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Genomic and phenotypic diversity of *Clostridium difficile* during long-term sequential recurrences of infection

F.E. Sachsenheimer[a,1], I. Yang[b,c,1], O. Zimmermann[a], C. Wrede[d], L.v. Müller[e], K. Gunka[a], U. Groß[a,*,2], S. Suerbaum[b,f,g,2]

[a] Institute of Medical Microbiology, University Medical Center Göttingen, Kreuzbergring 57, Göttingen, Germany
[b] Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Carl-Neuberg-Str. 1, Hannover, Germany
[c] Lower Saxony Centre for Biomedical Engineering, Implant Research and Development, Department of Prosthetic Dentistry and Biomedical Materials Science, Hannover Medical School, Stadtfelddamm 34, Hannover, Germany
[d] Institute of Functional and Applied Anatomy, Hannover Medical School, Carl-Neuberg-Str. 1, Hannover, Germany
[e] National Consulting Laboratory for Clostridium difficile, Germany
[f] Max von Pettenkofer Institute, Ludwig-Maximilians-Universität München, Pettenkoferstr. 9a, 80336 Munich, Germany
[g] DZIF German Center for Infection Research, Hannover-Braunschweig and Munich Partner Sites, Germany

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ABSTRACT

Infection with the emerging pathogen *Clostridioides (Clostridium) difficile* might lead to colonization of the gastrointestinal tract of humans and mammals eventually resulting in antibiotic-associated diarrhea, which can be mild to possibly life-threatening. Recurrences after antibiotic treatment have been described in 15–30% of the cases and are either caused by the original (relapse) or by new strains (reinfection).

In this study, we describe a patient with ongoing recurrent *C. difficile* infections over 13 months. During this time, ten *C. difficile* strains of six different ribotypes could be isolated that were further characterized by phenotypic and genomic analyses including motility and sporulation assays, growth fitness and antibiotic susceptibility as well as whole-genome sequencing. PCR ribotyping of the isolates confirmed that the recurrences were a mixture of relapses and reinfections. One recurrence was due to a mixed infection with three different strains of two different ribotypes.

Furthermore, genomes were sequenced and multi-locus sequence typing (MLST) was carried out, which identified the strains as members of sequence types (STs) 10, 11, 14 and 76. Comparison of the genomes of isolates of the same ST originating from recurrent CDI (relapses) indicated little within-patient microevolution and some concurrent within-patient diversity of closely related strains.

Isolates of ribotype 126 that are binary toxin positive differed from other ribotypes in various phenotypic aspects including motility, sporulation behavior and cell morphology. Ribotype 126 is genetically related to ribotype 078 that has been associated with increased virulence. Isolates of the ribotype 126 exhibited elongated cells and a chaining phenotype, which was confirmed by membrane staining and scanning electron microscopy. Furthermore, this strain exhibits a sinking behavior in liquid medium in stationary growth phase. Taken together, our observation has proven multiple CDI recurrences that were based on a mixture of relapses and reinfections.

1. Introduction

The Gram-positive, spore forming, anaerobic bacterium *Clostridioides (Clostridium) difficile* is the causative agent of pseudomembranous colitis. The severity of disease varies from mild diarrhea to severe colitis with toxic megacolon (Borriello, 1998; Rupnik et al., 2009). The main virulence factors are toxin A (TcdA) and toxin B (TcdB) (Kuehne et al., 2010; Voth and Ballard, 2005). Furthermore some strains express a binary toxin (CDT), which is associated with increased virulence (Inns et al., 2013; McDonald et al., 2005; Schwan et al., 2009). *C. difficile* infection (CDI) is transmitted by uptake of spores via the fecal-oral route. Following germination in the small

* Corresponding author.
E-mail address: ugross@gwdg.de (F.E. Sachsenheimer).
[1] Authors contributed equally to this study.
[2] Authors share senior authorship.

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intestine, the vegetative cells produce disease-causing toxins and finally sporulate in the large intestine before being released into the environment, which may cause infection of new individuals (Koenigsknecht et al., 2015; Paredes-Sabja et al., 2014; Shen, 2015). The sporulation pathway is partially conserved between different spore-forming species (*Bacillus* spp., *Clostridium* spp.) (Paredes-Sabja et al., 2014), this is especially the case for its global regulators (de Hoon et al., 2010). The master regulator of sporulation is Spo0A (Pereira et al., 2013), which might be also a regulator of other putative virulence factors as well (Mackin et al., 2013). Endospores are extremely resistant to environmental stress, such as heat, oxygen and even sanitizers. Moreover, they are able to survive for prolonged periods of time which predisposes for nosocomial transmission.

In recent years, the incidence and mortality of CDI have been increasing (Redelings et al., 2007). In addition, the frequency of community-acquired infections and CDI of the young and healthy have been rising (Kuntz et al., 2011; Lessa et al., 2012). In Europe, PCR ribotyping is the standard method for genotyping of *C. difficile* isolates; however, it is apparent that whole genome sequencing will become the method of choice in near future. In Germany, ribotypes 001, 027, 014 and 078 are the most prevalent ones (Seedat et al., 2013). Some of them, such as 027 (BI/NAP1) and 078 are binary toxin positive and have been associated with increased virulence (Goorhuis et al., 2007, 2008a; McDonald et al., 2005; Warny et al., 2005). Ribotype 126, which is less prevalent, is considered as potentially hypervirulent, since it shares 99.7% of its genes with ribotype 078 (Kurka et al., 2014). Ribotype 027 has spread around the world since its first emergence. In addition, the prevalence of the hypervirulent strains continues to increase (Freeman et al., 2010; Goorhuis et al., 2008a). Nevertheless, the available data on an association of ribotypes with severe infections is contradictory (Carlson et al., 2013; Goorhuis et al., 2007; Walk et al., 2012).

Ribotypes 078 and 126 show a high genetic divergence to many other known ribotypes (Kurka et al., 2014). For example, strains of the ribotype 078 lack one of the three flagella operons, designated as the F3 regulon, resulting in a non-flagellated and non-motile phenotype (Stabler et al., 2009; Stevenson et al., 2015). Infections with ribotype 078 strains are very common in animals (Goorhuis et al., 2008b; Hensgens et al., 2012), indicating that the incidence of person-to-person transmission might be overestimated (Eyre et al., 2012a); instead transmission may often happen via the food borne or zoonotic route (Hensgens et al., 2012; Rodriguez-Palacios et al., 2013).

CDI is strongly associated with antibiotic pretreatment affecting the intestinal microbiome (dysbiosis) (Borriello, 1998; Buffie et al., 2015). Due to high transmission rates in hospitals and increasing infection rates, the socioeconomic burden of CDI to the health systems rises steadily (DePestel and Aronoff, 2013; Dubberke and Olsen, 2012; Lessa et al., 2015). Furthermore, recurrent infections are difficult to treat as the microbiome may be persistently affected which is associated with re-emergence of CDI despite successful treatment. This often leads to an ongoing cycle of symptoms, treatment, relief of symptoms and recurrence.

The present study is based on long-term surveillance of a patient with eight episodes of CDI for whom even with antibiotic administration a permanent remission could not be achieved. Although recurrent episodes of CDI are difficult to manage and potentially life-threatening, the precise pathogenic mechanisms are only partially understood. The aim of this study was to compare ten isolates obtained from eight episodes of CDI in a single patient to contribute to the understanding of the phenomenon of reoccurring CDIs.

2. Material & methods

2.1. Patient

The patient from whom all isolates were derived, was a 73-years-old man with severe underlying conditions, including end-stage chronic kidney disease and congestive heart failure. There was no diagnosis of chronic inflammatory bowel disease in the patient. Initially, he suffered from staphylococci sepsis that was treated with oxacillin and rifampicin. During this hospitalization, he developed the first episode of CDI which was very severe with massive diarrhea, vomiting, nausea, abdominal pain and fever. Following CDI-specific treatment, he recovered and was released from the hospital. Within a time period of twelve months he experienced seven recurrences of CDI. Some of the episodes of CDI were hospital-associated, some community-acquired and the severity of symptoms was highly variable (see Results). Approval for this study was obtained from the Ethics Committee of the University Medical Center Göttingen (ID of Ethic Committee approval: 11/4/15).

2.2. Routine diagnosis

The ten clinical *C. difficile* isolates derived from the above characterized patient were isolated during routine diagnosis at the Institute for Medical Microbiology, Göttingen, Germany. For this, stool samples of the patient were directly placed on selective CLO agar (bioMérieux, Nürtingen, Germany) and incubated at 37 °C for 48 h under anaerobe conditions. Subcultures were incubated on Columbia blood agar (bioMérieux) at 37 °C for 48 h under anaerobic conditions. If different morphotypes were present, these were also subcultured separately. All routinely processed cultures were judged by an experienced analyst.

Stool samples were also analyzed for the presence of other intestinal pathogens, such as e.g. *Salmonella enterica*, *Yersinia* spp., *Shigella* spp., intestinal viruses and parasites by qualified methods according to good laboratory practice. Norovirus was present only in the period between episodes 5 and 6 of CDI, but not simultaneously to any of the CDI episodes.

2.3. Reference strains, media and growth conditions

C. difficile reference strains 630 and R20291 were kindly provided by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Other reference strains were obtained from the laboratory biobank of the authors (Supplementary Table S1). For all subsequent analyses, *C. difficile* isolates and reference strains were routinely grown in BHI (brain heart infusion, BD, Heidelberg, Germany) medium supplemented with 0.5% (w/v) yeast extract and 0.03% (w/v) cysteine (BHIS medium). All media were reduced before inoculation. Subcultivation on solid media was done on COS-Agar plates (bioMérieux, Nürtingen, Germany). *C. difficile* cultures were grown under respective conditions in an anaerobic chamber (Coy laboratory products, Grass Lake, Michigan, USA) at 37 °C with an atmosphere of 5% CO_2, 5% H_2, 90% N_2. To avoid the presence of spores, overnight cultures were diluted 1:100 in fresh medium. Cells were grown until an OD_{600} of 0.2- 0.4. These exponentially growing starter cultures were diluted 1:100 with previously reduced medium for sporulation assays.

2.4. Light microscopy

For microscopy studies, cultures were removed from the anaerobic chamber. Cells were pelleted by centrifugation at 12.000 rpm at ambient temperature. After centrifugation, the pellets were washed with phosphate buffered saline pH 7.4 (PBS) and centrifuged again. The supernatant was removed and the cells were resuspended in PBS and placed on a microscopic slide. For fluorescence microscopy, samples were harvested in PBS, washed and centrifuged. Pellets were resuspended in PBS containing lipophilic styryl membrane dye *N*-(3-triethylammoniumprpyl)-4-(*p*-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM 4–64 (10 µg/ml), AAT Bioquest, Sunnyvale, CA, USA) (Fimlaid et al., 2013). All microscopic studies were performed with an Axiovert 200 M (Carl Zeiss, Oberkochen, Germany) microscope.