ORIGINAL ARTICLE

Real-Time Polymerase Chain Reaction Detection of Asymptomatic *Clostridium difficile* Colonization and Rising *C. difficile*–Associated Disease Rates

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OBJECTIVE. To evaluate the accuracy of real-time polymerase chain reaction (PCR) for *Clostridium difficile*–associated disease (CDAD) detection, after hospital CDAD rates significantly increased following real-time PCR initiation for CDAD diagnosis.

DESIGN. Hospital-wide surveillance study following examination of CDAD incidence density rates by interrupted time series design.

SETTING. Large university-based hospital.

PARTICIPANTS. Hospitalized adult patients.

METHODS. CDAD rates were compared before and after real-time PCR implementation in a university hospital and in the absence of physician and infection control practice changes. After real-time PCR introduction, all hospitalized adult patients were screened for *C. difficile* by testing a fecal specimen by real-time PCR, toxin enzyme-linked immunosorbent assay, and toxigenic culture.

RESULTS. CDAD hospital rates significantly increased after changing from cell culture cytotoxicity assay to a real-time PCR assay. One hundred ninety-nine hospitalized subjects were enrolled, and 101 fecal specimens were collected. *C. difficile* was detected in 18 subjects (18%), including 5 subjects (28%) with either definite or probable CDAD and 13 patients (72%) with asymptomatic *C. difficile* colonization.

CONCLUSIONS. The majority of healthcare-associated diarrhea is not attributable to CDAD, and the prevalence of asymptomatic *C. difficile* colonization exceeds CDAD rates in healthcare facilities. PCR detection of asymptomatic *C. difficile* colonization among patients with non-CDAD diarrhea may be contributing to rising CDAD rates and a significant number of CDAD false positives. PCR may be useful for CDAD screening, but further study is needed to guide interpretation of PCR detection of *C. difficile* and the value of confirmatory tests. A gold standard CDAD diagnostic assay is needed.

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Clostridium difficile is emerging as the most important healthcare-associated pathogen in many hospitals. Recognition of the hypervirulent, epidemic North American pulsed-field gel electrophoresis type 1/polymerase chain reaction (PCR) ribotype 027 (NAP1/027) *C. difficile* strain in the United States in 2001 has been associated with a marked increase in the incidence of *C. difficile*–associated disease (CDAD).[1] The frequency of severe CDAD; complications including toxic megacolon, colonic perforation, colectomy, and septic shock; and mortality rates in the United States have been rising.[2] Despite conventional therapy with antibiotics, approximately 20%–30% of CDAD cases fail to respond. Another 25% of CDAD patients who initially respond to antibiotics suffer recurrent disease.[3]

Nucleic acid amplification tests (NAATs), which amplify *C. difficile* toxin genes, have been endorsed as an acceptable or preferred test of choice for CDAD diagnosis because of their reported high sensitivity and specificity and reduced time and labor requirements.[4-6] Thirty-five percent of hospitals in the Centers for Disease Control and Prevention's National Healthcare Safety Network currently employ these molecular diagnostic assays.[7] Additional reported benefits associated with real-time PCR include decreased hospital costs with earlier discontinuation of contact isolation and inappropriate antibiotics.[8] However, increasing CDAD rates have also been described following adoption of NAATs for CDAD, as a result of the increased sensitivity of the real-time PCR assay.[9-12] NAATs detect the presence of toxigenic *C. difficile*

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isolates but not toxin production and are unable to discriminate between asymptomatic *C. difficile* colonization and symptomatic infection. Following a rise in CDAD incidence rates associated with a change in diagnostic methods from cell culture cytotoxicity assay to real-time PCR for CDAD diagnosis, we conducted a hospital-wide, epidemiologic real-time PCR surveillance for asymptomatic *C. difficile* colonization and symptomatic infection at a 600-bed university hospital in Houston, Texas, to evaluate the accuracy of real-time PCR for the diagnosis of CDAD.

METHODS

Surveillance Study Population

Patients at least 18 years of age who were admitted to the study hospital were eligible for enrollment. Exclusion criteria included incapacitated individuals lacking a legal representative to consent on their behalf. A stool sample or a rectal swab was collected from each study participant. Rectal swabs were obtained if consenting subjects were unable to provide stool samples within 48 hours of enrollment. All enrolled subjects completed a brief questionnaire providing demographic and clinical information related to CDAD development. All subjects provided written, informed consent. The Committee for the Protection of Human Subjects at the University of Texas Health Science Center, the Baylor College of Medicine Institutional Review Board, and the Department of Research at the study hospital approved the study protocol.

Definitions

Diarrhea was defined as the passage of 3 or more unformed stools within 24 hours. Definite CDAD was defined as a positive real-time PCR or enzyme-linked immunosorbent assay (ELISA) confirmed by toxigenic culture in a patient with diarrhea. Probable CDAD was defined as a positive PCR, ELISA, or toxigenic culture with diarrhea. Asymptomatic colonization was defined as *C. difficile* detection by PCR, ELISA, or toxigenic culture in the absence of diarrhea.

C. difficile Detection Methods

Four different assays were used for *C. difficile* detection. Stool samples and rectal swabs were screened by DNA extraction and amplification by multiplex real-time PCR within 24 hours of specimen collection. Bacterial DNA was extracted from fecal specimens with the QIAamp DNA Stool Mini Kit (QIAGEN), according to the manufacturer's instructions. A real-time PCR assay, originally developed and used by the microbiology laboratory at Texas Children's Hospital, was modified and performed by our research laboratory at the Center for Infectious Diseases in the University of Texas School of Public Health.[9] A multiplex assay amplifying toxin A (*tcdA*) and B (*tcdB*) genes was performed on a StepOne platform (Life Technologies). The 20-μL reaction mixture consisted of 4.4 μL extracted DNA, 10.0 μL TaqMan Universal PCR Master Mix (Life Technologies), 0.4 μM *tcdA* forward and reverse primers and 0.08 μM hydrolysis probe, and 0.5 μM *tcdB* forward and reverse primers and 0.1 μM hydrolysis probe. DNA was amplified under the following conditions: initial denaturation for 15 minutes at 95°C; 45 cycles of template denaturation for 10 seconds at 95°C, followed by primer annealing and extension for 1 minute and 15 seconds at 60°C.

The study hospital microbiology laboratory is currently using the BD GeneOhm Cdiff PCR assay (Becton Dickinson) for *C. difficile* detection, which has been approved by the Food and Drug Administration for the detection of the *tcdB* gene.[13] To compare consistency, we tested stools and rectal swabs with both real-time PCR methods. However, stool samples from 10 subjects were tested only by the hospital microbiology laboratory commercial assay at the discretion of the primary physician. An ELISA assay (C. DIFFICILE TOX A/B II, TECHLAB) was used to screen stool samples for toxin A and B production. Stool samples and rectal swabs were also cultured anaerobically on cycloserine-cefoxitin-fructose agar for *C. difficile* after alcohol shock treatment with 100% ethanol to kill vegetative cells. Toxigenic *C. difficile* isolates were detected by conventional PCR amplification of *tcdA* and *tcdB* genes, as described previously.[14]

Statistical Analysis

The study hospital microbiology laboratory discontinued performance of the cell culture cytotoxicity assay and began testing with the BD GeneOhm Cdiff molecular assay in late April 2011. Overall CDAD incidence density rates and their 95% confidence intervals (CIs) at the hospital before and after the change in CDAD diagnostic testing were calculated assuming Poisson distribution with large sample approximation. Hypothesis testing was performed by normal theory test, which applies large sample approximation to binomial test for the 2 incidence rates.[15] Further, an interrupted time series design using monthly incidence data was performed to assess for any difference in CDAD incidence rates associated with the change in CDAD testing. Serial autocorrelation of CDAD rates was assessed with an autoregressive integrated moving average (ARIMA) model, using a Box-Jenkins-Tiao strategy.[16] Briefly, CDAD monthly rates were plotted over time for hospitalized patients. The graphs were visually inspected to assess trend or nonstationarity of the data and adjusted in the ARIMA model.

Significant proportional differences were evaluated with a Fisher exact test or χ^2 analysis for categorical variables. Continuous variables were compared by *t* test and Wilcoxon rank sum test. Statistical analyses were conducted using SAS (ver. 9.1) software.

RESULTS

Following the introduction of PCR testing for CDAD, CDAD rates significantly increased and currently remain elevated compared with CDAD rates prior to adoption of NAAT test-

ing (Figure 1). Assuming a Poisson distribution with normal approximation, the mean *C. difficile* detection rate at the hospital doubled from 13.4 per 10,000 patient days (May 2010–April 2011; 95% CI, 11.8–15.0) to 27.0 per 10,000 patient days (May 2011–April 2012; 95% CI, 24.7–29.3) after PCR initiation and without other apparent changes in the study population, physician practice, or infection control policy. Normal theory test shows $P < .0001$. A significant increase in the CDAD rate was also observed after implementation of the BD PCR assay with an interrupted time series design assessment (Wald χ^2, 225.21; $P < .0001$). The ARIMA model estimated that the change in diagnostic assay from cell culture cytotoxicity assay to PCR was associated with a rate increase of 13.6 cases of CDAD per 10,000 patient days (95% CI, 10.2–17.0 cases per 10,000 patient days).

The increased CDAD rates associated with PCR testing at the study hospital led us to conduct an epidemiologic survey to evaluate the prevalence of CDAD and asymptomatic colonization identification by PCR. Among 415 patients admitted to the study hospital, 22 patients were excluded because they were incapacitated with no available legal representative to consent on their behalf. One hundred ninety-nine (51%) of the eligible patients were enrolled January 8–11, 2013 (Figure 2). One hundred ninety-four patients declined to participate in this study. Stool samples or rectal swabs were collected from 101 (51%) enrolled subjects. *C. difficile* was detected in 18 (18%) subjects who provided a fecal specimen for testing (Table 1). The mean age of enrolled subjects was 60 years, and 52% were male. Two-thirds of subjects were receiving antibiotics at enrollment, and 45% were receiving an antacid medication. Diarrhea was reported by 12% of subjects; 7% of participants described a previous history of CDAD. No significant differences in clinical characteristics were observed between *C. difficile*–positive subjects and *C. difficile*–negative patients, although *C. difficile* subjects reported diarrhea more frequently than non–*C. difficile* subjects (Table 2).

Among the 18 *C. difficile*–positive subjects, 1 subject (6%) was identified with definite CDAD, 4 subjects (22%) with probable CDAD, and 13 patients (72%) with asymptomatic colonization. The majority of subjects identified with *C. difficile* were asymptomatic, irrespective of the detection method, including 8 of 12 (67%) *C. difficile*–positive subjects by PCR, 11 of 13 (85%) *C. difficile*–positive subjects by toxigenic culture, and 3 of 4 (75%) *C. difficile*–positive patients by ELISA. Concordant results were obtained for 71 of 74 (96%) stool samples tested by the 2 real-time PCR methods. Asymptomatically colonized subjects were more likely to have a history of residing in a healthcare facility within the past 6 months, to have a previous CDAD, and to be receiving antibiotics. However, the only significant difference between subjects with CDAD and *C. difficile*–colonized patients was the mean number of stools passed in the previous 24 hours (5.6 ± 3.8 vs 0.9 ± 0.8 stools; $P < .01$).

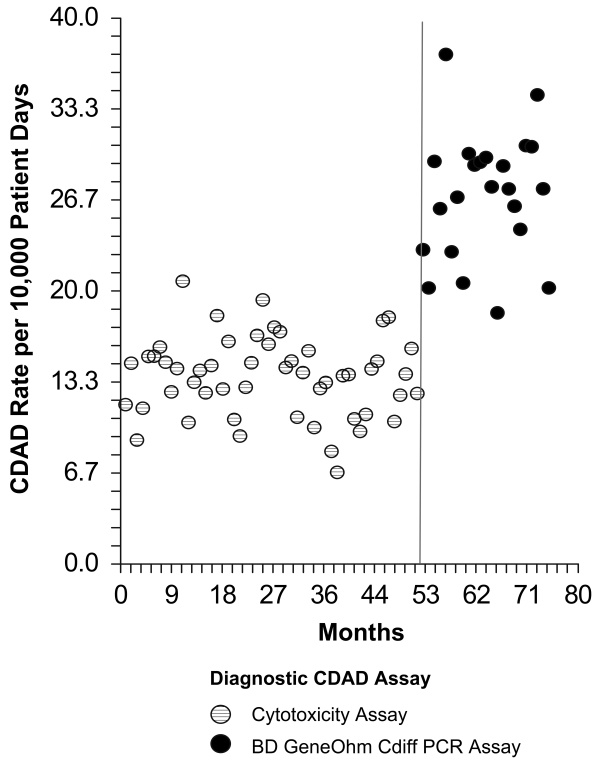

FIGURE 1. *Clostridium difficile*–associated disease (CDAD) incidence density rates at a 600-bed university hospital in Houston, Texas. Data are for CDAD incidence density rates from January 2007 to March 2013. Line indicates when CDAD detection methods were changed at the study hospital. Interrupted time series design was used to assess the change in CDAD rates before and after the change in diagnostic assay to real-time polymerase chain reaction (PCR). A significant increase in the CDAD rate was observed with the implementation of the BD PCR assay (Wald χ^2, 225.21; $P < .0001$).

DISCUSSION

Accurate diagnosis of CDAD is challenging. Although CDAD causes a tremendous burden of disease among hospitalized patients and is the most commonly identified cause of diarrhea-associated mortality in the United States,[17] the majority of healthcare-associated diarrhea is not attributable to CDAD. Approximately 75% of healthcare-associated diarrhea episodes are due to causes other than CDAD.[18] Often, no specific etiologic diarrheal agent is recognized.

NAATs are increasingly being used by clinical laboratories for CDAD identification. In the present university hospital, a significant rise in detection of *C. difficile* was observed following real-time PCR assay initiation. In this study, we attempted to examine the potential significance and implications of this dramatic rise in CDAD rates at the study hospital by investigating the relative prevalence of asymptomatic *C. difficile* colonization and CDAD identified by PCR.

Although greater frequency of asymptomatic *C. difficile* colonization compared with CDAD prevalence among patients

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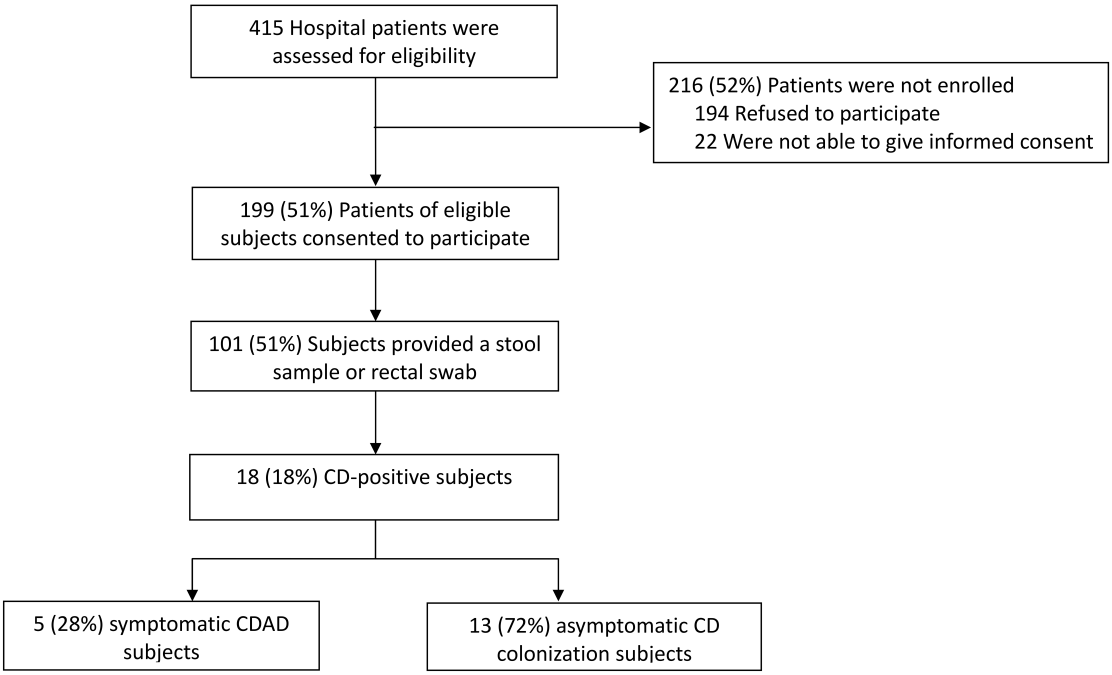

FIGURE 2. Study enrollment and results of *Clostridium difficile* (CD)–associated disease (CDAD) testing.

at healthcare institutions has been demonstrated, with asymptomatic *C. difficile* colonization ranging from 4% to 16% at acute care facilities[19-21] and up to 51% at long-term care facilities,[22] these previous studies of asymptomatic colonization used selective, anaerobic culture of stool samples or rectal swabs for *C. difficile* detection, with confirmation of toxigenic *C. difficile* isolates by cytotoxicity assay[19,21] or ELISA.[22] Epidemiologic surveillance for asymptomatic *C. difficile* colonization using NAATs has not been previously described. We examined whether the high sensitivity of the real-time PCR assay for *C. difficile* would lead to a greater frequency of asymptomatic *C. difficile* colonization at the study hospital compared with these previous reports. In this study, the overall prevalence of diarrhea was 12% among hospitalized patients, and the prevalence of *C. difficile* detection was 18%. Asymptomatic colonization with *C. difficile* was approximately 3 times more frequent than CDAD (13% vs 5%; $P < .05$). The prevalence of asymptomatic colonization by PCR was 8% versus 12% by toxigenic culture.

Although PCR for *C. difficile* toxin genes has been shown to correlate well with toxigenic culture, often considered the gold standard for CDAD diagnosis,[23] neither assay measures toxin production, the primary mechanism of *C. difficile* pathogenesis. Recognition of toxigenic isolates alone may not be sufficient to establish CDAD. At least half of patients with toxigenic isolates may remain asymptomatic.[19,21] The difficulty in interpreting the clinical significance of *C. difficile* detected by NAATs is emphasized by recent studies describing the importance of confirmation of *C. difficile* toxin production. Planche et al[24] demonstrated that worse CDAD clinical outcomes were significantly associated with toxin production, but no relationship between clinical outcomes and positive toxigenic culture alone was observed. Similarly, in another study, CDAD patients identified by PCR alone were significantly less likely to develop a CDAD complication than hosts who were positive by both PCR and a toxin assay.[11]

Understanding the clinical implications of an NAAT positive for *C. difficile* is further complicated by common CDAD host characteristics. CDAD hosts are typically sicker patients requiring hospitalization for prolonged periods of time, increasing their risk for acquiring *C. difficile*. However, CDAD patients often have multiple comorbidities and several potential underlying causes for diarrhea, including medications such as antibiotics and laxatives, tube feeding, altered intestinal motility related to gastrointestinal diseases such inflammatory bowel disease,[25] and enteric infection with other enteropathogens such as noroviruses.[26] In this study, established risk factors for CDAD—such as recent exposure to a healthcare facility, receipt of antibiotics or antacids, and past history of CDAD—were similarly identified in colonized patients and CDAD subjects. In spite of the high sensitivity of NAATs for *C. difficile* detection, PCR assays are unable to distinguish asymptomatic colonization from symptomatic disease. Reports are emerging that suggest a poor positive predictive value of real-time PCR for CDAD diagnosis when the endemic *C. difficile* prevalence is low.[27-29] Since there is no true gold standard for diagnosing CDAD, we cannot exclude the possibility that our CDAD-diagnosed patients had diarrhea from causes other than *C. difficile*.

With their high negative predictive value, NAATs serve as

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TABLE 1. *Clostridium difficile* Detection by Assay Performed

Test	*C. difficile* detected	*C. difficile* colonization	Probable CDAD	Definite CDAD	Probable or definite CDAD
UTSPH real-time PCR	8/91 (8.8)	7/91(7.7)	0	1/91 (1.1)	1/91 (1.1)
BD GeneOhm Cdiff PCR assay	9/84 (10.7)	5/84 (6.0)	4/84 (4.8)	0	4/84 (4.8)
ELISA for toxins A and B	4/75 (5.3)	3/75 (4.0)	0	1/75 (1.3)	1/75 (1.3)
Toxigenic culture	13/90 (14.4)	11/90 (12.2)	1/90 (1.1)	1/90 (1.1)	2/90 (2.2)
Cumulative test results[a]	18/101 (17.8)	13[b]/101 (12.9)	4/101 (4.0)	1/101 (1.0)	5/101 (5.0)

NOTE. Data are no. (%). The discrepancy in denominators for the different tests reflects an inadequate amount of stool from certain subjects to be tested by all assays. The 101 fecal samples collected include 10 stools tested only by the BD assay in addition to 91 fecal samples tested by the University of Texas School of Public Health (UTSPH) real-time polymerase chain reaction (PCR). CDAD, *Clostridium difficile*–associated disease; ELISA, enzyme-linked immunosorbent assay.

[a] Cumulative test results represent the total number of subjects for each *C. difficile* category identified by the different diagnostic methods, including some subjects who were positive by more than 1 assay.

[b] *C. difficile*–colonized subjects included patients positive by PCR alone ($n = 1$); toxigenic culture alone ($n = 5$); PCR and toxigenic culture ($n = 4$); PCR and ELISA ($n = 1$); and ELISA, PCR, and toxigenic culture ($n = 2$).

an ideal screening test. In this study, we also used an ELISA for detection of *C. difficile* toxin production, since ELISA has been proposed as an adjunct test to NAAT to establish disease pathogenesis in multistep algorithms, currently employed in England.[29] However, multistep testing with PCR has not been universally accepted as the recommended strategy for diagnosing CDAD, and many clinical laboratories in the United States are using NAATs alone to detect *C. difficile*.[30] Furthermore, in our study, 3 of 4 subjects positive for *C. difficile* toxin production by ELISA were only asymptomatically colonized. Toxin detection is also not necessarily indicative of active CDAD. Persistent toxin production after clinical resolution of CDAD symptoms is well described and has led to the recommendation to avoid using *C. difficile* assays as a test of cure.[31] Further study is needed to determine the optimal confirmatory test for NAATs. We are currently developing a quantitative real-time PCR assay for *C. difficile* toxin genes and plan to evaluate whether this assay and others—including toxin ELISA and detection of fecal inflammatory markers such as lactoferrin, calprotectin, or IL-8—can increase the positive predictive value and the specificity of CDAD diagnosis when performed in conjunction with NAATs.

Inappropriate CDAD diagnosis may lead to unnecessary antibiotic exposure and adverse effects, including increased risk of CDAD by alteration of the intestinal microbiota,[32] exclusion from certain healthcare facilities that deny access to CDAD patients, and potentially wasted resources for contact isolation. The benefit of contact isolation for asymptomatically colonized patients to reduce *C. difficile* transmission has yet to be demonstrated. In addition, falsely inflated CDAD rates with PCR assays may compromise hospital reimbursements in the future, if the Centers for Medicare and Medicaid Services consider CDAD as a preventable hospital-acquired condition. Mandatory reporting of hospital CDAD rates has been initiated in the United States and is required in other countries as well.

Limitations of this study include enrollment of 51% of eligible patients and fecal specimen collection from only half of enrolled subjects. Failure to enroll subjects was due to patient refusal to participate or inability to consent. Exclusion of incapacitated patients unable to provide consent may have excluded some potentially sicker patients. However, these patients represented only 5% of patients approached for consent. In addition, all patients unable to consent because of their underlying medical condition were visited multiple times to identify legal representatives who could consent on their behalf. Clinical information was not collected for unenrolled subjects; as a result, we cannot exclude potential selection bias in this study. Stool collection was limited primarily by our inability to collect specimens from subjects prior to their hospital discharge. Nevertheless, we believe that our study results are representative of the hospital inpatients. Previous surveillance at the hospital in 2004 also noted an overall diarrhea frequency of 12%, although diarrhea was defined by the passage of 2 or more stools within 24 hours in this earlier study.[33] In the present study, asymptomatic patients were included for *C. difficile* testing for the purpose of evaluating the relative frequency of asymptomatic *C. difficile* colonization to determine whether NAATs may be detecting false positives among diarrhea patients. The nearly 3-fold greater prevalence of asymptomatic *C. difficile* colonization than CDAD frequency among our hospitalized patients suggests that a significant proportion of patients diagnosed with CDAD by NAATs may actually only be colonized. For routine *C. difficile* testing, however, we advocate adhering to the recommendation for testing of only diarrheal stools for CDAD diagnosis.[6] Finally, this study was a prevalence study at a single institution.

In the healthcare setting, where the majority of diarrhea cases are not attributable to CDAD and the prevalence of asymptomatic *C. difficile* colonization is greater than the frequency of CDAD, NAAT detection of asymptomatic colo-

TABLE 2. Demographic Characteristics of Study Population by *Clostridium difficile* Status

Characteristic	*C. difficile* positive (n = 18)	*C. difficile* negative (n = 83)	P	CDAD (n = 5)	*C. difficile* colonized (n = 13)	P
Age, mean ± SD, years	57.0 ± 16.2	60.2 ± 15.8	.47	53.0 ± 16.8	58.5 ± 16.4	.59
Sex, male	8 (44.4)	44 (54.3)	.45	2 (40.0)	6 (46.2)	1.00
Residence in a healthcare facility within past 6 months	2 (11.1)	9 (11.4)	1.00	0	2 (15.4)	1.00
Hospitalized within past 3 months	7 (38.9)	35 (43.8)	.71	2 (40.0)	5 (38.5)	1.00
Antibiotic use	11 (61.1)	56 (68.3)	.56	2 (40.0)	9 (69.2)	.33
Antacid use	7 (38.9)	37 (45.1)	.63	2 (40.0)	5 (38.5)	1.00
History of previous CDAD	1 (5.6)	11 (13.6)	.69	0	1 (7.7)	1.00
Stools in past 24 hours, mean ± SD	2.2 ± 2.9	1.8 ± 2.5	.51	5.6 ± 3.8	0.9 ± 0.8	<.01
Diarrhea within past 24 hours	5 (27.8)	10 (12.2)	.09	...	...	...

NOTE. Data are no. (%), unless otherwise indicated. *C. difficile* positive denotes *C. difficile* detected in stool or rectal swab; CDAD denotes *C. difficile*–associated diarrhea (diarrhea plus a positive stool for *C. difficile*). SD, standard deviation.

nization among healthcare-associated diarrhea patients may be contributing to a significant number of CDAD false positives. Unfortunately, the precise extent of this misclassification of asymptomatically colonized individuals as CDAD is unknown and cannot be accurately assessed because a true gold standard for CDAD diagnosis is currently lacking. Better CDAD diagnostic methods—such as multistep algorithms including confirmation of PCR-positive stools with toxin production or intestinal inflammation—are urgently needed to avoid compromising patient safety with the administration of unnecessary antibiotics and future hospital financial reimbursements related to CDAD.

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