

# Conventional and alternative treatment approaches for *Clostridium difficile* infection

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## ABSTRACT

*Clostridium difficile*-associated disease continues to be one of the leading health concerns worldwide. *C. difficile* is considered as a causative agent of nosocomial diarrhea that causes serious infection, which may result in death. The incidences of *C. difficile* infection (CDI) in developed countries have become increasingly high which may be attributed to the emergence of newer epidemic strains, extensive use of antibiotics, and limited alternative therapies. The available treatment options against CDI are expensive and promote resistance. Therefore, there is urgent need for new approaches to meet these challenges. This review discusses the current understanding of CDI, the existing clinical treatment strategies and future potential options as antidiarrheal agents based on the available published works.

**Keywords:** Alternative treatment, *Clostridium difficile*, *Clostridium difficile* infection

## Introduction

*Clostridium difficile* is an anaerobic, spore-forming, Gram-positive bacterium, the most common pathogen that can cause antibiotic-associated diarrhea. Normal gut flora usually resists colonization and overgrowth of *C. difficile*.<sup>1</sup> *C. difficile* infection (CDI) represents a serious challenge to medical practice in many developed countries, however, information from developing countries on this infection is generally lacking. One report on CDI in China indicated that such infection was present but no systematic study was conducted to provide more complete epidemiology data.<sup>2</sup> One reason is the lack of affordable diagnostic kits to be used for clinical or epidemiology studies.

The uses of antibiotics usually modify the intestinal microbiome and permit propagation of *C. difficile*. It has been noticed that hospitalized patients are the prime target of CDI, although *C. difficile* also present as a colonizer in 2-3% of healthy individuals and 70% in healthy children.<sup>3</sup> CDI is associated with antibiotic treatments and could occur in every kind of clinical setting. Recent reports have shown that 20% of individuals who were hospitalized become colonized with *C. difficile* during hospitalization, and about 30% of those patients can develop diarrhea.<sup>4</sup> Although CDI is commonly regarded as mild-to-moderate diarrheal disease with abdominal pain, in severe cases CDI can present with acute abdomen as well as fulminant and life-threatening colitis. The diagnosis of *C. difficile* colitis should be suspected in any patient with diarrhea, who has been under antibiotics treatment over the previous 3 months or has been recently hospitalized.<sup>5</sup> However, it has been recently reported that *C. difficile* can be a cause of

diarrhea in community without any previous hospitalization or antibiotic exposure.<sup>6</sup> *C. difficile* produces heat-resistant spores that can persist in the environment for several months, thus providing the basis for nosocomial outbreaks even after extensive cleaning measures.

## Pathophysiology

Pathogenic strains of *C. difficile* produce multiple diverse toxins.<sup>7</sup> The preeminent categorized toxins are toxin A, which is an enterotoxin, and toxin B, a cytotoxin. Both toxins are of high molecular weight proteins (308 and 270 kDa, respectively) having the capability of binding to some specific receptors on intestinal mucosal cells of the host. Receptor-bound toxins usually gain their intracellular entry using catalyst specific Rho proteins that support in actin polymerization, cytoskeletal rearrangements and cell movement.<sup>8,9</sup> Both toxin A and B emerge to play an essential role in the pathogenesis of *C. difficile* colitis in humans through the initiation of apoptosis in the target mucosal cells. Toxin damage of the colonic mucosa always escort to an accumulation of fibrin, mucin, and dead cells, finally structuring a layer of debris in the colon. Subsequent inflammatory activation adjoins to the direct toxin-associated damage resulting in mild diarrheal disease up to extensive intestinal wall damage with septic shock and death.<sup>7,10</sup>

## Clinical Symptoms

It is important and crucial to differentiate between asymptomatic colonization and symptomatic CDI. Individuals who are

colonized by the organism may attain protection against development of the infection but they stay as a potential transmission source of the disease in health-care settings.<sup>11</sup> Symptom starts from simple irritation of mucosa, watery to soft diarrhea with a sweetish, and foul odor<sup>12</sup> to the full clinical symptom of pseudomembranous colitis with typical endoscopic findings, preferentially in the region of the sigmoid and rectum. CDI affects the right colon alone is rarer.<sup>13</sup> Furthermore, stool frequency of the patient may be increased  $\times 10/\text{day}$ . In older patients, the signs of toxicosis requiring treatment can take place rapidly. If such symptoms prolonged, hypoalbuminemia, and protein-losing enteropathy can occur.<sup>14</sup> Subfebrile temperatures are common.<sup>15</sup> On physical examination, the colon is swollen in the lower left abdomen. There is usually slight local pain on palpation.<sup>12,13</sup> Aggressive signs of complicated CDIs with ileus, toxic megacolon, perforation, or sepsis (<5% of cases) include absence of colonic peristalsis, sudden-onset constipation, extreme leukocytosis, and high fever.<sup>12-15</sup> Moreover, further diagnostic measures may be required, i.e., contrast computed tomography of the abdomen.<sup>13</sup> Different professional societies of gastroenterologists as well as infectious disease specialists have recently published up-to-date guidelines for CDI management.<sup>16-18</sup> The European Society of Clinical Microbiology and Infectious Diseases have recently published guidelines in 2014.<sup>18</sup> The definition of an event of CDI comprises a clinical picture (Table 1) compatible with CDI, microbiological evidence of free toxins, and the presence of the organism in stool without realistic evidence of another cause of diarrhea, or pseudomembranous colitis (diagnosed by endoscopy, after colectomy, or on autopsy).

**Table 1:** Patient characteristics correlating with disease severity when associated with CDI (adapted from Debast *et al.*, [2014])

Category	Signals/Symptoms
Physical examination	Fever, rigors, hemodynamic instability including signs of shock, respiratory failure with need for mechanical ventilation, peritonism, ileus
Laboratory tests	Leukocytosis >15 Gpt/L, left shift with >20% neutrophils, rise in serum creatinine >1.5 $\times$ baseline, lactate >5 mmol/L, albumin <30 g/L
Endoscopy imaging	Presence of pseudomembranes colonic distension >6 cm in transverse colon/toxic megacolon, colonic wall thickening, pericolic fat stranding, ascites due to CDI

CDI: *Clostridium difficile* infection

**Table 2:** Microbiological diagnostic tests for *C. difficile* and their value (Adopted from Lübbert *et al.*, [2014])

Test	Indication
Test GDH EIA (TAT <2 h)	Initial screening test with high sensitivity and high negative predictive value; GDH-positive samples must undergo a confirmation test for the toxigenic infection
Toxin A and B EIA (TAT <2 h)	Confirmation test for toxigenic infection in GDH-positive samples (two-step algorithm); good correlation with severe infections, limited sensitivity; NAAT (three-step algorithm) recommended if no toxin detected
CTNA (TAT <24 h)	Standard test for evidence of toxins in stool; CTNA is rarely used for routine diagnosis, however, due to its longer TAT and low potential for standardization and automation
NAAT of toxin genes (TAT <4 h)	Confirmation test for toxigenic infection. NAAT (e.g., PCR) not recommended as screening test, as asymptomatic <i>C. difficile</i> carriers not requiring treatment or isolation may also be detected.

GDH: Glutamate dehydrogenase, CTNA: Cytotoxin neutralization assay, TAT: Turn-around time, EIA: Enzyme immunoassay, NAAT: Nucleic acid amplification test, PCR: Polymerase chain reaction, *C. difficile*: *Clostridium difficile*, CDI: *Clostridium difficile* infection

## Diagnosis

The international CDI diagnosis guidelines recommend evidence-based, rapid detection of toxigenic CDI from stool samples.<sup>16-22</sup> Multistep diagnostic procedures are recommended (Table 2).<sup>23</sup>

Only the symptomatic patients should be tested. Repeated stool samples are not usually required. Rapid antigen tests and nucleic acid amplification tests are mostly important in routine diagnosis, due to their short turnaround time (TAT), ranges from 15 min to 3 h. The toxigenic culture, i.e., the anaerobic culture in a special media, combined with evidence of the toxin in the culture supernatant, is considered as the diagnostic gold standard. Anaerobic culture is required for further antibiotic resistance and ribotype testing. Cultures are not well suited for acute diagnosis, as they have a long TAT (>72 h). A macroscopic finding of pseudomembranous colitis is in many cases so characteristic that CDI can also be diagnosed via endoscopy or colonoscopy, though with limited sensitivity.

## *C. difficile* therapy

Having a confirmed CDI, it is crucial that proper infection control measures are in place to avoid further spread of the infection within the same ward or hospital. To avoid spreading of *Clostridium* spores, hands need to be washed, patients should be kept in isolation, and importantly, gloves and protective clothing must be worn by all staff along with continuous hand hygiene after each patient contact.<sup>24</sup> In all patients with CDI, it is necessary to stop the causing antibiotic therapy. This may be suitable as the only treatment in a patient with little symptoms. Patients need appropriate replacement and monitoring of fluids and electrolytes, and antimotility drugs should be avoided. Currently, all recommendations for antibiotic therapy are based on differentiation between mild-to-moderate or severe disease.<sup>18</sup> Other guidelines further differentiate a severe to a complicated course.<sup>16-18</sup> For successful treatment of CDI, it is important for clinicians to start screening for risk factors of severe disease and parameters linked with an unfavorable outcome. There are many clinical and laboratory variables that correlate with severity of outcome as already explained in Table 1.<sup>18</sup> In general, the most important prognostic indicators of severe

disease are; the age of >65 years, leukocytosis (>15 Gpt/L), decreased serum albumin (<3 g/L), rise in serum creatinine (>133  $\mu\text{mol/L}$  or >1.5 times of the premorbid level) and underlying comorbidities.<sup>25-27</sup> In a patient with strong suspicion of CDI, empirical treatment is required according to European and American guidelines. This approach is only recommended for the patients with severe disease and/or risk factors for an unfavorable outcome. Furthermore, cessation of excessive antibiotic treatment is compulsory.

## Antibiotics

Recent advances in the CDI antibiotic resistance have been reviewed by Spigaglia.<sup>28,29</sup> However, the following antibiotics are well known for the treatment of CDI and received a considerable attention by different researchers.

### Metronidazole (MTZ)

MTZ is considered as the first choice for mild-to-moderate CDI.<sup>30</sup> Although the percentage of *C. difficile* strains resistant to MTZ is, in general, low, several studies have emphasized treatment failure with MTZ.<sup>31</sup> An elevated linear mean of minimum inhibitory concentrations (MICs) to MTZ have recently been observed in different strains; RT027 (1.1-1.42 mg/L), RT001/072 (0.65 mg/L), RT106 (0.65 mg/L), RT356 (0.61 mg/L) and in the nontoxigenic RT010 (1.5 mg/L), compared with the values of the other RTs (0.13-0.41 mg/L).<sup>32</sup> A study in Saudi Arabia by Alqumber reports susceptibility of MTZ with MIC range between 3 and 8  $\mu\text{g/ml}$ .<sup>33</sup> Another study, however, reports a spread of strains RT027 with reduced susceptibility to MTZ in Jerusalem, where they cause severe infections and a wide outbreak in 2013.<sup>34</sup>

Detection of strains with reduced susceptibility to MTZ can be challenging. This resistance is often unstable and laboratory manipulation of strains frequently results in MIC decrease toward a susceptibility range.<sup>35</sup> Experimental methodology may affect the magnitude of measured MTZ MICs for *C. difficile*. The overall data reported in a recent study suggest the Agar Incorporation Method (AIM)<sup>36</sup> as the method of choice to detect strains with reduced susceptibility to MTZ compared with the Etest and the agar dilution method (AD).<sup>37</sup> Differences in the media used (Schaedlers broth and Wilkins-Chalgren agar for AIM and Brucella broth/agar for both Etest and AD) and in the duration of the precultured period (24 h for AIM and 48 h for both Etest and AD) seem to affect MIC determination.<sup>37,38</sup> MTZ susceptibility breakpoint for *C. difficile* defined by the CLSI and the European Committee on Antimicrobial Susceptibility Testing are not equivalent: The first is defined as 32 mg/L, the second >2 mg/L. Methodological variations and different interpretation categories may result in discrepancies, impacting therapeutic decision and comparison of data. Therefore, international committees are currently cooperating with the intention of harmonizing susceptibility testing and international breakpoints.

### Vancomycin (VAN)

VAN, the first-line antibiotic treatment of choice for moderate to severe CDI,<sup>18,30</sup> consists of a glycosylated hexapeptide chain and cross-linked aromatic rings by aryl ether bonds, with a reduced absorption in the gastrointestinal tract.<sup>39</sup> Its mechanism of action results in inhibition of the biosynthesis of peptidoglycan, an essential component of the bacterial cell wall envelope.<sup>40</sup> Resistance to VAN has frequently observed in *Enterococci* and *Staphylococci*, but it is not so largely diffused in *C. difficile*. Although, a number of *C. difficile* strains with reduced susceptibility to VAN (MICs range >2-16 mg/L) have recently been described.

The mechanism of resistance in *C. difficile* is still unclear. Several Tn1549-like elements have been found in *C. difficile*.<sup>41,42</sup> Differently from the original Tn1549 element described in *Enterococcus faecalis*, the Tn1549-like elements of *C. difficile* do not have a functional vanB operon. Recently, a vanG-like gene cluster, homologous to the cluster found in *E. faecalis*, have been described in a number of *C. difficile* isolates but, although this cluster is expressed, it is not able to promote resistance to VAN.<sup>43,44</sup> MurG converts lipid I to lipid II during the membrane-bound stage of peptidoglycan biosynthesis. Alterations in this pathway, in VAN-resistant mutants, may affect VAN activity since VAN inhibits cell wall formation by binding to the D-Ala-D-Ala portion of lipid II.<sup>45</sup> Biofilm formation could be also involved in VAN-resistance. *C. difficile* within biofilms have been found to be more resistant to elevated concentrations of VAN (20 mg/L), and biofilm formation seems to be induced in the presence of subinhibitory and inhibitory concentrations of the antibiotic.<sup>46</sup> The clinical significance of reduced susceptibility to VAN remains to be determined, since the fecal concentration of this antibiotic is very high, ranging between 520 and 2200 mg/L.<sup>47</sup>

Recommended treatment with MTZ and VAN according to disease severity is summarized in Table 3.

### Rifamycins and fidaxomicin (FDX)

An increased rate of treatment failure and recurrence of infection have been associated with MTZ and VAN treatment,<sup>31</sup> therefore, other therapy options for CDI have been proposed in the recent years. Rifamycins, in particular rifaximin (RFX), have recently been proposed as “chaser therapy” for the treatment of relapsing CDI,<sup>48</sup> while FDX is a bactericidal new narrow spectrum macrocyclic antibiotic that is used for the management of CDI with high risk for recurrences.<sup>49</sup> Both rifampins (RIFs) and FDX are inhibitors of bacterial transcription but they have different RNA polymerase (RNAP) target sites. FDX binds to the “switch region” of RNAP, a target site that is adjacent to the RIF target but does not overlap.<sup>50,51</sup>

Susceptibility to RIF by either Etest or AD correlated completely with susceptibility to RFX.<sup>52</sup> Thus, testing susceptibility to RIF, a rifamycin that is related to RFX,



**Table 3:** Summary of recommended treatment with MTZ and VAN according to disease severity (Adapted from Surawicz *et al.*, [2013])

Severity	Criteria	Treatment	Comment
Mild-to-moderate disease	Diarrhea, no signs or symptoms of severe disease	MTZ 500 mg p.o. 3×/day for 10 days	If no improvement in 5-7 days switch to VAN 4×125 mg p.o.
Severe disease	Two of the following: Albumin<30 g/L; leukocytosis>15 Gpt/L; creatinine>133 μmol/L; age>65 years; abdominal tenderness; comorbidities	VAN 125 mg p.o. 4×/day for 10 days	Other authors consider age<65 years and a rise in creatinine>1.5×baseline as equal risk factors for severe disease
Severe and complicated disease	Any of the following attributable to CDI: Admission to ICU for CDI; prolonged hypotension; ileus or significant abdominal distension; mental status changes; leukocytes>35 Gpt/L or <2 Gpt/L	VAN 500 mg p.o. 4×/day and MTZ 500 mg i.v. 3×/day and VAN per rectum (500 mg VAN in 500 ml Nalco 0.9%) 2-4×/day	Consider surgical consultation

ICU: Intensive care unit, CDI: *Clostridium difficile* infection, VAN: Vancomycin, MTZ: Metronidazole

can assess rifamycin class susceptibility in *C. difficile*. Data concluded from recent studies show that 11% of *C. difficile* clinical isolates are resistant to RIF and the rate of overall resistance appear to be rising.<sup>53-55</sup> *C. difficile* clinical isolates resistant to RIF have been detected in 17 out of 22 countries participating in a recent pan European surveillance and, in particular, high percentages of resistance (between 57% and 64%) have been observed in Italy, Czech Republic, Denmark and Hungary.<sup>32</sup> Prior exposure to RIFs has been reported to be a risk factor for RIF-resistant *C. difficile*<sup>52,56</sup> and resistant *C. difficile* strains may emerge even during therapy.<sup>57,58</sup> RIFs are commonly used as antituberculosis (TB) agents. Interestingly, all strains belonging to the emergent RT046 isolated in Poland from patients affected by TB and with a prolonged RIF therapy have been found highly resistant to these antibiotics.<sup>59</sup>

## Other antibiotics

### Tetracycline (TET)

Recent papers on the *C. difficile* resistance to TET show different results according to the countries where the studies took place. The resistance ranges between 2.4% and 41.67%.<sup>60-62</sup> In this pathogen, resistance is commonly due to protection of the ribosomes from the action of antibiotic. The most widespread TET class in *C. difficile* is tetM, usually found on conjugative Tn916-like elements.<sup>63-65</sup> The Tn916-like family is responsible for the spread of antibiotic resistance (usually referred to TET but also to MLSB and other antibiotics) to many important pathogens. In *C. difficile*, the best-known element of this family is Tn5397, a 21kb element able to transfer between *C. difficile* and *Bacillus subtilis* or *E. faecalis* *in vitro*.<sup>66,67</sup> Tn5397 differs from Tn916 for the presence of a Group II intron and for a different excision/insertion module. In fact, Tn916 contains two genes, xisTn and intTn, encoding an excisionase and a tyrosine integrase, whereas Tn5397 has a tndX gene that encodes a large serine recombinase.<sup>68</sup> Furthermore, Tn916 inserts into multiple regions of the *C. difficile* genome,<sup>64</sup> while Tn5397 inserts DNA predicted filamentation processes induced by cyclic adenosine monophosphate (Fic) domain.<sup>69</sup>

Although tetM is the predominant class in *C. difficile*, other tet genes have been identified. In particular, the presences of both tetM and tetW have been described in *C. difficile* isolates

from humans and animals.<sup>70,71</sup> Furthermore, other integrative mobile genetic elements probably have a role in resistance to TET. An interesting element of 106 kb, the Tn6164, has been identified in *C. difficile* strain M120, a RT078 isolate.<sup>72</sup> This transposon is composed by parts of other elements from different bacteria, particularly from *Thermoanaerobacter* sp. and *Streptococcus pneumoniae*. Even if M120 is susceptible to TET and streptomycin, Tn6164 contains tet (44) and ant (6)-Ib predicted to confer resistance to these antibiotics, respectively.

## Chloramphenicol (CHL)

Resistance to CHL is not so common in *C. difficile* and only 3.7% of European clinical isolates have been found resistant to this antibiotic.<sup>32</sup> *C. difficile* resistance to CHL is usually conferred by a catD gene, encoding for a CHL acetyltransferase.<sup>73,74</sup> The catD gene is located on the transposons Tn4453a and Tn4453b, structurally and functionally related to the *Clostridium perfringens* mobilizable element Tn4451.<sup>75</sup>

## Recurrent of the Disease

The possibility of CDI recurrence after an initial occurrence is reported to be between 10% and 20% within 8 weeks and further increases with every other event up to 40-65%.<sup>76</sup> It is measured similar for the treatment with MTZ and VAN. Fewer secondary recurrences are also reported after treatment with fidaxomicin for patients with mild-to-moderate disease.<sup>77</sup> The first recurrence event can be treated with the same regimens used for the initial occurrence, it all depends on the severity of disease especially in patients with high risk of additional recurrence treatment with fidaxomicin. For the second failure of CDI, MTZ is no longer option based on concerns about its side effects, especially neuropathy. In such kind of situation fidaxomicin (200 mg twice daily for 10 days) or VAN (125 mg 4 times daily for 10 days) followed by either a pulsed or tapered strategy is mostly highly recommended. McFarland *et al.*<sup>76</sup> were able to reduce the frequency of relapse to 14.3% by a pulsed regime and up to 31% by using a tapered strategy.<sup>78</sup> The pulsed strategy proposed a standard VAN course over 10 days followed by a course of 125 mg VAN every 2-3 days for 10 doses. Of the multiple strategies used for tapering VAN, the IDSA guidelines 2010 recommended stepping down to

125 mg twice daily for a week after the regular 10 days of VAN, followed by 125 mg once daily for a week which is then followed by pulse of 125 mg every 2-3 days for 2-8 weeks.<sup>16</sup> In case of patients with multiple recurrences, the European as well as the American guideline on CDI recommends that the intestinal microbiota transplantation (IMT) must be considered for those patients.

## The IMT

The IMT (Microbiome transfer, or fecal microbiota transplantation), is a procedure in which fecal matter is collected from a tested donor, mixed with a saline or other solution, strained, and placed in a patient, by colonoscopy, endoscopy, sigmoidoscopy, or enema, to cure the underline disease.

In early fourth century, the stool transfer treatment for diarrheal diseases was successfully practiced in China.<sup>79</sup> It was described first as a treatment option for pseudomembranous colitis in 1958.<sup>80</sup> It has been found that the intestinal microbiota in patients with CDI had a reduced bacterial diversity, as compared with healthy individuals. It has been also found that in patients with recurrent CDI, the IMT (infusion of donor feces) resulted in better treatment outcomes as compared with VAN therapy.<sup>81</sup> During the past few years, reports have shown that the treatment for recurrent CDI by endoscopically administering the feces in the duodenum, ileocolon, or by enema, the patient's cure rates were reported to increase for up to about 92%.<sup>82</sup>

Colonoscopic stool transfer was recommended on the strength of better acceptance and avoidance of bacterial contamination of the small intestine with intestinal microbes, in addition to its higher success rate. About 200 ml volume was used via the upper digestive tract.<sup>82</sup> Furthermore, for conservative application, the response was improved using 500 ml or more of suspension (80% vs. 97%).<sup>82</sup> A highly diverse protective donor flora develops within 2 weeks following stool transplantation, predominantly natural *Bacteroides* species.<sup>83</sup> To do this, a protocol-based treatment was schedule, which could be followed. This trail must be monitored for a long-term follow-up. In animal trails, a correlation between altered intestinal microbiome and the development of autoimmune diseases and obesity was observed.<sup>84</sup>

Despite its increased rate for successful curing advanced CDI cases, IMT has important limitations such as the reluctance of patients, and also physicians, to choose this treatment strategy at an early stage. Furthermore, there are legitimate concerns that IMT can spread other infectious diseases including HIV or hepatitis. There are also concerns that IMT could change the microbiome and consequently increase susceptibility to chronic conditions such as obesity or autoimmune disorders.<sup>85</sup>

## DNA Vaccination

DNA vaccination offers a unique platform to study the optimal antigenic regions from both toxins as this approach is able to test the immunogenicity of candidate antigens in animals directly without first producing actual antigenic proteins *in vitro*. Once high-level antibody responses are elicited, the same antigen region can be used to produce subunit-based recombinant toxin proteins as vaccines. Furthermore, the same toxin antigens can be used to produce hyperimmune sera that can be administered for passive antibody protection. Monoclonal antibodies can also be generated from a high responder host (animal or human volunteers) who received the novel N-terminal region from the *C. difficile* toxin B.<sup>86</sup>

Many efforts by different pharmaceutical companies to develop vaccines against CDI. Passive protection with antitoxin monoclonal antibodies has also been proven to be effective in reducing recurrent CDI.<sup>87</sup> The important element for both active and passive vaccination approaches is the discovery of high quality protective antibody responses against two key *C. difficile* toxins.<sup>88</sup> Although, antitoxin antibodies have been widely investigated, less is known about the immunogenicity potential of toxin B. It might be the size and the highly unstable nature of both toxins, especially toxin B, has made the use of full-length recombinant protein-based vaccines less practical. While it is generally well established that the C-terminal receptor binding regions of both toxins (A and B) are ideal candidates for eliciting protective antibody responses, other areas of both toxins in eliciting protective antibody responses are yet to be investigated.

N-terminal region of toxin B protein is found to be an excellent immunogen to elicit protective antibodies, effective not only in protecting cells in an *in vitro* cytotoxicity assay but also functional in improving the protection of mice against a lethal *C. difficile* toxin when used in combination with antitoxin A antibodies. The discovery of this novel N-terminal domain of TcdB as a protective immunogen will offer more options to design the next generation subunit-based TcdB or TcdA-TcdB combination vaccines.<sup>86</sup>

The production of a panel of mAb against the toxin A of *C. difficile* elicited by DNA immunization has been described. Due to the large size of *C. difficile* toxins and the toxicity of natural materials, it is appropriate to use a part of the toxin protein as the immunogen. DNA immunization is an ideal approach for such investigations as various subdomains of the toxins can be designed and tested while there is no need for the production and purification of actual recombinant protein immunogens *in vitro*.<sup>89</sup>

Toxin A-specific mAb showed high antigen specificity and high antibody affinity. They have preserved functional activity as protective antibodies based on both *in vitro* cell protection and *in vivo* protection. More significantly, these

mAb targeted different epitopes of toxin A, which allowed for the development of a “cocktail” formulation to improve the *in vivo* protein or a paired detection kit to measure the presence and levels of toxin A in testing samples, including clinical samples. One study reported a significant correlation between toxin B protein concentrations and severities of clinical CDI.<sup>90</sup>

Given the recent rapid progress in mAb technology, including the direct cloning of Ig genes from immunized B cells from both animal and human sources,<sup>91-94</sup> great opportunities to study and optimize the immunization approaches that can be effective in eliciting high titer and high affinity antibody responses in the hosts are becoming available. In combination with advanced sequencing technology, the entire process of antigen-specific B-cell development can be monitored.

## Natural Products

Failure rate of existing antibiotics in combating *C. difficile* seems to be high and increasing, and the recurrent infections are frequently observed. This may be attributed to the excessive and widespread use of antibiotics. Consequently, choices for *C. difficile* treatment using conventional antibiotics is becoming limited, and the development of alternative therapeutic approaches, including plant-source remedies that are usually used in traditional medicine, are undoubtedly needed to prevent and contain the spread of resistance and to ensure an effective therapy against CDI.<sup>28,95</sup>

Plant extracts were considered to be significant for various diseases by the ancient civilizations.<sup>96</sup> It is estimated that there are about 250,000 species of higher plants in the world, and pharmacological activities for most of them are yet to be investigated.<sup>97</sup> Natural products and their derivatives are the source for more than 50% of the drugs currently available worldwide, in which higher plants contribution is about 25%.<sup>98</sup> Flowering plants are producing a variety of potent drugs, for example pilocarpine to treat glaucoma and dry mouth is derived from *Pilocarpus* spp. Reserpine and other hypertensive and tranquilizing alkaloids have been isolated from *Rauwolfia* spp.<sup>99</sup> There is an international raising trend to shift resources from allopathic to traditional health-care systems.<sup>100</sup>

Microbial organisms and higher plants have been used as a natural source for the discovery of new drugs. Artemisinin, quinine, and licochalcone A are the examples for plant derived products and amphotericin B are most important antiparasitics components isolated from microorganisms. Many other natural plant products have demonstrated antiparasitic activity in the laboratory and have represent the interesting and novel structures for the development of new and immediate needed antiparasitics. The plants essential oils have demonstrated anti-inflammatory, antibacterial, antiprotozoan, and antifungal activities.<sup>100-102</sup>

In comparison with antibiotics, herbal extracts, and essential oils contain different antibacterial agents that could employ

a number of inhibitory mechanisms, making it difficult for pathogens to initiate resistance.<sup>103</sup>

Antiparasitic properties of many new natural product groups have been identified with their efficacy and selectivity such as plant-derived alkaloids, terpenes, and phenolics.<sup>104</sup> Natural products have been a productive source of new bioactive compounds, allowing the discovery of therapeutic agents to treat not only infectious diseases but also cancer and other immunodeficiencies.<sup>105,106</sup> Extracts and essential oils were effective in controlling the growth of a wide variety of microorganisms, including bacteria, parasites, yeasts, and filamentous fungi.

The increased popularity of using natural product as alternative curing approach may be attributed to the belief that these products are safe and they are widely available at low costs. In traditional medicine, the use of plants in the form of crude extracts, infusions or plasters is a well-known practice to treat common infections in many parts of the world. So far, there is limited literature available regarding the natural products used against *C. difficile*, and few reports available in which the researchers have used essential oils<sup>107-109</sup> and fruits extract<sup>110</sup> against *C. difficile*. An interesting study was conducted to determine the effect of essential oil compounds on mixed fecal microbiota. The study concludes that thymol and geraniol at around 100 ppm could be effective in suppressing pathogens in the small intestine, without damaging the beneficial commensal colonic bacteria.<sup>107</sup>

Another plant product; virgin coconut oil (VCO) is found to have an antimicrobial activity. VCO active fatty acids have been tested for their antimicrobial potential against *C. difficile in vitro* trail. The results have shown the inhibitory effects on growth ( $P < 0.001$ ) when exposed to lauric acid (C12) when it was determined by colony-forming units per milliliter. Capric acid (C10) and caprylic acid (C8) have shown lesser degree of growth inhibition. VCO could not inhibit the *C. difficile* growth. However, bacterial cells could not grow when exposed to 0.15-1.2% lipolyzed coconut oil. The results of transmission electron microscopy clearly showed that at 2 mg/mL of lauric acid exposure both cell membrane and cytoplasm of cells was disrupted. Furthermore, the physiological changes in bacterial cell membrane integrity were also confirmed using live/dead staining following treatment with selected fatty acids. Inhibition of *C. difficile* using medium-chain fatty acids derived from VCO has been also demonstration.<sup>109</sup>

Finegold *et al.*, have investigated the effect of pomegranate extract in the management or prevention of CDIs or colonization. The activity of pomegranate was tested against 29 clinical *C. difficile* isolates using the Clinical and Laboratory Standards Institute-approved AD technique. Total phenolics content of the pomegranate extract was determined by Folin-Ciocalteu colorimetric method and final concentrations of 6.25-400 µg/mL gallic acid equivalent were achieved in



the agar. All strains had MICs at 12.5-25 mg/mL gallic acid equivalent range. The results suggest antimicrobial activity to be a useful tool in the management and prevention of *C. difficile* disease or colonization.<sup>110</sup>

The chemical composition and the antimicrobial activity of the essential oil of *Angelica archangelica* L. (Apiaceae) roots from central Italy were analyzed. The major constituents of the oil were  $\alpha$ -pinene (21.3%),  $\delta$ -3-carene (16.5%), limonene (16.4%), and  $\alpha$ -phellandrene (8.7%). The oil shows a good antimicrobial activity against *C. difficile*, *C. perfringens*, *E. faecalis*, *Eubacterium limosum*, *Peptostreptococcus anaerobius*, and *Candida albicans* with MIC values of 0.25%, 0.25%, 0.13%, 0.25%, 2.25%, and 0.50% v/v, respectively. Interestingly, weak antimicrobial activity against the useful intestinal microflora; bifidobacteria and lactobacilli has been observed with MIC values >4.0% v/v.<sup>108</sup>

*Nigella sativa* known as black seeds (BSs) have been used traditionally for treating various diseases in the Middle East regions for more than 2000 years.<sup>111</sup> Different active compounds in BS have been demonstrated for their antibacterial and antifungal activities such as thymoquinone.<sup>112</sup> BS were found to inhibit growth of many Gram-positive and Gram-negative bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium, and *Shigella flexneri*.<sup>113-116</sup> Another traditional herbal product is *Commiphora myrrha* (Myrrh). It has been also used in different medical contexts as astringent, antiseptic, antiparasitic, and antitussive and for treating leprosy, syphilis, and cancer.<sup>117</sup> Previous reports have indicated antibacterial activity of Myrrh against *E. coli*, *S. aureus*, *B. subtilis*, *Bacillus circulans*, *E. faecalis*, *P. aeruginosa*, and *Helicobacter pylori*.<sup>118-120</sup> In a recently published study, we have investigated both herbal extracts of BSs and Myrrh for their antibacterial activity against two strains of *C. difficile*. We have found that BS oil (2%) and Myrrh water extract are effective natural antibacterial agents to inhibit *C. difficile*.<sup>121</sup> In the later study, *in vitro* investigation suggest that the acidic environment of the human stomach (i.e., extreme acidic environment) does not compromise the effectiveness of treating human infection with *C. difficile* by oral administration of BS oil (2%) and Myrrh water extract.

## Conclusion

While IMT is considered as a promising approach with better outcome for treatment, especially in patient with recurrent CDI,<sup>81</sup> different antibiotics such as VAN, metronidazole, rifamycins and VAN are proven to have effective activity against CDI. However, overuse of such antibiotics, leading to possible emergence of antibiotic-resistant strains is potential threat. Therefore, natural antibacterial products, such as herbal extracts and essential oils that employ different inhibitory mechanisms, making it difficult for pathogens to develop resistance are potential future option. Taking into consideration

that some of these natural products, including *C. myrrha* (Myrrh), could have more inhibitory activity on *C. difficile* compared with their no-observed effects on other gut normal flora.<sup>121</sup> Further investigation is needed to explore the effect of these natural products on *C. difficile* pathogenesis, including toxin gene expression and colonization, *in vivo*.

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