Title: Diagnosis of *Clostridium difficile* Infections in Children

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Abstract

The detection and diagnosis of Clostridium difficile infection in pediatric populations has some unique considerations in comparison to testing in adults. The testing methodologies, including toxigenic culture, cell cytotoxicity, antigen detection, and more recently, molecular testing, are the same in all age groups. However, limited data exist on specific performance characteristics in children. In this review, we focus on the challenges of testing in pediatrics and assess the available data on test performance in this population. Additionally, a review of the existing guidance for testing is provided.

Introduction

The roots of Clostridium difficile in pediatrics are deep, tracing back to the discovery of the organism. Hall and O’Toole first described the organism as part of the normal flora in neonates naming it Bacillus difficilis (1). Much knowledge has since been gained about C. difficile with its pathogenic potential ranging from asymptomatic carriage to mild diarrhea, severe colitis, and toxic megacolon. It is well recognized that C. difficile infection (CDI) is a leading cause of nosocomial and antibiotic associated illness in adults, posing a huge burden on the healthcare system, and thus is a major public health concern. Concomitant to the increase of CDI in adults in the past decade, the rates of hospitalized children with the infection have been increasing as well. In a study of hospitalized children in the US, Nylund et. al. showed that the number of CDIs has risen from 3,565 cases in 1997 to 7,779 cases in 2006 (2). The number of cases per patient-days in the hospital has increased (3) and overall there has been a rise in hospitalizations attributable to C. difficile in children (4). While rates of CDI have increased, the severity of disease (e.g. colectomy and in–hospital deaths) has not increased in pediatrics as it has in adults (3).
Different approaches have been employed in the clinical laboratory to aid in the diagnosis of CDI. The changes in methods and the algorithms used to detect toxigenic C. difficile are a result of the increase in sensitivity and specificity of these assays, as well as the demand for accurate and timely results. A number of reviews have been written on the diagnosis of C. difficile (1, 5), but few have dealt specifically with the performance characteristics and the use of testing in children (5). This mini-review is a summary of clinical guidelines and available data for diagnostic testing for C. difficile focused on pediatric populations.

**Pathogenesis and Epidemiology of Clostridium difficile in children**

C. difficile is an anaerobic, gram positive, spore forming bacillus that can be found in the environment and the gastrointestinal tract of animals and humans. The pathogenicity of C. difficile is attributed to the production of two protein toxins designated A (enterotoxin) and B (cytotoxin), encoded by the tcdA and tcdB genes, respectively. They are found on the pathogenicity locus (PaLoc) and both cause significant disease. Toxins A and B are glycosyltransferases that can inactivate GTP-binding proteins. The toxins enter the cytoplasm of the colonocytes by binding to receptors that are found on the luminal-facing side of these cells. Once inside the cells, they inactivate a number of proteins involved in cytoskeleton organization, triggering the apoptosis of the cells. This results in an acute inflammatory reaction, leading to diarrhea and colitis (1, 6).

**Colonization with C. difficile in Infants:** The colonization rate of healthy infants has been studied either by culturing techniques or by PCR. For infants less than 1 month of age the organism is recovered at high rates with an average colonization rate of 37% (7), and a range of 0 to 61% being cited (8). Between 1 and 6 months of age, the colonization rate is still high at 30%, and drops to about 10% by the end of the first year of life (7). The asymptomatic carriage.
rate continues to drop until about three years of age, where it stabilizes to carriage rates of 0-3%, similar to those found in adults (7). *C. difficile* toxin titers found in the stool of healthy infants are similar to those found in adults with *C. difficile* associated diarrhea (9). There is a progressive increase in serum IgG antibody concentrations against toxins A and B between birth and 24 months of age (7). Together these findings suggest that toxigenic strains are colonizing the gut of infants, but they remain healthy without developing acute colitis. The exact mechanism of protection of the infants against *C. difficile* toxins is unclear, but a number of theories have been proposed including the lack of toxin receptors on the surface of the intestinal cells, the protective action of breast milk, and the defense provided by other neonatal intestinal flora. In a newborn rabbit ileum model, researchers found that there were no binding sites for toxin A on the surface of the cells. Additionally, even with maximum concentrations of toxin A applied on newborn and young rabbit ileum cells, the effects were minimal compared to the severe mucosal damage caused in adult rabbits (10). Although these data derive from an animal model, they suggest a possible mechanism by which human infants may avoid disease due to lack of toxin receptors on the surface of their colonocytes, which in turn inhibits internalization of toxins that lead to colonic damage. More research is needed to better characterize the actual receptors present in human infant gut tissue. It may be that a lower number of toxin receptors are expressed on the surface of infant colonocytes or that the binding sites of these receptors are altered leading to lower or absent binding capacity. Formula-fed infants have higher rates of colonization with *C. difficile* compared to breast-fed infants, but these differences seem to be gone by 12 months of age when weaning occurs (7). The exact mechanism of protection provided by breast milk is unclear. Various components of breast milk (galactose and colostrum) have been shown to inhibit binding of *C. difficile* on colonic cells (11), while human milk was
found to bind and neutralize toxin A itself, rendering it inactive in the gut (12). Neutralization of toxin A would allow the asymptomatic colonization of infants with *C. difficile* without the side effects an active toxin A may have. Additionally, breast fed babies have lower fecal pH, which favors the growth of *Bifidobacterium* which inhibits the growth and binding of *C. difficile* to enterocytes (13). Gradually, other commensal colonic flora compete with *C. difficile* and give rise to the adult colonic flora that is carried by the individuals for the rest of their lives (14).

While in most instances detection of *C. difficile* in infants will be non-contributory to diarrheal illness and represents colonization, there are rare cases of *C. difficile* associated deaths in this age group (15). In a study by Kim *et al.*, the authors noted that 26% of the reported *C. difficile* associated disease cases in their cohort occurred in children <1 year of age. But even the authors acknowledge that this finding could either represent disease to a previously unrecognized degree or a large number of children received treatment unnecessarily (3). Studies are needed to determine how often *C. difficile* in the <1 year of age population causes true disease.

**Hospital-Associated *C. difficile* Infection:** The rate of symptomatic CDI in hospitalized children has been increasing, with a number of associated risk factors, including; antibiotic use, immunosuppression, and bowel dysfunction (2). Unlike adults, there is no data suggesting an increased severity of disease (e.g. colectomy and in–hospital deaths attributable to CDI) among pediatric patients (2, 3).

Hospitalized children diagnosed with CDI have been shown to have poor outcomes with significant morbidity and increased risk of death (2). Additionally, CDI in pediatric patients has also been associated with increased length of stay and overall higher total hospital costs (16). It is clear that in hospitalized patients and those with other comorbidities there is a need for a timely and accurate diagnosis of CDI so that the appropriate therapy and intervention measures
are in place to prevent unfavorable outcomes. In a multicenter study across 41 children’s hospitals, Vendetti et al. attempted to identify the risk factors associated with in-hospital mortality for children with diagnosed CDI. Factors that were independently associated with increased mortality included older age (>13 years), receipt of gastric acid suppression, underlying malignancy, cardiovascular disease, and hematologic/immunologic conditions (17). In this study, the laboratory data for the type of test used for diagnosis or confirmation of CDI were not available. Therefore, the differences in the performance characteristics of the various methodologies for the detection of CDI in children in these high risk populations were not assessed. In a separate study assessing risk factors for children with recurring CDI, malignancy, recent surgery, and antibiotic exposure were the most significant factors (18). Both enzyme immunoassays (EIA) and nucleic acid amplification tests (NAAT) were used as laboratory tests for the detection of C. difficile, however, no data were presented on the differences in test performance. Solid organ transplantation also appears to be a risk factor for a higher incidence of CDI in children, especially those between 1-4 years of age (19).

As with infants, high rates of asymptomatic colonization have been reported among hospitalized children. Using PCR for C. difficile detection in hospitalized patients with and without diarrhea, Leibowitz et. al. found that there was no statistically significant difference between the positivity rates of asymptomatic and symptomatic children (24 % vs 19% of samples respectively) (20). Pediatric oncology patients may also be colonized or have prolonged shedding following, or in conjunction, with diarrhea (21). The apparent asymptomatic colonization or shedding can complicate the diagnosis of diarrheal illness in hospitalized children.
Among patients with inflammatory bowel disease (IBD), the rates of CDI have increased between two- to fourfold in the past decades (22, 23). These patients can acquire *C. difficile* in an outpatient setting and the presence of *C. difficile* can contribute to increased morbidity and mortality (1). When patients present with diarrhea during a relapse of IBD, they are often evaluated for CDI, but the best testing method for these patients has not been determined. In children with IBD, studies to characterize CDI are limited. Lamousé-Smith *et al.* assessed the prevalence of *C. difficile* in patients with IBD and non-IBD gastrointestinal disease (controls), in both diarrheal and nondiarrheal stool. There was no statistically significant difference in the number of positive samples for *C. difficile* by NAAT between the patients with IBD and controls, and patients with active or inactive disease (24). Additionally, of the 28 PCR positive results in this study, only 3 were positive by EIA, suggesting the detection of *C. difficile* could reflect colonization and not necessarily the cause of symptoms (24).

**Community-Associated *C. difficile* Infection:** The rate of detection of *C. difficile* in children with community, or outpatient acquired illness, is increasing. A retrospective study in a tertiary pediatric center showed that there was a statistically significant increase in the community acquired (CA) CDI cases, 11% in a period of 5 years (25). Historically, outpatient populations have been thought to be at lower risk of CDI. In a laboratory based surveillance study by Wendt *et al.*, the rate of *C. difficile* detection was 71% from testing of children in the community (26). However, this study and others have not correlated the positive laboratory findings with clinical data, making the link between a positive test and causality of disease unclear (5). In a recent retrospective pediatric study comparing clinical presentation and associated risk factors in those diagnosed with hospital–acquired (HA) versus CA-CDI, the authors found that many of the children diagnosed with CA-CDI by PCR lacked risk factors and...
were more often <2 years of age, a group with a known high rate of asymptomatic colonization (27). The positive PCR results were not always confirmed with additional C. difficile testing and evaluation for an alternate etiology was often limited, particularly for viral causes of diarrhea (27). The overall conclusion of these authors was that many of the CA-CDI were misdiagnosed and treated inappropriately. Clearly, the impact of C. difficile detection in the pediatric community care setting warrants further study to determine if there is a true increase in CA-CDI, and if it is wise to routinely test for C. difficile, particularly with a nucleic acid amplification testing (NAAT), in the absence of any of the established risk factors.

Guidelines Addressing C. difficile Infection: General Testing Recommendations and Specific Recommendations for Children

The severity of the disease, coupled with the public health implications as the most common hospital acquired infection in the US and other countries (28), prompted a number of professional societies to publish guidelines regarding CDI with recommendations for its diagnosis and treatment. Below, we highlight the important aspects of these guidelines as they relate either generally or specifically to testing in pediatric populations.

All existing guidance documents for the diagnosis of CDI clearly indicate that the key criterion for clinical diagnosis is the detection of toxigenic C. difficile in loosely formed stools. All guidelines follow the World Health Organization definition for diarrhea (the presence of 3 or more loose stools in a 24-hour period) and state that testing should be performed only on diarrheal unformed stool, unless ileus is suspected. One of the most pressing issues now relates to what testing methodology to use. Concerns have emerged that NAAT testing is too sensitive, while the use of toxin enzyme immunoassay (EIA) alone lacks sensitivity. Currently there is no single test or algorithm that has been universally accepted.
Guidelines from the Infectious Disease Society of America and the Society for Healthcare and Epidemiology of America (IDSA/SHEA) do not recommend the use of toxin EIAs as a standalone test due to low sensitivity, however, other testing approaches for the detection of the organism are not clearly endorsed and issues specific to children are not addressed (29).

The American College of Gastroenterology published supplemental guidelines to those of IDSA/SHEA recommending NAAT testing or a glutamate dehydrogenase (GDH) screening test as part of a two or three step algorithm, but do not address diagnostic issues or implications of positive results within the pediatric population (30).

The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) also acknowledges that the best standard laboratory test for diagnosis of CDI has not been established. They endorsed a two stage algorithm where a positive first test (culture, antigen detection or amplified molecular test), is confirmed with other tests or a reference method. This recommendation is based on the findings of a large study where various methodologies and algorithms were employed to identify the best testing strategy. According to the authors of that study, a two stage algorithm and combination of assays improves the diagnosis of CDI, but the performance of those tests depends on the reference method (31). In these guidelines there is no discussion of the diagnostic performances of the tests in pediatric patients (32).

The National Health Services (NHS) in Britain published guidelines for testing algorithms and reporting of C. difficile infection based on the study by Planche et. al. (33). The NHS recommends testing all loose stools, irrespective of risk factors for CDI, using a two-step algorithm with a screening assay (NAAT or GDH EIA) followed by a toxin EIA if the screening test is positive. Confirmed positive results are indicative of CDI, while positives not confirmed
by the toxin EIA, are most likely from patients that are excreting *C. difficile*. However, these patients are not at risk of complications related to CDI, similar to those that test negative with the initial screening test. All confirmed positive results are reported to a national surveillance database. The guidelines suggest that children under 2 years of age should not be tested and, if the testing is performed and is found positive, it is not mandatory to report the result in the national database (34).

The American Academy of Pediatrics (AAP) published guidelines in 2013 regarding CDI in children that address diagnosis and testing. They recommend only testing for CDI in children with diarrhea (3 or more loose stools in a 24-hour period). Recognizing the high colonization rates of children up to 3 years of age, testing should be limited in the following manner; it is recommended to avoid performing any testing in children less than 1 year of age unless there are specific gut motility disorders, such as Hirschsprung’s disease, or in the setting of an outbreak. For children between 1 and 3 years of age, testing can be considered if alternative causes, such as viral infections, have been explored. For positive results in children between 2 and 3 years of age, an alternative etiology for symptoms should be pursued. Testing in those >3 years of age with a positive result indicates probable CDI. The guidelines further suggest that a test of cure is not recommended. With a recurrence of symptoms, repeat testing should not be performed before 4 weeks after initial positive testing. The use of a specific testing methodology is not clearly endorsed, however, NAAT is mentioned as the most sensitive method for the detection of the organism (35). The guidelines do acknowledge that there is not enough published data to assess the diagnosis of disease in children with NAAT test alone (35).

*C. difficile* Diagnostic Tests
A number of laboratory tests are available to aid in the diagnosis of CDI. They differ in what is being detected with some identifying the presence of the bacteria, some detecting the presence of toxins, and some detecting nucleic acid for genes associated with toxin production. All of the tests described below can be used both in adult and pediatric patients. It is important to understand the differences in these methods and the clinical implications for a positive result in children.

**Toxigenic Culture (TC):** Culturing for the recovery of *C. difficile* from stool specimens was historically the initial method used in the clinical laboratory to detect the presence of the pathogenic bacteria. In order to reduce the normal flora and increase the recovery of *C. difficile*, stool samples are processed by heat or alcohol pretreatment and then inoculated on *C. difficile* selective media (36). Both toxigenic and non-toxigenic strains may be isolated and a confirmatory test for the expression of toxin proteins is required. This approach is lengthy and technically complicated with a turnaround time of almost a week. Culture is mainly employed in research settings and for epidemiological studies where the isolation of the organism can provide useful information but it is not routinely used in the clinical laboratory.

**Cell Cytotoxicity Assay (CCTA):** A direct cell cytotoxicity assay relies on the neutralization of *C. difficile* toxins using an antitoxin to enhance the specificity of toxin detection. An aliquot of diluted, buffered and filtered stool sample along with the tested stool sample mixed with *C. difficile* antitoxin are added into different wells with monolayers of cultured cells and are observed for the presence of cytopathic effect (CPE). The test is considered positive if CPE is seen in ≥50% of cells at 48h and the effect is inhibited in the wells containing *C. difficile* antitoxin (36). Nonetheless, it may be negative if the concentration of the toxin in the sample is too low or has been degraded due to improper specimen handling or even pretreatment.
of the patient with antibiotics prior to specimen collection (1). The assay is labor intensive, requires expertise both in cell culture maintenance and interpretation of the results and has a long turnaround time. In studies comparing CCTA versus TC, CCTA has a sensitivity of 75-85% (37). The relatively long turnaround times of both TC and CCTA limit their clinical utility, particularly compared to other methods. They are, however, both endorsed as tests against which other testing methodologies are compared (1).

**Enzyme Immunoassays (EIAs):** Enzyme immunoassays used for the detection of *C. difficile* consist of those that either detect the presence of toxins A and/or B or the presence of the metabolic enzyme GDH. Both assays are available as microwell EIAs or in lateral flow immunochromatographic formats.

Toxin immunoassays use monoclonal or polyclonal antibodies against toxins A and/or B to detect free toxin in stool samples. Due to their low cost and ease of use, they had been adopted by a large number of clinical microbiology labs as the diagnostic test for *C. difficile*. It has become clear though that toxin EIAs display variable sensitivity and specificity ranging from 42.3-96.8% and 84-100%, respectively, depending on the comparator assay used for the evaluation of the EIA (1). IDSA/SHEA, the American College of Gastroenterology, and NHS guidelines do not recommend the use of these assays as a standalone test for the diagnosis of *C. difficile* (29, 30, 34).

In a study of pediatric inpatients, the use of a toxin EIA for diagnosis of *C. difficile* related disease was evaluated. A total of 112 stool samples positive by EIA were tested for the presence of toxigenic *C. difficile* with TC and one third of those (40 samples) were found to be negative. Specimens that were negative by TC were also tested by PCR and GDH EIA, while some were further processed by broth enrichment or serial dilution and plated again. None of the
reprocessed samples were positive, and only one of the tested specimens was positive by PCR. This high rate of false positives was attributed by the authors to the low incidence of CDI in their patient population, resulting in a PPV of 64% (38). The authors did not explore other possible explanations for the high false positive rate with EIA such as low culture sensitivity or technical issues. The study results raise concerns about the use of toxin EIAs in the diagnosis of CDI in children due to high rates of false positives, however, this seems in contrast with other publications demonstrating a lack of sensitivity with EIA (39). The AAP guidelines also question the usefulness of EIA in the pediatric population due to poor performance characteristics and the low positive predictive value (35).

GDH is produced in high levels by toxigenic and non-toxigenic strains of *C. difficile* and is known to cross-react with *C. sordellii* (non-toxigenic strain). GDH assays have a high sensitivity as a screening test, ranging from 87.6 to 100% (1), and a negative predictive value of >97% (1). In addition, their low cost and ease of performance make them an attractive screening method for ruling out *C. difficile*. Due to the confirmation step needed when there is a positive GDH, combination EIAs have come to market with the detection of GDH and toxin done in one test. These assays can reliably predict *C. difficile* disease when both results are positive and can rule out disease when both are negative. A confirmatory test needs to be performed when the assay results disagree. This 2-step algorithmic approach (GDH/Toxin EIA with reflex to another method, if discordant) was assessed in a pediatric cohort where the dual EIA system results were compared to CCTA and NAAT. It was shown that the dual EIA results can be effectively used for accurately reporting positive and negative samples. For the samples that were discordant, the discrepant results needed to be resolved by another method to maximize sensitivity (40). CCTA and NAAT, as the secondary method of result confirmation, performed similarly in this cohort,
suggesting that the user friendly NAAT assay is acceptable as a confirmatory test in a 2-step algorithm. In a large multicenter study in the UK that demonstrated increased mortality in association with the presence of toxins in the stool sample, the authors advocated for the use of a molecular or an enzymatic assay (GDH) as a screening test and confirmation with a toxin EIA for the most accurate prediction of disease (33). The study included patients older than 2 years, but the pediatric patients were not analyzed separately; therefore, the predictive value of a positive EIA for assessing disease in these patients is still unknown.

**Molecular methods:** A number of different molecular platforms using nucleic acid amplification methods have been FDA-cleared for the detection of *C. difficile* directly from stool samples. These include either standalone tests looking specifically for *C. difficile* or *C. difficile* as part of larger multiplex molecular panel utilized for the detection of a variety of gastrointestinal pathogens. The available assays target the genes encoding the toxins tcdA, tcdB, or a combination of both. Some also include the tcdC 117 nucleotide deletion, which can be used for the presumptive identification of the 027/NAP1/BI strain, a strain isolated both from adult and pediatric patients in North America and Europe (2, 3). This strain has been associated with increased severity of CDI in adults (1). The methodology used for the assays varies from qualitative real-time PCR using molecular beacons or fluorescent probe primers, loop mediated isothermal amplification, helicase dependent amplification, to multiplex real-time or conventional PCRs. There are currently 10 manufacturers with FDA-cleared single analyte detection assays and 2 with multiplex platforms that include the detection of *C. difficile* in their testing menu. Table 1 is a summary of the FDA cleared molecular assays and the available pediatric data from their package inserts (PI). Some of the PI provide stratified data by age, but only one (Illumigene, Meridian Bioscience, Cincinnati, OH) provides specific performance
characteristics by age group. Table 2 summarizes published studies with performance characteristics of FDA-cleared molecular assays that were assessed either exclusively in a pediatric cohort or in a majority of pediatric samples (<21 years of age).

To date there are limited studies looking specifically at assay performance in pediatric patients. Selvaraju et al. tested BD GeneOhm (BD Diagnostics Inc., Sparks, MD) and Prodesse ProGastro (Hologic Gen-Probe Inc., San Diego, CA) in pediatric patients. Both showed better sensitivity and specificity than EIA alone in comparison to TC. However, the authors suggest that a 2-step algorithm utilizing a dual EIA screening test (GDH and toxin) followed by confirmation with a molecular assay provides the potential of reducing laboratory test costs while at the same time reporting fast and accurate results (41). Hart et al. compared the BD GeneOhm and Illumigene assays in pediatric patients to a GDH/Toxin EIA, culture on cycloserine cefoxitin fructose agar (CCFA) and CCTA. They evaluated the performance characteristics of the assays as standalone tests and as part of a 2-step algorithm. Molecular methods had comparable performance to GDH alone and higher sensitivity than the EIAs and CCTA. The 2-step algorithms (GDH + PCR) showed reduced sensitivity compared to NAAT alone (83% vs. 89%). The authors postulate that this difference is probably due to the high prevalence of CDI in their patient population (mainly hematology/oncology patients) and they recommend the use of NAATs alone in high risk patients (42). In another study comparing Illumigene and Quidel AmpliVue (Quidel Molecular, San Diego, CA) in a large number of pediatric patients, both tests performed similarly, suggesting that these tests can be used with this patient population. This study included a subanalysis of those <2 years of age. The detection rate of *C. difficile* in this group was 23%. The authors note that these tests may be accurate in those <2 but may not represent CDI and clinical judgment is needed in interpreting results (43).
There are currently two FDA-cleared multiplex platforms that detect an array of gastrointestinal pathogens including *C. difficile* as one of the reported targets: xTAG gastrointestinal pathogen panel (xTAG GPP, Luminex Molecular Diagnostics Inc., Toronto, Canada) and BioFire FilmArray Gastrointestinal Panel (FilmArray GI, BioFire Diagnostics, Salt Lake City, UT).

The xTAG GPP tests for the presence of 15 different pathogens. For the clinical trial of xTAG GPP for FDA-clearance, samples from pediatric patients were included but the results were not analyzed per age group. According to the package insert *C. difficile* was detected in 3/192 samples from healthy volunteers (ages not provided) and may have represented asymptomatic carriage (44). Data from the clinical trial (xTAG GPP package insert) showed that *C. difficile* was present in 52.7% of all co-infections, with the most common being norovirus co-detected with *C. difficile* (44). In a study by Beckmann et al. using xTAG GPP testing on samples from pediatric patients presenting with symptoms of acute gastrointestinal disease, 7% were positive for *C. difficile*, of which 39% were <1 year of age. The authors do not provide any further data regarding whether the detection of *C. difficile* correlated with CDI in these infants but they do question whether the finding of *C. difficile* in this setting warrants treatment (45). In another study where xTAG GPP was used as the first line of detection of gastrointestinal pathogens in 211 prospectively collected specimens from a pediatric hospital, *C. difficile* was the number one pathogen detected (10.9%). There were 5 cases of co-infection and *C. difficile* was present in 2 of these (*C. difficile* with norovirus and *C. difficile* with *Salmonella* spp.). The authors did not provide a breakdown of results positive for *C. difficile* by patient age making it difficult to assess the *C. difficile* positivity rate in this cohort (46). Interestingly, in a recent study using samples from pediatric patients from a developing country where xTAG GPP was the
testing platform, a high number of gastrointestinal parasites and viruses were detected. Of the samples tested, 64.7% were positive for a pathogen, but *C. difficile* was not detected in any of the specimens. However, the sample size was small with only 35 samples included in the study and the patients were older, 5-15 years (47).

The FilmArray GI tests for the presence of 22 pathogens, with *C. difficile* being one of them. According to the package insert, *C. difficile* was detected in 5/100 asymptomatic volunteers, 4 of which were less than 12 years of age (48). From the clinical trial data, *C. difficile* prevalence was stratified per age group and in patients less than 1-year-old, the organism was present in 40.5% of the samples tested, while in patients between 1-5 years old it was present in 15.8% (49). *C. difficile* was detected in 41.6% of samples with mixed infections, making it the second most detected target in these samples (49). In a recent study by Stockmann et al., the diagnostic yield of physician ordering patterns in pediatric patients with suspected infectious gastroenteritis was examined using traditional testing methods versus the use of FilmArray GI. The results were categorized based on whether a physician ordered testing for *C. difficile* alone, other pathogens alone, or the physician ordered testing for both *C. difficile* and additional pathogens. Of the samples tested, 68% were from outpatients, of which 25% were positive for *C. difficile*, a number similar to the positivity rate of 24% seen in inpatients. For all cases where *C. difficile* alone was tested, 28% were positive for another pathogen that was not known to the physician based on the testing ordered. Additionally, in those children 1-4 years of age where *C. difficile* alone was ordered, another pathogen was detected in 23% of the cases (50). This study had limited clinical data for review in order to identify true infections with *C. difficile* or the impact of *C. difficile* in co-infections thus making it hard to assess whether clinical intervention would have been required. Additionally, the high prevalence of *C. difficile*
in the outpatient population is evident, but correlation with disease was not established and additional studies are needed to interpret a positive result for *C. difficile* in these patients.

Similar to the study above, we analyzed data from our own hospital comparing the ordering patterns of *C. difficile* testing as a standalone test versus the results of the FilmArray GI panel run in parallel as part of a clinical research study. The FilmArray GI results were not known to the physicians (Figure 1, unpublished data). Following the AAP recommendations to avoid testing in the 1 to 3 age group, the pattern shows that *C. difficile* testing is not widely ordered in younger patients in our institution. However, it is considered a causative agent of diarrhea for older pediatric patients and the number of orders increased for those > 8 years of age in this cohort. Also of note, there were a considerable number of patients under 3 years of age with a positive result for *C. difficile* by FilmArray GI (n=63, 20 %) in which standalone testing for *C. difficile* had not been ordered by the physician. If a multiplex panel that includes *C. difficile* is used for testing on all samples, results are available even when the physician is not considering CDI in the differential diagnosis. Positive *C. difficile* results need to be interpreted with caution and in correlation with the clinical picture given the high rate of asymptomatic carriage in young children and other populations such as hospitalized children as described previously. Careful consideration of reporting is needed, including instructive comments or possibly withholding results of *C. difficile* testing in certain patient groups in order to mitigate the risk of inappropriate treatment.

Further studies to differentiate the patients that are colonized versus those with true disease are warranted. This includes more studies utilizing data from gastrointestinal multiplex panels where the exclusion of other agents of gastroenteritis and diarrhea can be explored and correlated with the patients’ clinical picture. Additionally, novel algorithms investigating the
role of intestinal inflammatory biomarkers to aid in the diagnosis of CDI are needed. El Feghaly et. al. explored the utility of interleukin-8, lactoferrin, chemokine ligand -5 RNA, and phosphorylated –p38 in children with and without diarrhea and symptomatic versus asymptomatic carriage of *C. difficile*. They conclude that fecal inflammatory cytokines may be useful for risk stratification in children (51).

**Conclusions**

The diagnosis of CDI should be made in context of the clinical presentation of the patient, in correlation with results of laboratory testing for detection of *C. difficile*. This is of particular importance in pediatric practice due to the known high levels of colonization in young children. Testing is not routinely recommended in children under 1 year of age. Caution needs to be used to interpret results in patients between 1 and 3 years of age and additional agents of infection need to be investigated (35). Nonetheless, in patients with risk factors and in special pediatric populations such as those with IBD and immunosuppressed patients, (17, 18) prompt and sensitive identification of *C. difficile* is necessary to allow appropriate treatment, prevent transmission, and improve outcomes.

The sensitivity and specificity of testing methodologies for the detection of *C. difficile* has improved with a plethora of assays available. With the advent of various NAAT tests, including multiplex panels, there exists the possibility of increased detection of *C. difficile*. This higher sensitivity can be of benefit, but there is also a risk that testing may lack positive predictive value in those who are not at risk of CDI. While NAAT testing is the most sensitive analytically, questions remain about its use as a first-line, standalone test. The best test or testing algorithms for the diagnosis of CDI and the ability to reliably differentiate disease from
colonization are still in debate. For both pediatric and adult patients, further studies are needed to shed light on the impact of testing on clinical outcomes.
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Amy L. Leber, Ph.D., D(ABMM), SM(ASCP)MB received her B.S in Microbiology and Ph.D. in Medical Microbiology and Immunology at The Ohio State University. She completed the CPEP Medical and Public Health Microbiology Fellowship at UCLA Medical Center in Los Angeles, CA. She joined Nationwide Children's Hospital in 2007 first as Associate Director then in 2013 as Director of Clinical Microbiology and Immunoserology. Dr. Leber is currently Associate Clinical Professor of Pediatric and Pathology and Laboratory Medicine at The Ohio State University College of Medicine. She is the current Editor-in-Chief for the Clinical Microbiology Handbook (ASM Press). Her research interests include development of improved diagnostics and the understanding of the pathogenesis of gastrointestinal and respiratory infections in pediatrics.

Stella Antonara, Ph.D., D(ABMM) completed her Bachelor in Biology at the University of Patra, Greece and her Ph.D. in Molecular Microbiology at Tufts University School of Medicine. She completed her CPEP Medical and Public Health Microbiology Fellowship at the National Institutes of Health in Bethesda, MD in 2013. Upon completion of her fellowship, she became the Assistant director of Microbiology and Immunoserology at Nationwide Children’s Hospital in Columbus, Ohio and is currently Assistant Clinical Professor of Pathology and Laboratory Medicine at The Ohio State University College of Medicine. Her research interests include the epidemiology of Clostridium difficile in pediatrics and development of diagnostic methods in the pediatric population.
Figure 1: Results of *C. difficile* testing using a multiplex molecular gastrointestinal panel versus physicians ordering pattern for *C. difficile* testing

A total of 782 stool samples were enrolled from June to September 2013 at Nationwide Children's Hospital as part of a clinical trial for FDA clearance of a multiplex gastrointestinal panel (BioFire FilmArray Gastrointestinal Panel). Samples were enrolled based on adequate volume of a stool sample submitted for physician/ordered routine stool culture in Cary-Blair transport media. Testing for *C. difficile* with the multiplex panel was compared to the number of physician orders for *C. difficile* PCR on the same sample. The FilmArray GI results were not known to the physicians. Of note: testing for *C. difficile* for patient care was not limited by age.

CDT, *Clostridium difficile* testing; MP, multiplex gastrointestinal panel; pos, positive; neg, negative.
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<td>Focus Diagnostics</td>
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<td>Simplexa</td>
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<tr>
<td>Great Basin</td>
<td>Helicase dependent amplification</td>
<td>Direct and Enriched toxigenic culture</td>
<td>Data stratified by age group, patients included &gt;2yrs</td>
</tr>
<tr>
<td>Portrait</td>
<td></td>
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</tr>
<tr>
<td>Nanosphere</td>
<td>qPCR combined with nanoparticle array hybridization</td>
<td>Direct and Enriched toxigenic culture</td>
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</tr>
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<td>Verigene</td>
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<td>qPCR/Fluorescent probe primers</td>
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<td>(Modaplex)</td>
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<td>Biofire GastroArray</td>
<td>Multiplex nested PCR</td>
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<td>GIT Panel</td>
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<tr>
<td>Assay</td>
<td>Study</td>
<td>Comparative Standard</td>
<td>Number of patient samples (age range) (% pediatric)</td>
</tr>
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<td>-----------------------------------------------------</td>
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<td>Toxigenic culture</td>
<td>200 (unavailable) (100%)</td>
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<td>Hart et al. (2014) (42)</td>
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<td>150 (11 days-17 yrs) (100%)</td>
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<td>Selvaraju et al. (2011) (41)</td>
<td>Toxigenic culture</td>
<td>200 (unavailable) (100%)</td>
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<tr>
<td>Cepheid GeneXpert</td>
<td>Leibowitz et al. (2014) (20)</td>
<td>Toxigenic culture</td>
<td>262 (1-18 yrs) (100%)</td>
</tr>
<tr>
<td>Meridian Illumigene</td>
<td>Ota and McGowan (2012) (40)</td>
<td>Cytotoxigenic culture or composite</td>
<td>141 (1-18 yrs) (100%)</td>
</tr>
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<td>xTAG GPP Luminex</td>
<td>Beckmann et al. (2014) (45)</td>
<td>Dual EIA and DNA-hybridization toxin assay</td>
<td>120 (6 days-21 yrs) (100%)</td>
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<td></td>
<td>Patel et al. (2014) (46)</td>
<td>Cepheid Xpert and 16S sequencing</td>
<td>211 (unavailable) (100%)</td>
</tr>
<tr>
<td>Biofire FilmArray Gastrointestinal Panel</td>
<td>Stockmann et al. (2015) (50)</td>
<td>Meridian illumigene</td>
<td>378 (1-25 yrs) (&gt;75%)</td>
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<td></td>
<td>Buss et al. (2015) (49)</td>
<td>tcdA and tcdB PCR</td>
<td>1556 (&lt;1 - &gt;65 yrs) (62%)</td>
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</table>

*Pediatric patients defined as those up to 21 years old; studies were either exclusively in a pediatric cohort or in a majority of pediatric samples.

Additional notes:
- Toxigenic culture defined by the authors as growth on cycloserine cefoxitin fructose agar (CCFA) or Cdiff Chromagar (BioMerieux, France).
- Confirmed with toxin gene PCR.
- Composite standard defined by the authors as a positive result for both Meridian illumigene and positive by another enzyme immunoassay.
C. diff Quick Check Complete kit (Alere GmBH, Kolm, Germany) and DNA-hybridization assay (Hain Lifescience GmBH, Nehren, Germany).

Additional FDA cleared assays have not been evaluated exclusively in pediatric patients. (Simplexa Focus, Portrait Great Basin, Verigene, IMDx Abbott m2000 and Primera ICEPlex).

PPV, positive predictive value; NPV, negative predictive value; NA, non-applicable
Table 1: FDA-cleared molecular assays for the detection of *C. difficile* and Available Pediatric Data from package inserts

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<td>Focus Diagnostics Simplexa</td>
<td>qPCR/Fluorescent probe primers</td>
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Table 2: Published performance characteristics of available FDA-cleared molecular assays in studies including pediatric patients

<table>
<thead>
<tr>
<th>Assay</th>
<th>Study</th>
<th>Comparative Standard</th>
<th>Number of patient samples (age range) (% pediatric)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
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<td>BD GeneOhm</td>
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<td>89.6</td>
<td>96.7</td>
<td>89.6</td>
<td>96.7</td>
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<td></td>
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<td>99</td>
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<td>100</td>
<td>93.4</td>
<td>82.8</td>
<td>100</td>
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<td>Cepheid</td>
<td>GeneXpert</td>
<td>Leibowitz et. al. (2014) (20)</td>
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<td>95</td>
<td>91</td>
<td>69</td>
<td>99</td>
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<tr>
<td>Meridian</td>
<td>Illumigene</td>
<td>Ota and McGowan (2012) (40)</td>
<td>Cytotoxigenic culture or composite</td>
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<td>758 (36 days-98 yrs) (61%)</td>
<td>96.1</td>
<td>99.8</td>
<td>99.2</td>
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