An *in vitro* model of the human colon: studies of intestinal biofilms and *Clostridium difficile* infection

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Summary

The *in vitro* gut model is an invaluable research tool to study indigenous gut microbiota communities, the behaviour of pathogenic organisms and the therapeutic and adverse effect of antimicrobial administration on these communities. The model has been validated against the intestinal contents of sudden death victims to reflect the physicochemical and microbiological conditions of the proximal to distal colon, and has been extensively used to investigate the interplay between gut microbiota populations, antibiotic exposure and *Clostridium difficile* infection. More recently the gut model has been adapted to additionally model intestinal biofilm. Here we describe the structure, assembly and application of the biofilm gut model.

Key words: Chemostat, *Clostridium difficile*, biofilm, modelling, mucosa, microbiota,

1 Introduction

Clostridium difficile infection (CDI) continues to pose a major public health threat and is associated with significant patient morbidity and mortality. Recurrent disease is observed in ~20% of patients [1], which introduces difficulties in treatment management and places further burden on healthcare facilities. The pathophysiological mechanisms of recurrent CDI are not fully understood, but likely involve continued disruption of the host gut microbiota, persistence of *C. difficile* spores and inadequate host immune response.

The human colon is a highly complex and diverse ecosystem comprising planktonic communities and sessile bacteria within mucosal biofilms. Biofilms are associated with 65% of nosocomial infectious in the US [2] with treatment costs over $1 billion annually [3,4]. Studies on intestinal mucosal communities are limited due to the physical inaccessibility of the healthy gut; therefore little is known about intestinal biofilms. Sessile communities differ from their planktonic counterparts, most notably in terms of recalcitrance to therapeutic interventions. The mucosal biofilm and the behaviour of *C. difficile* within this biofilm is likely to be of key importance in the disease progression, but is poorly understood.

The importance of investigating the behaviour of *C. difficile* within biofilm has recently been recognised by numerous research groups. However, initial studies utilise simple *in vitro* systems focused on single or dual species biofilms, which cannot accurately simulate the complex bacterial communities and environment of the human colon. Investigation of *C. difficile* biofilms is hampered by the same constraints as investigation of *C. difficile* planktonic cultures; namely that behaviour in simplified laboratory conditions is markedly different from behaviour in the complex, multi-species environment of the colon. Like other areas of *C. difficile* research, investigation of mucosa-associated *C. difficile* communities has utilised a murine model of CDI. However, animal models of CDI, including the hamster and mouse model, are limited by their inability to accurately reflect conditions within humans, and possess an intestinal physiology and microbiome which differs markedly from humans.

We have previously reported the use of an *in vitro* model of the human colon, which is validated against the intestinal contents of sudden death victims [5]. This model has been successfully utilised to determine the propensity of antimicrobial agents to induce CDI, efficacy of CDI treatment therapies and the effect of these antimicrobial agents upon *C. difficile* and the indigenous gut microbiota. It is reflective of disease *in vivo* and correlates well with clinical findings, thus providing an excellent research tool. This model has recently been adapted to study the formation and roles of complex, mixed-species intestinal biofilms, by incorporating 18 removable glass rods into vessel 3 (representing the distal colon and of most physiological relevance to CDI), which promote formation and subsequent sampling and analysis of intestinal biofilm [6]. The biofilm model has been validated for reproducible

and consistent formation of mixed-species intestinal biofilms. Optimal biofilm formation and removal techniques have been determined. The biofilm gut model is currently the only *in vitro* platform enabling analysis of complex, multi-species intestinal biofilm and has previously been utilised to analyse sessile mixed-species communities and investigate the role of biofilms in CDI [7]. We describe here the structure, the assembly and application of the traditional gut model, and how this model can be adapted to additional allow the analysis of intestinal biofilm communities.

2   Materials

2.1   Traditional chemostat vessel

The triple stage biofilm gut model consists of three glass fermentation vessels connected in a weir cascade system (**Fig. 2**). Vessels are maintained at 37°C via a water jacketed system connected to a circulating heated water bath. Vessel contents are magnetically stirred and pH values of the vessels are maintained at 5.5 (±0.2), 6.2 (±0.2), 6.8 (±0.2) for vessel 1, 2 and 3, respectively. The anaerobicity of the system is maintained by the continuous sparging of oxygen free nitrogen into each vessel. The system is top fed with a complex growth medium at a controlled rate (flow rate; 13.2 mL/h) using a peristaltic pump (*see* Notes 1 and 2).

1. Chemostat vessels (Soham Scientific, Ely, UK): The working volumes are 280 mL, 300 mL and 300 mL, for vessel 1, 2 and 3, respectively. The vessel consists of a base, lid and various attachments (**Fig. 1a**). The vessel contains a waste outlet port, connected to 6.4 mm bore tubing (Marprene), which attaches to the growth media delivery glassware of the downstream vessel, or waste receptacle (vessel 3 – *see* Note 3).   An O-ring sits on the outer contact surface of the chemostat base where the lid is placed. Ensure the O-ring and glassware contact surfaces are greased (Glisseal Laboratory grease, Border Chemie). The lid is secured to the base with a metal clamp. A magnetic stirrer bar is placed within the chemosat vessel. The lid unit contains 5 open ports (B19) housing the attachments outlined below.
    a. A glass stopper, which can be removed at any time to allow access to the contents of the vessel for sampling.

b. A gas inlet consisting of glass gas delivery tube, which allows the delivery of nitrogen gas into the culture fluid of the vessel.

c. A gas outlet to allow gas to escape from the vessel. Both the gas inlet and gas outlet ports are connected to a 0.22 μm vent filter.

d. A pH probe (attached to a pH control unit) attachment allowing the probe to be inserted into the vessel contents.

e. A triple-pronged media feeder (central port), which delivers growth media (or the contents from an upstream vessel), acid or alkali (via autoclaved tubing attached to an automated pH control unit).

2. Stirring: The contents of each vessel and the growth media is constantly stirred magnetically.

3. Nitrogen delivery: Oxygen-free nitrogen is provided via a generator (Parker Hannifin Ltd, UK). A gas manifold connected by silicone tubing allows delivery and control of gas flow to all vessels.

4. pH Control: Each vessel is fitted with a pH probe (P200 Chemotrode, Hamilton, Reno, NV, USA – *see* Note 4) connected to a controller controlled by delivering 1M NaOH/HCl into the vessels using pH controller units (Biosolo 3, Brighton Systems, UK). NaOH is delivered into the vessels by silicone peristaltic tubing. HCl is delivered into the vessels by flexible peristaltic tubing.

5. Temperature control: A circulating water bath (Grant TC120, or similar) able to maintain 37°C.

6. Growth media: Media constituents are outlined in section 2.3. Media is fed into vessel (via the triple pronged media feeder glassware) using a peristaltic pump (Cole-Parmer Masterflex L/S Digital drive with Masterflex easy load pump head 77800-60 or similar) able to achieve a flow rate of 13.2mL/h (*see* Note 5).

7. Waste: The overflow from vessel 3 flows to an autoclavable waste vessel.

2.2 Biofilm chemostat vessel

1. Biofilm chemostat vessel (Soham Scientific, Ely, UK): The biofilm vessel replaces vessel 3 and has a 300 mL working volume, lid, attachments as outlined above (gas inlet changed to a circular pipe, with drilled holes interspersed on the surface to

encourage even distribution of gas flow across the rods) and 18 rod attachment ports. The ports for the biofilm vessel attachments are smaller (B14) to allow for rod attachments.

2. Biofilm rods: Ground glass rods (2mm diameter) are inserted through the lid screwed in place. Upon removal of rods, replacement screw caps are placed onto the lid to maintain the integrity of the system.

2.3   Growth medium

The model is top fed with a complex growth medium that is prepared in 2L volumes; constituents are listed in table 1. Growth medium is prepared in Büchner flasks plugged with a bung adapted to incorporate media delivery ports and magnetically stirred. Sterilisation of the growth media is achieved by autoclaving at 123° C for 15 minutes after which resazurin (*see* note 6) and glucose are filter sterilised into the medium through a 0.22 μm syringe filter.

2.4   Solid agar medium

A list of solid agar medium used during gut model experiments are provided in table 2.

2.5   Cytotoxin assay

1. Cells: Vero cells (African Green Monkey Kidney Cells, ECACC 84113001) purchased from NCTT

2. Culture medium: Dulbecco's Modified Eagles Medium (Sigma D6546) supplemented with 50 mL newborn calf serum (Invitrogen 16010), 5mL antibiotic/antimycotic solution (Sigma A5955) and 5 mL L-glutamine (Sigma G7513).

3. Culture vessels: Tissue Culture Flasks (75ml, Nunc) or Flat Bottom 96 well Trays (Nunc).

3   Methods

3.1   Vessel assembly

Vessels are assembled individually. The lid connection and all ports and attachments are greased and sealed. Connecting tubing is measured and attached to the upper vessel. All exposed openings are covered with aluminium foil and autoclave tape. Vessels are

autoclaved separately. Tubing for media, acid and alkali is measured and autoclaved. After autoclaving, vessels are connected in situ, tubing is attached, pH probes are calibrated and inserted, the water jacket tubing is connected to the circulating water bath and gas tubing is connected.

3.2   Preparation of the gut model

Each vessel of the gut model is inoculated with approximately 150 mL of pooled faecal emulsion. Vessel 1 is topped up to approximately 280 mL with growth medium and the media pumped started.

3.3   Preparation of faecal emulsion

Faecal material is donated by healthy elderly volunteers (n=3-5, ≥60 years) with no history of antimicrobial therapy for at least 3 months prior to donation. Faecal samples are stored in a sealed anaerobic zip lock bag for no longer than 12 hours during transportation and transfer to an anaerobic cabinet.   To ensure the absence of *C. difficile* populations within the faecal material, samples are plated directly onto CCEYL agar in duplicate and incubated anaerobically at 37°C for 48 hours. Faecal material from any donor testing positive for *C. difficile* should be discarded. Faecal emulsion is prepared by suspending 10% w/v *C. difficile*-negative faeces in pre-reduced PBS, and the suspension emulsified in a stomacher until a smooth slurry is formed. Any large particulate matter is removed by filtration through a sterile muslin cloth.

3.4   *C. difficile* spore preparation

C. difficile is inoculated onto a single CCEYL plate and incubated anaerobically at 37°C for 48 hours. All growth is removed and sub cultured onto 10 Columbia blood agar (CBA) plates and incubated anaerobically at 37°C for 10-14 days. All growth is removed and emulsified into 1 mL of sterile saline, to which 1 mL of 99.6% ethanol is added. The suspension is vortexed for 30 seconds and stored aerobically at room temperature.

3.5   Planktonic bacterial populations sampling and enumeration

The glass stopper is removed from the vessel and approximately 5 mL culture fluid removed from the vessel via a sterile Pasteur pipette and transferred to a sterile glass tube. One millilitre aliquots are transferred to sterile Eppendorf and stored at 4˚C (cytotoxin assay) or -20˚C (antimicrobial bioassay). The samples are transferred to an anaerobic workstation. Cultures are serially diluted in pre-reduced peptone water to 10^{-7}. Twenty microliters of four appropriate dilutions are inoculated to quarter plates of each culture medium (table 2) in triplicate and incubated anaerobically (obligate anaerobes) or aerobically (facultative anaerobes) at 37˚C for 48 hours. Colonies are identified to genus level on the basis of colony morphology, Gram reaction and microscopic appearance on selective and non-selective agars.

3.6   Biofilm analysis

Biofilm associated bacterial populations are enumerated following careful removal of rods from the biofilm model. Rods are transferred into 5 mL pre-reduced saline, thoroughly vortexed for 2 mins and the rod discarded. Re-suspended biofilm is centrifuged at 4000 rpm for 10 mins, the supernatant removed and pellet re-suspended in 2 mL pre-reduced saline. An aliquot (500 μL) is centrifuged and stored at 4˚C for cytotoxin assay. A second aliquot (1 mL) is transferred to a pre-weighed eppendorf, centrifuged at 16000 g for 15 minutes and supernatant discarded. The remaining pellet is weighed and pellet weight calculated by subtracting the eppendorf weight from the total. The remaining biofilm suspension is enumerated as described for planktonic organisms and units were reported as log_{10}cfu/g of biofilm.

3.7   Cytotoxin assay

Vero cell monolayers are cultured to ensure confluent growth upon examination under an inverted microscope. One hundred and sixty microlitres of the harvested Vero cells are inoculated into the wells to which antitoxin will be added and 180 μL of trypsinsed Vero cells are added to all other wells and incubated in 5% CO2 at 37°C for 2 days or until Vero cell monolayers are confluent upon examination under an inverted microscope. The Vero cells are continuously passaged for the duration of each experiment. One millilitre samples for toxin enumeration are centrifuged at 16000 g for 15 minutes and the supernatant stored at 4°C. Sample supernatant and positive cytotoxin controls (48 hour culture of *C. difficile*

strain 027 210 in Brain Heart Infusion broth) are serially diluted 10-fold in sterile phosphate buffered saline (PBS-pH7) to 10^{-5}. Serial dilutions are aseptically transferred into microtitre trays containing Vero cell monolayers. Twenty microlitres of *C. sordellii* antitoxin is used to neutralise any cytotoxic effect and ensure that cell rounding observed is specific to *C. difficile*. A sample is designated as positive if at least ~80% of the Vero cell monolayer cells are rounded, and the effect is neutralised by antitoxin. A relative unit is used to quantify the level of cytotoxin present by assigning a titre to the sample based on the greatest dilution of which a positive cytotoxic effect is observed (*i.e.* if toxin was present in the neat only dilution this was assigned a titre of 1; if a positive cytotoxin effect was observed in the 10^{-1} dilution then it was assigned a titre of 2, *etc*.).

3.8   Reduced antimicrobial susceptibility surveillance

Breakpoint agars are utilised to monitor for the development and proliferation of antibiotic resistant or tolerant organisms. Solid medium specific for the target species is used including an additional supplement of the antimicrobial agent of interest at four times the MIC of that antimicrobial agent for the target species, i.e. surveillance for vancomycin resistant Enterococci (typical *E. faecalis* vancomycin MIC 1 mg/L) – use kanamycin aesculin azide agar base supplemented with 10 mg/L nalidixic acid, 10 mg/L aztreonam, 20 mg/L kanamycin, plus 4 mg/L vancomycin.

3.9   Antimicrobial bioassay

The concentration of active antimicrobial agent is determined by an in-house, large plate bioassay. See table 3 for assay specificities.

Indicator organisms are inoculated onto fresh CBA and incubated aerobically (37°C) overnight. A 0.5 McFarland suspension (~10^8 cfu/mL) of indicator organism is prepared in sterile saline and 1 mL added to 100 mL molten (50°C) agar. Inoculated agars are mixed by inversion, poured into 245 mm x 245 mm bioassay plates and allowed to set. Inoculated agars are dried (37°C) for 10 min and 25 wells (each 9 mm diameter) removed from the agar using a cork borer. Twenty microliters of target agent calibrator (doubling dilution calibration series) or each filter-sterilised sample from the gut model are randomly assigned to bioassay wells in triplicate. Bioassay plates remain at ambient temperature for 4 h prior to overnight aerobic incubation at 37° C. Zone diameters are measured using callipers

accurate to 0.1 mm. Calibration lines are plotted from squared zone diameters and unknown concentrations from culture supernatants determined. Coefficient of variation values are typically 10% and R2 values for calibration lines should be ≥0.9. Quantification of biofilm-associated antimicrobial activity was determined using the assumption that 1 g of biofilm was equal to 1 mL of planktonic culture fluid.

3.10   Experimental design

For a typical gut model experiment 2 triple stage models are constructed and run in parallel. After the gut models are inoculated with faecal emulsion no further interventions are made for 14 days to allow the system to equilibrate (Fig. 3, period A – *see* Note 7). Once steady state is reached (~ day 14) an inoculum of approximately 10^7 cfu *C. difficile* spores (typically ribotype 027, strain 210) are added to vessel 1 of the gut models (period B) and left without intervention for 7 days. This serves as an internal control period whereby the gut flora remain stable and spores quiescent in the absence of antimicrobial exposure. Subsequent interventions are dependant on the aims of the study. Typical examples are listed below:

3.10.1 Induction gut model

After the internal control period a further inoculum of approximately 10^7 cfu *C. difficile* PCR ribotype 027 spores are added to vessel 1 of the gut models along with a dosing regimen of antibiotic of interest and dosed to reflect levels present with faeces (period C – *see* Note 8).

3.10.2 Treatment gut model

After the internal control period a further inoculum of approximately 10^7 cfu *C. difficile* PCR ribotype 027 spores are added to vessel 1 of the gut model along with a dosing regimen of clindamycin (33.9mg/L QD 7 days – period C, figure 3). Following commencement of clindamycin dosing no further intervention are made until *C. difficile* germination, proliferation and high toxin production is observed (period D), at which point instillation of the therapeutic agent (e.g. vancomycin 125 mg/L, QD, 7 days – *see* Note 9) commences. Following cessation of therapeutic instillation the model is left without intervention for 21 days (period F).

Three rod-associated biofilm samples are taken at key stages of the experiment. Due to the finite number of rods (n=18) within the vessel and precautions taken to minimise sessile

population bias (i.e. 3 rods samples per time point), a maximum of six biofilm analysis time points are included in each experiment.

4.0 Notes

1. Regular observation of gut models is advised to prevent problems. Gas flow, temperature, pH and fluid levels should be regularly monitored.

2. Excessive frothing of the culture fluid can significantly reduce culture volume. If this cannot be reduced by altering the gas flow, addition of polyethylene glycol (PEG) can help.

3. Blockages of connecting tubing between vessels can occur. This is usually due to build up of biofilm / cellular matter and can generally be removed by manipulation / massage of the tubing.

4. pH probes must be able to withstand high protein concentrations.

5. Growth media and growth media tubing can become contaminated. The resazurin indicator in the media may cause a colour change (red to yellow) if this occurs.

6. Resazurin must be delivered into the medium whilst still warm ($\geq 50°C$).

7. Steady State (Period A) can be flexible in length, and should last until microflora populations are stable. Typically this will be ~10-14 days, but can be extended if required.

8. Clindamycin instillation results in consistent CDI induction in the gut model and is therefore the agent of choice for CDI induction during experiments focussing on CDI treatment.   Clindamycin is instilled to achieve levels equivalent to those in bile [8].

9. Vancomycin is the first line treatment agent for CDI and used as a comparator agent to determine superiority/non-inferiority/inferiority of novel anti-CDI agents under investigation.

10. Glucose is unstable at excessive temperatures. Medium constituents, including glucose should be added to cooled, autoclaved distilled water and steamed (100° C) until molten. Once cooled (approx. 50° C) propionic acid should be added and pH adjusted using 10M NaOH and/or 10M HCl.

5.0 Acknowledgements

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