REVIEW ARTICLE

Virulence Factors Associated with *Clostridiodes difficile*: An Overview

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Abstract

Clostridiodes difficile is a health threat mainly acquired *via* the feco-oral route and colonizes the human gut. There is a wide range of clinical presentation of *C. difficile* infection (CDI). *C. difficile* can be accountable for 15–25% of antibiotic-related diarrhea and up to 100% of pseudo-membranous colitis. Clinically important *C. difficile* are evolving and increasingly being reported globally. The pathogenesis of *C. difficile* is associated with many established and potential virulence factors. They include toxins, surface layer proteins, cell wall proteins, flagella, fimbriae, spores, etc. The main virulent factors of CDI are toxin A and toxin B, both of which share a high structural and functional resemblance between them. Both these toxins are responsible for neutrophil infiltration marked by mucosal insult and colitis which is a significant feature of CDI. These toxins also influence the cytoskeletal features, despite the difference in activity potency. A third toxin, produced by some *C. difficile* strains, contains components of both toxin A and toxin B and is referred to as the binary toxin. The role of this toxin in CDI virulence is not clear. Besides the above described virulence features there are other probable factors that could be involved in *C. difficile* colonization. They are flagella, surface layer protein, production of tissue degradative exoenzymes, and sporulation. In this overview, the virulence factors associated with *C. difficile* shall be discussed to highlight their potential role in the disease.

Keywords: Binary toxin, *Clostridiodes difficile* infection, Flagella, Spores, Surface layer proteins, Toxin A, Toxin B, Virulence factors.

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Introduction

Clostridiodes difficile is an anaerobic, heat-resistant-endospore-producing gram-positive bacillus with peritrichous flagella. It belongs to the Phylum Firmicutes and Family *Clostridiaceae*. Toxigenic and epidemic *C. difficile* is a well-established health threat and a leading cause of infectious diarrhea in patients exposed to the hospital environment[1,2] as well as in persons in the community.[3]

The organism is mainly acquired *via* the feco-oral route and colonizes the intestinal tract of humans. The clinical presentation of *C. difficile* infection (CDI) ranges from asymptomatic carriage, diarrhea, simple colitis, pseudo-membranous colitis, acute severe colitis, and recurring CDI. *C. difficile* could be accountable for 15–25% of antibiotic-related diarrhea and up to 100% of pseudo-membranous colitis.[4,5] The severity of infection includes high rates of leukemoid reactions, severe hypoalbuminemia, toxic megacolon, need for colectomy, and ultimately shock and death.[6] Exacerbation of ulcerative colitis due to CDI[7] and a higher risk for CDI in pancreatic disease patients[8] have also been reported.

Heightened awareness of CDI outbreaks has led to an increase in the surveillance for the disease. Epidemic and clinically important *C. difficile* with several PCR (polymerase chain reaction) ribotypes are evolving and is increasingly being reported from all over the world. The hypervirulent NAP1/BI/027 (North American Pulse Field type I/Restriction Endonuclease Assay type BI/Ribotype 027) strain of *C. difficile* is linked with a higher incidence of the disease and an increased rate of morbidity and mortality. In this overview, the virulence factors associated with *C. difficile* shall be discussed to highlight their potential role in the disease.

Virulence Factors of *C. difficile*

The pathogenesis of *C. difficile* is associated with many established and potential virulence factors. They include toxins, surface layer proteins, cell wall proteins, flagella, fimbriae, spores, etc. The main virulent factors of CDI are the two exotoxins A and B, the genes for which are positioned closely to each other within a pathogenicity locus (PaLoc) in the pathogenicity island.[9] Both these toxins are responsible for neutrophil infiltration marked by mucosal insult and colitis which is a significant feature of CDI.[10] These toxins also influence the cytoskeletal features, despite the difference in activity potency.

Both the *C. difficile* exotoxins have a high molecular weight making them the largest bacterial protein toxins, along with some other clostridial proteins like those of *C. sordellii* and *C. novyi*. These large clostridial toxins and glycosylate small guanine triose phosphate (GTP)-binding proteins[11] are solely present in the Rho and Ras GTPases[12] which are a family of hydrolase enzymes. Zhu et al.[13] reported identification and characterization of a new cell wall hydrolase Cwl0971 from a *C. difficile* strain. The 0971 gene deletion mutant showed delayed cell autolysis and increased cell viability which impaired the release of toxin A and B and affected sporulation.

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Toxin A

Toxin A (TcdA) is a 308 kDa proteinaceous enterotoxin encoded by gene *tcd*A. It is a lethal enterotoxin causing hemorrhage and fluid secretion in the rodent gut. Toxin A induces extensive damage to the intestinal epithelial cells and therefore accounts for almost all of the gastrointestinal symptoms. It is considered as the main virulence factor of *C. difficile* as it causes severe damage to the gut.[14] Toxin A has also been reported to disrupt the tight junctions of the intestinal epithelial lining by acting as a cytotoxin and this might be a significant mechanism involved in toxin enterotoxicity.[15] Various cytokines and neurokinins, playing a significant role in CDI pathogenesis, are induced by toxin A.[16] Toxin A causes cell rounding and cell detachment from the basement membrane, leading to apoptosis. Rapid loss of macrophages, T cells, and eosinophils also occur. Massive inflammation due to neutrophil infiltration results in denuding of the gut mucosa and damage to the intestinal epithelium. Katyal et al.[17] observed disturbances in the intestinal brush border membrane enzymes of CDI patients.

Toxin B

Toxin B (TcdB) is a 269 kDa potent toxin encoded by *tcdB* gene. It is largely a cytotoxin identified by its cytopathic effect on tissue culture cells[18] and is 1,000 fold more potent than toxin A as a cytotoxin. Toxin B does not by itself damage the gut possibly because of its lack of ability to attach to particular receptors on the brush border membrane of the gut.[19] Toxin A bind to the specific receptors on the intestinal wall to bring about the damage. Next, toxin B connects to gain access to the underlying tissue.[19] Partial detachment of cells occurs due to development of neurite-like retraction. Later on the cell-spanning stress fibers wane and actin filaments gather in the perinuclear space.[20] It disorganizes the actin filaments, brings about a loss of intracellular potassium, and a reduced protein and nucleic acid synthesis.[21] Toxin B has been found to suppress interleukin-2 expression, disrupt tight junctions, and stimulate nitric oxide production.[22,23]

Structure and Function of Major *C. difficile* Toxins

Toxin A and toxin B share a high structural and functional resemblance between them with a 63% sequence of amino acids.[24] The structure of *C. difficile* toxins was earlier described as having three parts, namely, a binding domain of C-terminal, a catalytic domain of N-terminal,[24] and a central hydrophobic region.[25] However, later on this toxin structure representation was substituted with a structural model of four-domains comprising the glucosyltransferase, the cysteine protease, the translocation, and the receptor-binding domains.[26]

A number of messenger RNAs are transcribed from the toxinogenic element, including a 17.5 kb polycistronic transcript.[27] Owing to the sequence similarity and the position on PaLoc, both *tcd*A and *tcd*B genes are supposed to have a common ancestor and are the result of gene duplication.[24] Together with three additional genes, namely, *tcd*C, *tcd*D, and *tcd*E, the *tcd*A and *tcd*B genes form the 19.6 kb PaLoc found only in the toxinogenic isolates.[28] Gene *tcd*E is a cell wall hydrolase gene, and *tcd*R gene—an alternative sigma factor—helps in the positive transcriptional regulation while *tcdC* serves as a presumed negative regulator.[29] The *tcd*A gene with 8,133 nucleotides is found between *tcd*E gene and the divergently transcribed *tcd*C gene.[30]

Sequencing and transcription analysis has shown that *tcd*D encoding a 22 kDa protein necessary for transcription of the toxin genes[31] when interacts with *tcd*C works as a positive regulator for TcdA and TcdB expression.[27,30] This has therefore been renamed as *Tcd*R.[32] The *tcd*B gene having 7,098 nucleotides is located between *tcd*R and *tcd*E genes. Tan et al.[33] demonstrated the bactericidal effect of *tcd*E when expressed in *Escherichia coli*. *Tcd*E is structurally and functionally similar to holins. It may facilitate the release of toxins to the extracellular environment.[33] Olling et al.[34] reported that a *tcd*E mutant neither delays nor inhibits the release of toxins A and B.

Due to the lack of negative regulation there is an increased production of toxins A and B as a consequence of the deletions of 18 and 39 bp found in *tcd*C gene forming truncated TcdC proteins.[35] There is a marked increase in the virulence of the NAP1/BI/027 strains due to 18 bp deletion in the *tcd*C gene thus producing both toxins in higher quantities and at higher rates.[36] NAP1/BI/027 strains are reported to generate about 16 times more toxin A and 23 times more toxin B.[36]

A second deletion at position 117 of a single-base-pair in the *tcd*C gene was found in all Canadian NAP1/BI/027 strains, and in a United Kingdom reference strain.[37] This strain has enhanced toxin production, a faster sporulation rate, and increased antimicrobial resistance, particularly to fluoroquinolones.[36,38,39] Surprisingly hyper-production of toxins has also been reported in a *C. difficile* strain with no *tcd*C mutations and normal levels of toxin production in a strain with *tcd*C mutation.[40] Thus, it appears that mutation in the *tcd*C gene is not definitively related to increased clinical virulence[41,42] and there could be other regulators of toxin expression involved in the hyper-production of toxin in some isolates of *C. difficile*.

The superfamily Ras comprising Rho, Rac, and Cdc42 GTPases within the intestinal cells gets targeted for alteration *via* glycosylation by the toxins. When this alteration occurs, it leads to activation of the guanosine triphosphate (GTP) binding Rho proteins after the toxins gain entry into the cytoplasm, resulting in interruption of critical signaling pathways in the cell.[29] In addition to the intracellular inactivation of GTPases, toxins A and B also bring about other morphological and physiological changes to the intestinal epithelial cells. The Rho proteins are engaged in the creation of focal adhesion complexes and stress fibers. They polymerize the actin, maintain the cytoskeletal structural design, as well as the cell movement.[43] The actin cytoskeleton gets regulated by these GTPases. The changes in the epithelial cell wall *via* Rho protein glycolysation involves at least two pathways including disaggregation of actin microfilaments leading to increased permeability of tight junctions and untimely discharge of proinflammatory cytokines from the intestinal epithelium resulting in stimulation of mast cells, vascular endothelium, and immune cells.[44] F-actin cytoskeleton forms aggregates after the spherical cells become thin and rope-like.[45]

The Rac proteins are responsible for membrane ruffling as well as lamellipodia formation. In some cell types, this is also induced by Rho proteins. Cdc42 brings about the formation of filopodia or microspikes. This change activates the tiny regulatory proteins and causes interruption in the fundamental cell signaling pathways[29] and tight junctions, causing excessive fluid accumulation and destruction of the intestinal epithelial lining.[46]

After colonization of the gut, toxin A along with toxin B comes into play. Both these clostridial toxins bind to the surface

receptors present on the intestinal epithelial cells, damage them to undergo apoptosis, modify the actin cytoskeleton, and increase the permeability of the tight junctions.[9] In the beginning, TcdA forms homodimers to bind the carbohydrate groups. Then, the toxin appears in coated pits which are then internalized. Once the toxin B accesses the underlying tissue, it brings about widespread damage with the disease getting progressed further. Thus it appears that both the toxins work synergistically.[19] *C. difficile* enters the intestinal cells and thereby inactivates the important intracellular signals. Concurrently there is a release of pro-inflammatory interleukins and tumor necrosis factor-α with an increase in vascular permeability. Toxin A has been found to stimulate substance P—an inflammatory mediator—thus triggering inflammation.[47] Neutrophils and monocytes get recruited to the site of injury and tissue degradation starts due to the production of hydrolytic enzymes leading to the formation of pseudomembranous colitis. A severe inflammatory reaction occurs in the lamina propria, because of the activity of the toxins. This is followed by the development of tiny ulcerations in the mucosa of the colon enclosed by a pseudomembrane.[48]

Apart from their role in precipitating CDI, toxin A and toxin B together are the principal markers for the disease diagnosis and can be detected in the fecal samples of patients by laboratory assays. Strains of *C. difficile* that are nontoxigenic do not cause disease.

Typing of *C. difficile* isolates can be done by restriction endonuclease analysis, pulse field gel electrophoresis, or PCR ribotyping. *C. difficile* strains can be distributed into 34 currently known toxinotypes (I to XXXIV) depending on the changes in both toxin genes.[49] Singh et al.[50] reported toxigenic culture of 95 (54.6%) toxigenic and 79 (45.4%) nontoxigenic *C. difficile* isolates from stool samples of CDI patients. Toxinotyping revealed that 121 (69.5%) of these isolates were toxigenic with 76 (62.8%) belonging to toxinotype 0 and 45 (37.2%) to toxinotype VIII. PCR ribotyping revealed that 36.8% of these belonged to ribotype 001, 33.9% to ribotype 017, and 13.2% to ribotype 106.[51] Partial sequencing of genes from 10 isolates showed changes in toxin A sequences of 5 (50%) isolates with translated nucleotide substitution in just 3 (30%) of them.[51]

Binary Toxin

Since 1987, another iota-like toxin produced by some *C. difficile* strains was identified. This toxin known as the binary toxin (CDT) contains components of both toxin A and toxin B. The role of this toxin in CDI virulence is not clear. This toxin was not cytotoxic to tissue epithelial cells, nor it was found to be lethal to animals upon intraperitonial inoculation.[52] Despite this, cytotoxicity brought about by CDT appears to be analogous to that of both toxin A and toxin B.[53] It has the potential to act in conjunction with toxins A and B or to act alone in so-called “nontoxinogenic” strains.

Up to 2% of *C. difficile* produce only binary toxin and 4–12% of isolates are positive for this toxin.[40,54,55] Binary toxin could be a significant virulence factor of *C. difficile* as it is present in the epidemic NAP1 strain. It is envisaged that CDT alone is not enough to commence the disease, but might play a role in the later stages of infection. Cytotoxic activity to Vero cells[56] and significant morphological changes to Caco-2 cells *in vitro* by purified binary toxin have been demonstrated.[57] CDI patients infected with a CDT positive isolate compared to those with a CDT negative isolate have a higher case-fatality rate infection.[58] All upcoming *C. difficile* hypervirulent strains possess this toxin, suggesting that the binary toxin could be a marker for increased virulence or that it might contribute to increased virulence, by acting in synergy with toxin A and B, exacerbating the toxicity of the strain.[58] As a matter of fact, the binary toxin is linked with the majority of severe outbreaks of drug-resistant CDI in the present century.[59]

Structure and Function of Binary Toxin

Binary toxin has been encoded in a different region called CdtLoc outside the PaLoc. This toxin comprises two unlinked molecules—one, the 48 kDa enzymatic component encoded by the 1,392 nucleotides (*cdt*A gene) and the other is a binding component of 94 kDa encoded by the 2,631 nucleotide (*cdt*B gene). Both these genes act synergistically.[53] Upstream of the *cdt*AB genes, *cdt*R a regulator gene, belonging to the LytTR family of response regulators, is located. It has no detectable sensor kinase common to other members of the family and therefore is considered as an orphan response regulator.[60] The lack of a functional *Cdt*R results in a 15-fold decrease in binary toxin production.[61]

Due to proteolytic cleavage, the binding component of the binary toxin gets activated and binds to the exposed cell surface receptor forming heptamers and prepore. Next the enzymatic component of the toxin binds to prepore-receptor complex.[62,63] Subsequently the toxin-receptor complex gets endocytosed. A conformational change in the heptamers occurs due to the low pH of the endosome, leading to membrane insertion and pore formation. The enzymatic component then gets translocated into the cytosol with the help of the host chaperones.[64] Once entry is gained, the enzymatic component ribosylates adenosine diphosphate monomeric G-actin at Arg177 and thereby inhibits the polymerization of G-actin to F-actin.[65] This toxin induces the production of a new kind of microtubule structures, which consist of long microtubule-dependent protrusions on the epithelial cell surface which promote bacterial adherence and colonization.[57]

Additional Virulence Factors

Besides the above-described virulence features, there are other probable factors that could be involved in *C. difficile* colonization. They are flagella, surface layer protein (SLP), production of tissue degradative exoenzymes,[66] and sporulation. The surface proteins and the flagella of *C. difficile* adhere to the colonic wall, particularly in individuals with depleted normal gut flora. Fimbriae may also act as potential mediators of attachment to intestinal mucosa thus enhancing the pathogenesis.[67] However, these factors are not clearly understood and their roles in *C. difficile* virulence are greatly speculative.

Surface Layer Proteins

The SLP is an adhesion factor, represented as one of several potential surface associated genes present in a group of 17 open reading frames along with cwp66, a cell wall protein. SLP is paracrystalline, proteinaceous arrays that envelop the cell wall of all *C. difficile* strains. The unique *slp*A gene comprises 2,160 bp codes for the SlpA precursor protein of 73.4 kDa.[68]

The *C. difficile* S-layer is composed of the precursor protein, SlpA. The S-layer proteins are composed of a surface protein with a low molecular weight (32–38 kDa LMW-SLP) and a cell wall-associated protein with a high molecular weight (42–48 kDa HMW-SLP).[69] The two subunits of the protein self-assemble to form a lattice and are structurally placed over one another showing square symmetry of the external LMW-SLP layer and hexagonal symmetry of the inner HMW-SLP layer.[69–71]

Surface layer protein can cause binding of *C. difficile* to host intestinal brush border membrane and thereby permit targeted delivery of toxins to enterocytes. Next, after toxin-induced epithelial damage occurs, SLP binding to extracellular matrix components is also liable to add considerably to further the tissue damage. Calabi et al.[72] reported the existence of a high degree of variability in the molecular masses of the two proteins of the S-layer of *C. difficile*.

Cell-associated Protein

Cell-associated proteins help in *C. difficile* adherence to the intestinal epithelial cells and are also considered as virulent factors. Cell wall protein, CwpV, is a large SlpA homolog, expressed in a phase variable manner. Antibodies against cell wall proteins have been observed in sera of CDI patients signifying their immunogenicity and *in vivo* expression.[73] Emerson et al.[74] suggested that it may be associated with immune evasion.

Flagella

Before establishing infection, the bacteria need to adhere to the tissue to start colonization or else shall be immediately removed by nonspecific host defense means.[75] Flagellum is required for movement, adherence, and invasion of mucosal surfaces as well as direct interaction with the host immune system.[76] Bacterial flagella consist of three parts: basal body, hook, and helicoidal filament. Even though nonflagellated strains occur, flagella are found on the surface of most *C. difficile* strains.[77] The presence of amplified flagellum genes in nonmotile strains propose that the flagella expression could be phase-variable.[78] Environmental signals regulate the translation of flagellum proteins; those strains that appear nonmotile *in vitro* may actually be motile *in vivo*.[79] Tasteyre et al.[80] reported that flagellated *C. difficile* led to a ten times higher adherence to mouse cecum tissue compared to unflagellated strains.

The virulence factor associated with adherence is the flagellar filament, protein C (FliC), along with the flagellar cap protein D (FliD).[81] *fliC* gene comprises 870 bp and its corresponding protein of 290 amino acid.[75] *C. difficile* genome has only one copy of *fliC*. Quite a lot of conserved alanine residues accountable for the α-helical conformation of the filament are present in FliC. Its N-terminal responsible for secretion and C-terminal for polymerization are also conserved. High conservation of FliC has been found between clinical strains isolated over a short stretch of time.[78] Between different *C. difficile* strains the central region is divergent, as it is surface-exposed; antigenic drift causes selection of variants and is therefore a useful genetic marker for epidemiological studies.[82]

The 39 kDa protein of *C. difficile* flagella shows similarity in all flagellated strains and is therefore responsible for the cross-agglutination observed in serogrouping reactions.[77] The genetic differences for the analysis of *fliC* can be seen using a typing method involving restriction fragment length polymorphism (RFLP).[75,79] The *fli*D is a 1524 bp gene coding for the 56 kDa FliD cap protein and composed of 507 amino acids.[80] FliD is highly conserved, surface-exposed, and does not have variable domains. It has a very precise purpose of attaching to cell or mucus receptors. Two main RFLP patterns have been observed by treatment of *fli*D with a variety of restriction endonucleases highlighting the conservation of its genetic sequence.

Tissue Degrading Exoenzymes

Other virulence factors found in some *C. difficile* strains are protease, collagenase, hyaluronidase, and other hydrolytic enzymes. They also add to the adhesion and dissemination of organism *in vivo*.[83]

Fimbriae

Infrequently, the presence of fimbriae has also been implicated for their role in infection.[83] However, their absence does not suggest affecting colonization or infection.[84,85]

Capsule-like Material

Some strains of *C. difficile* also possess a capsule-like material which might be implicated in adhesion and evasion of the immune system through its antiphagocytic properties.[86]

Spores

Spores are also factors for *C. difficile* pathogenesis because of its hard coat which helps the organism to survive disinfectants, heat as well as drying conditions. The spores shield *C. difficile* from unfavorable situations like antibiotics, nutrient deficiency, and bactericidal immune response, thereby increasing the virulence of the organism. Spore formation is regulated by gene *spo0*A, a master regulator of the sporulation pathway. This is related with high spore production as also with formation of biofilms, which is a possible reservoir for the restitution of CDI after initial therapy of the patients.[87]

Conclusion

Even though *C. difficile* has several virulence factors associated with its pathogenesis, the most important ones are toxin A and toxin B. The role of binary toxin is also being delineated as a virulence factor as all emerging hypervirulent strains of *C. difficile* possess this toxin, suggesting that it might contribute to increased pathogenesis, by acting in synergy with toxin A and B. Other virulence factors like flagella, surface layer protein, sporulation, etc., add to the virulence of the organism.

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