A real-time quantitative PCR assay for evaluating *Clostridium difficile* adherence to differentiated intestinal Caco-2 cells

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Received 19 February 2010
Accepted 22 April 2010

Herein we describe a real-time quantitative PCR assay for evaluating the adherence of *Clostridium difficile* to differentiated human intestinal Caco-2 cells. Our investigations demonstrated that the method, employing the *C. difficile*-specific triose-phosphate isomerase gene, is as reliable but less time-consuming than counting c.f.u. We conclude that the method will be useful for evaluating the role of host cell adherence in the pathogenesis of *C. difficile* infection.

INTRODUCTION

Clostridium difficile is a Gram-positive anaerobic micro-organism that is the most important cause of antibiotic-associated diarrhoea, now generally referred to as *C. difficile* infection (CDI), in humans. To cause clinical illness, *C. difficile* first penetrates the mucus layer covering the epithelial surface of the gastrointestinal (GI) tract; a strategy that is most likely facilitated by flagella and proteases (Deneve *et al.*, 2009; Tasteyre *et al.*, 2001). Once established in the GI tract, *C. difficile* expresses two large exotoxins, toxin A (TcdA) and toxin B (TcdB) (Jank & Aktories, 2008; Voth & Ballard, 2005). Delivery of TcdA and TcdB to the cytoplasmic compartment of epithelial cells then leads to glucosylation of Rho family GTPases, resulting in disorganization of the host cell cytoskeleton (Deneve *et al.*, 2009). This results in the loosening of tight junctions between adjacent intestinal epithelial cells ultimately leading to inflammation and diarrhoea.

Although TcdA and TcdB represent primary virulence factors, whether adherence to host epithelial cells is also important in the *C. difficile* pathogenic strategy is less clear. However, *C. difficile* adherence to epithelial cells *in vitro* has been reported to be mediated by several colonization factors, including the low- and high-molecular-mass S-layer proteins which are derived by post-translational cleavage of the S-layer precursor protein, SlpA (Calabi *et al.*, 2001). The high-molecular-mass S-layer protein is largely responsible for *C. difficile* binding to both human GI tissues and to components of the extracellular matrix (Calabi *et al.*, 2002). In addition, Cwp84, a putative colonization factor and cysteine protease, has recently been

implicated in a process whereby SlpA is cleaved into low- and high-molecular-mass counterparts (Janoir *et al.*, 2007; Kirby *et al.*, 2009). Several other cell-surface colonization factors including Cwp66, Fbp68 and GroEL, may also all play a role in *C. difficile* adherence to intestinal epithelial cells (Hennequin *et al.*, 2001, 2003; Waligora *et al.*, 2001). Moreover, the flagellar proteins FliC (flagellin) and FliD (flagellar cap protein) bind to axenic mouse mucus possibly facilitating the initial penetration of the intestinal mucus layer (Tasteyre *et al.*, 2001). CDI patients are known to produce serum antibodies to one or more of these cell-surface proteins during infection, emphasizing their potential role in pathogenesis and generating a protective host immune response (Pechine *et al.*, 2005; Wright *et al.*, 2008).

Several *in vitro* *C. difficile* adherence assays have been described (Cerquetti *et al.*, 2002; Drudy *et al.*, 2001; Eveillard *et al.*, 1993; Waligora *et al.*, 1999). There is, however, a lack of standardization in these *C. difficile* adhesion protocols with respect to the type and state of confluency of the tissue culture cells employed in addition to the duration and atmospheric conditions of the assays. Regardless, *C. difficile* has been reported to adhere to a variety of cultured cell lines, including Vero cells, Caco-2 cells and HT29-MTX cells, *in vitro* (Eveillard *et al.*, 1993; Waligora *et al.*, 1999). Although counting c.f.u. is a standard method for evaluating *C. difficile* adherence to host cells, the accuracy and reliability of the assay can be affected by aggregation of the *C. difficile* organisms on the host cell surface thereby leading to potential inconsistencies when counting c.f.u. The agar plating method is also time-consuming since it typically requires 48 h of growth before colonies can be counted. Herein, we describe a less time-consuming and more reliable *C. difficile* adherence assay using differentiated intestinal Caco-2 cells to model the human intestinal epithelium and real-time quantitative

Abbreviations: CDI, *Clostridium difficile* infection; gDNA, genomic DNA; GI, gastrointestinal; qPCR, real-time quantitative PCR; TEER, trans-epithelial electrical resistance.

PCR (qPCR) to enumerate the number of bound organisms. The adherence assay is also performed in an anaerobic environment to promote the viability of vegetative *C. difficile*. We conclude that the method has potential to advance further studies into the role of adherence in the pathogenesis of CDI.

METHODS

***C. difficile* strains and culture methods.** *C. difficile* VLOO13, a NAP1/027 clinical outbreak strain, was kindly provided by Dr Thomas J. Louie at the Foothills Medical Centre, Calgary, AB, Canada. VLOO13 was grown on Y agar plates [Brain Heart Infusion (BHI) agar containing 5% sheep blood, 5 mg haemin and 1 mg vitamin K¹] from frozen stocks and incubated anaerobically in a Bactron III anaerobic chamber (Shel Lab) for 48 h at 37 °C. In all of the adherence assays, single colonies were inoculated into BHI culture medium and incubated at 37 °C anaerobically for 40 h, until cultures reached the late-exponential phase of growth.

Culturing Caco-2 cells. Caco-2 cells (between passage number 40 and 60) were routinely cultured at 37 °C in minimal essential medium supplemented with 20% fetal bovine serum. Caco-2 cells were differentiated using the BD BioCoat Intestinal Epithelial Differentiation Environment (BD Biosciences) in fibrillar collagen-coated Transwell inserts placed in 24-well tissue culture plates as instructed by the manufacturer. Caco-2 cells were considered differentiated when the transepithelial electrical resistance (TEER) recordings were equal to or greater than 150 Ω cm². TEER recordings were made using a Millicell-ERS electrical resistance system (Millipore).

Purification of TcdA. TcdA was purified from VLOO13 using modifications to the method previously described by Sullivan *et al.* (1982). Briefly, *C. difficile* was grown anaerobically in dialysis tubing, 6000–8000 MWCO (Fisher Scientific), suspended in 800 ml BHI for 144 h at 37 °C. The culture was then centrifuged at 12 000 g for 20 min at 4 °C. The crude supernatant solution was filter-sterilized and concentrated using an Amicon Ultra Centrifugal Filter Device (10 000 MWCO; Millipore). The resulting concentrated culture medium was loaded onto a DEAE-Sepharose (Sigma-Aldrich) column (1.0 cm × 5.0 cm; GE Healthcare Bio-Sciences) equilibrated with 50 mM Tris buffer (pH 7.4) and chromatography was performed using an AKTA Explorer FPLC apparatus (GE Healthcare Bio-Sciences). The column was subsequently washed with 12 column volumes of 50 mM Tris buffer to remove any non-adherent proteins. TcdA was eluted with a 6 column volume gradient of 0–0.3 M NaCl prepared in 50 mM Tris buffer. The flow rate of the column during the entire process was 0.5 ml min⁻¹. Eluted proteins were detected by a UV-absorption monitor at a wavelength of 280 nm and characterized by SDS-PAGE using an 8% separating gel. Fractions containing TcdA were subsequently pooled, concentrated and analysed for purity by SDS-PAGE. TcdA activity was assessed by the rabbit haemagglutination and Verocytotoxicity assays described previously (Dingle *et al.*, 2008).

***C. difficile* c.f.u. determination.** *C. difficile* cultures grown in BHI were diluted 10-fold in standard sterile sodium/potassium phosphate-buffered (pH 7.2) physiological saline (PBS). Dilutions were subsequently plated in triplicate on Y agar plates. The plates were then incubated at 37 °C for 48 h and colonies were counted at a dilution resulting in 30–300 colonies per plate.

qPCR technique. One millilitre samples from the *C. difficile* serial dilutions used for c.f.u. determination were centrifuged at 3000 g for 3 min at room temperature. The resulting cell pellets were then suspended in lysis buffer (20 mg lysozyme ml⁻¹; 20 mM Tris/HCl,

pH 8.0; 2 mM EDTA; 1.2% Triton X-100) and genomic DNA (gDNA) was extracted using the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's protocol for Gram-positive bacteria. qPCR was performed using the Bio-Rad iQ5 real-time PCR detection system. The Maxima SYBR Green qPCR Master Mix (Fermentas) was used according to the manufacturer's instructions. The number of bacteria was evaluated by performing qPCR of the *C. difficile*-specific housekeeping gene triose-phosphate isomerase (*tpi*) (Lemee *et al.*, 2004). Primers designed for *tpi* were: forward, 5'-TATGGACT-ATGTTGTAATAGGAC-3'; and reverse, 5'-CATAATATTGGGTC-TATTCTAC-3'. The standard curve was produced by performing five 10-fold dilutions of purified gDNA (purified as described above) from *C. difficile*. Purified gDNA was quantified using a NanoVue Plus Spectrophotometer (GE Healthcare). qPCR was performed according to the Fermentas Maxima SYBR Green instructions (three-step cycling protocol) using an annealing temperature of 55 °C. The number of organisms was then determined by multiplying the ng gDNA determined from the standard curve by 0.0000272 (the portion of the *tpi* gene amplified represents 0.00272% of the *C. difficile* genome). This was then converted to mol *tpi* DNA using the molecular mass (7.2 × 10¹³ ng mol⁻¹) of the portion of the *tpi* gene amplified. Since each organism contains only one copy of the *tpi* gene, the mol *tpi* DNA was then converted to number of bacteria using Avogadro's number.

Viability of Caco-2 cells. The MTT tetrazolium salt colorimetric assay was performed to determine the viability of the differentiated Caco-2 cells as described previously (Dingle *et al.*, 2008). Briefly, thiazolyl blue tetrazolium bromide (Sigma-Aldrich) was added to the apical compartment of anaerobically or aerobically grown differentiated Caco-2 cells to a final concentration of 0.5 mg ml⁻¹. The Caco-2 cells were then aerobically incubated for an additional 4 h at 37 °C before replacing the culture medium with 100 µl PBS and 100 µl 0.01 M HCl in PBS containing 10% SDS. The cells were incubated for an additional 18 h at 37 °C and the results were finally recorded using a Spectramax model 340 microtitre plate reader (Molecular Devices) set to a wavelength of 570 nm.

Caco-2 cell adherence assay. Caco-2 cells were differentiated using the BD Biocoat Intestinal Epithelial Differentiation Environment (BD Biosciences) until TEER recordings were equal to or greater than 150 Ω cm² (approx. 72 h). The Caco-2 cells were then placed in the anaerobic chamber and the medium in the lower and upper compartments was replaced with fresh enterocyte differentiation medium (EDM). All experiments were performed in triplicate. *C. difficile* cultures in the late-exponential phase (OD₆₀₀=0.65) of growth were diluted in overnight pre-reduced EDM and added to the apical compartment of the Transwell inserts at a concentration of approximately 1 × 10⁶ c.f.u. ml⁻¹ (m.o.i.=1:1). The tissue culture plates were incubated at 37 °C anaerobically for 3 h and TEER recordings were made at the beginning and completion of the experiments. The cells were subsequently washed five times with sterile PBS. Lysis buffer was then added to the apical side of the Transwell inserts and the plates were incubated at 37 °C until the Caco-2 cells lifted from the membrane (approx. 5 min). The Caco-2 cells were next transferred to a clean microcentrifuge tube and whole gDNA was extracted using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions for Gram-positive bacteria. qPCR was performed as described above to determine the number of adherent bacteria in each well. Wells containing only EDM (no Caco-2 cells) were also inoculated with 1 × 10⁶ c.f.u. ml⁻¹. The contents of these wells were also subjected to qPCR analysis to determine the number of bacteria in the inocula after the 3 h incubation period.

To determine the number of adherent bacteria by the plating technique, the Caco-2 cell adherence assay was performed as described above. However, after the final PBS washing step, the cells were treated

with 0.25 % trypsin containing 1 mM EDTA and then diluted 10-fold in sterile PBS. Dilutions were subsequently plated in triplicate on Y agar plates. The plates were incubated at 37 °C for 48 h and colonies were counted at a dilution resulting in 30–300 colonies per plate.

RESULTS AND DISCUSSION

Validation of qPCR as a technique to quantify *C. difficile* bacterial counts

To validate the qPCR technique, we produced three independent standard curves using *C. difficile* gDNA. These were highly reproducible, resulting in R^2 values consistently greater than 0.990 (Fig. 1). In addition, melt curves of qPCR products indicated that the primers were specific for the *tpi* gene and no other non-specific products were produced (data not shown). qPCR and the agar plating methods were then performed using *C. difficile* cultures diluted 10-fold in PBS. Tenfold dilutions of the cultures resulted in 10-fold differences in bacterial counts (Fig. 2), which were within the same order of magnitude by both techniques. These results confirm that the qPCR technique represents a sensitive and specific method for determining the number of *C. difficile* c.f.u. qPCR had the additional advantage of decreasing the time required to determine bacterial counts since the assay does not rely on growing the organisms.

Effect of anaerobic conditions on viability of differentiated Caco-2 cell monolayers

Since tissue culture cells are normally propagated in an aerobic environment, we employed the MTT tetrazolium salt colorimetric assay (Mosmann, 1983) to investigate the effect of anaerobiosis on the viability of the Caco-2 cells.

Fig. 1. Reproducibility of the qPCR standard curve. Standards represent 10-fold dilutions of *C. difficile* gDNA. The standard curve presented is the result of three independent qPCR experiments using primers specific for the *C. difficile* *tpi* gene. Error bars represent standard deviation of the means for each determination.

Fig. 2. Determination of the number of *C. difficile* VLOO13 organisms by qPCR (black bars) and by the agar plating method (white bars). Error bars represent standard deviation of the means.

Incubating differentiated Caco-2 cells for 3 h under anaerobic conditions had no effect on their viability relative to control cells incubated under aerobic conditions (Fig. 3a). Although the TEER declined slightly in Caco-2 cells incubated under anaerobic conditions (Fig. 3b), the Caco-2 cells remained differentiated since these recordings remained greater than 150 Ω cm². When differentiated Caco-2 cells were incubated anaerobically for 3 h with 1×10^6 c.f.u. of *C. difficile*, their viability did not decline relative to uninfected cells incubated aerobically, and the TEER recordings of these infected cells remained greater than 150 Ω cm² (Fig. 3a, b).

Indirect ELISA detected the presence of both TcdA and TcdB in the media harvested from *C. difficile*-infected Caco-2 cells after the 3 h incubation period (data not shown) but TEER recordings indicated that the toxins did not affect the Caco-2 cell barrier function. This may indicate that the toxins were produced later during the incubation period and therefore did not have sufficient time to disrupt the epithelial cell monolayers. TEER recordings revealed that the barrier function of differentiated Caco-2 cells treated with 0.8 μ g purified TcdA for 3 h or 48 h anaerobically was reduced to background values (Fig. 3b). The viability of cells treated for 3 h with TcdA remained comparable to that of untreated control cells whereas the viability of those treated for 48 h with TcdA decreased significantly. The majority of the cells (78 %), however, remained viable relative to untreated cells, after 48 h of exposure to purified TcdA. When we compared the sensitivity of conventionally grown Caco-2 and Vero cells to TcdA, it was apparent, by the MTT assay, that Vero cells are significantly more sensitive to TcdA (data not shown). These results indicate that differentiated Caco-2 cells incubated anaerobically for 3 h, even in the presence of TcdA, display good viability and therefore represent a useful *in vitro* system for investigating *C. difficile* adherence to eukaryotic cells.

Fig. 3. Effect of anaerobic conditions on differentiated Caco-2 cell (a) viability as determined by the MTT tetrazolium salt colorimetric assay and (b) barrier function as determined by TEER recordings. TEER values are relative to $T=0$ where cells display 100% TEER. Cells not treated with TcdA remain differentiated since the TEER recordings remained greater than $150 \Omega \text{ cm}^2$. Experiments were performed in triplicate. Error bars represent standard deviation of the means. *t*-tests were performed to determine significant differences relative to wells incubated aerobically for 3 h. * $P < 0.05$, ** $P < 0.01$.

Adherence of *C. difficile* to differentiated Caco-2 cell monolayers

The adherence of *C. difficile* organisms to differentiated Caco-2 cells was analysed by both the qPCR and agar plating methods in triplicate. The number of adherent organisms was determined to be $3.38 \times 10^3 \pm 6.70 \times 10^2$ by the qPCR method and $3.13 \times 10^3 \pm 1.19 \times 10^3$ by the conventional plating method. This observation indicates that both techniques are useful for determining the number of *C. difficile* organisms that adhered to differentiated Caco-2 cell monolayers. However, the qPCR technique was less time-consuming than the plating method because it did not depend on growing the organisms.

Variability in the number of *C. difficile* organisms adherent to differentiated Caco-2 cells was assessed in three independent experiments. The percentage of *C. difficile* bacteria bound at an m.o.i. of 1:1 varied from 0.5% to 0.8% of the inocula (Fig. 4). When the number of *C.*

Fig. 4. Adherence of *C. difficile* to differentiated Caco-2 cells. Percent adherence represents the number of adherent *C. difficile* bacteria relative to the number added. Each independent experiment was performed in triplicate. Error bars represent standard deviation of the means.

difficile organisms added to the apical compartment was increased 10- or 100-fold, the number of bound organisms did not increase, indicating that the bacterial binding sites were likely completely occupied (data not shown) at an m.o.i. of 1:1. At these higher concentrations of bacteria, however, the TEER recordings declined to background values and cell viability remained comparable to that of uninfected cells.

In conclusion, we describe herein a rapid and reliable assay for evaluating the role of *C. difficile* adherence in pathogenesis. We believe that this assay is superior to previously described methods. One advantage is that differentiated Caco-2 cells may be more representative of the environment that *C. difficile* encounters in the human GI tract. In addition, the assay is less time-consuming than the agar plating method and is performed under strict anaerobic conditions promoting the survival of the *C. difficile* organisms. Caco-2 cells are inherently more resilient to stressful conditions, such as low oxygen concentrations, and therefore are an ideal cell line to use for these investigations. We conclude that the method will be useful for further studies into determining the importance of *C. difficile* adherence to host cells in CDI.

ACKNOWLEDGEMENTS

Funding for this project was provided by the Alberta Ingenuity Centre for Carbohydrate Science (AICCS), the National Sciences and Engineering Council of Canada (NSERC) and the Canadian Institutes of Health Research (CIHR).

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