Incidence and importance of *Clostridium difficile* in paediatric diarrhoea in Brazil

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Clostridium difficile strains were detected in 14 of 210 (6.7 %) faecal samples from children in Rio de Janeiro, Brazil, by cultivating faeces on cycloserine/cefoxitin/fructose agar after alcohol-shock. Two main groups of children were studied: inpatients ($n = 96$) and outpatients ($n = 114$). The inpatient group consisted of children on antibiotics or immunosuppressors who presented with diarrhoea and other children who did not present with diarrhoea and were not under an antibiotic or chemotherapeutic regimen. Among the outpatients, two groups were examined: namely, a group that comprised children who presented with diarrhoea and were occasionally under an antibiotic regimen and another group that comprised patients who were not taking antibiotics. After cytotoxic assay, toxigenic *C. difficile* ($Cd\text{tox}^+$) strains were detected in 4.2 % of inpatients and 3.5 % of outpatients. Exclusion of other infectious causes of diarrhoea indicated a typical case of *C. difficile*-associated paediatric diarrhoea in the community. Among $Cd\text{tox}^+$ isolates, no variations were detected by PCR for toxin A that employed primers NK9 and NKVO11. No resistance was found to metronidazole or vancomycin among strains that were isolated from children who presented with diarrhoea, but the MIC_{50} and MIC_{90} values for clindamycin were 6–8 and 16 $\mu\text{g ml}^{-1}$, respectively. Resistance to clindamycin seems to be more disseminated in strains from outpatients than in those from inpatients ($P < 0.05$). In conclusion, these data suggest that investigation for *C. difficile* infection should be taken into account in paediatric diarrhoea in both inpatients and outpatients in developing countries.

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INTRODUCTION

Clostridium difficile has been recognized as the most important nosocomial pathogen in adults who manifest gastrointestinal symptoms subsequent to the use of broad-spectrum antibiotics (Brazier, 2001). *C. difficile* has not usually been considered to be clinically important in stool specimens from neonates (<1 month), as this organism can also be found as part of their normal gut microbiota (Rietra *et al.*, 1978). However, in infants (between 1 month and 2 years) and older children (>2 years), *C. difficile* colonization seems to become less frequent with increasing age, eventually reaching an isolation rate similar to that of an adult

(McFarland *et al.*, 2000). Nowadays, some authors also acknowledge that *C. difficile* can be an important cause of paediatric diarrhoea (McGowan & Kader, 1999; McFarland *et al.*, 2000). In such diarrhoea, the extent and degree of illness may seem to be worse in children than in adults, e.g. in fulminant enterocolitis (Price *et al.*, 1988). Changes in the composition of the intestinal microbiota have been implicated in the initiation or maintenance of *C. difficile*-associated diarrhoea (CDAD), which occurs predominantly in patients whose colonic microbiota has been disrupted by antibiotic therapy (Hopkins & MacFarlane, 2002). It has been established that the use of antibiotics by children presents the same risk as for adults. However, most literature in this field stems from the collection and interpretation of data from developed countries, where the use of antibiotics is under rigid control. Data collected in developing countries, on the other hand, can lead to a different interpretation, due to the widespread use of antibiotics.

Abbreviations: CDAD, *Clostridium difficile*-associated diarrhoea; ETEC, enterotoxigenic *Escherichia coli*; INCA, National Institute of Cancer; IPPMG, Instituto de Puericultura Professor Martagão Gesteira.

The purpose of this study was to evaluate the prevalence of toxigenic *C. difficile* strains in symptomatic outpatient and inpatient children. Antibiotic susceptibility levels of *C. difficile* strains were also assessed.

METHODS

Patients, stool samples and strains. We investigated the incidence rate of *C. difficile* strains (toxigenic or not) in the faeces of 210 children, aged between 3 months and 7 years, in the city of Rio de Janeiro, Brazil. Faecal samples were obtained from: (a) 114 outpatients, including 51 children with diarrhoea who were seen by doctors in several districts of Rio de Janeiro and were occasionally under an antibiotic regimen, 40 children without diarrhoea who were not using antibiotics and 23 children from day-care centres who did not present with diarrhoea; and (b) 96 inpatients, including 30 children with diarrhoea from the 'Instituto de Puericultura Professor Martagão Gesteira' (IPPMG) who were taking antibiotics, 49 neutropenic paediatric patients from the National Institute of Cancer (INCA) who were under a chemotherapeutic regimen and 17 inpatients from IPPMG who were taking antibiotics, but did not have diarrhoea. Specimens were collected after parental consent.

In 51 outpatients and 17 IPPMG patients with diarrhoea, other enteropathogens [enterotoxigenic *Escherichia coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), *Salmonella* sp., *Shigella* sp., *Vibrio cholerae*, *Yersinia enterocolitica*, *Aeromonas* sp., *Campylobacter* sp., enterotoxigenic *Bacteroides fragilis*, *Plesiomonas* sp., adenovirus, astrovirus, calicivirus, rotavirus and intestinal parasitic organisms] were investigated. Among INCA inpatients, there were no indications to investigate these other enteropathogens.

Diarrhoea was defined as three or more unformed stools in 24 h. Information was obtained by using a standardized questionnaire that was submitted to the children's parents and by review of clinical charts. Outpatients who presented with diarrhoea were evaluated by primary care physicians in the community.

Two control strains were included in this investigation: *C. difficile* ATCC 9689^T for culture in selective medium and cytotoxin and PCR analyses and *Clostridium perfringens* ATCC 10543 for susceptibility analysis.

Isolation and identification procedures. Stools were collected in sterile universal collectors and kept under refrigeration for no more than 18 h. Faecal samples were cultured directly or after enrichment (by using an alcohol-shock procedure) onto selective cycloserine/cefoxitin/fructose agar and incubated anaerobically for 48 h at 37 °C in jars filled with a gas mixture that consisted of N₂ (80 %), CO₂ (10 %) and H₂ (10 %). Several colonies (up to 10) from each patient were selected to investigate potential carriers of multiple strains. Isolates were identified as *C. difficile* by Gram-staining and standardized biochemical tests (Sumannen & Baron, 1993).

Toxin production

Cytotoxin assay. To determine *in vitro* production of toxin B, isolates were cultured for 72 h in brain heart infusion broth that had been pre-reduced and anaerobically sterilized (BHI-PRAS). Filtered (0.22 µm membrane) supernatants (0.1 ml) were tested for cytotoxin in Vero monolayer cells added to 0.9 ml Eagle's medium (Sigma) in 24-well microtitration plates. Plates were incubated for 24 h at 37 °C under an atmosphere that contained 5 % CO₂. *C. difficile* strains were considered to be toxin B-positive when >50 % of cells showed cell rounding.

Toxin A 'in situ'. Stool samples from outpatients with diarrhoea and

neutropenic inpatients were evaluated for toxin A 'in situ' by a commercial enzyme immunoassay method (VIDAS CDA; Vitek).

PCR assay to detect deletions of the repeating regions of the toxin A gene

Genomic DNA extraction by using guanidine. The procedure of Pitcher *et al.* (1989) was followed. Cultures of toxin B-positive *C. difficile* strains grown in BHI-PRAS for 18 h were centrifuged at 8000 g for 5 min. Supernatants were disposed and cells were washed with PBS (pH 7.0). Cells were then treated with lysozyme (50 mg ml⁻¹), resuspended in 100 µl TE (Tris, 10 mM; EDTA, 50 mM; pH 8.0) and incubated at 37 °C for 30 min. Afterwards, 5 M guanidine isothiocyanate (Life Technologies) was added and the tubes were agitated and incubated at room temperature for 10 min. Lysates were cooled on ice for 10 min, then 7.5 M ammonium acetate was added and the mixture was kept on ice. Chloroform/isoamyl alcohol (24:1) was added, the DNA was precipitated and washed and any remaining ethanol was evaporated.

DNA primers and PCR procedure. The procedure of Kato *et al.* (1999) was followed. Briefly, 2 µl DNA was added to 30 µl reaction that contained 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM each dNTP, 0.75 U *Taq* DNA polymerase (Life Technologies) and 4.5 ng of each primer (NK9, 5'-CCACCAGCTGCAGCCATA-3'; NKV011, 5'-TTTTGATCCTATAGAATCTAACTTAGTAAC-3'). Samples were amplified on a PE Applied Biosystems GeneAmp 9700 PCR system for 35 cycles of 95 °C for 20 s, 60 °C for 2 min and 74 °C for 5 min. Amplification products were analysed by electrophoresis in 1.5 % agarose gel on a horizontal gel electrophoresis apparatus (Horizon; Thistle Scientific). A molecular size marker (1 kb DNA Ladder; Life Technologies) was also used.

Antibiotic susceptibility by MIC determination. MICs for vancomycin, metronidazole and clindamycin were determined by Etest (AB Biodisk) for strains isolated from outpatients and by the agar dilution method (NCCLS, 1997) for strains isolated from inpatients.

Statistical analysis. Significance of differences in antibiotic susceptibility profiles was analysed by the χ^2 test or by Fisher's exact two-tailed test with the Epi Info 6.04 software (Centers for Disease Control and Prevention).

RESULTS

C. difficile strains were isolated from 14 of 210 children (6.7 %) among the different groups examined. Toxigenic *C. difficile* strains (*Cd* tox⁺) were isolated from 4.2 % of inpatients and from 3.5 % of outpatients (Table 1). Among children who harboured *Cd* tox⁺, especially those with diarrhoea as a symptom, concomitance of tox⁺ and tox⁻ strains was detected in five patients. Exclusion of other infectious causes of diarrhoea among symptomatic outpatients contributed to the detection of a case of paediatric CDAD in subject 14LS (aged 3 years, 5 months) (Table 2). All strains that were positive by cytotoxic assay were also positive by PCR with primers NK9 and NKV011, which yielded a PCR product of approximately 1200 bp and therefore excluded any variations in the *tcdA* gene; this identified all toxigenic *C. difficile* strains detected as positive for both toxin A and toxin B (A⁺, B⁺).

Table 3 shows the MIC ranges and the MIC₅₀ and MIC₉₀ values of metronidazole, vancomycin and clindamycin for 65 *C. difficile* isolates from children who presented with diar-

Table 1. Recovery of *Clostridium difficile* strains from Brazilian children

	Inpatients				Outpatients		
	Group 1	Group 2	Group 3	Total	Group 1	Group 2	Total
Intestinal disorders	Yes	Yes	No		Yes	No	
Positive cultures/no. stools tested	2/30*	3/49†	1/17‡	6/96	6/51§	2/63	8/114
<i>Cd</i> tox ⁺ in children aged:							
<24 months					1		
>24 months	1	3		4	1	2	4
Percentage of <i>Cd</i> tox ⁺ ¶				5.2			3.5
Under antibiotic or chemotherapeutic therapy#	A	C	No		A**	No	

From *, †, ‡, § and ||, respectively 7, 23, 5, 35 and 20 strains were isolated.

¶*Cd* tox⁺, toxigenic *C. difficile* strains.

#A, Antibiotic; C, chemotherapeutic.

**Occasionally.

Table 2. Characteristics of patients who harboured toxigenic *Clostridium difficile* strains

ND, Not detected; NE, not examined.

Patient	Status	Diarrhoea*	Antibiotic† (A) or chemotherapeutic agent (CA)	Age (months)	Toxin A 'in situ'	Other enteric pathogens	No. strains Tox ⁺ /Tox ⁻
14 LS	Outpatient	Yes	A	41	Yes	ND	1/4
4 LS	Outpatient	Yes	No	17	Yes	Astrovirus, ETEC	5/0
IPd 6	Inpatient	Yes	A	84	NE	ND	4/0
11D	Outpatient	No	No	28	NE	NE	1/0
12 LE	Outpatient	No	No	36	No	NE	4/2
9 LIN	Inpatient	Yes	CA	>24	Yes	NE	1/7
12 LIN	Inpatient	Yes	CA	>24	Yes	NE	7/1
21 LIN	Inpatient	Yes	CA	>24	Yes	NE	2/5

*More than 10 loose watery stools per day.

†Sulfametoxazole, trimethoprim, metronidazole.

rhoea. The antibiotics used for treatment of CDAD showed a narrow MIC range (0.047–2.0 and 0.25–1.5 µg ml⁻¹ for metronidazole and vancomycin, respectively), regardless of the origin of the isolates. MIC₅₀ values for vancomycin (0.38 and 1.0 µg ml⁻¹ for isolates from outpatients and inpatients, respectively) and metronidazole (0.094 µg ml⁻¹ for isolates from both outpatients and inpatients) were as low as the MIC₉₀ values (respectively 0.19 and 1.0 µg ml⁻¹ for metronidazole and 1.0 and 1.5 µg ml⁻¹ for vancomycin). On the other hand, clindamycin showed a relatively broad MIC range (3–16 µg ml⁻¹), notwithstanding the fact that the majority of isolates displayed similar sensitivity (MIC₅₀ and MIC₉₀ values were 6–8 and 16 µg ml⁻¹, respectively). Among toxigenic strains, 63 % of strains from outpatients and 28 % of strains from inpatients were resistant to

clindamycin, whereas resistance to clindamycin was detected in 70 % of non-toxigenic strains from outpatients and in 52 % of non-toxigenic strains from inpatients. These values revealed that strains from the community were more resistant to clindamycin ($P < 0.05$).

DISCUSSION

According to the World Health Organization, the aetiologies of a great number of diarrhoeal illnesses remain unknown (Bern *et al.*, 1992), which highlights the importance of monitoring the possible presence of novel pathogens.

C. difficile is widespread in nature and causes diarrhoea after the disruption of microbiota by antibiotic usage. The

Table 3. Profile of susceptibility to clindamycin, metronidazole and vancomycin among *Clostridium difficile* strains isolated from paediatric outpatients ($n = 35$) and inpatients ($n = 30$) who presented with diarrhoea

Antimicrobial agent (break-point)	MIC ($\mu\text{g ml}^{-1}$)		
	Range	MIC ₅₀	MIC ₉₀
Clindamycin (8 $\mu\text{g ml}^{-1}$):			
Outpatient	4–16	8	16
Inpatient	3–16	6	16
Metronidazole (32 $\mu\text{g ml}^{-1}$):			
Outpatient	0.047–0.19	0.094	0.19
Inpatient	1–2	1	1
Vancomycin (4 $\mu\text{g ml}^{-1}$):			
Outpatient	0.25–1.5	0.38	1
Inpatient	1–2	1	2

combination of self-medication and widespread use of over-the-counter drugs in countries where policies about medication are not strict might favour *C. difficile* colonization and, consequently, potential development of diarrhoea. Among the children examined in the present study, we detected a specific case of a toxigenic *C. difficile* strain present without any other enteropathogen in an outpatient child (aged 3 years, 5 months), which is characteristic of a true case of CDAD. A multicentre study carried out by our research group investigated enteropathogens that are prevalent in community diarrhoea and found that *C. difficile* was the third most frequently isolated species (Antunes *et al.*, 2002). Riley *et al.* (1991) found that *C. difficile* was the agent isolated most frequently from community-acquired diarrhoea, which was probably a result of the dimension of the population studied (which included stools from adults and children). In our study, in the inpatient group, *Cd* tox⁺ was isolated more often from children with intestinal disorders and recent antibiotic treatment than from carriers. Again, bearing in mind that CDAD in children has the same course as in adults or the elderly, colonic microbiota must be disturbed prior to *C. difficile* colonization. Cytotoxic drugs (aside from antibiotics) are prone to disturbing such colonic microbiota (Brazier & Borriello, 2000). Hence, paediatric neutropenic patients who are undergoing cancer chemotherapy should also be monitored by bacteriological investigation in diarrhoeal diseases. Histopathological evidence of enterocolitis in such neutropenic patients fails frequently (Anand & Glatt, 1993) and misdiagnosis can lead to complications in the treatment of these children. In our group of neutropenic patients, 6.1 % presented with both *C. difficile* tox⁺ strains and toxin A in their faeces, which suggests an association of *C. difficile* with this group of patients. Therefore, investigation for toxigenic *C. difficile* in such patients should be maintained in our cancer unit.

Immunoenzymatic tests (ELISA) for toxin A, such as the one we performed in paediatric faecal specimens, may lead to

misdiagnosis if this is the only type of test used to verify the presence of CDAD. On the other hand, toxin B detection by ELISA still has poor sensitivity and specificity (Kader *et al.*, 1998). Detection by cytopathic assay or even through cultural methods is still appropriate in epidemiological investigations to identify and characterize strains (Brazier & Borriello, 2000). It should be emphasized that the detection of phenotypic diversity of *C. difficile* strains in the same patient was due to cultural methods. By this procedure and by the larger number of colonies examined, it was possible to detect tox⁺ and tox[−] *C. difficile* strains in the same patient.

With the aim of investigating *C. difficile* variant strains (A[−], B⁺), a PCR assay with primers specific to the *tcdA* gene (Kato *et al.*, 1999) was employed, but no variant strains were detected among paediatric isolates.

Sensitivity to vancomycin and metronidazole among the 65 strains studied confirms the susceptibility of *C. difficile* to these antibiotics, although some reports suggest an increasing emergence of strains with reduced susceptibility to metronidazole and vancomycin (Pelaez *et al.*, 1998).

In spite of the fact that the break-points established for concentrations of drugs in serum are not usually achieved for intraluminal infections (Barbut *et al.*, 1999), MIC data indicated that all isolates were susceptible to metronidazole and vancomycin. The two methodologies employed – Etest and agar dilution – have been shown to have good correlation for anaerobic bacteria, regardless of the antibiotic or species used (Citron *et al.*, 1991; Wust & Hardegger, 1992; Bolmström, 1993). Having said that, Barbut *et al.* (1999) have demonstrated that Etest results are always underestimations when compared to agar dilution data.

Even though clindamycin usage has decreased dramatically due to its induction of CDAD in the developed world, we do not have such information about developing countries. Selective pressure for clindamycin resistance seems to persist even in the community, as *C. difficile* strains isolated from outpatients (regardless of toxigenic activity) were more resistant to clindamycin than those from inpatients ($P < 0.05$). The clinical impact of clindamycin resistance in the community should be further evaluated.

In conclusion, surveillance of paediatric diarrhoea in developing countries should take *C. difficile* into account in both inpatients and outpatients, especially when the symptoms are very pertinent and no other enteropathogen is detected.

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REFERENCES

- Anand, A. & Glatt, A. E. (1993). *Clostridium difficile* infection associated with antineoplastic chemotherapy: a review. *Clin Infect Dis* 17, 109–113.
- Antunes, E. N. F., Ferreira, E. O., Vallim, D. C., Paula, G. R., Seldin, L., Sabrá, A., Ferreira, M. C. S. & Domingues, R. M. C. P. (2002). Pattern III non-toxicogenic *Bacteroides fragilis* (NTBF) strains in Brazil. *Anaerobe* 8, 17–22.
- Barbut, F., Decré, D., Burghoffer, B. & 8 other authors (1999). Antimicrobial susceptibilities and serogroups of clinical strains of *Clostridium difficile* isolated in France in 1991 and 1997. *Antimicrob Agents Chemother* 43, 2607–2611.
- Bern, C., Martinez, J., de Zoysa, I. & Glass, R. I. (1992). The magnitude of the global problem of diarrhoeal disease: a ten-year update. *Bull World Health Organ* 70, 705–714.
- Bolmström, A. (1993). Susceptibility testing of anaerobes with Etest. *Clin Infect Dis* 16 (Suppl. 4), S367–S370.
- Brazier, J. S. (2001). Typing of *Clostridium difficile*. *Clin Microbiol Infect* 7, 428–431.
- Brazier, J. S. & Borriello, S. P. (2000). Microbiology, epidemiology and diagnosis of *Clostridium difficile* infection. *Curr Top Microbiol Immunol* 250, 1–33.
- Citron, D. M., Ostovari, M. I., Karlsson, A. & Goldstein, E. J. (1991). Evaluation of the E test for susceptibility testing of anaerobic bacteria. *J Clin Microbiol* 29, 2197–2203.
- Hopkins, M. J. & MacFarlane, G. T. (2002). Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection. *J Med Microbiol* 51, 448–454.
- Kader, H. A., Piccoli, D. A., Jaward, A. F., McGowan, K. L. & Maller, E. S. (1998). Single toxin detection is inadequate to diagnose *Clostridium difficile* diarrhea in pediatric patients. *Gastroenterology* 115, 1329–1334.
- Kato, H., Kato, N., Katow, S., Maegawa, T., Nakamura, S. & Lyerly, D. M. (1999). Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. *FEMS Microbiol Lett* 175, 197–203.
- McFarland, L. V., Brandmarker, S. A. & Guandalini, S. (2000). Pediatric *Clostridium difficile*: a phantom menace or clinical reality? *J Pediatr Gastroenterol Nutr* 31, 220–231.
- McGowan, K. L. & Kader, H. A. (1999). *Clostridium difficile* infection in children. *Clin Microbiol Newsl* 21, 49–53.
- NCCLS (1997). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*, 4th edn. Approved Standard M11-A4. Villanova, PA: National Committee for Clinical Laboratory Standards.
- Pelaez, T., Martinez-Sanchez, L., Alcalá, L., Muñoz, P., García-Lechuz, J. M., Rodríguez-Creixems, M. & Bouza, E. (1998). Metronidazole resistance in *Clostridium difficile*: a new emerging problem? In *Program and Abstracts of the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, pp. 219, abstract no. E-173. Washington, DC: American Society for Microbiology.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* 8, 151–156.
- Price, E. M., Wright, V. M., Walker-Smith, J. A. & Tabaqchali, S. (1988). *Clostridium difficile* and acute enterocolitis. *Arch Dis Child* 63, 543–545.
- Rietra, P. J. G. M., Slaterus, K. W., Zanen, H. C. & Meuwissen, S. G. M. (1978). Clostridial toxin in faeces of healthy infants. *Lancet* ii, 319.
- Riley, T. V., Wetherall, F., Bowman, J., Mogyrosy, J. & Golledge, C. L. (1991). Diarrhoeal disease due to *Clostridium difficile* in general practice. *Pathology* 23, 346–349.
- Sumannen, P. & Baron, E. J. (1993). *Wadsworth Anaerobic Bacteriology Manual*, 5th edn. Belmont, CA: Wadsworth.
- Wust, J. & Hardegger, U. (1992). Comparison of the E test and a reference agar dilution method for susceptibility testing of anaerobic bacteria. *Eur J Clin Microbiol Infect Dis* 11, 1169–1173.