ORIGINAL ARTICLE

High Proportion of False-Positive *Clostridium difficile* Enzyme Immunoassays for Toxin A and B in Pediatric Patients

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OBJECTIVES. To determine the frequency of false-positive *Clostridium difficile* toxin enzyme immunoassay (EIA) results in hospitalized children and to examine potential reasons for this false positivity.

DESIGN. Nested case-control.

SETTING. Two tertiary care pediatric hospitals.

METHODS. As part of a natural history study, prospectively collected EIA-positive stools were cultured for toxigenic *C. difficile*, and characteristics of children with false-positive and true-positive EIA results were compared. EIA-positive/culture-negative samples were recultured after dilution and enrichment steps, were evaluated for presence of the *tcdB* gene by polymerase chain reaction (PCR), and were further cultured for *Clostridium sordellii*, a cause of false-positive EIA toxin assays.

RESULTS. Of 112 EIA-positive stools cultured, 72 grew toxigenic *C. difficile* and 40 did not, indicating a positive predictive value of 64% in this population. The estimated prevalence of *C. difficile* infection (CDI) in the study sites among children tested for this pathogen was 5%–7%. Children with false-positive EIA results were significantly younger than those with true-positive tests but did not differ in other characteristics. No false-positive specimens yielded *C. difficile* when cultured after enrichment or serial dilution, 1 specimen was positive for *tcdB* by PCR, and none grew *C. sordellii*.

CONCLUSIONS. Approximately one-third of EIA tests used to evaluate pediatric inpatients for CDI were falsely positive. This finding was likely due to the low prevalence of CDI in pediatric hospitals, which diminishes the test's positive predictive value. These data raise concerns about the use of EIA assays to diagnosis CDI in children.

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Clostridium difficile is the most common cause of healthcare-associated diarrhea in adults in developed countries.[1] Although *C. difficile* has generally been considered a less important pathogen in the pediatric age group, recent studies suggest that the incidence of *C. difficile* infection (CDI) has increased significantly among hospitalized children.[2-4] During the past decade, the emergence of an epidemic *C. difficile* strain, termed North American pulsed-field gel electrophoresis type 1 (NAP1), has been associated with significant increases in the incidence and severity of CDI among adults in North America and Europe.[1,5,6] In a study conducted in 2 tertiary care pediatric hospitals in 2006–2007, the NAP1 strain accounted for 19% of all isolates cultured, although additional factors other than the introduction of the NAP1 strain may explain the increasing incidence of pediatric CDI.[7]

These epidemiologic observations highlight the requirement for accurate *C. difficile* diagnostic tests in the pediatric age group. Enzyme immunoassays (EIAs) for toxin A and B are the most commonly used tests for the diagnosis of CDI in the United States because they are easy to use and provide rapid results.[8,9] In our prior study assessing the frequency of NAP1 CDI in children, only approximately two-thirds of EIA-positive stool samples yielded organisms by culture, which at the time was attributed to low specimen volume.[7] In a subsequent prospective natural history study of CDI in hospitalized children,[10] we found that a similar proportion of EIA-positive stool samples were culture negative despite an attempt to collect larger stool volume, suggesting a false-positivity rate in *C. difficile* EIA toxin assays that had not been previously appreciated. Here, we report the incidence of false-positive EIA test results in hospitalized children and compare the characteristics of those with positive EIA and negative culture results with those with both positive EIA and culture results. In addition, we explore potential reasons for the false-positive EIA assays in this population.

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METHODS

Study Setting and Design

The study was conducted in conjunction with a larger program evaluating the natural history of CDI in hospitalized children. Inpatients at 2 referral pediatric centers (Rainbow Babies and Children's Hospital, Cleveland, Ohio, and Children's Hospital of Philadelphia, Pennsylvania) were enrolled if a stool specimen sent at the discretion of the attending pediatrician for evaluation of diarrhea was positive for *C. difficile* toxin A or B by EIA. Subjects who were discovered after enrollment to have been asymptomatic ($n = 6$) were excluded from further study. Each hospital used the Premier Toxins A&B assay (Meridian Bioscience). Patients were enrolled only once. To estimate the prevalence of CDI at the study hospitals among children tested for this pathogen, the total numbers of EIA tests performed in each site during the study period and the percentages that were positive by EIA, both within and outside the context of the natural history study, were extracted from clinical microbiology records.

Clinical data, including age, antibiotic use, comorbid illnesses, previous hospitalizations, previous CDI, signs and symptoms of the current illness, and outcome, were collected prospectively. Differences between subjects whose stool was EIA positive/culture negative and those whose stool was EIA positive/culture positive were tested using the χ^2 test and the Wilcoxon 2-sample test for discriminate and continuous variables, respectively. The positive predictive value (PPV) of the EIA test was calculated by dividing the number of specimens that were true positive (ie, positive by both EIA and toxigenic culture, the latter designated as the gold standard) by the total number that were positive by EIA (reflecting both true-positive and false-positive tests). The study was approved by the institutional review boards of both participating hospitals, and written informed consent was obtained from all participants.

Culture of *C. difficile* and Examination of Potential Reasons for False-Positive EIA Assays

Stool remaining after completion of the EIA assay was stored at −70°C and batch processed for isolation of toxigenic *C. difficile*. Specimens were inoculated onto prereduced cycloserine-cefoxitin-brucella agar containing 0.1% taurocholic acid and lysozyme adjusted to 5 mg/L (CDBA-TAL).[11] The plates were incubated anaerobically for 48 hours at 37°C. Colonies were confirmed to be *C. difficile* on the basis of typical odor and appearance and by a positive reaction using *C. difficile* latex agglutination (Microgen Bioproducts). All isolates were tested for in vitro toxin production using *C. difficile* Tox A/B II (Wampole Laboratories).

Several additional assays were performed to investigate potential causes for false-positive EIA tests. In these experiments, because specimen volumes were sometimes depleted by repeated assays, the samples from the natural history study were supplemented by EIA-positive/culture-negative specimens collected as part of a quality assurance initiative at the Cleveland site. First, specimens that did not yield *C. difficile* after the initial attempt were further processed by including an overnight enrichment step in CDBA-TAL broth prior to plating. Second, unenriched samples were serially diluted prior to inoculation to remove putative inhibitors. Culture-negative samples were also tested for the presence of *C. difficile* using non-culture-based methods, specifically C. DIFF QUIK CHEK (TechLab) EIA for glutamate dehydrogenase (GDH) and the BD GeneOhm C diff assay (Becton Dickinson) for detection of toxin B gene (*tcdB*) by polymerase chain reaction (PCR). Because *Clostridium sordellii* has been shown to be a potential cause of false-positive EIA results,[12,13] a subset of the stool specimens with negative cultures were cultured for this organism. Stool specimens were inoculated in Brucella broth containing 20 μg/mL of aztreonam, incubated anaerobically for 48 hours, ethanol shocked (50% v/v) for 10 minutes, and plated onto prereduced tryptic soy agar blood plates and incubated for 24–96 hours; colonies with morphology consistent with *C. sordellii* were subjected to speciation using the RapID AnaII system (Remel).

RESULTS

Of the 112 stool specimens cultured as part of the natural history study, 72 grew toxigenic *C. difficile* (ie, true positives) and 40 had negative cultures (ie, false-positives) after direct inoculation onto selective agar. The PPV of the EIA in our study population therefore was 64%. During the course of the study, 4,007 total stool specimens were submitted for *C. difficile* EIA testing at the 2 hospitals, of which 286 (7.1%) were positive (127 of 1,272 [10.0%] at Rainbow Babies and Children's Hospital and 159 of 2,735 [5.8%] at Children's Hospital of Philadelphia). If adjusted for the EIA PPV calculated from the natural history study specimens, the true prevalence of CDI among children tested for this pathogen was approximately 5%.

Overall, subjects had diarrhea for a median of 2.5 days (interquartile range [IQR], 1.0–6.0 days) prior to submission of stool for *C. difficile* EIA, and 98 (88.3%) were prescribed therapy for *C. difficile* infection once the positive EIA test was reported. Table 1 compares the characteristics of the children, based on whether their stool was positive or negative for *C. difficile* by culture. Children with culture-negative stool samples were significantly younger than those whose specimens yielded *C. difficile*, but no other differences between the two groups were detected (Table 1).

Twenty-four of the 40 stool specimens with EIA-positive/culture-negative specimens had sufficient volume remaining to allow additional testing to determine potential reasons for this apparent false positivity. To render the investigations more robust, 16 additional EIA-positive/culture-negative pediatric specimens collected as part of a quality assessment initiative at the Cleveland site, but not from children enrolled

TABLE 1. Comparison of Enzyme-Linked Immunosorbent Assay (ELISA)–Positive/Culture-Negative versus ELISA-Positive/Culture-Positive Subjects

	ELISA+/culture− (N = 40)	ELISA+/culture+ (N = 72)	P
Age, years	0.53 (0.09–4.13)	2.86 (1.04–9.80)	.002
Duration of diarrhea prior to enzyme immunoassay, days	2.0 (0.0–4.0)	3.0 (1.0–7.0)	.41
Clostridium difficile positive, past year	5 (12.5)	8 (11.4)	1.0
Hospitalized, past year	19 (57.6)	51 (73.9)	.11
Intensive care unit, past 30 days	9 (23.1)	17 (23.6)	1.0
Antibiotics, past 30 days	21 (55.3)	51 (70.8)	.14
Diarrhea on admission	15 (38.4)	29 (42.0)	.84
Gastric acid blocker	13 (33.3)	36 (50.0)	.11
Comorbidity[a]	27 (67.5)	58 (80.5)	.17
White blood cell count, $\times 10^3/\mu L$[b]	10.7 (6.8–15.9)	11.1 (7.6–14.5)	.67
Blood urea nitrogen, mg/dL[b]	9.5 (6.0–13.0)	11.0 (7.0–15.0)	.68
Maximum temperature, °C[b]	37.4 (36.9–38.2)	37.2 (36.8–38.2)	.70

NOTE. All data are presented as N (%), except for age, duration of diarrhea prior to enzyme immunoassay, total white blood cell count, blood urea nitrogen, and maximum temperature, which are presented as median (interquartile range).

[a] Comorbidity is defined as 1 or more of the following: prematurity, surgery during past 30 days, parenteral nutrition, cystic fibrosis, inflammatory bowel disease, human immunodeficiency virus infection, renal dysfunction, malignancy, transplantation, or immunosuppression.

[b] Refers to values obtained on the day the ELISA-positive sample was submitted ±24 hours.

in the natural history study, were added. Of the 40 putative false-positive EIA specimens, none yielded *C. difficile* when cultured after broth enrichment or serial dilution. One specimen was positive for GDH antigen and *tcdB* by PCR, indicating that it likely contained *C. difficile* despite the inability to culture the organism. One additional specimen had a positive EIA for GDH but was *tcdB* PCR negative. Fourteen randomly selected specimens were cultured for *C. sordellii*, and all were negative.

DISCUSSION

Prior concerns surrounding *C. difficile* EIA assays have focused on their intrinsically suboptimal sensitivity, as low as 80% in some kits.[14] In children, the utility of EIA tests has been further obscured by a relatively high incidence of asymptomatic excretion of toxigenic *C. difficile*, especially in infants.[15-17] The current study is unique in its aim to systematically document and investigate issues of false-positive EIA tests in children. The high frequency of false-positive *C. difficile* EIAs has several important clinical implications. First, a positive *C. difficile* toxin assay in a hospitalized child mandates isolation, which consumes bed space and other resources during times of high census. Moreover, treatment of CDI in children with false-positive test results may lead to adverse drug reactions and in selection of resistant microorganisms, including vancomycin-resistant enterococci.[18] Finally, false-positive test results undermine the validity of local hospital CDI surveillance as well as the accuracy of health department and public reporting.

The factor that most likely accounted for the high proportion of false-positive EIAs in the current study was the low prevalence of CDI in the tested population. Most commercial *C. difficile* EIA toxin assays exhibit a specificity ranging between 93% and 98%.[14] In a recent systematic review, the median specificity of the Premier Toxins A&B assay, the one employed in the current study, was 97% (IQR, 95%–98%).[14] With test specificity at this level, PPV diminishes to unacceptable levels as the prevalence of the tested population decreases, since positive samples become disproportionately represented by those that are falsely positive. Indeed, arithmetically a test with a specificity of 97% applied to a population with a prevalence of 5%–7% will result in a PPV of approximately 60%–70%, similar to that derived empirically in our study. It was additionally noted in the course of the current study that some attending pediatricians retested stool samples for *C. difficile* toxin when the initial result was negative. This practice, now largely discouraged,[19-22] almost certainly exacerbated the problem of falsely positive EIA tests, since the stools that were initially negative for *C. difficile* likely represented a subset of children with an even lower prevalence than the total tested population, further driving down the PPV.

Additional potential causes of false-positive *C. difficile* EIA assays that have been identified by others did not appear to be operative in the current study. These include faulty EIA assay lots[23] and improper handling of specimens.[24] These explanations appear unlikely in the current study, given the similarity of the results from 2 different hospitals over many months. Other potential causes for the high frequency of false-positive *C. difficile* toxin assays are colonization by *C. sordellii*, which produces exotoxins that are antigenically similar to those of *C. difficile*,[12,13] and the presence of growth

inhibitors in the stool of children. These also appear unlikely, since the search for *C. sordellii* colonization yielded negative results and the dilution of our specimens, which should have decreased the concentration of putative inhibitors of *C. difficile* growth, did not increase yield.

Our study was subject to limitations. First, while we compared children with true- and false-positive EIA tests to attempt to establish clinical characteristics that could reliably identify patients who were truly positive, the study was underpowered to detect small but biologically important differences. Second, subjects were tested for CDI at the discretion of the attending pediatrician rather than after meeting uniform criteria for significant diarrhea illness. Thus, some subjects may have been colonized rather than infected with toxigenic *C. difficile*. All subjects were assessed to be experiencing new-onset diarrhea by seasoned pediatricians, however, and in the marked majority of instances, therapy specific for CDI was prescribed; hence, we believe that in almost all cases, the subjects were exhibiting clinically significant symptoms. Third, although we believe that the sum of our observations implicates the low prevalence of CDI in our study population as the principal cause of the EIA's poor PPV in children, the true prevalence in the study hospitals could not be ascertained, since EIA was used exclusively for diagnosis. Clearly, however, the true prevalence was low. Finally, it is possible that our inability to cultivate *C. difficile* was due to a low concentration of organisms in the stool of infected children. Our lab has been adept at culturing small numbers of *C. difficile* in other contexts, however, such as from the skin of adults recovering from colitis.[25] Moreover, it is unlikely that stool samples with numbers of organisms too low to detect by culture and PCR would contain sufficient amount of toxin to detect by EIA.

The findings of this study suggest that alternative testing strategies should be standard for identifying *C. difficile* infection in children. PCR detection kits for the *C. difficile* toxin B gene, now commercially available, possess superior sensitivity compared with EIA, but their specificity is not significantly different;[26] hence, difficulties with poor PPV when applied to populations with low prevalence are not solved by this assay. Moreover, the enhanced sensitivity of PCR-based kits may result in reduced specificity in the diagnosis of CDI, since some patients asymptomatically colonized by a low burden of bacteria that would have gone undetected by EIA may be misclassified as infected. We suggest 3 non-PCR-based testing strategies that increase the likelihood of true infection in the tested population. First, *C. difficile* testing should be restricted to children in whom suspicion of CDI is high. Among older children, testing should be confined to patients whose stool is sufficiently unformed that it assumes the shape of the specimen container, as has been recommended in adults.[20] Among infants, testing should be pursued only when the stool pattern is unequivocally and persistently abnormal. Second, repeated testing after an initial negative test should be discouraged. Finally, testing schemes that increase test specificity should be employed. A 2-step strategy, in which stool specimens are initially screened by the relatively nonspecific GDH assay, followed by the gold standard toxigenic culture or cell culture cytotoxicity assay, as has been recommended for adult specimens,[20,27] may be appropriate for pediatric populations as well. Testing strategies that embrace these measures in children, however, await empiric validation.

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