Diagnostic Microbiology and Infectious Disease 94 (2019) 109–112

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Bacteriology

GDH and toxin immunoassay for the diagnosis of *Clostridioides* (*Clostridium*) *difficile* infection is not a 'one size fit all' screening test

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ARTICLE INFO

Article history:
Received 25 October 2018
Received in revised form 22 December 2018
Accepted 22 December 2018
Available online 29 December 2018

Keywords:
Glutamate dehydrogenase
Algorithm
EIA
PCR
Immunocompromised

ABSTRACT

Diagnosing *Clostridioides* (*Clostridium*) *difficile* infection is challenged by lack of a clear gold standard. We sought to determine if the two-step algorithm (screening GDH and toxin lateral flow assay followed by *tcdB* PCR) would have adequate clinical performance at a tertiary care center. Of 486 patients, 310 (63.8%) were immunocompromised. Of 150 PCR-positive specimens, 52 (34.7%) were toxin-positive and 126 (84.0%) were GDH positive. Positive GDH or toxin results corresponded to lower PCR cycle threshold values ($P < 0.01$). PCR-positive patients had more frequently documented antibiotic usage (78.4% vs 66.9%, $P = 0.05$) and diarrhea (91.0% vs. 79.4%, $P < 0.01$) and less frequent alternate etiologies of diarrhea (27.3% vs. 41.1%, $P = 0.004$) or laxative use (24.6% vs 36.1%, $P = 0.02$). Toxin positivity was associated with antibiotic use ($P < 0.01$), but not with neutropenia, diarrhea, malignancy, or chemotherapy ($P > 0.05$). The application of the 2-step algorithm should be thoroughly evaluated in immunocompromised patient populations before implementation.

1. Background

Clostridioides (*Clostridium*) *difficile* infection (CDI) is a major cause of healthcare-associated infection in the United States, with almost half a million estimated infections and 29,000 deaths annually (Lessa et al., 2015). Yet, the optimal approach towards diagnosis of CDI is controversial with no clear diagnostic gold standard. The most appropriate diagnostic approach in immunocompromised patients is even less clear. Although they are at higher risk of CDI and CDI recurrence, toxin testing performs especially poorly in these patients (Chung et al., 2016; Erb et al., 2015; Mani et al., 2016). One recommended, general diagnostic approach is to use a two-step algorithm beginning with *C. difficile* toxin A/B and glutamate dehydrogenase (GDH) antigen immunoassays, followed by PCR detection of toxigenic *C. difficile* for discordant results (Sharp and Gilligan, 2010). The aim of this study was to understand if such an algorithmic approach could be reasonably applied to a population with a substantial number of immunocompromised patients.

2. Material and methods

From January 2016–March 2017, stool samples from two facilities, a tertiary care center for adult patients predominantly requiring surgical care (Keck Medical Center) and a cancer care center (Norris Cancer Hospital) in Los Angeles, CA were studied. Fresh specimens having been tested for *C. difficile* PCR (Cepheid Xpert *Cdiff*/NAP1) per routine clinical care were tested by a GDH/toxin combination lateral flow assay within the manufacturer's stated stability limits of the assay (C. diff Quik Chek Complete, Alere, Waltham, MA). Cycle threshold values for the Xpert PCR *tcdB* positive results and presumptive positive results of NAP1 PCR (detection of both *tcdC*Δ117 and binary toxin (CDT) gene) were retrospectively recorded. Only stools with consistency of Bristol scale 6 or 7 and one stool per patient in a 7-day period were acceptable for routine clinical testing. Only the first positive and first negative *C. difficile* PCR result for a patient during the study period were included, with exclusion of any subsequent duplicate cases over the entire study period. Consecutive cases were enrolled until >300 PCR-negative specimens were reached (January – August 2016), and thereafter positive specimens were continued in enrollment until 150 positive cases meeting inclusion criteria were reached (March 2017). Medical charts were reviewed for patient data including demographic data, clinical presentation, antibiotic usage, history of solid tumor or hematologic malignancy, laxative use in past 24 h, and immune status including chemotherapy use, transplant history, and absolute neutropenia (absolute neutrophil count <500/μL).

Exposure to antibiotics was defined as any antibiotics within the 6 months prior to *C. difficile* testing. High-risk antibiotic exposure was noted separately and defined as exposure to cefepime, ceftazidime, carbapenems, fluoroquinolones, or clindamycin within the 6 months prior to *C. difficile* testing (Brown et al., 2013; Slimings and Riley,

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https://doi.org/10.1016/j.diagmicrobio.2018.12.010
0732-8893/

2014). Patients who lacked medical records for that time period were omitted from this specific analysis. Active chemotherapy and immunosuppression were defined as receipt of such agents in the 7 days prior to *C. difficile* testing. Laxatives included stool softeners and stimulant, osmotic, or bulking agents.

Statistical analysis for dichotomous data was performed using the Fisher exact test. We used the Student t test for comparisons of continuous outcomes with normal distributions and the Mann–Whitney U test for non-parametric continuous outcomes. All tests were two-tailed with an alpha level of 0.05 being considered as statistically significant. To examine independence of factors associated with PCR positivity more closely, we performed multivariate analyses using Cox proportional hazards regression based on variables from univariate analysis with $P < 0.10$ which included documented diarrhea, high risk antibiotic usage, antibiotic usage in past 6 months, and laxative use. We also performed the same multivariate analysis to examine variables associated with EIA toxin positivity within the PCR-positive data set. Variables included age, documented diarrhea, history of hematologic malignancy, high risk antibiotic usage, antibiotic usage in past 6 months, recent chemotherapy use, laxative use, and NAP1 PCR status. Statistical analysis was performed using R version 3.2.2. This study protocol was approved by the institutional review board of the University of Southern California.

3. Results

Of 545 total cases, 59 duplicates were excluded for a total of 486 cases. The median age of study subjects was 58 y (range 18–88 y). There were 138 outpatients (28.4%) and 348 inpatients (71.6%). The population tested included patients with a history of transplant (20.6%), malignancy (40.7%), active chemotherapy (19.8%), active immunosuppression (35.4%), and decompensated cirrhosis (3.7%), where 319 (65.6%) patients had at least one of these immunocompromising conditions. Three-hundred thirty-six cases were *C. difficile* PCR-negative and 150 were PCR-positive. Among PCR-negative samples, 14 of 336 (4.2%) were GDH-positive; none were toxin EIA-positive. Of the 150 PCR-positive specimens, 127 (84.7% (95% confidence interval (CI) 78.0–89.6%)) were GDH-positive, and 52 (34.7% (95% CI 27.7–42.6%)) were toxin EIA-positive. One PCR-positive sample was GDH-negative, toxin-positive. Twenty-three (15.3%) were NAP1 PCR-positive.

3.1. Patient characteristics based on PCR status

Antibiotic usage was documented in 78.4% of PCR-positive patients and 66.9% of PCR-negative patients ($P = 0.05$), although documented use of high-risk antibiotics was less frequent in PCR-positive than PCR-negative cases (56.2% vs. 68.3%, $P = 0.03$; Table 1). Documented diarrhea prior to testing was more frequent in PCR-positive than PCR-negative cases (91.0% vs. 79.4%, $P < 0.01$). Probable etiologies besides CDI for the patient's diarrhea were charted in 27.3% of PCR-positive cases vs. 41.1% of PCR-negative cases ($P = 0.004$). The most documented alternate reasons for diarrhea were medications (e.g., chemotherapeutic agents, non-CDI antibiotic-associated diarrhea, magnesium oxide) and inflammatory bowel disease. Recent laxative use occurred in 24.6% (33/134) of PCR-positive patients and 36.1% (113/313) of PCR-negative patients ($P = 0.02$). No statistically significant differences were noted between PCR-positive and -negative cases with regard to age, gender, history of malignancy or transplant, and recent receipt of chemotherapy or immunosuppressant agent. On multivariate analysis, only diarrhea was found to be independently associated with PCR positivity ($P = 0.008$), but not high risk antibiotic usage, any antibiotic usage in past 6 months, or laxative use. PCR-positive specimens were not statistically more likely to be GDH- or toxin-positive in hospitalized patients (100/114 [87.7%] and 43/114 [37.7%]) than outpatients (27/36 [75.0%] and 9/36 [25.0%]) ($P = 0.11$ and $P = 0.23$, respectively).

Table 1
Comparison of clinical data parameters between *C. difficile* PCR-positive and –negative cases.

	C. difficile PCR-positive	*C. difficile* PCR-negative	*P* value
n	150	336	NA
Mean age (SD), y	56.6 (17.1)	55.1 (16.0)	0.33
Male (%)	89 (59.3)	175 (52.1)	0.14
GDH-positive	127 (84.7%)	14 (4.2%)	NC
Toxin EIA-positive	52 (34.7%)	0	NC
Laxative use within 24 h	33/134 (24.6%)[a]	113/313 (36.1%)[a]	0.02
Absolute neutrophil count <500/μL	15 (10.0%)	24 (7.1%)	0.28
Documented diarrhea	111/122 (91.0%)[a]	162/204 (79.4%)[a]	<0.01
High risk antibiotic use	68/121 (56.2%)[a]	153/224 (68.3%)[a]	0.03
Any antiobiotic usage	87/111 (78.4%)[*]	121/181 (66.9%)	0.05
Any immunocompromised state	99 (66.0%)	220 (65.5%)	1.00
Hematologic malignancy	25 (16.7%)	53 (15.8%)	0.79
Solid tumor malignancy	39 (26.0%)	80 (23.8%)	0.65
History of transplant	34 (22.7%)	66 (19.6%)	0.47
Immunosuppressant agents	61 (40.7%)	114/334 (34.1%)	0.18
Chemotherapy usage	24 (16.0%)	72/335 (21.5%)	0.18

NA, not applicable; NC, not calculated.
[a] Cases without specific documentation were excluded from analysis.

3.2. PCR positive cases

Among PCR-positive cases, patients with hematologic malignancy were less likely to be toxin EIA-positive than other patients (7.7% vs. 38.4%, $P = 0.04$) on univariate analysis (Table 2). Patients who were toxin-positive were older on average than toxin-negative patients (mean 60.8 vs. 54.4 y, $P = 0.03$). Prior antibiotic usage was more frequent in toxin-positive than toxin-negative cases (92.7 vs. 70.0%, $P < 0.01$). EIA toxin results were not associated with neutropenia, solid tumor malignancy, or high risk antibiotic usage ($P > 0.05$). EIA toxin-positive cases demonstrated a negative trend towards association with recent chemotherapy ($P = 0.06$) and a positive trend towards association with recent laxative use ($P = 0.07$). On multivariate analysis, only NAP1 PCR positivity and prior antibiotic usage remained significant factors predicting toxin production ($P = 0.01$ and $P = 0.03$ respectively).

3.3. C. difficile PCR cycle thresholds (Ct)

GDH-positive specimens had lower *tcdB* PCR Ct values than GDH-negative ones (median 26.4 vs. 33.1 cycles, $P < 0.01$), but did not differ in frequency of being NAP1 PCR-positive ($P = 0.53$; Table 3). Toxin EIA-positive specimens had lower *tcdB* PCR Ct values than toxin-negative specimens, (median 24.1 vs. 30.5 cycles, $P < 0.01$) and were

Table 2
Comparison of clinical characteristics of cases positive and negative for toxigenic *C. difficile*-positive by EIA. All cases tested positive for toxigenic *C. difficile* by PCR.

	Toxin EIA-positive	Toxin EIA-negative	*P* value
n	52	98	NA
Outpatient status	9 (17.3%)	27 (27.6%)	0.23
Mean age (SD)	60.8 (15.1) y	54.4 (17.8) y	0.03
NAP1 PCR-positive	16 (30.8%)	7 (7.1%)	<0.01
Laxative use within 24 h	14/47 (29.8%)[a]	12/80 (15.0%)[a]	0.07
Absolute neutrophil count <500/μL	5 (9.6%)	10 (10.2%)	1.00
Documented diarrhea	30/42 (71.4%)[a]	72/81 (88.9%)[a]	0.02
High risk antibiotic use	25/39 (64.1%)[a]	43/82 (52.4%)[a]	0.25
Any antiobiotic usage	38/41 (92.7%)[a]	49/70 (70.0%)[a]	<0.01
Any immunocompromised state	31 (59.6%)	68 (69.4%)	0.28
Hematologic malignancy	4 (7.7%)	21(21.4%)	0.04
Solid tumor malignancy	12 (23.1%)	26 (26.5%)	0.70
History of transplant	11 (21.2%)	23 (23.5%)	0.84
Immunosuppressant agents	19 (36.5%)	42 (42.9%)	0.49
Chemotherapy usage	4 (7.7%)	20 (20.4%)	0.06

NA, not applicable.
[a] Cases without specific documentation were excluded from analysis.

Table 3
Comparison of PCR crossing threshold (Ct) values between GDH-positive and –negative results, toxin-positive and –negative results, and NAP1-positive and –negative results.

	n	median *tcdB* Ct	*P* value	No. (%) NAP1	*P* value
PCR+/GDH-	23	33.1	<0.01	2 (8.7)	0.53
PCR+/GDH+	127	26.35		21 (16.5)	
PCR+/Toxin-	98	30.5	<0.01	7 (7.1)	<0.01
PCR+/Toxin+	52	25.15		16 (30.8)	
PCR NAP1+	23	26.1	0.05	na	
PCR NAP1-	127	28.2		na	
All PCR+	150	27.7		23 (15.3)	

more likely to be NAP1 PCR-positive (31.4% vs. 10.4%, $P < 0.05$). NAP1 PCR-positive specimens had overall lower *tcdB* Ct values than non-NAP1 PCR-positive specimens (median 26.1 vs. 28.2 cycles, $P = 0.05$) and were independently associated with toxin positivity by EIA on multivariate analysis ($P < 0.01$).

3.4. PCR-positive, GDH-negative, toxin-negative cases

We further reviewed the 22 cases in which the 2-step algorithm would have missed PCR-positive specimens. All 22 patients were treated for CDI, including the 9 outpatients. By comparison, among PCR-positive/GDH-positive cases (n = 127), 10 were not treated for CDI, including 2 that were toxin EIA-positive. There was documented diarrhea in 16 of 18 (88.9%) patients (4 patients excluded due to lack of documentation or receipt of bowel preparation 24 h before specimen collection). PCR-positive/GDH-negative cases did not differ significantly from PCR-positive/GDH-positive cases in terms of frequency of neutropenia, recent use of immunosuppressants or chemotherapeutic agents, history of solid tumor or hematologic malignancy, laxative use, or antibiotic usage (Table 4). Usage of antibiotics, immunosuppressive agents, or chemotherapeutic agents was noted in 21 of 22 cases. Six of the 22 cases, five of which were outpatients, had colonoscopic exam and biopsies taken at the time of symptoms. None demonstrated pseudomembranous colitis, two showed only focal colonic erythema or edema, one demonstrated terminal ileitis in a patient with Crohn's disease, one demonstrated proctitis in a patient with ulcerative colitis, and two were negative for significant findings.

4. Discussion

As clinical laboratories are challenged with instituting laboratory stewardship practices to help control hospital CDI rates, the use of nucleic acid amplification tests (NAAT), which tend to have higher positivity rates than other CDI testing modalities (Kamboj et al., 2018), is being reconsidered. Some studies caution that NAATs are overly sensitive and that toxin positivity is a key indicator of clinically significant CDI (Longtin et al., 2013). Rapid tests that combine GDH and toxin EIA in a lateral flow format have become an increasingly plausible alternative. Used to exclude negative specimens, these combination assays can cut down on NAAT costs while also providing a toxin EIA result.

Despite these attributes, consideration must be taken before adopting such an algorithm for CDI testing. While some have found GDH to perform exceptionally well (Chung and Lee, 2017; Quinn et al., 2010), we and others found that a significant proportion of NAAT-positive specimens would be missed (Gomez et al., 2018; Grein et al., 2014; Novak-Weekley et al., 2010). In our study in which nearly two-thirds of tested patients were immunocompromised, the GDH component was only ~85% sensitive if applied as a screening assay for *C. difficile*. Clinical review of the GDH-negative, PCR-positive cases showed that these patients were symptomatic with diarrhea, had risk factors for CDI, and were all treated for CDI. The lack of significant pathology on the 6 cases with colonoscopic examination, however, suggests a lesser severity of disease in this group. Yet it would be difficult to institute an algorithm which would have missed detection of *C. difficile* in these symptomatic cases. Variability in GDH performance may depend on the local prevalence of *C. difficile* strains (Johansson et al., 2016; Tenover et al., 2010), as well as analytical sensitivity differences with NAAT. As corroborated by others, we found significantly higher PCR Ct values in samples that were GDH-negative (Chung and Lee, 2017; Dionne et al., 2013). GDH status was not related to the NAP1 strain in our study.

The toxin component of the rapid combination assay had only 35% sensitivity compared to *C. difficile* PCR which, while low, is within the range described by others (Gomez et al., 2018; Larson et al., 2010; Lee et al., 2014). This level of sensitivity is undesirable in a screening assay and false negative results have the potential to mislead providers. Toxin EIA is known to be less analytically sensitive than other methods of toxin detection such as the cytotoxin neutralization assay (Planche et al., 2008). Toxin results may be susceptible to strain prevalence, for example toxin hyperproducing NAP1 strains being more often associated with toxin-positive results (Gomez et al., 2018). The analytical sensitivity is also evident by the significantly lower organism load (higher *tcdB* Ct values) of toxin-negative than toxin-positive samples as found here and elsewhere (Dionne et al., 2013; Ota and Mcgowan, 2012). Although *tcdB* Ct values on the Xpert *Cdiff*/NAP1 PCR test are neither calibrated for quantification purposes nor intended for clinical reporting, one group was able to demonstrate acceptable lot-to-lot reproducibility among other performance characteristics (Senchyna et al., 2017).

Prior studies have noted decreased detection of toxin in *C. difficile*-positive specimens to be independently associated with immunocompromised states such as high-dose corticosteroid use and leukopenia. The cause of low toxin positivity rate in immunocompromised groups is unclear, but others have conjectured that the pathophysiology of CDI is altered in a weakened immune system and in microbiome disruptions from immunosuppressive or chemotherapeutic agents (Erb et al., 2015; Manian et al., 2007). Although we found hematologic malignancy, older age, and antibiotic usage in the prior 6 months to be associated with positive toxin EIA results on univariate analysis, of these, only antibiotic usage remained independently associated upon multivariate analysis. While this could have been biased by lack of medical record documentation in a minority of patients, our data suggest that toxin positivity was more likely after antibiotic exposure.

While the merits of NAAT testing have been called into question, consensus has converged on the real need to enforce judicious use of CDI testing, e.g., limiting testing to diarrheal samples, rejecting repeat test requests, screening for laxative use, etc. (McDonald et al., 2018). At our center, stool consistency and testing frequency were routinely screened; however, there was no systematic process of monitoring laxative use. Laxative use remained prevalent among tested patients.

Table 4
Comparison of clinical characteristics of GDH-positive and GDH-negative cases among those that were PCR-positive.

	PCR+/GDH+	PCR+/GDH-	*P*-value
n	127	23	NA
Mean age (SD)	57.1 (16.9)	53.9 (18.2)	0.41
Laxative use within 24 h	24/107 (22.4%)[a]	2/20 (10.0%)[a]	0.36
Absolute neutrophil count <500/μL	14 (11.0%)	1 (4.3%)	0.47
Documented diarrhea	94/103 (91.3%)[a]	17/19 (89.5%)[a]	0.68
High risk antibiotic use	59/104 (56.7%)[a]	9/17 (52.9%)[a]	0.80
Any antiobiotic usage	76/95 (80.0%)[a]	11/16 (68.8%)[a]	0.33
Any immunocompromised state	85 (66.9%)	14 (60.9%)	0.63
Hematologic malignancy	22 (17.3%)	3 (13.0%)	0.77
Solid tumor malignancy	33 (26.0%)	6 (26.1%)	1.00
History of transplant	33 (26.0%)	1 (4.3%)	0.03
Immunosuppressant agents	52 (40.9%)	9 (39.1%)	1.00
Chemotherapy usage	19 (15.0%)	5 (21.7%)	0.54

NA, not applicable.
[a] Cases without specific documentation were excluded from analysis.

Still, in PCR-positive cases, laxative use was a significantly less frequent occurrence, and recent prior antibiotic use a more frequent occurrence, than in PCR-negative cases. Likely there was lower clinical suspicion for CDI in PCR-negative cases, for which alternative causes for diarrheal illness were noted more often at the time of CDI testing. The higher rate of documentation of diarrhea in PCR-positive cases may reflect an actual higher rate of diarrheal episodes, increased severity of diarrhea prompting documentation, or other factors that were difficult to evaluate retrospectively. Regardless, these findings reflect the general clinical relevance of positive PCR results in our healthcare setting, and are similar to those described in studies involving immunocompromised patients (Erb et al., 2015; Kaltsas et al., 2012). All in all, the cause of diarrheal illness in a complicated patient may be multifactorial and the relative contribution of *C. difficile* could remain uncertain despite testing results. For a patient at risk for CDI, reliance on toxin test results alone for clinical decision making can lead to poor clinical outcomes, therefore test results must be subject to careful interpretation (Larson et al., 2010; Origüen et al., 2018).

As this was a retrospective study, some clinical data points were not captured in the course of clinical care, and case–controlled analysis was not performed. The enrollment periods for positive and negative specimens overlapped but was extended by 6 months for positive specimens, thus introducing potential bias in temporal exposures to infectious pathogens and antibiotics between groups. Our patient population was a mix of immunocompromised and predominantly surgical adult patients at a tertiary care center in the U.S., thus results may not apply to all other healthcare settings. The Xpert NAP1 PCR studies are considered presumptive results by the manufacturer, although studies have found high correlation with a variety of other molecular typing methods (Babady et al., 2010; McMillen et al., 2016).

NAAT remains a cornerstone in CDI testing for our patient population, of whom nearly two-thirds were immunocompromised. Additional toxin testing to stratify risk of disease progression may be useful clinically, but we feel that this should not be done at the exclusion of NAAT. For infection prevention purposes, symptomatic patients who harbor PCR-positive, toxigenic *C. difficile* but are toxin-negative are still important sources of CDI transmission (Mawer et al., 2017). With controlled criteria for laboratory CDI testing, rapid and sensitive NAAT serves to identify patients who either have CDI or are at increased risk for developing CDI. We suggest that the application of the 2-step algorithm be thoroughly evaluated before being widely implemented. Further studies of immunocompromised patients and significance of *C. difficile* GDH/toxin as compared to PCR positivity may elucidate optimal test practices among this specific population.

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