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Review Article

The role of gut microbiota in *Clostridium difficile* infection

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ABSTRACT

Clostridium difficile infection has emerged as a major health problem. Because it is a spore-forming microorganism, *C. difficile* is difficult to eradicate and recurrences of the infection are frequent. The strong association of CDI with prior use of antibiotics led to the recognition that disturbances in the gut microbiota apparently plays a central role in CDI. Except for antibiotics, several other risk factors for CDI have been recognised, such as advanced age and use of proton pump inhibitors. The common characteristic of these factors is that they are associated with changes in the composition of gut microbiota. Data from human studies have shown that the presence of *C. difficile*, either as a colonizer or as a pathogen, is associated with reduced microbiota diversity. *C. difficile* infection per se seems to be associated with changes in the representation of specific microbial populations (e.g. taxa) which either may act protectively against *C. difficile* colonization of the gut or may increase susceptibility for *C. difficile* infection. Therapeutic gut microbiota manipulation can be achieved by faecal microbiota transplantation, which is highly effective for the treatment of CDI.

1. Introduction

Clostridium difficile infection (CDI) represents a significant health problem, as it is the most frequently reported pathogen causing healthcare associated infections [1,2]. Since *C. difficile* was recognised as a major cause of antibiotic-associated diarrhoea, the role of the then called "gut microbial flora" became central in the pathogenesis of CDI. Advances in molecular methods allowed the detailed study of the human microbiota and there is growing interest in the disturbances of gut microbiota, which lead to *C. difficile* colonization and infection. Here we will review human data on the role of gut microbiota in *C. difficile* infection focusing on the role of some of the risk factors for CDI, such as age, antibiotics and proton-pump inhibiors (PPI). Therapeutic interventions will be only briefly reviewed. To retrieve the relevant literature, we have searched the MEDLINE database using the following search string: "*Clostridium difficile*"[Mesh] AND ("Microbiota"[Mesh] OR microbiome) Filters: Humans. We have also searched the SCOPUS database with the search string: KEYWORD (clostridium AND difficile AND (microbiota OR microbiome) AND human). We initially screened the abstracts of the resulting citations and we have obtained the full text of all articles we have considered relevant. Furthermore, we have obtained the full text of relevant articles that were cited by the initial batch of articles.

2. Microbiology and epidemiology of *C difficile*

Clostridium difficile is a Gram-positive, obligate anaerobic bacillus. *C. difficile* exist as a vegetative and as a spore form. The vegetative form cannot survive in the environment for long, as *C. difficile* is an obligate anaerobe. The spore form on the other hand, is metabolically inactive and it is resistant to oxidative stress, extremes of temperature, desiccation, and the acidic environment of the stomach as well as to the alcohol containing solutions used for hand hygiene [3,4]. *C. difficile* spores thus can survive in the environment for long periods, and they have been found in soil, feces, sewage, and food as well [5,6]. Spores are an essential determinant of the epidemiology of *C. difficile* as they are the transmissible form of the bacterium and they survive inside the host, causing the recurrences of the disease.

C. difficile was first isolated in the intestinal flora of newborn infants in 1935, and it was initially named *Bacillus difficilis* "because of the unusual difficulty which was encountered in its isolation and study". The researchers confirmed the pathogenicity of the new species on experimental animals and they also proved the presence of an exotoxin [7]. However, it was not until the 1970's that the pathogenic role of C difficile in antibiotic-associated diarrhoea was confirmed, when different research groups showed that the disease could be transferred by filtrates of cecal contents obtained from animals after administration of clindamycin, and from patients with pseudomembranous colitis to experimental animals, fulfilling thus the Koch's postulates [8,9].

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Although *C. difficile* was recognised as a cause of human disease only 40 years ago, CDI represents now a significant health problem. Approximately 1%–3% of healthy adults are colonized [10,11]. It has been estimated that in the USA during 2011 there have been approximately half a million CDI cases resulting in 29,000 deaths and *C. difficile* is the most frequently reported pathogen causing healthcare associated infections [1,2]. Although the majority of hospitalized patients infected by *C. difficile* are asymptomatic carriers, this population is important as it serves as a reservoir for continued *C. difficile* contamination of the hospital environment. Until relatively recently, CDI was considered an almost exclusively healthcare-associated infection. However, it is now apparent, that a significant part of CDI involves patients in the community [12]. Furthermore, these patients may lack the major risk factors such as antibiotic use or recent hospitalization [13].

3. The pathogenesis of CDI

C. difficile is transmitted via the faecal-oral route. Spores in the stools of patients or healthy carriers may remain in the environment for long periods, as they are resistant to heat and desiccation [3]. The vegetative form of *C. difficile* cannot transmit the infection because, being an obligate anaerobe, it is unable to survive outside the host, where the environment is oxygenated. Once ingested, *C. difficile* spores initially survive the acidic environment of the stomach and they subsequently reach the small bowel where they may germinate. The main factor triggering germinations is the presence of primary bile acids, although other triggers such as glycine have been described [14]. The vegetative forms of *C. difficile* move to the large bowel, where, under anaerobic environment, they multiply and colonize. C difficile can adhere to the colonic epithelium and secrete its virulence factors, mainly toxins A (TcdA) and B (TcdB) and the binary toxin (*Clostridium difficile* transferase – CDT). Other virulence factors have been described such as cell surface protein Cwp84, which is a mucolytic enzyme that can damage the colonic mucosa [15].

Toxins A and B are encoded on a Pathogenicity Locus (PaLoc) which also encodes the regulatory proteins TcdR, TcdE and TcdC. TcdR upregulates the transcription of TcdA and TcdB, while TcdC inhibits it. TcdE is probably involved on the secretion of TcdA and TcdB [16]. Binary toxin is encoded on a different locus (CdtLoc). TcdA and TcdB are large proteins with molecular weight of 308 kD and 270 kD respectively, with an amino acid sequence homology of 64% [17]. They have four functional domains: the glucosyltransferase domain (GTD), the autoprotease domain (APD), the pore-forming domain (POD) and the combined repetitive oligopeptides (CROPS) domain. The CROPS domain mediates binding to glycosylated receptors on the gut epithelial cells, although there is evidence that other domains participated in binding with other receptor such as polio-virus like receptor protein 3 [18]. Following binding to the cell surface the toxins are endocytosed and are after autocleavage by the APD, they are transferred into the cytoplasm. TcdA and TcdB glycosylate GTPases of the Rho family (RhoA, Rac1 and Cdc42) resulting in disaggregation of the actin cytoskeleton leading to epithelial cell death by apoptosis and necrosis. Epithelial cell death along with intercellular tight junction damage lead to intestinal barrier breakdown [19]. At the same time TcdA and TcdB induce inflammatory cytokine secretion (e.g. IL-8) resulting to neutrophil influx and formation of microabscesses and the characteristic pseudomembranes on the colonic mucosa. The role of binary toxin in the pathogenesis of CDI is not entirely clear. It has been suggested that it acts on the cytoskeleton resulting in the formation of protrusions on the epithelial cells, which facilitate C difficile adherence and colonization [19].

4. Gut microbiota

Advances in molecular laboratory techniques, especially the introduction of next-generation sequencing, have made possible the comprehensive study of the microbial population residing in the gut. Using whole-genome shotgun sequencing, we can now recognize the taxonomic profiles of between 70 and 100 bacterial species in the healthy gut microbiome [20]. However, profiling of 16 S rRNA gene sequences suggests that the number of species in the gut might be far larger, possibly up to 1000 species [21]. The composition of the microbiome can be described in terms of alpha and beta diversity. Alpha diversity is a measure of taxonomic (e.g. phylae or taxa) diversity within a subject and is expressed with the Shannon index. Beta diversity is a measure of taxonomic diversity between subjects and is expressed by Bray–Curtis beta diversity.

Moving from duodenum to the terminal ileum microbial diversity increases, approaching that of the proximal colon. In the terminal ileum Firmicutes predominate with genera such as *Streptococcus, Veilonella* and *Clostridium*. The dominant phylae in the colon are Bacteroidetes (genus Bacteroides) and Firmicutes (genus *Clostridium, Faecalibacterium, Lactobacillus, Ruminococcus*), followed by Actinobacteria and Proteobacteria. Established pathogens such as *Campylobacter jejuni* and *Salmonella enterica* comprise less than 0.1% of the human gastrointestinal microbiota. *E. coli* was detectable in 61% of the specimens; however, it comprised 0.1% or more of the microbiota in only 15% of them [22].

The gut microbiota compositions varies widely between individuals, however it is relatively stable within an individual. Thus, individuals can be classified according to their microbiota composition, into three "enterotypes" with predominance of *Bacteroides*, *Prevotella*, or *Ruminococcus*, respectively [23]. The clinical significance of these enterotypes is yet unknown.

5. The effect of risk factors for CDI on the gut microbiota

There is an extensive literature discussing the risk factors for CDI. Recognised risk factors include host-related risk factors such as patient age (with risk increasing with age of the patient), severity of underlying illness, recent abdominal surgery, long-term care residency, and exogenous factors such as use of antibiotics, use of PPI or Histamine receptors 2 blockers, prior hospitalization and nasogastric tube placement [3]. We will briefly discuss alterations in the gut microbiota brought upon by some of these risk factors.

5.1. Age

Age is a strong risk factor for CDI. It has been found that for every additional year of age after age 18, the risk of health care–associated *C. difficile* infection increases by approximately 2% [24]. On the other hand, there is sound evidence that the gut microbiota is also gradually changing with age. There is no age threshold beyond which the microbiota becomes different; however, the core gut microbiota of elderly subjects was distinct from that of younger adults, with enrichment of Bacteroides spp. and Clostridium groups. It is notable that the microbiota composition of the elderly was extremely variable between individuals, but stable over time in each individual [25]. Rea et al. analyzed the gut microbiota in elderly patients with and without CDI as well as in asymptomatic subjects with *C. difficile*. There was little difference at the phylum or family taxonomic level between asymptomatic carriers and culture negative individuals, however a marked reduction in microbial diversity at genus level was observed in patient with CDI caused by the hypervirulent strain R027 [26]. In a recent study of nursing home residents, the stability of the elderly gut microbiota was confirmed. Residents with C difficile positive stools (either colonized or infected) comprised 30.4% of the studied population and they had a relative abundance of Blautia, Flavonifractor and Lachnospiraceae (all belonging to the Firmicutes), while in *C. difficile* negative residents Akkermansia (Verrucomicrobiaceae) was more abundant [27]. This is an interesting finding as a decrease in Akkermansia has been associated with gut inflammation [28].