Increased environmental sample area and recovery of *Clostridium difficile* spores from hospital surfaces by quantitative PCR and enrichment culture

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Abstract

Objective: *Clostridium difficile* spores play an important role in transmission and can survive in the environment for several months. Optimal methods for measuring environmental *C. difficile* are unknown. We sought to determine whether increased sample surface area improved detection of *C. difficile* from environmental samples.

Setting: Samples were collected from 12 patient rooms in a tertiary-care hospital in Toronto, Canada.

Methods: Samples represented small surface-area and large surface-area floor and bedrail pairs from single-bed rooms of patients with low (without prior antibiotics), medium (with prior antibiotics), and high (*C. difficile* infected) shedding risk. Presence of *C. difficile* in samples was measured using quantitative polymerase chain reaction (qPCR) with targets on the 16S rRNA and toxin B genes and using enrichment culture.

Results: Of the 48 samples, 64.6% were positive by 16S qPCR (geometric mean, 13.8 spores); 39.6% were positive by toxin B qPCR (geometric mean, 1.9 spores); and 43.8% were positive by enrichment culture. By 16S qPCR, each 10-fold increase in sample surface area yielded 6.6 times (95% CI, 3.2–13) more spores. Floor surfaces yielded 27 times (95% CI, 4.9–181) more spores than bedrails, and rooms of *C. difficile*-positive patients yielded 11 times (95% CI, 0.55–164) more spores than those of patients without prior antibiotics. Toxin B qPCR and enrichment culture returned analogous findings.

Conclusions: *Clostridium difficile* spores were identified in most floor and bedrail samples, and increased surface area improved detection. Future research aiming to understand the role of environmental *C. difficile* in transmission should prefer samples with large surface areas.

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In this study, we sought to identify whether increased sample surface area was associated with increased detection of *C. difficile* from environmental samples collected from patient rooms in a tertiary-care hospital in a nonoutbreak setting. We explored whether this relationship existed for both floor and bedrail samples, and across several different microbiologic methods, including qPCR and enrichment culture.

**Methods**

**Design and setting**

We used an efficient split-plot sampling design to assess the impact of sample surface area on *C. difficile* detection from hospital environmental samples while controlling for room-level clustering of bacterial burden. Small surface-area and large surface-area pairs of bedrail and floor environmental samples were selected from 12 rooms (N = 48) in 3 inpatient units (2 general medicine, 1 intensive care), over the course of 2 days in September 2017 in a large tertiary-care hospital located in Toronto, Canada.

**Sample collection**

For sampling, patient rooms were selected based on a categorization of risk of contamination: high-risk rooms were those of patients with active *C. difficile* diarrhea identified by hospital infection control staff; medium-risk rooms were those of patients with a history of in-hospital antibiotic use in the last 14 days; and low-risk rooms were those of patients without a history of *C. difficile* in-hospital antibiotic use in the previous 14 days. All high-risk rooms available in the hospital were selected (N = 5), as well as a selection of medium-risk (N = 2) and low-risk (N = 5) on the same wards. All samples were collected independently of ward- and room-cleaning schedules, and the timing of most recent cleaning was not known or recorded. The cleaning was surface cleaning patterns using a defined protocol. We conducted quantitative polymerase chain reaction (qPCR) on 2 targets. A 157-bp conserved region of the *C. difficile* 16S rRNA gene (present in ~10 copies per genome) and an internal TaqMan probe (Applied Biosystems, Foster City, CA) were used for detection of all *C. difficile* strains. A 127-bp region of the toxin B gene (present in 1 copy per genome) and a corresponding TaqMan probe were used to specifically detect toxigenic *C. difficile* strains. Reactions (10 µL) containing 5 µL JumpStart Taq ReadyMix for qPCR (D7440, Sigma-Aldrich, St Louis, MO), 0.1 µL reference dye for qPCR (R4526, Sigma), 0.4 µL 25 mM MgCl₂, 0.5 µM (each) forward and reverse primers, 0.15 µM TaqMan probe, and 3.85 µL DNA were prepared. These were run in triplicate on an ABI 7900HT thermocycler (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles at 95°C for 15 seconds, 60°C for 1 minute. The other half of the sample was enriched and cultured using banana broth (Hardy Diagnostics, Santa Maria, CA). Samples positive by enrichment culture were ribotyped (Appendix 1).

We coded 5 outcome variables. First, 16S qPCR positivity and toxin B qPCR positivity were coded such that any threshold cycle (Ct) < 45 was considered positive, and any Ct ≥ 45 (or undetermined) was considered negative. The enrichment culture-based measure was coded as either positive or negative. Second, we converted qPCR Ct to spore counts for 16S qPCR and for toxin B qPCR based on their respective standard curves. These *C. difficile* standard curves were generated by serial dilution of a spore stock quantitated using a hemocytometer. As customary in occupational exposure assessment analyses, we truncated the estimated spore count distribution at 0.5 spores, corresponding to half the detection limit.

**Covariates**

We coded risk-factor variables corresponding to sample type (bedrail or floor) and room risk of contamination (low, medium, and high). Sample surface area was encoded as a categorical variable (small or large). To adjust for surface area in our multivariable models, we also encoded sample surface area in as a continuous, log₁₀-transformed variable, log₁₀(m²). Finally, we also coded 3 clustering variables: sample pair identifier, room identifier, and ward identifier.

**Statistical analyses**

For descriptive analyses, we measured the proportion of samples that were positive for the binary outcome variables and the geometric (log₁₀) mean spore count for the continuous outcome variables across the 3 risk-factor variables. For multivariable analyses, we used separate logistic mixed effects models for the presence versus absence of *C. difficile* by 16S qPCR, toxin B qPCR, and enrichment culture. We used negative binomial mixed effects models for the estimated spore count by...
16S qPCR and toxin B qPCR. All multivariable models included the same fixed effects and random intercepts. We used fixed effects for patient risk stratum, sample type, and sample surface area, and we used random intercepts corresponding to the ward, the patient room, and the sample pair. The random intercepts for patient room and sample pair accounted for the clustering inherent to the split-plot sampling design. All multilevel models were fit using Bayesian random-effects regression models in the R statistical programming language (rstanarm library) and default settings (4 chains of 1,000 warmup and 1,000 sampling draws, and default weak priors). Furthermore, 95% confidence intervals (95% CIs) were based on the sampling draws.

We conducted 2 secondary analyses. First, to assess whether the association between sample surface area and yield was consistent across the surface type (bedrail vs floor), we ran an additional negative binomial multivariable model that added the interaction term between surface area and type. Second, to assess the relatedness of the 3 different laboratory techniques for measuring *C. difficile*, we measured the association between 16S qPCR spore counts and toxin B qPCR spore counts using linear regression, and between 16S qPCR spore count and enrichment culture positivity using logistic regression.

**Ethics**

This study was approved by the Public Health Ontario and Sunnybrook Health Sciences Center research ethics boards.

**Results**

Of the 48 samples collected, 31 (64.6%) were positive for *C. difficile* by 16S qPCR, with a geometric mean of 13.8 spores per sample; 19 (39.6%) were positive by toxin B qPCR, with a geometric mean of 1.9 spores per sample; and 21 (43.8%) were positive by enrichment culture.

**Descriptive analyses**

Large surface-area samples were more likely to be positive and had higher estimated spore counts compared to small surface-area samples (Table 1, Appendix 2). By 16S qPCR, 17 of 24 large surface-area samples (70.8%) were positive, while 14 of 24 small surface-area samples (58.3%) were positive. The geometric mean estimated spore count was 27.0 for large surface-area samples and 7.0 for small surface-area samples. However, large surface-area bedrail samples (0.23 m²) were less likely to be positive (5 of 12, 41.7%) than small surface-area floor samples (0.10 m²; 9 of 12, 75%) (Fig. 1). Overall, 21 of 24 floor samples (87.5%) were positive compared to 10 of 24 bedrail samples (41.7%), and the geometric mean estimated spore count was 69.4 for floor samples compared to 2.7 for bedrail samples.

**Multivariable analyses**

After multivariable adjustment, each 10-fold increase in surface area was associated with a 4.5-fold (adjusted odds ratio [aOR],

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Table 1. Estimated Environmental Sample *Clostridium difficile* Positivity and Quantity Based on qPCR (16S target and Toxin B target) and Enrichment Culture From Environmental Samples Collected at a Tertiary Hospital

<table>
<thead>
<tr>
<th>Sample Area</th>
<th>No.</th>
<th>16S qPCR (N, %)</th>
<th>Toxin B qPCR (N, %)</th>
<th>Enrichment culture (N, %)</th>
<th>16S qPCR (Geometric Mean)</th>
<th>Toxin B qPCR (Geometric Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surface area</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small</td>
<td>24</td>
<td>14 (58.3)</td>
<td>7 (29.2)</td>
<td>9 (37.5)</td>
<td>7.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Large</td>
<td>24</td>
<td>17 (70.8)</td>
<td>12 (50.0)</td>
<td>12 (50)</td>
<td>27.0</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>Type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bedrail</td>
<td>24</td>
<td>10 (41.7)</td>
<td>5 (20.8)</td>
<td>6 (25)</td>
<td>2.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Floor</td>
<td>24</td>
<td>21 (87.5)</td>
<td>14 (58.3)</td>
<td>15 (62.5)</td>
<td>69.4</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>Surface area and type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small bedrail</td>
<td>12</td>
<td>5 (41.7)</td>
<td>2 (16.7)</td>
<td>3 (25.0)</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Large bedrail</td>
<td>12</td>
<td>5 (41.7)</td>
<td>3 (25.0)</td>
<td>3 (25.0)</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Small floor</td>
<td>12</td>
<td>9 (75.0)</td>
<td>5 (41.7)</td>
<td>6 (50.0)</td>
<td>23.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Large floor</td>
<td>12</td>
<td>12 (100)</td>
<td>9 (75.0)</td>
<td>9 (75.0)</td>
<td>206.5</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>Room Risk of Contamination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>20</td>
<td>10 (50.0)</td>
<td>5 (25.0)</td>
<td>7 (35.0)</td>
<td>4.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Medium</td>
<td>8</td>
<td>4 (50.0)</td>
<td>3 (37.5)</td>
<td>1 (12.5)</td>
<td>5.0</td>
<td>1.7</td>
</tr>
<tr>
<td>High</td>
<td>20</td>
<td>17 (85.0)</td>
<td>11 (55.0)</td>
<td>13 (65.0)</td>
<td>65.6</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**NOTE.** qPCR, quantitative polymerase chain reaction.

Low, occupant had no receipt of antibiotics in prior 14 days; medium, occupant had receipt of antibiotics in prior 14 days; high, occupant had confirmed *C. difficile* infection.
4.5; 95% CI, 0.79–34) increase in the odds of positivity and a 6.6-fold (adjusted count ratio [aCR], 6.6; 95% CI, 3.2–13) increase in the spore count (Table 2). Floor surfaces were 17-fold (OR, 17; 95% CI, 2.1–170) more likely to be positive than bed rails and had 27-fold (aCR, 27; 95% CI, 4.9–181) more spores. Compared to rooms of patients that received no antibiotics in the previous 14 days, samples from rooms of patients with C. difficile infection were 17-fold (aOR, 17; 95% CI, 1.2–246) more likely to be positive, with 11-fold (aCR, 11; 95% CI, 0.55–164) more spores. Compared to rooms of patients that received no antibiotics in the previous 14 days, samples from rooms of patients with C. difficile infection were 17-fold (aOR, 17; 95% CI, 1.2–246) more likely to be positive, with 11-fold (aCR, 11; 95% CI, 0.55–164) more spores.

Comparison of 16S qPCR versus toxin B qPCR and enrichment culture

Estimated spore counts by 16S qPCR were strongly associated with toxin B spore counts (Pearson’s ρ, 0.75) (Fig. 2, panel A) and with enrichment culture-based results. When the 16S spore count was 0, only 1 of 19 enrichment culture samples (5.3%) were positive. For comparison, 8 of 16 samples (50%) were culture positive for spore counts of 1 to 100; 7 of 8 samples (87.5%) were culture positive for spore counts of 100 to 1000; and 5 of 5 samples (100%) were culture positive for spore counts >1,000 ($P_{\text{Trend}} < .001$) (Fig. 2, panel B).

Ribotyping

We successfully ribotyped 20 of 21 samples (95%) that were positive by enrichment culture, and we detected 8 different ribotypes (Table 3). Single ribotypes were identified in 16 of 20 samples (80%), and 2 ribotypes were identified in 4 of 20 samples (20%). Ribotype 015 was found in 11 of 12 positive samples (92%) from ward B, but 0 of 8 samples (0%) were positive from ward A ($P_{\chi^2} = .04$). Of 20 samples, 17 (85%) contained ribotypes known to have toxin A and B genes.

Discussion

In this study, we considered the impact of increasing sample surface area on C. difficile recovery using culture and culture-independent techniques. We found that increased sample surface area was associated with increased counts of spores recovered and increased proportions of samples that were positive by all laboratory techniques. Additionally, we identified that 16S qPCR, toxin B qPCR, and enrichment culture are all acceptable techni-
ques for measurement of environmental *C. difficile*. We also found that *C. difficile* contamination was common on bedrails and floors and was significantly more common in the rooms of patients with *C. difficile* infection.

Several techniques have been used to measure environmental *C. difficile* contamination, including culture,

\[\text{24 enrichment culture,25 and qPCR.18 Culture-based techniques are commonly used and inexpensive, but they can be plagued by low detection limits, issues of nonculturability, and long turnaround times for results. Although qPCR is a more sensitive and culture-independent technique, it is also more labor intensive and costly, largely due to the DNA extraction step. However, it can produce results within 2–3 hours. qPCR is routinely used for clinical diagnostics and has also been adapted for environmental sampling.18 We found that 16S qPCR was more sensitive than toxin B qPCR. We also found that results from qPCR-based techniques and culture-based techniques were strongly correlated. Increasing levels of contamination by 16S qPCR were strongly associated with increased levels by toxin B qPCR and enrichment culture-based positivity. At levels beyond 1,000 spores by 16S qPCR, all toxin B qPCR samples and enrichment culture samples were positive, suggesting that toxin B qPCR and enrichment culture may have been negative due to a lack of sensitivity of the laboratory technique rather than a lack of toxigenic or live cells on those surfaces.}

Using real-world environmental samples, this study demonstrated that large surface-area samples yielded higher positivity and higher counts of spores across several different microbiologic techniques and 2 surface types. These findings provide further evidence of the advantages of maximizing surface area when conducting environmental sampling. For 16S qPCR, the effect of surface area on *C. difficile* capture may have been moderately stronger among floor samples than among bedrail samples. This finding may have been due to inconsistencies in the sampling of bedrails because bedrails have complex surfaces.

Previous studies have shown that, for *Bacillus atrophaeus*, large surface-area sampling (1 m²) translates into greater sensitivity for detection and necessitates fewer samples for testing exposure–outcome associations, compared with samples with smaller surface areas.26 A more recent study used large surface area samples (up to 0.22 m²) to quantify the microbial bioburden of several hospital pathogens.9 Our work showed that all techniques tested, including both culture and culture-independent techniques, reacted positively to increased sample size, though the clinical relevance of environmental *C. difficile* burden has not yet been established.

We found that *C. difficile* contamination was ubiquitous in the hospital rooms we sampled. All rooms, even those of patients without a history of *C. difficile* or antibiotic use, yielded positive samples by 16S qPCR. It has been shown that floor-surface samples are approximately twice as likely to be culture positive as bedrail samples,6 and surfaces more proximate to patients are more likely to be contaminated than those farther from patients.27 In our study, floor surfaces actually had 10 times more *C. difficile* contamination than bedrail surfaces. Also, 100% of the 12 large floor samples we collected were contaminated with *C. difficile* according to 16S qPCR. *Clostridium difficile* on floor surfaces may be a risk to patients because microbes on the floor may be transported within wards by shoe soles28 are continually resuspended by foot traffic.29 Furthermore, mobile patients can contaminate linens and themselves via their feet or shoes.13 Although our results do not allow us to assess directionality, the finding of strong relatedness of strains recovered within wards suggests that spatial dissemination involving floors may be a concern. These results also lend support to several studies that have shown the importance of ward-level effects for the transmission of *C. difficile* and other hospital-associated infections.30–32

The infective dose of *C. difficile* for a healthy but susceptible inpatient is not known33 and as such, safe levels of environmental *C. difficile* are not known. For mice, the environmental infectious
The dose required to infect 50% of mice (ID50) for *C. difficile* was 1 hour of exposure to 5–10 culturable spores per square centimeter of cage floor. Future research should seek to identify the infectious dose for *C. difficile* for both healthy individuals and those at a risk of *C. difficile* infection due to antibiotic receipt and to define the maximum threshold of environmental density of *C. difficile* that confers an acceptably low risk of infection. Such environmental density thresholds could be used to better ascertain the acceptability of current hospital cleaning protocols.

Our study has several limitations. First, it is a study of rooms in a single hospital over 2 days of sampling and may not be generalizable to other hospitals. We lacked several covariates including antibiotic history prior to hospital admission and timing of most recent cleaning prior to sample collection. However, these limitations were controlled for by the experimental design of our study, wherein sample surface area and sample type, our 2 exposures of primary interest, were compared to each other within rooms at the same point in time. Second, our small surface-area bedrail samples were selected from the head of the bedrail, while our large surface-area samples were chosen from the remainder of the bedrail, which could have led to a bias if spore density was higher or lower near the head of the bedrail.

In this study, we found that increased sample surface area was associated with increased counts of spores recovered and increased proportions of samples that were positive. We also found that *C. difficile* contamination was common on hospital bedrails and floors and even more so in the rooms of patients with *C. difficile* infection. Future studies attempting to quantify the environmental density of *C. difficile* should consider using large surface-area samples whenever possible. Further study is required to better understand the role of contaminated hospital floors in the transmission of *C. difficile* infection.

### Table 3. Characteristics of *Clostridium difficile* Samples Successfully Ribotyped (N = 20)

<table>
<thead>
<tr>
<th>Ward-Room</th>
<th>Room Risk of Contamination</th>
<th>Type</th>
<th>Surface Area</th>
<th>Ribotypes</th>
<th>Estimated Spore Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-2</td>
<td>High</td>
<td>Floor</td>
<td>Small</td>
<td>027, PR18099</td>
<td>28.1</td>
</tr>
<tr>
<td>A-2</td>
<td>High</td>
<td>Floor</td>
<td>Large</td>
<td>027</td>
<td>424.2</td>
</tr>
<tr>
<td>A-2</td>
<td>High</td>
<td>Bedrail</td>
<td>Small</td>
<td>027, PR18101</td>
<td>19.6</td>
</tr>
<tr>
<td>A-2</td>
<td>High</td>
<td>Bedrail</td>
<td>Large</td>
<td>027</td>
<td>778.0</td>
</tr>
<tr>
<td>A-4</td>
<td>High</td>
<td>Floor</td>
<td>Large</td>
<td>PR18099</td>
<td>41.8</td>
</tr>
<tr>
<td>A-4</td>
<td>High</td>
<td>Bedrail</td>
<td>Small</td>
<td>014-0</td>
<td>2.0</td>
</tr>
<tr>
<td>A-5</td>
<td>Low</td>
<td>Floor</td>
<td>Small</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>A-5</td>
<td>Low</td>
<td>Floor</td>
<td>Large</td>
<td>400</td>
<td>55.1</td>
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<tr>
<td>B-7</td>
<td>Low</td>
<td>Floor</td>
<td>Large</td>
<td>015</td>
<td>24.6</td>
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<tr>
<td>B-8</td>
<td>Low</td>
<td>Floor</td>
<td>Small</td>
<td>015</td>
<td>475.6</td>
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<tr>
<td>B-8</td>
<td>Low</td>
<td>Floor</td>
<td>Large</td>
<td>015</td>
<td>602.3</td>
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<tr>
<td>B-11</td>
<td>High</td>
<td>Floor</td>
<td>Small</td>
<td>015</td>
<td>7,863.5</td>
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<tr>
<td>B-11</td>
<td>High</td>
<td>Floor</td>
<td>Large</td>
<td>015</td>
<td>136,143.6</td>
</tr>
<tr>
<td>B-11</td>
<td>High</td>
<td>Bedrail</td>
<td>Small</td>
<td>015</td>
<td>1,101.1</td>
</tr>
<tr>
<td>B-11</td>
<td>High</td>
<td>Bedrail</td>
<td>Large</td>
<td>015</td>
<td>4,373.7</td>
</tr>
<tr>
<td>B-10</td>
<td>Low</td>
<td>Floor</td>
<td>Small</td>
<td>015</td>
<td>19.9</td>
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<td>B-10</td>
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<td>Floor</td>
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<td>B-12</td>
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<td>495.1</td>
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<td>Bedrail</td>
<td>Large</td>
<td>015, 010</td>
<td>31.2</td>
</tr>
</tbody>
</table>

NOTE. qPCR, quantitative polymerase chain reaction.

*No positive samples were obtained from ward C.*

Low, occupant had no receipt of antibiotics in prior 14 days; medium, occupant had receipt of antibiotics in prior 14 days; high, occupant had confirmed *C. difficile* infection.

In this study, we found that increased sample surface area was associated with increased counts of spores recovered and increased proportions of samples that were positive. We also found that *C. difficile* contamination was common on hospital bedrails and floors and even more so in the rooms of patients with *C. difficile* infection. Future studies attempting to quantify the environmental density of *C. difficile* should consider using large surface-area samples whenever possible. Further study is required to better understand the role of contaminated hospital floors in the transmission of *C. difficile* infection.

### Supplementary materials

To view supplementary material for this article, please visit https://doi.org/10.1017/ice.2018.103

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Conflicts of interest. All authors declare no conflicts of interest relevant to this article.

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