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REVIEW

Clostridium difficile infection in the twenty-first century

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Clostridium difficile **is a spore-forming gram-positive bacillus, and the leading cause of antibiotic-associated nosocomial diarrhea and colitis in the industrialized world. With the emergence of a hypervirulent strain of *C. difficile* (BI/NAP1/027), the epidemiology of *C. difficile* infection has rapidly changed in the last decade. *C. difficile* infection, once thought to be an easy to treat bacterial infection, has evolved into an epidemic that is associated with a high rate of mortality, causing disease in patients thought to be low-risk. In this review, we discuss the changing face of *C .difficile* infection and the novel treatment and prevention strategies needed to halt this ever growing epidemic.**
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INTRODUCTION

With the discovery of penicillin in 1928 in St. Mary's Hospital in London, Alexander Fleming made a contribution to mankind that would change the course of modern medicine.[1] Along with the discovery of antibiotics, vaccination, sanitation and nutrition, life expectancy changed in the developed world from around 50 years in 1945 to around 80 years in the twenty-first century.[2] Formerly fatal, bacterial infections such as syphilis, diarrheal illnesses and lower respiratory infections are routinely cured by taking a course of antibiotics. Unfortunately, due to the widespread use of such drugs and the lack of antibiotic stewardship, adverse consequences to the use of antibiotics have appeared in the form of multidrug resistant bacteria and emerging opportunistic infections.[3]

Several metagenomic studies as well as the National Institutes of Health (NIH)-funded Human Microbiome Project have begun to address the question of biodiversity of the human gut which contains an estimated 10^{14} bacterial cells representing thousands of bacterial species.[4] The lower intestine is the home of several major phyla of bacteria—*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and as well as less prevalent phyla such as *Proteobacteria* and *Fusobacteria*. These bacteria along with protozoans, fungi and bacteriophages play an important role in maintaining the microbiological diversity in the colon.[5] More importantly, autochthonous (resident) and allochthonous (transient) microbes maintain the delicate balance that prevents seemingly innocuous pathogens from gaining a foothold in the gut microbiological niche, leading to disease.[6]

Direct microbe–microbe interactions, competition for the same niche and nutrients, production of bacteriocins are some of the mechanisms that the normal gut microflora uses to maintain colonization resistance.[7] Administration of broad-spectrum antibiotics, leads to the elimination of the healthy microflora of the gut, followed by the loss of colonization resistance that may allow microbes such as *Clostridium difficile*, *Clostridium perfringens* and *Salmonella* spp. to colonize, adhere and replicate to substantial levels to cause disease. Of these, the most notorious and press-worthy culprit is *C. difficile*. *C. difficile* infection (CDI) has been around for more than 30 years, a hospital-acquired disease, treated as a somewhat annoying byproduct of antibiotic use.

Unfortunately, due to a rapidly spreading epidemic, it has gained tremendous notoriety in the last several years. An online search of the last 12 months of publications of *New York Times* yields several high-profile articles about *C. difficile*, from the humorous side of science (such as the beagle who can identify *C. difficile* in human stool) to the novel treatments for this fast-spreading, life-threatening epidemic that shows no sign of abating.[8] According to the Centre for Disease Control, 1 in 20 hospitalized patients will acquire a health care-associated infection and while most health care-associated infections such as methicillin-resistant *Staphylococcus aureus* are decreasing, *C. difficile* rates continue to rise rapidly.[9]

WHAT IS *Clostridium difficile* INFECTION?

C. difficile was first identified in the stool of neonates in 1935 by Hall and O'Toole[10] and was incorrectly thought to be part of the normal gut flora. It has been shown that 60%–70% of newborns and infants are colonized asymptomatically with *C. difficile*.[11] It has been speculated that newborns and infants lack the receptors for the disease-causing toxins secreted by *C. difficile*, and hence, are colonized, but remain disease-free.[12] Due to the difficulty in isolating and culturing of the bacterium, it was initially named *Bacillus difficile*. It was not until 1978 when Tedesco *et al.* and Bartlett *et al.* identified *C. difficile* as the toxin-producing causative agent of pseudomembranous colitis in patients receiving clindamycin.[13–15]

C. difficile is a spore-forming gram-positive anaerobic bacillus, and the leading cause of antibiotic-associated nosocomial diarrhea and colitis in the industrialized world.[16] Colonization of humans by *C. difficile* can produce enteric symptoms termed CDI, ranging from asymptomatic intestinal colonization to diarrhea, colitis, pseudomembranous colitis and death. Broad-spectrum antibiotic usage, hospitalization, advanced age and comorbidities increase the risk of developing CDI.[17] Antibiotic-associated diarrhea occurs in about 10% of patients

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receiving antibiotics, of which CDI accounts for 20%.[18] Antibiotic exposure, especially a course of clindamycin, cephalosporines and penicillins, leads to an increased risk of acquiring *C. difficile*.[19] Recently, fluoroquinolones have also been added to this growing list of offending antibiotics.[20] In some cases, even a single dose of antibiotics has led to CDI. Additionally, the use of proton pump inhibitors and H2 blockers, which decrease the acidity of the stomach, allow *C. difficile* spores to transit through the stomach into the gut where the anaerobic environment and the presence of bile salts, allow the spores to germinate into the toxin-producing vegetative state.[21,22]

Epidemiological studies have shown that up to 3% of healthy adults may be colonized asymptomatically with *C. difficile*. This number increases dramatically in the health-care setting. Elderly patients are especially at risk with as many as 10% of elderly patients (defined as greater than 65 years of age) colonized with *C. difficile* at hospital admission.[23] Studied have shown that within the first week, 13%–20% of inpatients in a hospital acquire *C. difficile* and by 4 weeks, 50% of all in-house patients are colonized.[24,25] Age-related immune senescence, comorbidities, need for stay at long-term care facilities and surgical procedures are primary risk factors for CDI.[26,27] Secondary risk factors include vitamin D deficiencies, Crohn's disease, irritable bowel disorders and the immunosuppressive medications including chemotherapy.[28,29]

C. difficile spores are transmitted via the fecal/oral pathway. These spores are ubiquitously present on inanimate objects, are resistant to commonly-used decontaminants and can persist for long periods of time in the spore form without the loss of viability.[30] In the absence of antibiotics, the normal gut microbiota prevents the overgrowth of ingested *C. difficile* spores. Antibiotic treatments, which can lead to the disruption of the gut microbiota, allow *C. difficile* to germinate sufficiently to establish infection. Germination of *C. difficile* spores to form vegetative cells followed by attachment to intestinal epithelial cells is a critical step in the pathogenesis of *C. difficile*.

The pathogenicity of *C. difficile* is mediated primarily through the release of two toxins—toxin A and toxin B. These large toxins (TcdA, 308 kDa; TcdB, 270 kDa) function as glucosyltransferases that inactivate small GTPases such as Rho, Rac and Cdc42 within eukaryotic target cells, leading to actin polymerization, opening of tight junctions and ultimately cell death.[31] These toxins have a tripartite structure consisting of an enzymatically active N-terminal domain, a middle translocation section and a C-terminal receptor-binding domain (RBD) consisting of repeating units of 21, 30 or 50 amino-acid residues. Although the receptors for these toxins have not been identified, the crystal structure for the RBD of TcdA has been resolved.[32,33] The 1.85-A resolution crystal structure reveals a β-solenoid fold containing 32 small repeats of 15–21 residues and 7 large repeats of 30 residues. The RBD of TcdB is thought to be similar to toxin A, although structural data are unavailable. Following receptor binding and endocytosis, the toxins translocate through the early endosomal compartments into the cytosol.[31] The toxins are processed by autocatalytic cleavage in the endosomal compartments, such that only the N-terminal enzymatic domain is released into the cytosol.[34] In the cytosol, Rho GTPases are glucosylated, which in turn result in the blocking of downstream signal transduction pathways. Previously, it was believed that TcdA initiated intestinal epithelial damage and mucosal disruption that allowed TcdB to gain access to underlying cells.[35] Recent studies using TcdA-negative *C. difficile* mutants demonstrate the importance of TcdB in a CDI animal model.[36] There have also been numerous reports of TcdA-negative/TcdB-positive strains of *C. difficile* isolated from patients with CDI and colitis.[37]

THE CHANGING EPIDEMIOLOGY OF *Clostridium difficile* INFECTION

The last two decades have overseen a dramatic change in the epidemiology of CDI. A disease considered to be an easily treated side effect of antibiotic usage, is now associated with outbreaks with increased mortality and morbidity. The incidence of CDI in hospitalized patients has risen dramatically from 31 cases per 100 000 patients in 1996 to 84 cases per 100 000 in 2005, with more than 300 000 cases reported per year in the USA.[38] Mortality rates have also jumped from 1.5% of cases in 1997 to 6.9% in 2004 and account for 14 000 deaths per year in the United States.[39] At the height of the epidemic in 2008, 93% of all CDI-related deaths were in patients older than 65 years of age, with *C. difficile* reported as the eighteenth leading cause of death in this age group.[40]

The severity of this new epidemic is not unique to North America. Across Europe, severe outbreaks of CDI have been documented in almost all countries, with the first cases reported in England followed by the Netherlands.[41] CDI with higher mortality and morbidity has also been reported in Australia, Asia and Central America.[42]

The new epidemic of CDI has been defined by community-based outbreaks affecting individuals considered to be at low risk and not on antibiotics, refractory to treatment and the emergence of *C. difficile* strain BI/NAP1/027, toxinotype III identified by North American Field Pulse Type Analysis and polymerase chain reaction ribotyping.[43] Although *C. difficile* BI/NAP1/027 was originally identified in 1980s, it was not until the outbreaks in Quebec, Canada in the early 2000 that this strain was identified as the epidemic strain.[44,45] The Quebec outbreak was associated with a mortality rate of 16.7%.[46] At the same time, several hospitals across six states in the USA reported outbreaks by *C. difficile* strain BI/NAP1/027.

C. difficile strain BI/NAP1/027 has certain unique characteristics that might explain the increased severity associated with this strain. The genes for *C. difficile* toxins, *tcdA* and *tcdB* are part of a 19.6-kb pathogenicity locus along with the regulatory genes *tcdR, tcdE* and *tcdC*.[41] Toxin production is regulated positively by *tcdR* and negatively by *tcdC*. *C. difficile* strain BI/NAP1/027 isolated during the North American outbreaks have an 18 bp deletion in *tcdC*, which along with a 1 bp deletion at position 117, may allow increased toxin production, although the exact mechanism is not clearly understood.[47–49]

In addition to the two large toxins, *C. difficile* strain BI/NAP1/027 expresses binary actin-ADP-ribosylating toxin, *C. difficile* toxin (CDT), an iota-like toxin such as *C. botulinum* C2 toxin and *C. perfringens* E toxin.[50] It is a two-component toxin encoded by two genes, *cdtA* (enzymatic domain) and *cdtB* (binding domain). All pathogenic *C. difficile* strain BI/NAP1/027 carry CDT.[51] CDT adds an ADP-ribose moiety to G-actin, thereby causing disruption of the actin cytoskeleton, leading to the formation of microtubule-based cell protrusions allowing enhanced colonization of the gut epithelium by *C. difficile*.[52] TcdA-negative/TcdB-negative *C. difficile* strains that express CDT are able to colonize hamsters, but are unable to cause symptomatic disease. It is possible that the presence of CDT in BI/NAP1/027 strains enhances the effects of TcdA and TcdB in an additive manner. Additionally, patients with CDI caused by strains producing CDT are at a higher risk for recurrent disease.[53]

Sporulation rates of hypervirulent *C. difficile* strains may contribute to the severity of epidemic of CDI. Patients with CDI excrete 1×10^4 and 1×10^7 spores per gram of feces.[54] These highly infectious spores can remain in the dormant state in the hospital environment for up to 6 months and following ingestion can cause disease in a susceptible host. Consequently, sporulation rates of BI/NAP1/027 strains and non-epidemic strains has been studied in great detail by several

groups.[55] Unfortunately, due to the differences in *in vitro* techniques used for the quantification of sporulation, results are inconsistent from study to study. In general, it is now accepted that varied rates of sporulation do exists among different *C. difficile* strains, but such rates do not correlate with certain subtypes that are considered to be hypervirulent.[55,56]

Flouroquinolone resistance of *C. difficile* strain BI/NAP1/027is a key contributing factor to the rapid spread of CDI. *C. difficile* strains carry many mobile genetic elements that are capable of conferring antibacterial resistance genes to susceptible strains.[43] In the early 2000s, the most commonly prescribed drugs in a hospital setting in the United States were flouroquinolones, which are broad-spectrum antibiotics active against a wide range of bacteria. The widespread use of flouroquinolones allowed for the selection and acquisition of flouroquinolones-resistance genes in *C. difficile* strain BI/NAP1/027which in turn likely promoted the world-wide spread of flouroquinolone-resistant *C. difficile* 027/BI/NAP1 strains.[57] The use of fluoroquinolones, in addition to age over 65 years, is now associated with the highest risk factor for CDI due to *C. difficile* strain BI/NAP1/027.[58] Additionally, these strains have higher resistance to cationic antimicrobial peptides, such as polymixin or nisin produced by the host microbiota.[59,60]

In addition to flouroquinolones mentioned above, circulating strains of *C. difficile* has varying degrees of resistance to antimicrobials depending on the country of circulation.[61] In general, most circulating strains are susceptible to metronidazole and vancomycin, although antimicrobial susceptibility testing has shown lower susceptibility to metronidazole in *C. difficile* 027/BI/NAP1 strains as well as ribotypes 106 and 001.[62] Erythromycin and clindamycin resistance is very common in *C. difficile* strains, with rates greater than 50%.[63] Tetracycline resistance is present in about 10% of the circulating strains, although in some countries, the rate may be as high as 39%.[64] Resistance rates to rifamycin, which is also used for the treatment of CDI, also varies from country to country with 3% of clinical isoaltes in the United States being resistant, whereas almost all clinical isolates in Canada are resistant. *C. difficile* 027/BI/NAP1 strains have close to 80% resistance as shown by a study in a large teaching hospital in the United States.[65] The mechanism behind the resistance to each antibiotic is similar to other gram-positive bacteria, which include localization of resistance genes on mobile elements, point mutations in genes, *etc.*

The new outbreaks of CDI are also characterized by community acquired CDI, in populations considered to be at low risk. Recent surveillance data suggest that approximately 20% or greater of all CDI cases are community associated.[66,67] Community-based patients of CDI appear to be younger with less comorbidity and fewer complications related to CDI.[68] In many cases, this patient population has had no previous exposure to antibiotics. Rates of CDI in children have also increased from 3565 cases in 1997 to 7779 cases in 2006.[69] Approximately 30% of newborns are colonized with *C. difficile* with this number decreasing to around 15% by 12 months of age. By age 3, 3%–5% of children are colonized with *C. difficile*, a percentage similar to adult asymptomatic colonization.[70] CDI, which historically has not been identified in pediatric patients less than 2 years of age, has been reported in children as young as 18 months of age caused by flouroquinolone-resistant *C. difficile* 027/BI/NAP1.[71] Pediatric patients that have associated risk factors for developing CDI such as antibiotic therapy, immunodeficiency, poor diet, and comorbidities such as super-infections and cancer, should be tested for CDI if presented with persistent diarrhea.[71] These new changes have been implemented to address the rising cases of CDI in children.

Since the early 2000s, severe outbreaks of CDI have been reported in peripartum women. The rates and severity of CDI in peripartum women have increased from 1998 to 2006 and mimic the epidemic seen in the general hospital population.[72] Delivery by caesarean section and hence, usage of antibiotics, are risk factors for developing CDI in postpartum women.[73] Health-care workers are also at risk of developing CDI. Health-care workers who have direct contact with patients such as doctors and nurses, as well as laboratory personnel who have indirect contact with patients but are involved in patient sample processing, have reported CDI due to hypervirulent *C. difficile* strains.[74]

The resistance of hypervirulent *C. difficile* strain BI/NAP1/027 spores to regular disinfectants has spurred research in infection control and infection management studies.[75] Commonly used disinfectants such as chlorine-releasing agents, peroxygen-releasing agents and quaternary ammonium compounds, either in the form of a disinfectant or a wipe, have been compared for efficient spore killing.[76] In general, chlorine-releasing agents are most effective than any other disinfectant for killing spores, but whether this leads to decreased CDI is unknown. Hand hygiene which includes washing with soap and water as opposed to alcohol-based hand rubs may lead to the reduction of transmission of hypervirulent spores from patient to patient in a hospital setting.[77] Although the spread of *C. difficile* spores may be reduced by strict adherence to hand hygiene and other contact precautions, such control practices are costly and difficult to enforce, and have yet to yield the desired results.

Although many efforts have been made to control the spread of the CDI epidemic caused by *C. difficile* strain BI/NAP1/027, CDI rates have not yet dropped to the pre-outbreak levels. Infection control barriers, antimicrobial stewardship, education of health-care workers and patients have led to 78% reduction in CDI incidence and a decrease in severity.[78] *C. difficile* strain BI/NAP1/027 has decreased from 51% of clinical isolates associated with CDI in 2001 to 13% in 2005.[78] While *C. difficile* strain BI/NAP1/027 is the current epidemic strain, it is possible that in future outbreaks new epidemic strains will emerge that may have a completely different profile.[79] Already there have been reports of the emergence of polymerase chain reaction ribotype 078 as the predominant circulating strain in parts of Europe where *C. difficile* strain BI/NAP1/027 has rapidly decreased from 55% of all circulating strains in 2007 to 21% in 2010.[80]

HOST IMMUNE RESPONSE AND THE DEVELOPMENT OF CDI

Clinical evidence suggests that host immune responses play a role in the manifestation of CDI.[81] Antibodies against *C. difficile* are present in a majority of adults and older children through transient exposure to *C. difficile* present in the environment, be it present ubiquitously in the soil, in meat meant for consumption or in domestic pets. *C. difficile* has been isolated from many domestic and wild animals, including pigs, calves, poultry, horses, donkeys and bears.[82] Whether or not *C. difficile* is a food-borne disease or a zoonotic disease still remains to be determined.[83]

Following exposure, the role of anti-toxin antibody in the prevention of primary disease is well established.[81] Patients with serum IgG antibodies directed against TcdA at the time of spore colonization generally remain asymptomatic, while those without such immune responses are at a higher risk of developing CDI following colonization.[81] Furthermore, acquired immunity after an initial infection, especially serum IgG antibodies directed against TcdA, is protective against recurrence of disease.[84] Over 25% of elderly patients may have recurrent CDI within 30 days of effective antibiotic therapy.[85]

Recurrent CDI correlates well with a failure to mount effective neutralizing anti-toxin antibodies by day 12 of initial infection. High mucosal anti-toxin A IgA antibody concentrations as well as the presence of anti-toxin antibodies in stool have been associated with protection against severe or recurrent CDI. Individuals who are immunocompromised, have renal failure, are greater than 65 years of age and those who continue taking antibiotics are at a higher risk of recurrence.[19] Patients infected with the hypervirulent epidemic strains expressing CDT are at a higher risk for recurrence.

Innate immune responses to *C. difficile* has been studied extensively in animal models and *ex vivo* studies using human cell lines, aided by limited studied in CDI patients.[86] Toxin production during CDI leads to an inflammatory response which includes the production of pro-inflammatory cytokines and chemokines, activation of Toll-like receptors, nucleotide-binding oligomerization domain-containing protein 1 and the interleukin 1-1β/inflammasome.[87] Neutrophil activation, aided by mast cell degranulation, causes extensive host cell damage.

Adaptive immune responses to non-toxin virulence factors such as the S-layers proteins, cell wall proteins and flagella have been reported in CDI patients. Recent studies show that antibody levels against surface proteins such as flagella and protease Cwp84 were significantly higher in a control group of patients than in a CDI patient group.[88,89] This suggests that these proteins are able to induce an immune response that could play a role in the defense mechanism of the host.[90] The role of such antibodies in the pathogenesis of CDI is unknown.

OVERVIEW OF TREATMENT AND PREVENTION OPTIONS FOR CDI

Given the importance of the host immune response against symptomatic disease, an effective *C. difficile* vaccine could be considered for both prophylactic and therapeutic applications in several target populations. Mathematical studies of CDI suggest the possibility that herd immunity may be important in disease control.[91] An effective *C. difficile* vaccine could be considered for both prophylactic and therapeutic applications in several target populations.[92] For example, patients prior to elective and urgent surgical procedures who get admitted to hospitals would be an important target population. Similarly, at-risk patients in long-term care facilities such as nursing homes and hospices could be vaccinated upon admission. Those with chronic illnesses (e.g., chronic obstructive pulmonary disease, renal failure and diabetes) that frequently lead to hospital admission with infections would also be excellent candidates for vaccination. Taken together, these factors suggest that development of a commercially viable *C. difficile* vaccine suitable for the broadest range of indications would likely depend upon a platform capable of inducing rapid, protective anti-toxin neutralizing responses within days to weeks of vaccination.

Over the past decade, a variety of vaccine and immunotherapeutic monoclonal antibodies for active and passive immunizations have been tested in preclinical animal models and clinical trials; none of these vaccines are approved by the Food and Drug Administration (FDA). A number of *C. difficile* vaccines are being developed; their utility has been hindered by the need for repetitive dosing, the use of formalin-induced detoxification and the stability of the vaccine constructs. For a vaccine to warrant clinical development, it must induce a strong protective immune response against autologous and heterologous strains, requiring the fewest immunizations with or without the added help of an adjuvant. Several key issues need to be addressed. Foremost is to demonstrate that immunization can induce rapid, high-level protection against both toxin and bacterial challenge while significantly improving on the number of immunizations, and time interval to protection. Addressing these issues will ensure the identification of a clinical vaccine candidate of sufficient efficacy and potency such that there can be high confidence that the vaccine will be clinically and commercially feasible for patients susceptible to *C. difficile* in a public health setting. Additionally, it costs upwards of $500 000 000 to $1 000 000 000 to take a vaccine or antibody, respectively, from the bench top to the clinics.[93]

In addition to vaccines, novel therapeutic agents have been developed in the last decade. Fidaxomicin, a narrow-spectrum antibiotic that inhibits bacterial transcription, has been approved in North America and Europe for the treatment of CDI.[94] Persistent high rates of recurrences following antibiotic therapy has led to additional novel treatment options such as stool transplants, intravenous immunoglobulin and probiotics.[95–97]

Active and passive immunization against *Clostridium difficile*

The ability to stimulate a toxin-neutralizing response may be a key property of any future *C. difficile* toxin-based vaccine. The importance of toxin-neutralizing antibody has been highlighted by Torres *et al.*[98] who have correlated protection against lethal *C. difficile* challenge in the hamster infection model with the presence of TcdA neutralizing antibody. To date, the best correlate of vaccine efficacy is the development of serum neutralizing antibodies against the both toxins.

A formalin-inactivated toxoid A and B vaccine, developed by Sanofi Pasteur, adjuvanted with alum, requiring at least three parenterally administered doses, has been tested in six phase 1 trials in more than 200 volunteers including young healthy adults, 18–55 years of age, and healthy adults greater than 65 years of age.[92] It has been found to be safe and immunogenic in all the healthy volunteers, with no vaccine-related adverse events reported. The requirement of three administrations makes this vaccine impractical for use in a public health setting requiring a rapid response. The issue raised by the toxicity of the formalin treatment also reduces the safety of this vaccine.[99] Sanofi Pasteur has initiated two phase II studies using an adjuvanted vaccine dose of 50 μg or higher. The first trial is designed to study the effect of the vaccine on patients with recurrent CDI. The second study is a randomized, placebo-controlled, double-blind, dose-ranging study to assess the effect of the vaccine on primary infection in at-risk volunteers. The performance of this vaccine in elderly patients with comorbidities remains to be determined.

Pfizer has also initiated a phase 1 placebo-controlled, randomized, observer-blinded study of an alum-adjuvanted vaccine containing toxoid A and B requiring at least three parenterally administered doses, in healthy volunteers, 50–85 years of age to evaluate the safety and immunogenicity of the said vaccine.[100] The toxin constructs were first rendered 10 000-fold less toxic than the wild-type toxins by *in vitro* cell-based toxicity assays by introducing targeted mutations in the glucosyltransferase cytotoxicity domains followed by formalin treatment to remove the residual toxicity.

In addition to above mentioned clinical trials, investigators are also targeting the RBDs of the toxins as potential vaccine candidates. These RBDs have been shown to be immunogenic, capable of inducing neutralizing antibodies and protective against bacterial challenge in a murine model of CDI.[101] Intercell has initiated a phase 1 open-label dose-escalation study of its recombinant protein vaccine consisting of truncated toxin A and toxin B proteins, with or without aluminium hydroxide as adjuvant, in healthy adults aged 18–65 years, as well as in healthy at-risk volunteers greater than 65 years of age.[102]

Passive immunotherapies for the treatment of recurrent CDI with antibodies that target the RBDs of the toxins have been tested in clinical trials. Two fully humanized monoclonal antibodies, CDA-1 and CDB-1, which target and neutralize TcdA and TcdB, respectively, in addition to standard antimicrobial therapy, were tested in 200 patients recovering from an initial episode of CDI in a phase 2 multicenter, randomized, double-blind, placebo-controlled trial.[103,104] Interestingly, in a phase 1 trial, the infusion of CDA-1 alone was not protective again emphasizing the importance of neutralizing both toxins. The performance of these antibodies for use as a prophylaxis still remains unclear. Although a reduced recurrence of *C. difficile* diarrhea was reported, this antibody therapy did not improve the severity of the diarrheal illness, the duration of hospitalization, or the time to resolution of the diarrhea. Additional evaluation of this therapy for *C. difficile* infection and recurrence in target older adults is needed. These biologics are being further developed for clinical use by Merck.

With the emergence of *C. difficile* BI/NAP1/027 strains expressing binary toxin, it may be prudent to also target CDT as a vaccine candidate. Immunization of hamsters with TcdA and TcdB does not afford protection against strains that express CDT. Investigators at Merck Research Laboratories have developed a multivalent vaccine that targets TcdA, TcdB as well as the two components of the CDT. Mutations in the active enzymatic sites of TcdA, TcdB and the CDT lead to reduced reactogenicity in animal models of CDI. Immunization of hamsters with the four-component vaccine leads to full protection against lethal challenge with epidemic *C. difficile* BI/NAP1/r027 strain. Clinical studies are needed to compare the efficacy of this vaccine against the others already advancing in clinical trials.

While a strong immune response against TcdA and TcdB may prevent the development of disease, it does not prevent colonization of the host by the bacterium. Therefore, an ideal vaccine, in addition to targeting the toxins, would eliminate carriage and dissemination of *C. difficile* spores. Whether host humoral immune responses to these spore or surface proteins of *C. difficile* can determine the clinical outcome of CDI by influencing bacterial colonization, persistence and toxin effects is an under-investigated area of *C. difficile* pathogenesis. Indirect evidence shows that breast-fed babies are colonized with *C. difficile* spores less often than formula-fed babies, suggesting the protective role of maternal immunoglobulin against *C. difficile* colonization.[105]

Colonization and adherence to gut tissue, especially in the terminal ileum and the cecum, by *C. difficile* is an important step in the pathogenesis of CDI. To date, a number of adherence factors have been identified and characterized, such as S-layer protein (SLPs), flagella, protease Cwp84, the Fbp68 fibronectin-binding protein, the GroEL heat-shock protein and certain hydrolytic enzymes.[106] Recently, the targeted ClosTron gene knockout system for the genus *Clostridium* has been successfully developed, which, in combination with *in vivo* models for CDI, provides a powerful technique to study factors involved in the pathogenicity of CDI.[107] Preliminary experiments suggest a redundancy of function within the surface-associated proteins that are associated with adherence and colonization.

C. difficile is surrounded by a paracrystalline S-layer that consists of a high molecular weight (HMW-SLP) and a low molecular weight SLP (LMW-SLP).[108] Extensive sequence variations among strains, and therefore, limited cross-reactivity among strains are found in the LMW-SLP, whereas the HMW-SLP is highly conserved among strains and are immunologically cross-reactive. Although SLPs are able to bind gastrointestinal tissues, active immunization with SLPS with various adjuvants provided partial protection against *C. difficile* challenge in hamsters.[109]

C. difficile have peritrichous flagella. In *C. difficile*, it is known that adherence of non-flagellated strains of *C. difficile* to mouse cecum is 10-fold lower than that of flagellated strains.[110] *C. difficile* flagellum is made up of two components, the 39 kDa FliC (flagellin) and the 56 kDa flagellar cap protein FliD.[111,112] Several studies have reported that the flagella proteins are highly immunogenic and during the course of natural infection, anti-flagella immune responses may play a role in protection against colonization.[88] Following active mucosal immunization and challenge in the human flora-associated mouse model, there was a decrease in intestinal colonization of *C. difficile* in the FliC–FliD immunized mice compared to the control group.[113]

The vegetative form of *C. difficile* cells express complex oligosaccharides, named PSI, PSII and PSIII, on the cell surface.[114] PSII is abundantly expressed by all *C. difficile* ribotypes, including the hypervirulent *C. difficile* BI/NAP1/r027 strains. Anti-PSII IgA antibodies have been found in stool of patients with CDI, thus making these polysaccharides potential vaccine targets.[115] The immunogenicity of conjugate vaccines of PSII fused with various carrier proteins has been studied in mice and hamsters and is found to be immunogenic. Further studies are currently underway to elucidate the role of anti-PSII adaptive immune responses against *C. difficile* colonization.[116]

Studies in patients and animal models show that spores of nontoxigenic *C. difficile* strains may prevent toxin-producing *C. difficile* strains from colonizing susceptible patients by providing barrier resistance. Hamsters inoculated with spores from nontoxigenic *C. difficile* strain M3, named VP20261, are protected from challenge with toxigenic *C. difficile* strain. Viropharma Incorporated, has completed a randomized, double-blind, placebo-controlled safety and colonization effectiveness study in adult subjects.[117] Healthy volunteers (18–45 years of age and older than 60 years of age) were orally administered single or multiple doses of VP20261 suspension containing 10^4, 10^6 or 10^8 spores. Following vancomycin treatment, all volunteers shed VP20261 in stool. No adverse events were reported and volunteers were colonized with VP20261. A phase II trial of VP20261 to prevent recurrence of CDI in patients previously treated for CDI has been initiated.

Antibiotic therapy with fidaxomicin

Until recently, oral vancomycin was the only FDA approved therapy for CDI. Due to its high cost and the added concern of vancomycin resistance of hospital-acquired *Enterococci*, oral metronidazole, although not FDA-approved for CDI, was the first-line therapeutic agent for CDI. Following antibiotic treatment and cessation of such treatment, 25% of patients with CDI experience recurrence within 30 days following treatment with these antibiotics.[85] High recurrence rates and metronidazole's poor colonic pharmacokinetics have significantly limited the use of these two antibiotics. Fidaxomicin, marketed as Dificid by Optimer Pharmaceuticals (New Jersey, USA), was approved by the FDA in 2011, the first new drug to be approved for CDI in over 25 years.

Fidaxomicin, is a novel narrow spectrum, oral macrocyclic antibiotic which has bactericidal activities against *C. difficile*.[94,118] It has no activity against gram-negative bacteria and has minimal activity against *Bacteroides* species, which allows for the normal flora to be somewhat suitably maintained.[119] In two phase 3 clinical trials in which patients with CDI were treated with either fidaxomicin or vancomycin, fidaxomicin has been shown to be non-inferior to vancomycin in the management of mild-to-moderately severe CDI.[120,121]

Subgroup analyses also show that there were significantly fewer CDI recurrences in patients who received fidaxomicin than those treated with vancomycin. However, this result was not seen in patients infected with the hypervirulent *C. difficile* BI/NAP1/r027 strains where the overall cure and recurrence rates were 86% and 27%, respectively, compared to 94% and 16% in patients infected with other *C. difficile* strains. The recommended dosage for treatment of CDI with fidaxomicin is 200 mg orally twice daily for 10 days is generally well tolerated. A 10-day course of Dificid costs $3000, which is twice the cost of vancomycin and 300 times more than metronidazole. Till date, fidaxomicin resistance *in vivo* has not been reported.

Fecal bacteriotherapy for recurrent CDI

Fecal microbiota transplantation has been used to treat patients with pseudomembranous colitis for the last 50 years.[122] Transfer of healthy donor feces (read normal microflora, e.g., *Bacteriodes* sp.) either by nasogastric tube, colonoscopy or enema directly to the lower gut allows the microflora of the donor to reestablish quickly in the patient following the cessation of antibiotic treatment. Although resolution of recurrent CDI following fecal microbiota transplantation has been reported in 92% of cases, this treatment for recurrent CDI has not become routine due to the lack of efficacy data from large clinical trials, although abundant data from case reports and case series do exist. Additional roadblocks include the unappealing nature of the process as well as the cumbersome procedure of screening of potential fecal donors. Fecal donors are first screened to prevent communicable diseases and the preferred donor is usually chosen from close family members who likely have similar microbiota to the patient. Additionally, the cost of such procedures is around $1300, most of which goes towards screening of donors for potential pathogens.

As the severity and number of recurrences following an initial episode of CDI in patients infected with the hypervirulent *C. difficile* BI/NAP1/027 strains increase, fecal microbiota transplantation will likely be adopted as routine therapy for recalcitrant recurring CDI. A recent randomized trial from the Netherlands compared three treatment modalities for recurring CDI.[97] One group received vancomycin treatment, followed with gastric lavage and duodenal infusion of donor feces, whereas the control groups received vancomycin treatment, with or without gastric lavage. Eighty-one percent of patients receiving fecal microbiota therapy had resolution of CDI following a single infusion compared to 31% of patients receiving vancomycin alone and 23% of patients receiving vancomycin followed with gastric lavage. These results corroborate previous anecdotal evidence for the use of fecal microbiota transplantation for treatment in recurring CDI patients.

Probiotics for the prevention and treatment of CDI

Probiotics with predefined mixtures of bacterial culture that can lead to the reestablishment of microflora in a patient's gut and may lead to the resolution of recurring CDI or prevent an initial episode of CDI. A number of probiotics, either single strains of *Lactobacillus rhamnos* GG and *Saccharomyces boulardii* or multistrain mixtures, have been evaluated for the treatment and prevention of CDI in both adults and children. A meta-analysis of 23 randomized controlled trials suggests that probiotics are safe and moderately effective at preventing CDI, although additional trials need to be conducted to identify probiotic mixtures that are most efficacious in preventing CDI.[123] In critically ill patients, adverse side effects such as bacteremia and sepsis may limit the use of probiotics.

CONCLUSION

The human microbiome, made up of a complex ecosystem representing billions of bacteria, plays an important role maintaining the balance between health and diseases such as inflammatory bowel disease, asthma and obesity. Perturbation of this delicate balance with the use of antibiotics has led to the rise of antibiotic resistance bacteria such as methicillin-resistant *Staphylococcus aureus*, *Acinetobacterbaumanii*, vancomycin-resistant enterococci, *etc.* Additional side effects of antibiotic overuse is the development of illnesses due to the loss of colonization resistance provided by the normal microbiome allowing pathogens such as *C. difficile* to establish infection and cause disease, often with life-threatening consequences.

Before the early 2000s, *C. difficile* was barely known outside of the medical community, typically associated with nosocomial diarrhea in elderly patients being given antibiotics. This little-known bacterium has now become a well-known pathogen that is the leading cause of nosocomial diarrhea worldwide, with cases being reported in the Americas, Europe and Asia. The incidence of CDI has doubled since the emergence of hypervirulent *C. difficile* BI/NAP1/027 strain, now including patients who were thought to be at 'low risk' in outpatient settings, in children, peripartum mothers and health-care workers, and also in the community in people with no previous exposure to antibiotics. Antibiotic-based therapies still remain the mainstay for the management of CDI, although new therapies such as fecal transplants and probiotics, aimed at restoring the normal microbiota in patients, are gaining momentum in the medical community. Active and passive vaccinations are currently being tested in clinical trials for initial and recurrent CDI. Antibiotic stewardship, in addition to strict standards of infection control in hospitals, are providing a multifaceted 'bundle' approach in successfully decreasing the spread of CDI in hospitals among patients. Collaborative approaches between patients, health-care workers and researchers can potentially stop the growth of the current CDI epidemic.

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