Original Investigation

Overdiagnosis of *Clostridium difficile* Infection in the Molecular Test Era

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IMPORTANCE *Clostridium difficile* is a major cause of health care–associated infection, but disagreement between diagnostic tests is an ongoing barrier to clinical decision making and public health reporting. Molecular tests are increasingly used to diagnose *C difficile* infection (CDI), but many molecular test-positive patients lack toxins that historically defined disease, making it unclear if they need treatment.

OBJECTIVE To determine the natural history and need for treatment of patients who are toxin immunoassay negative and polymerase chain reaction (PCR) positive (Tox−/PCR+) for CDI.

DESIGN, SETTING, AND PARTICIPANTS Prospective observational cohort study at a single academic medical center among 1416 hospitalized adults tested for *C difficile* toxins 72 hours or longer after admission between December 1, 2010, and October 20, 2012. The analysis was conducted in stages with revisions from April 27, 2013, to January 13, 2015.

MAIN OUTCOMES AND MEASURES Patients undergoing *C difficile* testing were grouped by US Food and Drug Administration–approved toxin and PCR tests as Tox+/PCR+, Tox−/PCR+, or Tox−/PCR−. Toxin results were reported clinically. Polymerase chain reaction results were not reported. The main study outcomes were duration of diarrhea during up to 14 days of treatment, rate of CDI-related complications (ie, colectomy, megacolon, or intensive care unit care) and CDI-related death within 30 days.

RESULTS Twenty-one percent (293 of 1416) of hospitalized adults tested for *C difficile* were positive by PCR, but 44.7% (131 of 293) had toxins detected by the clinical toxin test. At baseline, Tox−/PCR+ patients had lower *C difficile* bacterial load and less antibiotic exposure, fecal inflammation, and diarrhea than Tox+/PCR+ patients ($P < .001$ for all). The median duration of diarrhea was shorter in Tox−/PCR+ patients (2 days; interquartile range, 1-4 days) than in Tox+/PCR+ patients (3 days; interquartile range, 1-6 days) ($P = .003$) and was similar to that in Tox−/PCR− patients (2 days; interquartile range, 1-3 days), despite minimal empirical treatment of Tox−/PCR+ patients. No CDI-related complications occurred in Tox−/PCR+ patients vs 10 complications in Tox+/PCR+ patients (0% vs 7.6%, $P < .001$). One Tox−/PCR+ patient had recurrent CDI as a contributing factor to death within 30 days vs 11 CDI-related deaths in Tox+/PCR+ patients (0.6% vs 8.4%, $P = .001$).

CONCLUSIONS AND RELEVANCE Among hospitalized adults with suspected CDI, virtually all CDI-related complications and deaths occurred in patients with positive toxin immunoassay test results. Patients with a positive molecular test result and a negative toxin immunoassay test result had outcomes that were comparable to patients without *C difficile* by either method. Exclusive reliance on molecular tests for CDI diagnosis without tests for toxins or host response is likely to result in overdiagnosis, overtreatment, and increased health care costs.

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Invited Commentary

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Clostridium difficile is one of the most common causes of health care–associated infection in US hospitals, affecting almost 1% of hospitalized patients each year.[1-3] Since 2000, the incidence of *C difficile* infection (CDI) has increased more than 200% while the rates of other health care–associated infections have decreased.[1,2,4-6] More than 300 000 hospitalizations involve a CDI each year, at an annual cost of $1.0 to $4.9 billion to the US health care system.[2,7]

Initial increases in the rate of CDI were attributed to the emergence of a novel, hypervirulent strain during a period when at least 95% of hospitals used toxin immunoassays for diagnosis (2000-2008).[3,5,8-10] More recent increases have been linked to greater *C difficile* detection after the introduction of molecular tests, which are more sensitive and detect microbial DNA instead of toxin.[10-15] Individual hospitals have reported a 50% to 100% increase in the rate of CDI after switching from toxin tests to molecular tests.[11,12,14] Similar increases have been observed in the rate of publicly reported CDI as reporting facilities adopted molecular tests.[15]

For decades, toxin tests were favored over culture for diagnosis of CDI because toxins mediate disease and toxin detection was faster and provided evidence of toxin production in vivo that typically correlated better with clinical disease.[3,10,16-18] Molecular tests such as polymerase chain reaction (PCR) target toxin genes but are similar to culture in detecting *C difficile* bacteria regardless of toxin production, making it unclear whether positive PCR results reflect clinical disease.[3,10,19-21] The uncertain clinical significance of positive PCR results is problematic in inpatient health care facilities, where *C difficile* colonization is 5 to 10 times more common than CDI and noninfectious causes of diarrhea are also common.[22-26] Nonetheless, concern that patients with CDI were being missed by toxin tests prompted many laboratories to switch to molecular tests in 2009, when they became available.[10,19,27] As of the first quarter of 2014, a total of 44% of acute care hospitals participating in the National Healthcare Safety Network (NHSN) reported using molecular tests alone or in combination with other tests for diagnosis of CDI (NHSN, written communication, September 15, 2014). Therefore, there is an urgent need to determine whether patients with negative toxin test results and positive molecular test results have CDI or are simply colonized with another cause of symptoms.

To address this need, we prospectively tested hospitalized adults with suspected CDI at the University of California Davis Medical Center with molecular tests while maintaining our existing toxin test for clinical diagnosis. We then collected clinical outcome and treatment data to enable us to ask 3 related questions. First, what is the natural history of PCR-positive patients with negative toxin immunoassay results? Second, how do outcomes in these patients compare with outcomes in patients with positive toxin and PCR results or completely negative *C difficile* test results? Third, do PCR-positive patients with negative toxin results require treatment for CDI?

Methods

Study Design and Population

Hospitalized adults with a diarrheal stool sample submitted for *C difficile* testing 72 hours or longer after admission to the University of California Davis Medical Center between December 1, 2010, and October 20, 2012, were included in the study. Only the first sample was analyzed for each patient. Samples received after discharge were excluded. Patients with *C difficile* detected by culture and no other test were excluded from the study. The study protocol was approved by the University of California Davis Institutional Review Board. Informed consent was waived for the initial screening and symptom verification and overall outcome and safety analysis. A subset of patients had written informed consent obtained for additional in-person follow-up.

Laboratory Testing

All stool samples had a US Food and Drug Administration (FDA)–approved *C difficile* toxin immunoassay (*C difficile* Premier toxins A and B; Meridian Biosciences) performed and reported clinically. Formed stools were rejected. Eligible samples also had 1 or more FDA-approved molecular *C difficile* tests (Xpert *C. difficile*/Epi; Cepheid; and illumigene *C. difficile*; Meridian Biosciences) performed but not reported, allowing patients to be grouped by *C difficile* toxin immunoassay and PCR results as toxin immunoassay positive and PCR positive (Tox+/PCR+), Tox−/PCR+, or Tox−/PCR−. Additional tests were performed to characterize the nature of the *C difficile* colonization and host inflammatory response. The PCR-positive samples had toxin quantitated (xCELLigence System for Real-Time Cellular Analysis, version 2; ACEA Biosciences) and the concentration of *C difficile* DNA determined as a measure of bacterial load (Xpert *C. difficile*/Epi; Cepheid).[28-30] The Tox−/PCR+ samples were tested by a cell cytotoxin assay (*C. difficile* Tox-B; TechLab), the more sensitive historical standard for *C difficile* toxin detection and diagnosis, to determine the number of samples that would have been positive if this test had been used instead of the toxin immunoassay. Culture was performed to recover *C difficile* isolates for ribotyping and verification of capacity to produce toxins. Lactoferrin was measured in PCR+ samples and random PCR− samples as a marker of inflammation (Leuko EZ Vue; TechLab; and IBD-Scan; TechLab). Lactoferrin results were classified as high if they exceeded the 95th percentile of results in PCR− patients. See the eMethods in the Supplement for additional details.

Clinical Data Collection

Diarrheal symptoms were verified at the time of *C difficile* testing. Patients were considered to have diarrhea if they had at least 3 unformed bowel movements or at least 600 mL of rectal or colostomy output recorded in the electronic health record (EHR) within 24 hours on the day of or before sample collection. Patients not meeting the threshold for diarrhea in the EHR had their nurse called to verify diarrheal status. Other data were obtained from laboratory, EHR, and adminis-

trative databases. See the eMethods in the Supplement for additional details.

Outcomes and Clinical Case Attribution

The primary outcome was duration of diarrhea for the 15-day period encompassing the day of sample collection (day 1) and up to 14 days of treatment. Secondary outcomes included rate of CDI-related complications (ie, megacolon, colectomy for fulminant colitis, and intensive care unit [ICU] care related to CDI) and CDI-related deaths within 30 days. The CDI-related complications and deaths were analyzed separately to distinguish patients with complicated CDI disease of the colon from patients with CDI as a contributing cause of death but not necessarily complicated CDI of the colon. Repeat *C difficile* tests and treatment were analyzed within 14 days of day 1 as an indication of ongoing clinical suspicion or empirical treatment for CDI in Tox−/PCR+ patients and to determine how many became positive with repeat testing. *Clostridium difficile* tests and treatment 15 to 30 days after day 1 were analyzed as a proxy for recurrent or prolonged CDI occurring after the initial treatment period. Ten or more days of metronidazole or oral vancomycin therapy was considered full treatment. Duration of diarrhea was determined from nurse-recorded stool counts and rectal or colostomy outputs in the EHR, excluding formed stools. Each day was categorized as a diarrhea day if at least 3 unformed stools or at least 600 mL of fecal output was recorded. Days with less stool output were categorized as a no-diarrhea day. Duration of diarrhea was the sum of days from day 1 to the last day with diarrhea, followed by 2 or more days without diarrhea. Cases of CDI-related megacolon and colectomies were identified by searching for patients with a procedure or billing code for abdominal radiology, colonoscopy, colectomy, or diagnosis of megacolon or pseudomembranous colitis within 30 days (eTable 1 in the Supplement). Clinical and surgical notes and radiology, endoscopy, and pathology reports were reviewed to confirm or exclude CDI-related megacolon or colectomy. Partially treated complications diagnosed before day 1 were excluded. Intensive care unit care related to CDI was determined as follows. First, patients located in or transferred into the ICU on day 1 (±1 day) were identified. The ICU care was then determined to be CDI related (ie, attributable to or contributed to by CDI) or unrelated by blinded EHR review by 2 board-certified infectious diseases physicians (H.H.N., L.W.L., J.V.S., or S.H.C.). The physician adjudicators were blinded to PCR results but otherwise were provided with all relevant clinical, procedural, diagnostic, and outcome information available in the EHR. Disagreements were resolved by a third infectious diseases physician (H.H.N., L.W.L., J.V.S., or S.H.C.). Deaths were identified by discharge disposition codes and EHR review of PCR-positive patients with unknown mortality status at 30 days. Attribution of deaths as CDI related or unrelated was determined by blinded infectious diseases physician EHR review (L.W.L., J.V.S., or S.H.C.) in the same manner as for ICU care.

Statistical Analysis

Baseline data were summarized and tested for differences. The Kruskal-Wallis test was used for continuous variables except for age, which was compared with an analysis of variance. For categorical variables, including outcomes, a χ^2 test or Fisher exact test was used. Kaplan-Meier estimates were used to show time to resolution of diarrhea for each group, with censoring of patients who were discharged or died during the follow-up, and compared with the log-rank test. A Cox proportional hazards model was used to estimate the effect of Tox+/PCR+ or Tox−/PCR+ status compared with Tox−/PCR− status on the duration of diarrhea, adjusting for age, comorbidities, ICU status on day 1 (±1 day), prior antibiotic days, prior metronidazole or oral vancomycin exposure, maximum white blood cell count on day 1 (±1 day), *C difficile* ribotype, and fecal lactoferrin level. See the eMethods in the Supplement for additional details.

Results

Patient Cohort and Baseline Characteristics

An overview of the study design, patient cohort, and follow-up is shown in **Figure 1**. In total, 1416 hospitalized adults were analyzed, including 131 Tox+/PCR+ patients (9.3%), 162 Tox−/PCR+ patients (11.4%), and 1123 Tox−/PCR− patients (79.3%).

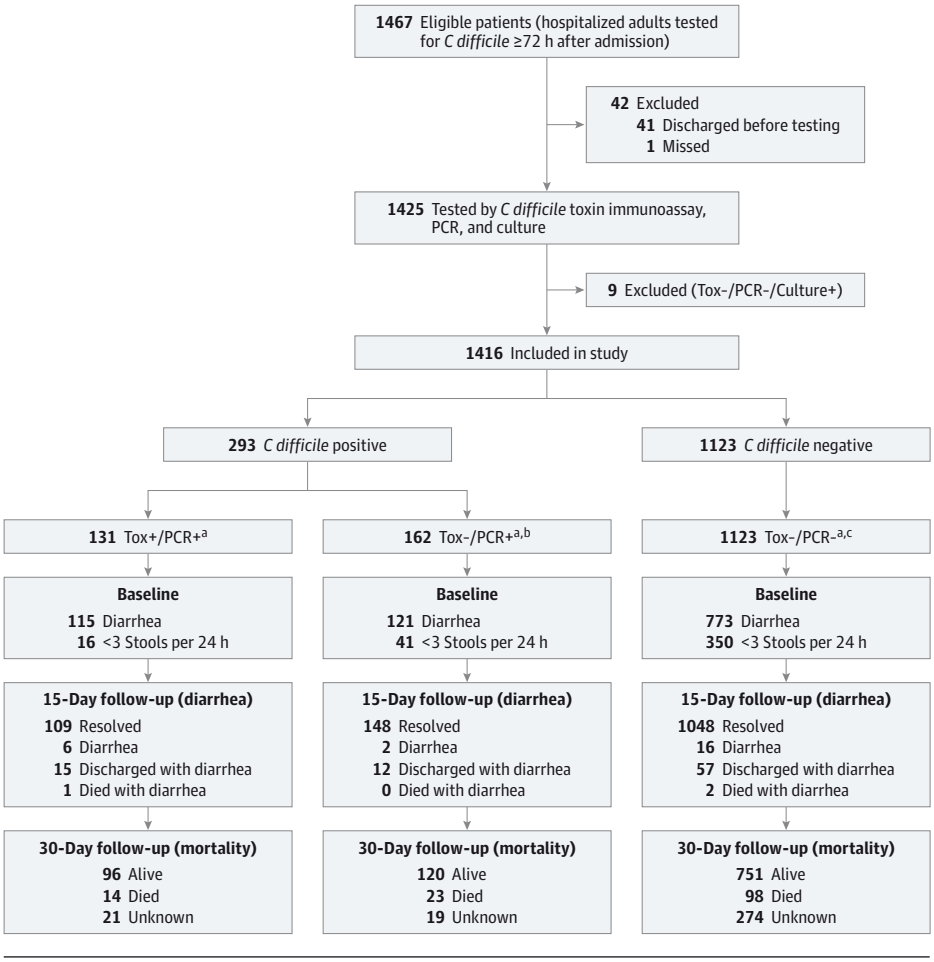
The groups were similar in age, sex, number of comorbidities, nonantibiotic medication exposures, and proportions with leukopenia, renal insufficiency, and hypoalbuminemia except for fewer comorbidities in Tox−/PCR− patients (**Table 1** and eTable 2 in the Supplement). However, the Tox+/PCR+ group had more prior antibiotic exposure, more patients with leukocytosis, and more diarrhea on day 1. In feces, Tox+/PCR+ patients had an increased *C difficile* bacterial load, higher toxin concentration, and greater frequency of hypervirulent *C difficile* strain than Tox−/PCR+ patients. Correspondingly, Tox+/PCR+ patients had significantly more fecal lactoferrin than Tox−/PCR+ patients, and 36.8% (43 of 117) had a lactoferrin level greater than the 95th percentile of Tox−/PCR− patients. In contrast, few Tox−/PCR+ patients (13.4% [19 of 142]) had a lactoferrin level above the 95th percentile of Tox−/PCR− patients, and 79.0% (15 of 19) of these patients had an alternative explanation for fecal inflammation, a previous diagnosis of CDI, or anti-*C difficile* treatment before testing (eTable 3 in the Supplement).

Duration of Diarrhea

The Tox+/PCR+ patients had a longer duration of diarrhea than Tox−/PCR+ patients and Tox−/PCR− patients ($P < .001$) and had a greater risk of diarrhea during the follow-up (**Figure 2** and **Table 2**). In contrast, Tox−/PCR+ patients and Tox−/PCR− patients had a similar risk of diarrhea on most days.

In the multivariable model, Tox+/PCR+ status had the strongest effect on duration of diarrhea, decreasing the probability of diarrhea being resolved by 37% each day relative to the Tox−/PCR− reference group (hazard ratio, 0.63; 95% CI, 0.48-0.83). Age, white blood cell count, and lactoferrin level were also significant predictors of duration of diarrhea, but their relative contribution was small (≤2% each) (eTable 4 in the Supplement). The Tox−/PCR+ status and pretest exposure to

Figure 1. Flow of Patients Through Testing and Follow-up



Tox+/PCR+ indicates *Clostridium difficile* toxin immunoassay positive and polymerase chain reaction positive; Tox−/PCR+, *C difficile* toxin immunoassay negative and polymerase chain reaction positive; and Tox−/PCR−, *C difficile* toxin immunoassay negative and polymerase chain reaction negative.

[a] *Clostridium difficile* test group based on US Food and Drug Administration–approved toxin immunoassay and polymerase chain reaction results.

[b] Includes one patient with false-positive immunoassay.

[c] Includes 20 patients with false-positive immunoassay.

metronidazole or oral vancomycin were not significant predictors in the multivariable model.

CDI-Related Complications and Mortality Within 30 Days

The frequency of CDI-related complications (ie, megacolon, colectomy for fulminant colitis, and ICU care related to CDI) and deaths is summarized in **Table 3**. The Tox+/PCR+ patients had more CDI-related complications than Tox−/PCR+ patients and Tox−/PCR− patients (10 [7.6%] of 131 vs 0 [0%] of 162 vs 3 [0.3%] of 1123, $P < .001$). In contrast, the rate of CDI-related complications was similar between Tox−/PCR+ patients and Tox−/PCR− patients (0% vs 0.3%, $P > .99$). The Tox+/PCR+ patients also had more CDI-related deaths than Tox−/PCR+ patients and Tox−/PCR− patients (11 [8.4%] of 131 vs 1 [0.6%] of 162 vs 0 [0%] of 1123, $P < .001$) while the rate was similar between Tox−/PCR+ patients and Tox−/PCR− patients (0.6% vs 0%, $P = .13$). Two deaths in the Tox+/PCR+ group were directly attributable to CDI, and 9 had CDI as a contributing factor. One Tox−/PCR+ patient (patient 1641 in eTable 3 in the Supplement) had an uncomplicated, recurrent CDI that resolved before care was withdrawn for severe underlying illness, but CDI was considered a contributing factor to death.

Repeat *C difficile* Testing and Treatment Within 14 Days

Repeat *C difficile* testing and treatment within 14 days of day 1 was analyzed as an indication of ongoing clinical suspicion or empirical treatment for CDI in Tox−/PCR+ patients (Table 3). During this period, 61 Tox−/PCR+ patients (37.7%) were retested, and 13 (8.0%) had toxins detected (mean time to positive result, 5.7 days; 95% CI, 3.2-8.2 days). None of these patients developed a *C difficile*-related complication. However, one patient (patient 1641 in eTable 3 in the Supplement) had CDI that was considered a contributing factor to death, although symptoms had resolved before care was withdrawn for other reasons. During the same period, most Tox−/PCR+ patients (59.3% [96 of 162]) received no treatment, 45 patients (27.8% [45 of 162]) received partial treatment (1-9 days), and 21 patients (13.0% [21 of 162]) received the equivalent of full treatment (≥10 days).

Clostridium difficile Testing and Treatment Between 15 and 30 Days

Clostridium difficile tests and treatment 15 to 30 days after day 1 were analyzed as a proxy for recurrent or prolonged CDI (Table 3). During this period, Tox+/PCR+ patients were retested almost twice as often as Tox−/PCR+ patients (19.8% vs

Table 1. Baseline Characteristics of the Study Population by *Clostridium difficile* Test Group

Characteristic	*C difficile* Positive		*C difficile* Negative	*P* Value[a]
	Tox+/PCR+[b] (n = 131)	Tox−/PCR+[b,c] (n = 162)	Tox−/PCR−[b,d] (n = 1123)	
Age, median (IQR), y	64 (52-71)	58 (48-68)	59 (47-71)	.12
Female sex, No. (%)	64 (48.9)	83 (51.2)	530 (47.2)	.61
Comorbidities, median (IQR)	4 (2-6)	4 (2-5)	3 (2-5)	.01
APR-DRG risk of mortality subclass 3 or 4, No. (%)	104 (79.4)	128 (79.0)	787 (70.1)	.008
Intensive care unit care on day 1 ±1 d, No. (%)[e]	30 (22.9)	57 (35.2)	435 (38.7)	.002
Hospital days before day 1, median (IQR)[e]	10 (6-24)	8 (5-12)	8 (5-12)	<.001
Admitted from health care facility, No. (%)	40 (30.5)	34 (21.0)	160 (14.2)	<.001
C difficile positive within 90 d before day 1[e]	5 (3.8)	10 (6.2)	13 (1.2)	<.001
Antibiotic days within 90 d before day 1, median (IQR)[e]	16 (7-32)	10 (4-27)	8 (4-18)	<.001
Other diarrheal or gastrointestinal inflammatory process, No. (%)[f]	8 (6.1)	27 (16.7)	161 (14.3)	.02
Metronidazole or oral vancomycin within 48 h before day 1, No. (%)[e]	3 (2.3)	32 (19.8)	184 (16.4)	<.001
WBC count ≥15 000 cells/μL on day 1 ±1 d, No./total No. tested (%)[e]	54/129 (41.9)	50/154 (32.5)	323/1101 (29.3)	.01
WBC count <4000 cells/μL on day 1 ±1 d, No./total No. tested (%)[e]	20/129 (15.5)	32/154 (20.8)	200/1101 (18.2)	.52
Creatinine level >1.5 mg/dL on day 1 ±1 d, No./total No. tested (%)[e]	36/127 (28.3)	45/156 (28.8)	297/1102 (27.0)	.85
Albumin level <2.5 g/dL on day 1 ±1 d, No./total No. tested (%)[e]	29/48 (60.4)	50/70 (71.4)	318/475 (66.9)	.46
Diarrhea present on day 1 ±1 d, No. (%)[e]	121 (92.4)	143 (88.3)	927 (82.5)	.004
Stool count on day 1, median (IQR)[e]	5 (3-6)	3 (2-5)	3 (2-5)	<.001
C difficile toxin B, median (IQR), ng/mL	640.8 (172.5-1194.0)	1.1 (0.3-2.5)	NA	<.001
Hypervirulent *C difficile* ribotype RT027/078, No. (%)	68 (51.9)	39 (24.1)	NA	<.001
C difficile binary toxin positive, No. (%)	71 (54.2)	45 (27.8)	NA	<.001
Log_{10} *C difficile* DNA copies/mL, median (IQR)	7.3 (6.6-7.7)	4.9 (4.4-6.2)	NA	<.001
Fecal lactoferrin level, median (IQR), μg/mL,	37.7 (8.8-261.5)	20.1 (5.0-50.3)	7.8 (0.5-32.6)	<.001
Normal lactoferrin level, No./total No. tested[g]	25/117 (21.4)	44/142 (31.0)	89/188 (47.3)	<.001
High lactoferrin level, No./total No. tested[h]	43/117 (36.8)	19/142 (13.4)	9/188 (4.8)	<.001

Abbreviations: APR-DRG, all-patient refined diagnosis-related group; IQR, interquartile range; NA, not applicable; Tox+/PCR+, *C difficile* toxin immunoassay positive and polymerase chain reaction positive; Tox−/PCR+, *C difficile* toxin immunoassay negative and polymerase chain reaction positive; Tox−/PCR−, *C difficile* toxin immunoassay negative and polymerase chain reaction negative; WBC, white blood cell.

SI conversion factors: To convert WBC count to $\times 10^9$/L, multiply by 0.001; to convert creatinine level to micromoles per liter, multiply by 88.4; to convert albumin level to grams per liter, multiply by 10.

[a] *P* value for significance across 3 groups except for characteristics not applicable to Tox−/PCR− group.

[b] *Clostridium difficile* test group based on US Food and Drug Administration–approved toxin immunoassay and PCR results.

[c] Includes one patient with false-positive toxin immunoassay.

[d] Includes 20 patients with false-positive toxin immunoassay.

[e] Day 1 is the day of sample collection for the *C difficile* toxin test.

[f] Includes inflammatory bowel diseases, functional diarrheal disorders, diverticulitis, appendicitis, ischemic colitis, other infectious or noninfectious enterocolitis, graft-vs-host disease, and peritoneal, mesenteric, or retroperitoneal infections.

[g] Normal fecal lactoferrin level defined as within the upper limit of a healthy person's reference range per the manufacturer's package insert.

[h] High fecal lactoferrin level defined as exceeding the 95th percentile fecal lactoferrin level in Tox−/PCR − patients (>89.05 μg/mL).

11.1%, *P* = .04) and were positive 3 times more often (10.7% vs 3.1%, *P* < .001). During the same period, most Tox−/PCR+ patients (78.4% [127 of 162]) received no treatment, while 13 patients (8.0% [13 of 162]) received treatment for at least 10 days.

Additional Analyses to Evaluate the Robustness of the Study Findings

Outcome differences between the Tox−/PCR+ and Tox+/PCR+ groups remained significant when comparisons were limited to the subgroup of Tox−/PCR+ patients who received full or partial treatment within 14 days (*P* = .04 for time to resolution of diarrhea and *P* = .004 for CDI-related complication or death) or no treatment (*P* = .003 for time to resolution of diarrhea and *P* < .001 for CDI-related complication or death). No significant outcome differences were observed between the Tox−/PCR− group and individual Tox−/PCR+ subgroups with or without treatment.

If the historical cell cytotoxin assay had been used for diagnosis instead of a toxin immunoassay, 48 additional Tox−/PCR+ patients (29.6%) would have been reported positive. However, this subgroup had a low toxin concentration (median, 10 ng/mL; interquartile range, 2-81 ng/mL) and outcomes that were similar to cell cytotoxin-negative Tox−/PCR+ patients (*P* = .47 for time to resolution of diarrhea and *P* = .30 for CDI-related complication or death), with no differ-

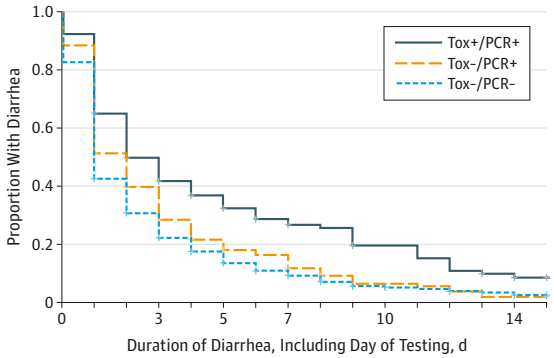
ence in treatment ($P = .61$), and better than Tox+/PCR+ patients ($P < .001$ for time to resolution of diarrhea and $P = .03$ for CDI-related complication or death).

Discussion

This study addresses an important question for physicians, hospitals, and policy makers: do toxin-negative patients with a positive *C difficile* PCR test result require treatment? To answer this question, we prospectively tested 1416 hospitalized patients with FDA-approved PCR tests while maintaining our existing toxin test for clinical diagnosis to determine the natural history of toxin-negative patients with positive PCR results. We found that 55.3% (162 of 293) of patients with a positive *C difficile* PCR test result lacked toxin by the clinical toxin immunoassay test and had outcomes that were comparable to patients with no *C difficile* detected. These Tox−/PCR+ patients had milder symptoms at the time of testing and a shorter duration of diarrhea than toxin-positive patients. In total, 58.7% (95 of 162) were never retested, and only 13.0% (21 of 162) received the equivalent of a full course of treatment. Repeat analyses with the treated Tox−/PCR+ patients removed did not change our conclusions. Overall, 18 of 19 *C difficile*–related complications and deaths (94.7%) occurred in toxin-positive patients. Only one of 162 toxin-negative patients (0.6%) was considered to have CDI as a contributing factor to death.

Our findings are consistent with the conventional view that CDI is a toxin-mediated inflammatory disease preceded by antibiotic exposure and *C difficile* overgrowth.[3] Toxin-negative patients had less antibiotic exposure, *C difficile* DNA, and inflammation and manifested milder symptoms and no complications, despite minimal or no treatment. These findings strongly suggest that most patients with negative toxin test results and *C difficile* detected by PCR do not need treatment for CDI. We suspect that most of these patients were colonized with *C difficile* and had another cause of diarrhea. This hypothesis is supported by studies[22-26,31] showing that *C difficile* colonization and immunity are common in hospitalized patients and most nosocomial diarrhea is noninfectious. It is possible that some toxin-negative patients have mild or early infection because clinical toxin tests can miss toxin at low concentrations, and occasional toxin-negative patients become positive on repeat testing.[3,10,18,32-35] Correspondingly, we detected toxin in 29.6% (48 of 162) of Tox−/PCR+ patients by the historical cell cytotoxin assay, and 8.0% (13 of 162) of Tox−/

Figure 2. Kaplan-Meier Curves of Time to Resolution of Diarrhea by *Clostridium difficile* Test Group



No. at risk

Tox+/PCR+	131	62	41	29	25	8
Tox−/PCR+	162	60	29	21	10	2
Tox−/PCR−	1123	328	172	99	42	23

The median duration of diarrhea for patients with at least 1 day was 3 days (interquartile range, 1-6 days) for Tox+/PCR+ (121 of 131), 2 days (interquartile range, 1-4 days) for Tox−/PCR+, and 2 days (interquartile range, 1-3 days) for Tox−/PCR− (927 of 1123) ($P < .001$). Log-rank P values are $P < .001$ for all groups, $P = .003$ for Tox+/PCR+ vs Tox−/PCR+, (143 of 162) $P < .001$ for Tox+/PCR+ vs Tox−/PCR−, and $P < .001$ for Tox−/PCR+ vs Tox−/PCR−. Tox+/PCR+ indicates *C difficile* toxin immunoassay positive and polymerase chain reaction positive; Tox−/PCR+, *C difficile* toxin immunoassay negative and polymerase chain reaction positive; Tox−/PCR−, *C difficile* toxin immunoassay negative and polymerase chain reaction negative.

Table 2. Relative Risk (95% CI) of Diarrhea Each Day

Day	Comparison		
	Tox+/PCR+ vs Tox−/PCR+	Tox+/PCR+ vs Tox−/PCR−	Tox−/PCR+ vs Tox−/PCR−
1	1.05 (0.97-1.13)	1.12 (1.06-1.18)	1.07 (1.01-1.14)
2	1.27 (1.03-1.56)	1.46 (1.29-1.73)	1.18 (0.99-1.40)
3	1.28 (0.98-1.67)	1.62 (1.32-1.98)	1.27 (1.02-1.58)
4	1.51 (1.07-02.13)	1.87 (1.46-2.40)	1.24 (0.93-1.66)
5	1.75 (1.15-2.65)	2.04 (1.53-2.73)	1.17 (0.82-1.67)
6	1.88 (1.17-3.02)	2.31 (1.67-3.20)	1.23 (0.81-1.85)
7	1.71 (1.02-2.85)	2.51 (1.73-3.64)	1.47 (0.95-2.29)
8	2.30 (1.25-4.22)	2.72 (1.82-4.06)	1.18 (0.69-2.04)
9	3.09 (1.54-6.20)	3.90 (2.52-6.03)	1.26 (0.66-2.42)
10	3.18 (1.37-7.38)	3.67 (2.18-6.19)	1.16 (0.53-2.53)
11	3.18 (1.37-7.38)	4.06 (2.39-6.90)	1.28 (0.58-2.81)
12	2.89 (1.14-7.30)	3.64 (2.00-6.62)	1.26 (0.54-2.96)
13	3.09 (0.99-9.63)	3.30 (1.63-6.68)	1.07 (0.38-3.02)
14	4.95 (1.07-22.90)	2.98 (1.36-6.53)	0.60 (0.14-2.53)
15	3.71 (0.76-18.08)	3.22 (1.28-8.07)	0.87 (0.20-3.73)

Abbreviations: Tox+/PCR+, *Clostridium difficile* toxin immunoassay positive and polymerase chain reaction positive; Tox−/PCR+, *C difficile* toxin immunoassay negative and polymerase chain reaction positive; Tox−/PCR−, *C difficile* toxin immunoassay negative and polymerase chain reaction negative.

Table 3. Nondiarrheal Outcomes and Treatment by *Clostridium difficile* Test Group

Outcome	*C difficile* Positive		*C difficile* Negative	*P* Value[a]
	Tox+/PCR+ (n = 131)	Tox−/PCR+ (n = 162)	Tox−/PCR− (n = 1123)	
***C difficile*-Related Complication or Death Within 30 d, No. (%)**				
Complication[b]	10 (7.6)	0	3 (0.3)	<.001
Death[c]	11 (8.4)	1 (0.6)	0	<.001
Complication or death	18 (13.7)	1 (0.6)	3 (0.3)	<.001
Repeat *C difficile* Testing Within 14 d, No. (%)				
Retested	14 (10.7)	61 (37.7)	374 (33.3)	<.001
Positive toxin test result	3 (2.3)	13 (8.0)	17 (1.5)	<.001
Repeat *C difficile* Testing at 15-30 d, No. (%)				
Tested	26 (19.8)	18 (11.1)	106 (9.4)	.001
Positive toxin test result	14 (10.7)	5 (3.1)	10 (0.9)	<.001
Treatment Within 14 d				
Metronidazole or oral vancomycin, No. (%)[d]	131 (100)	66 (40.7)	361 (32.1)	<.001
Duration of metronidazole or oral vancomycin, if treated, median (IQR), d	14 (11-14)	6 (3-11)	5 (2-9)	<.001
Non-*C difficile* antibiotic, No. (%)	98 (74.8)	141 (87.0)	912 (81.2)	.03
Duration of non-*C difficile* antibiotic, if treated, median (IQR), d	11 (3-14)	10 (4-14)	10 (4-14)	.13
Treatment at 15-30 d				
Metronidazole or oral vancomycin, No. (%)	75 (57.3)	35 (21.6)	137 (12.2)	<.001
Duration of metronidazole or oral vancomycin, if treated, median (IQR), d	9 (3-14)	4 (3-15)	6 (3-9)	<.001

Abbreviations: IQR, interquartile range; Tox+/PCR+, *C difficile* toxin immunoassay positive and polymerase chain reaction positive; Tox−/PCR+, *C difficile* toxin immunoassay negative and polymerase chain reaction positive; Tox−/PCR−, *C difficile* toxin immunoassay negative and polymerase chain reaction negative.

[a] *P* value for significance across 3 groups.

[b] Intensive care unit care, colectomy, or megacolon related to *C difficile* infection. The Tox+/PCR+ complications included 3 fulminant colitis or megacolon and 7 intensive care unit care related to *C difficile* infection. Two Tox−/PCR+ patients with partially treated complications diagnosed as having a positive toxin test result before day 1 were excluded. The Tox−/PCR− complications included 3 intensive care unit care related to *C difficile* infection. $P < .001$ for Tox+/PCR+ vs Tox−/PCR+ and $P > .99$ for Tox−/PCR+ vs Tox−/PCR−.

[c] All-cause mortality within 30 days was 14 (10.7%), 23 (14.2%), and 98 (8.7%), respectively, for the 3 groups. $P = .08$ for all groups and $P = .21$ for Tox+/PCR+ vs Tox−/PCR+. For *C difficile* infection–related death, $P < .001$ for Tox+/PCR+ vs Tox−/PCR+ and $P = .13$ Tox−/PCR+ vs Tox−/PCR−.

[d] Full treatment (≥10 days) and partial treatment (1-9 days) values were 119 (90.8%) and 12 (9.2%), respectively; 21 (13.0%) and 45 (27.8%), respectively; and 82 (7.3%) and 279 (24.8%), respectively, for the 3 groups.

PCR+ patients retested positive by the clinical toxin immunoassay in a subsequent sample. However, the relative lack of adverse events in this subgroup suggests that these patients are also at lower risk of complications than clinical toxin immunoassay–positive patients and routine treatment is unnecessary.

These results are consistent with a large retrospective study[36] that found no *C difficile*–related complications and lower mortality among hospitalized patients with negative toxin results. Our findings also agree with several smaller studies[11,14,37-41] and one large, multicenter study[21] that reported milder symptoms or a lower mortality rate in toxin-negative patients with positive PCR results. Other studies[42-45] that have investigated clinical characteristics of Tox−/PCR+ patients were generally underpowered or not designed to compare outcomes. Finally, there are reports of patients with severe or complicated CDI missed by toxin tests,[43,46] but our data suggest that such patients are rare.

Strengths of our study include the prospective study design, large sample size, nonreporting of PCR results, measurement of duration of diarrhea, inclusion of patients without *C difficile* for comparison, and rigorous evaluation of *C difficile*–related complications and deaths. We quantified fecal *C difficile* DNA, toxins, and inflammation to provide mechanistic insight into the reasons for the different test results and outcomes. The primary weakness of the study was the inability to achieve equivalent risk allocation between groups. In addition, we cannot exclude the possibility that empirical treatment affected outcomes in some Tox−/PCR+ patients, but the outcome differences we observed remained when these patients were removed. It is also possible that our outcome adjudicators were influenced by positive toxin results, but 26 of 42 Tox+/PCR+ patients with ICU care or death (61.9%) were judged not to have a CDI-related outcome, indicating that the adjudication was a highly discriminatory process overall. Finally, we cannot exclude the possibility that systematic un-

derrecording of stools in patients with negative toxin results could account for the shorter duration of diarrhea in these patients. However, our requirement of 2 or more days without diarrhea to end an episode would make it unlikely that underrecording by individual nurses would have a significant effect on our diarrhea measure.

Molecular tests have the potential benefits of decreasing the need for repeat testing and empirical treatment because of their high negative predictive value and may have a role in infection prevention if Tox−/PCR+ patients contribute to the spread of *C difficile* in health care facilities.[34,43,47] However, our results offer compelling evidence that as many as half of the patients with positive *C difficile* PCR test results are likely to be overdiagnosed and exposed to unnecessary treatment at institutions using molecular tests. The number of patients potentially affected by this issue is massive. Most institutions experience a 50% to 100% increase in reported CDI after switching to molecular tests, and the proportion of institutions using molecular *C difficile* tests has increased dramatically since initiation in 2009 of the first FDA-approved molecular test.[11-15] In 2014, almost 44% of NHSN acute care facilities reported using molecular tests for CDI diagnosis (NHSN, written communication, September 15, 2014).

Therefore, there is an urgent need to educate physicians that molecular tests are not specific for CDI, even in the presence of symptoms, and patients with positive PCR results do not necessarily need treatment. Similarly, while underdiagnosis may occur with lack of testing,[48] policy makers should be aware that molecular *C difficile* tests are a major cause of overdiagnosis and consider the potential costs of overtreatment in recommendations and analyses. Laboratories need to be aware that rejection of formed stool samples is not sufficient to ensure that all positive molecular *C difficile* results represent disease.

We concur with authors in the United Kingdom that molecular tests should not be used as a stand-alone diagnostic test for CDI and diagnostic recommendations should move back in the direction of defining clinical disease as a positive toxin result in patients with diarrhea.[21,49] Most toxin-negative patients with *C difficile* do not need specific treatment, although there may be a role for identifying carriers to prevent transmission.[21,43] Future studies should focus on developing diagnostic approaches to accurately distinguish patients with active infection vs colonization, which may include quantitation of *C difficile* DNA, toxins, or host response. In the meantime, 2-step testing with a screening test, such as PCR or glutamate dehydrogenase antigen detection, followed by a toxin test to confirm active infection is a reasonable diagnostic strategy.[21,49]

Conclusions

Up to half of the patients with positive molecular test results for *C difficile* do not experience adverse events without treatment and do not need treatment for CDI. Exclusive reliance on molecular tests for *C difficile* diagnosis is likely to result in overdiagnosis, unnecessary treatment, and increased health care costs.

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Clinical and Infection Control Implications of *Clostridium difficile* Infection With Negative Enzyme Immunoassay for Toxin

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In a prospective study of 132 patients with a diagnosis of *Clostridium difficile* infection (CDI) by polymerase chain reaction, 43 (32%) had enzyme immunoassay (EIA) results negative for toxin. EIA-negative patients with CDI did not differ in clinical presentation from EIA-positive patients and presented a similar risk for transmission of spores.

Enzyme immunoassays (EIAs) for toxin A and B are commonly used for diagnosis of *Clostridium difficile* infection (CDI) because they are easy to use and provide rapid results [1, 2]. However, EIAs for toxin have poor sensitivity compared with toxigenic culture, which is the gold standard for CDI testing [1, 2]. In a recent evaluation, 6 commercially available EIAs and 3 lateral-flow assays for detection of toxin had a mean sensitivity of 75% (range, 60%–86%), compared with that of toxigenic culture [3]. The recent development of commercial real-time polymerase chain reaction (PCR) assays for detection of toxin genes may provide a rapid and sensitive alternative method for CDI diagnosis [1, 4, 5]. However, PCR assays are more expensive than EIAs and require specialized equipment. In addition, it is unclear whether the use of sensitive testing methods will result in improved clinical or infection control outcomes. Because levels of toxin in stool may correlate with severity of diarrhea [6], it is plausible that many patients with CDI with negative EIA results might have low levels of toxin in the intestinal tract, resulting in less severe diarrhea and less shedding of spores. Here, we tested the hypothesis that patients with CDI diagnosed by PCR but with negative EIA test results have less severe illness and reduced shedding of spores, compared with those patients with positive EIA results.

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METHODS

The Cleveland Veterans Affairs Medical Center is a 265-bed tertiary-care hospital. During the study, diagnostic testing for CDI was performed using EIA for glutamate dehydrogenase (Wampole C. diff Chek-60, Inverness Medical) as an initial screen and a commercial PCR test for toxin B genes (Becton Dickinson) for confirmation. The laboratory rejected formed stool samples and performed repeat tests only if 7 days elapsed after a prior test. The Cleveland Veterans Affairs Medical Center's Institutional Review Board approved the study protocol.

From October 2009 through July 2010, we conducted a 10-month prospective study of all patients who received a diagnosis of CDI, defined as presence of unformed stool in the absence of another obvious cause and positive glutamate dehydrogenase and PCR test results. After PCR testing, samples were analyzed using a commercial EIA for toxin (Wampole *C. difficile* TOX A/B II, Inverness Medical); in comparison with toxigenic culture, the sensitivity and specificity of the assay are 88% and 94%, respectively [3]. PCR-positive samples were cultured for toxigenic *C. difficile* as previously described [7]. Medical record review was performed to obtain information regarding demographic characteristics, medical illnesses, medications, laboratory tests, and mortality. Information on duration of diarrhea and number of bowel movements per day was obtained through interviews with patients and nurses and by medical record review. CDI cases were classified as community-onset cases or community-associated cases and as mild–moderate, severe, or severe–complicated on the basis of definitions from current guidelines [2].

For inpatients, cultures were obtained within 3 days of the diagnosis of CDI to evaluate the potential for acquisition of spores on gloved hands after contact with skin (chest and abdomen, arm and hand, and groin) and environmental (bed rail, bedside table, and call button) sites. The cultures were obtained and processed as previously described [7].

All *C. difficile* isolates were tested for in vitro cytoxin production using *C. difficile* Tox A/B II (Inverness Medical); isolates that did not produce toxin were excluded. To determine the prevalence of epidemic ribotype 027 strains, a subset of stool isolates was subjected to PCR ribotyping [8]. PCR ribotyping

Table 1. Baseline Characteristics and Outcomes of the 132 Patients With *Clostridium difficile* Diagnosed by Polymerase Chain Reaction for Toxin B Genes

Characteristic	Enzyme immunoassay positive (n = 90)	Enzyme immunoassay negative (n = 42)	*P*
Age, mean years (range)	68 (30–91)	63 (28–93)	.12
Male sex	89 (99)	40 (95)	.24
Unformed bowel movements, mean no./d (range)	5 (1–17)	5 (2–18)	.50
<3 Unformed bowel movements/d	7 (8)	4 (10)	.74
Clinical conditions			
Diabetes mellitus	36 (40)	16 (38)	.83
Chronic pulmonary disease	32 (36)	10 (24)	.18
End-stage renal disease	4 (4)	2 (5)	>.99
Cancer	18 (20)	5 (12)	.33
Neurological disease	22 (24)	11 (26)	.59
Paraplegia	14 (16)	3 (7)	.18
Heart disease	24 (27)	14 (33)	.43
Long-term care facility residence	27 (30)	11 (26)	.66
Antibiotic use in past 90 days	78 (87)	35 (83)	.61
Hospitalized >48 hours in past 90 days	40 (44)	26 (62)	.06
Limited mobility[a]	30 (33)	8 (19)	.09
Classification of CDI			
Community-onset[b]	10 (11)	9 (21)	.12
Community-associated[c]	7 (8)	6 (15)	.20
Severe, uncomplicated[d]	25 (28)	9 (21)	.53
Severe, complicated[e]	3 (3)	2 (5)	.24
CDI therapy			
Metronidazole	67 (74)	35 (83)	.37
Vancomycin	20 (22)	5 (12)	.23
Metronidazole and vancomycin	3 (3)	2 (5)	.24
Outcome			
Recurrence[f]	15 (17)	10 (24)	.33
Death due to any cause	1 (1)	2 (5)	.23
Death due to CDI	0 (0)	1 (2)	…

NOTE. Data are no. (%) of patients, unless otherwise specified. CDI, *C. difficile* infection.

[a] Mobility score of ≤3 on the Braden score for prediction of pressure ulcer risk.

[b] Onset of diarrhea in the community or ≤48 hours after admission to a health care facility.

[c] Onset of diarrhea in the community or ≤48 hours after admission to a health care facility, provided that symptom onset was >12 wk after the last discharge from a health care facility.

[d] *C. difficile* infection associated with leukocytosis with a white blood cell count of ≥15,000 cells/mL or a serum creatinine level ≥1.5 times the premorbid level, but without hypotension, sepsis, ileus, or megacolon.

[e] *C. difficile* infection associated with hypotension or shock, ileus, or megacolon.

[f] *C. difficile* infection occurring ≤8 wk after the onset of a previous episode, provided that the symptoms from the earlier episode resolved with or without therapy.

was also performed to compare isolates from stool, skin, and environmental sites. PCR was performed to amplify 1 of the genes for binary toxin (*cdtB*) [9].

Distributions of clinical and demographic characteristics and proportions of contamination of skin and environmental sites were compared for EIA-positive and EIA-negative patients. Unpaired Student's *t* test was used for normally distributed data. Pearson's χ^2 and Fisher's exact tests were used for categorical data. Data were analyzed with SPSS statistical software, version 10.0 (SPSS) and STATA software, version 9.1 (StataCorp).

RESULTS

Of 132 patients who received a diagnosis of CDI based on the presence of unformed stool and positive glutamate dehydrogenase and PCR results, 90 (68%) had positive EIA results for toxin (sensitivity, 68% vs PCR). Table 1 provides a comparison of the characteristics and outcomes for the EIA-positive and EIA-negative patients. There were no significant differences between the 2 groups with regard to clinical characteristics, number of bowel movements per day, CDI therapy, severe or

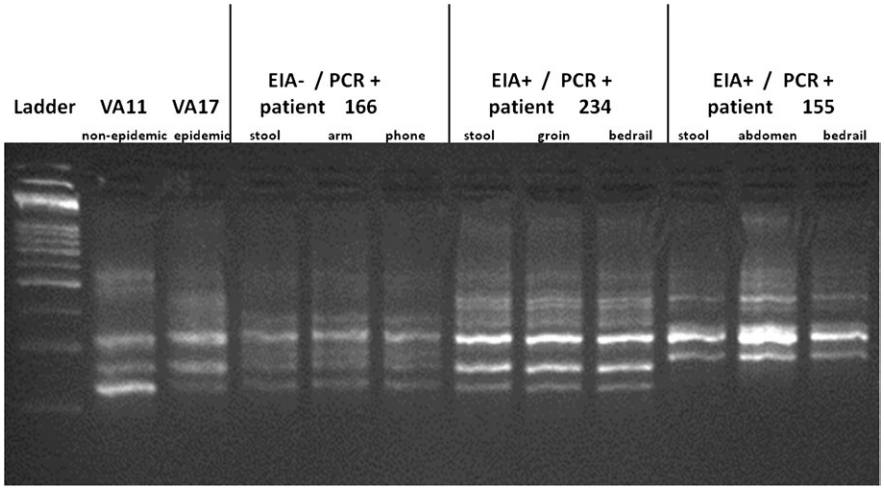

Figure 1. PCR ribotype analysis of *Clostridium difficile* isolates cultured from stool samples from 3 patients with *C. difficile* infection and concurrent isolates acquired on gloved hands after contact with skin and environmental sites. Patients 1 and 3 had binary toxin–negative, nonepidemic strains. Patient 2 had a binary toxin–positive, PCR-ribotype 027 strain. EIA, enzyme immunoassay for toxin A and B; PCR, polymerase chain reaction for toxin B genes (BD GeneOhm); VA 11, binary toxin–negative, nonepidemic control strain; VA 17, binary toxin–positive, PCR-ribotype 027 epidemic strain.

severe-complicated CDI, or recurrences. Nine (21%) EIA-negative patients presented with severe CDI, and 2 (5%) presented with severe-complicated CDI, including 1 patient who died of fulminant CDI.

Of 128 PCR-positive stool samples subjected to culture, 127 (99%) grew toxigenic *C. difficile*. The PCR-positive sample that did not grow toxigenic *C. difficile* was from an EIA-positive patient who received empiric oral vancomycin prior to stool collection and who had skin cultures positive for toxigenic *C. difficile*.

For 37 EIA-positive and 14 EIA-negative inpatients who had skin and environmental cultures, the frequencies of acquisition of *C. difficile* on gloved hands were not significantly different after contact with the skin (57% and 57%, respectively; $P = >.99$) and environment (30% and 29%, respectively; $P > .99$). For 5 patients with matched stool and hand acquisition isolates after contact with skin and/or environmental sites, stool and hand acquisition isolates had identical PCR ribotypes. Figure 1 shows PCR ribotype results of matched stool, skin, and environmental isolates for 3 patients.

Of 105 stool isolates subjected to typing, 53 (50%) were binary toxin-positive, PCR ribotype 027 strains. The proportion of patients infected with ribotype 027 strains was significantly higher in the EIA-positive than in the EIA-negative group (46 [58%] of 79 vs 7 [27%] of 26; $P = .007$).

DISCUSSION

We found that nearly one-third of patients with CDI diagnosed using a 2-step glutamate dehydrogenase and PCR testing algorithm would have been missed if only EIA for toxin testing had been performed. Patients with CDI with negative EIA test results were less likely than others to be infected with ribotype 027 strains. However, EIA-negative patients did not differ in clinical presentation from EIA-positive patients. Notably, 21% of EIA-negative patients presented with severe CDI, including 1 patient who died of fulminant CDI. Patients with CDI with negative EIA toxin results were also as likely as were EIA-positive patients to shed spores onto their skin and into the environment. These findings suggest that use of PCR-based CDI testing methods could potentially improve clinical and infection control outcomes, compared with the use of EIA for toxins A and B.

Several studies suggest that PCR may provide a single diagnostic test for CDI that combines excellent sensitivity (>90% sensitive, compared with toxigenic culture) and rapid results [4, 5, 10]. Alternatively, a 2-step testing strategy may be used that includes detection of glutamate dehydrogenase by EIA as an initial screening method [2, 11]. In some studies, glutamate dehydrogenase testing has demonstrated excellent sensitivity [12], but others have suggested that this test is not sufficiently sensitive to be used as an initial screen for CDI [1]. If EIA for toxin A and B is used as the second step in a 2-step algorithm, an additional test, such as toxigenic culture or PCR, should be available for testing of samples in cases where CDI is suspected but the EIA result is negative.

Effective prevention of *C. difficile* transmission is dependent on rapid and accurate identification of patients with CDI [13]. Our findings demonstrate that EIA-negative patients with CDI present a significant risk for transmission. Use of diagnostic tests

with increased sensitivity could therefore be beneficial for infection control efforts to control CDI. One caveat of the use of sensitive methods that detect *C. difficile* organisms (eg, PCR and toxigenic culture) is that low levels of organisms may represent colonization rather than CDI [1]. Therefore, it is recommended that laboratories not test formed stools and that testing should only be performed when patients have clinically significant diarrhea (ie, ≥3 loose stools per day) [1, 2].

Our study has some limitations. Our patient population included primarily men, and ribotype 027 was the predominant circulating strain. Additional studies are needed in other settings. Glutamate dehydrogenase and PCR were used for diagnostic testing. Therefore, we were unable to determine what the outcomes of the EIA-negative patients with CDI might have been if they had not been treated. In practice, some patients with suspected CDI may be treated empirically despite negative EIA toxin results. Although the laboratory rejected formed stool specimens, we did not require the presence of ≥3 unformed stools within 24 hours in our case definition of CDI. However, there was no significant difference in the percentage of cases with <3 unformed stools per day in the EIA-negative and EIA-positive groups (10% and 8%, respectively). Finally, we evaluated only 1 commercial EIA assay.

In conclusion, our findings demonstrate that patients with CDI diagnosed by glutamate dehydrogenase and PCR but with negative EIA toxin results do not differ in clinical presentation from EIA-positive patients and present a significant risk for transmission of spores. These findings suggest that EIA for toxin should not be relied upon as a sole test for diagnosis of CDI.

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