Prevalence and Duration of Asymptomatic *Clostridium difficile* Carriage Among Healthy Subjects in Pittsburgh, Pennsylvania

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Summary: Healthy subjects with no risk factors for *Clostridium difficile* infection were surveyed for *C. difficile* (CD) carriage by anaerobic culture. This study identified 7 (6.7%) individuals with asymptomatic CD colonization within a study population of 106 residents from the general Allegheny County, PA population and confirms prior CD colonization estimates in healthy individuals.
ABSTRACT

Previous studies suggest that 7-15% of healthy adults are colonized with toxigenic *Clostridium difficile* (CD). To investigate the epidemiology, genetic diversity, and duration of CD colonization in asymptomatic persons we recruited healthy adults in the general Allegheny County, PA population. Participants provided epidemiologic and dietary intake data and submitted a stool specimen. The presence of CD in stool specimens was determined by anaerobic culture. Stool specimens yielding CD underwent nucleic acid testing of the *tcd*A gene segment with a commercial assay; *tcd*C genotyping was performed on CD isolates. Subjects positive for CD by toxigenic anaerobic culture were asked to submit additional specimens. One hundred six (81%) of 130 subjects submitted specimens, 7 of whom (6.6%) were colonized with CD. Seven distinct *tcd*C genotypes were observed among 7 CD colonized individuals, including *tcd*C 20, which has been found in uncooked ground pork in our region. Two (33%) out of 6 CD colonized subjects who submitted additional specimens were positive for identical CD strains on successive occasions 1 month apart. The prevalence of CD carriage in this healthy cohort is concordant with prior estimates. CD-colonized individuals may be important reservoirs for CDI and may test falsely positive for infection due to CDI when evaluated for community acquired diarrhea caused by other enteric pathogens.
INTRODUCTION

*Clostridium difficile* infection (CDI) has shown dramatic increase in incidence and morbidity during the last decade. (1) While *C. difficile* (CD) is well established as a healthcare-associated pathogen, incidence estimates for community-acquired CDI (CA-CDI) vary between 6-30%. (2-4) The source of CD in community-acquired cases is unclear. Prevalence estimates for asymptomatic CD colonization range from 7-15% among healthy, non-health care workers (HCW) outside the US to 1-4% among HCW in the US and abroad. (5-9) Individuals colonized with CD could represent a potential reservoir of strains imported into hospitals as well as a source of false positive clinical testing for CDI among patients with community acquired diarrheal illness, especially norovirus and *Clostridium perfringens*. We studied the prevalence, genotypic distribution, and duration of CD carriage among healthy adults in Allegheny County and evaluated potential risk factors for asymptomatic colonization. We also examined the relative abundance of CD and the sensitivity of a commercial nucleic acid test for detection of CD in the stool of healthy colonized individuals.
MATERIALS AND METHODS:

Healthy adult residents (age ≥ 18 years) of Allegheny County, PA were recruited for participation via print and electronic advertisements September 2012 – April 2013. Individuals who reported chronic constipation or diarrhea, the presence of an ostomy or a history of colon resection, a history of CDI, recent or anticipated hospitalization or surgery, or employment involving direct patient contact in a health care facility were excluded. Eligible participants provided epidemiologic data via a written questionnaire and thereafter recorded and categorized all dietary intake for 10 days using an online food diary; at completion of the food diary subjects submitted a stool specimen. Specimens were shipped from subjects to the laboratory, where they were stored at room temperature and inoculated for incubation within 7 days of collection; aliquots of stool were frozen at -80 °C. Subjects whose stool yielded CD on at least one occasion were referred to as CD colonized and were asked to submit additional epidemiologic and dietary data along with additional stool specimens on a monthly basis.

Stool specimens (approximately 0.01 – 0.1 g using a 10µl inoculation loop) were cultured by broth enrichment using CCMB-TAL (Anaerobe Systems; Morgan Hill, CA) as previously described. (10) PCR and sequencing of tcdC was used to infer toxigenicity of isolates. CD isolates that were tcdC-negative were confirmed as non-toxigenic by lok1/lok3 PCR. (11) In addition, stool specimens positive for CD by culture underwent loop-mediated isothermal amplification of tcdA (illumigene C. difficile, Meridian Bioscience, Cincinnati, OH) per manufacturer instructions. Quantitative cultures to determine CD stool density were performed using serial 10-fold dilutions of approximately 0.1 g stool diluted in sterile deionized water followed by anaerobic culture in triplicate of 100 µl for each dilution on cycloserine-cefoxitin-mannitol agar (CCMA) for 72 hours. CD isolates were typed using tcdC genotyping. (12) The
tcdC genotypes were assigned according to the PubMLST database (http://pubmlst.org/cdifficile). Ethical approval was granted by the University of Pittsburgh Institutional Review Board.

Epidemiologic and dietary data submitted by study participants were analyzed to identify risk factors for CD carriage. P-values for differences in the prevalence of categorical variables were assessed using the Fisher’s exact test; differences in the distribution of continuous variables were assessed using the Wilcoxon test. All odds ratios and 95% confidence intervals for putative CD exposure risks were calculated using exact logistic regression and reported as either odds ratios or median unbiased estimates as appropriate. Analyses were conducted with SAS®Software (Version 9.3, SAS Institute, Cary, NC, USA).
RESULTS

One hundred six (81%) of 130 enrolled participants submitted stool specimens. Sixty-nine (65%) participants were females, 65 (61%) were aged <35 years, 71 (67%) identified their race as white, 57 (54%) had attained a college degree or higher, and 100 (94%) were self-reported omnivores. Seven (6.6%) of 106 participants were colonized with toxigenic CD. There was no recovery of non-toxigenic CD. The median ages of colonized and non-colonized individuals were 24 (range 19-37) and 30 (range 18-71), respectively (p=0.25). There was no association between CD colonization and race (p=0.26), ethnicity (p=0.64), consumption of raw beef (p=0.41) or seafood (p=0.65), exposure to a physician office, emergency department or urgent care clinic (p=0.88), antibiotics, (p=0.36) children under age 4 years, (p=0.56) or individuals known to have CDI (p=1.0). Participants who reported exposure to pets (median unbiased estimate 0.14, CI 0.00-0.74; p=0.05), a dentist office (OR 0.56, p=0.12) and those who reported public restroom use of ≥7 times/week (OR 0.47, CI 0.16-1.37, p=0.18) exhibited less prevalent CD colonization, but these associations did not reach statistical significance. The most frequent pet ownership among CD-uncolonized participants were mammalian species (40/43, 93%); no CD-colonized participants reported pet ownership. Two of 12 (17%) participants reporting antibiotic use in the 90 days prior to participation had CD colonization compared to 5 of 94 (5.3%) with no antibiotic use, but this observed difference was not significant (OR 3.5, CI 0.30-25.2, p=0.36).

The microbiologic details of the 7 CD colonized participants are described in the Table. Six (86%) colonized individuals submitted >1 stool specimen. Stool from 2 (29%) individuals yielded CD on 2 successive occasions 1 month apart. The median density of CD in stool specimens of colonized participants was $3.0 \times 10^4$ cfu/g. The quantity of CD in 2 stool specimens

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was less than $1 \times 10^1$ cfu/g. The *illumigene* assay detected 3/9 (33%) broth enrichment culture-positive stool specimens, each of which exhibited $\geq 3.0 \times 10^4$ cfu/g stool. A total of 7 *tcdC* genotypes and 7 multilocus sequence (MLST) were identified among colonized participants. These include *tcdC* 3, a genotype associated with the epidemic PCR ribotype 001 lineage, and *tcdC* 20, a genotype associated PCR ribotype 078, which has been detected in food animals. Genotype *tcdC* 1, associated with the epidemic PCR ribotype 027, was not observed in this study.

**DISCUSSION**

The prevalence of CD colonization in this small healthy cohort is concordant with contemporary values derived from Japanese populations and higher than estimates in the US, many of which date prior to the early 2000’s (5, 7-9). This difference may result from the well-documented increase in rates of healthcare-associated CDI within the US since 2000, resulting in spillover into outpatient populations (2). We believe, however, that it is more likely that the higher prevalence of CD colonization observed in our cohort and in prior Japanese studies compared to earlier US estimates results principally from the incorporation of essential CD spore germinants (sodium taurocholate and lysozyme) in the culture medium used compared to the CCFA medium used in prior US studies, which is now known to be substantially less sensitive (13). Our data support a potential role of asymptomatic carriers in the introduction of the organism into hospitals and the transmission to susceptible community dwellers.

It is notable that the mean stool density of CD among our healthy participants is $3.0 \times 10^4$ cfu/g, whereas reported values for stool CD density in symptomatic CDI patients has been reported as $4.0 \times 10^6$ (14). CD-colonized individuals with a low colonic density of organisms may not participate in the transmission cycle of CDI, however, this is unlikely because the
infectious dose for CDI is low in animal models (15), and CD colonized patients within hospitals are well-documented as responsible contamination of hospital rooms and in transmission to new patients (16-18). It is also notable that the majority (67%) of CD-colonized participants who submitted >1 stool specimen were negative for CD 30 days after submitting their positive sample. Further study is needed to quantify the duration of CD colonization in asymptomatic persons.

Of interest was the detection of *tcdC* genotype 20 (inferred ribotype 078) CD carriage in one participant. In a recent study, our laboratory identified 2% of uncooked ground pork sausage products were contaminated with this CD lineage in the Pittsburgh area (19). However, the current study was underpowered to identify risk factors that are significantly associated with food consumption or other participant characteristics, including the interesting lower observed prevalence of CD colonization among individuals with pet ownership and frequent public restroom use.

This investigation underscores the need for caution when interpreting the results of commercial nucleic acid-based CD detection assays. Three of 9 (33%) stool specimens from healthy CD-colonized individuals were positive by a commonly used commercial nucleic acid test for CD. These data reinforce the notion that nucleic acid-based CD assays lack specificity and may over-diagnose CDI in lower-risk populations when additional clinical criteria are not considered or when testing for other enteric pathogens is not performed (20, 21).

It is also notable that the nucleic acid test in this study was insufficiently sensitive to detect toxigenic CD in 67% of the broth-enrichment CD-positive stool specimens submitted by healthy colonized individuals. This finding should provide a note of caution to investigators seeking to screen healthy donors for fecal microbiota therapy (FMT) as a treatment for CDI.
without using toxigenic anaerobic culture. This finding probably reflects the limit of detection for the illumigene assay, which is not optimized for organism densities lower than those typically encountered in CDI patients (~10^6/g stool). Further, nucleic acid-based assays may be insufficiently sensitive to identify asymptomatic, CD-colonized hospital inpatients, the active surveillance of whom may have a role in hospital-acquired CDI reduction strategies (17).

The principal limitation of this investigation is the small sample size. A larger sample is necessary to identify risk factors for CD colonization among healthy individuals. Because we only performed broth enrichment culture on 0.01 to 0.1 g of stool, our culture method may have missed subjects with low CD colonization densities (i.e., less than 10^-100 cfu/g), but this culture method has recently been shown to be the most sensitive currently in use (13). Nonetheless, it is conceivable that our estimate of CD colonization prevalence would be higher if a greater quantity of stool were sampled.

In conclusion, the prevalence of asymptomatic CD colonization in this cohort was 6.7%, a value that is concordant with estimates in other studies from outside the US. Nucleic acid testing for CDI is not as sensitive as anaerobic toxigenic culture to identify carriers, but false-positive tests for CDI could result from testing of carriers with other causes of diarrheal illness.

ACKNOWLEDGEMENTS

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REFERENCES


Table. Stool testing characteristics of 7 healthy subjects testing positive for *C. difficile* in Pittsburgh, PA.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Visit</th>
<th>CD culture*</th>
<th>quantitative culture (cfu/g stool)</th>
<th>Nucleic acid test (illumigene)</th>
<th>tcdC</th>
<th>MLST</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>POS</td>
<td>2.7 x 10³</td>
<td>NEG</td>
<td>tcdC 5</td>
<td>58</td>
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<tr>
<td></td>
<td>2</td>
<td>NEG</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>NEG</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>POS</td>
<td>&lt; 10⁴**</td>
<td>NEG</td>
<td>tcdC 20</td>
<td>11</td>
</tr>
<tr>
<td></td>
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<td>-</td>
</tr>
<tr>
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<td>8.7 x 10³</td>
<td>NEG</td>
<td>tcdC 19</td>
<td>8</td>
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<tr>
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<td>POS</td>
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</tr>
<tr>
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<td>-</td>
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<td>POS</td>
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<td>110</td>
</tr>
</tbody>
</table>

* broth enrichment culture

** *C. difficile* did not grow in direct plating of 0.03 g stool, i.e. the quantity tested with three of the initial 10-fold dilutions (see methods)