New trends in *Clostridium difficile* virulence and pathogenesis

C. Denève*a, C. Janoir*a, I. Poilaneb, C. Fantinatob, A. Collignon*a,b, *

*aEA 4043, Université Paris-Sud, Faculté de Pharmacie, Châtenay-Malabry, France

bService de Microbiologie, Hôpital Jean Verdier, Assistance Publique-Hôpitaux de Paris, France

**ARTICLE INFO**

**Keywords:**

*Clostridium difficile*

Hypervirulent

Epidemic 027 strain

**ABSTRACT**

The disease spectrum caused by *Clostridium difficile* infection ranges from antibiotic-associated diarrhoea to life-threatening clinical manifestations such as pseudomembranous colitis. *C. difficile* infection is precipitated by antimicrobial therapy that causes a disruption of the normal colonic microbiota, predisposing to *C. difficile* intestinal colonisation. The pathogenicity of *C. difficile* is mediated by two exotoxins, TcdA and TcdB, both of which damage the human colonic mucosa and are potent cytotoxic enzymes. *C. difficile* must first be implanted in the gut and attach to epithelial cells, which are protected by a layer of dense mucus. Confirmed and putative accessory virulence factors that could play a role in adherence and intestinal colonisation have been identified and include proteolytic enzymes and adhesins. Recently, the epidemiology of *C. difficile* infection has radically changed and an increased incidence is associated with outbreaks in North America and Europe. Several reports suggest that disease severity is increasing to include sepsis syndrome and toxin megacolon. Elderly, debilitated patients in hospitals and nursing homes are particularly vulnerable. A hypervirulent, epidemic strain has been associated with the changing epidemiology and severity of disease. Here, we review the characteristics of the epidemic NAP1, PCR ribotype 027 *C. difficile* strain that could explain its hypervirulence and epidemic spread.

© 2009 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.

1. Infection risk factors

*Clostridium difficile* is an important nosocomial enteric pathogen causing post-antibiotic diarrhoea and pseudomembranous colitis. The disease spectrum caused by *C. difficile* infection (CDI) is highly variable, ranging from mild diarrhoea to life-threatening clinical manifestations.

There are many risk factors for CDI; the main one being antibiotic exposure, particularly to broad-spectrum antibiotics with activity against anaerobes. The first evidence for this was the link between clindamycin use and pseudomembranous colitis (PMC) in the 1970s. At that time aminopenicillins and cephalosporins were found to have a similar effect. Almost all antibiotics have been implicated in CDI and, recently, fluoroquinolones and new fluoroquinolones with activity against anaerobes have also been found to be associated with CDI.

Broad-spectrum antibiotics, particularly those with activity against anaerobic bacteria, alter the intestinal microbiota, leading to dysbiosis, and disrupt its barrier effect. In a human microbiota-associated (HMA) mouse model challenged with amoxicillin–clavulanic acid we have previously studied the dominant bacterial groups of the intestinal microbiota by fluorescence in situ hybridisation combined with flow cytometry using group-specific 16S rRNA targeted oligonucleotide probes. Antibiotic treatment did not quantitatively modify the level of the total microbiota as assessed with the *Eubacteria* oligonucleotide probe; however, during antibiotic treatment the *Bacteroides–Porphyromonas–Prevotella* group increased and, simultaneously, the *Clostridium cocoides–Eubacterium rectale* group decreased dramatically. This imbalance may be responsible for diarrhoea and may also favour *C. difficile* colonisation.

Hospitalisation and old age (>65 years) are also major risk factors. Elderly, debilitated patients in hospitals and nursing homes are particularly vulnerable and colonisation rates as high as 73% have been reported. During the course of their hospital stay 15–21% of inpatients are infected by this organism; two-thirds may remain asymptomatic.

Other risk factors have been described, such as treatment with proton-pump inhibitors, H2 antagonists and methotrex-
2. Pathogenesis

*C. difficile* infection is precipitated by antimicrobial therapy, which causes a disruption of the normal colonic microbiota. After endogenous or exogenous contamination by *C. difficile* spores, spores germinate and vegetative forms multiply.

*C. difficile* can then adhere to the mucus layer carpeting the enterocytes and penetrate the mucus layer with the aid of flagella and proteases. After this step *C. difficile* can adhere to the enterocytes by means of its multiple adhesins. This results in the first phase of the pathogenic process, colonisation (Figure 1).

*C. difficile* can adhere in vitro to various cell lines such as Caco-2 cells and Vero cells and in vivo to caecal mucus of mice. Confirmed and putative accessory virulence factors that could play a role in adherence and intestinal colonisation have been identified, including proteolytic enzymes such as the cysteine protease Cwp84 and adhesins involved in mucus and cell association. The latter include the S-layer P36 and P47 proteins, a 66 kDa cell-wall protein Cwp66, the GroEL heat-shock protein, a 68 kDa fibronectin-binding protein, and the flagella components FlIC (flagellin) and FlID (flagellar cap protein).

The genes encoding Cwp66, Cwp84, and the S-layer precursor are located close to each other in a 37 kb DNA region of the *C. difficile* genome. This genetic locus carries 17 open reading frames (ORFs) in the same orientation, 11 of which encode proteins which present a two-domain structure: a domain homologous to the cell wall-anchoring domain of the autolysin LytB of *Bacillus subtilis*, present as either the N- or the C-terminal domain, and a second domain, named the functional domain, displaying remote homologies with different enzymes or structural proteins from Gram-positive bacteria (Figure 2).

The second phase of the pathogenic process is the production of toxins. The main virulence factors, the two large clostridial toxins A and B (TcdA and TcdB), are then produced. TcdA and TcdB are potent cytotoxic enzymes which damage the human colonic mucosa. They specifically glucosylate the small Rho proteins using UDP-glucose as a sugar donor and lead to cell cytoskeleton disorganisation. The genes encoding these two components, CdtA and CdtB, and a regulatory protein, are co-located on a locus called *CdT*. This toxin might potentiate the toxicity of TcdA and TcdB and lead to more severe disease and could, thus, be considered to be an additional virulence factor. Its prevalence is about 6% among *C. difficile* isolates.

3. Epidemiology

The epidemiology of CDI has changed radically in the last 10 years; in North America there has been a five-fold increase in the incidence in the whole population and an eight-fold increase in the elderly. In addition, several outbreaks have been described. Similarly, the incidence has increased in Europe in association with outbreaks, first in the UK from 2003 to 2004, then in the Netherlands and Belgium from 2005, followed by France and other European countries.

Other epidemiological changes that have been seen are an increase in disease severity associated with septic shock, toxic megacolon, and intestinal perforation; a three-fold increase in mortality in Canada between 1991 and 2003; an increase in treatment failure with metronidazole plus several relapses; and spread to a population previously considered at low risk.

A hypervirulent and epidemic strain has been associated with this changing pattern of disease in North America and in Europe.
4. Epidemic, hypervirulent *C. difficile* 027

The epidemic *C. difficile* 027 strain has been characterised, by several typing methods, as toxino-type III, as BI by restriction endonuclease analysis (REA), as North American pulsotype 1 (NAP1) by pulsed field gel electrophoresis, and as 027 by PCR ribotyping. Only REA (BI-6 to BI-24) and multilocus variable number tandem repeat analysis can distinguish between subtypes among epidemic strains.

By comparative phylogenomics using DNA microarray, Stabler et al. have shown that the hypervirulent 027 isolates from North America and Europe cluster in a specific clade. This suggests that 027 strains belong to a specific lineage.

4.1. What makes the epidemic 027 strain hypervirulent?

The epidemic strain 027 has been shown to produce higher levels of TcdA (16-fold) and TcdB (23-fold) than the toxino-type 0 strain. This hyperproduction of toxins is associated with accelerated kinetics and sustained production. Toxins are produced by the 027 strain during both the exponential and stationary growth phases, by contrast with common strains, in which toxin synthesis increases as bacteria enter the stationary phase.

Strain 027 belongs to toxino-type III and is almost identical to the toxino-type 0 reference strain except for a few different restriction sites. However, sequence heterogeneity in tcdB has been demonstrated by microarray analysis. 027 strains have a different 3' end tcdB sequence, which can lead to a divergent C-terminal binding domain. This may affect its binding capacity and potentiate its toxic activity.

In addition to variability in tcdA and tcdB, several tcdC alleles have been reported among toxigenic strains. All 027 strains, including the historic CD 196 strain, are characterised by a non-specific in-frame 18 bp deletion and a specific point deletion at position 117 resulting in a frameshift mutation introducing a stop codon at position 196. This leads to a truncated, inactive TcdC protein, which would usually negatively regulate toxin production. The severe truncation of TcdC therefore seems responsible for the increased toxin production in these strains.

Another reason for the hypervirulence is the production of the binary toxin, CDT, which may potentiate the toxicity of TcdA and TcdB and lead to more severe disease. All 027 strains are binary toxin-positive and possess the entire CdtLoc of 6.2 kb as described by Carter et al., but other, non-027, strains also produce this toxin.

A further characteristic of 027 strains is an increased sporulation rate described in the absence or presence of non-chloride cleaning agents. This can lead to better survival and spread of the strain, but this feature is not unique to 027 strains. In addition, different protein profiles and increased adherence to epithelial cells have been described in a few epidemic 027 strains.

To conclude, the combination of several virulence factors appears to be responsible for the alarming phenomenon of hypervirulence. However, in a case–case study the epidemic 027 strain was not associated with severe disease.

4.2. What makes the hypervirulent 027 strain epidemic?

It is likely that the antibiotic resistance profile of the hypervirulent 027 strain is responsible for its spread. Historic 027 strains (CD 196, BI-1 to BI-5) are susceptible to fluoroquinolones and erythromycin. By contrast, epidemic hypervirulent 027 strains are resistant to fluoroquinolones with an MIC of >32 mg/L. This resistance has been associated with mutations in gyrA and gyrB. Epidemic strains are also resistant to erythromycin with an MIC of >256 mg/L. Recently, 027 strains with high-level resistance to clindamycin have been described in Europe. However, all the 027 strains are
susceptible to metronidazole and vancomycin; this antibiotype has also been found in non-027 strains.

Antibiotics lead to the disruption of the intestinal microbiota barrier, to the selection of resistant strains such as 027, and to the induction of growth and virulence factors. Gerber et al. demonstrated that sub-inhibitory concentrations of metronidazole, vancomycin and linezolid induce tcdA and tcdB gene transcription and toxin production. Studies in an ex vivo mouse model and a human gut model have shown that fluoroquinolones with anti-anaerobic activity promote C. difficile spore germination, vegetative cell growth, and toxin production.

We have studied the impact of sub-inhibitory concentrations of antibiotics on colonisation factor gene expression in C. difficile isolates. Sub-inhibitory concentrations of clindamycin and ampicillin up-regulate colonisation factor gene expression in several strains including 027. This correlates with increased adherence of C. difficile to cultured cells. The levels of gene regulation among the strains tested were highly variable; the cwp84 gene was the most up-regulated in all the strains tested, whatever their toxigenic potential. With sub-inhibitory concentrations of ofloxacin cwp84 was, again, the most up-regulated gene. However, this up-regulation was particularly notable in epidemic fluoroquinolone-resistant 027 isolates (personal data). These results suggest that epidemic hypervirulent 027 strains can be differentially regulated. Fluoroquinolones could favour C. difficile 027 infections, not only by disrupting the barrier microbiota, but also by enhancing the expression of virulence and colonisation factors.

5. Conclusions

The widespread success of the epidemic 027 strain can be related to three factors: first, its characteristics, virulence factors, and antibiotic resistance profile, which give it ecological advantages; second, susceptible hosts with intestinal dysbiosis and inefficient immune responses; and third, favourable environmental conditions such as the existence of antibiotic pressure and spore-contaminated environments, such as hospitals and nursing homes, but also sites outside healthcare settings.

Several questions remain unanswered: is the epidemic ongoing; are other strains emerging; and what other types of CDI will appear in the future?

Funding: CD was supported by a fellowship from the French "Minist`ere de l'Enseignement Sup`erieur et de la Recherche".

Competing interests: The authors have no conflicts of interest to declare.

Ethics approval: To be received.

References