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Microbiological factors affecting *Clostridium difficile* recurrence.

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Abstract

Background

Recurrent *Clostridium difficile* infection (rCDI) places a huge economic and practical burden on healthcare facilities. Furthermore, rCDI may impact quality of life, leaving patients in a 'rCDI cycle', and dependant on antibiotic therapy.

Aims

This article discusses the importance of microbiological factors in the development of rCDI.

Sources

Literature was drawn from a search of PubMed from 2000 onwards with the search term "recurrent *Clostridium difficile* infection"; and further references quoted within these articles.

Content

Meta-analysis and systematic reviews have shown that CDI and rCDI risk factors are similar. Development of rCDI is attendant upon many factors including immune status/function, comorbidities and concomitant treatments. Studies suggest that poor bacterial diversity is correlated with clinical rCDI. Narrow spectrum gut microflora-sparing antimicrobials (*eg* surotomycin, cadazolid, ridinilazole) are in development for CDI treatment; while microbiota therapeutics (faecal microbiota transplantation, non-toxigenic *C. difficile*, stool substitutes) are increasingly being explored. Recurrent CDI can only occur when viable *C. difficile* spores are present, either within the gut lumen post-infection, or re-acquired from the environment. *C. difficile* spore germination can be influenced by gut environmental factors resulting from dysbiosis; and spore outgrowth may be affected stage by some antimicrobials, (*eg* fidaxomicin, ramoplanin, oritavancin).

Implications

Recurrent CDI is a significant challenge for healthcare professionals, requiring a multi-faceted approach:optimised infection control to minimise re-infection; *C. difficile*-targeted antibiotics, to minimise dysbiosis; gut microflora restoration to promote colonisation resistance. These elements should be informed by our understanding of the microbiological factors involved: both *C. difficile* itself and the gut microbiome.

Introduction

Clostridium difficile infection (CDI) continues to be the leading infectious cause of antibiotic-associated diarrhoea, and a significant burden on healthcare systems worldwide.[1, 2] Disease recurrence following initial symptom resolution frequently arises, with recurrent *C. difficile* infection (rCDI) occurring in 20-30% of CDI patients.[3] In hospitalised patients, rCDI is responsible for increased mortality and decreased quality of life,[4] and a first recurrence greatly increases risk of subsequent recurrences, which doubles after $\geq$2 recurrent episodes.[5] This can result in patients trapped in a 'rCDI cycle', which is problematic to resolve (see Figure 1) and further increases the burden on healthcare facilities. A recent study suggested median costs associated with length of stay increased from $20,693 to $45,148 for primary CDI *vs* rCD I patients respectively ($P<0.0001$), with associated pharmacological treatment costs of $60 and $140 respectively.[6]

Recurrent CDI is currently defined as the reappearance of symptomatic CDI within 8 weeks after the onset of a previous episode, and following previous resolution of symptoms[7], although the validity of this definition has been questioned. [8]

Meta-analyses and systematic reviews indicate that the risk factors for CDI and rCDI are similar. Advanced age, additional antibiotic therapy during follow up, and PPI therapy were the most frequent independent risk factors for rCDI.[9-11] Risk of rCDI is also greater in patients with chronic renal insufficiency and those previously receiving fluoroquinolones.[9]

Factors including immune status/function, comorbidities and concomitant treatments are likely to influence rCDI development. However, this article will discuss the microbiological factors affecting rCDI, outlined in Figure 1, focussing on the intestinal microbiota and *C. difficile* spore germination.

The intestinal microbiota and recurrence of CDI

Evidence for gut microbiota link CDI and recurrence

The link between gut microbiota disruption and CDI is well-established. Highly significant risk factors for CDI include age >65yrs and prior antimicrobial use.[12] Increasing age has been associated with an altered gut microbiota profile,[13,14] while antibiotic-mediated disruption of intestinal microbiota and loss of "colonisation resistance" has long been associated with CDI. Increasing availability of sequencing technologies has enabled more accurate exploration of antibiotic-mediated microbiota

alterations associated with CDI. No single microbiota component has yet been linked to *C. difficile* susceptibility; many different dysbiotic populations exist, all of which may predispose to CDI.

Work in rodents and *in vitro* gut models indicated that clindamycin exposure resulted in decreased obligate anaerobic populations and a microbiota dominated by Enterobacteriaceae;[15-17] cephalosporin exposure in Pseudomonadacae- and Lactobacillacaeae-dominated microbiota,[15, 19, 20] and tigecycline exposure in decreased in Bacteroidetes and increased Proteobacteria populations.[21, 22] These changes have been linked with CDI susceptibility to varying degrees and can persist longer term; with microbiota populations taking up to a year to recover post-ciprofloxacin or clindamycin treatment.[23]

There is considerable inter-individual variability of human microbiota profiles and discrepancies between different clinical studies are evident.[14, 24] Defining microbiota changes associated with CDI susceptibility is difficult, due to the range of antibiotic exposures and patient co-morbidities. In general, CDI patients are reported to have decreased Bacteroides, Prevotella, Lachnospiraceae and Bifidobacteria spp, and increased Lactobacilli, Ruminococci, Enterococci and Enterobacteriaceae populations [24-26]

Studies suggest that decreased bacterial diversity is a common trait of all diarrhoeal samples, not only those of CDI patients.[24, 25, 27] However, loss of bacterial diversity has been correlated with rCDI clinically.[27, 28] Chang *et al.* demonstrated decreased species 'richness in faecal microbiomes of rCDI patients versus healthy controls and patients with a single CDI episode.[28]

Antimicrobials and CDI recurrence

While *C. difficile* was first identified as a pathogen in clindamycin-associated colitis,[29,30] most other antibiotics have been linked to CDI at some point, though the highest risk is associated with clindamycin, cephalosporins, penicillins and fluoroquinolones. [31,32]

The major paradox of CDI treatment is that while antibiotic therapy is a major risk factor for CDI, it is also the first-line therapeutic option.[33] Thus, while CDI treatment may successfully inhibit vegetative *C. difficile* populations, further disruption of the microbiota subsequent also occurs, increasing the risk of CDI and contributing to the rCDI cycle (Figure 1). Current guidelines recommend different strategies for the treatment of initial CDI *versus* rCDI and can be found in more detail in Debast *et al.*[7] However, a discussion of this topic is beyond the scope of this article.

Oral metronidazole and vancomycin were the primary CDI treatment options until recently. Both agents have been linked to further gut microbiota disruption. Vancomycin extended the disruption

primarily caused by clindamycin in both hamster s[33] (Bacteroidales , Clostridiales) and *in vitro* gut models (*Bacteroides fragilis* group spp, bifidobacteria, clostridia). Gut concentrations of metronidazole are low to undetectable (<0.25-9.5 mg/L), [34] and this was reflected in minor microbiota disruption and poor efficacy against simulated CDI an *in vitro* gut model.[35] The high recurrence rates associated with both these agents has led to development of narrower spectrum antibiotics, with potent anti-*C. difficile* activity, but largely sparing of the gut microbiota.

Fidaxomicin was introduced to the European market in 2012, and shows greater activity against clinical *C. difficile* isolates than vancomycin or metronidazole.[36] Fidaxomicin has a narrower spectrum of activity than vancomycin or metronidazole and is more sparing of the gut microbiota during treatment [37, 38],39 and *in vitro*. [18, 35] A meta-analysis of two large concurrent double-blind randomised non-inferiority trials [39] showed that fidaxomicin was non-inferior to vancomycin for initial resolution of symptoms. [40] Statistically fewer patients experienced a rCDI episode following fidaxomicin *vs* vancomycin. [41] Whole-genome sequencing (WGS) demonstrated a 2.5-fold lower cumulative risk of relapse (with the infecting *C. difficile* strain) fidaxomicin, and a 3-fold lower cumulative risk of reinfection (with a different strain) up to 28 days post-therapy . [41] After a first recurrence, fidaxomicin is associated with a lower risk of subsequent recurrence, [39, 43] however there are currently no data regarding vancomycin *vs* fidaxomicin use in patients with multiple recurrences.

Other novel non-absorbed, narrow spectrum antimicrobials are also in development for CDI treatment. Surotomycin (cyclic lipopeptide) shows potent antibacterial activity against *C. difficile* and other Gram positive bacteria, but limited effects on Gram negative organisms in phase I clinical trials and an *in vitro* gut model,.[44 45] However, this did not correlate with improved outcomes in phase III studies and the primary clinical endpoint of non-inferiority to vancomycin was not met.[46-48]

Cadazolid, (oxazolidinone antibiotic incorporating a fluoroquinolone side-chain) with potent anti- *C. difficile* activity,[16, 49] demonstrated similar time to resolution of diarrhoea but lower recurrence rate than with vancomycin (18.2 to 25.0% versus 50%) in a phase II study of 84 patients.[50] *In vitro* gut model studies suggest it is sparing of the microbiota (excepting bifidobacteria),[16,51], but clinical data are lacking.

Ridinilazole shows good anti-*C. difficile* activity,[52] and efficacy in hamster and *in vitro* gut models. [52,] and was sparing of healthy volunteers' gut microbiota in Phase I studies.[54] Phase II clinical data demonstrated ridinilazole superiority over vancomycin with sustained clinical response in 24 of 36 patients (67%) versus 14 (42%) of 33 respectively. [55] This was attributed to a lower rate of rCDI with ridinilazole (14%) compared with the vancomycin-treated group (35%).

Microbiota therapeutics

Tthere has been an increasing trend towards the use of microbiota therapeutics to restore the host microflora. Initially, this focussed on faecal microbiota transplantation (FMT), although recently, targeted microbiota therapies have emerged.

Faecal Microbiota Transplantation (FMT)

FMT involves the transfer of faecal material from donor to recipient with the aim of restoring a healthy gut microflora and re-establishing colonisation resistance to *C. difficile*. Donors are screened for enteric bacterial pathogens, viruses and parasites.[56] Donor faeces are diluted in water, saline, (or milk / yoghurt), coarse-filtered and administered into the recipient's gut via a nasogastric, nasoduodenal or nasojejunal tube, rectal enema or colonoscopically. A randomised, open-label trial. compared FMT, vancomycin and bowel lavage to vancomycin and bowel lavage; and vancomycin alone. [57] An overall cure rate of 94% was reported, with a primary cure rate of 81% (13/16 subjects) for FMT *vs* 23% (3/13) and 31% (4/13) cure rates for vancomycin and bowel lavage and vancomycin alone respectively (10 week follow-up). A systematic review of 25 studies reported similar overall success rates, with complete symptomatic resolution in 91% of patients (mean follow-up of 12.6 months), including 289 with refractory CDI treated by FMT.[58] Cure rates were unaffected by the route of administration[59] or use of fresh or frozen faeces.[60]

Studies indicate a diverse, balanced flora is important in restoration of colonisation resistance: 16S rRNA gene amplicon pyrosequencing, showed reduced bacterial diversity and compositional changes in microbiota samples from pre-FMT rCDI patients *vs* post FMT rCDI patients and healthy volunteers for up to a year following successful FMT.[61] No bacterial groups were invariably associated with either rCDI or successful FMT outcome, however, microbiota composition continued to change for at least 16 weeks post-FMT, indicating microbiota recovery may take considerably longer than symptomatic resolution. Similarly, Jalanka *et al*. performed microbiota profiling by phylogenetic microarray analysis on samples from 3 universal donors and 14 rCDI recipients pre- and post-FMT over 1 year, commenting on the similarity between post-FMT recipient flora, and universal donor's floras, which persisted for the duration of the study.[62]

Despite impressive success rates, concerns exist about the use of FMT. Most adverse effects are mild to moderate (eg, diarrhoea, flatulence, boating, abdominal discomfort) but a small number of serious adverse events have been reported (bacteraemia, perforations and death.[63] The long-term effects of FMT are unknown, particularly the theoretical risk of transmitting other biological agents

to the recipient, despite rigorous screening procedures. National guidelines (*e.g.* UK NICE.[56]) reflect this, while acknowledging the role of FMT for patients with rCDI that has failed to respond to other treatments

Biological agents

The undefined nature and possible long-term effects of FMT mean that the use of a defined microbiological agent or mixture for the treatment of CDI is an attractive approach.

Animal model studies have demonstrated that *Bifidobacterium bifidum,* [64] Lachnospiracea [19] and non-toxigenic *C. difficile* (NTCD) can all mitigate the pathogenic effects of toxigenic *C. difficile*. A bacterial 'cocktail' made up of six species (Staphylococcus, Enterococcus, Lactobacillus, Anaerostipes, Bacteroidetes and Enterorhabdus) also resolved rCDI and restored colonisation resistance in mice.[65]

The use of NTCD spores was evaluated in a Phase II, randomised, double-blind, placebo-controlled trial of 168 patients. CDI recurrence was 11% *vs* 30% in the NTCD *vs* placebo groups respectively, with successful NTCD colonisation associated with lower recurrence rates (2% *vs 31%* for placebo).[66] However, despite relatively few adverse events being reported, the possibility of PaLoc (pathogenicity locus, containing genes for *C. difficile* toxin production) transfer is a major concern and has been demonstrated in the laboratory [67] and further work is clearly.

Petroff *et al*. formulated a stool substitute using 33 representative bacterial species from healthy donor faeces. These were administered to 2 patients who had failed to respond to conventional antimicrobial treatments for CDI and in both cases, symptoms resolved.[68] A Phase Ib trial of SER-109 (a spore mixture from healthy, screened donors) prevented CDI recurrence in 86.7% of patients (26/30), noting increased gut microbiota diversity.[69] Interim Phase II results, however, showed that SER-109 failed to achieve the primary efficacy endpoint of reduced CDI occurrence after 8 weeks. [70]

Microbiota therapeutics is a promising area of CDI treatment, however, it is clear that the gut microflora is a highly complex entity, with myriad compositions, interactions and factors involved in colonisation resistance. Studies so far indicate that treatments promoting increased gut flora bacterial diversity rather than the use of a single species may be more successful.

Spore viability and CDI recurrence

Microbiota disruption will not lead to CDI/ rCDI unless viable *C. difficile* spores are present (Figure 1). Therefore, factors affecting the presence and viability of spores in the gut are important considerations in recurrent disease.

Reinfection vs Relapse

CDI can recur within two contexts; recrudescence of *C. difficile* spores persisting in the gut (relapse), or reinfection with spores from the environment. Relapse is likely to be affected by the amount or viability of *C. difficile* spores in the gut lumen; while reinfection is likely to be affected by *C. difficile* spore viability or environmental contamination. Furthermore identification of reinfection within the nosocomial environment has infection control implications.

Distinguishing between relapse and reinfection is challenging, particularly as PCR ribotyping may lack the power to discriminate between genotypically similar isolates. The picture is further complicated by patients harbouring multiple *C. difficile* genotypes.[71] Some studies using more discriminatory techniques suggest reinfection accounted for ~50% of recurrent infections, [72, ,73, 74, 77]

Varying rates for recurrence due to relapse have been reported in the literature, ranging from ~52-88% of rCDI episodes. [72, 73] Risk of relapse is greatest during the first 14 days post-treatment; [74] while greater time periods between initial and recurrent episodes tend to be associated with reinfection. [75, 76]

Effect of *C. difficile* strain type

C. difficile strains exhibit variable growth dynamics, sporulation and germination rates, [78-80] factors that may affect rCDI. Several studies have shown that certain strains, particularly PCR ribotype (RT) 027/ NAP1/BI (hereafter referred to as ribotype 027) carry a higher risk of recurrent disease.[71, 80-82] Marsh *et al* reported initial infection with RT027 as a significant risk factor for relapse (P = 0.008),[75] indicating an association of this ribotype with both recurrence and relapse due to spore recrudescence. This could be due to increased sporulation in this ribotype, [83] increasing the load of residual spores in the gut lumen post-treatment and increased 'shedding' of spores to the environment.

Other PCR ribotypes have also been linked with increased CDI rates, such as RTs106 [84], RT176 [85] and RT001.[86] However, it is also imperative to consider this against the underlying population demographic as regional differences in prescribing and initial infection characteristics may influence rCDI.

Persistence of *C. difficile* spores in the host gut

In recrudescent disease, spores must remain in the host gut and proliferate in response to agreeable conditions. *C. difficile* vegetative cells can adhere to Caco-2, HeLa and HT-29 cells and extracellular proteins *in vitro*,[87, 88] and two potential proteins responsible for this interaction have been identified.[89] However, interaction with human colonic epithelia does not trigger germination.[89] *C. difficile* spores were present in complex, mixed species biofilms within an *in vitro* gut model,[90] suggesting that intestinal biofilms may act as a reservoir. Recent work demonstrated the persistence of two different morphotypes of *C. difficile* spores produced from one culture[91]. It is possible that biofilm-associated and planktonic spores may have different properties, potentially altering their respective ability to attach to host cells. Although these experiments are *in vitro*, they suggest a potential role for biofilm-associated spores in recurrent disease.

Factors affecting spore viability

C. difficile spore viability and germination in the gastrointestinal environment is pivotal in transmission and recurrence (Figure 1). Germination begins when a germinant molecule interacts with the germinant receptor (GR). *C. difficile* spores do not share homologs of the GerA, GerB and GerK germinant receptors commonly recognised in *Bacillus* spp and other Clostridia,[92] and are therefore receptive to a different spectrum of germinants. Germination is completed by release of a vegetative cell from the ruptured spore coat/exosporium.

One receptor involved is CspC, a bile acid binding protein. Bile salts are the main germination factor identified for *C. difficile*, although the picture is complicated. Germination rates vary for different bile salts; primary bile acids taurocholate and glycocholate increase germination,[93] while the primary bile salt chenodeoxycholate inhibits germination.[94] Furthermore, the secondary bile acid deoxycholate is reported to stimulate germination, but inhibit vegetative cell growth. Stool extracts from antibiotic-treated mice have higher concentrations of primary bile acids, whereas stools from untreated mice have higher secondary bile acid concentrations.[95] Bile acid metabolism has been implicated as a factor in colonisation resistance. [96,97] However, while Buffie *et al* associated a specific bile acid 7 alpha-dehydroxylating intestinal bacterium, *Clostridium scindens*, with colonisation resistance, [96,] Allegretti *et al*. suggested that several organisms may be performing this metabolic function.[97] Varying bile acid composition, and primary bile salt metabolism by gut microbiota along the gastrointestinal tract may have a regulatory role in both spore germination and maintenance of colonisation resistance.

Treatment agents and spores

Spore germination can be affected by treatment agents, at least *in vitro*. Fidaxomicin , vancomycin[98, 99] and oritavancin[100] exposure inhibit *C. difficile* spore outgrowth, although early germination events are still evident. Thus, vegetative outgrowth remains supressed only while supra-MIC antibiotic levels are maintained in the colon. Interestingly. detectable fidaxomicin activity persisted in both an *in vitro* gut model, [16,35] and in patient stool samples.[43] Detectable fidaxomicin activity at supra-MIC levels (>4mg/L) persists on *C. difficile* spores following washing, preventing spore recovery.[99] Persistent fidaxomicin activity prevented vegetative outgrowth and toxin production in batch culture and similar observations were also made for ramoplanin[98] and oritavancin.[100] It is likely that fidaxomicin adheres to the exosporium of *C. difficile* (as for ramoplanin[101]), potentially due to electrostatic charges resulting from cross-linkages on the spore surfaces. The presence of the exosporium can increase hydrophobicity of *C. difficile* spores, affecting adherence to cells.[102] Thus, if antibiotic activity persists on spores *in vivo* (yet to be determined), this may result in reduced risk of spore recrudescence *in situ*, potentially affecting the viability of spores shed into the environment, with implications for transmission, and recurrence due to reinfection.

Some antibiotics including fidaxomicin,[103] cadazolid,[49,104] tigecyline[105, 106] and piperacillin/tazobactam[105] have been shown to inhibit spore formation *in vitro* at sub-inhibitory levels. There is conflicting evidence, due to the different strains and methodologies used, regarding the effects of vancomycin and metronidazole on spores.[49,103,105.]

Conclusion

Whilst our understanding of the risk factors for rCDI has increased, it remains a continuing challenge. Recurrent CDI is multifactorial, but two microbiological factors - the intestinal microbiota and *C. difficile* spore germination - are key. The microbiota has become a major focus for breaking the rCDI cycle, with novel narrow spectrum atimicrobials, FMT and next generation precision microbiota therapies showing great treatment potential. However, further research is needed into the long term implications of microbiota manipulation. The effects of treatment agents on spore production and germination; retention within the host and environmental disessmination are comparatively poorly understood, but crucial aspects of recurrent disease.

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Transparency Declaration

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