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Recurrent *Clostridium difficile* Infection in Children: Patient Risk Factors and Markers of Intestinal Inflammation

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Abstract

Background—The management of *Clostridium difficile* infection (CDI) in children is complicated by recurrence rates of 20–30%. The identification of risk factors associated with recurrent disease might allow early recognition of those children at highest risk.

Methods—Pediatric patients with CDI were identified through clinical laboratory records at two tertiary care children's hospitals from March 2013 through May 2014. Subjects were enrolled and followed for 60 days to assess for recurrent CDI (rCDI). Blood samples were obtained at enrollment to evaluate host interleukin (IL-) 8 polymorphisms and anti-toxin A antibody levels; stool samples were obtained for inflammatory markers (lactoferrin, calprotectin, IL-8), and *C. difficile* ribotype 027 strain status. Thirty days post enrollment another serum sample was obtained to compare antibody responses.

Results—Of the 28 pediatric patients enrolled, 27 completed follow-up and 8 (30%) experienced rCDI. At enrollment, children with malignancy had significantly lower stool calprotectin, lactoferrin, and IL-8 than those without malignancy. There was a trend toward increased stool inflammatory markers in those who later developed rCDI. The IL-8 A/A genotype was not associated with recurrent disease. No patients were found to have ribotype 027 or an antibody increase to toxin A.

Conclusions—The rate of rCDI in our pediatric cohort was 30%. Children with rCDI had a trend toward higher fecal inflammatory markers with the initial infection and these values were

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lower in children with malignancy. Fecal lactoferrin, calprotectin, and IL-8 should be further studied to determine their value in predicting the risk of rCDI in children.

Keywords

diarrhea; cytokines; malignancy; *Clostridium difficile*

INTRODUCTION

Clostridium difficile is an anaerobic, spore forming bacterium that is a frequent cause of antibiotic-associated diarrhea.(1) The incidence of *C. difficile* infection (CDI) is increasing in both adults and children.(2, 3) In the last ten years, the rate of pediatric hospitalization with CDI has nearly doubled. (4)

A critical clinical challenge with CDI is its propensity to recur. Recurrence rates of 20–30% have been reported in both children and adults.(5–7) The pathogenesis of recurrent CDI (rCDI) is incompletely defined and is likely multifactorial. Related factors may include a defective immune response to the infection, dysbiosis of the intestinal microbiome, or the persistence of clostridial spores in the intestinal lumen.(8) In adults, multiple risk factors for rCDI have been described and include older age, concomitant antacid use, concurrent antibiotic use with CDI treatment, virulence of the *C. difficile* strain, and lack of host antibody response to toxin A.(9–12)

There are limited data on risk factors for rCDI in the pediatric population. Although two retrospective studies, including one conducted at our center, identified malignancy, recent surgery, antibiotic exposures, and the presence of a tracheostomy tube to be significantly related to the risk of rCDI in children, to our knowledge no prospective studies of risk factors have been published. (5, 13) The purpose of this study is to prospectively identify those host and bacterial factors that might confer an increased risk of rCDI in the pediatric host, allowing a more comprehensive follow up of such patients.

MATERIALS AND METHODS

Study Design and Setting

This is a prospective, observational cohort study of children with CDI. Subjects were enrolled from March 2013 through May 2014 at two tertiary-care children's hospitals (Monroe Carrell Jr. Children's Hospital, Nashville, Tennessee and Texas Children's Hospital, Houston, Texas). The Institutional Review Boards of the participating institutions reviewed and approved the study.

Hospital laboratory records were used to identify individuals who tested positive for *C. difficile* at the time of diagnosis. Both hospitals employ molecular-based assays to test stool for the presence of toxigenic *C. difficile* (Nashville – Illumigene, ARUP laboratories; a loop-mediated isothermal amplification assay; Houston – a multiplex real-time polymerase chain reaction assay standardized in the laboratory).(14, 15)

Children aged 12 months through 18 years with CDI were eligible for enrollment. CDI was defined as a positive *C. difficile* diagnostic test and diarrhea (a change in bowel habits and 3 episodes of watery bowel movements in a 24 hour period). Subjects were excluded from the study if an alternative enteropathogen was identified by routine clinical testing requested by the primary medical team. Additionally, children who were diagnosed with CDI in the previous 6 months were ineligible.

The primary endpoint was recurrent CDI – a subsequent episode of CDI occurring within 60 days of the initial episode. Recurrent CDI was defined as a return of diarrhea (as defined above) with a positive *C. difficile* diagnostic test, provided that there were at least 72 hours of formed stool or 2 watery stools per day after the primary CDI episode.

Data Collection and Laboratory Testing

Blood samples were collected at baseline and 30 days later. Stool was collected at enrollment and at the time of a subsequent episode of CDI.

Fecal levels of lactoferrin (IBD-SCAN, TechLab, Blacksburg, VA), interleukin (IL)-8 (Quantikine, R&D Systems, Minneapolis, MN), and calprotectin (Calprotectin ELISA, Eagle Biosciences, Nashua, NH) were measured by an enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions. Stool was cultured for *C. difficile* on cycloserine cefoxitin fructose agar (CCFA) media for 48 hours under anaerobic conditions after an alcohol shock procedure. *C. difficile* colonies were confirmed with detection of the presence of a highly conserved region of the 16S rRNA gene and also with the presence of toxin A and B genes (*tcdA* and *tcdB*, respectively).(16, 17) To determine if the *C. difficile* isolated was ribotype 027, polymerase chain reaction (PCR) ribotyping was performed.(18)

Levels of serum antibodies isotypes (IgG, IgM, and IgA) to toxin A of *C. difficile* were measured at baseline and after 30 days by a direct ELISA using methods previously described.(19) All samples were assayed in duplicate.

Genetic testing on the patient was performed to identify a single nucleotide polymorphism (SNP) in the IL-8 promoter gene (–251 A/A allele). DNA purification of whole blood specimens was performed with the PureGene DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) and genotyping was completed in the Vanderbilt VANTAGE Genomics Core. All samples were run in duplicate and results confirmed.

Data Analysis

Summary statistics were computed for demographic and clinical characteristics, and laboratory tests completed. Categorical variables were summarized using frequencies and percentages. Continuous variables were summarized using mean, median, and interquartile range (IQR).

Subjects with and without rCDI were compared. Categorical data were analyzed using Fisher's exact test and continuous variables were analyzed using Wilcoxon rank-sum test. A two-tailed $P < 0.05$ defined statistical significance. Stata 12 (Statacorp, College Station, TX) was used for all statistical analyses.

RESULTS

Twenty-eight patients were enrolled in the study between March of 2013 and May of 2014. Fourteen patients were enrolled at each study site. Of the 28 patients, 24 (86%) were inpatients at the time of enrollment and 4 (14%) were enrolled as outpatients. Demographic and clinical data are summarized in Table 1. Antibiotic use in the first 6 months of life was reported in 12 (42.9%) patients in the cohort and 14 patients (50%) were breastfed during infancy. The comorbidity profile of the cohort is detailed in Supplemental Digital Content Figure 1. The most common comorbidities included hematologic malignancy in 8 (29%) and solid tumor malignancy in 8 (29%).

Although diarrhea was universally present, other symptoms were prominent. Twenty patients (71.4%) complained of abdominal pain, 15 (57%) had a fever ($> 38.5^{\circ}\text{C}$), 9 (32%) had vomiting, and 5 (18%) had bloody stools. The median fever duration was 2 days (IQR, 1–3) and median maximum number of diarrheal stools daily during illness peak was 7 (IQR, 5–9.5). Of the 27 patients that had a CBC obtained while symptomatic, 5 (17.9%) had leukocytosis (WBC $> 15,000$ cells/dL) (range: 19,200 – 31,450) and 16 patients (59%) had an abnormal hemoglobin with a median value of 10 g/dL (IQR, 8.4–12.8). No patients had an abnormal age-adjusted creatinine.

The majority of the cohort (92.9%) was treated with metronidazole at the time of diagnosis. Four patients received combination therapy with metronidazole and oral vancomycin and 2 patients received oral vancomycin alone. Of the 6 patients treated with oral vancomycin, 5 completed the 60 day follow-up and 2/5 (40%) had an episode of recurrence. There were no CDI related complications during the study, although one patient died during the follow-up period from complications of malignancy and unrelated to CDI.

Of the 28 patients, 26 (93%) completed the 60-day study follow-up. The patient who died during the follow-up period was included in the analysis as the patient had a recurrence prior to death. The other patient was available for 30-day follow-up but could not be reached for 60-day follow-up. This patient was excluded from further analysis as recurrence status could not be determined. Of the included 27 patients, 8 patients (30%) had an episode of rCDI.

Of the 28 patients enrolled in the study, 27 had stool samples available for culture. Twenty-one specimens (75%) were culture positive for *C. difficile*. Of these 21 *C. difficile* isolates, 14 (67%) tested positive for both *tcdA* and *tcdB* genes. We did not detect the ribotype 027 strain among the isolates. Of the 8 patients with rCDI, stools were collected during the recurrent episode in 3, two of which were culture positive for *C. difficile*. These two isolates tested positive for both *tcdA* and *tcdB* genes but were also negative on 027 ribotyping. All enrolled patients had stool available at the time of diagnosis for IL-8, lactoferrin, and calprotectin testing which is detailed in Table 1 and Figure 1.

Twenty-three patients (82%) had serum obtained at enrollment and on 30 day follow-up. No titer rises in anti-toxin A antibodies was seen in any of the paired samples. Positive controls and serum samples from an additional reference population that had previously tested positive were tested at the same time as the study samples and showed positive activities. Host genotyping was successfully performed on 27/28 patients (96%) (Table 1). A/T was the

most common genotype (13, 48%), but there were no host IL-8 genotype difference in those with or without rCDI.

Patients with and without rCDI were compared using multiple clinical and laboratory parameters (Table 1). There were no statistically significant differences between groups although patients with rCDI did have a trend toward higher stool inflammatory markers at the time of initial infection. In addition, at enrollment stool inflammatory markers were significantly lower in patients with a diagnosis of malignancy compared to those without (Table 2).

DISCUSSION

In our cohort of 27 pediatric patients with primary CDI that completed the study follow-up, 8 (30%) had an episode of recurrence within 60 days. This finding confirms earlier results with similarly high recurrence rates.(3, 20) Prior research by our group and others have identified malignancy, recent surgery, antibiotic exposures, and the presence of a tracheostomy tube to be independent risk factors for rCDI in children.(5, 13) No studies to date have prospectively evaluated inflammatory markers and other host exposures as risk factors for pediatric rCDI.

In our study, patients with and without recurrent CDI did not differ in multiple clinical parameters. However, in concordance with prior studies, children with rCDI did have a trend towards increased number of antibiotic days and antibiotic class exposures in the 30 day pre-primary CDI risk window. The presence of malignancy and recent surgeries did not differ, although the relatively small sample size may have restricted our ability to differentiate between the groups.

The intestinal inflammatory response with associated neutrophilic infiltrate is a well-established mechanism to account for the clinical manifestations of CDI. In particular, monocytes and macrophages exposed to *C. difficile* toxins have been shown to release pro-inflammatory cytokines including IL-8.(19) Additionally, fecal levels of lactoferrin and calprotectin, neutrophil-derived proteins, have been demonstrated to correlate with intestinal inflammation.(21, 22) In adults, studies have demonstrated high levels of serum C-reactive protein, a non-specific maker of inflammation, to be predictive of rCDI and higher fecal levels of lactoferrin to correlate with severe CDI.(12, 23) However, the correlation with fecal inflammatory markers and rCDI in children has not been evaluated.

We identified higher median IL-8, lactoferrin and calprotectin values in those with recurrent disease, although there was a notably wide range of results in these patients, and the differences were not statistically significant. In the majority of patients, IL-8 was undetectable. A prior study done by El Feghaly et al. identified lower levels of IL-8 in immunosuppressed patients.(24) As the majority of patients in our current study were immunosuppressed, our low levels of IL-8 is likely reflective of this.

In addition, when comparing inflammatory marker values in immunosuppressed versus non-immunosuppressed patients in our study, there was a trend toward increased stool inflammatory markers in those without immunosuppression. This trend was statistically

significant when comparing those with a diagnosis of malignancy versus those without malignancy, with children with malignancy having significantly lower inflammatory marker values. Multiple studies have identified malignancy as the most common comorbidity in children with CDI, with estimates of 25% of pediatric CDI occurring in children with cancer.(4, 25) Despite this, severity scores are poorly applicable to this population as most rely on the presence of leukocytosis (26), which is absent in neutropenic patients on chemotherapy. Our findings suggest that the fecal inflammatory response may also be blunted in children with cancer, and stool inflammatory markers may be less useful in this patient population. To further evaluate this point, we analyzed the stool inflammatory markers of the 5 children not on immunosuppression or with a malignancy diagnosis. The one child in this cohort with rCDI had higher levels of lactoferrin (63,289 vs. median of 9,072), calprotectin (354 vs. median of 10), and IL-8 (692 vs. median of 0) than the four patients that did not develop rCDI. Although this cohort is too small for additional sub-analyses, the trend is supportive of the stronger relationship between stool inflammatory markers and rCDI in pediatric patients without immunosuppression or a diagnosis of malignancy.

A specific host polymorphism associated with higher levels of fecal IL-8 concentrations (-251 A/A allele) has been implicated as a predictor of rCDI in adults in a single study.(27) However, the predilection for this polymorphism was not replicated in an additional study that demonstrated no difference in the AA genotype between CDI cases and non-infected controls.(28) Similarly, we did not confirm an association between an IL-8 A/A polymorphism and recurrent CDI in our pediatric cohort. Interestingly, in the 5 children with an A/A polymorphisms, none had detectable stool IL-8. This suggests that the mechanism for the host inflammatory response in children may be different than in adults.

The nature of the humoral immune response to *C. difficile* remains unsettled. Adult patients with rCDI have demonstrated lower levels of IgM and IgG against toxin A and a single case series of 6 children with rCDI found that all affected children had lower IgG levels against toxin A compared to healthy controls. In our study, of the 23 patients that had serum obtained on 30 day follow-up, none demonstrated antibody responses to toxin A despite the presence of positive controls and appropriate results in a previously tested reference population. The reasons for this are unclear, but likely reflect, at least in part, the large number of patients on immunosuppression (81.5%) in our cohort, who are unable to mount an appropriate immune response. In a previous study in pediatric patients with inflammatory bowel disease with the majority on immunosuppressive or immunomodulatory agents, 50/72 (69%) had a positive antibody response to Toxin A. However, most of these same patients (50/60, 83%) tested negative for *C. difficile* at the time as detection of the antibody. It is not clear whether the antibody had a role in clearance or whether the detection method for *C. difficile* was less sensitive.(29) The role of the host immune response in preventing rCDI needs further study.

Our study has several limitations. The relatively small sample size limited the ability to identify statistically significant difference between pediatric patients with and without rCDI. In addition, of the 27 patients with stool available for culture at the time of enrollment, *C. difficile* was unable to be cultured in 22%. Other studies have also demonstrated discordant

testing results between molecular based assays and culture. A single study by Kociolek et al. found higher PCR cycle thresholds for specimens that were culture negative. The authors suggested that their inability to grow the organism may have been reflective of a higher rate of *C. difficile* colonization in their outpatient cohort.(30) Issues of colonization may also be relevant in our patient population. To date, we have no available markers to distinguish colonization from disease. Finally, the high rates of immunosuppression in our cohort may make our findings less applicable to non-immunosuppressed patients, particularly when evaluating antibody responses and the presence of stool inflammatory markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Relationship between Host Variables and Recurrent CDI

	Total (N=28)	No recurrence (N=19)	Recurrent CDI (N=8)	P value
Age (mean)	7.2	6.7	8.4	0.49 ²
Male Gender	14 (50%)	8 (42%)	5 (63%)	0.42 ¹
Race White Non-white	26 (93%) 2 (7%)	17 (90%) 2 (10%)	8 (100%) 0	0.49 ¹
Recent hospitalization	20 (71%)	16 (84%)	4 (50%)	0.15 ¹
Recent surgery	15 (55%)	11 (58%)	4 (50%)	0.71 ¹
Presence of fever	16 (57%)	12 (63%)	3 (38%)	0.40 ¹
Max number of diarrheal stools (median, IQR)	7 (5–9.5)	7 (5–11)	6 (5–8.5)	0.10 ²
Type of CDI CA-CDAD HO-HCFA CO- HCFA	8 (29%) 13 (46%) 7 (25%)	3 (16%) 10 (53%) 6 (31%)	4 (50%) 3 (38%) 1 (12%)	0.26 ¹
Malignancy	16 (57%)	12 (63%)	4 (50%)	0.68 ¹
Antibiotic use	24 (86%)	17 (89%)	6 (75%)	0.56 ¹
Antibiotic days (median, IQR) *	13.5 (10–19)	13 (10–20)	16 (13–20)	0.40 ²
Antibiotic classes (median, IQR) *	2.5 (1.5–3)	2 (2–3)	3 (1–4)	0.76 ²
Proton pump inhibitor use *	8 (29%)	4 (21%)	4 (50%)	0.15 ¹
Immunosuppressant use *	22 (79%)	15 (78%)	7 (88%)	0.6 ¹
Concurrent use of antibiotic **	22 (79%)	16 (84%)	5 (63%)	0.32 ¹
Concurrent use of immunosuppressant **	14 (50%)	8 (42%)	6 (75%)	0.21 ¹
Stool Calprotectin ug/g (median, IQR)	9.4 (8.2–30.1)	8.6 (8.1–18.1)	14.4 (8.7–121.9)	0.38 ²
Stool Lactoferrin ng/mL (median, IQR)	1,407 (414–18,525)	825 (404–15,694)	9,966 (711–61,243)	0.24 ²
Stool IL-8 pg/mL (median, IQR)	0 (0–44.0)	0 (0–0)	44.0 (0–428.8)	0.25 ²
Interleukin-8 Genotype (n=26) A/A A/T T/T	(n=27) 5 (19%) 13 (48%) 9 (33%)	4 (21%) 8 (42%) 6 (31%)	1 (12%) 5 (63%) 2 (25%)	0.74 ¹

CDI: *Clostridium difficile* infectionCA-CDAD: Community associated *Clostridium difficile* associated disease

HO-HCFA: Healthcare facility-onset healthcare facility-associated

CO-HCFA: Community-onset healthcare facility-associated

* 30 days prior to symptom onset

** While antibiotic therapy for CDI ongoing

¹ Fisher's exact test;² Wilcoxon test

Table 2**Stool Inflammatory Markers and Immunosuppression**

	Stool Calprotectin ug/g (median, IQR)	Stool Lactoferrin ng/mL (median, IQR)	Stool IL-8 pg/ml (median, IQR)	
Malignancy Diagnosis				
Yes (N=16)	8.6 (8.0–13.2)	800 (0–5,800)	0 (0–0)	P<0.01
No (N=12)	24.6 (8.4–265.2)	23,049 (5,814–62,457)	106.0 (0–428.8)	
Immunosuppressant Use *				
Yes (N=22)	9.1 (8.2–18.1)	819 (414–18,330)	0 (0–0)	P=0.14
No (N=6)	37.0 (8.2–354.3)	20,691 (4,334–61,083)	62.0 (0–169.0)	

* 30 days prior to symptom onset

Wilcoxon test