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Intestinal Inflammatory Biomarkers and Outcome in Pediatric *Clostridium difficile* Infections

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Abstract

Objectives—To identify specific fecal biomarkers for symptomatic *Clostridium difficile* infection and predictors of poor outcomes.

Study design—We enrolled children with positive *C. difficile* testing (cases) and symptomatic controls. We also analyzed stool samples from colonized and non-colonized asymptomatic children. We performed enzyme immunoassays (EIA) to determine fecal interleukin (IL)-8, lactoferrin and phosphorylated-p38 protein concentrations, and quantitative polymerase chain reactions (PCR) to determine IL-8 and CXCL-5 RNA relative transcript abundances, and *C. difficile* bacterial burden.

Results—Of 68 asymptomatic controls, 16 were colonized with *C. difficile*. Phosphorylated-p38 was specific for *C. difficile* infection but lacked sensitivity. Fecal cytokines were elevated in samples from symptomatic children, whether cases or controls. In children with *C. difficile* infection, fecal CXCL-5 and IL-8 mRNA abundances at diagnosis correlated with persistent diarrhea after five days of *C. difficile* infection therapy and with treatment with vancomycin. When children with concomitant viral gastroenteritis were excluded, these correlations persisted. Time-to-diarrhea resolution was significantly longer in patients with elevated fecal cytokines at diagnosis. A logistic regression model identified high CXCL-5 mRNA abundance as the only predictor of persistent diarrhea. Conversely, fecal *C. difficile* bacterial burden was not different in symptomatic and asymptomatic children and did not correlate with any clinical outcome measure.

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Conclusions—Fecal inflammatory cytokines may be useful in distinguishing *C. difficile* colonization from disease and identifying children with *C. difficile* infection likely to have prolonged diarrhea.

Keywords

C. difficile; cytokines; inflammation; CXCL-5; Interleukin-8; biomarkers

Clostridium difficile is the leading cause of hospital-associated diarrhea in adults.¹ Traditionally, it was thought to be nonpathogenic in infants and young children^{2, 3} because the pathogen and even its toxins can be found in the stool of up to 70% of asymptomatic neonates.^{4–6} However, recent studies suggest that *C. difficile* is a bona fide cause of disease in children and patients with no known risk factors, such as antibiotic or healthcare exposure.^{7–11} Emergency room visits and hospitalizations with *C. difficile* infection in children are increasing,^{12–14} and recent data suggest that *C. difficile* infection is a risk factor for pediatric in-hospital mortality.^{15, 16} Without diagnostic tests that differentiate children with *C. difficile* infection from those simply colonized with *C. difficile*, physicians cannot make informed management decisions when confronted with a positive test for this pathogen.

There are many theories regarding the potential pathophysiologic difference between colonized and symptomatic children,¹⁷ but an excessive host inflammatory response is one of the major drivers of disease pathogenesis.¹⁸ *In vitro*, *C. difficile* toxins activate the p38 pathway¹⁹ and induce multiple pro-inflammatory cytokines and chemokines such as interleukin (IL)-8, IL-12, IL-18 and tumor necrosis factor (TNF)- α which may be responsible for host damage and many of the histopathologic features of severe illness.¹⁸

Although children are less likely than adults to develop severe complications from *C. difficile* infection,^{8, 20} diarrhea caused by this pathogen may nevertheless persist and be difficult to manage in this age group,¹⁴ recurrent symptomatic *C. difficile* infection is common,²¹ and pediatric *C. difficile* infection is associated with increased morbidity and mortality.^{15, 16} Clinical severity scores have attempted to detect adults predisposed to severe disease,^{22, 23} but such risk stratification has not been developed for children. Systemic inflammatory biomarkers, such as C-reactive protein (CRP) and white blood cell count (WBC) correlate with disease severity in adults,^{24–26} but the effect size and lack of specificity of these variables are problematic. We found that treatment failure correlates with persistent intestinal inflammation and is independent of the fecal *C. difficile* bacterial burden in adults with *C. difficile* infection.^{27, 28} The host response to *C. difficile* in children has not yet been investigated.

We conducted this case control study to compare stools of symptomatic children with *C. difficile* infection to symptomatic and asymptomatic controls, and attempt to identify fecal inflammatory biomarkers specific to *C. difficile* infection. We then followed our cohort of cases prospectively to assess the relationship between inflammatory cytokines and symptom persistence, and identify predictors of poor outcome in children with *C. difficile* infection.

METHODS

This study was approved by the Washington University School of Medicine Institutional Review Board (HRPO ID 201101801) and conducted at the St Louis Children's Hospital (SLCH), a tertiary pediatric center in St Louis, MO, between July 1, 2011 and July 4, 2012. It comprises two designs: The first is a case-control study comparing children with a positive *C. difficile* test (cases), children with diarrhea but no *C. difficile* (symptomatic controls) and asymptomatic controls, some of whom were colonized with *C. difficile*. The second is a prospective cohort study where we followed our cases for the duration of their illness and recorded *C. difficile* infection outcomes (diarrhea persistence and time-to-diarrhea-resolution). We obtained informed consent, in person or by telephone interview, from all enrolled children's caregivers. As children with diarrhea and a positive *C. difficile* test (cases) were identified, microbiology laboratory personnel stored stool samples at -80°C . SLCH microbiology laboratory uses a flowchart for the diagnosis of *C. difficile*, starting with a glutamate dehydrogenase (GDH) enzyme immunoassay (EIA) (the Wampole *C. diff* Quik Chek, Orlando, FL). If this test is positive, it is confirmed with the GeneXpert *C. difficile* polymerase chain reaction (PCR) (Cepheid, Sunnyvale, CA). We excluded children whose residual stools were $<1\text{mL}$ in volume. We included inpatient, outpatient and emergency department visits, and had no limitations on patient age or underlying disease. We followed this cohort of cases prospectively. We also enrolled a convenience sample of symptomatic controls: a study team member, when present, stored available stool samples from children diagnosed with bacterial gastroenteritis or who had diarrheal stools with negative bacterial cultures, within 48 hours of receipt. Additionally, we obtained 68 well-preserved samples from asymptomatic controls from a study conducted in Seattle, between 2001 and 2002.²⁹ Data available to us from these controls include age, sex, time of stool collection, date of last diarrheal episode and antibiotic exposure. We performed *C. difficile* PCR testing in our laboratory (as described below) on all patients' samples upon study entry, to confirm correct group allocation.

Clinical data

Data collection included demographic characteristics, clinical presentation with emphasis on diarrhea (onset, number of stools per day, consistency according to the Bristol Stool Chart,³⁰ associated symptoms), medications and healthcare exposure. We reviewed medical charts and recorded vital signs, medications, and laboratory findings (including stool cultures if performed) and telephoned cases within 2 weeks of treatment initiation to record diarrhea persistence and changes in *C. difficile* infection therapy. Our primary outcome was diarrhea persistence (defined as three or more bowel movements with a Bristol score ≥ 6) after five days or more of appropriate *C. difficile* infection therapy. Secondary outcomes were time-to-diarrhea resolution from start of *C. difficile* infection therapy, and vancomycin therapy. Physicians at SLCH generally use metronidazole as a first-line treatment for moderate disease and reserve vancomycin for severe cases or treatment failures.

Laboratory assays

Enzyme Immunoassay (EIA)—We suspended stools in Protease Inhibitor Lysis Buffer (Roche Diagnostics, Indianapolis, IN) and followed manufacturers' instructions for all EIAs.

We used 50µL of 1/100 w/v lysate and the Human CXCL8/IL-8 Quantikine kit (R&D Systems, Minneapolis, MN) to determine fecal IL-8 protein concentration. 100µL of 1/100 w/v lysate and IBD-Scan kit (Techlab, Blacksburg, VA) were used to determine fecal lactoferrin protein concentrations; and 100µL of 1:1 w/v lysate and the Phospho-p38α (T180/Y182) Immunoassay kit (R&D Systems, Minneapolis, MN) for fecal phosphorylated-p38 (pp38) concentrations.

Nucleic Acid Extraction—We extracted total nucleic acid using the NucliSENS® EasyMAG™ automated system, software 1.0.2 specific A protocol (bioMérieux, Marcy L'Etoile, France), according to the manufacturer's instructions for a final volume of 110µL of nucleic acid extract.²⁷

Nucleic Acid Amplification—We used 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) for all PCRs.

Cytokine PCR: We performed real-time reverse-transcription (RT)-PCR using Path-ID Multiplex one-step RT-PCR mix (Applied Biosystems, Foster City, CA), as described in detail in our recent study.²⁷ We reported CT values, noting the normalization of the cycle threshold (CT) of the detection of the cytokine to the CT of the internal control.

Quantitative *tcdB* DNA PCR: We performed SYBR Green-based real-time PCR. Conditions were based on Wroblewski et al³¹ with slight modification.²⁷ We reported CT values if the melting temperature (*T_m*) ranged from 70 to 72.5°C.

Viral Gastroenteritis PCR: We performed multiplex TaqMan real-time RT-PCR for norovirus genogroups 1 and 2, sapovirus, astrovirus, adenovirus group F, and rotavirus. Primers, probes and PCR conditions were based on published data,^{32, 33} and included positive and negative controls in each run.

Pp38 EIA and viral gastroenteritis PCR were only performed on cases and symptomatic controls. All other assays were performed on all samples.

Statistical analyses

To account for non-normal distributions, we used the Wilcoxon rank-sum test to compare mRNA CT and *tcdB* CT if outcomes were dichotomous, and Kruskal-Wallis test for comparisons in different groups. We used Chi-square test and, when appropriate, Fisher exact test, for categorical data comparison and for dichotomized IL-8, lactoferrin and pp38 protein concentrations. We fitted multivariate binary logistic regression and Cox proportional hazards models, through a backward selection process, to determine the predictors of poor clinical outcomes, assigned *a priori*. Variables offered in the regression models included elevated IL-8 mRNA, elevated CXCL-5 mRNA, WBC >15000 cells/µL, serum creatinine 1.5 χ baseline and *tcdB* CT. Proportional hazard assumptions were assessed visually from the plots. We used medians to define elevated cytokine mRNA abundances (−2.1 and −0.8 for CXCL-5 and IL-8 mRNA respectively), and manufacturer's recommendations for protein concentrations. In addition, we used Kaplan-Meier survival analysis to interrogate time-to-diarrhea resolution in children with elevated fecal cytokines

on admission compared with those with no elevation. The effect size is expressed as odds ratio (OR), relative risk (RR), hazard ratio (HR), or median difference (MD). A CT MD of x translates to a 2^x fold change in the cytokine abundances. Two-tailed $p < 0.05$ were considered significant.

RESULTS

During the study period, we identified 74 individual patients with diarrhea and positive *C. difficile* PCR; 10 were excluded because of inadequate sample volume ($n=7$), inability to reach a caregiver for consent ($n=2$) or refusal to participate ($n=1$). We also enrolled 42 symptomatic controls (who had bacterial gastroenteritis or diarrhea and negative stool cultures). Of those, three had a positive *tcdB* PCR in our laboratory, but no *C. difficile* testing in the microbiology laboratory, so they were included in the case category. We excluded two cases and two controls because of discrepancies between our *tcdB* PCR and the microbiology laboratory PCR results (Figure 1; available at www.jpeds.com). After taking these changes into account, our final study included 65 cases (*C. difficile* positive, symptomatic) and 37 *C. difficile* negative symptomatic controls (14 with bacterial gastroenteritis (*Campylobacter*, 5; *Salmonella*, 4; *Escherichia coli* O157:H7, 3; non-O157:H7 Shiga toxin-producing *E. coli*, 1; and concomitant *Campylobacter* and *Aeromonas*, 1), 4 with inflammatory bowel disease and 19 with unknown causes for their diarrhea). Cases were more likely to have cancer, recent exposure to immunosuppressives, antibiotics and healthcare than symptomatic controls (Table I).

Of the 68 samples from asymptomatic controls, *tcdB* PCR was positive in sixteen (colonized asymptomatic controls). These colonized patients were younger than all other groups (median age=0.8y, interquartile range (IQR)=(0.5,1)) ($p < 0.001$). Interestingly, only one (6%) of them had received antibiotics in the previous 4 weeks, compared with 11 (21%) of the 56 non-colonized asymptomatic controls.

Thirty-five (54%), 20 (31%), and 10 (16%) cases were initially treated with metronidazole, vancomycin or no therapy, respectively. Of those treated for *C. difficile* infection, 22 (40%) had persistent diarrhea, 31 (56%) had diarrhea that resolved, and two (4%) had an undeterminable diarrhea status at 5 days of therapy (Table II; available at www.jpeds.com). Sixteen (25%) cases had a concomitant viral gastroenteritis (Table I) and of those, 13 were treated for *C. difficile* infection. One child had concomitant *C. difficile*, *Shigella sonnei* and sapovirus infections. 23 (35%) cases did not have a fecal bacterial culture performed, nine (39%) of whom developed their diarrhea 5 days or more after admission. Eight (12%) cases had a history of inflammatory bowel disease (IBD).

Cytokines and bacterial burden

Fecal cytokines were higher in samples from symptomatic children (cases and symptomatic controls) than in samples from asymptomatic patients (MD=7.33 and 2.5 for CXCL-5 and IL-8 mRNA respectively, $p < 0.001$). Asymptomatic controls had robust amplification of the GAPDH internal control, with CT values similar to the fresh case samples collected during this study, indicating that the nucleic acids were well preserved in these samples. Interestingly, asymptomatic children colonized with *C. difficile* tended to have lower *tcdB*

CT than symptomatic cases, indicating slightly higher *C. difficile* burden (MD=3.48, $p=0.11$) (Figure 2, A). IL-8, Lactoferrin and CXCL-5 did not differentiate diarrhea causes and were elevated in both cases and symptomatic controls (Figure 2, B). Although none of the symptomatic controls had elevation in pp38, a subset of cases had an elevated level (Figure 2, C). That prompted us to perform a receiver-operating characteristics (ROC) curve to identify a cut-off of pp38 concentration that maximized specificity. We found that at 40pg/mL, pp38 was 100% specific for *C. difficile* infection (95% confidence interval [CI]=83–100%), although this cut-off value lacked sensitivity (25%, 95% CI=15–38%) ($p=0.009$).

Investigating our *C. difficile* infection cohort, children with persistent diarrhea had greater fecal CXCL-5 (MD=4.29, $p<0.001$) and IL-8 (MD=3.83, $p=0.004$) mRNA abundances and IL-8 protein (RR=2.16, $p=0.03$) concentrations than those whose diarrhea resolved by 5 days of *C. difficile* infection therapy. In addition, CXCL-5 and IL-8 mRNA abundances were higher in patients treated with vancomycin, presumably reflecting greater concern by their treating physician (MD=2.3, $p=0.03$ and MD=3.48, $p=0.02$ respectively). In contrast, *C. difficile* bacterial burden correlated with no clinical parameter or severity outcome (Figure 3). When we excluded children with concomitant viral gastroenteritis, children with persistent diarrhea had higher CXCL-5 (MD=4.72, $p<0.001$) and IL-8 (MD=4.78, $p=0.003$) mRNA abundances, and IL-8 (RR=3.08, $p=0.03$) and lactoferrin (RR=2.76, $p=0.04$) protein concentrations. CXCL-5 and IL-8 mRNA abundances were significantly higher in patients treated with vancomycin (MD=3.36, $p=0.009$ and MD=4.89, $p=0.001$ respectively) (Figure 4; available at www.jpeds.com). Immunosuppressed and non-immunosuppressed children had similar clinical presentations, and immunosuppressed children had lower IL-8 mRNA abundances (MD=4.36, $p<0.001$). When we excluded immunosuppressed children, CXCL-5 and IL-8 mRNA remained associated with persistent diarrhea (MD=3.05, $p=0.001$ and MD=3.71, $p=0.008$ respectively). Among children receiving immunosuppressants ($n=37$), 8 (22%) had a concomitant viral gastroenteritis; CXCL-5 mRNA at diagnosis was higher in immunosuppressed children with persistent diarrhea (MD=4.5, $p=0.002$), however IL-8 mRNA was not. Additionally, time-to-diarrhea resolution was longer among immunosuppressed children with high CXCL-5 mRNA at diagnosis (MD=4 days, $p=0.03$), but was not different with higher IL-8 mRNA levels. Children with IBD did not differ from other cases clinically, although they were more likely to be treated with vancomycin (RR=3, $p=0.001$). They also tended to have higher IL-8 mRNA abundances (MD=3.04, $p=0.055$). Multivariate logistic regression modeling identified elevated CXCL-5 mRNA as the only predictor of persistent diarrhea (OR=9.53, 95% CI =1.85–49.2, $p=0.007$), and elevated IL-8 mRNA as the only variable associated with vancomycin therapy (OR=20, 95% CI=1.61–247.98, $p=0.02$). Time-to-diarrhea resolution was longer in patients with elevated CXCL-5 mRNA and IL-8 mRNA at diagnosis (medians of 7 versus 2days $p<0.001$ for CXCL-5, medians of 5 versus 3days $p=0.02$ for IL-8). When patients were segregated into quartiles based on their initial *tcdB* CT, there was a paradoxical trend towards a longer interval to diarrhea resolution in children with a lower bacterial burden at diagnosis ($p=0.06$) (Figure 5). It is interesting to note that children treated with vancomycin had a longer time-to-diarrhea resolution (medians of 7 days versus 3 days, $p=0.03$). Cox proportional hazards model identified low CXCL-5 as a predictor of a quicker time-to-diarrhea resolution

(HR=3.2, 95% CI=1.35–7.57, $p=0.008$). Lower fecal bacterial burden at diagnosis was again associated with longer times to diarrhea resolution (HR=0.93, 95% CI=0.86–1, $p=0.058$). No major changes in these results were observed when we excluded infants younger than 1 year of age ($n=4$) from our cohort.

DISCUSSION

Fecal inflammatory markers were elevated in symptomatic patients with *C. difficile*, differentiating children with asymptomatic colonization from those with disease. These markers, however, did not distinguish children with *C. difficile* infection from those with other causes of diarrhea. Interestingly, *C. difficile* fecal burden was slightly higher in asymptotically colonized children than cases, suggesting that disease is independent of the amount of bacterial presence, and is due to the host injury and the ensuing inflammatory response. The elevation of pp38 in stools of children with *C. difficile* infection, but not in symptomatic controls, suggests that the p38 pathway is specific for *C. difficile*-associated injury. In the cohort of children with *C. difficile* infection, fecal biomarkers predicted treatment failure, and their elevation at diagnosis was associated with a longer time to symptom resolution. *C. difficile* bacterial burden was not associated with any clinical outcome, such as symptom persistence or severe disease.

Identifying a host biomarker specific to *C. difficile* infection would be the best way to differentiate children with disease from those with *C. difficile* colonization and diarrhea due to another cause. In our cohort, CXCL-5, IL-8 and lactoferrin were not specific for disease caused solely by *C. difficile*. Indeed, these cytokines are elevated in adults with inflammatory bowel disease and bacterial gastroenteritis.^{34–36} In contrast, the p38 pathway was activated exclusively in *C. difficile* infection. *In vitro* studies have implicated p38 in *C. difficile* infection pathophysiology,^{37, 38} and we recently reported that the p38 pathway and its downstream kinase target MK2 are major components of the inflammatory response elicited by *C. difficile* toxins in cells, mice and adults.¹⁹ We now complement these experimental and adult data by showing that p38 is activated in children with *C. difficile* infection. Unfortunately, pp38 is not a sensitive biomarker, and is only elevated in a subset of children with *C. difficile*. Larger studies and/or more sensitive assays, might clarify the implications of pp38 elevation in *C. difficile* infection.

We also demonstrate that fecal concentration of *C. difficile* is not associated with diarrhea. There are many theories as to why infants have *C. difficile* “disease resistance”: absence of intestinal toxin receptors,³⁹ protective factors from breast milk, and competition from other commensal flora¹⁷ have all been proposed. Our data complement studies that found similar bacterial counts and toxin titers in adults with *C. difficile* infection and neonates,¹⁷ and refute the hypothesis that infants have relatively low numbers of the pathogen. Our findings that asymptotically colonized children had low fecal cytokines raise the possibility that neonates lacking disease have an attenuated inflammatory response to toxigenic organisms in the gastrointestinal tract.

The second major finding of our study is that cytokine levels at the time of diagnosis correlate directly with prolonged diarrhea, which is typically considered a failure of

antibiotic treatment. Instead, our results, and those from our study in adults,²⁷ indicate that prolonged symptoms are a manifestation of intense intestinal inflammation present at the time of diagnosis. By excluding children with viral co-infections, we were able to assess children with no other explanation for their diarrhea and still found correlation between persistent diarrhea and elevated fecal inflammatory markers. It was interesting to find that children treated with vancomycin had a longer time to diarrhea resolution compared with those treated with metronidazole. This finding is probably related to the treating physicians' use of vancomycin for more severe cases,²¹ and does not by itself implicate vancomycin failure compared with metronidazole.

Many clinical scores attempt to identify adults at risk of severe disease such as colectomy or death. Although the morbidity and mortality associated with *C. difficile* infection is increasing in children,^{15, 16} they are less likely than adults to develop such severe complications.^{8, 20} It is therefore more important to identify patients at risk of clinically actionable *C. difficile* diarrhea in this age group. Host inflammatory responses in *C. difficile* infection are gaining more interest, as systemic biomarkers in blood, such as CRP, albumin and WBC were recently proposed as being associated with poor clinical outcomes in these infections.^{24, 26} Fecal inflammatory biomarkers may be more specific because they reflect the local response of the gastrointestinal tract to the effects of *C. difficile* toxins. We included in our regression models leukocytosis and elevated creatinine, described to be associated with severe disease in adults,⁴⁰ and found that fecal cytokines better predicted persistent diarrhea. Children with evidence of intestinal inflammation, like adults,²⁷ have a longer disease course than children who do not have elevated fecal inflammatory biomarkers. Identifying those children early may influence decisions about therapy, hospitalization, and follow-up, thus preventing some of the morbidity in children with *C. difficile* infection. Elucidating the causes and consequences of the host inflammatory response during *C. difficile* infection may enable targeted immune modulation as an adjunctive therapy in patients with unremitting disease.

This study does have limitations. First, because of the difficulty of obtaining samples on asymptomatic controls, we used samples from a study conducted a decade ago in a different geographic area that included limited exposure and medical history information on enrolled patients. However, these samples were frozen since acquisition, and the PCR internal control amplified as robustly as it did on fresh samples, indicating that target degradation likely did not affect our study results. Second, we did not type the *C. difficile* strains in the positive subjects, but such information does not influence physicians' management decisions and the data regarding strain type correlation with disease severity is controversial.^{25, 41, 42} Indeed, biomarkers are far more likely to be a useful guide to treatment strategies than strain type.^{24, 43, 44} Third, detecting cytokine transcripts in the stools does not necessarily correlate with cytokine protein levels, and more studies looking at these correlations are needed. Finally, except for asking families about treatment cessation, we have no corroboration of medication administration or compliance, such as antibiotic concentrations in host fluids.

In summary, fecal inflammatory cytokines differentiate asymptomatic *C. difficile* colonization from disease and are associated with poor outcome in children with *C. difficile* infection. Additionally, our data suggest that the host immune response to *C. difficile*, rather

than the burden of intestinal pathogen, is a major determinant of disease progression and symptom persistence. We believe the host-pathogen interaction should receive more attention in future *C difficile* infection studies in individuals of all ages.

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List of Abbreviations

EIA	enzyme immunoassay
CRP	C-reactive protein
CT	cycle threshold
HR	hazard ratio
IL	interleukin
IQR	interquartile range
MD	median difference
PCR	polymerase chain reaction
RR	relative risk
SLCH	St Louis Children's Hospital
WBC	white blood cells
CT	difference of cycle thresholds between cytokines and internal control, representing cytokine relative abundances

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Figure 1. online only: Study enrollment

*Patients who were originally enrolled as symptomatic controls but who did not have *C. difficile* polymerase chain reaction (PCR) testing in the microbiology laboratory, and had a positive *tcdB* PCR in our laboratory. (Met case definition and moved to cases)

†Patients whose *C. difficile* PCR from the microbiology laboratory was positive (originally enrolled as cases) but had a negative *tcdB* PCR in our laboratory. (Excluded for PCR discrepancies)

‡Patients whose *C. difficile* PCR was negative in the microbiology laboratory (originally enrolled as symptomatic controls) but had a positive *tcdB* PCR in our laboratory. (Excluded for PCR discrepancies)

Figure 2. Fecal cytokines and *tcdB* in the 65 cases (symptomatic children with *C. difficile*), 37 symptomatic controls, 16 *C. difficile* colonized asymptomatic controls, and 52 *C. difficile* negative asymptomatic controls

A. *tcdB* CT in asymptomatic controls colonized with *C. difficile* (dotted) tend to be lower (correlating to a higher fecal bacterial burden) than *tcdB* CT of symptomatic cases with *C. difficile* (vertical stripes). This finding did not reach statistical significance.

B. CXCL-5 mRNA abundances in asymptomatic controls, whether colonized with *C. difficile* (dotted) or not (clear), are lower than abundances in patients with diarrhea, whether *C. difficile* positive (vertical stripes) or not (oblique stripes).

C. Phosphorylated p38 is only elevated in cases (downward triangles) when compared with symptomatic controls (upward triangles).

For A and B: Boxes represent median and interquartile range; whiskers represent 5–95 percentiles; dots represent outliers; p is the p value of a two-tailed Kruskal-Wallis/ Dunn's multiple comparison test; *** p<0.001.

For C: Each shape represents an individual patient's value; bars represent medians and interquartile ranges.

Figure 3. Fecal cytokines and *tcdB* at diagnosis in cases (symptomatic children with *C. difficile*) grouped by outcome or antimicrobial therapy

A. CXCL-5 mRNA abundances in children with persistent diarrhea (vertical stripes) are higher than those whose diarrhea resolved by 5 days of *C. difficile* infection therapy (clear).

B. *tcdB* CT in patients with persistent diarrhea (vertical stripes) are not different from those whose diarrhea resolved by 5 days of *C. difficile* infection therapy (clear).

C. IL-8 mRNA abundances in patients treated with metronidazole (dotted) are lower than those treated with vancomycin (oblique stripes).

D. *tcdB* CT in patients treated with metronidazole (dotted) are not different from those treated with vancomycin (oblique stripes).

Boxes represent median and interquartile range; whiskers represent 5–95 percentiles; dots represent outliers; p is the p value of the two-tailed Wilcoxon rank-sum test; *p<0.05,

**p<0.01, ***p<0.001.

Figure 4. online only: Fecal cytokines and *tcdB* at diagnosis in cases (symptomatic children with *C. difficile*) grouped by outcome or antimicrobial therapy, after exclusion of children with gastrointestinal viral co-infections

A. CXCL-5 mRNA abundances in patients with persistent diarrhea (vertical stripes) are higher than those whose diarrhea resolved by 5 days of *C. difficile* infection therapy (clear).

B. IL-8 mRNA abundances in patients with persistent diarrhea (vertical stripes) are higher than those whose diarrhea resolved by 5 days of *C. difficile* infection therapy (clear).

C. CXCL-5 mRNA abundances in patients treated with metronidazole (dotted) are significantly lower than those treated with vancomycin (oblique stripes).

D. IL-8 mRNA abundances in patients treated with metronidazole (dotted) are significantly lower than those treated with vancomycin (oblique stripes).

Boxes represent median and interquartile range; whiskers represent 5–95 percentiles; dots represent outliers; p is the p value of the two-tailed Wilcoxon rank-sum test; *p<0.05, **p<0.01, ***p<0.001

Figure 5. Time-to-diarrhea resolution in patients with elevated markers at diagnosis compared with those with low markers

A. Time-to-diarrhea resolution is longer in patients with elevated CXCL-5 mRNA on admission (CT CXCL-5 mRNA > (-2.1), red line) (median 7 days) compared with those with low CXCL-5 mRNA (green line) (median 2 days).

B. Time-to-diarrhea resolution is longer in patients with elevated IL-8 mRNA on admission (CT IL-8 mRNA > (-0.8), red line) (median 5 days) compared with those with low IL-8 mRNA (green line) (median 3 days).

C. When patients were segregated into quartiles based on their initial *tcdB* CT (CT>30.68, green line; 25.84<CT<30.68, blue line; 21.66<CT<25.83, orange line; CT<21.66, red line), there was no statistically significant difference in the time-to-diarrhea resolution among the four groups. However, there was a trend to a slower resolution in children with higher CT values (which translate to a lower bacterial burden) (green and blue lines).

+ represents censored values; p is the p value of the Log Rank (Mantel-Cox) test; * p<0.05, ***p<0.001.

Table 1

Baseline Characteristics of cases (symptomatic children with *C. difficile*) and symptomatic controls (symptomatic children with no *C. difficile*)

	Cases N =65	Symptomatic controls N = 37	P value*
Age in years; median (IQR)	10.2 (3.3,15.1)	10.9 (3.9, 16.4)	0.61 [†]
Male	30 (46)	18 (49)	0.84
Cancer/ transplant	21 (32)	2 (5)	0.001
Type of visit			0.45
Inpatient	55 (85)	28 (75)	
Outpatient	5 (8)	3 (8)	
Emergency department	5 (8)	6 (16)	
Prior CDI in the past 90days	4 (6)	0 (0)	0.29
Prior CDI in the past 30days	3 (5)	0 (0)	0.55
Hospitalization in the past 30days	26 (40)	7 (19)	0.047
Antibiotic use in the past 90days	59 (91)	14 (38)	<0.001
Metronidazole use prior to diagnosis	6 (9)	3 (8)	1.0
Immunosuppressives in the past 90days	37 (57)	9 (24)	0.002
Steroids	35 (54)	7 (19)	0.001
Other immunosuppressants	27 (41)	8 (22)	0.052
Anti-acids in the past 90days	33 (51)	7 (20)	0.002
Non-steroidal anti-inflammatory medications in the past 90days	5 (8)	4 (11)	0.72
Viral agent of gastroenteritis found	16 (25)	2 (5)	0.015
Norovirus genogroup 2	11 (17)	1 (3)	0.051
Norovirus genogroup 1	1 (1)	0 (0)	1.0
Sapovirus	3 (5)	1 (3)	1.0
Astrovirus	1 (1)	0 (0)	1.0
Rotavirus	0 (0)	0 (0)	1.0
Adenovirus group F	0 (0)	0 (0)	1.0

Data are n (%) unless otherwise specified.

IQR= Interquartile range; CDI= *C. difficile* infection

* p value of Fisher's exact test unless otherwise specified

[†] p value of a two-tailed Wilcoxon rank-sum test

Table 2online only: Outcomes of the 65 cases (symptomatic children with positive *C. difficile*)

Cases n = 65	n (%)
<i>C. difficile</i> treatment at diagnosis	
Metronidazole	35 (54)
Vancomycin PO	20 (31)
No treatment	10 (15)
Switched from metronidazole to vancomycin	3/35 (8)
Diarrhea lasting $\geq$ 5 days after initiation of CDI therapy	22/55 (40)
Serum WBC $>15\,000/\mu\text{L}$	7 (11)
Serum creatinine >1.5 baseline	1 (1.5)
Toxic megacolon, colectomy or death	1 (1.5) death from underlying disease
Admitted or transferred to the intensive care unit	9 (14)