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Genetic relatedness, Virulence factors and Antimicrobial Resistance of *C. difficile* strains from hospitalized patients in a multicentric study in Brazil

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ABSTRACT

Background: *Clostridium* (*Clostridioides*) *difficile* infection (CDI) is recognized worldwide as a public health concern, related mainly with hypervirulent strains. In Brazil there are few studies about molecular epidemiology of *C. difficile*, for this reason, we aimed to characterize *C. difficile* isolates from a large cohort study of three different Brazilian states to identify virulence and resistance genes, specifically genes related to metronidazole and vancomycin resistance.
Methods: All 153 fecal samples were submitted to *C. difficile* culture in CM0601 broth. Identification of suspected colonies was confirmed by matrix-assisted laser desorption/ionization (MALDI-TOF/MS, Brucker Daltonics, Germany). The *tcdA* and *tcdB* toxin were searched by PCR. The sequence type (ST) was determinate by multilocus sequencing typing (MLST) and susceptibility profile was performed by agar dilution method.
Results: Among the 16 isolates, we identified fourteen different STs, five belonging to Clade 1, one to Clade 2 and eight news STs with high similarity levels. Resistance (*ermB, tetM, VanW* and *nimB*) and virulence genes (*cwp84, cwp66, cwp2, fbpA* and *secA*) were found in toxigenic strains.
Conclusion: Differently from other studies, we found high levels of resistance to vancomycin. These results suggest that the main circulating strains in Brazil belong to Clade 1 and have high pathogenicity and resistance profile.

1. Introduction

Clostridium (*Clostridioides*) *difficile* infection (CDI) is a serious disease that affects the inner surface of the colon, causing inflammation due mainly to the production of toxins (A and B), which can lead to the development of toxic colitis [1]. The A and B toxins genes are located in the region called PaLoc (Locus of Pathogenicity). PaLoc is a genomic island normally found at the same location on the chromosome, and is substituted in non-toxigenic strains by a highly conserved non-coding region of 115 / 75 bp. The regulation of A and B toxins genes is mediated by *tcdC* gene and mutations in this gene are associated to overproduction of both toxins [2].

The main risk factor for CDI is the previous use of broad-spectrum antibiotics by the patients, modifying the gut microbiota and allowing the proliferation of *C. difficile* [3]. This bacterium has been reported as the major cause of nosocomial diarrhea among adults in industrialized countries, and it is associated with significant morbidity, particularly among patients older than 65 years [4].

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To understand the epidemiological distribution of *C. difficile*, the typing methods applied are Ribotyping, Pulsed-field Gel Electrophoresis (PFGE), Multiple-Locus Variable number tandem-repeat Analysis (MLVA) and Multilocus Sequencing Typing (MLST) [1]. Currently, MLST analysis classify isolates into eight clades according to the phylogenetic evolution of *C. difficile*: Clades 1, 2, 3 and 5 consist mainly of toxigenic strains [5].

In Latin America, the dynamics of CDI have been studied mainly in Chile, Uruguay, Costa Rica, Argentina, Mexico and Brazil. However, there are few epidemiological data and surveillance systems for these regions [6]. Our group recently demonstrated a high frequency of *C. difficile* in a multicenter Brazilian study [7].

The study aimed to characterize *C. difficile* isolates from inpatients of three different Brazilian states by MLST, to identify virulence and resistance genes and to determine the susceptibility profile of these isolates to metronidazole and vancomycin.

2. Material and Methods

2.1. Data Collection

Overall, 153 stool specimens from eight Brazilian hospitals located in the Rio Grande do Sul state (H1, H2 and H3), São Paulo state (H4 and H5) and Paraná state (H6, H7 and H8) were collected in March and July, 2017. The study was initiated after approval from the local Ethics Committees of the participating hospitals (nº1.476.107).

2.2. Microbiological Methods

The stools samples were treated with absolute alcohol (proportion 1:1) at room temperature for 1 h and subcultured in CM0601 *Clostridium difficile* agar (Oxoid), enriched with 7% of blood horse, D- Cycloserine and Cefoxitin and incubated for 48 h in anaerobiosis atmosphere. The suspect colonies were identified by MALDI-TOF MS (Bruker) to confirm the isolate species as *C. difficile*.

2.3. PCR (polymerase chain reaction) test for PaLoc genes

DNA extraction was performed by thermal lysis. Briefly, few colonies were emulsified in TE (Tris-EDTA) buffer (Sigma-Aldrich) and heated at 80 °C for 20 min. Debris were removed by centrifugation and the supernatant was used. DNA was stored at −20 °C.

In house PCR was performed for *tcdA, tcdB* and *tcdC* genes. The primers and reaction conditions were described elsewhere [8]. Isolates positive for *tcdA* and/or *tcdB* genes were considered toxigenic. ATCC *C. difficile* 9689 was used as the negative control.

2.4. Multilocus Sequence Type (MLST)

Multilocus sequence typing was performed on all recovered isolates as described previously [8]. The Sequence Type (ST) assignments were defined using the *C. difficile* database at PubMLST. The Neighbor-Joining tree was performed using BioNumerics v7.3 software.

2.5. Whole Genome Sequence

The MiSeq platform (Illumina, San Diego, CA) was used for whole genome sequencing (WGS) only for toxigenic strains. The paired-end library was constructed with the Nextera XT DNA Library Prep Kit (Illumina). The run was performed with the MiSeq Reagent V2 kit (500 cycles) with a calculate coverage of 100 × . The paired-end reads were merged, quality trimmed and denovo assembled into contigs using SPAdes v3.6.2; http://cab.spbu.ru/software/spades/ (base quality scores > Q20, k = 21) [9]. The contigs were annotated by Prokka v1.12 (> 500bp; e-value cut-off 10^{-6}) [10]. In addition, reference annotation (*C. difficile* 630 reference strain) was carried out in Geneious 10.1. The resistance genes were predicted by ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) plus manual curator on Geneious 10.1.

2.6. Minimum Inhibitory Concentration (MIC)

The MIC for metronidazole and vancomycin were determined by Agar Dilution according to the Clinical and Laboratory Standards Institute (CLSI) document M10 [9]. For metronidazole, isolates with MIC ≤ 8 g/L were considered susceptible, 16 g/L intermediary and ≥32 g/L resistant. For vancomycin, MIC ≤ 2 g/L were defined susceptible and ≥ 4 g/L, resistant [11].

3. Results

3.1. Identification and Definition of Toxigenic Strains

Among the 153 fecal samples, 44 (28.75%) presented colonies with *C. difficile* characteristics (4-6 mm diameter irregular, raised opaque, grey-white). However, according to the MALDI-TOF MS, 16 (10.45%) were confirmed as *C. difficile*. Ten of them amplified the genes *tcdA, tcdB* and *tcdC* and were considered toxigenic: four from inpatients of hospital 1, three from hospital 2, two from hospital 3 (Rio Grande do Sul state) and one from hospital 5 (São Paulo state). Six samples did not amplify the PaLoc genes and were considered non-toxigenic: two from inpatients of hospital 1, two from hospital 2 (Rio Grande do Sul state) and two from hospital 7 (Paraná State).

3.2. Multilocus Sequence Type (MLST) and Phylogenetic Analyses

MLST revealed that toxigenic and non-toxigenic *C. difficile* encompassed 14 STs: four already described in Brazil (ST2, ST35, ST42, and ST67); two described for the first time in Brazil (ST15 and ST54) and eight new STs (ST541, ST542, ST543, ST544, ST545, ST546, ST547 and ST548).

Most STs were represented by only one isolate, except for ST42 (Clade 1) that included 2/16 isolates from the same hospital, and the ST54 (Clade 1), encompassing 2/16 isolates from different hospitals (Table 1).

Among toxigenic isolates, we found one isolate from hospital 3, in Porto Alegre, belonging to ST67, classified as hypervirulent strain [12].

The Neighbor-Joining tree (Fig. 1) showed high levels of similarity between strains (≥ 86.6 %), toxigenic and non-toxigenic, forming three distinct groups. ST545 and ST544 presented similarity of 97.2 %, encompassing group A; ST542 and ST546 had similarity of 98.3 % among them and ST 541 and ST11 had similarity of 97.4 %, defined group B. Finally group C were formed by STs 42, 15, 2, 54, 35, 67, 1 (BI/NAP1/ 027), 543 and 547 with similarities ≥ 99.1 %.

3.3. Virulence and Resistance Genes

Seven of the 10 toxigenic isolates were submitted to WGS, the assemble genome permitted the analysis of antibiotic resistance and virulence genetic determinants. As demonstrated in Table 1, the virulence factors linked to cell wall-binding proteins (CWP), *cwp84, cwp66* and *cwp2*, are present in three of the sequenced isolates. The *secA* gene associated to CWP cluster appears in five isolates and the gene of the shock-thermal *groEL* and fibronectin gene *fbpA* were found in four isolates. Genes codifying flagellum proteins appeared in all analyzed samples. Binary toxin genes are present in four isolates. Clinically important resistance genes, such as the *NimB*, and

Table 1
Sequence Type, Resistance and Virulence genes of *C. difficile* strains.

Sample	Hospital[a]	Collection date	ST[b]	Clade[c]	Allelic Profile[d]	Resistance Genes[e]	Virulence Factor[f]	MIC metronidazole (range 0.5 – 32 g/L)	MIC vancomycin (range 0.5 – 16 g/L)
Toxigenic samples submitted to the WGS									
T12	H1	March	35	1	2,5,8,1,1,3,6	*tetW, tet32, VanW, VanA, NimB, qnr*	*tcdA, tcdB, tcdC, cwp84, cwp66, fbpA, GroEL*	S (1 g/L)	R (4 g/L)
T113	H1	July	2	1	1,1,2,1,5,3,1	*mdtD, mdtG, NorM, VanB, MecI*	*tcdA, tcdB, tcdC, cdtB, cwp2, cwp66, cwp84, secA, slpA*	S (4 g/L)	R (8 g/L)
T26	H2	March	42	1	1,1,2,1,1,7,1	*tetW, tetO,VanW, VanZ, mefA, NimB, MdtG, qnr*	*tcdA, tcdB, tcdC, cwp2, cwp66, secA, slpA, GroEL*	NR	NR
T100	H2	July	42	1	1,1,2,1,1,7,1	*ermB, mefA, MecI, MdtA, NorM, MdtG, MarR, tetA, VanB*	*tcdA, tcdB, tcdC, cdtA, cdtB, cwp2, cwp66, cwp84, secA, slpA*	R (>32 g/L)	R (4 g/L)
T150	H3	July	54	1	1,4,7,1,1,3,3	*tetW, tetM, tetO, msrD, lnuC, ermB, VanW, NimB*	*tcdA, tcdB, tcdC, cdtA, cdtB, cwp2, cwp66, cwp84, secA, slpA, fbpA, GroEL*	NR	NR
T153	H3	July	67	2	1,1,9,9,1,3,5	*VanW, VanB, MarA, MecI, mecR, NorM, emrY, bmr3, mdtG*	*tcdA, tcdB, tcdC, cdtA, cdtB, secA, fpbA*	R (>32 g/L)	R (>16 g/L)
T66	H5	March	54	1	1,4,7,1,1,3,3	*ermB, tetW, tetM, VanZ, VanW, mefA, qnr*	*tcdA, tcdB, tcdC, secA, fbpA, GroEL*	S (1 g /L)	R (4 g/L)
Toxigenic and non-toxigenic samples non-submitted to WGS									
T01	H1	March	541[NS]	2	1,1,11,1,1,22,4	NA	*tcdA, tcdB, tcdC*	S (<0.5 g/L)	S (<0.5 g/L)
T08	H1	March	542[NS]	1	1,1,2,1,1,7,3	NA	*tcdA, tcdB, tcdC*	S (2 g/L)	S (<0.5 g/L)
NT02	H1	March	544[NS]	1	1,1,24,34,1,3,3	NA	Negative[g]	NR	NR
NT14	H1	March	545[NS]	1	1,1,24,16,1,3,1	NA	Negative[g]	R (>32 g/L)	R (16 g/L)
NT34	H2	March	546[NS]	1	1,1,51,35,1,1,1	NA	Negative[g]	NR	NR
NT36	H2	March	547[NS]	4	3,7,3,8,11,20,11	NA	Negative[g]	S (4 g/L)	R (8 g/L)
T48	H2	March	543[NS]	2	1,1,10,9,1,3,2	NA	*tcdA, tcdB, tcdC*	S (<0.5 g/L)	R (4 g/L)
NT101	H7	July	548[NS]	ND	13,46,22,40, 18,31,26	NA	Negative[g]	S (<0.5 g/L)	S (<0.5 g/L)
NT119	H7	July	15	1	1,1,6,1,8,5,1	NA	Negative[g]	S (1 g/L)	R (4 g/L)

S = susceptible; R = resistant; NR = Non-recovered: the isolates were not recovered from freeze to the tests; T = toxigenic strains; NT = non-toxigenic strains; NS = New ST; NA = Not applicable; ND = not defined.
[a]H1, H2 and H3 = Rio Grande do Sul state, Brazil; H5 = São Paulo state, Brazil; H7 = Paraná state, Brazil. [b]Sequence Type according to PubMLST; [c] Clade group according to PubMLST; [d] Allelic profile: *adk, atpA, drx, glyA, recA, sodA* and *tpi* [e] Resistance Genes performed by WGS: *tetO:* Tetracycline-resistant ribosomal protection protein; *VanW:* Vancomycin B-type resistance protein VanW; *VanZ:* Teicoplanin resistance protein; *mefA:* Macrolide efflux A domain protein; *MecI:* Methicillin resistance regulatory protein; *NimB:* Nitroimidazole antibiotic resistance protein NimB; *MdtA. MdtC, MdtD* and *MdtG*: Multridrug resistance protein; *qnr:* Quinolone resistance protein; *VanA:* Vancomycin/teicoplanin A-type resistance protein VanA; *msrD:* Macrolide resistance; *emrY:* Multidrug resistance protein; *bmr3:* Multidrug resistance protein; *mecR:* Methicillin resistance mecR1 protein; *lnuC:* Lincosamide nucleotidyltransferase; [f]Virulence Genes: *cwp2*: Cell wall binding protein; *cwp66:* Cell surface protein; *ermB*: Clindamycin resistance; *secA:* Protein translocase subunt secA; *slpA:* Precursor of the S-layer proteins; *tetW* and *tet32:* Tetracycline resistence protein; *GroEL:* Heat shock protein; *tcdA:* Toxin A; *tcdB*: Toxin B; *tcdC:* presence of *tcdC*. [g] Negative = The isolates were negative for toxin genes performed by PCR in house.

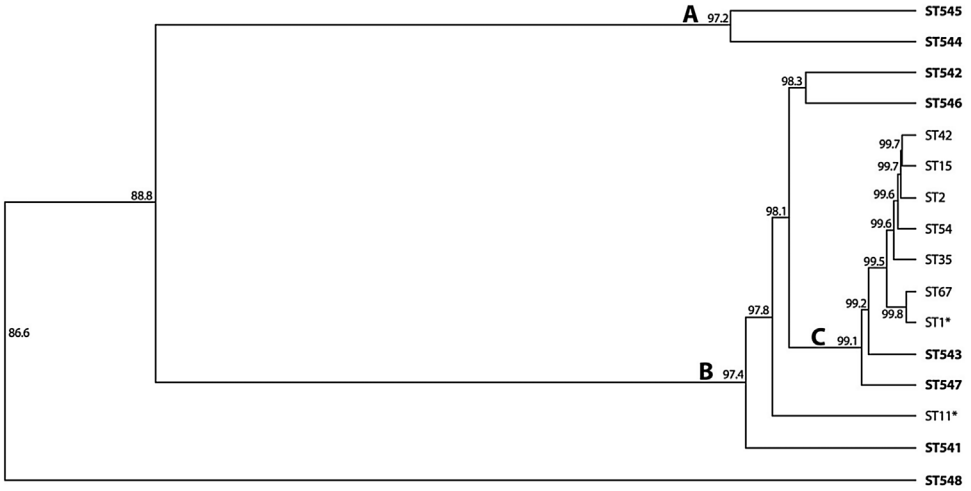

Fig. 1. Neighbor-joining tree constructed using the concatenated sequences of the seven *loci* used in MLST. Bootstraps were generated using 50 replicates, and low values were removed for clarity. The STs forming tree groups, designated A to C, with similarity of 97.2% (A), 97.4% (B) and ≥99.1% between them (C). * ST1/RT027 and ST11/RT078 hypervirulent strains used for comparison; Highlighted represent the new STs deposited in this study; Sequence Type deposited in PubMLST.org.

the *Van* family, which confer resistance to metronidazole and vancomycin, respectively appeared in samples T12 (ST35), T26 (ST42) and T150 (ST54). *Van* genes were also present in samples T66 (ST54), T100 (ST42), T113 (ST2) and T153 (ST67).

3.4. Minimum Inhibitory Concentration (MIC)

We were able to determine vancomycin and metronidazole MICs for 12 isolates (Table 1), 7 toxigenic and 5 non-toxigenic

strains. According to the CLSI breakpoints, two isolates were classified as resistant to metronidazole (≥32 g/L). On the other hand, only three isolates (two toxigenic and one non-toxigenic) were susceptible to vancomycin (≤2 g/L).

4. Discussion

Studies concerning epidemiology of *C. difficile* are scarce in Brazil. The methodologies applied are heterogeneous, i.e., ribotyping [13] and MLST [12; 14], the latter present greater inter-laboratory reproducibility.

We determined the genetic relatedness of isolates from stool samples of patients from different hospitals in different states of Brazil by MLST and found a population of *C. difficile* distributed in 14 STs. To the best of our knowledge, this is the first description of STs 15 and 54 in our country. ST15 has already been described in the UK [8], but because it is a non-toxigenic ST, there are not much data in the literature. ST54 is already widely disseminated in Latin America, causing outbreaks [15].

Besides, two toxigenic isolates from the same hospital (Hospital 1, in Porto Alegre), one collected in March and another in July, both identified as ST42, suggesting a persistence of this ST for at least 5 months in this hospital.

Most STs identified by us belonged to clade 1, which reinforces data described before by Diniz et al [14] and suggests this clade as the predominant in Brazil. This is a subject of major concern as clades 1 and 2 are considered the most virulent [16]. Specifically in this study we detected ST67, belonging to Clade 2 and considered hypervirulent. Our group has already described this ST in 2018, suggesting this strain is circulating in South Brazil [12].

While among toxigenic isolates we observed STs highly disseminated around the world, all but one non-toxigenic isolates belonged to unpublished STs. This fact is probably due to the paucity of epidemiological studies related to non-toxigenic strains. In this context, it should be emphasized that our study is describing for the first time in Brazil the circulation of a strain belonging to Clade 4, ST547. The Neighbor-joining tree showed high similarity among the isolates, suggesting common ancestry and possible horizontal transfer of toxins genes.

The virulence factors associated with typing are poorly explored in the literature, and there are gaps in the understanding between as CDI, virulence factors and Sequence Type are related. It has been increasingly difficult to identify strains of *C. difficile* that are associated with severe disease versus those related to mild diarrhea or asymptomatic colonization. This is because patients with *C. difficile* infection have a variety of other comorbidities, varying states of immune function, and generally have received various combinations of antibiotics that cause disruption of the microbiota, complex clinical conditions that make it difficult to distinguish the role of different strains in the severity of colitis. Therefore, only the determination of the presence/absence of toxin production is not sufficient to understand the bacterial pathogenicity potential [16].

In our study, most of the strains tested (5/7 toxigenic and 4/5 non-toxigenic) had a resistance profile to vancomycin. In other countries, E*test* was used for vancomycin, and a comparison cannot be made because we used Agar Dilution. Another study in Brazil that used agar dilution reported rates of resistance to vancomycin [17]. However, in our study, we found rates much higher than those already reported which increases the concern about the treatment of CDI in the country, since vancomycin is the main drug recommended.

5. Conclusions

This is the largest study ever performed in Brazil aiming to analyze molecular epidemiology of *C. difficile.* Although the number of isolates is limited, the multicentric design and the lack of broad data concerning typing of *C. difficile* in our country reinforces the importance of our results. Further surveillance studies must be performed to increase our knowledge about ST circulating in Brazil, including hypervirulent isolates.

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Competing Interests

All authors report no conflicts of interest relevant to this article.

Ethical Approval

This study was approved by ethical committee Santa Casa de Misericórdia de Porto Alegre (1.476.107).

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