Prevalence of *Clostridium* spp. and *Clostridium difficile* in Children with Acute Diarrhea in São Paulo City, Brazil

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Species of Clostridium *are widely distributed in the environment, inhabiting both human and animal gastrointestinal tracts.* Clostridium difficile *is an important pathogen associated with outbreaks of pseudomembranous colitis and other intestinal disorders, such as diarrhea. In this study, the prevalence of* Clostridium *spp. and* C. difficile, *from hospitalized children with acute diarrhea, was examined. These children were admitted to 3 different hospitals for over 12 months. Eighteen (20%) and 19 (21%) stool specimens from children with (90) and without (91) diarrhea respectively, were positive to clostridia. Only 10* C. difficile *strains were detected in 5.5% of the stool samples of children with diarrhea. None healthy children (without diarrhea) harbored* C. difficile. *From these 10* C. difficile, *9 were considered as toxigenic and genotyped as* tcdA+/tcdB+ *or* tcdA-/tcdB+, *and 1 strain as nontoxigenic* (tcdA-/tdcB-). *They were detected by the citotoxicity on VERO cells and by the multiplex-polymerase chain reaction. Thirty clinical fecal extracts produced minor alterations on VERO cells. The presence of* C. difficile *as a probable agent of acute diarrhea is suggested in several countries, but in this study, the presence of these organisms was not significant. More studies will be necessary to evaluate the role of clostridia or* C. difficile *in diarrhoeal processes in children.*

Key words: *Clostridium difficile* - acute diarrhea - toxins - diagnosis - São Paulo - Brazil

Few data are available concerning *Clostridium* inhabiting human gastrointestinal tract, and the interest for these organisms is due to acute diarrhea in children, and to colon cancer etiology (Falk et al. 1998, Freeman & Wilcox 1999).

Clostridium spp. are widely distributed in the environment. They are heterogeneous Gram-positive rods, anaerobic, fermentative, and spore forming inhabiting the human intestinal indigenous microbiota (Rocha et al. 1999).

Clostridia are distributed in 5 groups: group I, *C. perfringens, C. septicum, C. novyi* (type A), *C. bifermentans, C. histolyticum, C. sordellii*, related to mionecrosis or gas gangrene; group II, *C. tetani,* which causes tetanus; group III, *C. botulinum,* cause botulism; group IV, *C. difficile,* which causes acute diarrhea antibiotic-associated and pseudomembranous colitis; group V, *C. perfringens, C. ramosum, C. bifermentans,* and others linked to cerebral abscesses, abdominal and gynecologic infections, pneumonia, and bacteremies (Delost 1997).

C. difficile is an important nosocomial pathogen and is associated to outbreaks of pseudomembranous colitis (PMC) and other intestinal disorders, such as diarrhea in children and adults, although its role in the pathogenesis of gastroenteritis is not clear yet. However, it has been observed an association between the detection of *C.*

difficile toxin from stools and a previous treatment with antibiotics in children over 12 months old (Freeman & Wilcox 1999).

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 isolates are not available. The major recognized cause of antibiotic-associated colitis is the cytotoxigenic *C. difficile* (Bartlett 1984). *C. difficile* produces 2 toxins: A (enterotoxin) and B (cytotoxin), which are the major virulence factors of this organism and which are encoded by 2 separated genes located in close proximity on the chromosome (Karasawa et al. 1999). Toxin A causes fluid accumulation and mucosal damage in several animal models (rabbit ileal and colonic loops), as well as in hamster cecal segments and in mouse and rat intestines (Lyerly et al. 1985). Toxin B has no enterotoxic activity, but it is a more potent cytotoxin than toxin A in tissue culture line by approximately 1,000-fold (Banno et al. 1984).

Several methods are presently available for the laboratory diagnosis of *C. difficile*-associated diarrhea (CDAD), including cell culture assay to detect the presence of cytotoxin, anaerobic culture of stool specimens for the organism followed by tests of the production of toxin (toxigenic culture), latex agglutination to detect *C. difficile*-associated antigen in stools, and enzyme immunoassays (ELISA) to detect toxin A, toxin B or both (Knoop et al. 1993), but none of these methods have been able to offer, in an easy and quick way, a high sensitivity and specificity of test performance (Peterson et al. 1996). Recently, it has been developed a multiplex-polymerase chain reaction (PCR), which amplifies simultaneously toxins A and B genes and which can be used to distinguish both toxigenic and non-toxigenic *C. difficile* (McMillin et al. 1992).

In this study, the presence of *Clostridium* spp. and toxigenic *C. difficile* strains from stool samples of hospitalized children with acute diarrhea was examined.

MATERIALS AND METHODS

Patients and sample collection - From June 2000 to June 2001 a total of 181 stool samples were collected from 90 children with acute diarrhea and 91 children without diarrhea. Children aged 0-5, neither sex nor race distinguished, were selected. Patients with acute diarrhea were hospitalized at Hospital Infantil Darcy Vargas, Hospital Universitário at the Universidade de São Paulo and Hospital Infantil Cândido Fontoura (São Paulo, SP, Brazil). Diarrhea was defined as 3 loose stools per day, at least, for 2 days. Some patients were given an antibiotic therapy (penicillin, oxacillin, erythromycin, cephalexin, trimetropin, amikacin, chloramphenicol, amoxicilin). Healthy children (without diarrhea), at the same age group, were selected from a day-care center, as control. Stools from both studied groups were naturally obtained and immediately processed. The Ethic Commission of the hospitals and of the Instituto de Ciências Biomédicas, USP (Parecer 097/CEP) approved this study.

Bacterial isolation and identification - Stools were streaked on a selective cycloserine cefoxitin fructose agar (CCFA) (George et al. 1979) and incubated in anaerobic conditions (90% N_2/10% CO_2), at 37^oC, for 5 days. Approximately 8 to 9 characteristic colonies of each sample, showing a yellow fluorescence under ultraviolet light, were subcultured onto blood agar, and isolates were presumptively identified (Gram stain; lipase, lecithinase, catalase, H_2S, and indole production; gelatin, esculin and starch hydrolysis, and localization of spores). Definitive identification was performed by the fermentation of glucose, fructose, lactose, maltose and sucrose, in peptone-yeast extract (PY) broth (Holdeman & Moore 1977).

Cytotoxicity assay - Bacterial cytotoxicity was assayed on VERO (African green monkey kidney) tissue culture monolayers (Gulke et al. 2001). Cells were grown in a 96-well microtitration plate (Corning, USA) with L15 minimal medium (Cultilab Ltd.) added of 2% fetal bovine serum, and incubated in air with 5% CO_2, at 37^oC, for 48 h. All the 181 fecal extracts were prepared by mixing 0.5 ml of stool and 1 ml of phosphate buffered saline (PBS, pH 7.2), and by centrifugation (6,000 g, 15 min). Supernatants were filtered through 0.45 µm membrane filter (Millipore Corporation, Bedford, Mass). In addition to this operation, all the isolated clostridia were grown in brain-heart infusion (BHI), and they were centrifuged (13,000 g, 5 min). Supernatants were filtered through 0.45 µm membrane filters (Millipore) (Gilligan et al. 1993). Stool or supernatant filtrates (50 µl) were added, in duplicate, to VERO cells. After a 2-day incubation in air-5% CO_2 at 37^oC, the results were compared with control cells, to which 50 µl of PBS had been added. Cytotoxicity was considered when the presence of affected cells was detected. A cytotoxic reference strain *C. difficile* VPI 10463 (*tcdA*$^+$/*tcdB*$^+$), kindly provided by Dr Felicja Meisel Mikolajczyk (Department of Medical Microbiology, Center of Bio-structure Research, Poland) was used as control.

Detection of tcdA and tcdB genes - Chromosomal DNA was extracted from toxigenic *C. difficile* and non-toxigenic clostridia grown on blood agar. Bacteria were mixed with 300 µl of sterilized Milli-Q water and washed twice at 12,000 g, for 10 min. Pellet was resuspended in 300 µl of Milli-Q water and boiled for 17 min. After centrifugation (14,000 g, 10 min), supernatant was saved and used as template (McMillin et al. 1992). Positive (*C. difficile* VPI 19463) and negative control strains of toxigenicity were included in PCR assays.

Multiplex-PCR reaction - The primers used 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') (McMillin et al. 1992) (Gibco BRL Technologies). These primers amplified a characteristic 1,217-bp toxin A (gene *tcdA*) and 1,050-bp toxin B (gene *tcdB*) bands. DNA amplification was performed in a final volume of 25 µl containing 10X PCR Buffer (Gibco), 50 mM $MgCl_2$, 10 mM dNTP mix, 0.4 µM each primer, 0.5 U/µl *Taq* polymerase, and 10 ng DNA template. Amplification was performed in a DNA thermal cycler (Perkin Elmer, GenAmp PCR System 9700), programmed for 94^oC (5 min) followed by 37 cycles of 94^oC (30 sec), 50^oC (30 sec) and 72^oC (30 sec), and then 72^oC (5 min), in order to allow the completion of DNA extension.

Detection of amplified products - PCR products were detected by electrophoresis in 1% agarose gel in 1X TBE (Gibco), at 70 V, for 2 h, stained with ethidium bromide (0.5 µg/ml), and photographed on a UV transilluminator (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science). A molecular mass standard of 1 Kb DNA ladder (Gibco) was included.

RESULTS

Recovery of clostridia from feces samples - The prevalence of clostridia and *C. difficile* isolated from children with acute diarrhea is showed in Table I. In Table II it can be observed the isolation of *C. difficile* from stools of hospitalized children of different ages.

TABLE I

Distribution of clostridia in 37 stool samples from children with and without diarrhea

	Stool samples			
Species	Diarrhe (*n* = 18)	%	Normal (*n* = 19)	%
Clostridium difficile	5	28	0	0
C. septicum	1	5.6	4	21.1
C. bifermentans	0	0	1	5.3
C. ramosum	9	50	8	42.1
C. barattii	0	0	4	21.1
C. butyricum	1	5.6	2	10.5
C. clostridioforme	1	5.6	1	5.3
C. innocuum	1	5.6	2	10.5
C. sphenoides	0	0	2	10.5
C. paraputrificum	0	0	1	5.3
Clostridium sp.	3	16.6	0	0
C. limosum	2	11.1	0	0

C. difficile toxin detection and presence of toxin genes - Nine *C. difficile* strains showed cytotoxic activity on VERO cells, producing typical alterations such as increase of size and rounding of cells. Only one *C. difficile* strain did not show neither cytotoxic activity, nor toxin A or B genes. Thirty stool extracts showed cytotoxicity, including those from which *C. difficile* were isolated. Other strains as *C. ramosum* (23), *C. septicum* (7), *C. barattii* (4), *C. butyricum* (3), *C. clostridioforme* (2), *C. limosum* (3), *C. bifermentans* (1), *C. innocuum* (1), *C. paraputrificum* (1), *Clostridium* sp. (1), and *C. sphenoides* (1), produced slight alterations on VERO cells, differently from those produced by *C. difficile*, which become the cells oval without altering their size (data not shown).

Both the presence and the absence of *C. difficile* toxin genes were examined by multiplex-PCR. Only 6 *C. difficile* carried both the toxin A and B genes, three strains carried only the toxin B gene and one strain did not carry any gene (Table III). Other species of *Clostridium*, differently from *C. difficile*, did not carry any toxin gene.

TABLE II

Isolation of *Clostridium difficile* from stool samples of 90 hospitalized children of different age groups

Age	Children with diarrhea			
	n	%	*C. difficile*
Newborn - 5 months	44	4.5	2
6 - 11 months	22	9.1	2
1 - 5 years	24	4.2	1
Total	90	5.5	5

TABLE III

Cytotoxicity on Vero cells and genotypes of *Clostridium difficile* recovered of children with diarrhea

Strain	Vero cytotoxicity	Genes [a]		Genotypes
		tcdA	*tcdB*	
P2	-	-	-	$tcdA^-/tcdB^-$
P3	+	-	+	$tcdA^-/tcdB^+$
P27A	+	+	+	$tcdA^+/tcdB^+$
P27C	+	-	+	$tcdA^-/tcdB^+$
P27H	+	+	+	$tcdA^+/tcdB^+$
P29B	+	+	+	$tcdA^+/tcdB^+$
P29C	+	+	+	$tcdA^+/tcdB^+$
P77E	+	+	+	$tcdA^+/tcdB^+$
P77G	+	-	+	$tcdA^-/tcdB^+$
P77I	+	+	+	$tcdA^+/tcdB^+$
VPI 10463 [b]	+	+	+	$tcdA^+/tcdB^+$

a: multiplex-PCR detection; *b*: reference strain *C. difficile* VPI 10463

DISCUSSION

Clostridium spp. is part of the intestinal indigenous microbiota of young children and they can produce several endogenous infections (Van der Vorm 1999). *C. difficile* is considered the etiologic agent of PMC and the major cause of antibiotic-associated diarrhea in adults, but the attribution of this organism to enteric diseases in children is less clear (Karlsson et al. 2000).

Studies on *C. difficile*-associated diarrhea in Brazil have been limited, probably due to the lack of technology and facilities in the culture of anaerobic pathogens. In this study, the prevalence of *Clostridium* spp. and *C. difficile,* and the toxin production amongst hospitalized children with acute diarrhea and of a control group was investigated.

CCFA medium is recommended for the isolation of *C. difficile*; however, other species of clostridia also grow and produce a characteristic yellow fluorescence under UV light, according to Murray et al. (1995) and Lee et al. (2000).

C. difficile was isolated from 5.5% of the patients and no healthy children harbored this microorganism. Similarly, Soyletir et al. (1996) found this organism in 4.9% of Turkish children with diarrhea. In Brazil, Garcia and Uzeda (1988) isolated *C. difficile* from 13.8% of clinical samples from hospitalized children with acute diarrhea.

From the 18 hospitalized children with clostridia-positive stool samples, 4 of them were not taking antibiotics at the time of the sampling, but *C. difficile, C. ramosum, C. limosum, C. innocuum, and Clostridium* spp. were also recovered. The use of antimicrobial agents can alter the bacterial isolation range; however, no differences in the recovering of clostridia from hospitalized children who were given or not an antibiotic therapy were observed. On the other hand, it is suggested that some factors such as immunological alterations, age, nutritional conditions, genetic factors, pathologies or antimicrobial therapy, can also interfere on the clostridia isolation (Zhang et al. 1999). No other enteropathogenic organism was observed in stool cultures.

The recovery of *C. difficile* in patients with diarrhea could represent a small fraction of their intestinal microbiota, or it could be a fecal-oral contamination (Bressane et al. 2001). In this study, 9 *C. difficile* were toxigenic to VERO cells, but other isolated clostridia also produced minor tissue alteration. Clostridia produce cytotoxins that alter the eucariotic Rho proteins, producing a cytoskeleton disruption of the intestinal cells and modifying their shape (Lerm et al. 2000). From 37 stool samples positive to clostridia, 30 stool extracts produced alterations on VERO cells; however, it cannot be considered exclusively as caused by *C. difficile*, unless these alterations on cellular culture are neutralized by a specific antitoxin (McVay & Rolfe 2000).

Both toxins are consistently detected in fecal specimens from human beings and experimental animals; thus, both toxins are produced during the disease period (Lyerly et al. 1985). According to Kato et al. (2001), we also observed that, from 10 isolated *C. difficile,* only 1 nontoxigenic strain lacked the genes for both toxins A and B, while 6 toxigenic strains carried both toxin genes, and 3 toxigenic strains carried only toxin B gene.

On the other hand, it has been documented that *C. difficile* cytotoxin is inactivated by the myeloperoxidase system of neutrophils and by H_2O_2 of *Lactobacillus acidophilus* (Ooi et al. 1984). According to Niyogi et al. (1991), it may occur the absence of cytotoxic activity in fecal samples due to an in vivo inactivation of the toxin, despite the isolation of toxigenic *C. difficile* in this sample.

These findings indicate that both *tcdA* and *tcdB* or *tcdB* are stably expressed in *C. difficile*, suggesting that a definitive diagnosis of *C. difficile* infection can be accomplished by a multiplex-PCR detection of the toxin genes, rather than by tissue culture assay or enzyme immunoassay of isolates from stool (Karasawa et al. 1999).

The presence of *C. difficile* as a probable causal agent of acute diarrhea is suggested in several countries. However, our results show that the presence of *C. difficile* was not significant, suggesting that other enteropathogens such as rotavirus, enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli* or *Vibrio cholerae* could be implicated in diarrhoeal processes in Brazil (Bressane et al. 2001).

These results also suggest the need of more studies to evaluate the role of *C. difficile* in diarrhoeal processes, which could provide a better understanding of these infections, in ecological and pathogenic terms.

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