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 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 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 MIC50 and MIC90 values for clindamycin were 6–8 and 16 μg 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.

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.
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), entero-aggregative *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 9689T 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 discarded 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. Lyvates 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′-CCACACGTGCAGCCAT3′; NK011, 5′-TTTTGATCTTATAAGATCTAATTGAA-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 χ2 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 NKVO11, 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 diarr-
rhoea. The antibiotics used for treatment of CDAD showed a narrow MIC range (0.047–2.0 and 0.25–1.5 \( \mu \)g ml\(^{-1} \) for metronidazole and vancomycin, respectively), regardless of the origin of the isolates. MIC\(_{50}\) values for vancomycin (0.38 and 1.0 \( \mu \)g ml\(^{-1} \) for isolates from outpatients and inpatients, respectively) and metronidazole (0.094 \( \mu \)g ml\(^{-1} \) for isolates from both outpatients and inpatients) were as low as the MIC\(_{90}\) values (respectively 0.19 and 1.0 \( \mu \)g ml\(^{-1} \) for metronidazole and 1.0 and 1.5 \( \mu \)g ml\(^{-1} \) for vancomycin). On the other hand, clindamycin showed a relatively broad MIC range (3–16 \( \mu \)g ml\(^{-1} \)), notwithstanding the fact that the majority of isolates displayed similar sensitivity (MIC\(_{50}\) and MIC\(_{90}\) values were 6–8 and 16 \( \mu \)g 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

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

<table>
<thead>
<tr>
<th>Intestinal disorders</th>
<th>Inpatients</th>
<th>Outpatients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive cultures/no. stools tested</td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td><em>Cd tox(^+)</em> in children aged:</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>&lt;24 months</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>&gt;24 months</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Percentage of <em>Cd tox(^+)</em></td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>Under antibiotic or chemotherapeutic therapy#</td>
<td>A**</td>
<td>No</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Status</th>
<th>Diarrhoea*</th>
<th>Antibiotic† (A) or chemotherapeutic agent (CA)</th>
<th>Age (months)</th>
<th>Toxin A 'in situ'</th>
<th>Other enteric pathogens</th>
<th>No. strains</th>
<th>Toe +/Toe –</th>
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</thead>
<tbody>
<tr>
<td>14 LS</td>
<td>Outpatient</td>
<td>Yes</td>
<td>A</td>
<td>41</td>
<td>Yes</td>
<td>ND</td>
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<td></td>
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<tr>
<td>4 LS</td>
<td>Outpatient</td>
<td>Yes</td>
<td>No</td>
<td>17</td>
<td>Yes</td>
<td>Astrovirus, ETEC</td>
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<td>1PD 6</td>
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<td>Yes</td>
<td>A</td>
<td>84</td>
<td>NE</td>
<td>ND</td>
<td>4/0</td>
<td></td>
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<tr>
<td>11D</td>
<td>Outpatient</td>
<td>Yes</td>
<td>No</td>
<td>28</td>
<td>NE</td>
<td>NE</td>
<td>1/0</td>
<td></td>
</tr>
<tr>
<td>12 LE</td>
<td>Outpatient</td>
<td>No</td>
<td>No</td>
<td>36</td>
<td>No</td>
<td>NE</td>
<td>4/2</td>
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</tr>
<tr>
<td>9 LIN</td>
<td>Inpatient</td>
<td>Yes</td>
<td>CA</td>
<td>&gt;24</td>
<td>Yes</td>
<td>NE</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td>12 LIN</td>
<td>Inpatient</td>
<td>Yes</td>
<td>CA</td>
<td>&gt;24</td>
<td>Yes</td>
<td>NE</td>
<td>7/1</td>
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</tr>
<tr>
<td>21 LIN</td>
<td>Inpatient</td>
<td>Yes</td>
<td>CA</td>
<td>&gt;24</td>
<td>Yes</td>
<td>NE</td>
<td>2/5</td>
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</tbody>
</table>

*More than 10 loose watery stools per day.

†Sulfamethoxazole, trimethoprim, metronidazole.
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 falls frequently (Anand & Glatt, 1993) and misdiagnosis can lead to complications in the community, as C. difficile strains isolated from inpatients (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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