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C.difficile (including epidemiology)

Molecular epidemiology of *Clostridium difficile* infection in a Brazilian cancer hospital

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ABSTRACT

Clostridium difficile is a Gram-positive spore forming anaerobic bacterium and the main cause of healthcare-associated diarrhea. This study aimed to perform the phenotypic characterization and molecular typing of *Clostridium difficile* isolates among patients at a cancer hospital in Brazil. During 18 months, 48 diarrheic fecal samples were collected, of these 48% were positive in either one or both of the performed tests: detection of toxins A/B and culture. *Clostridium difficile* was recovered from four samples (17%). All strains carried toxin A and B genes, and the isolates belonged to PCR-ribotype 014/020, PGFE-type NAP4 and toxinotype XVIII. On the other hand, one isolate belonged to a novel PCR-ribotype, and PFGE-type, likewise to toxinotype IXb. The isolates showed susceptibility to metronidazole, vancomycin and moxifloxacin, and were resistant to ciprofloxacin. Finally, the findings indicate high positivity between the samples tested, suggesting an expressive importance of this infection, including detection of a novel ribotype/PFGE-type of *Clostridium difficile*, and show for the first time the detection of community-associated *Clostridium difficile* infection (CA-CDI) in these patients in Northeast Brazil. These data emphasize the importance to a better understanding of the epidemiological situation of this infection in Brazilian hospitals.

1. Introduction

Clostridium difficile is a strictly anaerobic, spore-forming, toxin-producing Gram positive bacillus. Currently, it is the main cause of healthcare associated diarrhea due to the use of antibiotic [1].

The major risk factors for the development of *C. difficile* infection (CDI) include advanced age and exposure to healthcare institutions and antimicrobial agents [1].

Patients with cancer can be particularly susceptible to CDI owing to the presence of multiple CDI risk factors including prolonged hospitalization and exposure to multiple antibiotics and chemotherapeutic agents [2,3].

Chemotherapeutic agents can cause CDI without concurrent use of antibiotics, because alter the intestinal mucosa, causing inflammation and necrosis, which are important factors that predispose these patients to CDI. Methotrexate and 5-fluorouracil have been commonly associated with CDI [2,3]. Moreover, these patients are often immunosuppressed and frequently use broad spectrum antibiotics, which can alter the gut microbiota [2–5].

Diarrhea is a frequent side effect of antineoplastic agents. In cancer patients receiving chemotherapy, it is difficult to distinguish chemotherapy-associated diarrhea from antibiotic-associated CDI with that *C. difficile* infection can be neglected in these patients, resulting in further compromise of general health

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and poor response to chemotherapy, increasing the risk of death [2,3,5].

In the last two decades, CDI has emerged as a challenge in healthcare settings because of a dramatic rise in infection rate, increased virulence, and resistance to treatment and an expanding at-risk population [2–5].

Considering the lack of information and diagnosis of *C. difficile* infection in Brazilian hospitals, particularly in the Northeast region, and the globalization of the infection, this study aimed to determine the perform phenotypical and molecular characterization of strains of *C. difficile* isolated from cancer patients in an oncological hospital in Brazil.

2. Materials and methods

2.1. Fecal isolates

Forty-eight fecal samples were collected between May 2013 and November 2014 from inpatients admitted in Haroldo Juaçaba Hospital of Cancer Institute of Ceará, Fortaleza, Brazil. Adult (>18 years old) patients showing ≥ 3 liquid stools over a 24-h period were considered for inclusion. All samples were collected during active diarrhea episodes. All samples were tested for the presence of toxins A/B (ProSpecT *Clostridium difficile* Toxin A/B Microplate, Remel®), according to the manufacturer's instructions.

Stool samples were also cultured using the standardized process of isolation and identification [6]. Briefly, alcohol shock was performed on the stool sample, followed by culture on cefoxitin–cycloserine–fructose agar (CCFA, Oxoid®) and fastidious anaerobe broth (FAB) for 5 and 15 days, respectively, incubated under anaerobic conditions (anaerobic jar 90% N_2, 10% CO_2). Then the FAB was inoculated onto another CCFA. Characteristic *C. difficile* colonies on CCFA appeared yellowish, with a ground-glass appearance; they were circular with slightly filamentous edges and flat to low with a rounded elevation. The colonies were lipase- and lecithinase-negative using an egg-yolk agar (EYA). These colonies were inoculated onto Brucella agar with 5% lysed sheep blood and vitamin K (5 mg/ml) for identification and molecular tests.

The identification was confirmed by testing with a RapID ANA II system (Remel) and by *tpi* gene PCR amplification.

2.2. Toxigenic profile of the isolates

Genomic DNA from each strain was obtained from overnight cultures in Brain Heart Infusion broth (BHI; Oxoid) using the InstaGene™ reagent (Bio-Rad). Fragments of *tcdA, tcdB, cdtB*, and *tcdC* were amplified by PCR using known primers and condition [6].

2.3. PCR-ribotyping

For ribotyping, intergenic spacer regions were amplified using Bidet primers as described previously [7]. PCR-ribotypes were determined by submitting data to the web-database WEBRIBO (http://webribo.ages.at).

2.4. PFGE typing

The PFGE procedure used was derived from published protocols [8]. Images were analyzed with the BioNumerics software (version 5.1. Applied Maths) and the resulting macrorestriction patterns were compared to those deposited in the databases of the National Microbiology Laboratory of the Public Health Agency of Canada (Winnipeg, Canada).

2.5. Toxinotyping

For toxinotyping, A1 and B3 regions of *tcdA* and *tcdB* were analyzed with a method described previously [9].

2.6. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) for ciprofloxacin, ceftriaxone, moxifloxacin, levofloxacin, clindamycin, metronidazole, rifampin, and vancomycin were determined using E-test (bioMérieux) [10]. Resistance breakpoints were set in agreement with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (M11-A8) as follows: ceftriaxone ≥ 64 μg/ml; moxifloxacin ≥4 μg/ml; clindamycin ≥8 μg/ml; metronidazole ≥ 32 μg/ml; rifampin ≥32 μg/ml and vancomycin >2 μg/ml.

For the fluoroquinolones we used the moxifloxacin breakpoint (ciprofloxacin and levofloxacin), for the vancomycin were used the EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/) and for the rifampicin the breakpoint used by O'Conner et al. [11].

2.7. Statistical analyses

Data are presented as means ± standard error (SEM) or as medians when appropriate. The descriptive analysis of the characteristics of the patients was performed using univariate analysis and the Chi-square test. *P* values of <0.05 were considered statistically significant. All analyses were performed using IBM SPSS® Statistics 20 software.

Table 1
Clinical data of patients with diarrhea admitted to the Haroldo Juaçaba Hospital, Fortaleza, Ceará (2013–2014).

Characteristics	Total, n (%) 48 patients	CDI, n (%) 23 patients
Age, median (range), y	58 (28–84)	57 (29–84)
Sex		
Male	20 (42)	9 (39)
Female	28 (58)	14 (61)
Cancer		
Breast cancer	10 (21)	5 (22)
Colon cancer	5 (10)	2 (9)
Gastric cancer	5 (10)	1 (4)
Rectal cancer	3 (6)	2 (9)
Others	25 (52)	14 (61)
Therapy		
Chemotherapy	39 (81)	19 (83)
Radiotherapy	21 (44)	9 (39)
None	5 (10)	4 (17)
Use of antibiotics		
Piperacillin + tazobactam	13 (27)	8 (35)
Metronidazole	9 (19)	7 (30)
Ciprofloxacin	5 (10)	4 (17)
None	11 (23)	4 (17)
Others	9 (19)	7 (30)
Comorbidities		
Hypertension	24 (50)	12 (52)
Diabetes	6 (13)	4 (17)
Cardiovascular (Stroke)	2 (4)	2 (9)
None	22 (46)	4 (17)
Others	8 (17)	6 (26)
Reasons for hospitalization		
Diarrhea	13 (27)	8 (35)
Chemotherapy	9 (19)	3 (13)
Surgery	8 (17)	3 (13)
Others	18 (37)	9 (39)
Hospitalization in the last 30 days	8 (17)	5 (22)