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A Review – Elucidating the Virulence Factors of *Clostridiodes difficile*

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ABSTRACT

Clostridiodes difficile a spore forming, Gram positive anaerobic bacterium, has been observed to be the most prevalent infectious cause of antibiotic associated diarrhea (AAD) which has both mild and chronic cases. Virulence factors of *C. difficile* include Toxin A and Toxin B (TcdA and TcdB) and CDT toxin. Toxigenic *C. difficile* may produce two major toxins which act as glycosyltransferases, toxin A (308 kDa) and toxin B (270 kDa). These toxins on release from the cell modify through glycosylation the Ras superfamily of small GTPases within enterocytes, thus inactivating these proteins and, hindering important cell signaling pathways. Thus this review aimed to collate and discuss the virulence factors of *C. difficile. C. difficile* from literature search was observed to have multiple virulence factors and multidimensional in functionality which could be contributing to its adverse clinical manifestation. Thus, therapeutic handling of infected cases has to be multidimensional in approach, not just aimed at destroying causative organisms but also targeting other virulence factors such as damages caused by its toxins and its sporulation capacity.

Keywords: *C. difficile*, Virulence, Toxins, Binary Toxin Genes, Glycosyltransferases, Pathogenicity Locus, Antibiotics.

Introduction

Clostridiodes difficile a spore forming, Gram positive anaerobic bacterium, has been observed to be the most prevalent infectious cause of antibiotic associated diarrhea (AAD) (Mutlu *et al*., 2007, Lynch *et al*., 2013). The clinical manifestations of *C. difficile* can range from mild diarhoea to mortality, and antibiotic therapy has been affected by the pathogen's capacity to develop resistance, which is being researched, e.g. determining the presence of *nim* genes as a potential contribution to resistance to metronidazole, the effect of hemin on metronidazole susceptibility, (Mohammed & Baines 2024a, Mohammed & Baines 2024b), and nitroreductase association to resistance e.t.c. The understanding of *C. difficile* virulence factors could aid determination of more therapeutic measures. This review is hereby aimed at collation of the virulence factors of *Clostridioides difficile* thereby aiding the management of infections.

Virulence factors of *C. difficile* include Toxin A and Toxin B (TcdA and TcdB) and CDT toxin. Toxigenic *C. difficile* may produce two major toxins which act as glycosyltransferases, toxin A (308 kDa) and toxin B (270 kDa). Cellular activities such as actin cytoskeleton organisation are controlled by the Rho GTPase family. However, TcdA and TcdB are part of the clostridial glucosylating toxin family and inhibit the action of Rho, Rac and Cdc42 which are of the GTPases family within colonic epithelial cells (Voth and Ballard, 2005).

C. difficile may possess a pathogenicity locus on its chromosome on which both toxin genes can be encoded. The toxins are approximately 66% similar and the genes have a low guanine and cytosine (G+C) content compared to the rest of the *C. difficile* genome. The similarity between toxins can be seen in N and C terminals (Voth and Ballard, 2005).

On the pathogenicity locus both toxin genes are separated by a 1350 nucleotide sequence and on the same locus there are 3 other open reading frames that might aid the regulation of toxin removal from the cell or toxin synthesis. Both genes of the toxins are transcribed in the same direction.

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The *tcdC* gene is also present in the pathogenic locus and is expressed in the early exponential phase, its gene transcription is in the opposite direction to *tcdA* and *tcdB* and is situated downstream of *tcdA* (Voth and Ballard, 2005). TcdC has been suggested to be a negative regulator to the production of these two toxins because the onset of stationary phase leads to a decrease in the production of TcdC and increase in production of the two toxins.

However, there has been some dispute on the role of TcdC. It was reported that TcdC in *C. difficile* 630Δerm did not significantly regulate toxin expression (Bakker *et al*, 2012). One major positive regulator of both toxins is TcdD whose gene is found upstream of *tcdB.* TcdD is a sigma factor whose response is dependent on environmental factors (Hundsberger *et al.*, 1997, Voth and Ballard, 2005). TcdE has been reported to possibly promote toxin release through *C. difficile* cell wall and it is structurally similar to a bacteriophage holin protein. The *tcdE* gene is located between *tcdA* and *tcdB* genes. Environmental factors also contribute to toxin production which could be classified under catabolite repression and stress. Sub-inhibitory levels of some antibiotics such as vancomycin could lead to toxin production, in limiting levels. Biotin has also been observed to induce toxin production (Yamakawa *et al*, 1996, Voth and Ballard 2005). These toxins on release from the cell modify through glycosylation the Ras superfamily of small GTPases within enterocytes, thus inactivating these proteins and, hindering important cell signaling pathways. The toxins need an acidified endosome for translocation and access the interior of the cell by receptor mediated endocytosis. On gaining entry into the cell, the inactivation of GTPases involves the monoglucosylation of an active threonine responsible for the binding to GTP thereby causing actin condensation, cell rounding and death (Voth and Ballard, 2005). Recent studies have succeeded in generating mutants for both toxins' in single toxin knockouts, meaning both toxins can act independently to cause disease, as demonstrated by Toxin A^-B^+ strains *in vivo* (e.g. ribotypes 078). Kuehne *et al.* (2010) generated a strain lacking both toxins and the double knockout was avirulent (Kuehne *et al.*, 2010).

With regards to the CDT toxin, Perelle *et al*. (1997) reported in *C. difficile* CD196 the presence of protein encoded by a binary toxin gene *cdtA* and *cdtB.* This protein was an ADP–ribosyltransferase, was observed to function in initiating actin alterations. Both genes encode for different function in the protein, *cdtA* encodes for CDTa which functions as the ADP-ribosyltransferase enzyme, whereas the binding component is carried out by CDTb (encoded by *cdtB*). These genes are similarly transcribed and spaced as a result of the presence of 52 non-coding nucleotides between them in *C. difficile* (Perelle *et al.*, 1997).

CDT was observed to cause depolymerisation of the actin cytoskeleton, which generates microtubules based membrane protrusions that creates a network on epithelial cells and facilitate bacterial adherence (Gerding *et al.*, 2014). CDT binary toxin gene was identified in human *C. difficile* isolates in Brazil for the first time in a 2015 report (Silva *et al.*, 2015).
Goncalves *et al.*, 2004, observed CDT in 6% of 369 strains analysed. These strains were isolated in Paris from AAD patients in healthcare facilities (Goncalves *et al.*, 2004). CdtR is a LytTR family response regulator, and was observed to be responsible for maximal production of CDT. The *cdtR, cdtB* &*cdtA* gene locus is referred to as Cdt loc (Carter *et al.*, 2007). The role of *CdtR,* was questioned in another research study on *C. difficile* ribotype 078, a mutation was observed in the *cdtR* gene which might have prevented the secretion of the binary toxin but this was not the case (Metcalf & Wease, 2010).

The role of CDT in causing disease has been suggested in some research. A report in Australia of a 15-year - old hospitalised boy with diarrhoea and other health complications, showed a negative stool test for toxin A and toxin B using the Chek complete rapid membrane enzyme immunoassay kit (Androga *et al.*, 2015). However, a secondary analysis was performed and *C. difficile* was isolated anaerobically and typed using PCR ribotyping. The strain of *C. difficile* was of ribotypes 033 and was confirmed to be toxin A^- and toxin B^- but was positive for the binary toxin. The presence of *C. difficile* RT014/020 known to lack CDT but produce toxins A and B was later also detected using a different test. These findings intimate that CDT in absence of toxin A and B might be able to contribute to CDI however this is yet to be confirmed (Androga *et al.*, 2015).

Furthermore,, in France *C. difficile* toxinotype XI of zoonotic origin only possessing binary toxin genes has been observed in patients with apparent CDI.

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The presence of toxinotype XI is rare in humans but this could largely be due to it not being noticed since the routine test for *C. difficile* test is a test fortoxin A and B. So Cdt^+ only strains could have been classified as not toxigenic (Eckert *et al.,* 2015). This study showed that infection of *C. difficile* strain, positive only to the binary toxin was pathogenic giving an inclination to the severity of the disease elicited by this toxin (Eckert *et al.,* 2015).

Other factors contributing to the virulence of *C. difficile*

Quorum sensing

The production of toxins in *C. difficile* has also been observed to be facilitated and regulated by quorum sensing. The presence of antimicrobial therapy increases the presence of *C. difficile* due to death of the intestinal microbial flora (Darkoh *et al*, 2015). Thus a thiolactone signal is synthesized by these *C. difficile* as the population increases; the thiolactone signal is a form of quorum sensing done by *C. difficile* to quantify its population in an environment before toxin production. Due to continuous cell increase the thiolactone signal reaches a break point which leads to the transcriptional activation of toxin genes as a result of the activation of a 2 component system Agr C2A2 (Darkoh *et al*, 2015).

Colonisation Factors

Surface layer proteins (SlpA) have been observed to be a major adherence factor in *C. difficile* to epithelial cells (Merrigan *et al*, 2013). An anaerobic quantitative assay was used to detect the level of attachment to host epithelial cells by vegetative cells of *C. difficile* strains. It was observed that the *C. difficile* isolates analysed which included isolates obtained during CDI outbreaks, have high adherence capability, partially as a result of the presence of SlpA (Merrigan *et al.,* 2013).

C. difficile surface associated protein Cwp84 protease, observed to be associated to S – layer proteins, was characterised and reported to be an inactive proprotein which can be expressed as an active form after a process of maturation as a result of trypsin action or the reducing environment within the colon (Janoir *et al*, 2007). Cwp84 was reported to be responsible for the degradation of vitronectin and laminin as well as the cleaving of fibronectin, thus aiding toxin diffusion (Janoir *et al*, 2007).

Biofilm

Biofilms have been earlier reported to protect bacteria from adverse environments such as antibiotic environments due to therapy. Not much information is known with respect to biofilms produced by anaerobic bacteria, this could be as a result of challenges encountered in creating the required environment for *in vitro* formation of biofilms. *C .difficile* biofilms have also been proposed to have the potential to prevent the effect of cellular immune response during CDI thus contributing to pathogenesis and *C. difficile* recurrence (Dapa & Unnikrsihan 2013). Additionally, biofilms may protect *C. difficile* vegetative cells and spores from antimicrobial agents and therefore facilitate recurrence following the cessation of antimicrobial therapy.

Certain features have been observed to influence *C. difficile* biofilm formation, e.g. glucose was observed to increase biofilm formation in *C. difficile* 630 but not in R20291 (ribotype 027), thus the gut nutritional environment can impact on *C. difficile* biofilm formation. A well-developed S layer was observed to be required for biofilm formation probably due to its role in early stage biofilm formation in holding proteins that aid surface binding. *C. difficile* motility was also observed to be an important factor in biofilm formation, since a flagellin knockout mutant, which is a major protein component of flagella, was observed to be defective in formation of biofilm *in vitro* (Dapa *et al.,* 2013). The data obtained in the research also indicated the involvement of quorum sensing mediated by *lux Sin vitro*. Dapa *et al.,* 2013 also speculated that initiation of biofilm formation, sporulation and toxin production could be a role done by *spoOA.*

Biofilms have been demonstrated to possess a gradient of nutrient availability and are often nutrient limited within microcolonies. Therefore, sporulation in *C. difficile* may be initiated within biofilms and several studies have demonstrated the presence of *C. difficile* spores in microtitre plate biofilms and in more complex fermentation vessel models (Dapa *et al.,* 2013, Crowther *et al.,* 2014). Sporulation in *C. difficile* is controlled by the master regulator of sporulation, Spo0A, and this regulatory protein has been observed to be involved in biofilm formation. Spo0A has been observed in *B. subtilis* to influence biofilm matrix formation and phosphorylated Spo0A has been observed to activate biofilm and sporulation formation.

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Biofilm matrix is activated by Spo0A-P action via SinI a depressor of matrix formation by SinI binding to SinR, a repressor of matrix formation (Chai *et al*., 2011, Viamakis *et al*., 2013). Thus the switch from matrix formation to sporulation is dependent on SpoA-P levels which if high, turn off matrix formation and switch on sporulation. Additionally, this switch is also affected by the number of copies of the Sin I – SinR complex in a cell (Viamakis *et al*, 2013).

Sporulation

Stress conditions such as depletion of nutrients enhance the formation of the dormant form of *C. difficile* commonly referred to as spores (Awad *et al.*, 2014). *C. difficile* sporulation ability enables this pathogen to withstand the stomach acidity and aerobic environment when not inside a host (Smith *et al.*, 2016). Awad *et al.*, 2014 also referred to spores as infectious particles necessary for transmission of the infection. The spore consists of inner and outer coat, with the outer coat made up of four layers approximately 75nm in width, surrounding the inner coat (Driks, 1999). These coats enclose a core that contains the organisms entire genome copy (Awad *et al.*, 2014). A *Clostridium difficile* strain of hypervirulent ribotype 027 was observed to have diverse sporulation features and similar sporulation rates to other ribotypes analysed (Burns *et al.*, 2011).

Membrane vesicles

C. difficile was observed to secrete membrane vesicles (MVs) consisting of 262 proteins. In Caco-2- cells, these MVs stimulated the expression of proinflammatory cytokine genes, also surface associated proteins, flagellin and GroEL proteins were also observed using proteomic analysis. Cytotoxicity in cells of colonic epithelium was observed to be induced by *C. difficile* MVs. However the role of MVs in CDI is yet to be elucidated (Nicholas *et al.*, 2017).

Conclusion

The effectiveness of *C. difficile* as a pathogen is aided by the expression of the multiple virulence factors as elucidated above. Future research into these virulence factors and antimicrobial targeting of them with inhibitor molecules may add to the therapeutic interventions available to manage *C. difficile* infection and alleviate the need for, or supplement conventional antimicrobial therapy targeting vegetative bacterial cells.

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