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## infection in Polish pediatric outpatients with inflammatory bowel disease

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**Abstract: Purpose.** The prevalence of Clostridium difficile infection (CDI) in pediatric patients with inflammatory bowel disease (IBD) is unknown. We assessed the prevalence of CDI in outpatients with IBD. In addition, the influence of IBD therapy on CDI and antimicrobial susceptibility of C. difficile strains was assessed.

**Materials and methods.** All specimens were obtained between January 2005 and January 2007 from the IBD outpatient service and screened for C. difficile infection. C. difficile isolates were genotyped by PCR-ribotyping.

**Results.** One hundred forty three fecal samples from 58 pediatric IBD patients (21 Crohn's disease and 37 ulcerative colitis) were screened. The risk of C. difficile infection was 60% and was independent of disease type (CD or UC) ( $\chi^2 = 2.5821$ ,  $df=3$ ,  $p=0.4606$ ) and disease activity ( $\chi^2 = 1.3058$ ,  $df=1$ ,  $p=0.2532$ ). About 17% of pediatric IBD patients experienced a recurrence of CDI. All C. difficile strains were susceptible to metronidazole, vancomycin and rifampin.

**Conclusions.** A high prevalence of C. difficile infection and recurrences in pediatric outpatients with IBD was observed, independent of disease type or disease activity. There was no significant correlation between C. difficile infection and IBD therapy. PCR ribotyping revealed C. difficile re-infection and relapses during episodes of IBD in pediatric outpatients.

Response to Reviewers: To the Editor of EJCMIID

I reviewed our manuscript according to reviewer's and I'm sending of list all revisions below:

**Abstract:**

1. It is reported that the prevalence of CDI in pediatric patients is unknown. However, a referenced article #10 by Pascarella et al in the Journal of

Pediatrics suggests that this is not the case.-I changed this sentence. However, The prevalence of Clostridium difficile infection (CDI) in children with inflammatory bowel disease (IBD) seems to be much higher than in adults.

2. The abbreviations UC/CD may be expanded prior to their use.-I expanded these abbreviations.

#### Methods:

1. Redefine clinical recurrences as 10 days after resolution of the prior episode.-I redefine recurrence according to European Society for Clinical Microbiology and Inf. Dis. Guidance document of Clostridium difficile infection (CDI) (Martijn Bauer, E. Kuijper etc.)

2. The discussion comments on the use of several different scales to assess disease activity by the authors (line 39, page 9. These may be referenced in the methods.- I deleted this discussion comment.

3. Dose of metronidazole 22.5 mg.- I change this sentence in the text.

#### Results:

1. It may be useful to detail the demographics in Table 1 to include the number of patients, age, sex, distribution among UC/CD and those with silent and active disease.-I change several point and expanded of presented results in this table according with reviewer's suggestion.

2. Number of patients who tested positive for C.difficile infection may be enumerated.- I give number of tested patients.

3. The lack of a comparator population (patients without IBD) limits the strength of the study and the significance values for patients with C.difficile infection (line 51, page 7). It is suggested that this be recognized as a limitation of the study in the discussion.- I put this sentence in discussion.

4. All previous studies have reported the association between C.difficile Infection and disease activity. The authors note significant limitations in assessing the disease activity in their study. Hence, it is suggested that no conclusions be drawn regarding CDI and disease activity in this study including the abstract, results and discussion section.- I deleted all suggestion concerning lacking of association IBD activity and CDI: in the abstract, results and discussion.

5. The use of genotyping in this study adds to its strength. Table 2 may include the distribution of common genotypes in patients with recurrences / relapse. Table 2 also may be appropriately formatted for better display and include the number of patients with recurrences - The table 2 is formatted and complete with another results for better display and include common PCR-genotype in patients with recurrences.

#### Discussion:

1. Omission of headers in the discussion makes for easier reading - I removed of headers in discussion.

2. Delete the paragraph on rationale of therapy with metronidazole line 12, page 11 as it is not relevant to the study question. In addition line 49, page 11

on the use of rifampin and rifaximin testing may be deleted for the same reason.-I deleted this paragraph.

3.Line 1, page 11 regarding the efficacy of a 10-14 day course may be deleted as there is insufficient supportive evidence.-I deleted this sentence

4.Was the relation between the use of proton pump inhibitors and prior antibiotics on CDI studied?- Out patients are not getting the antibiotics (only metronidazole for treatment of IBD) and were not treatment PPI so that we could not do such any analysis.

Sincerely yours  
Dr Hanna Pituch

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***Clostridium difficile* infection in Polish pediatric outpatients with inflammatory bowel disease**

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Running title: *C. difficile* in pediatric IBD outpatients.

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**Key Words:** *Clostridium difficile* infection, recurrence, inflammatory bowel disease, children, adolescents.

## Abstract

**Purpose.** The prevalence of *Clostridium difficile* infection (CDI) in pediatric patients with inflammatory bowel disease (IBD) is still unknown. We assessed the prevalence of CDI and recurrences in outpatients with IBD. In addition, the influence of IBD therapy on CDI and antimicrobial susceptibility of the potentially causative *C. difficile* strains was assessed.

**Materials and methods.** This was a prospective, single-centre, observational study. All specimens were obtained between January 2005 and January 2007 from the IBD outpatient service and screened for *C. difficile* and its toxins. *C. difficile* isolates were genotyped by PCR-ribotyping. Diagnosis of Crohn's disease and ulcerative colitis was based on Porto criteria. Severity of disease was assessed using the Hyams scale (for Crohn's disease) and Truelove-Witts scale (for ulcerative colitis).

**Results.** One hundred forty three fecal samples from 58 pediatric IBD patients (21 Crohn's disease and 37 ulcerative colitis) were screened. The risk of *C. difficile* infection was 60% and was independent of disease type (CD or UC) ( $\chi^2 = 2.5821$ ,  $df=3$ ,  $p=0.4606$ ) and disease activity ( $\chi^2 = 1.3058$ ,  $df=1$ ,  $p=0.2532$ ). About 17% of pediatric IBD patients experienced a recurrence of CDI. All *C. difficile* strains were susceptible to metronidazole, vancomycin and rifampin.

**Conclusions.** A high prevalence of *C. difficile* infection and recurrences in pediatric outpatients with IBD was observed, independent of disease type or disease activity. There was no significant correlation between *C. difficile* infection and IBD therapy. PCR ribotyping revealed *C. difficile* re-infection and relapses during episodes of IBD in pediatric outpatients.

## Introduction

*Clostridium difficile* (*C. difficile*) strains cause severe diarrhea and colitis in both adults and children [1, 2]. Clinically important *C. difficile* strains usually produce two toxins: toxin A (TcdA) - an enterotoxin and toxin B (TcdB) - a cytotoxin. These toxins are the main virulence factors of *C. difficile*, responsible for symptoms of diarrhea and inflammation [3, 4]. The epidemiology of *C. difficile* infection (CDI) has changed during the past decade. Traditional risk factors for *C. difficile*-associated disease are use of broad-spectrum antibiotic, chemotherapy, age over 65 years, and long-term hospitalization [5]. It was recently demonstrated that use of immunomodulators and presence of inflammatory bowel diseases (IBD) are important risk factors for development of CDI [5, 6, 7].

The two major types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Patients with IBD are often hospitalized with worsening diarrhea, which is attributable to progression of their underlying disease. Whether infection plays a role in this process is ill-defined. Potential risk factors for the acquisition of *C. difficile* infection in IBD patients include drugs used in medical treatment (e.g. sulfasalazine) which might alter the intestinal flora and promote colonization, altered immune status possibly related to therapeutic agents, nutritional status and frequent hospitalizations [8]. IBD patients with colonic involvement exhibited a significant association with development of *C. difficile* infection, with both Crohn's disease and ulcerative colitis [6].

CDI may be difficult to distinguish from an IBD onset or relapse, given the similar symptoms of diarrhea, abdominal pain and low-grade fever. In addition, CDI might be more commonly community-acquired than hospital-acquired [6, 7, 9]. The frequency of *C. difficile* infection in IBD pediatric patients has not been established precisely, but seemed to be higher than in adults [10].

1 The aim of the study was to determine the prevalence of *C. difficile* infection in  
2 pediatric outpatients with active and inactive (in remission) IBD. Also this study  
3  
4 determined if diarrhea episodes were due to *C. difficile* re- infection or relapse. In addition,  
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6 the influence of treatment on *C. difficile* infection and antimicrobial susceptibility of *C.*  
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10 *difficile* strains were assessed.

## 14 **Materials and methods**

### 19 *Patients and specimens*

21 This was a prospective, single-center, observational study. All specimens were submitted  
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23 and processed immediately from the outpatients service of the Department of Pediatric  
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25 Gastroenterology and Nutrition in Warsaw, Poland between January 2005 and January  
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27 2007. We collected data on patient age and gender, antibiotic treatment and hospitalization  
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29 within 3 months before the time of *C. difficile* diagnosis. All drugs used by patients were  
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31 recorded. Diagnosis of Crohn's disease and ulcerative colitis was based on clinical signs  
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33 and symptoms combined with endoscopical, histological and radiological results which  
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35 were interpreted according to the Porto criteria [11]. We used the modified Truelove and  
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37 Witts activity index for ulcerative colitis and pediatrically modified Hyams activity index  
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39 (PCDAI) for Crohn's disease. Active disease was defined as symptomatic UC with a  
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41 Truelove-Witts score greater than 4 points and symptomatic CD with a PCDAI score  
42  
43 greater than 10 points. The Truelove–Witts activity score is partly based on the patient's  
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45 symptoms (number of stools, blood presence in stool, fever) but also on laboratory data  
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47 (hemoglobin level, ESR) and nutritional assessment. The Hyams activity score assesses  
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49 patient's symptoms (abdominal pain, bowel habits, overall well-being), laboratory findings  
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1 (ESR, hemoglobin level, albumin level), physical examination of abdomen, presence of  
2 perianal complications, extraintestinal manifestation and nutritional assessment.  
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4 Clinical recurrences were defined when resurgence of symptoms after therapy cessation  
5 occurred at least 10 days after the first episode. The fecal samples from patients with an  
6 active or an inactive form of IBD were collected in sterile universal collectors and send to  
7 the diagnostic laboratory. Stools samples were investigated for enteropathogenic organisms  
8 such as toxigenic *Escherichia coli*, *Salmonella* sp., *Shigella* sp., rota- and adenoviruses  
9 (VIKIA ROTA ADENO KIT, bioMerieux, France). Patients with IBD were treated with  
10 sulfasalazine or mesalazine, azathioprine, cyclosporine, steroids and infliximab according  
11 to accepted clinical protocols. Patients with *C. difficile* infection (TcdA/TcdB toxins and/or  
12 toxigenic strains detection) were treated with metronidazole for 10-14 days per one episode  
13 (22,5 mg/kg per day in 3 doses).  
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### 31 *Diagnosis of C. difficile infection (CDI)*

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34 Diagnosis of CDI was based on a positive stool enzyme immunoassay (EIA) and/or on the  
35 isolation of toxigenic *C.difficile* strains. Either or both of the *C. difficile* toxins TcdA/TcdB  
36 were detected in the fecal samples with an enzyme immunoassay (*C. difficile* TOX A/B II  
37 <sup>TM</sup>; TechLab, Inc., Blacksburg, VA, USA). All fecal samples were inoculated after  
38 enrichment (by using an alcohol-shock procedure) onto selective Columbia Agar  
39 supplemented with cycloserine-cefoxitine and amphotericin B (CCCA medium,  
40 bioMerieux, France) for detection of *C. difficile*. Plates were incubated in an anaerobic  
41 chamber (Forma Scientific, USA) at 37°C for two days [12]. The isolates were identified  
42 as *C. difficile* by the characteristic morphology of colonies, the specific horse odour, the  
43 yellow-green fluorescence under UV light (365 nm), Gram staining results and  
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biochemical tests (API 20A; bioMerieux, France). For detection of *tcdA/tcdB* and binary toxin genes (*cdtA* and *cdtB*) genes, PCR was performed as described previously [12].

### *PCR ribotyping*

*C. difficile* isolates were typed by the PCR-ribotyping methods described earlier [12]. Banding patterns were compared with those of the library of PCR ribotypes at the ARL, Cardiff [13].

### *Determination of antibiotic susceptibility*

MICs for metronidazole (MZ), vancomycin (VA), ciprofloxacin (CI), gatifloxacin (GA), moxifloxacin (MX) and rifampin (RI) were determined by E-test (AB Biodisc, Solna, Sweden) according to the manufacturer's instructions, as described previously [12]. According to the Clinical and Laboratory Standard Institute (CLSI) recommendations (formely NCCLS), antibiotic resistance was defined as follows: MIC $\geq$ 32 mg/L for metronidazole, MIC $\geq$ 32 mg/L for vancomycin, MIC $\geq$ 4mg/L for ciprofloxacin, MIC $\geq$ 4 mg/L for gatifloxacin and MIC $\geq$ 4mg/L for moxifloxacin [14]. Rifampin resistance was defined as MIC $\geq$ 32 mg/L in accordance with O'Connor et al. [15].

### **Statistical Analyses**

The Chi-square test for independence was used to verify the hypothesis that infection *C. difficile* was independent of the type of illness. The gender proportions in groups were investigated by Chi-square test; for multiple comparisons LSD *post hoc* Fisher test was used. The constancy of variance across the four-patient groups was checked using Bartlett test. We used Classical Multidimensional Scaling based on Hamming distance to

1 identify common treatment schemes. We examined infection risk and explanatory variables  
2 using logistic regression. The Yates' correction for continuity was used when needed.  
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## 7 **Ethical Considerations**

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9 This work was approved by the Ethics Committee of Clinical Investigation of the  
10 Medical University of Warsaw.  
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## 14 **Results**

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16 One hundred forty three fecal samples collected from 58 IBD pediatric patients were  
17 screened for *C. difficile* toxin TcdA/TcdB, and toxigenic *C. difficile* bacteria. Twenty five  
18 females and 33 males, age between 3 and 18 years (mean age 11,9 years), participated in  
19 this study. From all patients in the IBD group 21 had a Crohn's disease (CD) and 37 had an  
20 ulcerative colitis (UC). Baseline characteristics of stool samples are shown in table 1. All  
21 stool samples were negative for enteropathogenic organisms such as toxigenic *Escherichia*  
22 *coli*, *Salmonella sp.*, *Shigella sp.*, enterotoxigenic *C. perfringens*, rota- and adenoviruses  
23 (data not shown). No patient required hospitalisation for more than three months before  
24 fecal sample collection.  
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41 *C. difficile* toxins were identified in 60% (86/143) of the stool samples. The risk of *C.*  
42 *difficile* infection was independent of disease type (Crohn's disease or ulcerative colitis) ( $\chi^2$   
43 =2.5821, df=3, p=0.4606) and disease activity ( $\chi^2$  =1.3058, df=1, p=0.2532). TcdA/TcdB  
44 detection rates and *C. difficile* culture data are shown in table 2. A highly significant  
45 relationship between the presence of *C. difficile* toxins and IBD, both active (p<0.0001)  
46 and inactive (p<0.0001), was observed. In addition, no significant correlation was found  
47 between *C. difficile* infection and IBD therapy, which included sulfasalazine (p=0.1856),  
48 mesalazine (p=0.129), azathioprine (p=0.341) and steroids (p=0.8255). Cyclosporine and  
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infliximab were excluded from the statistical analysis because of their insignificant numbers.

From EIA positive stool samples we cultured 18 *C. difficile* strains. PCR-ribotyping identified five different ribotypes: three among the toxigenic (014, 018, 046) and two among the non-toxigenic strains (010, 035). The most predominant ribotype was 014 (A<sup>+</sup>B<sup>+</sup>CDT<sup>-</sup>) accounting for 50% of all strains. Two to seven clinical recurrences of *C. difficile* infection were observed during the study period among IBD patients and 17% of all 58 patients had recurrences. Strains isolated during the first episode of *C. difficile* infection usually belonged to PCR-ribotype 014, and strains from the re-infections belonged to different ribotypes (ribotype 018 or 046). Colonization of the gastrointestinal tract with non-toxigenic *C. difficile* strains (ribotype 010 or 035) was very frequent among the IBD patients.

All *C. difficile* strains were susceptible to metronidazole (MIC range 0.023-0.38 mg/L), vancomycin (MIC range 0.25-1.5 mg/L), rifampin (MIC=0,002 mg/L for all strains), moxifloxacin (MIC range 0.064-1.5 mg/L) and gatifloxacin (MIC range 0.5-1.5mg/L), but were resistant to ciprofloxacin (MIC $\geq$ 32 mg/L).

## Discussion

### *Prevalence of C. difficile infection in children with IBD*

In our study, the prevalence of *C. difficile* infection in pediatric outpatients with IBD was assessed at 60%. It is clearly higher than reported in the only previously published pediatric study (27.8%) [10] and among adults with IBD (4.2-5.8%) [6, 7]. Several factors could explain our results. First, the majority of our children were outpatients and previous studies showed that the majority of IBD patients contract *C. difficile* infections outside the hospital [6, 7]. A second possible reason is the way we controlled our patients: a fecal

1 samples for toxin detection was collected during every follow-up visit, which are regularly  
2 arranged even in the absence of aggravation. Finally, the incidence of *C. difficile* infection  
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4 in Polish pediatric IBD patients is unknown, but it could simply be higher than in Western  
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6 Europe or the United States.  
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### 8 9 10 11 *Type and severity of IBD*

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14 Large population-based studies have demonstrated that CDI is more common in  
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16 ulcerative colitis (UC) than Crohn's disease (CD) [6,7]. Our data showed that no specific  
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18 type of IBD in our pediatric outpatients predisposed to *C. difficile* infection; the risk of *C.*  
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20 *difficile* infection was the same both in UC and CD. This can be explained by the fact that  
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22 majority of our CD patients had colonic disease. IBD patients with colonic involvement  
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24 exhibited a significant association with the development of *C. difficile* infection [16].  
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29 We observed no significant association between the presence of *C. difficile* toxin in  
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31 stools and disease activity, both in UC and CD - our data do not confirm previous reports  
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33 [6, 7, 10]. The possible explanations for our findings are as follows: firstly, most of the  
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35 children in our study were outpatients – probably, severity of the disease was significantly  
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37 less severe than in hospitalized patients. Secondly, different scales to assess disease activity  
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39 were used by different authors cited above, so data comparison could be unreliable. This  
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41 finding was different from our earlier study, where *C. difficile* positive patients had active  
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43 disease more frequently [17]. This might be due to the limited size of the population of  
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45 pediatric patients.  
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### 50 51 52 53 *IBD therapy*

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55 We detected no significant correlation between the risk of *C. difficile* infection and  
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57 IBD therapy including anti-inflammatory drugs (sulfasalazine, mesalazine) or  
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1 immunomodulatory drugs (azathioprine, systemic steroids). These findings confirmed  
2 results from the published pediatric study [10] but not those from adult studies, which had  
3 shown that immune modulators were risk factors for *C. difficile* infection in hospitalized  
4 IBD patients [6, 18]. The impact of ongoing immune modulation on *C. difficile* infection in  
5 adult outpatients is yet undefined.  
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#### 11 *Re-infection and relapses of C. difficile infection*

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17 Two, three, four and seven recurrences of *C. difficile* infection were observed during  
18 the study period in one, four, four and one patients, respectively. Recurrences of *C. difficile*  
19 infection among IBD patients are a serious problem especially for patients with three or  
20 more episodes [19]. Issa et al demonstrated that 59% of the patients with IBD had a  
21 clinical recurrence within 1 month after initial resolution of symptoms using metronidazole  
22 or vancomycin [20]. Another 22% had persistent symptoms with frequent relapses. The  
23 data regarding the recurrence rate of *C. difficile* in the adult IBD population is limited and  
24 there is no data at all for pediatric IBD patients [16]. Recurrences of *C. difficile* may be  
25 explained either by the endogenous persistence of a *C. difficile* strain (relapse) or by  
26 acquisition of a new strain from environment (re-infection) [21]. We have shown both  
27 scenarios to be relevant. However, our data are limited because of small numbers of *C.*  
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by the same strain suggested that a 10 (14)-day treatment with a constant dose of metronidazole was insufficient, despite in vitro susceptibility of the strain involved. Metronidazole and oral vancomycin have been shown to be effective in the treatment of *C. difficile*- associated diseases, but symptomatic recurrences occur in 15% to 30% of cases [22, 23, 24].

Several factors influenced the use of metronidazole in our patients. Firstly, there is no recommended treatment of *C. difficile* infection in children with IBD, but current guidelines still recommend metronidazole as the first line therapy in mild or moderate disease and primary recurrence of the infection in adults with IBD. The second reason is that rifaximin is not registered for children younger than 12 years old. Besides that, the data relating to the use of rifaximin in adults are not insufficient to extrapolate that to the treatment of children. Rifaximin is not recommended in patients with bloody diarrhea, which occurred in some of our patients.

#### *Antimicrobial susceptibility of C. difficile strains*

Currently, there are no specific antibiotic regimens recommended for IBD patients suffering from CDI. Metronidazole is still considered the first-line therapy for *C. difficile* infection, so all of our patients were treated with metronidazole. In spite of reports that metronidazole treatment for *C. difficile* infection is effective in only 50% of patients [6], we did not observe resistance to metronidazole and vancomycin in pediatric *C. difficile* strains, [25]. We observed that all strains isolated from pediatric IBD outpatients were susceptible to rifampin. O'Connor et al. have shown that the rifampin E-test is a suitable surrogate for rifaximin susceptibility testing by agar dilution [15].

Fluoroquinolones are a mainstay of treatment in patients with IBD, particularly for management of suppurative complications such as abscesses in Crohn's disease [6]. Our

1 findings confirmed that the resistance of *C. difficile* to ciprofloxacin was very frequent [9,  
2 25, 26]. However, resistance to newer fluoroquinolones such as gatifloxacin and  
3  
4 moxifloxacin was not observed among strains isolated from our patients with IBD.  
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### 8 9 *Conclusions*

10 We report a high prevalence of CDI in pediatric outpatients with inflammatory bowel  
11 disease, a clear risk of CDI in both ulcerative colitis and Crohn's disease and that there  
12 was no association between the presence of *C. difficile* toxins in stools and disease activity,  
13 both in UC and CD. In addition, there was no significant correlation between *C. difficile*  
14 infection and IBD therapy and PCR ribotyping revealed both *C. difficile* re-infection and  
15 relapses during episodes of IBD in pediatric outpatients.  
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29 **Declaration of conflict of interest:** The authors report no conflicts of interest. The authors  
30 alone are responsible for the content and writing of the paper.  
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Table 1. Therapeutic treatment and number of fecal samples from pediatric outpatients in active (A) and inactive (I) IBD.

|                        | Fecal samples<br>(n=143) |                       | CD <sup>a</sup><br>(n=49) |                       | UC <sup>a</sup><br>(n=94) |                       |
|------------------------|--------------------------|-----------------------|---------------------------|-----------------------|---------------------------|-----------------------|
|                        | A <sup>b</sup> (n=62)    | I <sup>b</sup> (n=81) | A <sup>b</sup> (n=18)     | I <sup>b</sup> (n=31) | A <sup>b</sup> (n=44)     | I <sup>b</sup> (n=50) |
| Therapeutic treatment: |                          |                       |                           |                       |                           |                       |
| Sulfasalazine          | 23                       | 43                    | 3                         | 3                     | 20                        | 40                    |
| Mesalazine             | 33                       | 36                    | 12                        | 26                    | 21                        | 10                    |
| Azathioprine           | 38                       | 53                    | 14                        | 30                    | 24                        | 23                    |
| Cyclosporine           | 3                        | 1                     | 0                         | 0                     | 3                         | 1                     |
| Steroids               | 41                       | 22                    | 7                         | 5                     | 34                        | 17                    |
| Infliximab             | 1                        | 1                     | 1                         | 1                     | 0                         | 0                     |

Abbreviations: <sup>a</sup> CD or UC - patients with Crohn's disease or ulcerative colitis, respectively

<sup>b</sup> number of fecal samples in active (A) or inactive (I) IBD.

Table 2. Summary of detection of TcdA/TcdB toxins and/or toxigenic *C. difficile* strains in specimens of pediatric outpatients with IBD.

| Number of fecal samples <sup>a</sup> | Number (%) of positive faecal samples for CDI <sup>c</sup> |
|--------------------------------------|--|
| IBD <sup>b</sup> n= 143              | 86/143 (60%)   |
| n= 62 (A)                            | 42/86 (49%)  |
| n=81 (I)                             | 44/86 (51%)  |
| CD <sup>b</sup> n=49                 | 29/49 (59%)  |
| n=18 (A)                             | 12/29 (41%)  |
| n=31 (I)                             | 17/29 (59%)  |
| UC <sup>b</sup> n=94                 | 57/94 (61%)  |
| n=44 (A)                             | 30/57 (53%)  |
| n=50 (I)                             | 27/57 (47%)  |

Abbreviations: <sup>a</sup> number of fecal samples received from patients with active (A) IBD or inactive (I), <sup>b</sup> IBD -inflammatory bowel disease, CD-Crohn disease, UC-ulcerative colitis,

<sup>c</sup> *C. difficile* toxins and/or toxigenic *C. difficile* strains

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***Clostridium difficile* infection in Polish pediatric outpatients with inflammatory bowel disease**

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Running title: *C. difficile* in pediatric IBD outpatients.

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**Key Words:** *Clostridium difficile* infection, recurrence, inflammatory bowel disease, children, adolescents.

## Abstract

**Purpose.** The prevalence of *Clostridium difficile* infection (CDI) in paediatric patients with inflammatory bowel disease (IBD) is still not sufficiently recognized. We assessed the prevalence of CDI and recurrences in outpatients with IBD. In addition, the influence of IBD therapy on CDI and antimicrobial susceptibility of the potentially causative *C. difficile* strains was assessed.

**Materials and methods.** This was a prospective, single-centre, observational study. All specimens were obtained between January 2005 and January 2007 from the IBD outpatient service and screened for *C. difficile* and its toxins. *C. difficile* isolates were genotyped by PCR-ribotyping. Diagnosis of Crohn's disease (CD) and ulcerative colitis (UC) was based on Porto criteria. Severity of disease was assessed using the Hyams scale (for Crohn's disease) and Truelove-Witts scale (for ulcerative colitis).

**Results.** One hundred forty three fecal samples from 58 paediatric IBD patients (21 Crohn's disease and 37 ulcerative colitis) were screened. The risk of *C. difficile* infection was 60% and was independent of disease type (CD or UC) ( $\chi^2 = 2.5821$ ,  $df=3$ ,  $p=0.4606$ ). About 17% of paediatric IBD patients experienced a recurrence of CDI. All *C. difficile* strains were susceptible to metronidazole, vancomycin and rifampin.

**Conclusions.** A high prevalence of *C. difficile* infection and recurrences in paediatric outpatients with IBD was observed, independent of disease type. There was no significant correlation between *C. difficile* infection and IBD therapy. PCR ribotyping revealed *C. difficile* re-infection and relapses during episodes of IBD in pediatric outpatients.



## Introduction

*Clostridium difficile* (*C. difficile*) strains cause severe diarrhea and colitis in both adults and children [1, 2]. Clinically important *C. difficile* strains usually produce two toxins: toxin A (TcdA) - an enterotoxin and toxin B (TcdB) - a cytotoxin. These toxins are the main virulence factors of *C. difficile*, responsible for symptoms of diarrhea and inflammation [3, 4]. The epidemiology of *C. difficile* infection (CDI) has changed during the past decade. Traditional risk factors for *C. difficile*-associated disease are use of broad-spectrum antibiotic, chemotherapy, age over 65 years, and long-term hospitalization [5]. It was recently demonstrated that use of immunomodulators and presence of inflammatory bowel diseases (IBD) are important risk factors for development of CDI [5, 6, 7].

The two major types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Patients with IBD are often hospitalized with worsening diarrhea, which is attributable to progression of their underlying disease. Whether infection plays a role in this process is ill-defined. Potential risk factors for the acquisition of *C. difficile* infection in IBD patients include drugs used in medical treatment (e.g. sulfasalazine) which might alter the intestinal flora and promote colonization, altered immune status possibly related to therapeutic agents, nutritional status and frequent hospitalizations [8]. IBD patients with colonic involvement exhibited a significant association with development of *C. difficile* infection, with both Crohn's disease and ulcerative colitis [6].

CDI may be difficult to distinguish from an IBD onset or relapse, given the similar symptoms of diarrhea, abdominal pain and low-grade fever. In addition, CDI might be more commonly community-acquired than hospital-acquired [6, 7, 9]. The frequency of *C. difficile* infection in IBD pediatric patients has not been established precisely, but seemed to be higher than in adults [10].

1 The aim of the study was to determine the prevalence of *C. difficile* infection in  
2 pediatric outpatients with active and inactive (in remission) IBD. Also this study  
3  
4 determined if diarrhea episodes were due to *C. difficile* re- infection or relapse. In addition,  
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6 the influence of treatment on *C. difficile* infection and the antimicrobial susceptibility of *C.*  
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10 *difficile* strains were assessed.

## 14 **Materials and methods**

### 19 *Patients and specimens*

21 This was a prospective, single-centre, observational study. All specimens were submitted  
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23 and processed immediately from the out-patients service of the Department of Pediatric  
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25 Gastroenterology and Nutrition in Warsaw, Poland between January 2005 and January  
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27 2007. We collected data on patient age and gender, antibiotic treatment and hospitalization  
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29 within 3 months before the time of *C. difficile* diagnosis. All drugs used by patients were  
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31 recorded. Diagnosis of Crohn's disease and ulcerative colitis was based on clinical signs  
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33 and symptoms combined with endoscopical, histological and radiological results that were  
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35 interpreted according to the Porto criteria [11]. We used the modified Truelove and Witts  
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37 activity index for ulcerative colitis and paediatrically modified Hyams activity index  
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39 (PCDAI) for Crohn's disease. Active disease was defined as symptomatic UC with a  
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41 Truelove-Witts score greater than 4 points and symptomatic CD with a PCDAI score  
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43 greater than 10 points. The Truelove–Witts activity score is partly based on the patient's  
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45 symptoms (number of stools, blood presence in stool, fever) but also on laboratory data  
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47 (hemoglobin level, ESR) and nutritional assessment. The Hyams activity score assesses  
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49 patient's symptoms (abdominal pain, bowel habits, overall well-being), laboratory findings  
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1 (ESR, hemoglobin level, albumin level), physical examination of abdomen, presence of  
2 perianal complications, extraintestinal manifestation and nutritional assessment.  
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4 CDI recurrences were defined on the basis of defecation frequency as perceived by the  
5 patient. Increases should at least last for two consecutive days and stools should become  
6 progressively looser or new signs of severe colitis should develop. Microbiological  
7 evidence of the presence of toxins of *C. difficile* and/or the actual presence of toxin-  
8 producing *C. difficile* strains after an initial CDI treatment response should be presented.  
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10 The faecal samples from patients with an active or an inactive form of IBD were collected  
11 in sterile universal collectors and send to the diagnostic laboratory. Stools samples were  
12 investigated for enteropathogenic organisms such as toxigenic *Escherichia coli*,  
13 *Salmonella* sp., *Shigella* sp., rota- and adenoviruses (VIKIA ROTA ADENO KIT,  
14 bioMerieux, France). Patients with IBD were treated with sulfasalazine or mesalazine,  
15 azathioprine, cyclosporine, steroids and Infliximab according to accepted clinical  
16 protocols. Patients with *C. difficile* infection (TcdA/TcdB toxins and/or toxigenic strains  
17 detection) were treated with metronidazole for 10-14 days per one episode, which was the  
18 standard antimicrobial treatment protocol.  
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#### 41 *Diagnosis of C. difficile infection (CDI)*

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43 Diagnosis of CDI was based on a positive stool enzyme immunoassay (EIA) and/or on the  
44 isolation of toxigenic *C. difficile* strains. Either or both of the *C. difficile* toxins TcdA/TcdB  
45 were detected in the fecal samples with an enzyme immunoassay (*C. difficile* TOX A/B II  
46 <sup>TM</sup>; TechLab, Inc., Blacksburg, VA, USA). All faecal samples were inoculated after  
47 enrichment (by using an alcohol-shock procedure) onto selective Columbia Agar  
48 supplemented with cycloserine-cefoxitine and amphotericin B (CCCA medium,  
49 bioMerieux, France) for detection of *C. difficile*. Plates were incubated in an anaerobic  
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1 chamber (Forma Scientific, USA) at 37°C for two days [12]. The isolates were identified  
2 as *C. difficile* by the characteristic morphology of colonies, the specific horse odour, the  
3 yellow-green fluorescence under UV light (365 nm), Gram staining results and  
4 biochemical tests (API 20A; bioMérieux, France). For detection of *tcdA/tcdB* and binary  
5 toxin genes (*cdtA* and *cdtB*) genes, PCR was performed as described previously [12].  
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### 11 *PCR ribotyping*

12 *C. difficile* isolates were typed by the PCR-ribotyping methods described earlier [12].  
13 Banding patterns were compared with those of the library of PCR ribotypes at the ARL,  
14 Cardiff [13].  
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### 26 *Determination of antibiotic susceptibility*

27 MICs for metronidazole (MZ), vancomycin (VA), ciprofloxacin (CI), gatifloxacin (GA),  
28 moxifloxacin (MX) and rifampin (RI) were determined by E-test (AB Biodisc, Solna,  
29 Sweden) according to the manufacturer's instructions, as described previously [12].  
30 According to the Clinical and Laboratory Standard Institute (CLSI) recommendations  
31 (formely NCCLS), antibiotic resistance was defined as follows: MIC $\geq$ 32 mg/L for  
32 metronidazole, MIC $\geq$ 32 mg/L for vancomycin, MIC $\geq$ 4mg/L for ciprofloxacin, MIC $\geq$ 4  
33 mg/L for gatifloxacin and MIC $\geq$ 4mg/L for moxifloxacin [14]. Rifampin resistance was  
34 defined as MIC $\geq$ 32 mg/L in accordance with O'Connor et al. [15].  
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### 51 **Statistical Analyses**

52 The Chi-square test for independence was used to verify the hypothesis that  
53 infection *C. difficile* was independent of the type of illness. The gender proportions in  
54 groups were investigated by Chi-square test; for multiple comparisons LSD *post hoc* Fisher  
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1 test was used. The constancy of variance across the four-patient groups was checked using  
2 Bartlett test. We used Classical Multidimensional Scaling based on Hamming distance to  
3 identify common treatment schemes. We examined infection risk and explanatory variables  
4 using logistic regression. The Yates' correction for continuity was used when needed.  
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## 10 11 **Ethical Considerations**

12 The research presented in this manuscript was approved by the Ethics Committee  
13 on Clinical Investigation of the Medical University of Warsaw.  
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## 21 **Results**

22 One hundred forty three fecal samples collected from 58 IBD pediatric patients were  
23 screened for *C. difficile* toxin TcdA/TcdB, and toxigenic *C. difficile* bacteria. Twenty-five  
24 females and 33 males, age between 3 and 18 years (mean age 11,9 years), participated in  
25 this study. From all patients in the IBD group 21 had a Crohn's disease (CD) and 37 had an  
26 ulcerative colitis (UC). Baseline characteristics of paediatric patients and stool samples are  
27 shown in table 1. All stool samples were negative for enteropathogenic organisms such as  
28 toxigenic *Escherichia coli*, *Salmonella sp.*, *Shigella sp.*, enterotoxigenic *C. perfringens*,  
29 rota- and adenoviruses (data not shown). No patient required hospitalisation for more than  
30 three months before fecal sample collection.  
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45 *C. difficile* toxins were identified in 60% (86/143) of the stool samples and in 69%  
46 (40/58) of the paediatric patients participating in this study. The risk of *C. difficile* infection  
47 was independent of disease type (Crohn's disease or ulcerative colitis) ( $\chi^2 = 2.5821$ ,  $df=3$ ,  
48  $p=0.4606$ ). TcdA/TcdB detection rates and *C. difficile* culture data are shown in table 2. A  
49 highly significant relationship between the presence of *C. difficile* toxins and IBD, both  
50 active ( $p<0.0001$ ) and inactive ( $p<0.0001$ ), was observed. In addition, no significant  
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1 correlation was found between *C. difficile* infection and IBD therapy, which included  
2 sulfasalazine (p=0.1856), mesalazine (p=0.129), azathioprine (p=0.341) and steroids  
3 (p=0.8255). Cyclosporine and Infliximab were excluded from the statistical analysis  
4 because of their insignificant numbers.  
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9 From EIA positive stool samples we cultured 18 *C. difficile* strains. PCR-ribotyping  
10 identified five different ribotypes: three among the toxigenic (014, 018, 046) and two  
11 among the non-toxigenic strains (010, 035). The most predominant ribotype was 014  
12 (A<sup>+</sup>B<sup>+</sup>CDT<sup>-</sup>) accounting for 50% of all strains. Two to seven clinical recurrences of *C.*  
13 *difficile* infection were observed during the study period among IBD patients and 17% of  
14 all 58 patients had recurrences (table 2). Strains isolated during the first episode of *C.*  
15 *difficile* infection usually belonged to PCR-ribotype 014, and strains from the re-infections  
16 belonged to different ribotypes (ribotype 018 or 046). Colonization of the gastrointestinal  
17 tract with non-toxigenic *C. difficile* strains (ribotype 010 or 035) was very frequent among  
18 the IBD patients.  
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33 All *C. difficile* strains were susceptible to metronidazole (MIC range 0.023-0.38  
34 mg/L), vancomycin (MIC range 0.25-1.5 mg/L), rifampin (MIC=0,002 mg/L for all  
35 strains), moxifloxacin (MIC range 0.064-1.5 mg/L) and gatifloxacin (MIC range 0.5-  
36 1.5mg/L), but were resistant to ciprofloxacin (MIC $\geq$ 32 mg/L).  
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## 46 Discussion

47 In our study, the prevalence of *C. difficile* infection in pediatric outpatients with IBD  
48 was assessed at 69%. This is clearly higher than reported in the only previously published  
49 paediatric study (27.8%) [10] or among adults with IBD (4.2-5.8%) [6, 7]. Several factors  
50 could explain our results. First, the majority of our children were outpatients and previous  
51 studies showed that the majority of IBD patients contract *C. difficile* infections outside the  
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1 hospital [6, 7]. A second possible reason is the way we controlled our patients: a fecal  
2 samples for toxin detection was collected during every follow-up visit, which are regularly  
3 arranged even in the absence of aggravation. Finally, the incidence of *C. difficile* infection  
4 in Polish pediatric IBD patients is unknown, but it could simply be higher than in Western  
5 Europe or the United States.  
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11 Large population-based studies have demonstrated that CDI is more common in  
12 ulcerative colitis (UC) than Crohn's disease (CD) [6,7]. Our data showed that no specific  
13 type of IBD in our pediatric outpatients predisposed to *C. difficile* infection; the risk of *C.*  
14 *difficile* infection was the same both in UC and CD. This can be explained by the fact that  
15 majority of our CD patients had colonic disease. IBD patients with colonic involvement  
16 exhibited a significant association with the development of *C. difficile* infection [16].  
17 However, the lack of comparator population (patients without IBD) limits the strength of  
18 the study.  
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31 We detected no significant correlation between the risk of *C. difficile* infection and  
32 IBD therapy including anti-inflammatory drugs (sulfasalazine, mesalazine) or immune-  
33 modulating drugs (azathioprine, systemic steroids). These findings confirmed results from  
34 the published pediatric study [10] but not those from adult studies, which had shown that  
35 immune modulators were risk factors for *C. difficile* infection in hospitalized IBD patients  
36 [6, 17]. The impact of ongoing immune modulation on *C. difficile* infection in adult  
37 outpatients is yet undefined.  
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48 Two, three, four and seven recurrences of *C. difficile* infection were observed during  
49 the study period in one, four, four and one patients, respectively. Recurrences of *C. difficile*  
50 infection among IBD patients are a serious problem especially for patients with three or  
51 more episodes [18]. Issa *et al* demonstrated that 59% of the patients with IBD had a  
52 clinical recurrence within 1 month after initial resolution of symptoms using metronidazole  
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1 or vancomycin [19]. Another 22% had persistent symptoms with frequent relapses. The  
2 data regarding the recurrence rate of *C. difficile* in the adult IBD population is limited and  
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4 there is no data at all for paediatric IBD patients [16]. Recurrences of *C. difficile* may be  
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6 explained either by the endogenous persistence of a *C. difficile* strain (relapse) or by  
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8 acquisition of a new strain from environment (re-infection) [20]. We have shown both  
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10 scenarios to be relevant. However, our data are limited because of small numbers of *C.*  
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Two reports have previously shown that 38% to 56% of recurrent *C. difficile* infection were in fact due to re-infections [21, 22]. Barbut *et al* showed that 48,4% of clinical recurrences among patients hospitalized in different clinical units in France, were in fact, re-infections with a different strains of *C. difficile* [20]. Our results point to a frequent (re-)infection caused by one type of *C. difficile* (PCR-ribotype 014). Relapses followed each other more closely (less than 1 month between episodes) than re-infections. Metronidazole and oral vancomycin have been shown to be effective in the treatment of *C. difficile*- associated diseases, but symptomatic recurrences occur in 15% to 30% of cases [21, 22, 23].

Currently, there are no specific antibiotic regimens recommended for IBD patients suffering from CDI. Metronidazole is still considered the first-line therapy for *C. difficile* infection, so all of our patients were treated with metronidazole. In spite of reports that metronidazol treatment for *C. difficile* infection is effective in only 50% of patients [6], we did not observe resistance to metronidazole and vancomycin in pediatric *C. difficile* strains, [24]. We observed that all strains isolated from paediatric IBD outpatients were susceptible to rifampin.

Fluoroquinolones are a mainstay of treatment in patients with IBD, particularly for management of suppurative complications such as abscesses in Crohn's disease [6]. Our



1 findings confirmed that the resistance of *C. difficile* to ciprofloxacin was very frequent [9,  
2 24, 25]. However, resistance to newer fluoroquinolones such as gatifloxacin and  
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5 moxifloxacin was not observed among strains isolated from our patients with IBD.  
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### 9 *Conclusions*

10 We report a high prevalence of CDI in paediatric outpatients with inflammatory  
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12 bowel disease, a clear risk of CDI in both ulcerative colitis and Crohn's disease. In  
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14 addition, there was no significant correlation between *C. difficile* infection and IBD  
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16 therapy and PCR ribotyping revealed both *C. difficile* re-infection and relapses during  
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18 episodes of IBD in pediatric outpatients.  
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26 **Declaration of conflict of interest:** The authors report no conflicts of interest. The authors  
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28 alone are responsible for the content and writing of the paper.  
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Table 1. Therapeutic treatment, demographic and number of patients and faecal samples from paediatric outpatients in active (A) and inactive (I) IBD.

| Demographic of patients and therapeutic treatment | CD <sup>a</sup> (n=21) |                       | UC <sup>a</sup> (n=37) |                       | Number of faecal samples from female and male and therapeutic treatment | Faecal samples (n=143) from IBD patients |                       | CD <sup>a</sup> (n=49) |                       | UC <sup>a</sup> (n=94) |                       |
|---|------------------------|-----------------------|------------------------|-----------------------|---|--|-----------------------|------------------------|-----------------------|------------------------|-----------------------|
|   | A <sup>b</sup> (n=11)  | I <sup>b</sup> (n=10) | A <sup>b</sup> (n=16)  | I <sup>b</sup> (n=21) |   | A <sup>c</sup> (n=62)                    | I <sup>c</sup> (n=81) | A <sup>c</sup> (n=18)  | I <sup>c</sup> (n=31) | A <sup>c</sup> (n=44)  | I <sup>c</sup> (n=50) |
| Male (n=33)                                       | 6                      | 8                     | 10                     | 9                     | Male (n=82)   | 36                                       | 46                    | 11                     | 25                    | 25                     | 21                    |
| Female (n=25)                                     | 5                      | 2                     | 6                      | 12                    | Female (n=61)   | 26                                       | 35                    | 7                      | 6                     | 19                     | 29                    |
| Age 3-18 yr                                       |                        |                       |                        |                       | Age 3-18 yr   |  |                       |                        |                       |                        |                       |
| Sulfasalazine                                     | 3                      | 0                     | 4                      | 16                    | Sulfasalazine   | 23                                       | 43                    | 3                      | 3                     | 20                     | 40                    |
| Mesalazine  | 6                      | 7                     | 13                     | 6                     | Mesalazine  | 33                                       | 36                    | 12                     | 26                    | 21                     | 10                    |
| Azathioprine                                      | 8                      | 7                     | 10                     | 10                    | Azathioprine  | 38                                       | 53                    | 14                     | 30                    | 24                     | 23                    |
| Cyclosporine                                      | 0                      | 0                     | 2                      | 1                     | Cyclosporine  | 3  | 1                     | 0                      | 0                     | 3                      | 1                     |
| Steroids  | 6                      | 3                     | 13                     | 6                     | Steroids  | 41                                       | 22                    | 7                      | 5                     | 34                     | 17                    |
| Infliximab  | 1                      | 1                     | 0                      | 0                     | Infliximab  | 1  | 1                     | 1                      | 1                     | 0                      | 0                     |

Abbreviations: <sup>a</sup> CD or UC - patients with Crohn's disease or ulcerative colitis, respectively

<sup>b</sup> number of patients (female or male) in active (A) or inactive (I) IBD.

<sup>c</sup> number of fecal samples in active (A) or inactive (I) IBD.

Table 2. Summary of detection of TcdA/TcdB toxins and/or toxigenic *C. difficile* strains in specimens of pediatric outpatients with IBD and recurrences of CDI.

| Number of faecal samples <sup>a</sup>            | Number (%) of positive faecal samples for CDI <sup>c</sup> | Number of patients <sup>d</sup>                | Number (%) of positive patients for CDI <sup>c</sup> | Number of patients with recurrences <sup>e</sup> | Distribution of common genotypes in patients with recurrences of CDI <sup>f</sup> |
|--|--|--|--|--|---|
| IBD <sup>b</sup> n= 143<br>n= 62 (A)<br>n=81 (I) | 86/143 (60%)<br>42/86 (49%)<br>44/86 (51%)                 | IBD <sup>b</sup> n=58<br>n=27 (A)<br>n=31 (I)  | 40/58 (69%)<br>18 (66%)<br>22 (71%)                  | 10/58 (17%)<br>5/27 (18%)<br>5/31 (16%)          | 014<br>014<br>014/010   |
| CD <sup>b</sup> n=49<br>n=18 (A)<br>n=31 (I)     | 29/49 (59%)<br>12/29 (41%)<br>17/29 (59%)                  | CD <sup>b</sup> n=21<br>n=11 (A)<br>n= 10 (I)  | 13 (62%)<br>7 (64%)<br>6 (60%)                       | 3/21 (14%)<br>1/11 (9%)<br>2/10 (20%)            | 014<br>014<br>014/010   |
| UC <sup>b</sup> n=94<br>n=44 (A)<br>n=50 (I)     | 57/94 (61%)<br>30/57 (53%)<br>27/57 (47%)                  | UC <sup>b</sup> n=37<br>n= 16 (A)<br>n= 21 (I) | 27 (73%)<br>12 (75%)<br>15 (71%)                     | 7/37 (20%)<br>4/16 (25%)<br>3/21 (14%)           | 014<br>014<br>014   |

Abbreviations: <sup>a</sup> number of fecal samples received from patients with IBD active (A) or inactive (I), <sup>b</sup> IBD -inflammatory bowel disease, CD-Crohn disease, UC-ulcerative colitis, <sup>c</sup> *C. difficile* toxins and/or toxigenic *C. difficile* strains, <sup>d</sup>-number of patients with IBD (CD or UC) active (A) or inactive (I), <sup>f</sup> PCR-ribotype of *C. difficile* strains; <sup>e</sup> number of patients with recurrences with IBD (A) or (I); <sup>f</sup> PCR-ribotypes caused recurrences in patients with IBD (A) or (I)