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Review

Invisible steps for a global endemy: molecular strategies adopted by *Clostridioides difficile*

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***Abstract*:** *Clostridioides difficile* infection (CDI) is on the rise worldwide and is associated with an increase in deaths and socio-health burden. *C. difficile* has become ubiquitous in anthropized environments because of the extreme resistance of its spores. Based on the epidemiological data and knowledge of molecular pathogenesis of *C. difficile*, it is possible to predict its progressive colonization of the human population for the following reasons: first, its global spread is unstoppable; second, the toxins (Tcds) produced by *C. difficile*, TcdA and TcdB, mainly cause cell death by apoptosis, but the surviving cells acquire a senescence state that favours persistence of *C. difficile* in the intestine; third, proinflammatory cytokines, tumour necrosis factor-α and interferon-γ, induced during CDI, enhance the cytotoxicity of Tcds and can increase the survival of senescent cells; fourth, Tcds block mobility and induce apoptosis in immune cells recruited at the infection site; and finally, after remission from primary infection or relapse, *C. difficile* causes functional abnormalities in the enteric glial cell (EGC) network that can result in irritable bowel syndrome, characterized by a latent inflammatory response that contributes to *C. difficile* survival and enhances the cytotoxic activity of low doses of TcdB, thus favouring further relapses. Since a 'global endemy' of *C. difficile* seems inevitable, it is necessary to develop an effective vaccine against Tcds for at-risk individuals, and to perform a prophylaxis/selective therapy with bacteriophages highly specific for *C. difficile.* We must be aware that CDI will become a global health problem in the forthcoming years, and we must be prepared to face this menace.

Keywords: apoptosis, *Clostridioides difficile*, colonization, endemy, molecular strategy, proinflammatory cytokine, senescence, toxins

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Introduction

Over time, *Clostridioides difficile*, an anaerobic, Gram-positive, spore-forming bacterium, has become widespread in industrialized countries and is now ubiquitous in the environment, commodities, food items, and domesticated and farm animals.[1–3] *C. difficile* reportedly colonizes approximately 4–15% of healthy individuals,[4] and approximately 18–90% healthy newborns and infants.[5] *C. difficile* is transmitted person to person *via* the faecal–oral route,[6] and hospitals and community healthcare settings may become an important source of infection after high-grade environmental contamination due to the presence of *C. difficile*-infected patients,[7,8] and high resistance of its spores to strong disinfectants[9,10] and radiation.[11]

Epidemiological data indicate a progressive increase in the incidence and severity of *C. difficile* infection (CDI)[12] with clinical outcomes ranging from asymptomatic carriage or mild self-limiting diarrhoea to fulminant pseudomembranous colitis, toxic megacolon, and even death.[1,6–8,12] These manifestations are mostly associated with antibiotic therapies that, by altering the intestinal

microbiota (and causing dysmicrobism), can attenuate a series of factors that prevent *C. difficile* from germinating to its vegetative form[13,14] and producing two *C. difficile* toxins (Tcds), TcdA and TcdB.[15] These Tcds are mainly responsible for most clinical manifestations of CDI.[16] Other factors,[17] such as advanced age,[17,18] obesity,[19] renal disease,[20] hypoalbuminaemia,[17] and impairment of the immune system[21] also favours CDI.

Since its first microbiological identification, the continuous and progressive diffusion of *C. difficile* in anthropized environment[2,3,7,8] and its ability to produce more virulent forms,[22] may enable *C. difficile* in few years to colonize a greater part of the human population, thereby leading to unavoidable issues which can summarized into the following points:

1. Spread of *C. difficile* is uncontrollable because of the extreme resistance of its spore to the external environment.[9–11] Although the spore is an indispensable form for survival and spread of several microorganisms, *C. difficile* has a process of spore germination that is both complex and unique compared with that of two other well-studied organisms (e.g. *Bacillus anthracis* and *Clostridium perfringens*)[23] and for its peculiar interaction with the host by means of its Tcds and cellular microbial factors, which will favour its colonization and successively infection and recurren ces.[1,4,6–8,23–27] Mortality gradually increases with relapses and the epidemiological data reported over 500,000 deaths/year and a progressive colonization and induction of disease even in the absence of antibiotic therapies.[1,4,6–8]
2. Once the large bowel has been colonized by *C. difficile*, the bacterium waits for appropriate conditions that favour its passage to the vegetative form.[27] These favourable conditions are more prevalent in 'developed countries' due to increasing antibiotic therapies[7] across all ages and changes in microbiota due to heterogeneous external factors.[28] Therefore, a progressive increase of CDI and CDI-related deaths (at present in the range of 5–30% with primary infection)[8] is predictable, and with a further progressive increase of death rates following CDI relapses.[29,30] At present, the total number of CDI-related deaths in the USA and Europe is approximately 500,000 per year,[31,32] and this number may be more than 1 million worldwide.[33]
3. Development of targeted antibiotics toward *C. difficile*, even if effective, increases dysmicrobism and inflammation, which in turn favours relapse.[34] In fact, persistent dysmicrobism facilitates the overgrowth of various intestinal pathogens, including *C. difficile*, but there are some peculiarities of *C. difficile* facilitating its growth in such an altered environment with a low-grade inflammation.[7,24–26,28,34–38] The first episode of CDI is due to changes in gut microbial flora that favours overgrowth of *C. difficile*, also compared to other various intestinal pathogens (e.g. *C. perfringens*), and in turn the dysmicrobism depends primarily on the type of antibiotic therapy used but also on several other factors such as age, proton-pump inhibitors, types of foods, medication use, physical environment, the genetic and immune system of individuals and could be also linked to conditions such as obesity, autoimmune and allergic diseases, diabetes, and inflammatory bowel disease (IBD).[1,13–16,34–38] All these predisposing factors widen the range of individuals susceptible to colonization/infection by *C. difficile*.[1,13–16,34–38] However, the role of gut flora in regulating *C. difficile* is more complex than previously supposed and changes both in terms of biomass (how many microorganism are present) and composition (which taxonomic groups are present) rather than the simple reduction of some taxonomic groups are likely more important in preventing *C. difficile* colonization, disease, and recurrence, by preserving sufficient density of the correct type(s) of species to create an environment unfavourable to *C. difficile* expansion.[1,13–16,34–38] Then, after initial alteration of the intestinal microbial population that leads to *C. difficile* colonization, *C. difficile* can also lead to a perturbation in the gut microflora that amplify dysmicrobism and inflammation, favouring CDI and CDI relapse.[1,13–16,34–38] Moreover, the first episode of *C. difficile* by altering the native gut microflora could predispose individuals to recurrent CDIs, and prolonged antimicrobial therapy for *C. difficile*, in perturbed gut microbiome, can give rise to further and persistent dysbiosis and inflammation.

Additionally, although after the first CDI episode the bacterial community is restored with time, some bacterial taxa do not recover completely and gut microbiome keep a reduced resistance to colonization, which encourages the growth of pathogenic microbes such as *C. difficile*, changing the structure of the gut microbial flora in the individual. Periodic use of antibiotics and antibiotics toward *C. difficile* induces an increase in the reservoir of antibiotic-resistant genes in the gut microbiome, which are conditions that favour recurrent CDIs.[1,13,14,34–40] Regarding the molecular mechanisms responsible for the colonization of *C. difficile* and then CDI/CDI relapse, it has been reported that the following can play a key role: an increase of primary bile acids and a decrease of secondary bile acids, an increase of succinate that *C. difficile* metabolizes into butyrate, an increase of disaccharide trehalose, and increased production of particular substances from taxonomic groups present in perturbed microbiome that could favour *C. difficile* overgrowth against other pathogens.[23,24,38,41–45]

Further, the cytotoxic synergism (demonstrated by Fettucciari *et al.*[46]) between the low doses of TcdB of C. difficile and the proinflammatory cytokines, tumour necrosis factor (TNF)-α and interferon (IFN)-γ,[46–48] rapidly modify the cellular microenvironment of the colon with a toxic action.

Another important factor is the difficulty of eradicating C. difficile with antibiotic therapy, which also contributes to favour persistent low-grade inflammation.[1,7,8,12,34]

4. The emergence of epidemics due to novel strains of *C. difficile* that are or epidemics or hypervirulent or multidrug-resistant strains (e.g. ribotypes 015, 027, 078 or 176), many of them also produce *C. difficile* transferase toxin (CTD), and the emergence of *C. difficile* strains producing Tcd variants is worrisome and has elevated the threat of *C. difficile* in the current general antimicrobial crisis outlined by the World Health Organization,[22,37,45,49] because these may represent an additional tool for more selective host manipulation. In fact, the incidence of CDIs between hospital-acquired CDI (HA-CDI) in adults but also for community-acquired CDI (CA-CDI) effectively increased due to the emergence of the hypervirulent ribotypes 027 and 078 strain of *C. difficile*,[22,45,49–53] which showed high production/germination rate, expression of different Tcd variants and increased toxin production, leading to increased disease severity, recurrence, and a 15% increase in CDI-related mortality.[22,24,36,39,45,49–53] Further, the hypervirulent ribotype 078 was significantly higher in patients over 65 years of age, and it is recurrently detected in swine, cattle and retail meat.[24,36,39,45,52–55] Its increased detection in humans and ability to cause severe disease and mortality, suggest the possibility of animal contamination or transmission.[45,52,54,55] Epidemiological data have revealed that CDI outbreaks around the world has been associated with ribotype 027.[22,24,36,39,45,49–53] Overall, the prevalence of different ribotypes, in particular ribotypes 027 and 078, in different geographical areas demonstrates the genetic diversity of *C. difficile* and its recognition as a progressive threat to public health.

Moreover, the *C. difficile* strains that produces the Tcds variant may contribute to the expansion of CDIs because many of them are hypervirulent and produce the binary toxin CTD, which is associated with increased morbidity and mortality.[45,49–51,56–60] It has been shown that CTD promotes the formation of long cellular filaments, which become anchor points for new *C. difficile* to epithelial cells and thus potentiate the infection.[45,49–51,56–61] Further, the Tcd variants are highly diverse in terms of enzymatic activity, immunogenicity and in their receptor preference, with relevant implications on the colonic pathology.[45,49–51,56–60,62]

5. The surface antigens of *C. difficile*, (i.e. SlpA, cell wall proteins, pili, flagella, fimbria, and biofilms), which have the properties of colonizing factors or are involved in innate immunity,[36,56–60] can have an important impact on persistence of *C. difficile*, for the following reasons: first, they promote adhesion to mucosal epithelial cells and the penetration of *C. difficile* within the mucus layers;[36,56–60] second, they antagonize some aspects of the natural immune respo nse;[36,56–60] and third, they contribute to an inflammatory state with cytokine induction

such as TNF-α and IFN-γ that enhance the cytotoxic action of low doses of TcdB.[46–48]
6. Tcd-neutralising antibodies have had limited success and clinical application.[63–67]
7. Immunization toward Tcds has yielded scarce results because of low immunogenicity and inability of immune responses to eliminate *C. difficile*.[68,69]

Considering the above-mentioned aspects, it is crucial to understand the molecular mechanisms of the two Tcds, particularly TcdB, wherein TcdB is approximately 1,000 times more powerful than TcdA, and whether certain molecular aspects of Tcd action may favour the progressive diffusion of *C. difficile* to cause a "global endemy" with severe health and economic consequences.

Molecular characteristics of TcdA and TcdB

Tcds are single-chain proteins, and TcdA and TcdB are 308 kDa and 270 kDa, respectively. They share a 48% sequence identity and 66% sequence similarity, and their most diverse sequence is confined to the C-terminal binding domain.[57–60] TcdA and TcdB have four domains: a glucosyltransferase *N*-terminal domain, an autoprotease domain, a pore-forming and translocation domain, and C-terminal binding repetitive oligopeptides (CROPs) domain,[57–60] where each domain is characterized by specific biological and functional properties. The CROPs domain and other amino acids outside this domain allow the binding of Tcds to the cells for subsequent internalization.[57–60,70] Although TcdA and TcdB CROPs display the solenoid fold, they present distinct spatial and sequential arrangements of their repeat units. This is in agreement with findings that suggest that both TcdA and TcdB bind to different receptors;[57] therefore, TcdA and TcdB do not follow the rule of one toxin, one receptor.[57–60,70]

While two different receptors have been proposed for TcdA, rabbit sucrase isomaltase, and gp96,[57–60] three receptors have been identified for TcdB,[70] chondroitin sulphate proteoglycan 4 (CSPG4), poliovirus receptor-like 3 (PVRL3), and Wnt receptor frizzled family (FZD). The binding of TcdB to CSPG4 receptor induces cell rounding and apoptosis in HeLa and HT29 cells at picomolar concentrations of TcdB, while binding of TcdB to CSPG4 receptor mediate necrotic effects at higher concentration of TcdB.[70–72] The binding of TcdB to PVRL3 receptor induces necrosis cell death at high concentrations (the nanomolar range) of TcdB.[70,72,73] The FZD functions as an alternative receptor to CSPG4; indeed the binding of TcdB to FZD receptor induces cytopathic effects and apoptosis at picomolar concentrations of TcdB.[70,72,74] Another important peculiarity of TcdB is that TcdB can bind to the membrane receptor with amino acid sequences that extend beyond the CROP sequences.[70,72]

This picture underlines the heterogeneity of the receptors linked by TcdB and the diversity of the effects in relation to the receptor binding and concentrations of TcdB.[62,70,72–74] Then, TcdB may utilize multiple receptors with different binding sites to broaden the selection of mammalian cells it can target.[62,70,72–74] Moreover, TcdB variants are highly diverse for their receptor preference, with relevant implications on the colonic pathology.[45,53,56,62,72]

Antibodies against the CROPs domains of both TcdA and TcdB can block internalization,[57–60,70] and excess TcdA CROPs domain can compete with TcdA holotoxin for cell binding.[57–60,70] However, TcdA and TcdB that lack the CROPs domains are still able to enter cells.[57–60,70] Thus, the type of receptor on target cells is a very important consideration for some fundamental aspects of the pathogenic strategy of *C. difficile*. Receptors for *C. difficile* are not well-characterized molecular structures; however, they are likely to be constituted by a configuration of the polysaccharide chain recognized by the TcdA- or TcdB-binding domains that behave as a lectin-like structure[75] and probably possess some characteristics of intrinsically disordered proteins, and are able to modulate their conformation to adapt and more effectively bind with the target structure. The complex structural characteristics of the Tcd receptor allows the Tcds to bind to several and extremely different cell types,[57–60] such as the surface epithelium of the human colon,[76] hepatic cells,[77] nerve cells,[78] EGCs,[46] cardiac cells,[79] and colonic cells.[80]

However, it is noteworthy to consider why Tcds cause damage not only to cell types present at the primary site of infection the large bowel (epithelium of the human colon,[76] colonic cells,[80] EGCs[46]) but also to cell types that are not present in the large bowel (hepatic cells,[77] nerve cells,[78] cardiac cells[79]), as reported above).

It is hypothesized that when *C. difficile* spores convert into their vegetative forms and replicate, they are more sensitive to the activity of innate immune cells,[81] such as macrophages, polymorphonucleates,[51,82] and lymphocytes,[83] which induce and strengthen the inflammatory response that is characterized by secretion of several proinflammatory cytokines such as interleukin (IL)-1,[84] IL-6,[85] IL-8,[86] IFN-γ)[87] and TNF-α.[88] To fight against these immune cells, the Tcds must be able to bind to receptors with a pattern of carbohydrates that have different configurations and this result is obtained by the three types of receptors recognized by TcdB.[70] This explain why TcdB recognized such a broad range of carbohydrate patterns. Therefore, because of incidental molecular homology, Tcds could be cytotoxic to other cell types that express one or more of the receptors recognized by Tcds such as endothelial, hepatic, nerve, EGCs, and cardiac cells that are unrelated to the infection site.[46,57–60,77–79] This also explains why Tcds can cause toxic systemic effects once they reach the circulation in some patients with CDI.[17,47,89,90]

In addition, the ability of Tcds to bind to colonocytes deepens the tissue damage within and beyond the submucosa and damaging the muscle and enteric nervous system cells creates conditions to expel (for instance, *via* diarrhoea with liquid faeces) the vegetative forms of *C. difficile* that rapidly become *C. difficile* spores and can colonize other hosts to start new infection cycles as soon as appropriate conditions for germination occur. Furthermore, if the Tcd receptor domain mutates, it is possible that the pathogenicity of *C. difficile* may become more systemic.

Intracellular effects of Tcds

After binding to the cell membrane, the Tcds stimulate their internalization by an endocytic vacuole in which pH decrease favours a conformational change of the Tcds itself. This allows the Tcds to insert the catalytic domain outside the membrane for cleavage, and thus activate the glycosylation process of the catalytic site of Rho-GTPase to inhibit its activity.[57–60] This inhibition causes several biological consequences such as cytoskeleton disruption,[46,57–60] cell cycle arrest,[46,57–60] and cell death, which occurs after cycle arrest.[46,57–60] These phenomena are strictly dependent on the dose of Tcds, wherein at high concentration, cell death occurs by necrosis.[59,70–73,91]

It is essential to understand what the molecular strategy underlying cytoskeleton alterations and cell cycle arrest is. All immune cells that reach the replication area of the *C. difficile* vegetative form possess intrinsic motility; therefore, their immobilisation drastically decreases their functional effectiveness.[57–60] Moreover, the intracellular action of Tcds in these immune cells modifies the cytokine secretion pattern, which is shifted toward a greater production of proinflammatory cytokines such as IFN-γ and TNF-α,[47,57–60] and anti-inflammatory cytokines such as IL-10.[92] Cytoskeletal alterations are an early event (appearing in some cells after 30 min) that cause cell rounding in most cell types *in vitro*[93] with cell detachment; whereas, *in vivo* there is retraction of colonocytes and cells of the basal membrane, which allows the more in-depth penetration of *C. difficile* and contributes to the making of a highly inflammatory setting that stimulates expulsion in the external environment by diarrhoea.[94]

Following cell cycle arrest, a part of the infected cell dies by apoptosis,[57–60] a phenomenon that highlights another interesting aspect of the molecular strategy of this bacterium. First, while caspase-dependent Tcd-induced apoptosis has been extensively investigated,[46,57–60,95] there is evidence that TcdA can also induce caspase-independent apoptosis following cathepsin[96] and calpain activation (i.e. calcium-dependent).[96] These three apoptotic pathways are strongly interconnected at some points during their signalling and may display further converging points in the induction of reactive oxygen species and mitochondrial damage. This ability to activate three different pathways of cell death is a very important strategy adopted by *C. difficile*, compared with a stressful stimulus inducing cell death, because a cell may display resistance to a definite apoptosis pathway depending on the cell type. Therefore, Tcds, which can activate different pathways of apoptosis, have a higher likelihood of inducing apoptosis if the target cell possesses an intrinsic resistance to one, or two of the three pathways. In fact, Tcds are capable of inducing death of enterocytes,[57–60] colonocytes,[57–60] neuronal cells,[57–60,78] EGCs,[46,95] and different immune cells such as macrophages,[57–60,97] lymphocytes,[57–60,98] and eosinophils.[57–60,98]

Second, other cells exposed to low doses of Tcds could return to their normal functionality after a short arrest of their cell cycle, as we have previously demonstrated for EGCs.[46,99] Conversely,

EGCs that survive apoptotic concentrations of TcdB become senescent as a survival response to stressful stimulus mediated by TcdB.[46,99] The capability of cells surviving the cytotoxic activity of Tcds to become senescent may be found on cell types that have a long-life span, such as EGCs, intestinal neurons and myocytes, that guarantee bowel motility.[100] The acquisition of a senescence state by these cell types could cause irritable bowel syndrome (IBS) and IBD due to persistent inflammation, transfer of senescence status, and stimulation of pre-neoplastic cells.

Thus, we can hypothesize that, after recovery from an acute CDI, the number of EGCs decrease, their network is impaired, and their functionality is subsequently altered; further, EGCs and other cells with a long-life span, which survive toxicity, may become senescent. Following CDI, the structural and functional abnormalities induced by the Tcds might be long-lasting in a considerable percentage of patients with IBS, and cause low-grade inflammation and persistent dysmicrobism.[101] Residual *C. difficile* bacteria that persist after remission of an acute infection may take advantage of this situation, and induce relapse that can appear even after months, without any apparent trigger.[102] *C. difficile* modifies the large bowel environment to persist for a long time and induces easier relapses; this implies a continuous increase in *C. difficile* carriers in the large bowel environment characterized by an IBS-like status and a latent inflammatory condition.

Another aspect that highlights the sophisticated molecular strategy of *C. difficile* is that among the components of the inflammatory response, IFN-γ and TNF-α are of paramount importance as they potentiate *in vitro* cytotoxicity of TcdB.[46] Therefore, it is possible that IFN-γ and TNF-α act as drivers of infection by amplification, from the very beginning of infection, of apoptotic activity of low doses of Tcds, and pave the way for infection progression.[46–48]

Thus, it is likely that antibiotic therapy, in addition to causing dysmicrobism, creates an inflamed environment within the large bowel due to release of bacterial components from the cells killed by bacteriolytic antibiotics. Moreover, an inflammatory environment in the absence of antibiotic therapy could favour CDI in some patient subtypes such as those with obesity, or various pathologies accompanied by an inflammatory state.[103]

Conclusions

The key elements of the molecular strategy adopted by *C. difficile* can be summarized as follows: (1) progressive global diffusion due to strong resistance of *C. difficile* spores to the external environment that associated with the peculiar characteristics of the complex interaction of *C. difficile* with the host will favour its colonization. Further, it has the ability to spread after CDI and its relapses, and its episodes are significantly increasing on an annual basis due to the emergence of *C. difficile* strains of the hypervirulent ribotypes, producing Tcd variants, and/or producing binary toxin CTD, all of which are associated with increased colonization, morbidity and mortality rate; (2) progressive colonization of human hosts favoured by endogenous conditions (dysmicrobism and inflammation). In fact, dysmicrobism favour *C. difficile* overgrowth, creating an environment conducive to *C. difficile* expansion, while inflammation enhancing cytotoxic activity of low doses of Tcds could damage the cellular microenvironment of the colon increasing *C. difficile* colonization of human hosts; (3) production of two Tcds that are not only capable of causing *C. difficile* spread in external environments during infection, but can also modify the large bowel environment to ensure its persistence in a more inflamed milieu, which can harbour the bacterium more easily, with brief periods of replication and with mild symptoms, and without causing a full-blown disease. This strategy ensures that *C. difficile* as an opportunistic pathogen can become a progressive colonizer of human beings and animals, waiting for suitable growth conditions for its growth and can sometimes cause fatality. Thus, *C. difficile* may become a serious global health issue with enormous economic costs.

We must urgently adopt a strategy to counter *C. difficile*, keeping in mind that at present we lack truly efficacious counter strategies for the following reasons: (1) it is not possible to stop the spread or eradicate *C. difficile* from the external environment because it is not possible to eliminate it from hospitals or nursing homes, which represent some of the most contaminated environments, and which are the most important causes of diffusion;[104] (2) until now there has been no available antibiotic or eradication treatment that would prevent the simultaneous onset of developing favourable conditions for subsequent relapse;[66] (3) although very effective, faecal transplantation

is still a limited therapeutic option with several limitations in its widespread use;[50,105] (4) immunotherapy with monoclonal antibodies directed toward TcdA and TcdB have yielded limited results;[63–67] and (5) vaccination toward TcdA and TcdB has not produced significant results clinically and for eradicating *C. difficile*.[68,69]

In conclusion, assuming that *C. difficile* diffusion and human colonization will be unstoppable, desirable interventions with wider applications could include: (1) development of an effective vaccination strategy against Tcds for high-risk categories; (2) the availability of a selective prophylaxis against *C. difficile* based on highly specific bacteriophages, to be used as a therapeutic tool. Today, more than ever, we are realising that the greatest enemies of humanity are pathogens, and thus combating pathogen-related diseases is increasingly becoming a priority.

Author contributions

Specific author contributions are as follows. Conceptualizing of the work: KF, PM, GB; design of the work: KF, PM, GB, AM, AF, and AS; drafting of the manuscript: KF, PM and GB; and critical review of the manuscript: KF, PM, AM, GB, AF, and AS. All authors approved the final version of the manuscript. All authors participated sufficiently in the work to take public responsibility for appropriate portions of the content.

Conflict of interest statement

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