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Short communication

C.difficile (including epidemiology)

Detection and molecular characterization of *Clostridium difficile* ST 1 in Buenos Aires, Argentina

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ABSTRACT

Thirty one *C. difficile* isolates recovered in 2015 were characterized. Nineteen/31 were positive for *tcdA/B*, among them, 4 isolates were also positive for CDT coding genes. Two/4 *cdtA/B* positives isolates corresponded to ST 1 resembling BI/NAP1/027/ST 1 strain, while the others corresponded to ST 226 and ST 377.

Clostridium difficile infection (CDI) is a leading cause of hospital-associated gastrointestinal illness, which ranges from mild diarrhea to severe pseudomembranous colitis, and even death. In the last decade CDI has become more frequent, severe, refractory to standard therapy and more likely to relapse. As reported in the United States, Canada and Europe, these CDI have been associated to the emergence of a highly virulent fluoroquinolone resistant *C. difficile* strain referred as NAP1/BI/027 for North American pulsed-field type one (NAP1), restriction-endonuclease analysis type BI and PCR ribotype 027 [1,2].

Previous studies on CDI are scarce in Argentina. Although some of them described the detection of toxin A and/or B coding genes (*tcdB* and *tcdA*), to the best of our knowledge, binary toxin (CDT) coding genes (*cdtA* and *cdtB*) have never been reported in indexed literature [3–7], in our region. The aim of this study was to characterize *C. difficile* isolates including the detection of different toxin coding genes, the determination of the susceptibility profile of the isolates and their multi-locus sequence type (MLST, ST).

There were included a total of 31 *C. difficile* isolates recovered from diarrheal stools samples from patients with presumptive CDI at two different hospitals in Buenos Aires, during 2015. All these diarrheal samples had rendered positive for glutamate dehydrogenase (GDH) and 13 of them had been positive for toxins A/B using the C. Diff Quik Chek Complete Enzyme Immunoassay (EIA) (Techlab, Blacksburg, VA, USA) (Table 1).

To recover *C. difficile*, the stool specimens were subjected to ethanol shock with 1:1 vol/vol absolute ethanol/stool sample, mixed and incubated for 30 min at room temperature. After cultured on Brucella agar supplemented with 5% lysed blood (BA), they were incubated under anaerobic conditions at 35 °C, and examined after 48 h. The recovered *C. difficile* were confirmed by MALDI-TOF MS (Biotyper version 3.1, Bruker Daltonics GmbH, Bremen, Germany) [8].

PCR amplification of toxin coding genes, *tcdA, tcdB, cdtA* and

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Table 1
Characterization of *C. difficile* isolated from GDH positive samples.

C. difficile isolate		Quick Check complete[a]		Toxins genes by multiplex PCR				FQ susceptibility[b]/Mutations in GyrA-QRDR	MLST [clade[c]]
		GDH	Tox A/B	*tcdA*	*tcdB*	*cdtA*	*cdtB*		
Hospital I	HE782/15	+	−	−	−	−	−	S	
	HE1036/15	+	−	−	−	−	−	S	
	HE1046/15	+	−	−	−	−	−	S	
	HE1064/15	+	−	+	+	+	+	S	ST 1 [2]
	HE1098/15	+	−	−	−	−	−	S	
	HE1871/15	+	−	+	+	−	−	S	
	HE1935/15	+	−	+	+	−	−	S	
	HE2281/15	+	+	+	+	+	+	S	ST 1 [2]
	HE2299/15	+	−	+	+	−	−	R/WT	ST 42 [1]
	HE2322/15	+	+	−	−	−	−	S	
Hospital II	HA1	+	+	+	+	−	−	R/Thr82Ile	ST 375 [4]
	HA2	+	+	+	+	−	−	S	
	HA3	+	+	−	−	−	−	R/WT	ST 376 [1]
	HA4	+	+	−	−	−	−	R/Thr82Ile	ST 15 [1]
	HA5	+	+	+	+	−	−	S	
	HA6	+	−	−	−	−	−	S	
	HA7	+	−	+	+	−	−	S	
	HA8	+	+	+	+	−	−	S	
	HA9	+	+	+	+	−	−	S	
	HA10	+	+	+	+	−	−	S	
	HA11	+	−	−	−	−	−	S	
	HA12	+	−	−	−	−	−	S	
	HA13	+	+	+	+	+	+	S	ST 226 [2]
	HA14	+	+	+	+	−	−	S	
	HA15	+	−	+	+	−	−	S	
	HA17	+	−	+	+	−	−	S	
	HA18	+	−	+	+	+	+	R/Thr82Ile	ST 377 [ND]
	HA19	+	−	−	−	−	−	S	
	HA20	+	−	+	+	−	−	S	
	HA21	+	+	+	+	−	−	S	
	HA24	+	−	−	−	−	−	S	
	Total of positives	31	13/31	19/31	4/31				

[a] Quik Chek complete was performed on stool samples.
[b] S: susceptible; R: resistant; FQ: fluoroquinolones; WT: wild type, no mutation in QRDR region of *gyrA*.
[c] According to https://pubmlst.org/cdifficile/. ND: Not determined at https://pubmlst.org/cdifficile/, in S1 it is shown a dendrogram for different STs including ST 377.

cdtB, was conducted by multiplex PCR in all isolates according to Persson et al., using genomic DNA as template [9,10]. The amplicon sizes for *tcdA*, *tcdB*, *cdtA* and *cdtB* were 629 bp, 410 bp, 221 bp and 262 bp, respectively. The amplicons were purified using a commercial kit (INBIO HIGHWAY, Argentina), sequenced (Macrogen Inc, Korea) and compared with databases using NCBIs BLASTn tool. Nineteen from 31 isolates (61%) were positive for *tcdA* and *tcdB,* compared with 13 toxins A/B positive samples (41%) detected by EIA. No *tcdB* positive/*tcdA* negative isolates could be detected despite they have been previously reported in our country [5]. It is appropriate to point out that in the present study the toxigenic cultures were inferred by detecting the toxin coding genes but not the toxins itself, probably contributing to the discrepancies observed with the EIA test performed on stool samples [11–13].

cdtA/B were detected in 4/31 (13%) isolates, corresponding 2 isolates to each hospital (Table 1), 2 of them were categorized as negative for toxin production by the commercial kit. It has been suggested that the presence of binary toxin is linked to more severe disease outcomes [14]. One patient, from which *cdtA/B* positive isolate was recovered, presented recurrence and death attributable to this infection after 47 days. The others did not show signs associated with severe CDI (Table 2).

Minimum inhibitory concentration (MIC) of different antimicrobials were determined according to CLSI 2015 [15]. Current antimicrobial options for CDI treatment, such as vancomycin and metronidazole were included. Also, moxifloxacin, levofloxacin and gatifloxacin, were assayed as the hypervirulent strain, e.g. *C. difficile* BI/NAP1/027, has been associated with resistance to these drugs. MIC_{50} and MIC_{90} values of vancomycin, metronidazole, moxifloxacin, levofloxacin and gatifloxacin are shown in Table 3. Interpretative breakpoints for metronidazole and moxifloxacin were applied according to CLSI and EUCAST guidelines, while for vancomycin those from EUCAST for *C. difficile* (http://www.eucast.org/clinical_breakpoints/) [15]. All isolates were susceptible to metronidazole and vancomycin, and 5/31 exhibited resistance to moxifloxacin (Table 1). In good agreement with global reports, the lowest MIC ranges were observed for vancomycin (0.125–0.5 μg/ml) and metronidazole (≤0.125–1 μg/ml), even if isolates with reduced susceptibility to metronidazole and vancomycin, mainly related to ribotype 027 and 018, and 356, respectively, have been already reported [16–19]. Moxifloxacin resistant isolates (16–32 μg/ml), showed comparable MIC values for gatifloxacin (16–32 μg/ml) and levofloxacin (>32 μg/ml). From the 5 fluoroquinole resistant (FQR) isolates, HA18 was positive for *tcdA, tcdB* and binary toxin coding genes resembling the BI/NAP1/027 strain, while HA1 and HE2299/15 rendered positive results for *tcdA* and *tcdB*, but did not harbored binary toxin genes [1]. The remaining FQR isolates (HA3 and HA4) were non-toxigenic according to the multiplex PCR (Table 1).

The presence of mutations in the quinolone-resistance determining region (QRDR) of both DNA gyrase coding genes, *gyrA* and *gyrB,* was investigated according to Dridi et al., using genomic DNA as template [20]. Amino acid substitutions in QRDR of GyrA are shown in Table 1. No mutations were detected in QRDR of *gyrB.*

Table 2
Clinical features of patients with *cdtA/B* positive *C. difficile*.

Characteristics	HE1064/15 *tcdA/B* (+), *cdtA/B* (+), FQS, ST 1	HE2281/15 *tcdA/B* (+), *cdtA/B* (+), FQS, ST 1	HA13 *tcdA/B* (+), *cdtA/B* (+), FQS, ST 226	HA18 *tcdA/B* (+), *cdtA/B* (+), FQR, ST 377
Age	62	2	65	97
Gender[a]	M	F	F	F
Underlying disease	multiple myeloma	bone autoinflamatory diseases	recent kidney transplant	heart failure
Medical service	hematology	emergencies	transplants	medical clinic
Admitted for CDI diagnosis	no	yes	yes	no
Hospitalization's days before CDI	3	0	0	0
Days of diarrhea	5	1	13	1
Bloody diarrhea	no	yes	no	no
Inmunosupressors[b]	yes	yes	yes	no
Antimicrobial therapy[b]	yes	yes	yes	yes
WBC/mm^3[c]	591	10900	2500	7500
Fever	no	yes	yes	no
CDI treatment	metronidazole	autolimited	metronidazole	probiotics
Recurrence	no	no	yes	no
Death after 30 days of CDI	no	no	yes[d]	no
Stay after CDI (days)	14	0	47	0

[a] M: male, F: female.
[b] Exposure 90 days previous to CDI.
[c] WBC: white blood cells.
[d] Death after 47 days of hospitalization with CDI, FQS: fluoroquinolones susceptible, FQR: fluoroquinolones resistant.

Table 3
In vitro antimicrobial activities against 31 isolates of *C. difficile*.

Antimicrobials	MIC (μg/ml) (n = 31)		
	Range	MIC_{50}	MIC_{90}
Vancomycin	0.125–0.5	0.125	0.5
Metronidazole	≤0.125–1	0.125	0.5
Levofloxacin	1 - > 32	4	4
Moxifloxacin	1–32	1	16
Gatifloxacin	0.5–32	1	32

Thr82Ile substitution was identified in GyrA of 3/5 FQR samples (HA1, HA4 and HA18). In the remaining resistant isolates no mutations were identified (Table 1), probably involving other amino acid changes outside the QRDR of GyrA and/or GyrB, as proposed for *C. difficile* and other bacteria [21,22]. A Thr82Ile substitution in GyrA has been described as responsible for the acquisition of FQR in *C. difficile* epidemic clone BI/NAPI/027 and has also been reported in other ribotypes including those virulent and worldwide spread. Exposure to fluoroquinolones has been reported as an independent risk factor for CDI associated to FQR epidemic ribotypes, enhancing their persistence and increasing the outbreak occurrence. Even, it has been demonstrated that BI/NAPI/027 clone had the ability to quickly replace endemic strains such as ribotype 001 [23–26].

MLST was conducted on *cdtA/B* positive isolates and on those displaying FQR. The following housekeeping genes: *adk, atpA, dxr, glyA, recA, sodA* and *tpi* were targeted as previously described by Griffiths et al. 2010 [27]. Amplicons sequences were compared with MLST database (https://pubmlst.org/cdifficile/) to identify the allelic profiles and the corresponding ST. According to Griffiths et al. some STs correspond to a single PCR ribotype such as the hypervirulent strain BI/NAP1/027 which was related to ST 1, and currently is named BI/NAP1/027/ST 1 [27]. Recently, it has been reported the emergence of this epidemic strain in Latin America [28]. In our study, there were identified different STs such as ST 1, ST 15, ST 42 and ST 226, and also 3 new ST: ST 375 which corresponds to clade 4 and ST 376 which corresponds to clade 1. No clade was assigned for ST 377 (Table 1). A neighbor joining tree was constructed with all the concatenated formats for all ST deposited on data base (https://pubmlst.org/cdifficile/) using the MEGA 5 program (S1). ST 377 groups with ST122 for which no clade is assigned in https://pubmlst.org/cdifficile/. The nearest branch of the tree includes ST 210, ST 211 and ST 284 with no assigned clade, and ST 287, ST 414 and ST 429 which belong to clade 1. The isolate HA18, which was positive for *cdtA/B* and FQR, resembling the BI/NAP1/027/ST1 strain, was associated to ST 377 but not ST 1. On the other hand, 2 *cdtA/B* positive isolates, recovered at the same hospital (HE1064/15 and HE2281/15), were typed as ST 1 and displayed susceptibility to all tested antimicrobials. HE2281/15 was recovered from a child of two years old who developed auto limited bloody diarrhea (Table 2). Although the modern hypervirulent strain BI/NAP1/027/ST1 is highly resistant to fluoroquinolones, it has been related to the "historic" ribotype 027 strain, reported in Asia, which did not display FQR [29]. The isolate HA1 corresponded to ST 375, a single locus variant of ST 37 which is related to ribotype 017, previously detected in our country.

This study reports *cdtA/B* positive *C. difficile* producing healthcare-associated CDI, in Argentina. Although remaining susceptible to fluoroquinolones, *cdtA/B* positive isolates corresponded to ST 1, which resembles the hypervirulent epidemic clones; dissemination of this strain seems to be less frequent than in other regions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anaerobe.2017.10.007.

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