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Outcome of relapsing *Clostridium difficile* infections do not correlate with virulence-, spore- and vegetative cell-associated phenotypes

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ABSTRACT

One of the main clinical challenges of *Clostridium difficile* infections (CDI) is the high rate of relapse episodes. The main determinants involved in relapse of CDI include the presence of antibiotic-resistant *C. difficile* spores in the colonic environment and a permanent state of dysbiosis of the microbiota caused by antibiotic therapy. A possible scenario is that phenotypes related to the persistence of *C. difficile* spores might contribute to relapsing infections. In this study, 8 *C. difficile* isolates recovered from 4 cases with relapsing infection, and 9 isolates recovered from single infection cases were analyzed for PCR ribotyping and the presence of *tcdA*, *tcdB* and *cdtAB* genes. Factors associated to spore persistence, sporulation, spore adherence and biofilm formation and sporulation during biofilm formation were characterized. We also evaluated motility and cytotoxicity. However, we observed no significant difference in the analyzed phenotypes among the different clinical outcomes, most likely due to the high variability observed among strains within clinical backgrounds in each phenotype and the small sample size. It is noteworthy that *C. difficile* spores adhered to similar extents to undifferentiated and differentiated Caco-2 cells. By contrast, spores of all clinical isolates tested had increased germination efficiency in presence of taurocholate, while decreased sporulation rate during biofilm development in the presence of glucose. In conclusion, these results show that, at least in this cohort of patients, the described phenotypes are not detrimental in the clinical outcome of the disease.

1. Introduction

Clostridium difficile is a major cause of nosocomial antibiotic-associated diarrhea in developed and developing countries [1]. Clinical manifestations normally associated with *C. difficile* infection (CDI) range from mild to severe diarrhea, which can lead to fulminant colitis and toxic megacolon, bowel perforation, sepsis and death [1]. Mortality of CDI reaches 5% of total cases, but in several outbreaks it may raise up to 20% [2,3]. Although single episodes of CDI (S-CDI) resolve with metronidazole and/or vancomycin treatment, the main current clinical challenge to control CDI is derived from the high rate of recurrent infections that may reach 20%, 40% and 60% after a first, second and third episode, respectively [4]. Recurrent CDI episodes may be caused by the same strain that caused the first episode, termed relapsing CDI (R-CDI); or by

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subsequent re-infections with a new strain from the environment, termed re-infection of CDI [5]. Either with relapse or reinfection, the main host-associated factor involved in recurrent infection of CDI is a non-recoverable antibiotic-induced dysbiosis of the colonic microbiota which is unable to act as a resistance barrier against *C. difficile* colonization [6].

The main virulence factors responsible for the clinical symptoms of CDI are two exotoxins, the enterotoxin TcdA and the cytotoxin TcdB [1]. Both, TcdA and TcdB, glucosylate the Rho family of GTPases of intestinal epithelial cells, causing cell death and an acute inflammatory response [1]. A third toxin, the binary toxin (CdtAB), present in nearly 35% of clinical isolates (mainly belonging to 027 ribotype), contributes to the adherence of *C. difficile* vegetative cells to intestinal epithelial cells *in vitro* [7]. A recent clinical study identified the binary toxin as a predictor of recurrent infections [8]. Besides the toxins, *C. difficile* exhibits other virulence traits, some of which have been suggested to be essential of auxiliary to R-CDI. In this regard, it is recognized that the most important traits that contribute to R-CDI are those associated to the persistence of *C. difficile* spores in the host. For example, *C. difficile spo0A* mutant strains, which are unable to form spores, are incapable to persist in a murine model of R-CDI, indicating that spore formation during infection is essential for R-CDI [9]. Interestingly, several studies that have correlated the sporulation rate and the clinical outcome have provided controversial data [5,10–13]. By contrast, although spores of *C. difficile* isolates display a diverse germination response to bile salts [14], germination does not seem to correlate with the severity of CDI [11], but rather with R-CDI [5]. Oka et al. (2012) demonstrated that R-CDI spores exhibited higher germination efficiency than those from S-CDI cases in absence of the nutrient germinant, taurocholate, and are likely to be more prone to germinate after antibiotic therapy [5].

The aforementioned spore-related phenotypes conceive the hypothesis that other spore-related phenotypes might be contributing to the persistence of spores in the host, including colonization, biofilm formation, sporulation during biofilm development and spore adherence to intestinal epithelial cells [9,15–17]. Gut colonization by *C. difficile* is mediated by the flagella, which is essential for biofilm formation [18]. During the course of infection, biofilm formation has been demonstrated to increase the persistence of *C. difficile* in a mouse model of CDI [15]. Remarkably, during the development of biofilms, *C. difficile* also forms spores within biofilms [19,20] and might contribute to R-CDI. This is reinforced by the fact that a *spo0A* mutant, also has reduced formation of biofilm [18,20], suggesting that biofilm and spore persistence might be linked in perseverance. Furthermore, spore adherence to intestinal epithelial cells [17] might also be an additional trait that contributes to persistence of *C. difficile*. Despite the abovementioned evidence, the clinical relevance of these bacterial virulence traits in the R-CDI remains to be elucidated.

In this study, we recovered *C. difficile* clinical isolates from patients with single or relapsing episodes of CDI. In both groups of isolates, PCR ribotyping, the presence of *tcdA*, *tcdB*, and *cdtAB* genes, and metronidazol and vancomycin susceptibility were determined. Classical virulence phenotypes (i.e., toxin production, sporulation and spore germination) as well as phenotypes recently conceived to be involved in the host persistence of *C. difficile* (i.e., motility, biofilm formation, sporulation during development of biofilm and spore adherence to epithelial cells), were also analyzed.

2. Material and methods

2.1. Ethics approval and clinical setting

This study was approved by the Bioethics Committee of the University Hospital of the Pontificia Universidad Católica de Chile and informed consent to the use of their specimen for scientific research was obtained from all patients. Hospital Clínico de la Pontificia Universidad Católica de Chile is a tertiary-level health care center, with 450 beds of which 60 correspond to critical care services.

2.2. Patients and specimens

During an 11-month period (August 2011–June 2012), all patients were reviewed at the University Hospital of the Pontificia Universidad Católica de Chile. Inclusion criteria: adults at least 18 years of age, had at least one GeneXpert-positive *C. difficile* test within 72 h and agreed the informed consent. Clinical records of included patients were analyzed to ensure that there were no reported cases of CDI outside of our institution previous to their admission. All patients were followed for up to 12 month to ensure that those with R-CDI had no further episodes of CDI, and those patients with a S-CDI were truly single episodes cases. All patients started standard of care (SOC) therapy with oral vancomycin. S-CDI was defined as the onset of diarrhea ($\geq$3 loose stools/day for more than 24 h), one positive laboratory test and no *C. difficile* positive test within 1 year of surveillance prior to CDI. R-CDI was defined as a new episode of diarrhea after a symptom-free period, of 3 $\geq$ days to 1 year, after the completion of one round of SOC therapy. In this study's time frame, from a total of 94 patients recruited, four patients had R-CDI. Clinical isolates from 9 patients with S-CDI episode were selected based on the levels of C-reactive protein and leukocytes and Charlson index that would be similar to those found in patients with R-CDI episodes. Liquid or soft stool specimens from hospitalized patients suspected of having CDI were prospectively subjected to multiplex real-time PCR GeneXpert *C. difficile* (Cepheid). In addition 1 ml from each specimen was identified and frozen at −80 °C, until used for the recovery of *C. difficile*.

2.3. C. difficile reference strains and clinical isolates

C. difficile was routinely grown under anaerobic conditions in a anaerobic chamber (Bactron III-2, Shellab, OR, U.S.A.) in 3.7% Brain Heart Infusion supplemented with 0.5% yeast extract (BHIS) broth or on BHIS agar plates. *C. difficile* strains R20291 and M120 have been described elsewhere [21,22]. A total of seventeen clinical isolates of *C. difficile* were used in this study to compare genotype and/or phenotypic characteristics between strains isolated from S-CDI cases (n = 9) and those isolated from R-CDI cases (n = 4) (a total of 8 isolates, four isolates from the first R-CDI episode and four isolates from the second episode of R-CDI).

C. difficile was isolated from stool specimens using previously described protocols [23]. Briefly, stool samples were treated with ethanol for 60 min at room temperature, plated into Taurocholate Cycloserine Cefoxitin Fructose Agar (TCCFA) plates and incubated for 48 h at 37 °C in a Bactron III-2 anaerobic workstation (Shellab, OR, U.S.A.). To ensure that patients were infected with one ribotype, 3 colonies per sample were selected and inoculated into three BHIS broth tubes supplemented with 250 μg/ml cycloserine and 16 μg/ml cefoxitin. Glycerol stocks and cook meat stocks of pure cultures were stored at −80 °C until use.

2.4. Presence of tcdA, tcdB and cdtAB and PCR ribotyping

Genomic DNA was extracted using described protocols [24]. Presence of *tcdA*, *tcdB* and *cdtAB* genes was detected by PCR as described elsewhere [25,26]. PCR ribotyping was performed as previously described with minor modifications [27]. In brief, chromosomal DNA of 3 colonies from each stool sample was isolated as previously described [28] and subjected to standard