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### Performing Reflexive Toxin A/B Enzyme Immunoassay in a Two-Step Algorithm for the Diagnosis of *Clostridium difficile* Infection Has Limited Clinical Utility

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## 1 ABSTRACT

2 The differentiation of *Clostridium difficile* infection (CDI) from colonization is challenged by the  
3 suboptimal clinical specificity of nucleic acid amplification tests (NAAT). In this study, we  
4 examined the utility of testing for toxin via enzyme immunoassay (EIA) in specimens already  
5 tested by NAAT for the diagnosis of CDI in an attempt to differentiate colonization from infection.  
6 We tested 59 stool samples for the presence of *C. difficile* toxin B gene by NAAT followed by  
7 EIAs for glutamate dehydrogenase (GDH EIA) and toxins A and B (Toxin EIA). Two infectious  
8 disease physicians independently reviewed the patients' electronic medical records  
9 retrospectively to categorize each patient as CDI-Likely, CDI-Unlikely, or CDI-Indeterminate.  
10 Clinical sensitivities and specificities were calculated using 3 definitions of "true" CDI status,  
11 being: (1) concordance between both reviewers, (2) concordance, and CDI-  
12 Indeterminate/discordant cases classified as CDI-Likely, and (3) concordance, and CDI-  
13 Indeterminate/discordant cases classified as CDI-Unlikely. Based on these definitions, clinical  
14 sensitivity and specificity for NAAT was 100% and 49-94%, GDH EIA was 83-85% and 43-89%,  
15 and Toxin EIA was 39-42% and 83-100%, respectively. 85% (22 of 26) of patients who were  
16 NAAT-positive but Toxin EIA-negative symptomatically benefited from treatment for CDI. The  
17 addition of EIA to NAAT for CDI diagnosis had limited utility for differentiating colonization from  
18 CDI and could have led to under treatment of patients with CDI.  
19

## 20 INTRODUCTION

21 *Clostridioides* (formerly *Clostridium*) *difficile* is the most commonly recognized cause of  
22 infectious diarrhea in healthcare settings, and is the most commonly reported pathogen among  
23 healthcare-associated infections of adults in the United States (1). However, asymptomatic  
24 colonization has been found in acute care hospitals at an incidence of 3%-26% in adult inpatients  
25 (2-4). Differentiating patients who are colonized from those who have active CDI remains a  
26 diagnostic challenge. While NAAT has a high clinical sensitivity, its clinical specificity is

27 suboptimal for diagnosing patients who have CDI(5). An array of algorithms and protocols have  
28 been created to improve diagnostic accuracy of CDI.

29 The 2017 Infectious Disease Society of America (IDSA) and Society for Healthcare  
30 Epidemiology of America (SHEA) guidelines for *C. difficile* recommend the use of a stool toxin  
31 test as a part of a multistep algorithm (i.e. GDH plus toxin; GDH plus toxin, arbitrated by NAAT; or  
32 NAAT plus toxin) at institutions without a formal process to submit stool specimens from only  
33 patients with unexplained new onset diarrhea with 3 or more unformed stools in a 24 hour period.  
34 At institutions that do have a policy to only submit stool for *C. difficile* testing in patients with 3 or  
35 more unformed stools in a 24-hour period, testing by NAAT alone is provided as an alternative  
36 diagnostic option (6).

37 Accurate diagnosis of CDI is essential. A false positive test result would lead to unnecessary  
38 antibiotic exposure and increases in costs of care, while missing a diagnosis of CDI would likely  
39 lead to increased morbidity and mortality. We undertook this study to evaluate the impact of  
40 adding toxin testing to NAAT to differentiate colonization from CDI (7).

41

## 42 METHODS

43 This study was performed at a 500-bed, tertiary care, academic medical center in the  
44 Midwestern United States. As part of routine diagnostic workup for CDI, unformed stool  
45 specimens were submitted to the clinical microbiology laboratory to detect the presence of *C.*  
46 *difficile* toxin B gene via the Xpert *C. difficile*/Epi NAAT (Cepheid, Sunnyvale, CA). For this study,  
47 unformed stool samples, nonselectively chosen based on investigator availability from January to  
48 May 2017, were subsequently tested with the TECHLAB C. DIFF QUIK CHEK COMPLETE EIA  
49 (TECHLAB, Blacksburg, VA) according to manufacturer instructions to evaluate for the presence  
50 of GDH, and toxins A and B.

51 Two infectious disease physicians independently reviewed electronic medical records of the  
52 corresponding patients. Reviewers were blinded to the EIA results and to each other's

53 assessment. Blinding for NAAT results was not possible since the results could be readily seen  
54 within the patients' electronic medical records. The following criteria adapted and modified from  
55 the 2010 IDSA guidelines were utilized by the reviewers in assessing disease status: presence of  
56 diarrhea defined as 3 or more unformed stools within a 24-hour period, antibiotic use within 90  
57 days of symptom onset, prior history of CDI, improvement of symptoms with CDI treatment, and  
58 colonoscopy findings, if performed (8, 9). Since there is no gold standard for diagnosing CDI,  
59 each reviewer used the constellation of above factors, along with their clinical judgment to group  
60 patients into one of three categories: (1) CDI-Likely, (2) CDI-Unlikely, and (3) CDI-Indeterminate.  
61 Clinical sensitivity and specificity were calculated using 3 definitions of "true" CDI status: [1] both  
62 reviewers were concordant in assigning CDI-Likely or CDI-Unlikely to a patient (CDI-  
63 Indeterminate results were excluded in this calculation); [2] both reviewers were concordant in  
64 assigning CDI-Likely or CDI-Unlikely, and remaining patients (CDI-Indeterminate and discordant  
65 categorization) were included in the CDI-Likely group; and [3] both reviewers were concordant in  
66 assigning CDI-Likely or CDI-Unlikely, and remaining patients (CDI-Indeterminate and discordant  
67 categorization) were included in the CDI-Unlikely group.

68

69 **RESULTS**

70 59 patients were included in this study. Independent categorization of CDI status by two  
71 infectious disease specialists is shown in **Table 1**. Overall percent agreement between the  
72 reviewers was 78% (46 of 59 concordant), and Kappa was 0.64 (95% confidence interval 0.47-  
73 0.80). Notably, Reviewer 1 categorized more patients as CDI-Likely (n=35) than Reviewer 2  
74 (n=26), while Reviewer 2 categorized more patients as CDI-Indeterminate (n=12) than Reviewer  
75 1 (n=4). Both reviewers had a similar number of CDI-Unlikely patients (n=20 and n=21). Patient  
76 age and gender were similarly distributed among each CDI status group; median age was 60  
77 years (ranging 1 to 97 years) and there were 58% (n=34) males (**Table 2**). NAAT was positive in  
78 71% (42 of 59) of patients, GDH EIA in 66% (37 of 56), and Toxin EIA in 27% (16 of 59); the

79 results of 3 GDH EIA tests could not be retrieved (**Table 3**). Of CDI-Likely patients, 58% (14 of  
80 24) were Toxin EIA-negative. Of NAAT-positive specimens, 62% (26 of 42) were Toxin EIA-  
81 negative, and 85% (22 of 26) of these patients symptomatically benefited from treatment for CDI.  
82 There were no (0%) instances where GDH EIA and/or Toxin EIA was positive and NAAT was  
83 negative. Using 3 different definitions of “true” CDI status, clinical sensitivity and specificity for  
84 NAAT was 100% and 49-94%, GDH EIA was 83-85% and 43-89%, and Toxin EIA was 39-42%  
85 and 83-100%, respectively (**Table 4**).

86

## 87 **DISCUSSION**

88 The high sensitivity of NAAT in diagnosing CDI has been well documented (5). This attribute  
89 has been instrumental in directing CDI treatment and avoiding progression of disease for patients  
90 with true CDI. However, it is also recognized that molecular tests may lead to overdiagnosis of  
91 CDI due to suboptimal clinical specificity and result in increases in both direct and indirect costs  
92 related to unnecessary treatment and isolation precautions, overutilization of infection prevention  
93 and control resources, and reporting of inflated CDI rates (10-13). Furthermore, CDI treatment  
94 with antibiotics has been reported to increase the risk of vancomycin-resistant enterococci (14,  
95 15). Because of this, supplementation of NAAT with Toxin EIA as part of a 2-step testing  
96 algorithm has been proposed by IDSA/SHEA and European Society of Clinical Microbiology and  
97 Infectious Diseases guidelines (6, 16). Prior studies have reported that performing both NAAT  
98 and Toxin EIA may provide clinically useful information. For instance, patients with diarrhea who  
99 are NAAT-positive but Toxin EIA-negative may not develop adverse outcomes, even without  
100 specific therapy for CDI (17). Also, recurrence of CDI may be more common when both NAAT  
101 and toxin assays are positive than when NAAT alone is positive (17). And, complications are  
102 more common among patients who are positive by both NAAT and a 3-step algorithm – including  
103 GDH, toxins A and B, and cell culture cytotoxicity assay – than when NAAT alone is positive (18).

104        Nevertheless, others have found that addition of Toxin EIA to NAAT does not reliably  
105        distinguish patients with CDI from those who are colonized. Rios et al. found that 75% of their  
106        NAAT-positive specimens were Toxin EIA-negative, and that Toxin EIA-negative results were  
107        seen just as frequently in CDI as in colonized patients with positive NAAT results (19). We  
108        similarly found that most (62%) NAAT-positive specimens were Toxin-EIA-negative, and most  
109        (85%) of these patients still benefited symptomatically from CDI treatment. Toxin-EIA results were  
110        not contributory to the diagnosis of CDI, because of CDI-Likely patients, Toxin-EIA was negative  
111        in 58%. Consistent with this, informal polling (data not shown) of healthcare providers at our  
112        institution revealed that virtually all would treat for CDI if it was in the differential diagnosis and the  
113        patient had a positive NAAT result, regardless of the Toxin-EIA result.

114        Because there is currently no gold standard for the diagnosis of CDI, assay performance is  
115        particularly challenging to assess. When defining “true positives” and “true negatives” as  
116        concordance between both reviewers in categorizing patients as CDI-Likely and CDI-Unlikely,  
117        respectively, NAAT performed well (clinical sensitivity 100% and specificity 94%). However, when  
118        applying this definition, which uses only the most clinically apparent CDI patients, Toxin EIA had  
119        low clinical sensitivity (42%). When including CDI-Indeterminate patients and patients that had  
120        discordant categorization into the “true positives”, the clinical sensitivities and specificities of  
121        NAAT and Toxin EIA were essentially unchanged; however, when including CDI-  
122        Indeterminate/discordant patients as “true negatives”, clinical specificity of NAAT dropped  
123        markedly (94% to 49%), and it also decreased for Toxin EIA (100% to 83%). Regardless of how  
124        “true” CDI status was defined, the clinical sensitivity of Toxin EIA (range of 39-42%) clearly  
125        demonstrated a need for improvement, which was consistent with the findings of others (15, 19,  
126        20).

127        This study has several limitations. The lack of diagnostic gold standard for CDI resulted in  
128        variability between the two reviewers when categorizing patients into the three different CDI  
129        status groups. Upon unblinding, the discordant categorizations were thought to be attributable to

130 differences in interpretation of what constituted clinical improvement with treatment for CDI, and  
131 lack of agreement as to whether or not the presence of a rectal tube was indicative of a patient  
132 having 3 or more loose stools per day. Furthermore, due to the retrospective nature of this study,  
133 it was challenging to obtain complete information from the medical records; in particular,  
134 frequency of diarrhea, character of stools, and duration of symptoms prior to hospital presentation  
135 were difficult to ascertain for some patients. Another limitation of this study was that specimen  
136 selection was not completely randomized and was more dependent on when the investigator  
137 performing EIA testing was available. Our sample set is likely enriched with NAAT-positive  
138 patients; thus, the calculated clinical sensitivities and specificities may not be representative of an  
139 unbiased population.

140 In summary, the diagnosis of CDI remains challenging, but the addition of Toxin EIA testing  
141 to NAAT-positive specimens as part of a two-step diagnostic algorithm would provide minimal  
142 clinical benefit. At our institution, we have implemented multiple layers of checks to optimize *C.*  
143 *difficile* NAAT utilization, from decision support during ordering to rejection of formed stools during  
144 laboratory receipt, and in this setting, NAAT testing alone is currently the best solution for our  
145 healthcare system.

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**Table 1.** Retrospective categorization of patients with diarrhea as CDI-Likely, CDI-Unlikely, or CDI-Indeterminate based on clinical parameters by two infectious disease physicians. Percent agreement 78%, Kappa = 0.64 (95% confidence interval: 0.47-0.80).

		Reviewer 1			
	CDI Category	CDI-Likely	CDI-Unlikely	CDI-Indeterminate	Total
Reviewer 2	CDI-Likely	24	2	0	26
	CDI-Unlikely	3	18	0	21
	CDI-Indeterminate	8	0	4	12
	Total	35	20	4	59

**Table 2.** Age and gender of study patients and distribution by CDI category.

	CDI Category			Overall (n=59)
	CDI-Likely (n=24)	CDI-Unlikely (n=18)	CDI-Indeterminate or Discordant (n=17)	
Number Male (%)	13 (54.2)	11 (61.1)	10 (58.8)	34 (57.6)
Number Female (%)	11 (45.8)	7 (38.9)	7 (41.2)	25 (42.4)
Median Age (std dev) in Years	62.5 (19.8)	61.0 (21.0)	60.0 (21.9)	61.0 (20.5)

**Table 3:** NAAT, GDH EIA, and Toxin EIA results for patients with diarrhea distributed by CDI category.

CDI Category	NAAT			GDH EIA				Toxin EIA		
	Positive	Negative	Total	Positive	Negative	Unknown	Total	Positive	Negative	Total
CDI-Likely	24	0	24	20	4	0	24	10	14	24
CDI-Unlikely	1	17	18	2	13	3*	18	0	18	18
CDI-Indeterminate or Discordant	17	0	17	15	2	0	17	6	11	17
Total	42	17	59	37	19	3	59	16	43	59

\* GDH EIA results for 3 patients were unable to be retrieved.

**Table 4.** Clinical sensitivity and clinical specificity of NAAT, GDH EIA, and Toxin EIA calculated using 3 different definitions of "true" CDI status.

Test	Definition of "True" CDI Status					
	Concordance Between Both Reviewers in Categorizing CDI-Likely and CDI-Unlikely		Concordance, and CDI-Indeterminate/Discordant Considered CDI-Likely		Concordance, and CDI-Indeterminate/Discordant Considered CDI-Unlikely	
	Clinical Sensitivity	Clinical Specificity	Clinical Sensitivity	Clinical Specificity	Clinical Sensitivity	Clinical Specificity
NAAT	100% (24 of 24)	94% (17 of 18)	100% (41 of 41)	94% (17 of 18)	100% (24 of 24)	49% (17 of 35)
GDH EIA	83% (20 of 24)	72-89%* (13-16 of 18)	85% (35 of 41)	72-89%* (13-16 of 18)	83% (20 of 24)	43-51%* (15-18 of 35)
Toxin EIA	42% (10 of 24)	100% (18 of 18)	39% (16 of 41)	100% (18 of 18)	42% (10 of 24)	83% (29 of 35)

\* GDH EIA results for 3 patients were unable to be retrieved, and all 3 had been categorized as CDI-Unlikely. Clinical specificity was consequently calculated first assuming all 3 GDH EIA results were positive, and second assuming all 3 GDH EIA results were negative.