

Disparate prevalence of toxigenic and nontoxigenic *Clostridium difficile* among distinct adult patient populations in a single institution

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Clostridium difficile (CD) disease remains a costly and important hospital-associated infection. Although nontoxigenic CD is detected by some CD testing methods, can interfere with some detection algorithms and has been suggested as a treatment for CD disease, little is known about the relative occurrence of toxigenic and nontoxigenic CD in a single institution. We used both chromogenic and selective agar media to recover CD isolates and a molecular method to detect the toxin B gene from over 2400 fresh unformed stool specimens with isolates further tested for the toxin B gene. We recovered 74 nontoxigenic and 306 toxigenic CD isolates for which a collection site could be assigned. The frequency of recovery of toxigenic and nontoxigenic CD for each hospital location and the ratio of toxigenic to nontoxigenic CD were calculated. Although the overall prevalence of toxigenic and nontoxigenic CD was 12.7 % and 3.1 %, respectively, on some wards, 48 % of all CD were nontoxigenic, while on other wards, ≤ 5 % were nontoxigenic. The disparate ratios of nontoxigenic CD to toxigenic CD presented here for the various 'groups' within the adult veteran population are important to the ongoing discussion and reexamination of other published work on the occurrence of toxigenic and nontoxigenic CD, for evaluating the performance of CD detection tests, for designing infection control strategies and in ultimately understanding both CD carriage and disease.

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INTRODUCTION

Clostridium difficile (CD), a Gram-positive, spore-forming anaerobic bacillus, is a major nosocomial pathogen causing a range of symptoms from mild to severe diarrhoea and is the aetiological agent of pseudomembranous colitis. The difficulties and strategies for diagnosing CD disease have been well documented and reviewed (Bamber *et al.*, 2012; Barbut *et al.*, 2003; Delmee, 2001; Fenner *et al.*, 2008; Tenover *et al.*, 2011; Wren *et al.*, 2009a, b). There have also been many studies comparing methods of detecting CD, particularly the value of toxin detection versus culture (Dalpke *et al.*, 2013; Dubberke *et al.*, 2011; Hernandez-Rocha *et al.*, 2013; Peterson *et al.*, 2011) and the relative performance of different media for the recovery of CD (Carson *et al.*, 2013). Cepheid Xpert CD assay (XCD) has been shown to be a rapid and accurate system for detection of the toxin B gene directly from stool specimens with reported sensitivities and specificities in

the 96 % to 100 % range (Babady *et al.*, 2010; Culbreath *et al.*, 2012; Dalpke *et al.*, 2013; Goldenberg *et al.*, 2010; Shin *et al.*, 2012; Viala *et al.*, 2012; Williamson *et al.*, 2013; Zilberberg *et al.*, 2010). In addition to toxin detection, it is sometimes necessary to isolate CD for susceptibility testing, epidemiological investigations and evaluation of new CD detection methods. Therefore, it is important to continue to pursue improvements in culture methods for CD. Although detection of CD by culture is typically less expensive than toxin detection by molecular methods, the turnaround time can be significantly longer (about 2–4 days) compared to detection of CD toxin by molecular methods such as the XCD system (turnaround time less than 2 h). However, a chromogenic medium (bioMérieux) (ChromID) has been developed, which allows good growth and easier discernment of both toxigenic and nontoxigenic CD colonies and which can be read at 24 h. Using these methods, we were able to correlate recovery of both toxigenic and nontoxigenic CD with the location of the patient in our hospital system. Our objective was to assess the prevalence of toxigenic and nontoxigenic CD isolates for each of our wards and patient groups for infection prevention and epidemiological purposes.

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Abbreviations: BMTU, bone marrow transplant unit; CD, *Clostridium difficile*; SCI, spinal cord injury; XCD, Xpert *C. difficile* assay.

METHODS

Specimens. A total of 2467 fresh unformed stool specimens were examined by culture for the recovery of CD and detection of CD toxin. Information regarding the ward of collection was available for 2401 of these specimens. All specimens were from patients at the Puget Sound Veterans Affairs Medical Center in Seattle, Washington. We tested only unformed stools and requested that these be submitted only from symptomatic patients. We studied only one isolate per specimen. All isolates were from unique patients except for seven patients who had two positive specimens each, which were collected at least 1 month apart. The ward for our purposes was the location of the patient at the time the specimen was collected.

Growth and identification of isolates. PML CD agar (formerly PML), the previously validated medium used by our laboratory for over 15 years, was a proprietary medium which combined an enriched anaerobic brain–heart infusion blood agar with cycloserine-cefoxitin. The ChromID CD agar (ChromID) (bioMérieux) consists of an enriched nutritive base combining different peptones and taurocholate which favours the germination of spores (Carson *et al.*, 2013; Eckert *et al.*, 2013). It also contains a proprietary chromogenic substrate and a mixture of antibiotics which enable the detection and identification of β -glucosidase-producing CD strains based on the colour of colonies and the inhibition of most Gram-positive and Gram-negative bacteria, yeasts and moulds. It is examined after 24 h incubation for typical grey to black colonies with an irregular or smooth border. CD isolates were selected by colony morphology and identified by Gram staining and the proline test [the production of L-proline-aminopeptidase (PRO discs; Carr-Scarborough Microbiologicals)], which were performed directly from the isolated colonies. To validate that both PML and ChromID were performing as expected in our laboratory, we also subcultured a representative colony to anaerobic brain–heart infusion, performed the indole test and looked for apple-green fluorescence of the colony with UV light. Anaerobic incubation was performed in jars with a gas pouch which generates an environment of less than 0.1 % of oxygen and more than 15 % of CO₂ (Mitsubishi Gas). In addition to identifying CD isolates by biochemical testing, 16S rRNA gene sequencing was performed on 32 isolates (Mahlen & Clarridge, 2011).

Before PML medium was discontinued by the manufacturer, we validated the equivalency of PML medium and the new ChromID medium using 204 fresh stool specimens. Overall, there were 1002 stool specimens cultured only on PML agar, 1261 stool specimens cultured only on ChromID agar and 204 specimens cultured on both media. Toxinogenic culture was accomplished by combining these growth methods with the toxin detection method below.

Toxin detection from diarrhoeal stools. The GeneXpert CD assay (XCD) (Cepheid) was used for the detection of the toxin B gene (*tcdB*) (Dalpke *et al.*, 2013). The system automates and integrates sample purification, nucleic acid amplification and detection of the target sequence using real-time PCR assays for qualitative detection of CD toxin B directly from unformed (conform to the container) stool specimens of patients suspected of having CD infection.

Toxin detection from colonies. The XCD test for the detection of CD toxin B in stool specimens was adapted for toxin detection from colonies. Instead of using stool samples, two to three colonies were emulsified into 200 μ l of sample diluent in a test tube, and 100 μ l of the dilution was tested. All CD isolates from CD toxin B-negative stools were tested for toxin production by XCD. Fifty CD isolates from CD toxin B-positive stools were tested for toxin production by XCD.

For *tcdA* and/or *tcdB*, the detection of a 235 (*tcdA*) or 204 (*tcdB*) bp amplicon was accomplished by melt curve of Real-Time (RT)-PCR performed on a BioRad MyIQ I-Cycler. The test was developed by Dr Xuan Qin, University of Washington, and performed in her laboratory using

the original primers: *toxA*-F: CAT GGG ATA GAT ATC AGG GC; *toxA*-R: ATT TCC CAA CGG TCT AGT CC; *toxBI*-F: CAA TTG GAG AAG CAA TTG AAA and *toxBI*-R: GCC TTG TAA ACC ATC TTG ATT TG. This test was used on 28 isolates to validate the off-label use of XCD to determine the toxigenicity of colony isolates of CD.

Statistical analysis. Sensitivities, specificities, positive predictive values and negative predictive values were used to measure reliability for the two toxin assays and two culture methods. The statistical analyses were performed using MedCalc for Windows, version 12.7.5 (MedCalc Software), to generate the sensitivity, specificity, positive predictive value and negative predictive value where appropriate. The chi-squared test using GraphPad QuickCalcs software was used to compute significant values (*P* values).

RESULTS

To evaluate the equivalency of PML medium and ChromID medium, we cultured 204 consecutive unformed stools on both PML and ChromID media. For the recovery of CD, PML and ChromID are equivalently sensitive (Table 1). Although ChromID recovered more isolates (44 total with 8 nontoxigenic) compared to PML (39 total with 5 nontoxigenic), the differences were not significant ($P \geq 0.10$ for both comparisons). For the remainder of the study objectives (i.e. to determine the prevalence of both toxigenic and nontoxigenic CD isolated in our hospital), we utilized results from either agar for recovery of CD.

To validate the off-label use of XCD for the presence of toxin B gene, we evaluated 28 isolates; 23 of the presumptive nontoxigenic strains and 5 toxigenic strains were further analysed by an independent laboratory-developed test for the *tcdA* or *tcdB* toxin gene; more nontoxigenic strains were examined, as false-negative results are more probable than false-positive results. All 28 agreed with the toxin B gene detection results using XCD. Of the 50 colonies isolated from toxin B gene-positive stools, 49 were toxin B gene positive. Of the 84 colonies isolated from toxin B gene-negative stools, 9 were toxin B gene positive, and 75 were toxin B gene negative.

Of the 2467 stool specimens, information regarding the ward of collection was available for 2401 specimens. From these 2401 specimens, we recovered nontoxigenic CD from 74 (3.1 %) and toxigenic CD from 306 (12.7 %). For further analysis, we used only locations from which ≥ 70 total specimens were received. There were 188 specimens

Table 1. Comparison of PML and ChromID agars for recovery of CD

	ChromID positive	ChromID negative
PML positive	36 (4 strains nontoxigenic)	3 (1 strain nontoxigenic)
PML negative	8 (4 strains nontoxigenic)	157

All strains were CD by biochemical analysis; nine strains did not produce toxin.

from wards or locations with fewer than 70 submissions; of these, there were 1 nontoxigenic and 18 toxigenic isolates which were not counted in the final correlation. Thus, there were 73 nontoxigenic and 288 (13.0 %) toxigenic CD isolates with which to study prevalence patterns in this institution. Table 2 shows the variation in prevalence of toxigenic and nontoxigenic CD by location and the type of patient populations. The lowest rates of recovery of toxigenic CD were on the bone marrow transplant unit (BMTU), with a rate of 6.2 %, and the medicine intensive care unit, with a rate of 7.2 %. The highest rates of toxigenic CD were found in the spinal cord injury (SCI) and long-term care wards, which are physically next to each other in the hospital, with an average rate of about 23 %. These rates were significantly lower and higher, respectively, when compared to the group as a whole. The

remaining wards had rates of toxigenic CD varying between 11 % and 13 %. The sites identified as clinics or outpatient locations varied from 11 % to 18 %.

The highest rates of nontoxigenic CD were found in the SCI ward, with a rate of 8.3 %, followed by BMTU, with a rate of 5.8 %, which were significantly higher when compared to the group as a whole. The remaining wards had rates of nontoxigenic CD varying between 0 % and 5.6 %.

Although the rates of isolation of both toxigenic and nontoxigenic CD by ward varied, the ratio of the nontoxigenic CD to all CD isolated showed wider variability (Table 2). Almost half (48 %) of the BMTU isolates were nontoxigenic (significantly different from any other ward) ($P=0.0001$). Five locations, four inpatients and one outpatient, had ratios of nontoxigenic to all isolates ranging from 21 % to

Table 2. Disparate prevalence of toxigenic and nontoxigenic CD among patient populations

Type of ward	O/ I	No. of specimens tested	Nontoxigenic CD isolates (<i>n</i>)	% of total patient specimens with nontoxigenic CD (<i>P</i> value*)	Toxigenic CD isolates	% of total patient specimens with toxigenic CD (<i>P</i> value*)	% of total patient specimens negative by both culture and toxin tests	% nontoxigenic CD to total CD isolates (<i>P</i> value*)
Surgery ICU	I	119	4	3.4	15	12.6	81.5	21
3 E surgery	I	89	0	0.0	11	12.4	84.3	0
SCI	I	72	6	8.3 ($P=0.017$)	16	22.2 ($P=0.02$)	65.3	27
Bone marrow transplant	I	260	15†	5.8 ($P=0.026$)	16	6.2 ($P=0.001$)	86.5	48 ($P=0.0001$)
6 W medicine	I	278	15	5.4	37	13.3	78.4	29
Cardiac care	I	143	5	3.5	16	11.2	80.4	24
Long-term chronic	I	73	1	1.4	18	24.7 ($P=0.003$)	71.2	5
Medicine ICU	I	166	2	1.2	12	7.2 ($P=0.02$)	84.3	14
Medicine: chronic and acute	I	251	3	1.2	32	12.7	81.3	9
Seattle clinics	O	126	7	5.6	14	11.1	84.1	33
Outside clinics	O	406	8	2.0	59	14.5	80.3	12
Walk-in, ER	O	230	7	3.0	42	18.3 ($P=0.018$)	75.7	14
Total or average for this table		2213	73	3.3	288	13.0	83.7	20.2
Total for all wards including those from which we received <70 specimens		2401	74	3.1	306	12.7	84.2	19.6

I, inpatient; O, outpatient; ICU, intensive care unit; SCI, spinal cord injury; ER, emergency room.

Of the 2401 evaluable specimens, 188 were from wards or locations with fewer than 70 specimens and were not tabulated leaving 2213 specimens and 361 isolates.

**P* values included for statistically significant differences only.

†Fifteen specimens and 11 patients but cultures from same patient were taken more than 1 month apart.

33 %. The six remaining locations, four inpatients and two outpatients, had ratios less than 14 %.

DISCUSSION

The reported prevalence rates of toxigenic and nontoxigenic CD found in different populations vary greatly, even when only recent studies with more accurate methods are included. In general, reported rates are higher for the elderly and for infants, but this is not consistent (Boone *et al.*, 2012; Delmee *et al.*, 1988; Poilane *et al.*, 2007; Rousseau *et al.*, 2011, 2012). For example, in healthy infants, Rousseau *et al.* (2011, 2012) found that 32 % carried nontoxigenic CD and 13 % carried toxigenic CD. In a study using clinically indicated specimens, Boone *et al.* (2012) showed that nontoxigenic CD rates were lowest for nursing home patients and highest for patients younger than 20 years. However, the toxigenic CD rate was very high (40 %) for nursing home patients in their study. We did not find the differences in CD rates between inpatient and outpatient specimens (with outpatients being lower) which some reported (Boone *et al.*, 2012). One explanation may be that patients in our healthcare system receive care repeatedly and over longer periods in both inpatient wards and outpatient clinics. Another unique factor due to our hospital system may be that patients in the BMTU or SCI services are designated as receiving care as from an inpatient location regardless of the physical location where their care is received. Our data indicating differences within one hospital may lead to reevaluation of the publications citing ratios of toxigenic to nontoxigenic CD, as the exact populations within the institution that were sampled are often not mentioned.

We examined only specimens from symptomatic patients. Because we collected no data on patient disease, we could not make any association with disease states such as pseudomembranous colitis or carriage. Our aim was to gather a baseline rate of relative frequency of toxigenic to nontoxigenic CD in the various locations in the hospital for infection prevention and epidemiological purposes. In addition, the isolation of nontoxigenic CD is generally accepted to mean carriage or colonization without being associated with disease. However, Furuya-Kanamori *et al.* (2015) noted that the definition of asymptomatic colonization and carriage of CD is not firm and that outcomes subsequent to colonization with toxigenic and nontoxigenic CD are not known.

Studies have postulated a possible role for nontoxigenic CD in colonization and as a probiotic strain to prevent the colonization of toxigenic CD; however, many questions remain as to the significance of these isolates (Nagaro *et al.*, 2013; Sambol *et al.*, 2002; Shim *et al.*, 1998). Recovery and identification of both toxigenic and nontoxigenic CD strains may be important in defining routes of transmission of CD and in investigating their role in the gut microbiome. Although treatment of CD disease has generally involved antibiotics (e.g. vancomycin, metronidazole and

fidaxomicin), more recently, faecal biotherapy (transplants of healthy stool) or defined organism transplants or probiotics and even nontoxigenic CD strains have been used as treatment (McFarland, 2011; Surawicz *et al.*, 2013). Clinical laboratories might play a larger role in the differentiation of toxigenic and nontoxigenic organisms as the evolution of treatment modalities progresses.

The relative frequency of occurrence of toxigenic and nontoxigenic CD is important in evaluating and instituting testing strategies for the detection of CD: two- and three-step diagnostic algorithms, which are based on first detecting all CD (e.g. a test for glutamate dehydrogenase) and then determining toxigenicity, will perform differently with populations having different ratios of toxigenic to nontoxigenic CD. From a diagnostic perspective, a two-step diagnostic algorithm would be less cost-effective and efficient in a patient population where there is a high frequency of nontoxigenic CD, as in, for example, our BMTU. In addition, the prevalence of nontoxigenic CD might also be a marker for general faecal contamination or deficiencies in decontamination procedures. The persistence of these strains within a facility can easily be overlooked without a robust monitoring strategy and baseline data with which to compare them to. Ongoing and longitudinal analysis will likely elucidate transmission and persistence differences within and between units at our institution.

Our data were gathered under common clinical laboratory conditions which may have allowed rare errors. Our method detected only the toxin B gene and thus would not have detected the rare CD strains which cause disease with only toxin A or the binary toxin. It also would register a false positive if the toxin B gene was present, but for other reasons, toxin was not produced. However, the correlation of XCD gene detection with the presence of toxin has been studied elsewhere (Tenover *et al.*, 2010) and found to be excellent. In addition, only one CD colony was studied per subject, although co-infection by both toxigenic and nontoxigenic strains has been reported; however, there is no reason this would have biased this collection of toxigenic and nontoxigenic CD.

We measured the frequency of recovery of nontoxigenic and toxigenic CD in various populations in our medical centre. We had initially hypothesized that rates of both toxigenic and nontoxigenic CD would rise and fall together and correlate with infection control practices. However, that was not the case. In contrast, our study demonstrates that there are disparate ratios of toxigenic and nontoxigenic CD among the adult patient populations within the same healthcare facility. A likely reason for this is the different ordering patterns and causes of diarrhoea on the wards. For example, on the BMTU, multiple medications given to patients cause diarrhoea, yet the CD test is routinely ordered by clinicians due to its excellent negative predictive value and the fact that a missed diagnosis, even if unlikely, would be disastrous. Examples of different ordering patterns which would lead to different rates are those used by

BMTU, which samples continuously, and SCI ward, which samples more frequently when there is thought to be a problem of transmission. The SCI ward shares the problem with the BMTU in that new occurrences of diarrhoea are difficult to distinguish.

Overall, we found a wide variation in the proportion of nontoxigenic to toxigenic CD at our institution: in some locations (e.g. BMTU), 48 % of all CD were nontoxigenic, while in other locations, only 0 % to 9 % were nontoxigenic. The disparate ratios of nontoxigenic CD to toxigenic CD presented here for the various 'groups' within the adult veteran population are important to the ongoing discussion and reexamination of other published work on the occurrence of toxigenic and nontoxigenic CD, for evaluating the performance of CD detection tests, for designing infection control strategies and in ultimately understanding both CD carriage and disease.

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