Enterotoxigenic *Clostridium perfringens* infection and pediatric patients with inflammatory bowel disease☆

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Abstract

**Background and aims:** *Clostridium difficile* is the major cause of antibiotic-associated diarrhea and is the most well-known bacterial pathogen associated with inflammatory bowel disease (IBD). Enterotoxigenic *Clostridium perfringens* has also been detected in up to 15% of antibiotic-associated diarrhea cases, and it has not been found in healthy people. The aim of this study was to investigate the prevalence of *C. perfringens* infection in pediatric patients with IBD.

**Methods:** This was a prospective, controlled study evaluating pediatric IBD patients in the Department of Pediatric Gastroenterology and Nutrition in Warsaw, Poland. All of the patients were diagnosed according to the Porto criteria. There were two control groups: (1) non-IBD patients that were suspected for bacterial diarrhea and (2) healthy children. Stool samples were collected on the day of admission. *C. perfringens* infection diagnosis was based on a positive stool enzyme immunoassay (*C. perfringens* enterotoxin test kit TechLab).

**Results:** 91 fecal specimens from patients with IBD were collected. The average patient age was 11.7 years in IBD group, 7.4 years in non-IBD patients with diarrhea, and 7.4 years in healthy children. The prevalence of *C. perfringens* infection was 9% (8/91; CI 95% 4.6–16.4). There were more Crohn's patients (6/8) in the *C. perfringens* positive group. There was no *C. perfringens* infection in the two control groups.

**Conclusion:** Our pilot data add evidence to the hypothesis that *Clostridia* other than *C. difficile* may play a significant role in the clinical course of IBD. However, further studies are needed to confirm this.

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1. Introduction

The etiology of inflammatory bowel disease (IBD) is poorly understood. One hypothesis is that inflammation results from altered or pathogenic microbiota in genetically susceptible hosts. Several factors could confirm this hypothesis. First, several mouse models have documented that the presence of gut microorganisms is necessary for development of colitis. Second, the content of the gut microflora is different in IBD patients than in healthy controls. Third, it has been shown that the modification of the microflora by probiotics, antibiotics and fecal transplantation can improve the course of IBD. Finally, growing scientific evidence suggests that superimposed infections of pathogenic microorganism may have a deleterious effect on the clinical course of IBD.

The role of the intestinal microbiota in IBD appears important. However, whether dysbiosis is a cause or a consequence of inflammation is still unknown. Hansen et al. examined the mucosal microbiota of twenty-five children diagnosed with IBD before the start of treatment. The authors found a reduction in gut bacterial diversity in Crohn's disease (CD) but not ulcerative colitis (UC) patients. These results confirmed previous observations of a similar reduction in bacterial diversity in the oral microbiota of children with CD but not of children with UC. For the first time, the authors showed that IBD developed based on gut dysbiosis. Furthermore, there is evidence that links functional changes in commensal bacteria to the pathogenesis of IBD. For example, adherent/invasive Escherichia coli were found in up to 38% of patients with active ileal CD but in a very low percentage of patients with colonic CD.

The impact of the bacteria–bacteria and bacteria–host interactions in the human gut is more complex than our current understanding, even in healthy people. IBD is much more complicated and may involve defective host immunoregulation, requiring immunosuppressive drugs.

Clostridium difficile (C. difficile) is the primary cause of antibiotic-associated diarrhea (AAD) and is the most well known bacterial pathogen associated with IBD. Recent epidemiological data report more cases of C. difficile infection (CDI) in IBD patients than in controls. Current studies have also suggested a trend towards increasing rates of CDI in IBD patients. The data regarding pediatric patients with IBD are limited, but it appears that the prevalence of CDI is higher in this group of patients than among adults with IBD.

Clostridium perfringens (C. perfringens) is an anaerobic, gram-positive, spore-forming bacillus in the Clostridia genus. These bacteria can be found in soil, sediments, and the intestinal contents of animals and humans. C. perfringens isolates are categorized into five types based on toxin expression (A to E). Approximately 1–5% of C. perfringens type A strains produce the clinically important enterotoxin (CPE). As CPE has not been found in healthy people, the diagnosis of C. perfringens infection (CPI) is based on the detection of the enterotoxin in stool samples. Experimental diarrhea can be induced in healthy volunteers by the oral administration of CPE, which confirmed its role in the development of infection.

Similar to C. difficile, C. perfringens was also identified as a cause of AAD, sporadic diarrhea and nosocomial diarrhea. Evidence of CPE was found in up to 15% of AAD cases and 31% of sporadic (in the absence of antibiotic treatment) diarrhea cases. Another similarity between C. perfringens and C. difficile is that their presence in the gut does not indicate infection or disease. Colonization (fecal carriage) of C. perfringens is estimated to be 60% in healthy adults and can be as high as 90% in the elderly. Data on the pediatric population are very limited, but colonization was assessed as 4% in healthy Mexican infants and 6.5% in adolescents. Several risk factors have been recognized for CPI in adults: older age, immunosuppression and use of antacids, all of which are also well known risk factors for CDI.

Although epidemiological and microbiological studies suggest that C. difficile plays a substantial role in the clinical initiation and relapse of IBD, the precise relationship between the clinical assessment of IBD and the presence of C. difficile in the stool remains unknown. Furthermore, there are no clinical data for the association of CPI and IBD. This study was conducted to address accumulating evidence of the importance of microbes for the development and maintenance of IBD. This study further aimed to examine the prevalence of CPI and CDI on the IBD course in children.

2. Materials and methods

This was a prospective study with a control group, conducted in the Department of Pediatric Gastroenterology and Nutrition in Warsaw, Poland. The diagnoses of CD and UC were based on clinical signs and symptoms as well as endoscopic, histologic and radiologic results according to the Porto criteria. The severity of CD and UC was evaluated using the Pediatric Crohn's Disease Activity Index (PCDAI) and the Pediatric Ulcerative Colitis Activity Index (PUCAI), which incorporate symptoms, physical examination findings and laboratory test results. A PCDAI score ≤10 for CD and a PUCAI score <10 for UC were defined as remission. The current treatment was noted.

There were two control groups. The control group 1 consisted of non-IBD patients hospitalized with suspected bacterial diarrhea. The control group 2 consisted of healthy children of hospital employees. All parents and children >16 year old gave their written or oral consent to participate in the study.

All stool samples were collected on the day of admission. CDI and CPI diagnoses were based on a positive stool enzyme immunoassay (C. difficile TOX A/B II, TechLab, Blacksburg, VA, USA and C. perfringens enterotoxin test kit TechLab, respectively). CPI was diagnosed if the CPE was found in the stool sample.

The odds ratio and confidence interval were used as a measure of effect size. The confidence intervals for the difference between two independent binomial proportions were estimated using the Agresti–Caffo method. The Hodges–Lehman estimator (pseudo-median) was used as a location parameter. The Sn statistic was computed as a measure of variability: Sn = med[med(x|xj − xi|; j = 1...n)]. The confidence intervals were estimated using a bootstrap approach. The p-values were computed using Monte Carlo simulations.

3. Results

Between March 2011 and October 2012, 91 fecal specimens from IBD patients were collected, including 46 Crohn’s (14 boys) and 45 ulcerative colitis (23 boys) patients. The average patient
age was similar in both the C. difficile and C. perfringens groups (12.7 vs. 11.7 years, respectively). A total of 56% of Crohn’s patients and 40% of ulcerative colitis patients were in remission. The patient characteristics, including age, type of treatment and disease activity, are shown in Table 1. Control group 1 consisted of 34 non-IBD children with diarrhea (mean age 7.4 years) and control group 2 consisted of 18 healthy children (mean age 8.4 years).

The overall prevalence of C. perfringens infection in this study was 9% (8/91; CI 95% 4.6–16.4). The prevalence was 13% (6/46; CI 95% 6.2–25.7) in Crohn’s patients and 4.4% (2/45; CI 95% 1.4–14.8) in ulcerative colitis patients (p = 0.17; OR 3.22; CI 95% 0.49–20.7); the difference was not statistically significant. Fig. 1 presents the prevalence of CPI and the difference between the two groups of patients. There were no statistically significant differences in age between C. perfringens-infected and non-infected patients (difference −0.75; CI 95% −3–1.5; p = 0.74). Girls were more than twice as likely to be infected with C. perfringens (OR 2.19; CI 95% 0.49–7.27; p = 0.149), the difference was not statistically significant (CI 95% −5.6–15.3; p = 0.391). Disease activity was not associated with CPI. The pseudo-median disease activity index was 10 (CI 95% 5–22) in infected patients and 10 (CI 95% 5–17.5) in non-infected patients (p = 0.73). The type of treatment was also not associated with CPI (shown in Table 1). There was no CPI in the two control groups.

The overall prevalence of CDI infection was 24% (22/91; CI 95% 15.8–34.3). The prevalence was 20% (9/46; CI 95% 9.3–33.9) in CD patients and 29% (13/45; CI 95% 16.4–44.3) in UC patients (p = 0.427; difference −9.3%; CI 95% −29%–10.4%); the difference was not statistically significant. The prevalence of CDI was 41% (14/34; CI 95% 24.6–59.3) in non-IBD patients with diarrhea. There was no CDI in healthy controls. There were no statistically significant differences in age (p = 0.17; p = 0.99), sex (p = 0.42; p = 0.74), disease activity (p = 0.32; p = 0.71) and treatment of immunomodulators (p = 1; p = 1) and steroids (p = 0.47; p = 0.31) between patients with and without CDI, both in CD and UC, respectively.

Two specimens from girls with Crohn’s disease in remission contained both C. difficile toxins and CPE.

4. Discussion
This prospective pilot study showed that there was a 9% prevalence of CPI in children with IBD.

**Table 1** Characteristics of patients.

<table>
<thead>
<tr>
<th></th>
<th>Crohn’s disease patients</th>
<th>Ulcerative colitis patients</th>
<th>IBD patients</th>
<th>Non-IBD patients with diarrhea</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>46 (50.5)</td>
<td>45 (49.5)</td>
<td>91 (100)</td>
<td>34 (65.4)</td>
<td>18 (34.6)</td>
</tr>
<tr>
<td>Men</td>
<td>14 (30.4)</td>
<td>23 (51.1)</td>
<td>37 (40.7)</td>
<td>22 (64.7)</td>
<td>10 (55.5)</td>
</tr>
<tr>
<td>Immunomodulators</td>
<td>37 (80.4)</td>
<td>27 (60)</td>
<td>64 (70.3)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Steroids</td>
<td>7 (15.2)</td>
<td>13 (28.9)</td>
<td>20 (22)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C. difficile</td>
<td>6 (13)</td>
<td>13 (28.9)</td>
<td>22 (24.2)</td>
<td>14 (41.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>6 (13)</td>
<td>2 (4.4)</td>
<td>8 (8.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Med_{HL} 5</td>
<td>13 2.5</td>
<td>Med_{HL} 7</td>
<td>13 4</td>
<td>Med_{HL} 7.5</td>
</tr>
<tr>
<td>PCDAI</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PCDAI &gt; 0</td>
<td>12.5</td>
<td>7.5</td>
<td>7.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PUCAI</td>
<td>–</td>
<td>10</td>
<td>10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PUCAI &gt; 0</td>
<td>–</td>
<td>25</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Med_{HL}; location parameter (pseudo-median).  
S_{n}; average dispersion.  
PCDAI; Pediatric Crohn’s Disease Activity Index.  
PUCAI; Pediatric Ulcerative Colitis Activity Index.  
IBD; inflammatory bowel disease.
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There are no data concerning the prevalence of CPI in IBD patients, so our results can only be compared to those for otherwise healthy patients with diarrhea. Other studies found CPI in 0.14%, 3.3%,34 and 40%15 of adults with antibiotic-associated diarrhea, which are 2–4 times less common than *C. difficile* toxins.13,14,30 In all of these studies, CPI was detected using the same ELISA test, and the fecal specimens were collected from in-patients. The discrepancy between data may suggest local differences in the incidence of CPI-positive strains. In sporadic diarrhea, CPI was detected in 2.5–6.8% of patients,21,31 but another study found the toxin in 31% of patients.16 We also compared our CPI and CDI results in our IBD children. We found the presence of *C. difficile* toxins (24%) more than two times frequently than CPI (9%). The prevalence of *C. difficile* toxins, we found, was similar to that observed in previous studies in pediatric IBD population.32

We found CPI more frequent in CD than in UC children, however the difference was not significant. Until now there is no data on CPI prevalence in IBD patients. Similarly, we did not find significant difference of CDI prevalence between our CD and UC patients. Our findings were in line with results of all previous studies in IBD children. On the contrary, in adults CDI was more common in patients with UC. In adults with IBD, Frank et al. observed differences in the mucosa-associated microbiota in approximately three-quarters of Crohn's patients and two-thirds of UC patients compared to healthy controls.33 Changes in the gut microflora were not dependent on treatments either in children (performed at IBD diagnosis) or in adults.3,33 Significant microbiota differences exist between CD and UC patients.33,34 Recently, Joossens et al. identified five bacterial species that characterized the predominant dysbiosis in CD patients compared to unaffected relatives and healthy individuals.35 *C. perfringens* is an "ileal" bacterium, and we expected that CD, which involves the whole gut, would be more predisposed to this infection. CPI studies using experimental animals identified the ileum as the most sensitive region of the small intestine.36

We did not observe a correlation between age and both CPI and CDI. In adult patients with diarrhea, an increased risk for CPI was observed in the elderly.21,24,31 However, it had been previously demonstrated that the microbiota composition of healthy elderly people was significantly different than that of healthy young people.37 In all previous studies age was not associated with higher prevalence of CDI in IBD children.32

We observed no correlation between the prevalence of CPI and the treatment of IBD. Although immunosuppression is a known risk factor for infections, no strict correlations have been observed between a specific immunomodulatory drug and a certain type of infection, even for opportunistic infections.38 Among enteric infections, the best studied is *C. difficile*. Issa et al. reported that immunosuppression, defined as thiopurines, methotrexate or steroids, doubled the risk of CDI in adults with IBD.8 Schneeweiss et al. did not find such a correlation for immunomodulators and infliximab, but they did find one for steroids.39 In pediatric studies, IBD therapies that predisposed patients with IBD to CDI were not identified,13,40 what we confirmed in our study for both CD and UC patients.

We did not find a correlation between CPI or CDI and disease activity. Previous microbiological studies have suggested that IBD reflects overall gut dysbiosis rather than the effect of a pathogenic organism.41 In light of this hypothesis, a single bacterial species would not be able to disturb the gut microbial balance enough to influence disease activity. Until now, only several studies have reported a positive association between CDI and disease activity.32 This strongly suggests that *C. difficile* toxins do not worsen the course of IBD on their own. *C. difficile* toxins most likely require a microbial imbalance of the gut in predisposed hosts to cause a relapse of IBD. Similar mechanisms could explain the lack of association between CPI and disease activity observed in our study. Further investigation is required to explain the absence of CPI-associated gastrointestinal symptoms, which most commonly include diarrhea and abdominal cramps (which increase the disease activity index), during IBD.

In summary, we did not observe any association between prevalence of CPI and CDI and factors such as type of IBD, age, sex, disease activity or type of treatment. It may confirm our baseline hypothesis of similarity of the two infections, however further studies are needed.

Considering the pathogenesis of CPI, it is worth noticing that only 5% of *C. perfringens* types produce enterotoxin, which is responsible for diarrheal symptoms. Smedley et al. suggested that CPI first binds to receptors at the villus tips.36 Then, through the membrane pore formation and Ca2+ influx, CPI damages of villus tip cells. As the process continues, the intestinal villi become increasingly blunted and denuded, causing net fluid secretion into the small intestine and impairs intestinal absorption. It manifests clinically as diarrhea and abdominal cramps.42 Thanks to rapid PCR cpe-genotyping assays, we know that the cpe gene can have either a chromosomal or a large plasmid location.35 *Cpe*-positive *C. perfringens* type A isolates causing non-foodborne human diarrhea carry their cpe gene on a large plasmid.43 Plasmids can be transmitted to cpe-negative *C. perfringens* isolates by gut microflora, so the feces of healthy humans may be a reservoir for cpe-positive plasmid isolates. Moreover, unusual variants of cpe loci have been found amongst type A isolates in the feces of healthy humans.44 Although epidemiologic evidence implicates CPI as the virulence factor responsible for most (if not all) diarrheal symptoms associated with CPI, the findings of unusual variants of cpe loci and the transmission of plasmids may explain the presence of CPI in the feces of IBD patients with and without diarrhea. However, this hypothesis requires further investigation.

The enterotoxigenic *C. perfringens* found in our in IBD patients was probably a result of dysbiosis, which is considered as a one of important factors in the immunopathogenesis of IBD.6 Dysbiosis is defined as an abnormal ratio of beneficial and aggressive bacterial species.3 A detailed mechanism of how dysbiosis leads to gut inflammation is unknown, but it may be similar to all bacterial infections, including CPI and CDI. Although the dysbiosis in IBD seemed to be an important pathogenic factor, its significance is unknown. It needs advanced both clinical and molecular research.

Our trial assessed the prevalence of CPI in IBD children for the first time. Our group of patients was homogenous and large enough for statistical analysis. Moreover, we evaluated both CD and UC patients. As there is no data on prevalence of CPI in Polish children, we decided to have two control groups to exclude the possibility that CPI is common in this population. A significant shortcoming of the present study is the lack of follow-ups, which could help explain whether long-lasting CPI infection changes the course of IBD.
5. Conclusion

There was a 9% prevalence of CPI in pediatric IBD patients. The results of our study provide new data on gut infections during the course of IBD. However, the clinical impact of the data is still limited.

Contributors

AB, AG, JK, HP, PA and AR carried out the studies and data analyses and drafted the manuscript. JK and POW carried out the samples analyses. AB, AG, HP and GM participated in the design of the study and performed the statistical analysis. AB, HP and AR conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

We wish to confirm there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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