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In vitro models to study *Clostridioides difficile* infection: current systems and future advances

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Abstract

Purpose of review

Clostridioides difficile infection (CDI) is the most common cause of healthcare-associated diarrhoea in western countries, being categorised as an urgent healthcare threat. Historically, researchers have relied on the use of *in vivo* animal models to study CDI pathogenesis; however, differences in physiology and disease prognosis compared to humans limit their suitability to model CDI. *In vitro* models are increasingly being used as an alternative as they offer excellent process control, and some are able to use human *ex vivo* prokaryotic and/or eukaryotic cells.

Recent findings

Simulating the colonic environment *in vitro* is particularly challenging. Bacterial fermentation models have been used to evaluate novel therapeutics, explore the re-modelling of the gut microbiota, and simulate disease progression. However, they lack the scalability to become more widespread. Models which co-culture human and bacterial cells are of particular interest, but the different conditions required by each cell type make these models challenging to run. Recent advancements in model design have allowed for longer culture times with more representative bacterial populations.

Summary

As *in vitro* models continue to evolve, they become more physiologically relevant, offering improved simulations of CDI and extending their applicability.

Keywords

Clostridioides difficile, Clostridioides difficile infection, in vitro, gut model, gut microbiota

Introduction

Clostridioides difficile is a Gram-positive, endospore producing anaerobe which can colonise the intestinal microbiota. When the complex gut microbial ecosystem is perturbed, it allows the bacterium's rapid expansion, leading to symptomatic *C. difficile* infection (CDI). Disease severity can range from mild diarrhoea to toxic megacolon, perforation of the colon and ultimately death. CDI poses a significant healthcare burden across the globe, as the leading cause of healthcare-associated diarrhoea [1].

The pathogenesis of CDI is largely associated with the use of broad-spectrum antibiotics, which limit the competitive exclusion afforded by the endogenous microbiota, allowing *C. difficile* proliferation. Fluoroquinolones, clindamycin, and beta-lactams (particularly cephalosporins) are associated with high CDI risk [2, 3]. The recommended antibiotic treatments for *C. difficile*, vancomycin and fidaxomicin, [4] may compound the factors which caused the initial *C. difficile* proliferation, leading to a significant number of patients relapsing or developing recurrent CDI (rCDI).

Faecal microbiota transplant (FMT) is a relatively crude but effective restorative therapy, shown to prevent rCDI. FMT acts by reseeding the microbiota, restoring microbial and functional diversity. Autologous transplants are effective [5], but not always feasible. Due to the risk of transplanting pathogens which may lead to further complications for the patient or potentially unknown long-term health consequences, allogenic transplants are usually reserved for patients who have suffered from multiple instances of rCDI. Alternatives to FMT are defined as restorative therapies that reseed pre-selected beneficial communities of the endogenous microbiota [5-7]. However, none of these novel treatments are yet authorised for use clinically [6].

With interactions between *C. difficile*, host and microbiota having a key role in CDI pathogenesis, the ability to accurately model these interactions will be crucial to future *C. difficile* research. Current studies often rely on the use of *in vivo* animal models – namely hamster and mouse models [8, 9]. They are particularly used when studying immunological aspects of *C. difficile*; however, immune responses vary

greatly between species and disease progression in these models is often dissimilar to that in humans [10]. Further limitations include the differences in anatomy and microbiota composition [11], plus there is a general need to reduce and refine the use of animals in research. This has promoted the use of *in vitro* models to simulate CDI.

The complex multifaceted aetiologies behind CDI mean that model systems which can maintain complex and stable microbial communities and model their interactions with host physiology will be invaluable in further understanding *C. difficile*/CDI and for the development of novel therapies.

Current *in vitro* models for studying CDI

Bacterial fermentation models

Batch fermentation

Batch fermentation models are the simplest *in vitro* model and consist of a reaction vessel with controlled internal conditions [12]. They provide a quick and relatively inexpensive screening tool for the metabolization of specific substrates. However, depletion of nutrients, media acidification, and build-up of metabolites limit the experiment duration – typically less than 48h – making these models unsuitable for longitudinal studies [13]. Nonetheless, batch fermentation is used to study CDI. For instance, a batch model was used to assess the efficacy of a "Bacteriophage Cocktail" to clear CDI [14], while a separate study used a simple batch model consisting of six-well plates, to investigate the sporulation of *C. difficile* in faecal emulsions from different patients, showing that a dysbiotic microbiota is more susceptible to CDI, and this susceptibility is strain-dependent [15].

Continuous single stage (CSS) models

CSS models also consist of a single reaction vessel, but the continuous or semi-continuous influx of nutrient-rich media and efflux of waste products allows for longer culture times, where bacterial

populations are allowed to stabilise and can form trophic chains [16]. Its main disadvantage is that only simulates a single colonic region, so microbial dynamics across the entire gastrointestinal tract cannot be characterised. However, their simplicity and low cost compared to multi-stage models make repeats more feasible. For example, the Mini BioReactor Arrays (MBRAs) allows for 24 CSS to be run simultaneously, promoting the growth of stable microbial communities [17]. The MBRAs have been used to demonstrate that an *in vitro* gut microbiota modulated with polyphenols has decreased *C. difficile* colonisation resistance [18], and that *Fusobacterium nucleatum* acts synergistically with *C. difficile* in the formation of biofilms [19].

Continuous multi-stage (CMS) models

CMS models were first devised in the 1980s and subsequently validated against the colonic contents of sudden death victims [20]. In brief, the original model consisted of three vessels arranged sequentially to simulate the proximal, medial, and distal colon. Each vessel is maintained at conditions (pH, temperature, $\%O_2$) specifically designed to mimic each colonic region [21, 22]. This arrangement has become the standard reference from which other models have been developed.

A variation of this triple-stage model has been extensively used for studying CDI pathogenesis. Recent work includes evaluating the propensity of oral antibiotics to induce CDI: omadacycline, first-generation cephalosporins and eravacycline are among those that showed a low CDI association [22-24]. These models have also been used to evaluate the potential of a novel antibody therapy to prevent CDI [25], with results showing good efficacy at neutralising toxin production and rCDI prevention when combined with vancomycin. A further study showed that trehalose-induced remodelling of the gut microbiota can lead to colonisation resistance against *C. difficile* [21]. Furthermore, it is possible to modify the standard CMS to support biofilm growth and study sessile populations – using this model it was demonstrated that biofilms can harbour *C. difficile* and cause rCDI [26].

Although the majority of CDI studies using CMS models used the described triple-stage setup, other *in vitro* systems are available. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) [27, 28] is a commercial system which builds upon the triple-stage model by adding two fermentation vessels to simulate the stomach and small intestine. In doing so, it is one of the few models which simulates the entire gastrointestinal (GI) tract; however, the entire system is initiated with derived faecal matter which is unlikely representative of the physiological conditions of the upper GI tract.

CMS models have been extensively used for pre-clinical research and correlate well with patient outcomes [22, 24]. They allow for long-lasting longitudinal studies and sampling of different colonic regions to study microbiota spatial variation, which can only be achieved through invasive surgery in *in vivo* models. Despite showing greater control and fewer ethical restraints than animal models, CMS are resource intensive and thus impractical to run in large numbers, limiting the possibility of repeats. A summary of bacterial fermentation set-ups is shown in **Figure 1A**.

Other* in vitro *models

Variations of the multi-compartmental model TNO Gastro-Intestinal model (TIM) which simulates the GI tract lumen conditions, have been used to study the fermentation of foods and supplements [12, 29, 30]. TIM focusses on the meal transit time and it controls physiological parameters to reflect the conditions of the upper GI; thus, experiments have a short timeframe (>72h). Similarly, models such as EnteroMix [31] and ECSIM [32] also have reduced run times when compared to the triple-stage model and SHIME. As these models aim at investigating the digestion process, they do not offer the experimental durability of weeks/months that CDI studies often require.

Human interaction models

Bacterial fermentation models support solely microbial growth. Although suitable to investigate microbe-microbe and drug-microbe interactions, these models lack information on how microbial composition relates to the host physiology. Human interaction models aim to bridge this gap (**Figure 1B)** but are limited by the different culture requirements of prokaryotic and eukaryotic cells. Many fastidious anaerobes composing the intestinal microbiota are sensitive to low oxygen concentrations, which contrasts with the high oxygen requirements of human cells.

Three-dimensional (3D) models

3D organoids consist of ex-vivo culture of organ cells which mimic the source tissue architecture. Intestinal organoids developed through the culture of intestinal stem cells can accurately portray the physiological composition of the human intestinal epithelium. Intestinal organoids vary with the nature of the stem cell origin; enteroids derive from adult stem cells isolated from tissue biopsies, while induced human intestinal organoids are developed from pluripotent stem cells. The later provide a more robust representation of the intestinal epithelium and remove the need for biopsy samples but have increased culture times (months rather than days).

Organoids have proved useful in exploring the interactions between *C. difficile* and the intestinal epithelium [33, 34]. The pathogenicity of CDI is driven largely by toxin A (TcdA) and toxin B (TcdB). These are known to alter the cytoskeletal structure of the intestinal epithelium causing cell death and disrupting the epithelial barrier. Exposing organoids to *C. difficile* toxins demonstrated that microRNAs suppression in colonic tissues during CDI may be attributable to the actions of *C. difficile* TcdB [34].

However, the use of spheroid organoids for *C. difficile* research is not very common. Intestinal organoids form closed 3D spheres with an inner apical side representing the intestinal lumen and an outer basal side representing the submucosal of the intestinal epithelium. This orientation reduces access to the luminal

surface, impacting cell line consistency and physiology, and limiting 3D models suitability for *C. difficile* related studies.

Two-dimensional (2D) monolayers and "gut-on-a-chip" models

2D monolayers derive from fragmented 3D organoids, as patient-derived cell lines are plated onto extracellular matrix-coated wells known as Transwell plates [35]. The 2D organoid-derived monolayer allows access to the apical side and provides a simple and inexpensive method of co-culturing epithelial and bacterial cells. Jejunal human intestinal organoids have been used to study the action of TcdA and TcdB, revealing mucin might reduce toxin binding in the human epithelium [33]. Transwell models have recently been developed which allow extended co-culture of human epithelial cells with oxygen-sensitive bacterial species [36]. Although these models allow for longer culture times compared to 3D organoids and promote bacterial differentiation to form villus-like structures, they lack fluid flow and peristalsis-like motion, important *in vivo* characteristics of the intestinal environment.

To address this, various so-called "gut-on-a-chip" models have been developed. They typically consist of two channels representing the lumen and blood vessels, separated by a semi-permeable membrane on which epithelial cells can be grown [37]. Each channel can be perfused with separate culture media to support each cell type, and recent developments have transluminal hypoxia gradients, allowing the culture of strict anaerobes [36] and complex faecal-derived microbiota [38]. The flexible construction of these models allows the application of fluid flow to mimic *in vivo* environment [39], and the co-culture of human cells [40].

Current challenges and future advances

The biggest challenges with all the above models remain the culture of a stable bacterial community which is representative of the original inoculum, while maintaining epithelial and immune cell viability over

extended periods [41]. For the study of CDI, the host-microbe interaction is of critical importance as it links bacterial composition and/or the presence of *C. difficile* toxins to host outcomes. Incorporating relevant human cells rather than relying on animal-derived or immortalised cell lines would simulate a more physiologically accurate response to CDI *in vitro* [37]. Although co-culture of human cells with selected bacterial species may be beneficial to investigate individual mechanisms of action, cell culture with a "complete" microbiota would be more relevant to simulate host-microbiota interactions.

The currently available systems often compromise high throughput - which enable repeats- with limited complexity, and vice-versa. Therefore, the complex GI models which most closely mimic the colonic environment are difficult to scale up; while the most easily scalable assay-based methods have little relation to host physiology (**Table 1**). Future models should aim to address this issue by reducing complexity and resource requirements, and increasing automation, while maintaining clinical reflectiveness.

Many of the above *in* vitro models have been developed within single research groups and institutions, using bespoke equipment and custom culture media, which limits standardisation and poses financial restrictions as development costs are high. These differences can make it difficult to draw comparisons between studies, and so it would be beneficial to reduce method variability going forward.

It has been shown that biofilms play a critical role in the pathogenesis of CDI, particularly in recurrent infections [26, 42]. However, methods for growing and sampling intestinal biofilms *in vitro* are poorly standardised and vary greatly between studies. The SHIME model can be modified by the addition of mucin-coated microcosms [43] which facilitate biofilm growth, while other studies have use mucin-coated coverslips suspended within a bioreactor or bacterial suspension [19, 44, 45]. Recent studies have also shown that specially fabricated structures, more similar to the *in vivo* environment, can provide a greater surface area for biofilm attachment and growth [46, 47]. Models with defined flow characteristics and a

more representative luminal environment will be key for studying biofilm formation and its role in CDI (**Figure 2A**).

Other advances such as integrated sensing, inclusion of 3D structures, and extending culture times past 48h will all aid in the wide-scale adoption of *in vitro* models, as no device can currently simulate all the characteristics of the human colonic environment *in vitro* [12]. A more pragmatic approach would be to develop a modular system whereby different *in vitro* "modules", each with their own set of features, can be used as required (**Figure 2B**). This is exemplified in the SHIME model where a flow cell can be coupled to study host-bacteria interactions [48].

Future applications

Popularity of *in vitro* models as an ethical alternative to *in vivo* models is increasing. Their excellent process control and rapidly evolving ability to model both microbial and human components of the GI tract have the potential to revolutionise research of GI diseases.

As our understanding of the gut microbiota increases, the systemic implications of intestinal disease are becoming more relevant. To better support health-related studies and characterise mechanisms of disease, *in vitro* platforms must also evolve to accurately model inter-organ interactions. Multi-organ platforms are in development and will likely grow in popularity and relevance as they are refined [49, 50].

The relationship between CDI and intestinal dysbiosis is well established [21, 24-26], however dysbiosis is still poorly defined. Variation in the microbiota of individuals means there is likely a significant amount of redundancy in characterising dysbiosis, as numerous organisms can fill similar roles. *In vitro* models offer the capacity to monitor the intestinal environment throughout disease progression, thus they provide means for studying unique microbial activities. Exploring the microbiota from a functional perspective through the implementation of omics technologies and defining dysbiosis as a family of

functional disorders may redefine critical healthcare approaches. Understanding the functional capacity of a patient's microbiota may also provide valuable insights when curating treatment plans, by minimising microbiota disruption and reducing the risk of CDI.

In vitro models are well suited to longitudinal studies examining how the intestinal microbiota can be reseeded using restorative therapies to re-introduce functional diversity and increase ecological robustness, in patients at risk of CDI. *In vitro* models can also be used to investigate the transfer of mobile genetic elements within bacterial populations, and the microbial metabolization of therapeutic agents, particularly those delivered enterally.

The final hurdle in the development of *in vitro* models will likely be the integration of functional immune systems for vaccine development. *In vivo* models remain the only viable system for studies involving adaptive immunity.

Conclusions

There are currently several *in vitro* platforms which are capable of modelling microbial and human mechanisms of CDI. Simpler bacterial fermentation models are highly controllable and scalable systems suited for screening of potential therapeutic agents, whereas continuous multistage models are fit for longitudinal studies examining remodelling of the microbiota and rCDI studies. Advances in tissue culture techniques and evolution of 3D and 2D organoid systems offer the possibility to investigate CDI mechanisms in a controllable host environment, but are still limited in their potential. A likely evolution of *in vitro* models will be the integration of organoids into bacterial fermentation models, providing insight into how changes in microbiota composition and function may impact host cells, leading to intestinal disease.

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Key Points

- *In vivo* animal models are used for studying CDI; however, there are differences between animal and human physiology, microbiota composition, and disease progression.
- Bacterial fermentation models are widely used to study *C. difficile*, with increasingly complex models being used to successfully simulate different colonic regions.
- Studying the host-microbe interaction *in vitro* is particularly challenging but has been achieved through the co-culture of bacteria and epithelial cells in specially designed devices.
- Main advances in models of CDI will focus on solving the scalability issues faced by current fermentation models, and in improving the host interaction interface.
- Systems able to maintain complex microbial communities and model their interactions with host physiology will be key to further *C. difficile*/CDI research.

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Figure 1. Overview of the *in vitro* models currently available for studying *C. difficile* and CDI. (a) shows the bacterial fermentation models, (b) shows the human interaction models. Left to right indicates models of increasing complexity and corresponding decreasing scalability. Figure created using BioRender.com.

Figure 2. Future advances that would benefit *in vitro* modelling: (a) shows a gut-on-a-chip device with the inclusion of a functional surface for biofilm attachment; (b) shows how a bacterial fermentation bioreactor could be coupled with multiple organ-on-chip devices to study host interactions. Figure created using BioRender.com.

Table 1. Summary of *in vitro* models used for studying CDI and the human gut microbiota.