CHAPTER 1

1. GENERAL INTRODUCTION

1.1. *Clostridium difficile*: General Background

*Clostridium difficile* is a gram-positive bacterium belonging to the genus *Clostridium*, which is a member of phylum Firmicutes. The genus *Clostridium* contains more than 120 species that include both common free-living bacteria and important pathogens. Toxin producing *Clostridium difficile* is one such bacterial pathogen, well-known as a causative agent of antibiotic-associated diarrhea and pseudomembranous colitis (PMC) in human (Freeman, et al. 2010; Sebaihia, et al. 2006).

In 1935, scientists Hall and O’Toole were the first to describe *C. difficile* as an anaerobic, gram-positive, spore-forming, cytotoxin-producing bacillus from microbial flora of faeces of healthy newborn infants (Hall and O’Toole 1935). Initially, it was believed that *C. difficile* had no deleterious effects in human (Smith and King 1962). Later on, during the period of Second World War, while studying the function of penicillin in the treatment of gas gangrene induced by *Clostridium perfringens*, Hamre and his colleagues (Hamre, et al. 1943) reported that penicillin caused typhlitis in rodent model was more lethal compared to *C. perfringens*-induced gas gangrene. It might be due to a direct action of penicillin on the gut mucosa. In 1974, Green (Green 1974), while investigating penicillin-induced death in guinea pigs, noticed that there were cytopathic changes in stool samples and this was presumably due to the activity of some latent virus. But this event, in retrospect, may appear to be the first detection of *C. difficile* cytotoxin (Bartlett 2008).

In 1893, pseudomembranous colitis (PMC) was first described by Finny (Finny 1893). He reported pseudomembranous changes in the intestinal tract of a 22 year old postoperative patient, who developed severe diarrhea and died following gastric polyp resection. PMC, was then recognized as a common complication of antibiotic use, primarily among surgeons, in the early 1950s. The rate of occurrence of PMC was reported to be high as 14%-27% among postoperative patients (Altemeier, et al. 1963; Hummel, et al. 1964). From the early work in 1960s it was suspected that *Staphylococcus aureus* was an etiological agent for PMC (Khan and Hall 1966).

In 1974, the era of *C. difficile* started when Tedesco and his colleagues reported high rates of PMC among the patients undergoing clindamycin treatment at
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Bames Hospital, USA. Out of 200 patients, who were receiving clindamycin, 42 (21%) developed diarrhea and twenty (10%) patients of clindamycin recipients had PMC which was diagnosed through endoscopy study (Tedesco, et al. 1974). Thus, a clear association between antibiotic use and PMC was observed in 1974. However, *C. difficile* was not clearly identified as the cause of PMC in human at that time. Only in 1978, Bartlett and his colleagues (Bartlett, et al. 1978) identified toxin producing *C. difficile* as the primary cause of PMC in human.

1.2. *Clostridium difficile* Infection

A wide spectrum of gastrointestinal diseases, collectively referred as *C. difficile*-associated diarrhea (CDAD) or *C. difficile* infection (CDI), is caused by only toxigenic strains of *Clostridium difficile*.

1.2.1. Pathogenesis

*C. difficile* bacterium is ubiquitous in nature and dwells mainly in soil (Ahn and Simonne 2014). However, the organism is also found as a part of the normal microbiota in infants and healthy adult individuals (Hookman and Barkin 2009). *C. difficile* exists in two forms- vegetative form and spore form. In its vegetative form, the bacterium is capable of using nutrients for its growth and multiplication. However, in harsh conditions, it enters into spore form, which is metabolically dormant and extremely resistant to various environmental stimuli (Ahn and Simonne 2014). Thus, bacterium’s spore forming ability enables it to persist for long periods (several months) in patients and in the harsh physical environment, thereby aiding its transmission. The major mode of transmission of *C. difficile* is the fecal-oral route. When the conditions become favorable *C. difficile* spores germinate and grow as vegetative cells, mediating toxin production, thereby causing diseases (Ahn and Simonne 2014).

Normally, gut microbiota (gut flora), a set of bacterial species, dwelling in intestinal tract of human (Eckburg, et al. 2005; Marchesi and Shanahan 2007), provides a significant protective and mucosal defense mechanism (Bien, et al. 2013). In healthy individuals, microbiota is generally resistant to *C. difficile* colonization (Francis, et al. 2013; Wilson and Perini 1988). However, there is ample evidence pointing out that a slight disturbance in normal microbiota (dysbiosis) by several
factors can be deleterious to human enhances the risk of *C. difficile* colonization. In fact, the alteration in the composition of normal microbiota by antibiotic treatment initiates the pathogenesis of *C. difficile*, following fecal-oral transmission (Fig. 1.1) (Poutanen and Simor 2004). Most of the ingested vegetative cells are killed in the stomach by gastric acid (Wilson 1993; Wilson, et al. 1985). However, spores are highly resistant to acid and thus can survive in acidic environment of the stomach. Spores thereafter can germinate in response to specific bile acids in the small intestine, and produce vegetative cells, allowing the bacterium for colon colonization and proliferation (Poutanen and Simor 2004). This mediates the release of a number of virulence factors, including toxin A, toxin B, flagellae and hydrolytic enzymes that are associated with the progression of disease (Seddon, et al. 1990; Tasteyre, et al. 2000). Both toxins lead to the production of tumour necrosis factor-alpha (TNFα) and proinflammatory interleukins-8 (IL-8), elevated vascular permeability, neutrophil and monocyte recruitment, opening of tight epithelial cell junctions and apoptosis of epithelial cells (Borriello 1998; Poxton, et al. 2001). The production of hydrolytic enzymes causes the degradation of connective tissues (Poutanen and Simor 2004). All these events contribute to intestinal diseases (CDAD/CDI), including diarrhea and pseudomembranous colitis in susceptible human. Thus, alteration of normal gut microbiota, colon colonization with toxigenic strain of *C. difficile*, multiplication of the organism and production of virulence factors take place during pathogenesis of *C.-difficile*. The pathogenesis of *C. difficile* is illustrated in Fig. 1.2. The recurrence of *C. difficile* infection, due to the intricacy in eradicating the highly resistant spores from patients or environment, is a major cause of concern (Ahn and Simonne 2014; Barbut, et al. 2000; Wilcox, et al. 1998).
Fig. 1.1: Anatomy of a *C. difficile* infection (CDI). a) Normal healthy colon of an individual, b) Disturbance of gut microbiota by antibiotics, c) Ingestion of spores of virulent *C. difficile*, d) Development of CDI, e) Recurrent infection (Borody and Khoruts 2012).
Fig. 1.2: The pathogenesis of *C. difficile*. (1) Vegetative cells produce virulence factors (toxins A and B), and hydrolytic enzymes, (2) Production of virulence factors, causes the production of tumour necrosis factor-alpha, proinflammatory interleukins, increased vascular permeability, neutrophil and monocyte recruitment, (3) Opening the junctions of epithelial cell of colon, (4) Apoptosis of epithelial cell, and (5) Hydrolytic enzymes production causes the degradation of connective tissues, leading to pseudomembrane colitis (Reproduced with permission) (Poutanen and Simor 2004).

1.2.2. Clinical manifestation

*C. difficile* is recognized as a nosocomial pathogen. Clinical manifestations of *C. difficile* infection are very diverse, ranging from asymptomatic carrier stage to life-threatening toxin megacolon. The major clinical manifestations of CDI are delineated briefly:
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1.2.2.1. Asymptomatic carrier stage

Majority of people exposed to *C. difficile* infection are asymptomatic carriers of the organism (Johnson, et al. 1990; McFarland, et al. 1989). According to Lawrence (Lawrence 2007), about 50% hospitalized patients become carriers following a prolonged stay in hospitals. Asymptomatic carriers are silent reservoir of infection and are probably responsible for spreading the infection very quickly in the hospital/nursing home environments (Fekety, et al. 1981; Kim, et al. 1981). Perturbation of normal microbiota due to the use of antibiotics and the proliferation of *C. difficile* within the intestinal tract can make the intestine susceptible to infection (Bien, et al. 2013).

1.2.2.2. *C. difficile*-associated diarrhea (CDAD)

In most of the cases, the bacterium is accountable for causing diarrhea among patients who are undergoing antibiotic treatment in hospital environment (Adams and Mercer 2007). Diarrhea generally occurs after 2-3 days following the infection and is accompanied by severe abdominal cramping and pain. More number of bowel movements per day can alter the water and electrolyte balance in the body. It is evident that CDAD enhances mortality and morbidity rates especially in post-operative patients with severe conditions (Kazanowski, et al. 2014).

1.2.2.3. *C. difficile*-associated colitis (CDAC)

Symptoms associated with CDAC are very similar to those observed in CDAD. In addition pyrexia and leukocytosis are also noticed. Some patients complain of abdominal guarding, significant levels of dehydration and blood in the stool. At an early stage of CDAC, specific changes are noticed in intestinal wall, which include distinct erythematous mucosae with friability and bleeding on contact (Kazanowski, et al. 2014; Wanahita, et al. 2003).

1.2.2.4. Pseudomembranous colitis

Pseudomembranous colitis is a more serious illness than CDAD and CDAC. Around half of the patients with CDAC develop a severe form of colitis, known as pseudomembranous colitis. At an early stage of diagnosis, yellow plaque is usually
observed in colon mucosa and occasionally in the terminal ileum. These types of plaques are characterized by ulcerations of mucous membrane, which induces the release of mucus, inflammatory cells and serum proteins (Kazanowski, et al. 2014; Riegler, et al. 1995). White blood cells are observed in biopsies of the lesions (Price and Davies 1977). Accompanying symptoms of PMC are mainly fever, severe abdominal pain, bloody diarrhea, dehydration, a swollen colon and abdomen, and often hypoalbuminemia (Bartlett 1992; Kelly and LaMont 1998; Mylonakis, et al. 2001). Nearly 10-25% of cured patients experience relapses, which can be more severe and sometimes lead to death.

### 1.2.2.5. **Fulminant colitis**

Approximately 3-8% of patients with *C. difficile* infection develop fulminant colitis, which can cause more serious complications (Adams and Mercer 2007; Rubin, et al. 1995). Patients with fulminant colitis may have severe abdominal pain and diarrhea, high fever, chills, distension and marked peripheral leucocytosis (Wanahita, et al. 2003). Diarrhea may be minimal or absent if the patients develop an ileus, and they are at higher risk of developing toxin megacolon. A severe protein-losing enteropathy can cause hypoalbuminemia, which can lead to ascites in patients (Bartlett 1992). Complications associated with fulminant colitis include colonic perforation (rupture), prolonged ileus, ascites, toxin megacolon and even death (Kelly and LaMont 1998). The mortality rate associated with toxin megacolon is high, varying from 24% to 38% (Dobson, et al. 2003; Lipsett, et al. 1994; Morris, et al. 1990).

### 1.2.2.6. **C. difficile-associated enteritis**

*C. difficile*-associated enteritis (or small bowel enteritis) is a rare condition. However, it may occur in patients after total colectomy and also in patients with end ileostomies. Also, disease accompanied by sepsis and fluid shifts can be life threatening (Kazanowski, et al. 2014; Vesoulis, et al. 2000).

### 1.3. **Treatment regimen**

The important first step in treatment for patients with *C. difficile* diarrhea is discontinuation of antibiotic therapy, if it is medically appropriate, and as a supportive
therapy, fluids and electrolytes should be provided, if needed. Full recovery is often possible in case of mild infection (Florea, et al. 2003; Viswanath and Griffiths 1998). For moderate and more severe illness antibiotic therapy is recommended against *C. difficile* (Bartlett 2002). Metronidazole and vancomycin are the most commonly used antibiotics. The former one (250 mg orally four times daily/500 mg orally twice daily for 10-14 days) is recommended as first-line therapy, whereas latter one (125 mg orally four times daily for 10-14 days) is prescribed as second-line therapy (Guerrant, et al. 2001; Wenisch, et al. 1996). In fact, oral vancomycin is recommended for those patients who fail to respond to metronidazole and cannot tolerate oral metronidazole, and also for pregnant patients and critically ill patients (Bartlett 2002; Wenisch, et al. 1996).

For patients with fulminant colitis (critically ill), who cannot tolerate oral therapy, intravenous metronidazole or administration of vancomycin via rectal enema or nasogastric tube has been used (Apisarnthanarak, et al. 2002). Intravenous vancomycin cannot be suggested as this medicine is not excreted into colon. Surgical intervention is necessary for those patients who are unable to respond to medical treatment, and also for those who develop toxin megacolon and colonic perforation (Dallal, et al. 2002; Kelly, et al. 1994; Viswanath and Griffiths 1998).

It is unfortunate that nearly 10%-20% of patients even after the treatment with metronidazole or vancomycin develop recurrent *C. difficile* diarrhea/colitis (Bartlett 2002). In case of initial recurrence, an additional 10-14 days course of oral metronidazole or vancomycin is recommended (Schroeder 2005). For patients with multiple relapses a tapering course of metronidazole or vancomycin (for 4-6 weeks) has been recommended. However, it is a major cause of concern that *C. difficile* strains resistant to antibiotic therapy are being emerged out (Kelly and LaMont 1998; Kelly, et al. 1994).

### 1.4. Risk factors

The key risk factors associated with CDI have been reported by researchers. These include use of antimicrobial agents, host associated factors and environmental factors.
1.4.1. **Use of antimicrobial agents**

Traditionally, the predominant risk factor for developing CDI involves the use of antibiotics and consequent disruption of normal gut flora following the exposure of *C. difficile* spores from environment or infected individuals (Bignardi 1998; McFarland, et al. 1989). Almost all types of antibiotics can cause CDI, however, some antibiotics, especially, clindamycin, penicillins, cephalosporins and fluoroquinolones are associated with elevated risk of developing CDI (Bartlett 2002; Bignardi 1998; Gerding, et al. 1995). Chemotherapeutic agents and proton pump inhibitors are also implicated in some studies (Pepin, et al. 2005). Prolonged receipt of antibiotic therapy and use of combination antibiotic therapy are also associated with increased risk of CDI acquisition (Bignardi 1998; Chang and Nelson 2000). However, it is evident that even short-term receipt of prophylactic antibiotic courses is a risk factor (Bartlett 2002).

1.4.2. **Host associated factors**

Advanced age, suppressed immune system, abdominal surgery, reduced gastric acid, comorbid conditions are recognised as major contributors for the growing incidence of CDI (Bartlett and Gerding 2008; McFarland 2008). Infection rate for elderly patients (above 65 years) is 20-fold higher than that for younger patients. However, children and peripartum women are recognised as new at-risk populations (Centers for Disease and Prevention 2005; Klein, et al. 2006; McFarland, et al. 2000; Rouphael, et al. 2008).

1.4.3. **Environmental factors**

The environment plays a critical role in spreading the infection (Gerding, et al. 2008; Huang, et al. 2006). *C. difficile* spores are competent to persist on hard surfaces for long periods (Fekety, et al. 1981; Fordtran 2006; Owens 2006). The surfaces or devices contaminated with *C. difficile* can serve as reservoirs for spores. The incidence of *C. difficile* spores is relatively high in hospital environment. Thus, patients in this environment are at high risk of acquiring CDI. In hospital wards and intensive care units, the rooms occupied by patients with CDI have high contamination rates (~50%), followed by the rooms occupied by asymptomatic carriers (~30%) (McFarland, et al. 1989; Samore, et al. 1996). Healthcare workers are
the important carrier for transmission of *C. difficile* among patients (Gerding, et al. 1995; McFarland, et al. 1989; National Clostridium difficile Standards 2004). Also, prolonged hospital stay can increase the patient's susceptibility to CDI. It has been reported that *C. difficile* infection rates are around 10% following 2 weeks of hospitalization and can reach up to 50% after 4 or more weeks (Loo, et al. 2011).

### 1.5. Strain types and impact

Toxigenic (toxin producing) and non-toxigenic (non-toxin producing) *C. difficile* strains are present in nature, however people exposed to a toxigenic strain are vulnerable to develop CDI. Several toxigenic isolates (*eg.*, PCR ribotypes 001, 002, 012, 017, 027, 078 *etc.*) have been reported from different countries (Freeman, et al. 2010).

It is of great concern that during the last decade, the incidence of CDI has been increased significantly with emergence of a particular epidemic highly virulent strain (R20291) belonging to PCR ribotype 027 (sometimes referred as BI/NAP1) (Stabler, et al. 2009). This represents a huge clinical and economic burden worldwide. A number of studies have shown that this hypervirulent strain is accountable for causing more severe diarrhea, higher rate of mortality, and more recurrent occurrences compared to the other non-epidemic strains, ribotype 027 strain CD196 and ribotype 012 strain 630 (Hubert, et al. 2007; Loo, et al. 2005). The epidemic strain is highly resistant to fluoroquinolones and has higher sporulation capacity. It has caused several hospital outbreaks of CDI in Europe, Canada and United States and even in Asia (Mooney 2007; Redelings, et al. 2007; Stabler, et al. 2009).

### 1.6. Genome of *C. difficile R20291*

The complete genome sequence of *C. difficile* strain R20291 (PCR-ribotype 027), an epidemic hypervirulent strain, was first published in 2009 by Wellcome Trust Sanger Institute, UK. In 2006, *C. difficile R20291* was isolated from Stoke Mandeville Hospital, UK and is closely related to the North American hypervirulent BI strain ([http://www.ncbi.nlm.nih.gov/genome/browse/](http://www.ncbi.nlm.nih.gov/genome/browse/)) (F:\C.difficile basic details\all sequence CD\c.difficile r20291_all sequence\NC_013316_faa.mht). Its genome information is shown in Table 1.1.
Table 1.1: Genome information, morphology and environment of C. difficile

<table>
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1.7. Virulence factors

Normally, toxigenic strains of C. difficile harbor a 19.6 kb genomic island, known as pathogenicity locus (PaLoc). This PaLoc of C. difficile is comprised of five genes, namely toxin genes tcdA and tcdB, two regulatory genes tcdC and tcdD and a porin gene tcdE (Fig. 1.3) (Voth and Ballard 2005). Toxin genes, tcdA and tcdB encode the potent glucosyltransferase toxins, toxin A, an enterotoxin (309 kDa) and toxin B, a cytotoxin (267 kDa), respectively. Several studies have reported that toxin B is the key determinant of virulence in C. difficile (al-Barrak, et al. 1999; Barbut, et al. 2002;

Fig. 1.3: Pathogenicity locus (PaLoc) of C. difficile

Besides all these genes C. difficile R20291 strain also contains binary toxin genes. It is worth mentioning at this point that this hypervirulent strain harbors genetic mutations in tcdC, resulting in increased toxin production, which may be accountable for increased virulence of C. difficile R20291.
1.8. Objective of the study

*C. difficile* is currently considered to be one of the most important causes of healthcare-associated infections around the globe. The epidemiology of CDI has changed noticeably over the past decade and there have been a remarkable worldwide increase in its incidence with much more severity. The CDI imposes a substantial burden on healthcare facilities due to its higher rate of recurrence. Recurrence of CDI is usually due to the emergence of antibiotic resistance and persistence of spores in the gastrointestinal lumen and/or environment which are formed owing to discontinuation of antibiotic doses. The accuracy of current diagnostic tests remains insufficient and the optimal diagnostic testing algorithm has not yet been defined. The failure rate of CDI treatment with preferred first-line therapeutic agents, such as metronidazole and vancomycin has been increasing and also *C. difficile* strains resistant to these antibiotics are beginning to emerge, emphasizing the need to find alternative therapies. All these factors have led to the increased research interest in the pathogen and urged the need for the development of anti-*C. difficile* drugs.

Towards this end, the thesis work is aimed to identify potential therapeutic targets in *C. difficile R20291*, an epidemic hypervirulent strain and also to identify promising lead candidates against the identified (selected) target candidates. Furthermore, the work intends to discover promising lead candidates against an important virulence factor toxin B of the organism. The systematic *in silico* approach implemented here can be utilized for other pathogens of clinical interest in order to design novel therapeutic interventions for curbing the diseases.