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Pathogenesis

Cytotoxicity and antimicrobial susceptibility of *Clostridium difficile* isolated from hospitalized children with acute diarrhea

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Abstract

Clostridium difficile is an important pathogen associated with outbreaks of pseudomembranous colitis and other intestinal disorders such as diarrhea. In this study, 181 stool samples from children with and without acute diarrhea were analysed. Eighteen children with acute diarrhea were positive to *C. ramosum*, *C. difficile*, *C. limosum*, *C. clostridioforme*, *C. septicum*, *C. butyricum*, *C. innocuum* and *Clostridium* sp. Nineteen children without diarrhea harbored *C. ramosum*, *C. septicum*, *C. barattii*, *C. butyricum*, *C. innocuum*, *C. sphenoides*, *C. bifermentans*, *C. clostridioforme* and *C. paraputrificum*. No patient with diarrhea harbored *C. barattii*, *C. bifermentans*, *C. paraputrificum* and *C. sphenoides.* In addition, ten *C. difficile* strains were detected in 5 (5.5%) of the children with diarrhea. Also, no children from control group harbored *C. difficile*, *C. limosum* and *Clostridium* sp. Most of the tested strains were resistant to all the used antimicrobial. Nine *C. difficile* were toxigenic on VERO cells and by multiplex PCR, six strains showed both toxin A and B genes and three strains showed only toxin B gene. In this study, the presence of *C. difficile* was not significant, and it is suggested the need of more studies to evaluate the role of clostridia or *C. difficile* play in the childhood diarrhea and these organisms must be looked for routinely and a periodic evaluation of antimicrobial susceptibility should be performed.

Keywords: *Clostridium difficile*; Diarrhea; Cytotoxicity; Antimicrobial susceptibility

1. Introduction

The interest in the clostridia as intestinal pathogens, particularly in children, is due to the few data are available on the species of *Clostridium* inhabiting their gastrointestinal tract, and also because these organisms have also been implicated in the etiology of cancer of the colon [1,2]. *Clostridium* spp. is widely distributed in the environment; they are a heterogeneous Gram-positive rods, anaerobic, fermentative and spore-forming that inhabit the intestinal tract as human intestinal indigenous microbiota [3].

Clostridia are distributed in five groups: group I, *Clostridium perfringens*, *C. septicum*, *C. novyi* (type A), *C. bifermentans*, *C. histolyticum*, and *C. sordellii*, associated to mionecrosis or gas gangrene; group II, *C. tetani*, tetanus; group III, *C. botulinum*, botulism; group IV, *C. difficile*, acute diarrhea antibiotic-associated and pseudomembranous colitis (PMC); and group V, *C. perfringens*, *C. ramosum*, *C. bifermentans* and others, associated to cerebral abscesses, pneumonia, bacteremies, and abdominal and gynecologic infections [4].

C. limosum produces a transferase closely related to the *C. botulinum* C_3 exoenzyme and have a common substrate protein Rho which are involved in the regulation of the microfilament cytoskeleton and the formation of adhesion plaques [5].

C. difficile is an important nosocomial pathogen and is associated with outbreaks of PMC and other intestinal disorders such as diarrhea in children and adults, but its role in the pathogenesis of gastroenteritis is still the subject of controversy. In Brazil, only a small number of clinical laboratories are able to reach a definitive diagnosis of *C. difficile* infection, maybe because simple reliable assays for toxins in the isolates are not available.

The major recognized cause of antibiotic-associated colitis is cytotoxigenic *C. difficile* [6]. *C. difficile*

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produces two toxins: A (enterotoxin) and B (cytotoxin) which are the major virulence factors of this organism and are encoded by two separated genes that are located in close proximity on chromosome [5]. Toxin A causes fluid accumulation associated with mucosal damage in several animal models (rabbit ileal and colonic loops), hamster cecal segments and mouse and rat intestine [7]. Toxin B has no enterotoxic activity, but is a more potent cytotoxin than toxin A in tissue culture line by approximately 1000-fold [6].

The definitive diagnosis of *C. difficile* is accomplished by the isolation of toxigenic *C. difficile* from stool and detection of the toxin [5]. Several methods are presently available for the laboratory diagnosis of *C. difficile*-associated diarrhea (CDAD) including cell culture assay for the presence of cytotoxin, anaerobic culture of stool specimens for the organism followed by testing for the production of toxin (toxigenic culture), latex agglutination for the detection of *C. difficile*-associated antigen in stools and enzyme immunoassays (ELISA) for the detection of toxin A, toxin B or both [8], but none of those methods have yet been able to offer a high sensitivity and specificity with ease and rapidity of test performance [9]. The developing of a multiplex-PCR amplifying simultaneously genes that encode the toxins A and B production, and it can be used to distinguish toxigenic and non-toxigenic *C. difficile* [10].

C. difficile decrease with age in young children and it allow reaching the similar levels found in adults at about 2 years of age. However, it is suggested an existence of an association between recovery of *C. difficile* toxin from stools and previous treatment with antibiotics, at least after 12 months of age [11]. The antibiotic resistance patterns in *C. difficile* have been mentioned and several resistance genes have been described [12].

In this study, the presence of *C. difficile* and other clostridia in children with acute diarrhea hospitalized at the children's hospitals have been examined.

2. Materials and methods

2.1. Patients and sample collection

During the period from June 2000 through June 2001 a total of 181 fecal samples: 91 from children with acute diarrhea and 90 children without diarrhea, were collected. Children aged 0–5 years, without sex or race distinction, were selected. Children with diarrhea were hospitalized at the Children Hospital Darcy Vargas, University Hospital at the University of São Paulo and Children Hospital Cândido Fontoura (São Paulo, SP, Brazil). Diarrhea was defined as at least three loose stools per day within 2 days of the sample. Stools were naturally evacuated and immediately processed. Some patients were in antibiotic therapy (penicillin, oxacillin, erythromycin, cephalexin, trimetropin, amikacin, chloramphenicol, amoxicilin). Healthy children without diarrhea from a day care center, in the same age group were selected as control. The Ethic Commission from each hospital approved this study.

2.2. Bacterial isolation and identification

All the stool samples were streaked onto a selective cycloserine cefoxitin fructose agar (CCFA) [13] and incubated in anaerobic conditions (90% N_2/10% CO_2), at 37°C, for 5 days. Approximately, 8–9 characteristic colonies showing a yellow fluorescence under ultraviolet light from each sample were subcultured onto blood agar. Isolates were presumptively identified (Gram stain; lipase, lecithinase, catalase, H_2S, and indole production; gelatin, esculin and starch hydrolysis and spore localization). The definitive identification was performed by fermentation of fructose, glucose, lactose, maltose and sucrose, in peptone-yeast extract (PY) broth [3]. A cytotoxic reference strain *C. difficile* VPI 10463 kindly provided by Dr. Felicja Meisel Mikolajczyk from Department of Medical Microbiology, Center of Biostructure Research, Poland, was also used.

2.3. Cytotoxicity assay

Bacterial cytotoxicity was assayed on VERO (African green monkey kidney) tissue culture monolayers. Cells were grown in 96-well microtitration plate (Corning, USA) with L15 minimal medium (Cultilab Ltd.) contained 2% fetal bovine serum, in air plus 5% CO_2, at 37°C, for 48 h. Fecal extracts were prepared by mixing 0.5 mL of stool and 1 mL of phosphate buffered saline (PBS, pH 7.2), centrifuged (6000*g*, 15 min) and supernatant was filtered through 0.45 μm membrane filter (Millipore Corporation, Bedford, MA). In addition, all the isolated strains were grown in brain heart infusion (BHI), centrifuged (13,000*g*, 5 min) and supernatants filtered through 0.45 μm membrane filters (Millipore). Fecal or supernatant filtrates (50 μL) were added in duplicate to VERO cells, and after incubation for 2 days in air-5% CO_2 at 37°C, results were compared with control cells (VERO cells plus 50 μL of PBS). Cytotoxicity was considered when there were affected cells.

2.4. Detection of tcdA and tcdB genes in C. difficile

Chromosomal DNA were extracted from 10 *C. difficile*, 1 *C. barattii*, 2 *C. butyricum*, 1 *C. clostridioforme*, 1 *C. innocuum*, 1 *C. limosum*, 1 *C. ramosum*, 2 *C. septicum* and 1 *C. sphenoides*. Three colonies from each species grown on blood agar were mixed with 300 μL of sterilized Milli-Q water and washed twice at 12,000*g*, for 10 min. Pellets were resuspended in 300 μL

of Milli-Q water and boiled for 17 min. After centrifugation (14,000*g*, 10 min) supernatants were saves, transferred to a new tube and used as template.

2.5. Multiplex-PCR

The used primers were: Tox-A1 (5′-GGA AAT TTA GCT GCA GCA TCT GAC-3′); Tox-A2 (5′-TCT AGC AAA TTC GCT TGT GTT GAA-3′); Tox-B1 (5′-GGT GAT ATG GAG GCA TCA CCA CTA G-3′) and Tox-B2 (5′-TCC AGG ATA AGT CTC CTC TAC GTT G-3′) (Gibco BRL Technologies) [10]. These primers amplified a characteristic 1217-bp toxin A (gene *tcdA*) and 1050-bp toxin B (gene *tcdB*) bands. DNA amplification was performed in 25 μL containing 2.5 μL of 10 × PCR Buffer (Gibco), 1.5 μL of $MgCl_2$ (50 mM), 1.0 μL of dNTP mixture (10 mM), 1 μL of each primer (0.4 μM), 0.5 μL of *Taq* polymerase (0.5 U), 5.5 μL of ultrapure water (Milli-Q plus) and 10 μL of DNA template. Amplification was performed in a DNA thermal cycler (Perkin Elmer, GenAmp PCR System 9700), programmed for 94°C (5 min) followed by 37 cycles of 94°C (30 s), 50°C (30 s) and 72°C (30 s), and then 72°C (5 min) to allow the completion of DNA extension. A negative control without DNA template was included in each PCR run.

2.6. Detection of amplified products

PCR products were visualized by electrophoresis in 1% agarose gel in 1 × TBE (1 M Tris, 0.9 M boric acid, 0.01 M EDTA, pH 8.4), at 70 V, for 2 h, and then stained with ethidium bromide (0.5 μg/mL). DNA bands were photographed on an UV transilluminator (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science). Molecular mass standard 1 kb DNA ladder (Gibco) was included.

2.7. Antimicrobial susceptibility

The minimal inhibitory concentration (MIC) was determined by a standard agar dilution method [14] using a Wilkins and Chalgren agar (Difco). A total of 10 antimicrobial agents were used: amoxicillin, ampicillin, cephalexin, clindamycin (Lupper Ind. Farm. Ltd., SP, Brazil); aztreonam (Bristol-Myers Squibb, SP, Brazil); cefoxitin and imipenem (Merck, Sharp & Dohme, SP, Brazil); chloramphenicol and penicillin G (Prodotti Lab. Farm. Ltd., SP, Brazil) and metronidazole (Aventis Ltd., SP, Brazil). The bacterial inoculum was standardized to 5×10^8 cells/mL, by using a 0.5 McFarland scale. Then, plates were inoculated with a Steer's replicator (Cefar Ltd., SP, Brazil). Plates were incubated in anaerobiosis, at 37°C, for 48 h. The antimicrobial concentrations varied from 0.25 to 512 μg/mL. Media without antibiotics were used as control. The MIC was defined as the lowest concentration of agent that completely inhibited visible bacterial growth.

3. Results

3.1. Recovery of clostridia and C. difficile

Eighteen stool samples from children with acute diarrhea were positive to *C. ramosum* (27 isolates); *C. difficile* (10); *C. limosum* (10); *Clostridium* sp. (3); *C. clostridioforme* (2); *C. septicum* (2); *C. butyricum* (1) and *C. innocuum* (1). Also, 19 stool samples from children without diarrhea harbored *C. ramosum* (15), *C. septicum* (6), *C. barattii* (5), *C. butyricum* (2), *C. innocuum* (2), *C. sphenoides* (2), *C. bifermentans* (1), *C. clostridioforme* (1) and *C. paraputrificum* (1). Although, *C. difficile* and *C. limosum* were observed in children with diarrhea, but not in healthy children. Also, *C. barattii*, *C. bifermentans*, *C. paraputrificum* and *C. sphenoides*, were only observed in children without diarrhea (Table 1). Distribution of *C. difficile*-positive hospitalized children with acute diarrhea with different age is showed in Table 2.

3.2. C. difficile toxin detection and presence of toxin genes

All the isolated clostridia including *C. difficile* were examined for cytotoxicity against VERO cells. Nine of the 10 isolated *C. difficile* strains showed cytotoxic activity producing typical alterations with increase of size and rounding of VERO cells (Table 3). Only a *C. difficile* strain show neither cytotoxic activity nor toxin A or B genes. Extract from the 37 clostridia-positive stools were tested for their cytotoxicity. Thirty

Table 1
Isolation of clostridia of 37 feces samples from children with and without diarrhea

Species	Patient ($n = 18$)		Control ($n = 19$)	
	No. strains	%	No. strains	%
C. ramosum	27	47	15	42
C. difficile	10	18	0	0
C. limosum	10	18	0	0
Clostridium sp.	3	5	0	0
C. septicum	2	4	6	17
C. clostridioforme	2	4	1	3
C. butyricum	1	2	2	6
C. innocuum	1	2	2	6
C. barattii	0	0	5	14
C. bifermentans	0	0	1	3
C. paraputrificum	0	0	1	3
C. sphenoides	0	0	2	6
Total	56	100	35	100

Table 2
C. difficile-positive hospitalized children with acute diarrhea in different age groups

Age	Children with diarrhea		
	n	*C. difficile*	%
Newborn–5 months	44	2	2.2
6–11 months	22	2	2.2
1–5 years	24	1	1.1
Total	90	5	5.5

Table 3
Detection of toxigenic *C. difficile* by cytotoxic assay and multiplex PCR

Strain	Cytotoxic assay on VERO cells	Toxin genes	
		tcdA	*tcdB*
P2	–	–	–
P3	+	–	+
P27A	+	+	+
P27C	+	–	+
P27H	+	+	+
P29B	+	+	+
P29C	+	+	+
P77E	+	+	+
P77G	+	–	+
P77I	+	+	+
VPI 10463[a]	+	+	+

[a] Reference strain.

Table 4
MIC values and percentage of resistance of *Clostridium* spp. isolated from children with and without diarrhea

Antibiotics	MIC (μg/mL)			Resistance %
	Range	50%	90%	
Children with diarrhea				
Amoxicillin	⩽0.25–256	256	256	87.5
Ampicillin	1–256	256	256	92.8
Aztreonam	1–256	256	256	91.1
Cephalexin	16–256	256	256	100
Cefoxitin	4–256	256	256	98.2
Chloramphenicol	64–256	256	256	100
Clindamycin	⩽0.25–256	256	256	66
Imipenem	⩽0.25–256	8	16	25
Metronidazole	⩽0.25–256	128	256	67.9
Penicillin G	⩽0.25–256	256	256	80.4
Children without diarrhea				
Amoxicillin	⩽0.25–256	256	256	91.4
Ampicillin	⩽0.25–256	256	256	82.9
Aztreonam	1–256	256	256	91.4
Cephalexin	⩽0.25–256	256	256	97.1
Cefoxitin	⩽0.25–256	256	256	74.3
Chloramphenicol	⩽0.25–256	256	256	91.4
Clindamycin	⩽0.25–256	16	256	54.2
Imipenem	⩽0.25–256	1	16	14.3
Metronidazole	⩽0.25–256	128	256	62.9
Penicillin G	⩽0.25–256	16	256	45.7

Breakpoint: Amoxicillin, 16 μg/mL; Ampicillin, 16 μg/mL; Aztreonam, 32 μg/mL; Cephalexin, 8 μg/mL; Cefoxitin, 16 μg/mL; Chloramphenicol, 16 μg/mL; Clindamycin, 8 μg/mL; Imipenem, 8 μg/mL; Metronidazole, 16 μg/mL; Penicillin G, 16 μg/mL (NCCLS, 1988).

of them were cytotoxic, including fecal extracts from which *C. difficile* were isolated. Although, *C. ramosum*, *C. septicum*, *C. barattii*, *C. butyricum*, *C. clostridioforme*, *C. limosum*, *C. bifermentans*, *C. innocuum*, *C. paraputrificum*, *Clostridium* sp. and *C. sphenoides* produced a little alterations on VERO cells but different than those produced for *C. difficile* become the cells oval and without alter their size (data no showed). Also, both *C. difficile* and *C. limosum* were isolated in 18% of the children with diarrhea and both species produced cytotoxic alterations on VERO cells with exception of seven *C. limosum* strains.

The presence or absence of *C. difficile* toxin genes was examined by multiplex-PCR. Six *C. difficile* carried both the toxins A and B genes and three strains carried only the toxin B gene (Table 3). Clostridia other than *C. difficile* did not carry any toxin gene.

3.3. Antimicrobial susceptibility

Most of the isolated clostridia from patients and healthy children showed resistance to amoxicillin, ampicillin, aztreonam, cefoxitin, cephalexin, clindamycin, chloramphenicol, metronidazole and penicillin G. Imipenem was an effective drug to inhibit the most of tested clostridia. However, isolated strains from patients (25%) and from control group (14%) showed resistance to this antibiotic. The MIC values to the isolated clostridia from both children groups ranged from ⩽0.25 to 256 μg/mL (Table 4). In Table 5 is observed the antimicrobial resistance profile of the isolated clostridia. Only isolated clostridia from diarrhea showed resistance to all the tested agents. However, a *C. ramosum* strain isolated from a child without diarrhea showed resistance to only amoxicillin, aztreonam and cephalexin. Also, *C. difficile* and *C. limosum* strains showed, respectively, resistance from six to 10 and from four to nine antimicrobials.

4. Discussion and conclusions

Clostridium spp. are part of the intestinal indigenous microbiota of young children and they can produce endogenous infections. *C. difficile* is considered as etiologic agent of PMC and the main cause of antibiotic-associated diarrhea in adults, but its association with enteric disease in children is less clear [15].

Table 5
Resistance profile of 91 *Clostridium* spp. isolated from patients with acute diarrhea and healthy children

Organisms	No. of isolated strains		Resistance profile
	Children with diarrhea	Children without diarrhea	
C. butyricum	1	0	Amo, Amp, Azt, Ce, Cefo, Cli, Chlo, Imi, Pe G, Mz
C. clostridioforme	1	0	
Clostridium sp.	1	0	
C. difficile	1	0	
C. ramosum	3	0	
C. ramosum	2	0	Amo, Amp, Azt, Ce, Cefo, Cli, Chlo, Imi, Pe G
C. barattii	0	1	Amo, Amp, Azt, Ce, Cefo, Cli, Chlo, Pe G, Mz
C. bifermentans	0	1	
C. clostridioforme	1	1	
C. difficile	6	0	
C. limosum	5	0	
C. ramosum	10	1	
C. septicum	0	1	
C. difficile	1	0	Amo, Amp, Azt, Ce, Cefo, Cli, Chlo, Imi
C. ramosum	1	1	Amo, Amp, Azt, Ce, Cefo, Cli, Chlo, Mz
Clostridium sp.	1	0	Amo, Amp, Azt, Ce, Cefo, Cli, Chlo, Pe G
C. difficile	1	0	
C. innocuum	1	0	
C. limosum	2	0	
C. ramosum	2	2	
C. barattii	0	1	Amo, Amp, Azt, Ce, Cefo, Chlo, Mz, Pe G
C. paraputrificum	0	1	
C. ramosum	3	4	
C. barattii	0	1	Amo, Amp, Azt, Ce, Cefo, Chlo, Mz
C. butyricum	0	1	
C. ramosum	1	3	
C. septicum	2	0	
C. ramosum	1	0	Amo, Amp, Azt, Ce, Cefo, Chlo, Pe G
C. sphenoides	0	1	
C. ramosum	0	1	Amo, Amp, Ce, Cefo, Chlo, Mz, Pe G
C. septicum	0	1	Amo, Amp, Azt, Ce, Cefo, Chlo
C. ramosum	0	1	Amo, Amp, Azt, Ce, Chlo, Pe G
C. difficile	1	0	Amp, Azt, Ce, Cli, Chlo, Mz
C. innocuum	0	1	Amo, Amp, Azt, Ce, Cli, Chlo
C. septicum	0	1	Amo, Amp, Azt, Ce, Cli, Mz
Clostridium sp.	1	0	Amo, Azt, Ce, Cefo, Chlo, Pe G
C. butyricum	0	1	Amo, Ce, Cefo, Cli, Chlo, Pe G
C. ramosum	2	0	
C. barattii	0	1	Amp, Azt, Ce, Cefo, Chlo, Mz
C. innocuum	0	1	
C. limosum	1	0	
C. septicum	0	1	Amo, Amp, Ce, Cefo, Cli
C. septicum	0	1	Amo, Amp, Ce, Cli, Chlo
C. sphenoides	0	1	
C. barattii	0	1	Amo, Azt, Ce, Cli, Chlo
C. limosum	1	0	Amp, Azt, Ce, Cefo, Chlo
C. ramosum	2	0	
C. ramosum	0	1	Amp, Azt, Cli, Chlo, Mz
C. septicum	0	1	Amo, Azt, Ce, Cli
C. limosum	1	0	Azt, Ce, Cefo, Chlo
C. ramosum	0	1	Amo, Azt, Ce
VPI 10463	—	—	Amp, Azt, Ce, Cefo, Cli, Chlo, Pe G, Mz
Total	56	35	

Amo: Amoxicillin; Amp: Ampicillin; Azt: Aztreonam; Ce: Cephalexin; Cefo: Cefoxitin; Cli: Clindamycin; Chlo: Chloramphenicol; Imi: Imipenem; Mz: Metronidazole; Pe G: Penicillin G.

Studies about CDAD in Brazil have been limited, probably due to the lack of technology and facilities for culturing anaerobic pathogens. In this study, the occurrence of clostridia and *C. difficile* and the toxin production amongst hospitalized children with acute diarrhea and in a control group was investigated.

The CCFA medium is recommended for *C. difficile* isolation, however, other clostridia different than *C. difficile* were also able to growth over it and producing a characteristic yellow fluorescence under UV light in accordance with other authors [16].

Children with and without diarrhea harbored clostridia in 18 and 19 stool samples. In both studied groups *C. ramosum* was the most predominant. From that samples, 9 (50%) and 8 (42.10%) from children with and without diarrhea, respectively, were *C. ramosum*-positive in accordance to Van der Vorm [17]. On the other hand, *C. difficile* was isolated in children with diarrhea (5.5%) and no healthy children harbored this micro-organism (Table 1). These results are in accordance with Soyletir et al. [18] who found *C. difficile* in Turkian children (4.9%). In Brazil, has been observed that children with acute diarrhea harbored *C. difficile* in 13.8% from stool samples [19].

In this study, some hospitalized children were on antimicrobial therapy at the time of sampling, and it could be altered the bacterial isolation. In addition, it is well known that factors such as immunological alterations, age, nutritional conditions, genetic factors, pathologies or antimicrobial therapy, can also interfere on the clostridia isolation [2]. On the other hand, no other enteropathogenic organism was observed in stool cultures.

The recovery of *C. difficile* from children with diarrhea could represent a small fraction of their intestinal microbiota or it could be a fecal-oral contamination [20]. Moreover, nine out of 10 isolated *C. difficile* were toxigenic to VERO cells, but other clostridia different than *C. difficile* also produced minor tissue alteration. Clostridia can produce several cytotoxins producing alterations in the Rho proteins from eucariotic cells, causing a cytoskeleton disruption of the intestinal cells and modifying their shape [21]. On the other hand, from 37 clostridia-positive stool samples 30 stool extracts also produced alterations on VERO cells. However, according to Peterson et al. [9] it can not be considered exclusively caused for *C. difficile*, unless that these alterations on cellular culture are neutralized for specific antitoxin to this micro-organism.

In this study, we did not use a specific antitoxin with antibodies against toxins A and B. Also, three *C. limosum* strains produced cytotoxic acivity on VERO cells and it is possible because that species also produce a similar *C. difficile* transferase able to destroy Rho protein. Both toxins are detected in stool specimens from humans and experimental animals because both toxins are produced during the disease [7]. In according with Kato et al. [15] we also observed that from 10 isolated *C. difficile* only one non-toxigenic strain lacked the genes for both toxins A and B, while six toxigenic strains carried both the toxin genes. Also, three toxigenic strains carried only toxin B gene (Table 3).

On the other hand, it has been documented that *C. difficile* cytotoxin is inactivated by the myeloperoxidase system of neutrophils and H_2O_2 from *Lactobacillus acidophilus* [22]. The absence of cytotoxic activity in fecal samples, despite isolation of toxin producing *C. difficile* from the same sample may be assumed to be due to such an in vivo inactivation of the toxin [22].

These finding indicate that either both *tcdA* and *tcdB* or one (*tcdB*) are stably expressed in *C. difficile*, suggesting that a definitive diagnosis of the *C. difficile* infection can be accomplished for the toxin genes detection by multiplex-PCR or enzyme immunoassay. However, ELISA or PCR assay remains an extremely useful way to identify *C. difficile* rather than tissue culture [5].

In several countries, *C. difficile* has been considered as a possible agent of acute diarrhea. However, our results show that the presence of *C. difficile* was not significant, and it suggest that other enteropathogens such as rotavirus, EPEC, ETEC or *Vibrio cholerae* could be implicated in diarrheal process in Brazil [20].

Most of 91 isolated clostridia from patients (56) and healthy (35) children showed resistance to amoxicillin, ampicillin, aztreonam, cefoxitin, cephalexin, chloramphenicol, clindamycin, and penicillin G, in accordance with Wexler et al. [23]. In addition, imipenem showed a good activity against most of tested clostridia (75% for patients and 86% for controls), in accordance with Hecht et al. [24]. The MIC values to antimicrobials of isolated *Clostridium* spp. in both patient and control groups are shown in Table 4.

Isolated clostridia from children with diarrhea showed a high level of resistance and it can be explained because of antibiotics used in their treatment. The increase of antimicrobial resistance in anaerobes has been observed [11]. Clostridia such as *C. ramosum*, *C. clostridioforme* and *C. butyricum* are resistant to β-lactams because of β-lactamase production codified by genes which can be transferred to other bacterial species [24]. Usually, *C. difficile* strains are susceptible to metronidazole, however our isolates were resistant to this drug in accordance with Kink and Williams [25]. In Brazil, metronidazole is used as a choice drug to treatment of CDAD because of low cost than vancomycin. However, vancomycin is primarily used in patients who do not respond to metronidazole or in relapsing *C. difficile* cases [25]. On the other hand, all *C. difficile* strains were more resistant than *C. limosum* strains to antimicrobials and it is suggest that *C. difficile* present mechanisms for resistance to several drugs.

Clostridium spp. or *C. difficile* could be important etiological agents of acute diarrhea in hospitalized children and they must be looked for routinely. In addition, it is need more studies to know better the role that this microbial group play in the children intestinal indigenous microbiota and to evaluate the role of

C. difficile play in the diarrheal process. Certainly, it could provide a better understanding of these infections in ecological and pathogenic terms.

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