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Pathogenesis and toxins

Clostridium difficile infection among immunocompromised patients in Rio de Janeiro, Brazil and detection of moxifloxacin resistance in a ribotype 014 strain

Danielle Angst Secco [a, *], Ilana Teruszkin Balassiano [b], Renata Ferreira Boente [a], Karla Rodrigues Miranda [a], Jon Brazier [c], Val Hall [c], Joaquim dos Santos-Filho [a], Leandro Araujo Lobo [a], Simone Aranha Nouér [d], Regina Maria Cavalcanti Pilotto Domingues [a]

[a] *Laboratório de Biologia de Anaeróbios, Departamento de Microbiologia Médica, Instituto de Microbiologia Prof. Paulo de Góes, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, CEP 21941-900 Rio de Janeiro, RJ, Brazil*
[b] *Laboratório de Zoonoses Bacterianas, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, RJ, Brazil*
[c] *Anaerobe Reference Unit, Public Health Wales Microbiology, University Hospital of Wales, Cardiff, Wales, United Kingdom*
[d] *Coordenação de Controle de Infecções Hospitalares - HUCCF/UFRJ, Rio de Janeiro, RJ, Brazil*

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ABSTRACT

Clostridium difficile is a Gram-positive spore forming anaerobic bacterium, often associated with nosocomial diarrhea and pseudomembranous colitis. The acquisition of this organism occurs primarily in hospitals through accidental ingestion of spores, and its establishment and proliferation in the colon results from the removal of members of the normal intestinal flora during or after antibiotic therapy. In this study, stool samples from patients admitted to the University Hospital Clementino Fraga Filho (HUCCF/UFRJ) were screened for *C. difficile* toxins with an ELISA test and cultured with standard techniques for *C. difficile* isolation. A total of 74 stool samples were collected from patients undergoing antibiotic therapy between August 2009 and November 2010, only two (2.7%) were positive in the ELISA test and culture. A third isolate was obtained from a negative ELISA test sample. All cases of CDI were identified in patients with acute lymphoid or myeloid leukemia. Genotypic and phenotypic characterization showed that all strains carried toxins A and B genes, and belonged to PCR-ribotypes 014, 043 and 046. The isolated strains were sensitive to metronidazole and vancomycin, and resistant to ciprofloxacin and levofloxacin. Resistance to moxifloxacin, was present in the strain from PCR-ribotype 014, that showed an amino acid substitution in *gyrB* gene (Asp 426 → Asn). This is the first time that this mutation in a PCR-ribotype 014 strain has been described in Brazil.

1. Introduction

Clostridium difficile is a Gram-positive spore forming anaerobic bacterium. *C. difficile* infection (CDI) is highly prevalent in hospitals and is the most common cause of nosocomial diarrhea in adults, mainly in elderly and immunocompromised patients [1,2]. CDI cases in solid organ transplant recipients, hematopoietic stem cell or bone marrow transplant recipients and HIV-seropositive individuals are reported to be higher in units caring for these patients [3].

Proliferation of *C. difficile* in the colon results from the removal of members of the normal intestinal flora in consequence of antibiotic therapy [4,5]. Practically any antibiotic can be associated with CDI, but the antibiotics most commonly associated are clindamycin, cephalosporins and penicillins [6,7]. However, many studies have also described a high association between fluoroquinolones use and CDI, that may be related, even in part, to the development of fluoroquinolones resistance among *C. difficile* isolates in the last years [8–12].

C. difficile pathogenic strains are able to produce two toxins, enterotoxin A (TcdA) and cytotoxin B (TcdB), which constitute the major virulence factors of this species [7]. These toxins elicit several effects on the host, such as chemokine and cytokine production,

* Corresponding author. Tel.: +55 21 2560 8344.
E-mail address: daniangstsecco@gmail.com (D.A. Secco).

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neutrophil infiltration, mast cell activation, disruption of the tight junctions and actin depolymerization that lead to an extended inflammatory process [13]. In addition to toxins A and B, some strains produce *C. difficile* binary toxin (CDT), that is encoded by *cdtA* and *cdtB* genes [14]. The role of CDT in disease is not well understood, but it seems to potentiate the toxicity of the toxins A and B [7,15].

In the past decade a hypervirulent epidemic strain, classified as PCR-ribotype 027, has emerged and has been involved in more severe *C. difficile* associated diarrhea outbreaks in the United States, Canada, UK and other European countries [7,8,16–20]. This strain is resistant to fluoroquinolones, has deletions in *tcdC* gene that seem to be related to an overproduction of A and B toxins, produces binary toxin, shows alterations in surface layer protein A (SlpA) associated with an increased adherence to human intestinal cells and has increased sporulation rates, which contribute to its spread and survival at the hospital environment [7,15,21,22]. In Brazil, the detection of this hypervirulent strain has not been reported to date.

Fluoroquinolones are a broad-spectrum antibiotic class, which has been strongly associated to CDI [23]. This class of drug has favorable pharmacokinetic properties that have encouraged their widespread use. They have good tissue penetration and are well absorbed when taken orally, which facilitates their use for treatment of many diseases [24]. *C. difficile* isolates resistant to moxifloxacin, a fourth-generation fluoroquinolone, have one or more single nucleotide point mutation within the quinolone resistance-determining regions (QRDR) of their DNA gyrase genes. At least seven mutations within the QRDR of *gyr*A and seven more in *gyr*B of *C. difficile* are known to occur [25,26]. The most frequent amino acid change is Thr82 → Ile in *gyr*A, which is also present in the hypervirulent PCR-ribotype 027 strain [27]. A study conducted in South Korea showed that the use of fluoroquinolones was highly related to resistance to moxifloxacin in *C. difficile* strains [28]. In the past decades, CDI has increased in number of cases and rates of mortality and morbidity, associated in part to the emergence of the PCR-ribotype 027 strain in many countries. It has been hypothesized that fluoroquinolone-resistance may have facilitated spread of this strain potentially providing it a survival advantage in the hospital environments where fluoroquinolones are extensively used [29,30].

In Brazil, there are few studies addressing the role of *C. difficile* as an agent of nosocomial diarrhea, as well as their incidence and spread [2,31–34]. Thus, the aim of this study was to isolate and characterize genotypically and phenotypically *C. difficile* strains from fecal samples of inpatients of a university hospital in Rio de Janeiro, Brazil.

2. Materials and methods

2.1. Fecal isolates

Seventy-four fecal samples were obtained and analyzed between August 2009 and November 2010 from inpatients admitted in 15 different wards of "Hospital Universitário Clementino Fraga Filho (HUCFF)", affiliated to "Universidade Federal do Rio de Janeiro (UFRJ)" in Brazil. All patients were under broad-spectrum antibiotic therapy and the samples were collected during active diarrhea episodes. All samples were tested for the presence of toxins A and B by Ridascreen *C. difficile* Toxin A/B (R-Biopharm)® enzimatic assay (ELISA), according to manufacturer's instructions.

Stool samples were also cultured using the standardized process of isolation and identification [35]. Faecal samples were cultured directly or after an enrichment step (alcohol shock procedure) onto selective cycloserine/cefoxitin/fructose agar (CCFA). The plates were incubated anaerobically at 37 °C for 48 h. *C. difficile* isolates were identified based on morphology characteristics of this species in CCFA plates, Gram staining and characteristic odor. Isolates were confirmed as *C. difficile* by polymerase chain reaction (PCR) targeting *tpi* gene, a species-specific triose phosphate isomerase gene [36].

2.2. Toxigenic profile of the isolates

PCR for detecting the *tcdA* [36], *tcdB* [33], *cdtA* and *cdtB* [37] genes was performed as previously described. Amplification and sequencing of *tcdC* [38] was also performed to investigate deletions in this gene and the sequences were analyzed using the BLAST server of National Center for Biotechnology Information (NCBI).

2.3. PCR-ribotyping

C. difficile isolates were analyzed at the Anaerobe Reference Laboratory by PCR-ribotyping as previously described [39]. Gel images were analyzed using Gel Compar II Software (version 4.0; Applied Maths, Kortrijk, Belgium).

2.4. Minimal inhibitory concentration determination

MICs for clindamycin (CLI), metronidazole (MTZ), vancomicyn (VAN), moxifloxacin (MX), levofloxacin (LX) and ciprofloxacin (CIP) were determined for each *C. difficile* isolate by using E-test strips (AB Biodisk), according to the manufacturer's instructions. Quality control strains (*Bacteroides fragilis* ATCC 25285 and *Staphylococcus aureus* ATCC 29213) were used. The breakpoints for CLI (≥8 mg/L) and MTZ (≥8 mg/L) were determined in accord to CLSI guidelines (2007). For antibiotics, for which no standard breakpoints have been defined, breakpoints were considered as follows: VAN ≥8 mg/L, MX ≥ 4 mg/L, LX ≥ 4 mg/L, CIP ≥8 mg/L [27,40].

2.5. Detection of mutation in resistance genes

A quinolone resistance-determining region (QRDR) of DNA gyrase genes *gyrA* and *gyrB* [25] was amplified by PCR and the resulting amplicons were purified (GFX PCR DNA & Gel Band® (*GE* Healthcare)) and sequenced. The amino acid sequence alignments from *C. difficile* strain 630 were produced for comparison, whose sequences are available at NCBI databank.

3. Results

The seventy four stool samples obtained from patients of HUCFF were cultured and tested for the presence of toxins A and B by ELISA to detect active CDI (characterized as the detection of toxins and/or presence of the microorganism). Three samples obtained a positive result using an ELISA commercial assay, and *C. difficile* could be recovered from two of these samples. Besides that, *C. difficile* was also isolated from a sample that had a negative result in ELISA, presenting a total of four cases of CDI during the study. All cases of CDI were identified in patients from the hematology ward. These patients had acute lymphoid or myeloid leukemia and had made use of antimicrobial agents and chemotherapy. Table 1 shows the clinical information of the four patients.

Detection of *tcdA* and *tcdB* genes by PCR confirmed that all strains were toxigenic. The genes of CDT (*cdtA* and *cdtB*) were not detected, and no deletions in *tcdC* genes from the isolates were observed. PCR-ribotypes 014 [isolate DHU 22 (A)], 043 [DHU 3 (A)] and 046 [DHU 5] were identified among the isolates. PCR-ribotype 027 was not detected.

All strains were sensitive to metronidazole and vancomycin, but resistant to the fluoroquinolones ciprofloxacin and levofloxacin. Resistance to moxifloxacin, a fourth-generation fluoroquinolone, was demonstrated only in DHU 22 (A) strain, from PCR-ribotype

Table 1
Characterization of CDI patients from "Hospital Universitário Clementino Fraga Filho – HUCFF".

Patient identification	Clinical conditions	Age (gender)	Chemotherapy	Antibiotics	AHSCT[m]	Period
DHU 3	AML[a]	33 (M)	+	TRI[c]; IMI[d]; VAN[e]; POL[f]; AMP[g]; MTZ[h]	+	August/2009
DHU 5	AML	44 (M)	+	CEF[i]; IMI; TEI[j]; CIP[k]	+	September/2009
DHU 22	ALL[b]	33 (F)	+	TRI; MER[l]	–	December/2009
DHU 50	AML	34 (M)	+	CEF; MTZ; TRI, IMI	+	July/2010

[a] AML: Acute myeloid leukemia.
[b] ALL: Acute lymphoblastic leukemia.
[c] TRI : Trimetropim.
[d] IMI: Imipenem.
[e] VAN: Vancomicin.
[f] POL: Polimixin.
[g] AMP: Ampicilin.
[h] MTZ: Metronidazole.
[i] CEF: Cefepime.
[j] TEI: Teicoplanin.
[k] CIP: Ciprofloxacin.
[l] MER: Meropenem.
[m] AHSCT: Allogeneic hematopoietic stem cell transplantation.

014. Only one isolate [DHU3 (A)] was sensitive to clindamicyn (Table 2).

After sequencing of QRDR regions of *gyrA* and *gyrB* genes, an analysis of the sequence translation demonstrated an amino acid substitution in *gyrB* of the isolate from PCR-ribotype 014. This substitution led to a codon change, from GAT to AAT, which resulted on the substitution of the amino acid aspartic acid (Asp) to asparagine (Asn), at position 426 (Asp 426 → Asn).

4. Discussion

In recent years, *Cdifficile* infection has gained importance due to the greater frequency and severity of cases and is considered the main etiological agent of cases of diarrhea associated with antibiotics [41,42]. In Brazil, there is limited information about the infection promoted by *C. difficile*, especially regarding the incidence of CDI, dissemination of the pathogen in hospitals and its resistance to various antibiotics. This lack of information seems to be directly related to limited technologies of national laboratories available to investigate this organism, leading to underreporting of CDI cases [2]. Although our work used a small number of samples, the presence of CDI was detected in 5.4% (4/74) of patients under broad-spectrum antibiotic therapy enrolled in this study, and *C. difficile* could be recovered from 3 of the stool samples from these patients. The other ELISA-positive sample was repeatedly subjected to culture, but we were not able to recover *C. difficile*. All cases of CDI in this study were detected in patients with acute myeloid (3 out of 4 patients with this disease in the study) or lymphoblastic leukemia (1 out of 2). Patients with hematologic diseases are very susceptible to CDI as most of them present a combination of risk factors for CDI such as bone narrow and peripheral blood stem cell transplantation, recent receipt of chemotherapy and use of antimicrobial agents [43–45].

Table 2
MIC value (mg/L) obtained for the *C. difficile* strains from HUCFF.

MIC (mg/L)						
Strain	MTZ	VAN	CLI	CIP	LX	MX
DHU 3 (A)	0.5	2.0	4.0	**≥32.0**	**16.0**	1.0
DHU 5	0.25	2.0	**16.0**	**≥ 32.0**	**16.0**	2.0
DHU 22 (A)	0.25	1.0	**8.0**	**≥32.0**	**≥32.0**	**≥32.0**

Breakpoints: MTZ – metronidazole (breakpoint ≥ 8 mg/L); VAN – vancomycin (breakpoint ≥ 8 mg/L); CLI – clindamycin (breakpoint ≥ 8 mg/L); CIP – ciprofloxacin (breakpoint ≥ 8 mg/L); LX – levofloxacin (breakpoint ≥ 4 mg/L); MX – moxifloxacin (breakpoint ≥ 4 mg/L); MICs greater or equal to breakpoint are in bold.

The culture of diarrheal feces for isolation of *C. difficile* is not performed routinely in the clinic, even though the combination of a CDI diagnosis based on toxin detection and the culture and isolation of *C. difficile* is considered the most accurate methodology for the diagnosis of CDI [2]. Culture is extremely useful because it allows the realization of further typing of strains and epidemiological studies [6]. A commonly used typing methodology is PCR-ribotyping, which is high discriminative, reproducible, relatively rapid and easy to perform [39]. Our PCR-ribotyping analysis revealed the presence of three distinct PCR-ribotypes, 014, 043 and 046. The hypervirulent strain belonging to PCR-ribotype 027, was not found in these samples, and considering the works published in the country so far, this PCR-ribotype is not yet in circulation in Brazil [2,31–34,46–50].

The PCR-ribotypes 043 and 046 are more rarely found than PCR-ribotype 014. These two PCR-ribotypes had been described in Brazil many years ago by Alcides et al. [32], in a study conducted at a public children's hospital "Instituto de Puericultura e Pediatria Martagão Gesteira (IPPMG)", a HUCFF neighboring building.

The PCR-ribotype 014 is widely distributed throughout the world and it has been reported in almost all European countries [51,52]. The PCR-ribotype 014 has been also described in Brazil, by Balassiano et al., in 2009 [33], when strains were isolated from feces of hospitalized patients between 2006 and 2007 at "Instituto de Pesquisa Clínica Evandro Chagas – IPEC", located at "Fundação Oswaldo Cruz", also in Rio de Janeiro. The isolation of a strain of this PCR-ribotype in HUCFF could suggest a spread of *C. difficile* between hospitals and medical centers, which reinforces the need of more surveillance studies to monitoring this important pathogen.

The three isolates were sensitive to metronidazole and vancomycin, drugs used to treat the disease. This was expected since reports of resistance to these drugs are extremely rare [27]. Resistance to clindamycin is common among strains related to outbreaks, but among the non-epidemic strains, high levels of resistance (MIC > 256 mg/ml) are found in only 10% [53]. All strains in this study had low to moderate resistance (MIC 4–8 mg/ml) to this antibiotic. Currently, fluoroquinolone-resistance characterizes not only the epidemic *C. difficile* strain PCR-ribotype 027, but also other types of strains, epidemic or not [26,54]. Several studies have associated this resistance with an increase of virulence potential [16,18]. A case–control study conducted in 2003 found an association between fluoroquinolones and CDI stronger than the association between clindamycin and this disease [9]. In this study all strains were resistant to ciprofloxacin and to another second generation fluoroquinolone, levofloxacin. These results are in

agreement with those obtained by several research groups [2,27,33,34,37,55]. Resistance to moxifloxacin, a fourth-generation fluoroquinolone, is a hallmark of PCR-ribotype 027, also present in other multi-resistant strains of *C. difficile*. In this study, only the strain belonging to PCR-ribotype 014 was resistant to this antibiotic. When this PCR-ribotype was first isolated in Brazil by Balassiano et al. [33] in a study conducted between 2006 and 2007 in IPEC, this strain was susceptible to moxifloxacin.

To investigate the molecular mechanisms associated with fluoroquinolone-resistance regions of the genes that encode the subunits of DNA gyrase (*gyrA* and *gyrB* genes) were analyzed. So far, at least seven different amino acid substitutions in the *gyrA* gene and seven other in *gyrB* gene have been described, principally conferring resistance to moxifloxacin [27,56]. Mutations in *gyrA* occur more frequently than those observed in *gyrB* and $Thr_{82} \rightarrow Ile$ in *gyrA* is the most common replacement in fluoroquinolone resistant strains [57]. Despite all isolated strains being resistant to ciprofloxacin and levofloxacin, only the DHU 22 (A) isolate, belonging to PCR-ribotype 014, was resistant to moxifloxacin with an MIC of $\geq$32 mg/L, which indicates a high level of resistance to this drug. This strain was also the only one to demonstrate an amino acid substitution, the exchange $Asp_{426} \rightarrow Asn$ in *gyrB*. This type of mutation is usually associated with low and intermediate levels of resistance to moxifloxacin, while high levels of resistance are mainly related to the substitution Thr82 → Ile in *gyr*A [27]. Resistance to moxifloxacin in *C. difficile* PCR-ribotype 014 as well as the point mutation ($Asp_{426} \rightarrow Asn$) has been described previously by Spigaglia et al., in 2010 [57]. However, this is the first report of this mutation in a PCR-ribotype 014 strain in Brazil. Since moxifloxacin resistance may be regarded as an important factor in *C. difficile* spread, aided by such indiscriminate use of this antibiotic, the fact that we have isolated a PCR-ribotype 014 strain showing moxifloxacin resistance only three years after the first report of this PCR-ribotype in the country, which at that time were sensitive to moxifloxacin, may suggest that resistance to fluoroquinolones could be contributing to permanence, survival and prevalence of certain clones of *C. difficile* in the hospital environment.

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