PERSPECTIVE

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Nonantimicrobial drug targets for *Clostridium difficile* infections

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Clostridium difficile infection (CDI) is a major public health problem worldwide. Treatment has become complicated due to the emergence of strains with increased toxigenicity and sporulation rate, together with rampant antibiotics use that disrupts colonization resistance of the colonic microbiota. As a result, there is a critical need for nonantibiotic treatments. Therapies based on inhibiting the toxins, bacterial structures responsible for colonization, virulence and restoration of the gut microbiota are the most important nonantibiotic targets to combat CDI. This report outlines these targets and how they could become the focus of future therapeutic agents. Inhibiting colonization and virulence factors during CDI will disrupt pathogen persistence and decrease exposure to the inflammatory toxins, allowing the immune system to clear the infection.

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Clostridium difficile infection (CDI) is the most common cause of hospital-acquired and antibiotic-associated diarrhea worldwide. A conservative estimate by the US CDC in 2011 reported that about 500,000 diarrheal patients had CDI and this was linked to approximately 29,000 deaths in the USA [1]. Furthermore, a point-prevalence survey performed by the European Centre for Disease Prevention and Control based on data from physician requests for CDI diagnostic test between 2011 and 2012 reported that about 124,000 patients develop healthcare-associated CDI within the European Union annually [2]. The yearly cost of treatment of CDI in the USA is estimated to be up to USD\$4.8 billion [3]. *C. difficile*, a Gram-positive obligate anaerobic pathogen is responsible for 90–100% of the cases of pseudomembranous colitis, 50–75% of antibiotic-associated colitis and 10–25% of antibiotic-associated diarrhea in the USA [4,5]. Mortality and morbidity resulting from CDI-associated diseases have also increased significantly over the last decade, making *C. difficile* the most important emerging pathogen in the USA [6]. *C. difficile*, which has been designated as an urgent threat by the CDC, proliferates in the colon after the distribution and species diversity of the normal gut microbiota has been altered by antibiotic therapy. Thus, previous antibiotic therapy is the highest risk factor for CDI [7]. The problem of CDI is important in the USA, Europe and in Canada while largely unstudied in the developing world.

In the colon, *C. difficile* undergoes active binary division into oxygen-sensitive vegetative cells, which produce toxins and cause disease. To survive under aerobic conditions during dissemination

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from host to host, *C. difficile* forms oxygen-resistant spores (Figure 1). The vegetative cells must transform into spores in order to survive aerobic conditions and so the spores are generally acknowledged to initiate the infection process by serving as the disseminating form of this obligate anaerobic pathogen. Because only the vegetative cells can cause disease, germination of the spores into vegetative cells inside the GI tract is critical in *C. difficile* pathogenesis.

Sporulation occurs under nutrient-limitation conditions or when the cells can no longer sustain vegetative growth. During sporulation, an asymmetrically formed division septum divides the sporulating cell into two unequal compartments (the mother cell and a forespore). These two compartments share identical copies of the cell's genome. The forespore develops into a mature spore following engulfment by the mother cell compartment [8]. The maturation process involves addition of a peptidoglycan cortex and several layers of proteins that coat around the forespore. The mother cell finally lyses and the spore is released into the environment [9]. The spore is metabolically dormant, but resistant to harsh environmental conditions such as high temperature, pH and

alcohols [10]. The spores germinate and grow as vegetative cells under suitable conditions such as nutrients, presence of germinants, and anaerobic conditions [10]. The cholates families of bile acids, such as taurocholate and the amino acid glycine, act as cogerminants of the spores [11] and improve the germination of *C. difficile* spores [12]. However, little is known about the exact mechanism by which these molecules stimulate germination.

Pathogenic *C. difficile* strains encode a 19.6-kb pathogenicity locus consisting of *tcdR*, *tcdB*, *tcdE*, *tcdA* and *tcdC*. This locus is responsible for toxins A and B production, encoded by *tcdA* and *tcdB*, respectively [10]. These toxins are central in *C. difficile* pathogenesis to the extent that only strains that are able to produce either of the toxins cause disease [13–17]. Some *C. difficile* strains also produce a third toxin called binary toxin that is encoded at a specific *CdtLoc* locus in their genomes. The binary toxin is an ADP-ribosyltransferase and has been suggested to play a role during colonization, but has not been associated with disease [18]. However, the regulator of binary toxin production, *CdtR*, has been reported to also regulate production of toxins A and B in a strain-dependent manner [19].

Figure 1. *Clostridium difficile* has two life forms. It grows vegetatively by binary fission during favorable conditions and forms spores when effort to maintain vegetative growth fails. As an anaerobic pathogen, the spores serve as its disseminating agent from host to host and that germination in the GI tract is critical for infection. Thus, drugs that inhibit sporulation and germination may help thwart the infection process.

Both toxins A and B have similar enzymatic cleavage activities [20–22] and are cytotoxic to cultured cells; however, toxin B is more potent than toxin A [16,23–24]. During infection, the toxins are released into the colon, where they undergo internalization by the host cells through receptor-mediated endocytosis [25,26]. The internalized toxins monoglucosylate low-molecular weight GTPases of the Rho family in the cytosol [21,24]. This monoglucosylation interrupts the normal function of the GTPases leading to various deleterious effects including cell rounding, altered cellular signaling, actin cytoskeleton dysregulation and apoptosis [21,24,27–29]. Intoxication of the cells by the toxins also induces the release of various immunomodulatory mediators from mast cells, epithelial cells and phagocytes resulting in inflammation and accumulation of neutrophils [30]. Early detection of *C. difficile* during infection is critical to prevent further damage by the released toxins.

Current treatment of CDI is with antimicrobial drugs, which in as many as 25% of cases, is ineffective resulting in recurrence of the infection [31]. The intrinsic damaging effect of antimicrobials on indigenous microbiota is a major limiting factor of antimicrobial therapy in CDI [32]. As a result, there is an urgent need for nonantibiotic treatments, either as stand-alone therapies or as augmented therapies. The antibiotics currently used for CDI treatment (metronidazole, fidaxomicin and vancomycin) have good activity against the vegetative cells; however, these antibiotics are neither effective at eliminating the spores nor preventing recurrence. We envision that treatment of CDI will continue to be a challenge until new antibiotics or biologic agents are developed that kill the spores and prevent relapse of the infection.

Patients with circulating antibodies to the toxins or individuals who mount a rapid and effective immunologic response during infection are generally asymptomatic and do not exhibit CDI-associated symptoms [33–35]. These patients often experience less severe CDI and a lower risk of recurrence. However, patients who fail to mount a robust immune response to the toxins develop symptoms of CDI such as diarrhea and colitis. Furthermore, patients who are able to restore their natural gut microbiota or mount an effective immune response to the toxins and/or the bacterium recover from the infection, whereas patients who fail to do so are susceptible to recurrent CDI [35]. Given the

importance of the immune system and the native gut microbiota in clearing CDI, therapies that are based on inhibiting either the toxins or cellular structures on the bacterium responsible for colonization, virulence, and restoration of the gut microbiota are the most important strategies to combat this multidrug-resistant pathogen. This report outlines the various nonantibiotic drug targets against CDI and how these targets could be further developed therapeutically as shown in **Figure 2**.

Antigermination agents

Dormant *C. difficile* spores remain viable in the colon and when released from patients stools on hospital surfaces, continue to be viable for weeks to months; this facilitates the spread of CDI and recurrence [36,37]. As a result, sporulation by *C. difficile* is a major barrier in the prevention of hospital-acquired *C. difficile*-associated diseases and recurrence. The spores are not killed following antibiotic therapy and institutional treatment of surfaces with disinfectants is only partially effective in eliminating viable spores. This promotes *C. difficile* reinfection, colonization, and overgrowth in the intestinal tract [38]. There is a growing interest in utilizing agents that inhibit germination as a therapeutic strategy for *C. difficile* infections. **Figure 1** shows agents that could be harnessed to inhibit sporulation and as antigerminants. Also, Howerton *et al.* have demonstrated that a bile salt analog, CamSA, inhibits *C. difficile* spore germination *in vitro*. They further showed that a single dose of CamSA (50 mg/kg) protected mice from *C. difficile* infection and lower doses of CamSA resulted in delayed onset of the infection and less severe disease symptoms [39]. However, it remains to be seen whether CamSA can be utilized as a viable antigermination agent for CDI treatment.

Targeting the toxins

• Toxin-binding agents

Anion-binding resins can be designed to sequester the toxins from the intestinal lumen by binding to the *C. difficile* toxins A and B. An example of such binding agents are X-aptamers, which are small nucleic acid molecules isolated from repeated rounds of *in vitro* selection based on their binding to specific targets such as proteins, small molecules, cells, and tissues [40,41]. X-aptamers may have wide range of applications in therapeutics because they have similar recognition characteristics comparable to antibodies.

Figure 2. Nonantibiotic targets for *Clostridium difficile* infections.

Unlike antibodies, they are generated *in vitro* with no immunogenicity, extremely stable and a wide range of modifications can easily be employed to improve specificity. X-aptamers designed to bind with high affinity to the N-terminal glucosyltransferase domain and/or the C-terminal receptor-binding domain of the *C. difficile* toxins A and B may be able to rapidly sequester and inactivate the toxins.

Another group of potential toxin-binding agents are bile salts such as taurocholate [42], tolevamer, cholestyramines, and colestipol. Tolevamer was evaluated in conjunction with standard therapy or as a single therapy in a clinical trial to compare its efficacy to various CDI treatments. Louie *et al.* [43] demonstrated that patients who received tolevamer, a toxin-absorbing anion polymer designed to sequester toxins A and B, exhibited reduced severity of CDI. However, tolevamer was found to be inferior to vancomycin and metronidazole therapies [44] and hence, its clinical benefit is unclear. One of the limitations of toxin-binding agents is their potential to interact with antimicrobials when used simultaneously. For instance, cholestyramine and colestipol bind nonspecifically to

vancomycin [43]. Nonetheless, toxin- or spore-binding agents could be useful in preventing recurrent CDI following antibiotic therapy.

• Small-molecule-based inhibition of toxin production & toxin activity

The accessory gene regulator (Agr) quorum signaling system regulates virulence in many Gram-positive bacteria [45]. The quorum signaling regulatory mechanism enables bacteria to cooperatively express energetically expensive processes to increase the effectiveness of such processes on the environment or host. Generally, the Agr component genes are transcribed as a four-gene operon consisting of *agrA*, *B*, *C* and *D* [46]. The *agrD* gene encodes an auto-inducer prepeptide, which post-translationally processed by the AgrB transmembrane protein. This leads to release of the functional autoinducer signaling peptide into the extracellular milieu. The AgrC histidine kinase protein senses and binds the extracellular autoinducer signaling peptide and turns on its ATPase activity to phosphorylate the AgrA response regulator. Phosphorylated AgrA regulates transcription of target genes.

The *C. difficile* toxins are regulated by the Agr1 quorum signaling system [47,48]. Thus, the components of the Agr system (Figure 3) can be targeted to develop novel nonantimicrobial therapies to combat CDI. Each of the Agr system components could potentially be targeted to block toxin production and hence, eliminate the direct cause of *C. difficile*-associated disease. As shown in Figure 3, this can be accomplished by: blocking the synthesis of the toxin-inducing (TI) autoinducer peptide (TI signal) through AgrB1; competitive elimination of the TI signal using analogs or antibodies to sequester it; prevent binding of the sensory histidine kinase (AgrC2)

to the TI signal to jam the signaling pathway; and by preventing the phosphorylation or dimerization of the response regulator (AgrA2). The striking degree of homology and unique functional features shared between the Agr family members and orthologs among the Firmicutes offers the prospect of developing therapies that can inhibit virulence in numerous pathogens. Drugs that inhibit the Agr pathway in one bacterial species such as *C. difficile* may likely be useful in other bacteria that utilize similar Agr system to control virulence, such as methicillin-resistant *Staphylococcus aureus*, *C. perfringens* and *C. botulinum*.

Figure 3. A model for the quorum signaling regulation of *Clostridium difficile* toxin synthesis [47].

The genomes of some *C. difficile* strains encode two *agr* loci, designated *agr1* and *agr2*. The *agr1* locus encodes only the quorum signal-generation genes (*agrB1* and *agrD1*) that are responsible for producing the quorum signaling autoinducer peptide, whereas the *agr2* locus contains both quorum signal-generation and response genes (*agrB2D2* and *agrC2A2*, respectively). Hypervirulent strains, such as R20291, encode both *agr1* and *agr2* loci, but the nonhypervirulent strains, such as 630, encode only *agr1*. In the strain R20291, AgrD1 and AgrB1 synthesize the toxin-inducing (TI) autoinducer peptide (TI signal). Then, a two-component AgrC2 histidine kinase senses the TI signal and activates its autokinase activity leading to phosphorylation and dimerization of the AgrA2 response regulator, which may directly or indirectly induce toxin production. Each of the Agr component could be a promising therapeutic target to block toxin production and hence, eliminate the direct cause of *C. difficile*-associated disease.

We anticipate that blocking the synthesis and activity of the toxins, which are directly responsible for disease, with potent nonantimicrobial inhibitors delivered into the intestinal lumen will have a major impact on disease severity and treatment outcomes. Toxin-induced damage and inflammation likely generate a fertile niche for the vegetative cells and its spores to thrive. Therefore, inhibiting the *C. difficile* toxins without altering the colonic microbiota should maintain a normal intestinal physiology and homeostasis, facilitate immune-mediated clearance of the bacterium, and decrease the risk of recurrence. We expect that antimicrobial resistance should also be greatly reduced due to the lack of selective pressure, which promotes drug resistance.

Bacteriotherapy

C. difficile behaves as a commensal in the presence of the normal gut microbiota. The protection provided by the gut microbiota is altered following antibiotic therapy, which disrupts their composition and diversity and as a result, the colonization resistance. Thus, reconstituting the gut microbiota has gained popularity recently. One of such therapy is the fecal microbiota transplantation (FMT), in which fecal suspension from a healthy donor is delivered into the recipient's colon to help restore the gut microbiota. While used in veterinary world for a century, FMT was first reported in 1958 [49] in patients with postantibiotic colitis well before *C. difficile* had been identified as the important etiologic agent for this syndrome. Lately, FMT has emerged as an accepted and effective treatment for recurrent *C. difficile* infection. The specific constituents of the fecal microbiome that provide resistance against *C. difficile* are not known, but the phyla Bacteroidetes and Firmicutes are thought to be the critical components [50,51]. The fecal microbiota can be introduced into the colon via endoscopy, enema, lyophilized or frozen in the form of capsules. Several clinical trials have reported positive outcomes from treating patients with recurrent or refractory CDI by FMT [52–55]. Kocielek and Gerding [56] recently analyzed three randomized trials of more than 500 case reports of patients who received FMT and their results showed a cure rate of nearly 90% in patients with recurrent CDI. However, there are potential risks associated with FMT despite its increasing popularity. The procedure is not readily available and improperly screened donor stools could expose recipient patients to dangerous pathogens such

as HIV, hepatitis, among others [53,57]. Efforts are underway to develop suitable mixtures of culturable colonic bacteria as alternatives to donor stools. Nonetheless, there are no known reports of serious infectious complications resulting from an inappropriately screened donor stool used in FMT. Furthermore, this procedure while effective for multiple recurrent CDI has not yet been applied therapy for primary CDI treatment, where the greatest need of effective treatment is seen. Information about the use of FMT in primary CDI treatment is scanty, but recent reported case studies appear promising [58–60]. Further research and clinical trials are needed to fully evaluate the potential of FMT in primary CDI treatment.

Another method to reconstitute the gut microbiota is the use of probiotics. The live microorganisms in probiotics are thought to have immunoprotective properties, hamper adherence of *C. difficile* in the intestinal lumen and modulate the host's immune response. Probiotics promote increased intestinal secretion of IgA antitoxin production and inhibit the production of IL-8, a proinflammatory cytokine [61]. *Lactobacillus* and *Bifidobacterium* strains are the most frequently utilized probiotics, in addition to the yeast *Saccharomyces boulardii* as adjuvant treatment in CDI or as primary prevention therapy for patients receiving vancomycin [62,63]. *S. boulardii* has been shown to be effective in preventing recurrence when given after antibiotic therapy, but it has high risk in inducing fungemia in immunocompromised patients [62,64]. Now with the human intestinal microbiome initiative, it may be possible to identify multiple strains of culturable anaerobic probiotic strains that can reverse dysbiosis in CDI. Intense research is ongoing in this area.

The use of nontoxigenic *C. difficile* strains to outcompete the pathogenic toxigenic strains has also been proposed. Phase II clinical trial of a nontoxigenic *C. difficile* strain M3 (NTCD M3) developed by Viropharma, Inc. demonstrated efficacy in reducing CDI recurrence, with possible restoration of the intestinal flora to its normal state [65]. It was reported that 22 weeks after administration, NTCD M3 strains cannot be detected in stools. This observation suggests that colonization of the NTCD M3 strains may be transient and presumably, occurs as a result of restoration of the normal microbiota, which may then provide protection against subsequent CDI [65].

Immunotherapy

• Monoclonal antibodies against the toxins

The use of antibodies as therapeutic agents against bacterial infections is a rational choice because the immune system has naturally evolved to combat bacterial infections. More than 45 therapeutic monoclonal antibodies have been approved by the US FDA for the treatment of different diseases, including cancer, autoimmune disorders, cholesterol reduction and infectious diseases. Majority of the monoclonal antibodies are isolated using the hybridoma technology [66] in mice, but these antibodies usually have to be humanized for safe human use. Transgenic animals that produce humanized antibody repertoires have recently been developed, enabling isolation of human monoclonal antibodies [67]. These antibodies are usually specific with high target affinity and longer serum half-lives of up to 21 days at 37°C, making them ideal for use in human [68].

Due to their important role in the disease process, toxins A and B have been the most important targets for therapeutic antibodies against CDI. As a result of their inherent specificity, these antitoxin monoclonal antibodies are less likely to generate broad resistance in bacteria or alter the protective colonic microbiota. This may potentially help reduce the recurrence rates of CDI. The FDA recently approved bezlotoxumab as the first prophylactic antibody treatment against recurrent CDI. This fully humanized monoclonal antibody targets the *C. difficile* toxin B [69]. Related actoxumab antibody targets the C-terminal receptor-binding domain of toxin A and it is reported to bind twice on the toxin A protein and ultimately, neutralizes it [69,70]. On the other hand, bezlotoxumab recognizes the C-terminal receptor-binding domain of toxin B and seemingly overlaps with the presumed toxin B carbohydrate-binding region [70,71]. Actoxumab, when combined with bezlotoxumab, was reported to be protective in multiple mouse and hamster models [69,72]. Also, prophylactic administration of bezlotoxumab alone or combined with actoxumab in a piglet model of CDI provided 100% protection [73]. Piglets given actoxumab alone demonstrated no significant efficacy compared with placebo with a mortality rate of 67%, suggesting that much of the protection was driven by the antitoxin B monoclonal antibody [73].

In human, combining both actoxumab and bezlotoxumab significantly reduced the rate of CDI relapse compared with standard-of-care antibiotic therapy [74]. Treatment of CDI with

bezlotoxumab in conjunction with standard-of-care antibiotic therapy reduced CDI recurrence compared with placebo during the Phase III trial [75]. Treatment with both actoxumab and bezlotoxumab provided no added efficacy and actoxumab alone did not prevent *C. difficile* recurrence [76]. Following initial CDI episode, actoxumab and bezlotoxumab do not reduce diarrheal severity, time to resolution of diarrhea or the duration of hospitalization [74]. However, these Phase II and III clinical studies were not designed to evaluate these parameters, leaving the door open for developing next-generation antibody-based therapeutics with improved efficacy. Such therapies should be designed to reduce treatment cost, must have high efficacy, eliminate CDI recurrence and reduce the severity of diarrhea, time to resolution of diarrhea and duration of hospitalization. Moreover, a number of toxins A and B antibody combinations are in the early stages of development and have been reported to be more efficacious in reducing CDI recurrence and severity of diarrhea relative to actoxumab/bezlotoxumab during preclinical studies [70]. Recently, an antibody delivery technique was demonstrated in rats in which 72.6% of biologically active anti-A/B toxin IgY-loaded chitosan-Ca pectinate oral microbeads was successfully released specifically in the colon [77]. Such novel delivery techniques will be useful in delivering anti-*C. difficile* agents directly into the colon.

• Immunization

Immunization against *C. difficile* toxins, cell wall structures or outer membrane proteins offers the prospect of a relatively low-cost approach to CDI prevention. Phase III clinical trials are underway testing multidose inactivated *C. difficile* toxin-based (toxoids) vaccines. Sanofi Pasteur's ACAM-CDIFF vaccine contains formalin-inactivated toxoid and preliminary results from the Phase I trial demonstrated that it was safe and successful in eliciting adequate neutralizing antibody response [56,62,78]. After a successful Phase II study with their toxoid vaccine, Pfizer is currently initiating a large-scale multicenter Phase III trial. Currently, in Phase II clinical trial, Valneva has also developed a vaccine using a recombinant fusion-protein containing cell-binding domains from truncated forms of toxins A and B. It is not clear whether vaccination will be effective for primary or secondary prevention and whether it will prevent or reduce disease

severity. Clinical utilization will also depend on efficacy, cost and safety. Vaccination is unlikely to eliminate colonization and so patient isolation will be important in preventing CDI transmission.

• Antibodies to other *C. difficile* targets

Even though the *C. difficile* toxins A and B are the primary targets of most of the immunotherapies in development, several other virulence and colonization factors such as the flagella, surface-layer proteins, Cpw84 proteins and pilin (Figure 2) are promising avenues for therapeutic intervention. Success in the development of drugs that target these virulence and colonization factors may guide the next generation of therapies for CDI. Currently, there are no therapies for CDI that are based on direct inhibition of toxin production, toxin activity or colonization factors. Our laboratory and others [79] are actively investigating these novel approaches for CDI treatment. Since the use of antitoxin antibodies does not prevent the initial *C. difficile* colonization step [74], complementing them with, for instance, antibodies that target cell wall proteins or adherent factors to limit their dissemination appears attractive. Previous studies have shown that mice immunized with anti-flagellin (FliC) and flagellin filament cap protein (FliD) antibodies exhibited significant decrease in *C. difficile* colonization [80]. Also, hamsters orally administered with purified FliD-specific antibodies were protected from CDI when challenged with *C. difficile* strain 630 [81]. Furthermore, mice rectally vaccinated with a flagella preparation, FliD, Cpw84 and cell wall extracts showed significant decrease in *C. difficile* colonization [80]. These studies support the idea that targeting colonization factors may be promising as therapeutic agent against CDI.

While antimicrobial drugs are excluded from this review, it may be possible to use poorly absorbed antimicrobial drugs in concentrations below that required for inhibition of bacterial growth making them gut cytoprotective biologic agents rather than antibiotics. The most encouraging drug for such use is rifaximin [82] with activity against bacterial virulence while preserving colonic microflora because of low water solubility [83].

Conclusion & future perspective

CDI is one of the most significant causes of hospital-acquired infections and antibiotic-associated

diarrhea in the world with increased mortality in elderly patients and the immunocompromised. The emergence of hypervirulent strains and rampant antibiotic use, which predispose people to increased CDI risk has necessitated the development of nonantibiotic therapies. Some of the major promising targets for nonantibiotic therapy include inhibition of toxin production, toxin activity, colonization factors and the use of antispore agents. Using drugs that inhibit these factors that are critical in *C. difficile* pathogenesis would thwart colonization and reduce exposure of the intestinal mucosa to the inflammatory toxins. This may facilitate the host immune response and clear the infection. This approach is unlikely to alter the colonic microbiota, as it does not impose enormous selective pressure on the pathogen.

Currently, there are no therapies for primary CDI that are based on direct inhibition of toxin production, toxin activity or colonization factors. Majority of the efforts in nonantibiotic therapies are antibody-based. A major concern about antibody therapy is their exorbitant costs and acceptance. It is also possible for some recipients of these antibodies to generate secondary antibodies against the primary antibodies received. This may lead to major autoimmune problems with potentially serious consequences. Furthermore, a lot of neutralizing antibodies may be required to be physically present in the colon to thwart the infection during acute CDI. Such antibodies when administered intravenously must also travel rapidly to the site of infection in the colon to neutralize the toxins. Until these challenges and limitations have been overcome, antibody-based treatments of CDI will only be relegated to patients with multiple recurrences and unlikely as primary treatments where the need for effective treatment is greatest.

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EXECUTIVE SUMMARY

- *Clostridium difficile* infection (CDI) has been designated an urgent threat by the US CDC.
- Antibiotic-based treatment of CDI is increasingly becoming ineffective and severely alters the diversity and quantity of the native microbiota. This creates a conducive environment for recurrence of the infection (in as many as 25% of cases). Therefore, there is an urgent need for nonantibiotic treatments, either as stand-alone therapies or as augmented therapies.
- Therapies that are based on restoring the gut microbiota, inhibiting either the toxins or cellular structures on the bacterium responsible for colonization, virulence are the most important strategies to combat this multidrug-resistant pathogen.
- The most promising nonantibiotic targets include:
 - Toxin-binding agents to sequester the toxins.
 - Antibodies to prevent inactivate the toxins.
 - Small molecules to inhibit toxin production and/or toxin activity.
 - Agents to prevent germination and sporulation.
 - Vaccination against the toxins or cell wall structures.
- Use of defined collection of microbes as probiotics to restore dysbiosis associated with CDI and as an alternative to fecal material transplant.
- Antibodies that target important virulence and colonization factors such as the flagella, surface-layer proteins, Cpw84 proteins and pilin.

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