The *Clostridium Difficile* Toxins: Mechanism of Action and Immunopathogenesis

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1 Introduction

*Clostridium difficile* infection (CDI) is now the most common definable cause of hospital-acquired and antibiotic-associated diarrhea in the United States, with the total cost of treatment estimated between 1 to 4.8 billion U.S. dollars annually (Dubberke and Olsen, 2012; DuPont, 2011; Kyne et al., 2002; Magill et al., 2014; O’Brien et al., 2007; Wilkins and Lyerly, 2003). This bacterium is responsible for 10–25\% of the cases of antibiotic-associated diarrhea, 50–75\% of antibiotic-associated colitis, and 90–100\% of pseudomembranous colitis (Bartlett, 2002; Elliott et al., 2007). Morbidity and mortality resulting from CDI-associated diseases have also increased significantly over the past ten years, making *C. difficile* the most common emerging pathogen in the US (Ananthakrishnan, 2011; McDonald et al., 2005; O’Brien et al., 2007; Redelings et al., 2007). *C. difficile* overpopulates the colon after the normal gut microbiota has been altered by antibiotic therapy. Therefore, the highest risk factor for CDI is previous antibiotic therapy (Bartlett and Perl, 2005). Treatment of CDI has been hampered by increased virulence of the causative strains, sporulation, recurrence of the infection, and antibiotics used in treatment that further alter the composition and colonization resistance of the normal colonic microbiota. Moreover, treatment with antimicrobials is, in as many as 25\% of cases, ineffective resulting in recurrence of the infection (Burke and Lamont, 2013; Hansen et al., 2013).

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Pathogenic strains of C. difficile possess a 19.6 kb pathogenicity locus, which is composed of tcdR, tcdB, tcdE, tcdA, and tcdC. This locus is responsible for the production of toxin A and toxin B, encoded by tcdA and tcdB, respectively. These toxins are essential in C. difficile pathogenesis to the extent that only strains that are able to produce either of these toxins cause disease (Geric et al., 2004; Kuehne et al., 2010; Lyerly et al., 1985; Rupnik et al., 2001; Voth and Ballard, 2005). This section specifically discusses recent data on the properties of the C. difficile toxins A and B, their mechanism of action, and the immunopathogenesis of these toxins.

2 The History of C. difficile

Clostridium difficile was first isolated in 1935 by Hall and O’Toole (Hall, 1935), who were investigating the development of normal bacterial flora in neonates. They collected feces of new-born babies from sterile diapers, suspended the feces in sterile water, and examined prepared slides microscopically using the Gram’s method (Bass et al., 2013) and methylene blue staining. Primary cultures were prepared and tested for the presence of aerobic bacteria on blood agar and eosin methylene blue lactose agar plates. The presence of anaerobic Bacilli was also tested using dextrose broth constricted tubes and deep iron brain medium. Different bacterial species were isolated and identified from the feces of these neonates including various species of Streptococcus, Micrococcus, Lactobacillus, and anaerobic Bacilli. The most interesting among them was an obligate anaerobic Bacillus (Figure 1) that was observed to be an actively motile, heavy-bodied rod with elongated sub-terminal or nearly terminal spores of about the same diameter as the rods (Hall, 1935). This obligate anaerobic Bacillus was associated with Kopfchenbacterien in feces from three of the infants. Both bacteria appeared and disappeared at about the same time under similar conditions. According to Hall and O’Toole (Hall, 1935), the isolation of each of the two was complicated when both were present, but this anaerobic Bacillus was more difficult to isolate than the Kopfchenbacterien. This bacterium was also much more difficult to study, due to its slower growth and lack of distinctive morphological properties.

Based on Hall and O’Toole’s account (Hall, 1935), the colonies of this anaerobic Bacillus generally emerged in deep agar after 48 hours of incubation and initially appeared minute, flat, and opaque. After 3 days, the colonies appeared lobulated in shape with a diameter of about 1 mm. Single colonies on blood agar slants under alkaline pyrogallol were irregular in form, flat, and non-hemolytic.

Dextrose broth cultures of 13 isolated strains of this new anaerobic Bacillus were incubated for 48 hours under anaerobic conditions at 37 °C and subcutaneously inoculated into guinea pigs. Following inoculation, the guinea pigs refused to eat and exhibited moderate to marked edema on the belly, breasts, and at the site of inoculation. Post-mortem observation showed marked subcutaneous edema with or without congestion, but with no emphysema. Smears from the subcutaneous site of inoculation in one animal that died within 4 hours showed numerous Gram-positive rods, but no spores or leukocytes were observed (Hall, 1935). In all the animals, no evidence of septicemia was
apparent, but phagocytic leukocytes were found with few free bacteria. A guinea pig injected with a culture filtrate died within 24 hours, whereas boiling of the culture filtrate for one minute completely destroyed the toxicity. These observations indicated that the new anaerobic Bacillus produced and released soluble exotoxins into the medium. Hall and O’Toole (Hall, 1935) thus, named this new anaerobic bacterium *Bacillus difficilis* (now called *Clostridium difficile*), due to the difficulties in its isolation and culture.

3 The Life Cycle of *C. difficile*

*C. difficile* exist either as actively dividing vegetative cells or as spores. The vegetative cells are obligate anaerobes and are sensitive to oxygen. In order to survive under aerobic conditions, *C. difficile* forms oxygen-resistant spores. Thus, the vegetative cells of *C. difficile* excreted in feces must transform into spores to survive aerobically (Jump *et al*., 2007). It is therefore, widely acknowledged that the *C. difficile* spores initiate the infection process by serving as the disseminating form of this obligate anaerobic pathogen.
As a result, germination of the spores into vegetative cells inside the gastrointestinal tract is essential in *C. difficile* pathogenesis. This is because only the vegetative cells cause disease.

Little is known about sporulation and germination in Clostridial species. However, the physiological and morphological changes that occur during sporulation in the well-studied Bacillus species are similar to that of Clostridial species (Lofland *et al.*, 2013). Sporulation is initiated under conditions of nutrient limitation or other unfavorable conditions, when the cells can no longer maintain vegetative growth. When *C. difficile* initiates sporulation, an asymmetrically placed division septum is formed that divides the cell into two unequal compartments (the mother cell and a forespore). Each of these two compartments contains one copy of the chromosome. The larger mother cell compartment engulfs the forespore leading to maturation (Hilbert and Piggot, 2004). The process of maturation 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 (Henriques and Moran, 2007).

The spore is metabolically dormant when released from the mother cell, but resistant to various harsh environmental conditions, including high temperature, oxygen, pH, alcohols, among others. The spores germinate and grow as vegetative cells under suitable conditions such as the presence of germinants, abundance of nutrients, and anaerobic conditions. In *B. subtilis*, germination can be induced by different compounds such as L-alanine or a mixture of asparagine, glucose, fructose, and potassium ions (Kelly *et al.*, 2012). The *B. subtilis* receptors that have been identified to be involved in sensing these environmental signals are GerA, GerB, and GerK. A large amount of calcium dipicolinate is released subsequent to sensing of the germinant, leading to hydration of the core, degradation of the cortex, and resumption of metabolism (Setlow, 2003). Homologs of GerA, GerB, and GerK have been identified in many Clostridial species except *C. difficile*. This suggests that *C. difficile* spores respond to a different kind of environmental signal for germination (Sebaihia, Wren *et al.*, 2006).

Cholate derivatives of bile salts, such as taurocholate and the amino acid glycine, act as co-germinants of *C. difficile* spores (Sorg and Sonenshein, 2010) and improve the germination of *C. difficile* spores from environmental surfaces and stool samples (Bliss *et al.*, 1997; Weese *et al.*, 2000). Lysozyme and thioglycolate also improve the colony formation of *C. difficile* spores (Kamiya *et al.*, 1989). However, the exact mechanism by which these molecules stimulate germination of *C. difficile* spores is unknown. Patients infected with *C. difficile* shed dormant spores in their stools. These spores remain viable on hospital surfaces for months and facilitate the spread of CDI and recurrence (Gerding *et al.*, 2008; Rupnik *et al.*, 2009). Thus, the formation of spores by *C. difficile* is a significant impediment in overcoming hospital-acquired *C. difficile*-associated diseases and recurrence. The spores contribute to the survival of this bacterium after treatment of surfaces with antiseptics and disinfectants as well as antibiotic therapy, which disrupts the colonic microflora and precipitates *C. difficile* infection, colonization, and overgrowth in the intestinal tract (Montoya and Detorres, 2013). As a result, there is a growing interest in utilizing agents that inhibit germination as a therapeutic strategy for *C. difficile* infections. For instance, Howerton *et al.* (2013) have demonstrated that a bile salt
analog, CamSA, inhibits *C. difficile* spore germination *in vitro*. They further demonstrated that a single dose of CamSA (50-mg/kg) protected mice from *C. difficile* infection and that lower doses of CamSA resulted in delayed onset of the infection and less severe disease symptoms (Howerton *et al*., 2013).

### 4 Epidemiology of *C. difficile* Infection

The incidence of *C. difficile* infections in acute care hospitals in the United States during the early 1990s was maintained at stable rate of 30 to 40 cases per 100,000 population (McDonald *et al*., 2005). By 2001, the incidence of CDI had risen to almost 50 per 100,000 population. The incidence rate in 2005 (84 per 100,000) was almost triple that of 1996 (31 per 100,000) with concomitant increases in the severity and fatality of this infection (Ji *et al*., 1997; Loo *et al*., 2005). Currently, the number of cases of CDI in hospitals in the United States exceeds 250,000 per year (over 80 cases per 100,000), with the total cost of treatment estimated between 1 billion and 4.8 billion U.S. dollars annually (Dubberke and Olsen, 2012; DuPont, 2011). The number of cases occurring in the community and non-hospital healthcare facilities appears to make CDI the most common form of bacterial diarrhea in the United States. Morbidity and mortality resulting from CDI in recent years have increased significantly as a result of changes in the virulence of the causative strains, the expanding number of the elderly and immunocompromised patients in the population, improved diagnostics, and antibiotic usage patterns (Gould *et al*., 2013; Longtin *et al*., 2013; McDonald *et al*., 2005; O’Brien *et al*., 2007; Redelings *et al*., 2007; Zilberberg *et al*., 2008).

It is generally felt that antibiotic therapy allows *C. difficile* to overcome the normal gut microbiota colonization resistance mechanisms against CDI, which include occupying the space required for *C. difficile* proliferation, direct impairment of *C. difficile* growth or germination, siphoning nutrients or germinants from *C. difficile*, and shaping the host’s innate and adaptive immune responses. CDI predominantly affects the elderly and immunocompromised patients in hospitals and nursing homes (Ji *et al*., 1997; McDonald *et al*., 2005). It is a frequent cause of morbidity and mortality among elderly hospitalized patients. Nevertheless, other populations are also at risk of the infection, such as young and healthy individuals who have not undergone antimicrobial therapy or were not exposed to a healthcare environment. Severe CDI that results in either death or colectomy has also been described in young women.

Asymptomatic carriage of *C. difficile* in children is estimated to be about 50% or higher (Schutze and Willoughby, 2013; Viscidi *et al*., 1981). Interestingly, infants have been reported to have high levels of toxigenic *C. difficile* and toxins in their stools, but exhibit no clinical symptoms (Jafari *et al*., 2013; Karadsheh and Sule, 2013; Rogers *et al*., 2013). Although, it is not known why neonates are unusually refractory to CDI, several theories have been proposed. First, the membrane receptors required for toxin binding in the colon may be absent in neonates. Secondly, mucins directly inactivate or neutralize the *C. difficile* toxins (Vyas *et al*., 2013). Third, the thick layer of colonic mucus in neonates may mask the toxin receptors. Fourth, the intestinal epithelial cells in neonates
have reduced sensitivity to intoxication than adult cells, and this may contribute to the asymptomatic carriage. In fact, infant hamsters are also insensitive to CDI (Deshpande et al., 2013; Lyerly et al., 1988).

The emergence of hypervirulent high toxin producing strains of C. difficile has contributed to the increasing incidence of CDI. C. difficile isolates collected between 2000 and 2003 from eight health care facilities in six states (Illinois, Pennsylvania, Maine, Georgia, New Jersey, and Oregon) during CDI outbreaks and analyzed by McDonald et al. (2005) showed that a single strain accounted for half of the isolates from five of the facilities (McDonald et al., 2005; Warny et al., 2005). Moreover, 82% of the stool samples from another outbreak in Quebec, Canada were positive for the same strain (McDonald et al., 2005). In the 1980s, this epidemic strain was initially identified by restriction endonuclease analysis and named BI, but it is currently referred to as North American Pulsed Field Type 1 (NAP1) and PCR ribotype 027 (i.e., BI/NAP1/027, or NAP1/027) (McDonald et al., 2005). The unique characteristics of this virulent NAP1/027 strain are increased toxins A and B production, fluoroquinolone resistance, and production of the binary toxin. Also, high-level of gatifloxacin and moxifloxacin resistance has been reported in recent isolates, but not in the original NAP1 strains. Resistant strains may have a competitive advantage in the hospital environment where fluoroquinolone use is widespread (Khodaverdian et al., 2013).

5 The C. difficile Pathogenicity Island

The main virulence factors of C. difficile essential for disease are two large toxins that are chromosomally encoded by the genes tcdA (toxin A) and tcdB (toxin B). The toxin genes together with the genes that encode the proteins TcdR, TcdE, and TcdC (Figure 2) are located within a 19.6 kb pathogenicity locus (PaLoc) in the C. difficile genome (Braun et al., 1996; Hammond and Johnson, 1995). Toxin A (308 kDa) is known to function as an enterotoxin causing gastrointestinal damage, whereas toxin B (269 kDa) is a highly potent cytotoxin (Lyerly et al., 1985).

![Figure 2: The pathogenicity locus of C. difficile. The tcdA and tcdB genes encode toxins A and B, respectively. The tcdR gene encodes a sigma factor that controls the transcription of the toxin genes, whereas the tcdC encodes a protein that has been proposed to play a negative role in toxin gene regulation by antagonizing TcdR. TcdE codes for a protein similar to holin, which is suggested to be involved in the release of the toxins.](image-url)
The tcdR gene, which is located upstream of tcdB in the pathogenicity locus, encodes an RNA polymerase sigma factor that regulates transcription from the toxin promoters and from its own promoter (Mani & Dupuy, 2001; Mani et al., 2002). TcdR is homologous to transcriptional activators of several Clostridium species and families of RNA polymerase sigma factors found in many organisms (Moncrief, Barroso et al. 1997). Proteins that have been found in other pathogenic Clostridia similar to TcdR include the sigma factors that control the tetanus neurotoxins (TetR) in Clostridium tetani, botulinum toxin (BotR) in Clostridium botulinum, and UV-inducible bacteriocin (UviA) in Clostridium perfringens (Dupuy et al., 2005; Raffestin et al., 2005). The first evidence for the role of TcdR in C. difficile toxin regulation was reported by Moncrief (Moncrief et al., 1997) and his co-workers using E. coli as a surrogate host. These results were supported by similar experiments using C. perfringens as a surrogate host and later in C. difficile (Mani & Dupuy, 2001; Mani et al., 2002). Biochemical and genetic evidence suggest that the role of TcdR is indispensable for initiation of transcription from the tcdA and tcdB promoters. Furthermore, the expression of TcdR and the toxin genes are both influenced in parallel by the growth phase, growth temperature, and the composition of the growth medium (Karlsson et al., 2003). The same expression pattern is observed for all the genes in the pathogenicity locus except tcdC, which is highly expressed during the rapid exponential growth phase and less expressed during the stationary phase (Hundsberger et al., 1997).

Genetic evidence suggests that TcdC negatively regulates toxin production by disrupting the capability of TcdR-containing RNA polymerase to recognize the tcdA and tcdB promoters (Mani & Dupuy, 2001; Mani et al., 2002). Thus, it has been proposed that tcdC encodes a negative regulator of toxin production (Hundsberger et al., 1997) and this concept has been supported by qualitative functional genetics and in vitro protein interaction studies (Carter et al., 2011; Matamouros et al., 2007). Evidence to support this hypothesis includes the inverse transcription pattern of tcdC in relation to the toxin genes, and the emergence of epidemic strains (NAP1/027 strains) with deletions or frame-shift mutations in the tcdC gene that produce high toxin levels (Carter et al., 2011; Curry et al., 2007; Hundsberger et al., 1997; Matamouros et al., 2007; McDonald et al., 2005; Warny et al., 2005). Moreover, all NAP1/027 isolates from the 1980s and 1990s, like those from recent outbreaks, carry tcdC mutations (Loo et al., 2005; McDonald et al., 2005). These reports highlight the importance of tcdC in the pathogenesis of C. difficile. However, Cartman and co-workers (Cartman et al., 2012) found no association between toxin production and the tcdC genotype when they deleted the tcdC gene by allelic exchange. Furthermore, restoration of the Δ117 frame-shift mutation and the 18-nucleotide deletion that occur naturally in the tcdC gene of some virulent C. difficile strains such as R20291 did not alter toxin production (Cartman et al., 2012). These reports suggest that the regulation of C. difficile toxin synthesis is more complex than previously thought and that the mechanism may involve other key regulatory elements.

Indeed, our laboratory recently reported that C. difficile toxin synthesis is regulated by an accessory gene regulator quorum signaling system (Darkoh et al., 2015). Using an unbiased biochemical and genetic approach and a classic quorum signaling bioassay, our laboratory determined that C. difficile toxin synthesis is regulated by a novel cyclic quorum-signaling thiolactone peptide. The thiolactone was purified from the stationary
phase supernatant by acetone precipitation, anion exchange chromatography, and HPLC. The purified thiolactone induced early transcription of the *C. difficile* toxin genes and stimulated elevated toxin production. Furthermore, the thiolactone was detected in stools from *C. difficile*-infected patients, but not in *C. difficile*-negative stools from patients with diarrhea. This underscores the clinical relevance of the cyclic thiolactone in *C. difficile* pathogenesis during infection. An isogenic toxin synthesis mutant was generated by Himar-based random mutagenesis, which was determined to contain an insertion in the accessory gene regulator (Agr) response regulator gene, *agrA*. This mutant is able to generate the thiolactone, but does not respond to the thiolactone. Response to the thiolactone and toxin synthesis was restored by complementation with the wild-type *C. difficile* *agrA* gene. These findings provide direct evidence that *C. difficile* toxin synthesis is regulated by an Agr quorum signaling system and offers new avenues for both rapid CDI detection and development of quorum signaling-based non-antibiotic therapies to combat this life-threatening emerging pathogen.

Between the toxin genes is a small open reading frame, *tcdE*, which encodes a putative holin, a protein whose activity is thought to allow the release of the toxins from the cell (Tan *et al.*, 2001). The *tcdE* open reading frame encodes a small, hydrophobic protein with 166 amino acids comprising a short hydrophilic stretch at the N-terminus and a series of charged residues at the C-terminus. TcdE is predicted to contain three transmembrane domains with structural features and a primary sequence similar to class I holins. Holins are small membrane proteins encoded by double-stranded DNA phages required for lysis of host cells following completion of intracellular phage development (Wang *et al.*, 2000; Young *et al.*, 2000). Holins oligomerize in the plasma membrane of the host cell forming a disruptive lesion, which enables the transport of prophage-encoded endolysin (a muralytic enzyme) across the membrane (Desvaux *et al.*, 2009). The prophage-encoded endolysin hydrolyzes the murein of the host cell leading to cell lysis and release of the phage particles. Even though, most holins are associated with terminal lysis of phage-infected bacteria, some holin-like proteins are known to be involved in the release of proteins from uninfected bacteria (Desvaux *et al.*, 2009).

TcdE was initially suggested to play a role in the secretion of *C. difficile* toxins due to its homology to holins. Govind and Dupuy (Govind and Dupuy, 2012) demonstrated empirically that TcdE is required for efficient secretion of the *C. difficile* toxins and facilitates release of toxins without inducing cell lysis or general membrane permeability. On the other hand, Olling *et al.* insertionally inactivated the *tcdE* gene and observed no delay or inhibition of toxin release (Olling *et al.*, 2012). Olling, *et al.* further stated that inactivation of TcdE did not either alter the kinetics of toxin release or the absolute level of secreted toxins A and B, suggesting that TcdE does not account for the pathogenicity of *C. difficile*. Moreover, no significance difference was observed between the wild-type and *tcdE*-deficient *C. difficile* when the secretome was analyzed by mass spectrometry, thus, excluding the proposed secretory role of TcdE. In *C. difficile*, *tcdE* encodes a 19-kDa protein but when expressed in *E. coli*, TcdE appears as a 19 and 16-kDa protein. The truncated 16-kDa protein was associated with bacterial cell death, suggesting that TcdE does not exhibit pore-forming function in *C. difficile*, since only the non-lytic full length 19-kDa protein is present (Olling *et al.*, 2012).
6 The Large C. difficile Toxins

The toxins A and B are the essential virulence factors in C. difficile pathogenesis and belong to a family of the large Clostridial glucosylating toxins (Geric et al., 2004; Lyerly et al., 1985; Rupnik et al., 2001; Voth and Ballard, 2005). Strains that do not produce either of these toxins are not associated with disease (Elliott et al., 2007; Voth and Ballard, 2005). Both toxins have similar enzymatic cleavage activities (Dillon et al., 1995; Just et al., 1995a; Just et al., 1995b) and are cytotoxic to cultured cells; however, toxin B is 100-1,000-fold more potent than toxin A (Just and Gerhard, 2004; von Eichel-Streiber et al., 1996; Voth and Ballard, 2005). C. difficile toxins A and B share high amino acid sequence identity and similar in structure (Pruitt et al., 2010). These toxins are structurally similar to each other (Figure 3) with an N-terminal enzymatic domain composed of a glucosyltransferase domain and an autocatalytic cysteine proteinase domain, a central translocation domain encompassing a hydrophobic region, and a C-terminal receptor binding domain made up of Clostridial repetitive oligopeptides (CROPs) (Jank and Aktories, 2008; von Eichel-Streiber et al., 1996).

Figure 3: Structural comparison of C. difficile toxins A and B. These toxins have three domains: an N-terminal enzymatic domain consisting of a glucosyltransferase domain and an autocatalytic cysteine protease domain (CPD); a central translocation domain (TMD) encompassing a hydrophobic region (HR); and a C-terminal receptor binding domain containing the Clostridial repetitive oligopeptides (CROPs). The DXD (Asp-X-Asp) motif and a conserved tryptophan (W102) present in the glucosyltransferase domain are involved in Mn²⁺ and UDP-glucose binding. The DXG (Asp-X-Gly) motif in the TMD region of TcdB has an aspartate protease activity, which may be involved in toxin cleavage (Sun et al., 2010).

The N-terminus of the toxins harbors the glucosyltransferase activity, which is the biologically active domain, and a domain with conserved catalytic triad (Asp587-
His653-Cys698) of a cysteine protease, which mediates toxin autocleavage during internalization in the host cell (Sun et al., 2010).

The crystal structure of the TcdB glucosyltransferase domain has been determined and the essential amino acid residues involved in the glucosyltransferase reaction or substrate binding have been identified (Reinert et al., 2005). The Asp-X-Asp (DXD) motif and a conserved tryptophan (W102) play a role in Mn\(^{2+}\) and UDP-glucose binding (Reinert et al., 2005). There is limited information concerning the transmembrane domain and its function. The transmembrane domain comprises more than 50% of the total amino acid content of the toxins. It also includes a hydrophobic region whose role may be for membrane insertion.

The CROPs of the receptor binding domain has 21-, 30-, and 50- repetitive amino acid residues. The CROPs of TcdA contains between 30 and 38 contiguous repeats, whereas that of TcdB has between 19 and 24 repeats (Greco et al., 2006; Ho et al., 2005). The CROPs may be involved in the initial target cell interaction and receptor binding by the toxins. The crystal structure of the receptor binding domain of TcdA showed a solenoid-like structure that has been proposed to increase the surface area of proteins and enable protein-protein or protein-carbohydrate interactions (Greco et al., 2006; Ho et al., 2005). TcdA has been reported to bind to the trisaccharide, Galα1-3Galβ1-4GlcNAc, carbohydrate antigens, components in human milk, and glycosphingolipids (Kriván et al., 1986; Rolfe and Song, 1993; Teneberg et al., 1996; Tucker and Wilkins, 1991). The crystal structure of TcdA was solved in complex with the synthetic carbohydrate, Gal-α1-3Gal-β1-4GlcNAc (Greco et al., 2006). On the contrary, a functional α-galactosyltransferase does not exist in humans, suggesting that Gal-α1-3Gal-β1-4GlcNAc cannot be an intestinal receptor in human (Jank et al., 2007). The disaccharide Gal-β1-4GlcNAc, which is present in humans, has therefore been suggested to be part of the host receptor (Jank et al., 2007). Little is known about the TcdB receptor, but it has been suggested that the TcdB receptor appears to be at the basolateral sites, whereas the TcdA receptor is on the apical sites on the host intestinal cells (Stubbe et al., 2000). However, researchers have been unsuccessful in identifying the actual host receptor for the toxins. The interaction between the receptor binding domain of the toxins and the host cell receptors initiates receptor-mediated endocytosis (Florin and Thelestam, 1983; Karlsson, 1995; Tucker and Wilkins, 1991).

7 The C. difficile Binary Toxins

Some C. difficile isolates including the epidemic NAP1/027 strain also produce a third toxin that is unrelated to the pathogenicity locus called the C. difficile binary toxin (CDT). The binary toxin was first isolated by Popoff et al. in a patient with severe pseudomembranous colitis (Popoff et al., 1988). This toxin is detected in 17% to 23% of C. difficile strains in the general population (Eckert et al., 2013) and encoded by two genes, cdtA and cdtB, located on the CDT locus. C. difficile strains carrying the CDT locus have been grouped into specific toxino-types; e.g. toxino-types III, IV, V and XI, or more rarely, to strains for which the pathogenicity locus is absent (Geric et al., 2003).
The binary toxin is a two-component ADP ribosyltransferase comprising the enzymatic component (CDTa) and binding component (CDTb) and its genes were first sequenced in 1997 (Perelle et al., 1997). It has emerged that the CDT locus is present either as a whole or as a truncated version (Stare et al., 2007). There is a unique 68 bp sequence in the chromosomal location of the CDT locus in strains that lack the whole or truncated CDT (Gerding et al., 2014). The mature enzymatic component (CDTa) has two domains with a mass of ~48 kDa (Gerding et al., 2014). Both domains exhibit analogous folding and this has been suggested to be due to duplication of an ancient ADP-ribosyltransferase gene (Han et al., 1999). The N-terminal region of mature CDTa consists of residues 1–215 and has 5 α-helices and 8 β-strands, probably involved in the interaction with the binding component (CDTb). The C-terminal region covers residues 224–420 and houses the ADP-ribosyltransferase activity. The binding component CDTb has 4 domains and consists of 876 amino acids with a molecular mass of about 98.8 kDa and expressed with a signal sequence of 42 amino acids (Gerding et al., 2014).

The role of the binary toxin in C. difficile pathogenesis is not clear. Toxins A- and B-negative binary toxin-positive strains cause fluid accumulation in rabbit ileal loops, but no diarrhea or death in hamster models (Doder et al., 2013). Moreover, C. difficile strains that produce the binary toxin in the absence of toxins A and B do not appear to cause disease. The production of the binary toxin by NAP1/027 epidemic strains has renewed speculation that this toxin may act synergistically with toxins A and B in causing severe colitis (Loo, Poirier et al. 2005; McDonald, Killgore et al. 2005).

8 The Mechanism of C. difficile Toxins A and B Entry into the Host Cell

During infection, toxins A and B are released into the intestinal lumen where they bind to surface receptors on colonic epithelial cells via their receptor-binding domain (Figure 4). They are then internalized by the host cell by receptor-mediated endocytosis (Dingle et al., 2008; Ho et al., 2005). The acidic environment within the endosomes activates the cysteine protease activity of the toxins, which cleaves and releases the glucosyltransferase domain located at the N-terminus into the cytosol of the mammalian host (Egerer et al., 2007; Hofmann et al., 1997; Pfeifer et al., 2003; Reineke et al., 2007; Rupnik et al., 2005). A host cofactor, inositol hexakisphosphate, has been suggested to serves as a trigger of the cysteine protease-mediated autocatalytic cleavage of the toxins (Reineke et al., 2007).

Small GTPases (8–28 kDa) are characterized by their C-termini that are polyisoprenylated, and an intrinsic ability to bind to guanine nucleotides. In addition, they serve as molecular relays that transmit signals when bound to GTP and discontinue signal transmission when bound to GDP (Sun et al., 2010). Small GTPases are subdivided into the subfamilies of Rho, Rab, Ras, Ran and Arf, and Ran. The Rho subfamily members (Rho GTPases or Rho proteins) such as RhoA, Rac1, and Cdc42 are the major known intracellular targets of TcdA and TcdB (Just and Gerhard, 2004). Once in the cytosol, the glucosyltransferase effector domain of the toxins mono-O-glucosylates low molecular weight GTPases of the Rho family (RhoA, Rac1, and CDC42) using cellular
Figure 4: Mechanism of *C. difficile* toxins entry into host cells. The toxins bind to unknown host cell receptors and are subsequently internalized by receptor-mediated endocytosis (Reineke *et al.*, 2007). The low pH within the endosomes stimulates the autocatalytic cysteine protease activity of the N-terminally located enzymatic domain, resulting in the release of the glucosyltransferase domain. The released glucosyltransferase inactivates small molecular weight GTPases by monoglucosylation (Voth and Ballard, 2005).

Uridine diphosphoglucose (UDP-glucose) as the glucose donor (Just and Gerhard, 2004; Just *et al.*, 1995a). In the GDP-bound form, Rho GTPases are inactive and associate with guanine nucleotide dissociation inhibitors, which keep the GTPases in the cytosol. Rho GTPases interact with different effectors to control several signaling processes upon activation by guanine nucleotide exchange factors (Sun *et al.*, 2010). They regulate many host cell functions, such as epithelial barrier functions, adhesion, phagocytosis, cytoskeleton, immune cell migration, and immune cell signaling (Jank and Aktories, 2008; Just and Gerhard, 2004).

Monoglucosylation of the Rho GTPases by the toxins interrupts their normal functions leading to various deleterious effects including massive fluid secretion, apoptosis, cell rounding, actin cytoskeleton dysregulation, loss of tight junction integrity, acute inflammation and necrosis of the colonic mucosa, and altered cellular signaling (Genth *et al.*, 2008; Hofmann *et al.*, 1997; Huelsenbeck *et al.*, 2009; Just and Gerhard, 2004; Just *et al.*, 1995a). Cellular intoxication by the toxins also induces the release of various immunomodulatory mediators from epithelial cells, phagocytes, and mast cells resulting in inflammation and accumulation of neutrophils (Pothoulakis, 2000; Thelestam and Chaves-Olarte, 2000). The clinical manifestations of *C. difficile* infections are highly vari-
able: ranging from asymptomatic carriage, mild self-limiting diarrhea, to severe and mostly fatal pseudomembranous colitis.

9 Characterization of Toxin A and B Activity

Purified toxins A and B were tested for their ability to cleave p-nitrophenyl-β-D-glucopyranoside (PNPG), a substrate similar to the native substrate of the toxins, uridine diphosphogluucose (UDP). From this analysis, the PNPG substrate was established to be useful for the detection of the C. difficile toxin A and B activity (Darkoh et al., 2011). To confirm the cleavage of the PNPG substrate by each of the toxins, Western immunoblot analysis was used. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed on purified PNPG-active toxins A and B and transferred onto a membrane. Monoclonal antibodies specific for each toxin were used to probe the membrane for the presence of each toxin. Single bands were observed in each of the samples that had PNPG activity and contained either purified toxin A or toxin B due to their specific reactivity with monoclonal antibodies that recognize toxin A or B, respectively. These results demonstrate that both toxins A and B cleave the PNPG substrate, and consistent with the reported in vivo activity of the toxins, in that they both cleave the same cellular substrate, UDP-glucose (Just and Gerhard, 2004; Just et al., 1995a; Just et al., 1995b).

Both toxins A and B demonstrate optimal PNPG cleavage activities within a pH range of 7–9 (Figure 5A). In contrast to toxin A, which showed significant activity within the pH range of 6 to 12, toxin B displays a more narrow range of PNPG cleavage activity within the pH range of 7 to 10. This is consistent with the pathophysiological environment of the colon, where C. difficile causes disease. The pH of the colon varies from 6.4 ± 0.6 to 7.5 ± 0.4 (Khan et al., 2010). Both toxins shows activity optima at a temperature range of 35–40°C, with toxin A showing a broader range of activity than toxin B (Figure 5B) (Darkoh et al., 2011).

Based on the ProtParam analysis (Gasteiger E., 2005) of the amino acid sequences of the toxins, toxin A has a total of 588 charged residues out of 2710 residues, of which 54% and 46% are negatively and positively charged, respectively. Toxin B has more charged residues (597 out of a total of 2366 residues); 66% and 34% are negatively and positively charged, respectively. These data support the lower isoelectric point (IEP) of 4.42 estimated for toxin B compared to that of toxin A (5.51). The implication of this lower IEP for toxin B is a wide pH range for the maintenance of its overall negative charge at physiological pH. Toxin A is computed to be more stable with an instability index (Guruprasad et al., 1990) of 29.6 compared to that of 36.5 for toxin B. However, both toxins are estimated to have relatively long in vitro half-lives based on the N-terminal end rule (Bachmair et al., 1986; Gonda et al., 1989; Tobias et al., 1991) of 30 hours. These computational data suggest that toxin A should function in and tolerate a wider range of physiological and environmental conditions than toxin B (Darkoh et al., 2011).
Figure 5: Effect of pH (A) and temperature (B) on the PNPG cleavage activities of toxins A and B. For the pH experiment, the toxin activity assay was performed by incubating 100 µg of toxin A or B with 3 mM PNPG at 37 °C for 4 hrs in buffers at the various pH values shown. For the temperature experiment, the toxin activity assay was performed by incubating 100 µg of toxin A or B in 50 mM Tris-HCl containing 50 mM NaCl (pH 7.4) with 3 mM PNPG at the temperatures indicated for 4 hrs. The assay was monitored by absorbance at 410 nm. Error bars represent standard deviation between two replicate experiments (Darkoh et al., 2011).
The activity of both toxins follow the Michaelis-Menten curve (Figure 6), indicating that the toxins have a single active site. The Michaelis-Menten constant (Km) values of the toxins for the PNPG substrate as determined by the non-linear regression are 1.04 mM for toxin A and 0.24 mM for toxin B. The maximum velocity (Vmax) for toxin A for the PNPG substrate is 1.5 µmoles/mg/min, whereas that for toxin B is 6.4 µmoles/mg/min. These data indicate that the affinity of toxin B for the PNPG substrate is more than 4-fold higher than toxin A. Moreover, the rate of cleavage of the PNPG substrate was 4.3-fold faster for toxin B than toxin A. These results agree with assays of the relative damage by toxins A and B to tissue culture cells, in which toxin B was found to be more potent than toxin A (Just et al., 1995a; von Eichel-Streiber et al., 1996; Voth and Ballard, 2005).

![Figure 6: The Michaelis-Menten plot for the PNPG cleavage by C. difficile toxins A and B based on non-linear regression method. For toxin A: Km = 1.04 ± 0.06 mM and Vmax = 1.50 ± 0.03 µmoles/mg/min. For toxin B: Km = 0.24 ± 0.02 mM and Vmax = 6.40 ± 0.12 µmoles/mg/min. Error bars represent standard deviation from four replicate experiments (Darkoh et al., 2011).](image)

10 Inhibition of the C. difficile Toxins A and B Activity

To further characterize the toxin-substrate interactions, our laboratory has identified bile salts and their derivatives as potent inhibitors of the C. difficile toxin activity. For instance, the addition of 300 mM of sodium taurocholate reduced the activities of toxins A and B within one hour of incubation by 71% and 86%, respectively (Figure 7). Interestingly, taurocholate and phosphatidylserine (both negatively charged lipids) have been reported to inhibit β-glucosidases in a non-competitive manner (Choy and Davidson, 1980; Grabowski et al., 1984; Holleran et al., 1992; Peters et al., 1976). These results support the idea that the cleavage of the PNPG substrate is due to the glucosyltransferase/hydrolase activities of the toxins (Darkoh et al., 2011).
Figure 7: Dose-response inhibition by sodium taurocholate of toxin A and B PNPG cleavage activities. These experiments were performed by incubating 55 µg of each toxin for 1 hr with the amount of sodium taurocholate indicated at 37 °C in 30 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, and 10 mM of the PNPG. Error bars indicate standard deviation from three different experiments (Darkoh et al., 2011).

Treatment of C. difficile infections has been hampered by the recurrence of the infection, multi-drug resistance, emergence of strains with increased toxin production, and the indiscriminate use of antibiotics that further alter the colonization resistance of the gut microbiota. As a result, there is an urgent need for non-antibiotic treatments, either as stand-alone therapies or as therapies designed to augment the efficacy of currently used antibiotic regimens. An important potential treatment approach is to inhibit the activities of toxins A and B, which are directly responsible for the intestinal damage and subsequent inflammation associated with the infection. This approach, which targets the toxins without affecting bacterial cell growth, may be ideal since it is unlikely to impose selective pressure on the intestinal microbiome, thereby minimizing the risk of developing resistance.

Physiologic concentration of taurocholate (5 mM) was able to protect human colonic epithelial Caco-2 cells from C. difficile toxin B-mediated damage (Darkoh et al., 2013). When taurocholate and toxin B (16 µg) are added simultaneously to confluent Caco-2 cell monolayers, toxin-mediated cytopathic effects is prevented (Figure 8).
Figure 8: Effect of C. difficile toxin B and taurocholate on Caco-2 Cells. Confluent Caco-2 cell monolayers were incubated with 8 and 16 µg of toxin B in the presence or absence of 5 mM taurocholate in a total medium volume of 2 ml in 24-well plates for 24 hrs. Images were captured using an EVIS XL microscope. Magnification 10X. Tox B, purified toxin B; TC, taurocholate (Darkoh et al., 2013).

One of the mechanisms by which C. difficile toxins mediate cell damage is by inducing apoptosis. Specifically, toxin A has been reported to induce cell death in human epithelial cells ex vivo by activating caspases (Brito et al., 2002; Carneiro et al., 2006; Gerhard et al., 2008). Toxin B also induces caspase-3 production in Caco-2 cells in a dose-dependent manner (Figure 9) (Darkoh et al., 2013). Moreover, taurocholate reduced caspase-3 production in the presence of lethal toxin B concentrations.

The mechanism of taurocholate-mediated inhibition of C. difficile toxin activity remains to be determined. Brandes et al. (Brandes et al., 2011) reported that tauroursodeoxycholic acid, a modified conjugated bile acid, affect the host cell by inducing phosphorylation of Rac1/Cdc42 that inhibited C. difficile toxin B-mediated monoglucosylation of this GTPase. Taurocholate may also function through hydrophobic interactions to saturate the Caco-2 cell membranes, thereby inhibiting toxin entry and/or toxin activity. Other inhibitory mechanisms may involve direct effects of taurocholate on toxins such as alterations to toxin structure leading to loss of activity, or binding of taurocholate to the toxins leading to the prevention of entry into the host cell. Further research is ongoing to identify the mechanism of taurocholate action.
Figure 9: Effect of C. difficile toxin B and taurocholate on caspase-3 activity in Caco-2 cells. Caco-2 cells were incubated for 48 h with 0, 4, 8, 12 and 24 µg of toxin B in the presence or absence of 5 mM taurocholate. Cell monolayers were scraped from the bottoms of wells of a 24-well plate and lysed to obtain crude protein lysates. Caspase-3 activity was determined using Caspase-3 Colorimetric Kit (Invitrogen, Carlsbad, CA). Crude protein lysates (75 µg) were incubated with DEVD-NA caspase-3 substrate reagent for 8 h at 37 °C and absorbance at 410 nm was measured. A molar extinction coefficient for p-nitrophenol of ε = 17700 M⁻¹cm⁻¹ was used in the calculations (Shikita et al., 1999). The error bars represent the standard deviation from three different experiments.

The majority of nutrient absorption in the gastrointestinal tract occurs in the small intestine, where bile salts are at much higher concentrations compared to the colon. This difference in bile salts concentration is due to the reabsorption of more than 95% of the total human bile via the enterohepatic circulation in the ileum (Dowling, 1973), which is directly proximal to the colon. Clearly, only a small amount of bile salts enter the colon where C. difficile most frequently colonizes. An intriguing explanation for the C. difficile pathology being mostly limited to the bile salt-deficient colon and the associated sparing of the bile salt-rich small intestine is that toxin activity may be inhibited in the small intestine by the high bile salt concentrations. The C. difficile toxins may be active in the colon because of its low bile salt concentrations and not active in the small intestine due to its high bile salt concentration. Thus, bile salt concentration may represent a host-
mediated mechanism that naturally protects the absorptive surfaces of the small intestine from deleterious microbial products produced by pathogens such as *C. difficile* and acts to inhibit bacterial growth. Moreover, the lack of bile salts in the small intestine in diseased states (such as cirrhosis of the liver) may lead to bacterial overgrowth and result in competition for the essential nutrients required for normal human growth and function. The therapeutic benefits of bile salts are well documented; they prevent hepatocyte injury and cholestasis (Heuman *et al.*, 1991a; Heuman *et al.*, 1991b; Poupon *et al.*, 1991), drug-induced cholestasis (Queneau *et al.*, 1993), and endotoxin absorption (Bailey, 1976; Gouma *et al.*, 1986). Uncovering a mechanism to deliver higher concentrations of bile salts and/or their derivatives, perhaps in conjunction with antibiotics into the colon of individuals suffering from recurrent *C. difficile* infections may help protect the colon from the damaging effects of the *C. difficile* toxins and facilitate clearance of the pathogen. This line of research may result in a novel treatment of *C. difficile* infections that can produce recurrent and chronic disease or death.

11 Immunopathogenesis of the *Clostridium difficile* Toxins A and B

Major gaps still remain in our understanding of the immunopathogenesis of *C. difficile* infections (CDI), despite its increasing prevalence rates and poor patient outcomes. Our laboratory evaluated the relative amounts of 36 major biomarkers from stools collected from *C. difficile* infected- and non-*C. difficile*-infected patients. The data demonstrate that the immunopathogenesis of *C. difficile* infections is complex and elicits both Th1 and Th2 response, with an increased expression of pro-inflammatory proteins (Figure 10) (Darkoh *et al.*, 2014).

Interleukin-8 (IL-8) and IL-23 appear to be important in the immunopathogenesis of CDI. These two cytokines were detected in the majority of the CDI-positive stools compared to the CDI-negative stools. The average concentration of IL-8 in the CDI-positive stools was significantly (*p* < 0.05) higher than that of the stools from CDI-negative and hospitalized controls without diarrhea. IL-8 is a chemoattractant involved in the recruitment of neutrophils to sites of infection and has been implicated to play a key role in the pathogenesis of CDI (Tixier *et al.*, 2005). Increased levels of IL-8 is associated with more severe forms of CDI (Steiner *et al.*, 1997). Moreover, a single nucleotide polymorphism (SNP) in the promoter region of the IL-8 gene that increases its expression is associated with susceptibility to CDI (Jiang *et al.*, 2007). These reports are consistent with our data showing high levels of IL-8 in the majority of CDI-positive stools compared to the stools from the CDI-negative and hospitalized controls without diarrhea.

The average concentration of IL-23 in the CDI-positive stools was lower than that of the CDI-negative and hospitalized controls without diarrhea, even though IL-23 was present in a larger number of the CDI-positive stools. IL-23 is produced by activated macrophages and dendritic cells and plays an important role in host defense against
Figure 10 (A): Comparison of Th1 and Th2 cytokines in CDI-positive and CDI-negative stools. Fold change in amount of Th1 (IFN-γ, IL-2, IL-12, TNF-α) and Th2- (IL-4, IL-5, IL-6, IL-10, IL-13) cytokines obtained using Proteome Profiler Human Cytokine Array assay (R&D Systems, Minneapolis, MN). Stools (300 mg) from 100 antibiotic-associated diarrheal patients (50 CDI-positive and 50 CDI-negative) were evaluated for the presence of 36 inflammatory proteins using the Proteome Profiler Human Cytokine Array Panel A kit (R&D Systems, Minneapolis, MN). Data expressed as the mean of the relative band intensity of each cytokine. Stools from the hospitalized controls without diarrhea were not evaluated by the initial assay, but were included retrospectively in the quantitative ELISA for comparison. Error bars represent the standard error of measurement between two replicates per sample. * = \( P < 0.05 \).
Figure 10 (B): Comparison of Th1 and Th2 cytokines in CDI-positive and CDI-negative stools. Concentrations of IFN-γ, TNF-α, and IL-13 in CDI-positive stools and stools from CDI-negative diarrheic patients and hospitalized controls without diarrhea, determined by quantitative ELISA (R&D Systems, Minneapolis, MN). The Krustal-Wallis test showed significant differences between the means (p< 0.0001). Horizontal bar = mean concentration in μg/ml. Key: IFN-γ, interferon-gamma; IL-2, interleukin-2; IL-12, interleukin-12; TNF-α, tumor necrosis factor-alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-10, interleukin-10; IL-13, interleukin-13.
bacterial infections and development of chronic inflammation (Iwakura and Ishigame, 2006). During bacterial infection, antigen-stimulated dendritic cells and macrophages produce IL-23 that promotes the development of Th17 cells leading to enhanced priming of memory T cells (Iwakura and Ishigame, 2006; Yen et al., 2006). This results in induction and production of a variety of inflammatory mediators that triggers potent inflammatory responses. IL-23 also stimulates generation of pro-inflammatory cytokines such as IL-1, IL-6, IFN-γ, and TNF-α through its effects on dendritic cells and macrophages (Lankford and Frucht, 2003; Oppmann et al., 2000; Parham et al., 2002). The average fecal concentration of IL-23 in the stools of CDI-positive patients was significantly (P<0.05) lower than that of hospitalized controls without diarrhea and CDI-negative patients. This suggests that the amount of IL-23 produced during CDI may be inadequate to sustain the cellular immunity conferred by this cytokine in promoting the induction and proliferation of effector memory T cells. Thus, decreased production of IL-23 may explain the lack of robust immunological response exhibited by a proportion of CDI patients and may also relate to recurrence. Perhaps, boosting the level of IL-23 may help activate the cellular immune response required for a robust response to CDI.

The average concentrations of lactoferrin and calprotectin in healthy adults range between 1.45–4.6 µg/ml (Joshi et al., 2010; Kane et al., 2003) and ≤ 10 µg/ml (Dolwani et al., 2004), respectively. This is consistent with the concentrations obtained from our study; 6.8+/−0.85 µg/ml (for lactoferrin) and 10.2+/−0.92 µg/ml (for calprotectin). Elevated levels of lactoferrin and calprotectin in stools is associated with colonic inflammation (Konikoff and Denson, 2006). Our data shows that 88% and 80% of the CDI-positive stools had average lactoferrin and calprotection concentrations, respectively, higher than the hospitalized controls without diarrhea. These results agree with that of Shastri et al. (Shastri et al., 2008), in which 85.1% and 82.8% of CDI patients stools had higher levels of lactoferrin and calprotectin, respectively, than healthy adults. Lactoferrin and calprotectin serve as part of the innate inflammatory response and so their overexpression during CDI may provide insight into the extent of inflammation associated with this infection.

The differentiation of naïve CD4+ helper T-cells into either Th1 or Th2 cells is critical in the development of adaptive immune response (Murphy and Reiner, 2002). Th1 inflammatory response usually induces IFN-γ production leading to activation of phagocytes, whereas Th2 response results in humoral immunity, allergic inflammation, and stimulates host resistance to intracellular infections or agents (Abbas et al., 1996; Finkelman et al., 2004). The patterns of Th1- and Th2-associated cytokines found in the CDI-positive stools were not distinct from that of the CDI-negative stools (Darkoh et al., 2014). However, the concentrations of TNF-α and IL-13 in the stools of CDI-positive patients were significantly higher than that of the CDI-negative and hospitalized controls without diarrhea. This suggests a mixed Th1/Th2 response during CDI and infers that the host response to CDI is complex, pro-inflammatory, and encompasses both the innate and the adaptive arms of the immune system. These results may also denote a probable intracellular response to the toxins and an extracellular response to the bacterium.
12 Conclusion Remarks

Our understanding of the immunopathogenesis of the *C. difficile* toxins and their mechanisms of action has advanced as a result of the various exciting developments in the field. However, many important questions remain unanswered in spite of the progress made to date. These include the respective roles of toxins A and B in the host inflammatory response, a thorough understanding of the host immune response to the toxins, the exact function of the binary toxins in pathogenesis, identification of the specific host receptors for toxins A and B, generation of anti-toxin vaccines, and the development of non-antibiotic therapeutics for the treatment of the infection. With the increasing importance of *C. difficile* infections in public health, considerable research is warranted to fill these gaps in our knowledge of the immunopathogenesis in order to better prevent and treat the disease to reduce its public health burden.

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