Detection of *Clostridioides difficile* by Real-time PCR in Young Children Does Not Predict Disease

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**ABSTRACT**

**OBJECTIVES:** Diagnosing *Clostridioides difficile* infections in young children with high asymptomatic colonization is challenging. We compared the frequency of *C difficile* detection by polymerase chain reaction (PCR) in healthy control (HC) children with those with acute gastroenteritis (AGE) and evaluated fecal-lactoferrin and organism load as possible indicators of true *C difficile* infection disease.

**METHODS:** Stool was collected from children <2 years old with AGE and from HCs. *C difficile* was detected by real-time PCR, and lactoferrin was measured by enzyme-linked immunosorbent assay. Clinical data were obtained via interviews and chart review. Mann–Whitney *U* test and *χ²* tests were used for group comparisons.

**RESULTS:** Of 524 stools collected from 524 children (250 with AGE, 274 HCs), *C difficile* was detected less in children with AGE (14%, 36 of 250) than in HCs (28%, 76 of 274) stools (*P* < .0001). Among infants <1 year old (*n* = 297), *C difficile* was detected in 18% of children with AGE versus 32% of HCs (*P* < .005), and among children 1 to 2 years old (*n* = 227), *C difficile* was detected in 10% of children with AGE versus 21% of HCs (*P* < .02). There was no significant difference in *C difficile* PCR cycle threshold values between children with AGE and HCs or lactoferrin levels in *C difficile* PCR-positive versus -negative stools.

**CONCLUSIONS:** HC children <2 years of age had higher rates of *C difficile* detection by PCR than children with AGE; *C difficile* detection by real-time PCR alone is not a reliable means to diagnose *C difficile* disease in children <2 years old.

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“Clostridium difficile,” formerly Clostridioides difficile, manifests differently in children and adults. Although several risk factors for disease are similar (e.g., antibiotic exposure, underlying malignancy), asymptomatic detection, called colonization in this article (presence of pathogen in absence of disease), is more frequent in young children. Epidemiological studies indicate high rates of *C. difficile* detection in children <3 years old, ranging from 80% in neonates to 60% in infants <1 month old, decreasing to 10% in the second year of life, and reaching adult-like prevalence (0%–3%) by 3 years of age.4–7 Young children are not widely thought to be as susceptible to true disease associated with *C. difficile* (referred to as infection) in part because of lack of receptor for *C. difficile* toxin,6 making the clinical significance of detection an ongoing debate. Despite the general belief that most detections in young children primarily indicate colonization, some testing in young children continues in clinical settings. The literature has not clearly differentiated *C. difficile* colonization from *C. difficile*-caused diarrheal disease or infection. The terminology “*C. difficile* infection” has been used in published studies to represent colonization, disease associated with *C. difficile*, or both.11,12 More importantly, continued *C. difficile* testing in young children, often as part of multiplex gastrointestinal pathogen panel polymerase chain reaction (PCR) assays, can lead to positive results for *C. difficile*, leading to antibiotic treatment based on a test whose results have unclear clinical implications.

The American Academy of Pediatrics (AAP) recommends against routine *C. difficile* stool testing in infants <12 months old because of high colonization rates but recommends testing in children >3 years on the basis of similar criteria for adults.8 The difficulty for clinicians is that, for children 1 to 3 years old with diarrhea, the AAP guideline states testing can be considered after excluding other causes of diarrhea. The new Infectious Diseases Society of America guideline also does not recommend testing children aged 1 to 2 years unless other causes are ruled out.15

There is no laboratory confirmatory test that differentiates infection associated with *C. difficile* from colonization. PCR-based methods are preferred because of rapid turnaround and high analytical sensitivity. However, PCR has limitations, with false-positives resulting from detection of the *C. difficile* toxin gene even when the gene is not actively producing toxin or remaining detectable after treatment. Thus, a positive PCR result does not differentiate infection from colonization, presenting clinical challenges in diagnosing children <3 years old. Differing interpretations can be found in literature regarding the meaning of a positive PCR result in a child with or without diarrhea. The emergence of multiplex molecular-based assays to detect gastrointestinal pathogens has increased detection rates of *C. difficile*. Positive results are frequently interpreted as infection regardless of age or symptoms, adding unnecessary burden to the health care system, including isolation precautions for hospitalized children and antibiotic overuse. A reliable biomarker could assist in differentiating infection from colonization. In adults, fluoroquinolone-resistant North American pulsed-field gel electrophoresis type 1 (NAP1) strains have been associated with more severe disease and hypervirulence. Thus far, NAP1 strains appear uncommon in children. Another potential disease indicator in adults is high *C. difficile* burden, suggested by a low PCR cycle threshold (Ct) value.21 The Ct is the PCR cycle number when the fluorescence of the PCR product can be detected above the background signal. A Ct value is inversely proportional to pathogen load; for example, a Ct value of 37 indicates lower pathogen load, whereas a Ct value of 20 indicates a higher pathogen load. In adults, the potential role of *C. difficile* toxin B PCR Ct value to predict infection and poor outcome has been reported.21 Finally, fecal levels of lactoferrin, a neutrophil-derived protein that closely correlates with bowel mucosal injury, has also been proposed as a potential biomarker of *C. difficile* infection in children and adults.22–24 Our goal was to compare *C. difficile* detection rates in children <2 years of age with acute gastroenteritis (AGE) to rates in healthy controls (HCs) as well as evaluate NAP1 strains, PCR Ct values, and lactoferrin concentrations as potential biomarkers.

**METHODS**

The New Vaccine Surveillance Network (NVSN) includes 7 medical institutions across the United States conducting active, prospective surveillance for pediatric AGE hospitalizations and emergency department (ED) visits. NVSN methods have previously been published. The test results and data reported in this article are derived from a randomly selected subset of patients previously described.27 We tested stools collected at enrollment and analyzed parental interview information and selected data from medical chart reviews from 4 NVSN sites (Seattle, Kansas City, Memphis, and Rochester). The NVSN protocol was approved by the institutional review board at each site and the institutional review board at the Centers for Disease Control and Prevention. Written informed consent was obtained before collection of data and stool.

**Study Population**

This study included only the subset of enrolled NVSN subjects who were <2 years old and had been prospectively enrolled per the original NVSN protocol between January 1 and December 31, 2012, into 1 of 2 groups.

**AGE Group**

Per the original NVSN protocol, patients were eligible if there were no reported pre-existing chronic conditions and they presented with AGE (either diarrhea ≥3 episodes or vomiting ≥1 episode within a 24-hour period or both for ≤10 days’ duration) for hospital admission or in the ED. Children presenting with vomiting alone without diarrhea were excluded from this study (Fig 1). Stool samples were collected within 10 days of AGE symptom onset.

**HC Group**

Per the original NVSN protocol, HCs had no known pre-existing chronic conditions and no diarrhea or vomiting within 14 days of enrollment. HCs were enrolled from
outpatient clinics during routine well-child visits. Stool specimens were collected within 5 days of enrollment.

**Sample Preparation and C difficile Testing**

Whole stool samples were tested by US Food and Drug Administration–cleared Xpert C difficile/Epi PCR assay (Cepheid Inc, Sunnyvale, CA) per manufacturer’s instructions. C difficile/Epi PCR is a multiplex real-time PCR for qualitative detection of toxigenic C difficile and its toxins. These toxins include toxin B gene (tdcB), binary toxin gene (cdt), and tcdC gene deletion at nucleotide 117 (which identifies a strain as being 027/NAP1/B1). PCR testing is valid in frozen stools. Frozen stools were used for testing as previously described.22 PCR testing is valid in frozen and thawed stool samples. Results were reported as toxigenic C difficile positive and 027 presumptive negative, C difficile positive and 027 presumptive positive, and C difficile negative and 027 presumptive negative. Samples with invalid or error results were tested 1 additional time.

**Lactoferrin Measurement**

Thawed stool was used to measure lactoferrin by IBD-SCAN enzyme-linked immunosorbent assay (Tech Laboratory, Blacksburg, VA) on the same day, per manufacturer’s instructions. Enzyme-linked immunosorbent assay testing is valid in frozen and thawed stool samples. Lactoferrin values >7.5 μg/mL (g) feces were considered elevated.

**Analyses**

Descriptive statistics, including means, medians, SDs, interquartile ranges, and proportions, were used to summarize the data. The χ² tests were used to compare groups on categorical variables. The t tests and Wilcoxon Rank Sum tests were used to look for group differences in continuous variables. Because lactoferrin values were not normally distributed, their natural log values were used for all analyses. A whisker box plot was created to demonstrate potential differences in Ct values and lactoferrin levels between groups. Logistic regression models stratified by C difficile status were used to examine whether lactoferrin and breastfeeding were predictors of AGE. SAS version 9.4 (SAS Institute, Inc, Cary, NC) and SPSS version 23 (IBM SPSS Statistics, IBM Corporation) were used for analysis.

**RESULTS**

**Study Population**

A total of 615 <2-year-old children enrolled initially in the larger NVSN overall study met eligibility requirements for this substudy (Fig 1). However, 82 of 615 children with AGE were excluded because of having only vomiting without diarrhea, 5 were excluded for having inconclusive C difficile results, 2 were excluded for not consenting to further testing, and 2 were excluded for having insufficient sample. The final cohort (n = 524) included 250 children with AGE and 274 HCs.

**Demographics and Host Factors**

Demographic information of HCs and AGE groups can be found in Table 1. HC children were significantly younger than children with AGE. The increased proportion of female children in the HC group was not significant (P = .054). The HC group contained more African American children (P = .034), and the AGE group contained more Hispanic children (P = .004). Previous antibiotic use (oral or systemic) within 30 days of enrollment was reported in 12% (31 of 250) of the AGE group, but this question was not asked of HCs. Being breastfed or receiving breast milk was reported equally in the AGE and HC group (73% both groups). Ongoing breastfeeding at the time of enrollment was more common in the HC group (AGE: 20% [49 of 250] versus HCs: 26% [71 of 274], P = .086), and day care attendance was more common in children with AGE (AGE: 22% [56 of 250] versus HCs: 16% [44 of 274], P = .065), but these differences were not statistically significant.

**C difficile Detection**

Overall, C difficile was more frequently detected in HC children (28% vs 14%; P < .001) (Table 2). Among infants <1 year old, significantly more HCs tested C difficile positive (32% vs 18%; P < .005), and this was also true for children 1 to 2 years of age (21% vs 10%; P < .02). Of the
TABLE 1 Demographics of Study Population

<table>
<thead>
<tr>
<th></th>
<th>AGE (n = 250)</th>
<th>HC (n = 274)</th>
<th>P*</th>
<th>Total (n = 524)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), mo</td>
<td>11 (6.8)</td>
<td>9 (6.0)</td>
<td>.006</td>
<td>10 (6.4)</td>
</tr>
<tr>
<td>Sex, female, n (%)</td>
<td>104 (42)</td>
<td>137 (50)</td>
<td>.054</td>
<td>241 (46)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
<td></td>
<td>.034</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>113 (48)</td>
<td>112 (41)</td>
<td>—</td>
<td>225 (43)</td>
</tr>
<tr>
<td>African American</td>
<td>78 (32)</td>
<td>116 (42)</td>
<td>—</td>
<td>194 (37)</td>
</tr>
<tr>
<td>Other</td>
<td>59 (22)</td>
<td>46 (17)</td>
<td>—</td>
<td>105 (20)</td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
<td></td>
<td>.004</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>73 (29)</td>
<td>51 (19)</td>
<td>—</td>
<td>124 (24)</td>
</tr>
<tr>
<td>Insurance, n (%)</td>
<td></td>
<td></td>
<td>.040</td>
<td></td>
</tr>
<tr>
<td>Public</td>
<td>178 (72)</td>
<td>209 (76)</td>
<td>—</td>
<td>387 (74)</td>
</tr>
<tr>
<td>Private</td>
<td>60 (24)</td>
<td>58 (21)</td>
<td>—</td>
<td>118 (23)</td>
</tr>
<tr>
<td>Other</td>
<td>12 (4)</td>
<td>7 (3)</td>
<td>—</td>
<td>19 (3)</td>
</tr>
</tbody>
</table>

—, not applicable.

* A t test was used for age comparison; all other comparisons used χ² tests.

250 children with AGE, 66% reported ≥3 days of diarrhea, and 34% had >3 days of diarrhea at time of enrollment; however, they had similar rates of C difficile positivity (15% and 13%, respectively). Lactoferrin levels did not differ statistically in C difficile–positive versus –negative status (P = .79). Sixteen percent (5 of 31) of children with AGE whose parents reported antibiotic exposure (within 30 days of enrollment) were C difficile positive compared with 14% (30 of 215) of the children with AGE without recent antibiotic exposure (P = .746).

C difficile NAP1 Strains

Three NAP1 strain types were identified in positive C difficile samples. Two were from HC subjects (ages 4 and 7 months), and 1 was from a child with AGE (16 months) who also reported antibiotic use within 30 days of enrollment.

C difficile Ct Values

Among the C difficile–positive samples, there was no difference in C difficile PCR Ct values between AGE and HC groups (Table 2). PCR Ct values among hospitalized children who tested positive versus those seeking care at an ED also were not statistically significantly different (P = .482).

Lactoferrin Results

Lactoferrin testing was only performed on 308 samples that had sufficient residual sample for testing (AGE = 137, HC = 171). Children with AGE had significantly higher levels of lactoferrin (P = .002) (Fig 2A). Children breastfeeding at the time of the study (n = 40) had statistically higher lactoferrin levels (n = 268) (P < .001) (Fig 2B). No differences in lactoferrin levels were observed when analyzing groups on the basis of whether the stool PCR result was C difficile positive (n = 82) or negative (n = 226) (P = .788) (Fig 2C). In the C difficile–positive group, lactoferrin was not a significant predictor of AGE status with and without adjusting for breastfeeding status. In the C difficile–negative group, lactoferrin was a significant predictor of AGE status even after adjusting for breastfeeding status (P = .001).

DISCUSSION

Recognizing the difficulty of differentiating infection from colonization in infants who tested positive for C difficile, the AAP recommends against routine C difficile real-time PCR testing in children <1 year of age unless the infant has a severe motility disorder or is in an outbreak situation. Although our findings support this recommendation for children <12 months, our data reveal that infection associated with C difficile cannot be differentiated from colonization in children 1 to 2 years old with a positive C difficile real-time PCR test result. HC children in both age groups had C difficile detected approximately twice as often as children with AGE. Our results question the predictive value of real-time PCR testing among these age groups and raise uncertainty as to the etiologic role of C difficile in symptomatic, diarrheal children up to 2 years old. In our results, it is suggested that neither Ct values nor lactoferrin levels help distinguish colonization from infection in our populations. High lactoferrin levels were associated with breastfeeding and with AGE in children who were C difficile PCR negative, yet neither a low Ct value (higher abundance of C difficile in the sample) nor a high lactoferrin level correlated with clinical disease or C difficile PCR positivity status. Because lactoferrin is associated with bowel mucosal injury, the lack of an association between lactoferrin and C difficile positivity further supports the broader conclusion that C difficile positivity

### TABLE 2 C difficile Detection and Ct Values in Children With AGE Versus HCs by Age

<table>
<thead>
<tr>
<th></th>
<th>AGE (n = 250)</th>
<th>HC (n = 274)</th>
<th>P</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C difficile positive, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>36 (14)</td>
<td>76 (28)</td>
<td>&lt;0.001</td>
<td>112 (21)</td>
</tr>
<tr>
<td>&lt;1 y (n = 297)</td>
<td>24 (18)</td>
<td>52 (32)</td>
<td>0.005</td>
<td>76 (26)</td>
</tr>
<tr>
<td>1–2 y (n = 227)</td>
<td>12 (10)</td>
<td>24 (21)</td>
<td>0.023</td>
<td>36 (16)</td>
</tr>
<tr>
<td>Ct values for C difficile–positive samples, n = 112</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>30 (4.5)</td>
<td>30 (4.3)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>28.5 (26.2–34.6)</td>
<td>30 (26.0–33.1)</td>
<td>.663</td>
<td>—</td>
</tr>
</tbody>
</table>

IQR, interquartile range; —, not applicable.
Our overall finding was that 14% of children with AGE and 28% of HC children had C. difficile detected by PCR, which is consistent with other studies. Nicholson et al.²⁸ found that 16% of Tennessee pediatric inpatients or ED patients <6 years old who received care for diarrhea and/or vomiting tested positive for C. difficile by PCR in 2008 to 2011. Eight percent of HCs aged 0 to 51 months tested positive. González-Del Vecchio et al.²⁹ reported no statistically significant difference among children with diarrhea <2 years old when comparing those that tested positive for C. difficile to those testing negative.

Leibowitz et al.¹⁷ studied children aged 1 to 18 years who tested positive for C. difficile with or without diarrhea who were recruited from the pediatric oncology inpatient and outpatient setting. A higher positivity rate for C. difficile toxin B (tcdB) by PCR was noted in the asymptomatic group (24%) versus the symptomatic group (19%), although it was not statistically significant (P = .31). Although these results suggest colonization is also common in older children and adolescents, this chronically ill population differs from our immunocompetent cohort because of more frequent medical care visits and likely higher antibiotic exposures.

In an active surveillance study from 10 states participating in the Emerging Infections Program in 2010 to 2011,¹⁴ all positive C. difficile test results (by toxin or molecular assay) were assessed from laboratories serving the pediatric population within defined areas. The highest incidence of positivity was observed in children 1 to 2 years old (66.3 per 100,000).¹² Unlike our findings, Wendt et al.¹² concluded that the detection of C. difficile in their population (of which only 72% reported diarrhea within 1 day of stool collection) represented infection (ie, C. difficile disease) because the clinical presentation, severity, and outcomes in many of these 1- to 3-year-olds were similar to the 5- to 18-year-old

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FIGURE 2 Lactoferrin comparison by boxplots by (A) breastfeeding status, (B) HC versus AGE, and (C) C. difficile status.
groups. The recurrence rate was 11%, and antibiotic use in the 2 weeks before testing was documented in 33% of those that tested positive for *C. difficile*. Although no HCs were evaluated, Wendt et al. suggested they would have expected milder clinical symptoms in the younger children if these simply represented colonization. Information on *C. difficile* copathogens was available for 57% of their subjects. Only 3% were found to be coinfectected with other diarrheal pathogens, mostly among children ages 2 to 9. It appears that some cases of *C. difficile*–positive test results did not have diarrhea in this study and that a copathogen might explain the diarrhea in some of the younger children.

With our findings, we suggest that other etiologies be considered even when *C. difficile* is detected in symptomatic children <2 years old given that young children have multiple AGE episodes in the first few years of life. In the era before multiplex stool testing, ≤50% of these diarrheal illnesses had no detectable pathogen by conventional testing. Indeed, viral agents have been detected in half to two-thirds of US pediatric AGE hospital admissions and ED visits, making viral testing more likely to yield a true etiology than *C. difficile* testing alone. With our findings, we suggest that treatments specifically targeting *C. difficile* in young children who tested positive rarely be used.

The wide spectrum of symptomatic and asymptomatic *C. difficile* infections is not fully understood. Researchers of most adult studies find low colonization rates and have speculated that host factors may explain this wide spectrum. For example, relatively high colonization of *C. difficile* has been reported in adult long-term care facilities and statistically associated with previous *C. difficile*–associated disease (*P < 0.001*) and previous antibiotic use (*P = 0.017*). Among our asymptomatic HC children <2 years old, we observed a higher positivity rate than adults; more than one-quarter of these infants and children yielded a positive *C. difficile* test result without an apparent effect of recent antibiotic use among children with diarrhea. This indicates that *C. difficile* colonization in healthy young children may be part of their nonpathogenic gut microbiome. Whether a putative or protective health outcome later in life is related to this carriage is unknown. Questions regarding potential relationships between maternally derived immunologic protection, age-dependent commensal microbiotic relationships, and/or the immunologic and structural characteristics of the young gut that may support *C. difficile* colonization without producing symptoms deserve further study.

Given the lack of a true gold standard to confirm *C. difficile* disease in children, we evaluated a laboratory assay that could help differentiate colonization from disease in children <2 years old. Researchers of a pediatric study suggested lactoferrin as a potential tool to distinguish between disease and colonization. Our data suggest that lactoferrin can be an indicator of gut inflammation and injury because it was elevated in children presenting with AGE compared with HC children without diarrhea. However, a caveat regarding lactoferrin surrounds its known association with breastfeeding. The highest lactoferrin levels in our subjects were associated with breastfeeding regardless of whether they were in the AGE or HC group. For nonbreastfed children, modestly elevated lactoferrin levels were associated with any diarrheal disease, not just for *C. difficile*–positive AGE. Our data do not support lactoferrin as a useful biomarker for differentiating disease versus colonization in children <2 years of age. Still, some suggest lactoferrin may be protective against disease during *C. difficile* infection by reducing toxin production. Our findings were strengthened by the prospective, multicenter nature of our study design as well as the comparison of otherwise healthy children with AGE and HC children from the same community setting. The study was limited by the absence of information on antibiotic use in HC children and specific details about antibiotic use in those with AGE. Additionally, we did not test for other bacterial diarrheal pathogens, include stool samples from children >2 years old, or use the previously considered gold standard test (ie, cell culture cytotoxic assays) in this study because they are no longer used in the clinical setting and they are labor intensive. The HC group was younger than the AGE group, which may have skewed the *C. difficile* detection rate to more carriage in the younger HC group than in the slightly older AGE group. However, clinically, a 2-month mean difference (Table 1) should not account for a doubled detection rate in HC. Finally, our sample size was not sufficient to power detailed stratifications of children who tested positive versus those who tested negative by PCR.

**CONCLUSIONS**

In conclusion, positive *C. difficile* PCR results in children <2 years old are indicative of colonization, not infection, and are not sufficient as the sole diagnostic proof of *C. difficile* as the cause of a current diarrheal episode. Neither lactoferrin nor PCR Ct values help differentiate infection from colonization. Our study findings make us question the sole use of the rapid, increasingly available, and highly sensitive (but not disease-specific) PCR tests to accurately confirm *C. difficile* as an etiologic pathogen in children <2 years old.

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