea to severe colitis and pseudomembranous colitis, which can often be fatal [4]. The spore-forming ability of this organism makes it difficult to eradicate from the hospital environment – thus hindering infection control measures, allowing cross-

infection, and resulting in increased morbidity and mortality [5–7].

Adherence of bacteria to intestinal epithelium is known to be a prerequisite for colonisation and infection of the gastrointestinal tract by many gastrointestinal pathogens [8–10]. The intestinal epithelium is the primary site of contact for pathogens with host cells and plays an important role in the cross-talk between epithelial cells, luminal micro-organisms and immune cells. The role played by pathogens that adhere to epithelial cells and invade the underlying mucosa has been well documented [11–18]. Furthermore, nuclear factor kappa B (NF- κ B), the transcription factor that regulates the expression of many pro-inflammatory cytokines, inflammatory enzymes and adhesion molecules, is activated following bacterial adherence, suggesting a common pathway for activation of several mechanisms [19–24].

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C. difficile has been shown to associate with colonic mucus from hamsters [25]. Previous reports have demonstrated adherence to human intestinal cell lines by light microscopy [26, 27], although the levels of adherence observed were low (<1 bacterium/cell at 37°C). Adherence levels increased following bacterial heat-shock treatment but the relevance of bacterial heat shock to in-vivo infection is unclear. Adherence of *C. difficile* to non-intestinal epithelial cell lines has also been demonstrated [27]. Again low levels of adherence were observed at 37°C, with increased adherence seen after bacterial heat shock. Waligora *et al.* studied environmental factors that might influence *C. difficile* adherence [28]. Acidic pH, decreased iron availability and high calcium and sodium concentrations all increased *C. difficile* adherence to Vero cells. Furthermore, these authors found no significant difference in adherence of toxigenic and non-toxigenic *C. difficile* strains. However, for most of these studies, non-intestinal epithelial cell lines were used, rather than intestinal epithelial cells which may be more relevant physiologically.

The ability of *C. difficile* to attach to human primary colonic and small bowel epithelial cells has not been examined, despite the fact that adherence of this organism is likely to be the primary step in pathogenesis, preceding toxin release. This is mainly due to the difficulties associated with the long-term culture of primary human epithelial cells. The present study examined adherence of a toxin-positive and a toxin-negative strain of *C. difficile* to human intestinal cells. A method for removal of viable intestinal epithelial cells from colonic and small bowel epithelium was devised in a previous study [29]. Cells prepared by this methodology were used to develop a flow cytometric method for assessing adherence of fluorescently labelled bacteria to human intestinal epithelial cells.

Materials and methods

Bacterial strains

A toxin-producing *C. difficile* strain (ATCC 9689) was obtained from Oxoid (Basingstoke, Hampshire). A toxin-negative strain (R10567) was obtained from the Public Health Laboratory Service Anaerobe Reference Unit (Cardiff, Wales). Both strains were tested with the *C. difficile* Tox AB test (Tech lab, Blacksburg, VA, USA) to determine toxin production. Bacteria were cultured anaerobically on Columbia Blood Agar (Oxoid) plates for 36 h at 37°C, and the strains were stored on bacterial preserver beads at -70°C (Technical Services Consultants, Lancashire). For each assay, bacteria were grown on blood agar and then transferred into Fastidious Anaerobe Broth (LabM, Bury, homes) and incubated anaerobically for 36 h. Cultures were harvested by centrifugation at 1500 g for 10 min and resuspended in phosphate-buffered saline (PBS, pH 6.8; Oxoid). Bacteria in suspension were quantified by

optical density reading at 490 nm, comparison with McFarland standards and viable plate counts. Appropriate dilutions of bacterial suspensions were spread on Columbia blood agar plates and incubated anaerobically at 37°C for 48 h.

Preparation of fluorochrome

The fluorochrome BCECF/AM 2', 7'-bis-(Carboxy-ethyl)-5(6')-carboxyfluorescein acetomethyl ester (Calbiochem-Novabiochem, Nottingham) was used in this study. It diffuses into viable cells and is cleaved by cytosolic esterases to yield intracellularly trapped indicator BCECF. A 1 mM stock solution of BCECF/AM was prepared in acetone. This was sterile-filtered, dispensed into small volumes and stored at -20°C.

Fluorochrome labelling of bacteria

Bacteria were labelled with fluorochrome by incubating 1×10^9 bacteria/ml with 1 mM BCECF/AM in acetone at 37°C to give a working solution of 1 μ M BCECF/AM. Time course experiments in which bacteria were incubated with fluorochrome for 20, 40 and 60 min, respectively, revealed optimal labelling at 60 min. Excess fluorochrome was removed by washing in PBS five times at 1500 g. Fluorescent label uptake was assessed by microscopy and flow cytometry.

Isolation of human intestinal epithelial cells

Ethical approval for this study was obtained from the Hospital Ethics and Research Committee at St Vincent's University Hospital (Dublin, Ireland) and patient consent was obtained on every occasion. Biopsy tissue was taken from patients undergoing routine endoscopy/colonoscopy for various bowel diseases. Two biopsies were fixed for routine histological examination to exclude abnormal pathology. Results were only taken from adhesion experiments with biopsies from patients in whom pathology was excluded. The remaining (four) colonic or small bowel biopsies were placed immediately in Hanks's Balanced Salts Solution (HBSS; Gibco, Paisley) pH 6.8 with fetal calf serum (FCS; Gibco) 5% and transported directly to the laboratory. The biopsies were then placed in a 1 mM solution of ethyl diamine tetra-acetic acid (EDTA; BDH)/di-tri-threitol (DTT; Sigma) in HBSS without calcium or magnesium at 37°C, placed on a rotator and allowed to rotate freely for 60 min. The supernate was removed with a sterile pasteur pipette and cells were pelleted by centrifugation, washed once in Roswell Park Memorial Institute (RPMI; Gibco) medium. The cells were counted with a haemocytometer and viability was assessed with an ethidium bromide/acridine orange stain (EB/AO; Sigma). Cells were diluted in a known volume of EB/AO and when viewed under UV light, viable cells were identified by green fluorescence whereas dead cells emitted orange

fluorescence [30]. Cells were adjusted to a 1×10^6 /ml suspension and stored on ice until used.

Cell culture

Caco-2 cells (a human ileocaecal epithelial cell line) were obtained from the European Collection of Cell Culture (ECACC). Cells were grown in Dulbecco's medium (Gibco) containing glucose supplemented with fetal calf serum 5%, amphotericin B, penicillin/streptomycin, HEPES buffer and non-essential amino acids (NEAA) (all from Gibco), in polystyrene tissue-culture flasks until confluent. HT29 cells (a human colonic epithelial cell line) were obtained from the American Tissue Culture Collection (ATCC). Cells were grown in modified McCoy's medium (Gibco), supplemented with fetal calf serum 5%, amphotericin B, penicillin/streptomycin and HEPES buffer in polystyrene tissue-culture flasks until confluent. Before adherence assays, both cell lines were incubated with antibiotic-free medium for 24 h. Cells were removed from tissue-culture flasks with an EDTA-trypsin suspension, counted in a haemocytometer and adjusted to the relevant concentration.

Adherence assay

Fluorescently labelled bacteria (1×10^8) were incubated with 1×10^5 epithelial cells for 30 min at 37°C in PBS. Negative controls included epithelial cells in PBS (Sigma) and epithelial cells incubated with non-fluorescently labelled bacteria. After incubation, cells were washed three times in PBS to remove non-adherent bacteria. Cells were then fixed with paraformaldehyde (PFA; Sigma) 1%, before examination by flow cytometry.

Flow cytometry

Human intestinal epithelial cells with adherent *C. difficile* were measured in a FACScan flow cytometer (Becton Dickinson, Oxford). With use of a dot plot display of forward scatter (FSC) versus side scatter (SSC), the machine was gated to include epithelial cells and to exclude cellular debris, intra-epithelial lymphocytes and non-adherent bacteria. Cells inside the gated area were stained with FITC-labelled Ber Ep 4 antibody (DAKO, Glostrup, Norway) to confirm their epithelial origin. A total of 10 000 events was acquired and the data were analysed with the Cell Quest software program from Becton Dickinson. This program produces histograms of each individual particle sample and calculates the mean and median fluorescence intensity for each cell population. Median fluorescent intensity (MFI) values were compared for test populations and negative controls. Cells with fluorescently labelled bacteria attached show a shift in fluorescence as a result of bacterial binding when compared with the autofluorescence expressed by epithelial cells alone.

Semi-quantification of bacterial adherence

The approximate number of bacteria adhering to each epithelial cell was determined as follows. Colony counts were performed on the bacterial suspensions added to the epithelial cells and bacterial suspensions removed from the epithelial cells at the end of the adherence assay (the pooled washings obtained from the washing steps). Serial dilutions of bacteria were spread on Columbia agar plates, which were incubated anaerobically for 48 h. Colony-forming units (cfu) were then counted for both bacterial suspensions. The number of epithelial cells per sample was determined by the Cell Quest program on the flow cytometer with a specific anti-epithelial antibody. The total number of adherent bacteria was determined by subtracting the bacteria removed from those initially added. The total number of adherent bacteria was divided by the known number of epithelial cells. This allowed calculation of the number of adherent bacteria per epithelial cell.

Preparation of *C. difficile* antiserum and determination of specificity of adherence

C. difficile grown on Columbia blood agar was heat-treated by autoclaving at 121°C for 20 min. Rabbits were inoculated subcutaneously with 1×10^9 bacteria/ml emulsified in Freund's complete adjuvant and given two subsequent injections of bacteria with Freund's incomplete adjuvant 2 weeks apart. The rabbits were bled 1 week after the last immunisation. The presence of *C. difficile* antibodies in serum was confirmed by ELISA. To determine the specificity of the interaction between bacteria and epithelial cells, bacteria were pre-incubated with diluted anti-*C. difficile* antibodies for 60 min before the adherence assay.

Statistical analysis

All data were analysed with Statview (1989) and Microsoft Excel packages. Student's *t* test was used to compare differences in the adherence of toxin-producing and non-producing *C. difficile* strains to various cell types.

Results

BCECF labelling of *C. difficile*

Optimal uptake of label was achieved at 60 min (data not shown). The MFI was calculated for unlabelled and fluorescently labelled bacteria as mean 3.32 SD 1.55 and 26.44 SD 3.47; $n > 200$ (Fig. 1), respectively.

Isolation of epithelial cells from human intestinal biopsies

Epithelial cells were identified on the basis of cell size and morphology. Cell viability was examined by EB/AO staining and UV microscopy and was $>80\%$

Fig. 1. Fluorochrome uptake by bacterial populations. Dot plot profiles of (a) unlabelled and (b) fluorescently labelled *C. difficile*. The histogram plot (c) shows the shift in fluorescence seen with the labelled bacteria (open peak) versus the unlabelled bacteria (shaded peak).

and >90% for colonic and small intestinal epithelial cells, respectively. Colonic epithelial cell yield varied from 0.8 to 1.9×10^6 from four biopsies (mean = 1.4); small intestinal epithelial cell yield varied from 0.6 to 3.85×10^6 from four biopsies (mean = 1.6).

Adherence to primary colonic and small intestinal epithelial cells

Primary colonic epithelial cells incubated with fluorescently labelled *C. difficile* could be distinguished easily from cells with non-labelled bacteria attached and cells alone (Fig. 2). The adherence was confirmed by light and UV microscopy. The toxigenic strain of *C. difficile* was found to adhere to human colonocytes (Fig. 3a). The difference between fluorescently labelled *C. difficile* binding to primary cells and cells which had been exposed to non-fluorescently labelled bacteria was statistically significant ($p < 0.0001$). The ability of toxigenic *C. difficile* to adhere to primary small intestinal epithelial cells was also assessed (Fig. 3b) and significant adherence was observed ($p < 0.005$).

The adherence of the toxin-positive strain to primary colonic epithelial cells was not significantly different from adherence to primary small intestinal epithelial cells. Small intestinal tissue from 20 patients and colonic tissue from 30 patients was studied. The degree of variation in adherence to both types of primary intestinal epithelial cells was considerable.

Adherence to human intestinal epithelial cell lines Caco-2 and HT29

A toxin-producing *C. difficile* strain was assessed for its ability to adhere to the human intestinal epithelial cell lines, Caco-2 (ileocaecal) and HT29 (colonic) (Fig. 4). Adherence was statistically significant when compared with the negative controls ($p < 0.0001$ for Caco-2 cells and $p < 0.0001$ for HT29 cells). There was no significant difference in adherence to Caco-2 cells when compared to HT29 cells. Adherence of either *C. difficile* strains to these cell lines, while significantly lower than adherence to primary human colonic epithelial cells, was consistent.

Fig. 2. Flow cytometric analysis of *C. difficile* adherence to human colonic epithelial cells and confirmation of epithelial cell status. (a) Dot plot profile of size versus granularity – forward scatter (FSC) versus side scatter (SSC) – analysis of cell populations. The gate drawn (R1) allows analysis of the epithelial cells while eliminating cellular debris, intra-epithelial lymphocytes and non-adherent bacteria. (b) Analysis of *C. difficile* adherence to human primary colonic epithelial cells. The first peak (dark grey) represents cells that have not been exposed to *C. difficile*. The second peak (open) represents cells that have been exposed to unlabelled *C. difficile*. The relative fluorescence expressed by these two populations is similar. The third peak (light grey) represents cells that have been exposed to fluorescently labelled *C. difficile*. The shift in cell population to the right along the fluorescence axis represents bacteria bound to epithelial cells. A median fluorescent intensity (MFI) value can be calculated for each peak. Adherence is expressed as change in MFI (Δ MFI, the difference in MFI values between the test and control populations). (c) Dot plot profile of gated (R1) epithelial cell population (FSC versus SSC). (d) Confirmation of epithelial origin using Ber Ep 4. The gated region (R1) in C demonstrates that >85% of the cells stained positive (open peak). The shaded peak shows cells stained with an IgG1 FITC-matched control.

Semi-quantification of adherent bacteria

Colony counts were done and the approximate numbers of adherent bacteria were estimated; cfu values for the numbers of adherent bacteria observed by flow cytometry confirmed the degree of variation of adherence to primary intestinal epithelial cells when compared with cell lines.

Adherence to primary epithelial cells showed variation in both MFI values (mean, 58.8 SD 47.8; range, 15.8–103.6; $n = 4$) and the numbers of organisms adhering to 10 epithelial cells (mean, 218 SD 176.6; range 56–400; $n = 4$). Alternatively, adherence to cell lines was much more consistent whether measured by flow cytometry when MFI values (12.9 SD 3.9; 9.0–18.4; $n = 4$) or cfu were used to estimate the numbers of organisms adhering to 10 epithelial cells (28 SD 7.5; 21–38; $n = 4$).

Adherence of toxigenic versus non-toxigenic *C. difficile*

Toxin-producing and non-toxin-producing *C. difficile* adhered to primary human intestinal epithelial cells in

a similar fashion. The difference between binding by toxigenic and non-toxigenic organisms was not statistically significant with either colonic ($p = 0.28$) or small bowel ($p = 0.24$) epithelial cells (Fig. 3). There was no significant difference between these two strains when comparing adherence to either Caco-2 cells ($p < 0.07$) or HT29 cells ($p < 0.06$) (Fig. 4).

Inhibition of adherence

Pre-incubation of bacteria with anti-*C. difficile* specific antisera partially blocked the adherence of *C. difficile* to HT29 cells (Fig. 5). Labelled bacteria adhering to HT29 cells gave a mean MFI value of 7.61 SD 3.37 ($n = 6$). Adherence decreased significantly to 4.10 SD 1.21 for bacteria that had been pretreated with *C. difficile* antisera ($p < 0.03$). Bacteria incubated with pre-immune antisera showed no change in adherence (7.80 SD 2.53).

Discussion

In-vitro adherence of *C. difficile* to primary human colonic and small intestinal epithelial cells has not been

Fig. 3. Adherence of *C. difficile* to human primary colonic and small intestinal epithelial cells. **(a)** Adherence of *C. difficile* (toxin-positive, $n = 33$; toxin-negative, $n = 17$) to cells isolated from colonic biopsy specimens was assessed by flow cytometry. Epithelial cells were gated by size and the results of each separate experiment (o) and the mean value (●) and SD for each group of cells are plotted. Colonic epithelial cells that had not been exposed to bacteria were included for comparison. **(b)** Adherence of *C. difficile* (toxin-positive, $n = 19$; toxin-negative, $n = 16$) to cells isolated from small bowel biopsy specimens was assessed by flow cytometry. Epithelial cells were gated by size and the results of each separate experiment (o) and the mean value (●) and SD for each group of cells are plotted. Small bowel epithelial cells that had not been exposed to bacteria were included for comparison. EC, epithelial cells; CD, *C. difficile*; (u), unlabelled; (l, tox⁺), labelled toxin positive; (l, tox⁻), labelled, toxin negative.

examined previously. Animal models of bacterial adherence have been described but the lack of an in-vitro model for *C. difficile* infection of the human gut has hindered research in this area. In particular, viable functional epithelial cells from human biopsy specimens have not previously been used for *C. difficile* adherence studies. In this study, flow cytometry has been exploited

for the first time to examine *C. difficile* adherence to human intestinal epithelial cells. Large numbers of cells are not required for flow cytometry, so optimal use can be made of the relatively low epithelial cell yields from biopsy specimens. With this approach, it is possible to distinguish differences in cell populations based on changes in fluorescent intensities for test and control

Fig. 4. Adherence of toxin-positive and toxin-negative *C. difficile* to human intestinal cell lines Caco-2 (a) and HT29 (b) assessed by flow cytometry. Mean values and SD are plotted for each group of cells: □, cells that had been exposed to fluorescently labelled toxigenic *C. difficile*; ■, cells exposed to non-toxicogenic *C. difficile*; ■, intestinal epithelial cell populations that had not been exposed to bacteria.

Fig. 5. Inhibition of toxigenic *C. difficile* adherence by polyclonal *C. difficile* antibodies; Median fluorescent intensity of HT29 cells (□) and HT29 cells with adherent *C. difficile* (■). Partial inhibition of *C. difficile* adherence to HT29 cells with *C. difficile* polyclonal antiserum (■) compared with lack of inhibition with pre-immune serum (■). Results represent the mean of three separate experiments and SD.

populations by means of this technical approach. When compared with conventional microscopy, it is also possible to examine much larger numbers of cells in a shorter time period. Moreover, directly labelling the

bacteria with a fluorochrome eliminates the need for expensive monoclonal antibodies [31].

Analysis of adherence by light microscopy, electron

microscopy and radiolabelling has been described previously. Light microscopic methods, while useful, are tedious and time-consuming and may be prone to observer error. Electron microscopy studies require lengthy fixation processes and specific technical expertise. Ideally, the use of radioactive labels should be avoided if possible. Flow cytometry allows analysis of cell populations by virtue of their physical characteristics and has been used previously to assess the adherence of *Helicobacter pylori* [32]. Initial studies of bacterial adherence by flow cytometry used specific antibodies to the bacterium under investigation. However, direct labelling of the bacteria with a fluorochrome eliminates the need for expensive monoclonal antibodies [31]. Flow cytometry also allows the optimal use of the relatively small numbers of cells isolated from intestinal biopsies and, therefore, may provide a more suitable technical approach to the analysis of bacterial adherence.

C. difficile adherence to intestinal and non-intestinal immortalised epithelial cells has been demonstrated previously [27]. Waligora *et al.* [28] used a microscopy technique and found that toxigenic and non-toxigenic strains adhered to a kidney epithelial cell line. The present study used flow cytometry to demonstrate adherence of *C. difficile* to two intestinal epithelial cell lines. *C. difficile* consistently adhered to both Caco-2 and HT29 cell lines. Unlike primary human intestinal epithelial cells, which can survive for only a limited period once they are removed from their natural environment, immortalised cells may be more extensively manipulated *in vitro* and, therefore, are useful to study. However, many cell lines do not differentiate in culture and may not express all of the functional receptors seen in mature human colonocytes [33]. Thus, while these cell lines are useful for extensive screening studies, primary human enterocytes may have important physiological features, which may explain the significantly higher levels of adherence seen with primary epithelial cells.

In contrast to the consistent patterns of adherence to cell lines, considerable variability in adherence was observed with primary epithelial cells from individual patients. Neither histology reports nor patient charts revealed any apparent explanation for the lack of consistency of adherence to the cells from certain individuals, nor why, in some instances, no adherence at all could be observed. However, in some cases significantly more adherence to the primary cells was seen, with as many as 40 bacteria binding to each cell. The consistent adherence to immortalised cells, albeit at lower levels than that observed with primary cells, suggests genetic or environmental influences on the expression of the intestinal *C. difficile* receptor. Variation in receptor expression and bacterial adherence may also be relevant to the outcome of clinical disease, as not all colonised patients develop *C. difficile* disease [34, 35]. It is likely that adherence may

promote other virulence factors that influence whether patients remain asymptotically colonised or develop manifestations of disease.

The present study showed that a toxin-positive and a toxin-negative strain of *C. difficile* demonstrated similar adherence to either primary colonic and small intestinal epithelial cells or intestinal epithelial cell lines. Until recently, it was generally accepted that non-toxigenic strains of *C. difficile* that do not possess genes for either toxin are non-pathogenic. However, a number of studies have described symptomatic patients from whom only toxin-negative isolates were cultured. Wren *et al.* found that 28 of 172 strain types from 62 symptomatic patients did not produce either enterotoxin A or cytotoxin B [36]. McFarland *et al.* found no significant difference in toxin production between isolates from asymptomatic *versus* symptomatic patients [37]. In their symptomatic patient group, three of 35 strains were not toxin producers. While it is possible that other virulence factors are involved in the pathogenesis of this disease, the adherence of bacterial strains – whether toxigenic or non-toxigenic – is likely to be a prerequisite to pathology.

In vitro, *C. difficile* binds to small intestinal epithelial cells almost as well as to colonic epithelial cells; however, *C. difficile* infection *in vivo* is generally restricted to the colon. Isolation of *C. difficile* from the small bowel indicates that this region of the gut may act as a reservoir for *C. difficile* infection, although it is rarely a site of infection. Testore *et al.* isolated *C. difficile* from jejunal specimens from 3 of 100 patients, none of whom had gastrointestinal symptoms [38]. However, cases of pseudomembranous enteritis with no colonic involvement have been reported [39, 40] and we have seen a similar case. Several factors including pH, peristalsis, bile acids and normal flora may influence differences in manifestations of adherence at these intestinal sites, thus explaining the rarity of this pathology in the small bowel *in vivo*.

The ability of *C. difficile* to adhere to intestinal epithelial cells is likely to be the primary step in pathogenesis and this characteristic is exhibited by both toxigenic and non-toxigenic strains. The ability to produce toxins does not appear to affect adherence, but binding to primary colonic epithelial cells was significantly greater than adherence to either Caco-2 or HT29 cells, or to Vero cells, as shown by others [28]. Adhesion may allow bacteria to target toxins. It is possible that toxins may be involved in the promotion of mucosal association or that toxin-induced cellular damage might expose masked receptor sites. The method described here would be suitable for screening the ability of clinical *C. difficile* strains to adhere to intestinal epithelial cells. The use of primary intestinal epithelial cells and the increased adherence seen with this cell type demonstrates their optimal use as a more physiologically relevant model of gut epithelium than

immortalised intestinal cells. This assay could also be adapted for screening novel therapeutic agents for their ability to block or interfere with adherence.

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