**Clostridium difficile** shows no trade-off between toxin and spore production within the human host

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**Abstract**

**Purpose.** This study aimed to describe the correlation between **Clostridium difficile** spore and toxin levels within the human host. In addition, we assessed whether overgrowth of **Candida albicans** modified this association.

**Methodology.** We measured toxin, spore and **Candida albicans** levels among 200 successively collected stool samples that tested positive for **C. difficile**, and PCR ribotyped these **C. difficile** isolates. Analysis of variance and linear regression were used to test the association between spore and toxin levels. Kruskal–Wallis tests and t-tests were used to compare the association between spore or toxin levels and host, specimen, or pathogen characteristics.

**Results.** **C. difficile** toxin and spore levels were positively associated (P<0.001); this association did not vary significantly with **C. albicans** overgrowth [≥5 logs of **C. albicans** colony-forming units (c.f.u.) g⁻¹]. However, ribotypes 027 and 078–126 were significantly associated with higher levels of toxin and spores, and **C. albicans** overgrowth.

**Conclusion.** The strong positive association observed between *in vivo* levels of **C. difficile** toxin and spores suggests that patients with more severe **C. difficile** infections may have increased spore production, enhancing **C. difficile** transmission. Although, on average, spore levels were higher in toxin-positive samples than in toxin-negative/PCR-positive samples, spores were found in almost all toxin-negative samples. The ubiquity of spore production among toxin-negative and formed stool samples emphasizes the importance of following infection prevention and control measures for all **C. difficile**-positive patients during their entire hospital stay.

**INTRODUCTION**

**Clostridium difficile** infection (CDI) is one of the most important healthcare-associated infections (HAIs) worldwide. In the United States, CDI accounts for 12% of all HAIs, leading to almost 500,000 infections and approximately 29,000 deaths per year [1–3], while costing an estimated 4.8 billion dollars annually [4]. Similarly, in Europe there are on average 7 CDI cases for every 10,000 overnight patients [5].

The severity of CDI symptoms is expected to increase with toxin levels [6]; however, whether symptom severity increases with spore levels is less certain. Carlson et al. reported that cultured isolates from severe CDI cases (*n*=34) produced more spores after 24 h than isolates from non-severe CDI cases (*n*=72) [7]. The potential explanation for this observed association is that more spores will germinate into more vegetative cells, which will eventually produce more toxin – resulting in more severe disease [7]. For the latter to be true, toxin and spore production must be directly correlated. However, both positive and negative correlations between toxin and spore levels have been reported [6, 8–10].

Akerlund et al. compared toxin yield and spore levels among 13 clinical isolates *in vitro* and observed an inverse correlation between toxin and spore levels [6]. By contrast, Koenigsknecht et al. observed that spores and toxins were
produced simultaneously 24 h after infection in a mouse model (n=30), suggesting a positive correlation between faecal toxin and spore levels [9]. However, the in vivo correlation between toxin and spore levels, particularly in humans, remains to be confirmed.

Although toxin production is essential for pathogenicity, it is not sufficient. Toxin can be detected in the absence of symptoms, suggesting that other factors modify the effect of the toxin on the host [11]. One factor that may potentially play a role is the presence of Candida spp. in the gut microbiota. C. difficile is a strict anaerobic organism; nevertheless, it has been shown to persist and grow under aerobic in vitro conditions when in the presence of Candida albicans [12], suggesting that both micro-organisms may interact with each other within the human gut. Both positive and negative associations between CDI and Candida spp. have been reported [13, 14]; however, whether the presence of Candida spp. is associated with C. difficile spore and toxin production has not been investigated.

Our study aimed to understand the in vivo correlation of C. difficile spore and toxin levels by directly testing clinical samples and comparing them to previously published in vitro results. Additionally, we aimed to describe the association between C. difficile spore and toxin levels and overgrowth of C. albicans.

METHODS

Sample collection

We collected 200 successive C. difficile-positive stool samples from the microbiology laboratory at Saint Joseph Mercy Hospital (SJMH) in Ann Arbor, Michigan (MI), USA, a reference laboratory for several hospitals, outpatient healthcare centers and urgent care facilities within the Saint Joseph Mercy Health System. The Institutional Review Boards at SJMH and the University of Michigan (IRB Health Sciences and Behavioral Sciences) approved our study protocol.

We included all samples submitted for CDI testing between 1 February and 20 July 2015 that were determined to be positive using the SJMH laboratory algorithm and had sufficient volume left for our testing protocol. The SJMH laboratory algorithm includes an initial glutamate dehydrogenase (GDH) and toxins A (TcdA) and B (TcdB) screening using C. Diff Quik Chek Complete (Alere, Waltham, MA/Technlab, Blacksburg, VA, USA). In cases of indeterminate results (GDH+, Toxin−), samples were further tested using a tcdB PCR assay (Cepheid Gene Xpert II, Sunnyvale, CA, USA) for the presence of the toxin genes (Fig. 1). We recorded the age, gender and location code (inpatient vs outpatient) of the individual providing the sample, and qualitatively assessed the physical consistency of the study stool samples.

Samples were classified as (1) formed/semiformed, (2) soft and unformed, (3) liquid and (4) mucoid. As diarrhoeic samples were the focus of our study, we considered all hard or soft samples that retained their shape in the collection cup to be formed or semiformed. Stools that were loose and took the shape of the collection cup were identified as soft and unformed, while samples that could be pipetted and resembled the consistency of water were defined as liquid. Soft or liquid samples that contained significant amount of mucous were considered mucoid (modified from [15]).

Determination of toxin levels

Following determination of positivity by the SJMH laboratory, we measured C. difficile toxin A and B levels in a known amount of stool (0.05 g) using the C. Difficile Tox A/B II Immunoassay (Techlab, Blacksburg, VA, USA). We quantitated the colour intensity of the reaction by measuring the optical density at 450 nm using an Victor X3 Multi-label Plate Reader (PerkinElmer, 2030 Multilabel Reader, Waltham, MA, USA) according to the kit manufacturer’s instructions [16]. Due to the known variability of this assay, we normalized each sample value (V) by subtracting its corresponding negative control (NC) and dividing this value by its positive control (PC), also corrected by subtracting its negative control value (i.e. V–NC/PC–NC). Therefore, we report the ratio of toxin levels in each sample relative to that in the positive control. In addition, due to the high intra-assay coefficient of variation (10 % across faecal specimens [16]), we categorized our toxin levels based on quantiles. We created three toxin categories: (1) non-detectable (ND) levels with an upper threshold of 0.07 (p50), (2) low levels with an upper threshold of 0.36 (p75) and (3) high levels with an upper threshold of 3.94.

Determination of spore levels

We prepared 1:1 dilutions with 1 ml pre-autoclaved distilled water and 1 g of stool. Following a heat treatment (65°C for 20 min), the spore levels were quantified by culturing serial dilutions of the faecal specimens on cycloserine cefoxitin fructose agar with horse blood and taurocholate (Anaerobe Systems, Morgan Hill, CA, USA) for 24 h at 37°C under anaerobic conditions. Spore levels were reported as colony-forming units (c.f.u.) per gram of faeces (c.f.u. g−1).

Detection of Candida albicans

DNA was extracted in duplicate from 200 to 250 µl of stool using the PowerMag Soil Isolation kit (Mo Bio, Carlsbad, CA, USA) optimized for epMotion (Mo Bio, Carlsbad, CA, USA). C. albicans levels were quantified in triplicate for each extraction using RT-PCR (BioRad, CFX96 Realtime System, Hercules, CA, USA) using previously described conditions and primers [17]. Because stool is not a microbiologically and chemically homogeneous sample, we analysed six replicates of each sample: if at least two out of the six replicates of the same sample were positive, this sample was considered positive and an average level of C. albicans was calculated from the detected estimates. C. albicans overgrowth was defined as the growth of ≥5 logs (base 10 logarithm) of c.f.u. g−1 of stool, as described by Raponi et al. [13].
Ribotyping of isolates

A fluorescent PCR ribotyping technique using capillary gel electrophoresis was performed to ribotype the C. difficile isolates recovered from our study samples as previously described [18]. Our strains were compared to a reference strain database recently updated by the European collection in Cardiff. Strain ribotyping was performed at the Walk Lab at Montana State University.

Determination of dry weight of samples

Samples from severe cases are expected to be more diarrhoeic, which may dilute spore and toxin levels, resulting in an underestimation of toxin and spore production, especially in more severe cases. To determine whether this bias occurred, we desiccated the 159 samples, with sufficient material remaining in a vacuum concentrator (Eppendorf Vacufuge AG 22331, Hamburg, Germany) at 60°C and 1400 revolutions per minute for 3 h. The wet gramme weight of each stool sample used for toxin and spore quantification was adjusted to quantity per dry gramme weight. For the remaining 41 specimens without sufficient sample, we imputed water content, dry spore and dry toxin levels, using multiple imputation. Water content results also were used to validate our qualitative classification of stool consistency.

Statistical analysis

To test whether the distribution of spores was significantly different across toxin categories for both wet and dry samples, we used analysis of variance (ANOVA) and linear regression. The Kruskal–Wallis (KW) or chi square ($\chi^2$) tests were used to compare toxin levels or water content between groups of different age, gender, location, stool consistency, C. difficile ribotype and Candida growth. The T test or ANOVA was used to compare log-transformed spore levels (using a logarithm with base 10) between the different groups, including laboratory diagnosis at SJMH (toxin-positive or toxin-negative/PCR-positive). Finally, univariate and multivariate ordered logistic regression and linear regression were used to estimate the measures of association linked to toxin or spore (log-transformed using an logarithm with base 10) levels with/without adjustments for confounders. SAS 9.4 (Cary, NC, USA) and STATA 14 (College Station, TX, USA) were used to perform all analysis and graphics.

RESULTS

Among 200 successively collected CDI positive stools samples, 90 were from inpatients and 110 were from outpatients.
(including emergency room patients). Most samples were from females (60.5%) and 50.5% were from individuals aged 65 or older. The stool sample consistencies were primarily soft (40.5%), liquid (32.0%), mucoid (in addition to liquid or soft) (19.5%), or semiformal/formed (8.0%).

There were a total of 42 different ribotypes among our collected isolates (Table 1). The most common ribotypes were 027 (17%) and 014–020 (13%). Ribotype 078–126 also occurred in six samples (3%). Ribotype 027 and ribotype 027 (17%) and 014

lected isolates (Table 1). The most common ribotypes were

Table 1. Distribution of toxin and spore levels by ribotype in order of prevalence. 200 consecutive CDI positive stool samples were collected from the Microbiology Laboratory of the Saint Joseph Mercy Hospital, Ann Arbor, MI, USA from 1 February to 20 July 2015

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>N (%)</th>
<th>Normalized toxin levels</th>
<th>Spore levels (c.f.u. g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td>027</td>
<td>34 (17.0)</td>
<td>0.10 (−0.06–3.94)</td>
<td>3.6·10⁴ (2.0·10⁻¹–5.5·10⁶)</td>
</tr>
<tr>
<td>014–020</td>
<td>26 (13.0)</td>
<td>0.00 (−0.01–0.51)</td>
<td>3.0·10⁵ (5·4·10⁶)</td>
</tr>
<tr>
<td>002</td>
<td>11 (5.5)</td>
<td>0.02 (−0.05–0.90)</td>
<td>4.0·10⁴ (2.0·10⁻¹–9·10⁸)</td>
</tr>
<tr>
<td>053–163</td>
<td>8 (4.0)</td>
<td>0.02 (0.00–2.33)</td>
<td>6.2·10⁴ (3.5·10⁻¹–2·2·10⁹)</td>
</tr>
<tr>
<td>054</td>
<td>7 (3.5)</td>
<td>0.00 (0.00–0.29)</td>
<td>6.3·10⁴ (3.5·10⁻¹–1·6·10⁹)</td>
</tr>
<tr>
<td>106</td>
<td>7 (3.5)</td>
<td>0.41 (−0.01–1.96)</td>
<td>3.5·10⁴ (7·5·10⁻¹–2·4·10⁶)</td>
</tr>
<tr>
<td>005</td>
<td>6 (3.0)</td>
<td>0.39 (0.06–1.35)</td>
<td>1.4·10⁴ (7·5·10⁻¹–1·4·10⁶)</td>
</tr>
<tr>
<td>078–126</td>
<td>6 (3.0)</td>
<td>0.23 (−0.02–0.96)</td>
<td>8.8·10⁴ (7·5·10⁻¹–7·5·10⁶)</td>
</tr>
<tr>
<td>103</td>
<td>6 (3.0)</td>
<td>1.16 (0.00–1.58)</td>
<td>3.3·10⁴ (1·4·10⁻¹–2·3·10⁹)</td>
</tr>
<tr>
<td>015</td>
<td>5 (2.5)</td>
<td>0.00 (−0.01–0.06)</td>
<td>2.6·10⁴ (1·5·10⁻¹–5·0·10⁶)</td>
</tr>
<tr>
<td>255</td>
<td>5 (2.5)</td>
<td>0.01 (−0.02–1.15)</td>
<td>8.0·10⁴ (1·2·10⁻¹–8·5·10⁹)</td>
</tr>
<tr>
<td>019</td>
<td>4 (2.0)</td>
<td>0.77 (0.00–1.05)</td>
<td>4.7·10⁴ (2.0·10⁻¹–3·6·10⁹)</td>
</tr>
<tr>
<td>010</td>
<td>3 (1.5)</td>
<td>0.00 (−0.06–0.01)</td>
<td>8.5·10⁴ (3·5·10⁻¹–1·5·10⁹)</td>
</tr>
<tr>
<td>012</td>
<td>2 (1.0)</td>
<td>0.01 (0.00–0.01)</td>
<td>1.8·10⁴ (3·8·10⁻¹–3·3·10⁹)</td>
</tr>
<tr>
<td>153</td>
<td>2 (1.0)</td>
<td>0.00 (0.00–0.00)</td>
<td>4.5·10⁴ (1·5·10⁻¹–9·10⁹)</td>
</tr>
<tr>
<td>001</td>
<td>1 (0.5)</td>
<td>0.01 (−)</td>
<td>1.5·10⁴ (−)</td>
</tr>
<tr>
<td>017</td>
<td>1 (0.5)</td>
<td>0.00 (−)</td>
<td>3.5·10⁴ (−)</td>
</tr>
<tr>
<td>018</td>
<td>1 (0.5)</td>
<td>1.81 (−)</td>
<td>1.6·10⁴ (−)</td>
</tr>
<tr>
<td>056</td>
<td>1 (0.5)</td>
<td>0.12 (−)</td>
<td>3.6·10⁴ (−)</td>
</tr>
<tr>
<td>075</td>
<td>1 (0.5)</td>
<td>0.00 (−)</td>
<td>1.5·10⁴ (−)</td>
</tr>
<tr>
<td>087</td>
<td>1 (0.5)</td>
<td>1.48 (−)</td>
<td>8.1·10⁴ (−)</td>
</tr>
<tr>
<td>097</td>
<td>1 (0.5)</td>
<td>0.02 (−)</td>
<td>1.2·10⁴ (−)</td>
</tr>
<tr>
<td>137</td>
<td>1 (0.5)</td>
<td>0.00 (−)</td>
<td>5·10⁴ (−)</td>
</tr>
<tr>
<td>Others</td>
<td>46 (23.0)</td>
<td>0.00 (−0.04–1.96)</td>
<td>9.3·10⁴ (5·4·10⁻¹×10⁹)</td>
</tr>
<tr>
<td>No ribotype data*</td>
<td>14 (7.0)</td>
<td>0.00 (−0.04–0.37)</td>
<td>2.5·10⁴ (0·4·10⁶)</td>
</tr>
</tbody>
</table>

*The category referred as 'No ribotype data' includes stool samples from which C. difficile could not be cultured and isolates that could not be ribotyped.
Fig. 2. Distribution of spore levels across 200 CDI-positive stool samples. (a) The distribution of log-transformed (using a base 10 logarithm) spore levels (c.f.u. g⁻¹) by categories of increasing toxin levels: non-detectable levels (≤0.007 UA ml⁻¹), low levels (0.008–0.367) and high levels (0.368–3.941) (ANOVA, P<0.001). (b) The distribution of log-transformed (using a base 10 logarithm) spore levels (c.f.u. g⁻¹) across categories of increasing toxin levels and by ribotype: 027–078 (ANOVA, P<0.001), 014–020 (ANOVA, P=0.232) and other ribotypes (ANOVA, P<0.001). ND, non-detectable.
patient location ($\chi^2$, $P=0.06$). Stool samples infected with ribotypes 027–078 compared to other ribotypes (excluding 014–020) were 2.12 (95 %, CI: 1.00–4.49) times more likely to have high levels of toxins. This association remained strong after adjustment by age, gender, location and stool consistency [OR=2.28 (95 %, CI: 1.04–5.01)]. By contrast, those with ribotype 014–020 compared to other ribotypes (excluding 027 and 078) had lower toxin levels, even after controlling by host variables [OR=0.12 (95 %, CI: 0.02–0.90)]. Mucoid samples were 3.14 (95 %, CI: 1.36–7.25) times more likely to have higher toxin levels than soft samples (Table 2). These associations remained after controlling for gender, age, patient location and ribotype [OR=2.89 (95 %, CI: 1.15–7.22)].

Spore levels did not differ significantly by gender ($t$-test, $P=0.35$) or patient location ($t$-test, $P=0.83$). However, patients who were 65 or older had higher average levels of log-transformed spores than younger patients ($t$-test, $P=0.04$), even after adjustment for ribotype, location, stool consistency and gender. Although not statistically significant, mucoid samples also tended to have higher numbers of spores than samples of soft consistency (LR, $P=0.14$) (Table 2). Furthermore, the distribution of spores overlapped across ribotypes (Fig. 4). Only ribotype 014–020 was significantly associated with spore levels, and the association was negative (LR, $P=0.02$), and remained after adjustment for age, gender, location and stool consistency (LR, $P=0.02$).

Water content was significantly associated with stool consistency, which validates our qualitative assessment (KW test, $P<0.001$); however, there was no statistically significant difference between water content and whether the sample was from an inpatient or outpatient (KW test, $P=0.64$). Stool consistency was distributed differently by diagnostic test ($\chi^2$, $P=0.04$): toxin-positive cases were more likely to have mucoid stool (versus a soft consistency) than PCR-positive cases were more likely to have soft stool consistency than PCR-positive cases were more likely to have soft stool consistency than PCR-positive cases were more likely to have soft stool consistency than PCR-positive cases were more likely to have soft stool consistency than PCR-positive

**Table 2. Median levels of spores by stool consistency across 200 consecutive CDI-positive stool samples**

<table>
<thead>
<tr>
<th>Stool consistency</th>
<th>Spores levels (c.f.u. g$^{-1}$) Median (range)</th>
<th>Unit change in spore levels*</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft</td>
<td>$2.6 \times 10^4$ (0–4.0 $\times 10^6$)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Formed/semiformed</td>
<td>$7.0 \times 10^3$ (0–2.4 $\times 10^4$)</td>
<td>0.97</td>
<td>0.95</td>
</tr>
<tr>
<td>Mucoid</td>
<td>$6.2 \times 10^4$ (0–5.5 $\times 10^6$)</td>
<td>1.49</td>
<td>0.14</td>
</tr>
<tr>
<td>Liquid</td>
<td>$7.5 \times 10^3$ (0–2.8 $\times 10^4$)</td>
<td>0.74</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*Unit change in spore levels compared to soft stool consistencies.
patients after adjustment for age, gender, patient location and ribotype [OR=3.10 (95 %, CI: 1.28–7.52)]. Furthermore, stool samples infected with ribotypes 027–078 were 4.18 (OR 95 %, CI: 1.52–11.50) times more likely to be mucoid than soft after controlling for host variables.

C. albicans were quantified for 166 samples (83 %) of our study specimens. Among those 166 samples, 30 (18 %) were positive for C. albicans. Samples where C. albicans could be quantified were not significantly different from those where it could not be quantified (due to lack of sample) with respect to gender, age, location, or stool consistency. The quantities of C. albicans did not differ by age (KW test, P=0.80), gender (KW test, P=0.40), stool consistency (ANOVA, P=0.80), or spore levels (LR, P=0.63). Although not statistically significant, stools with high levels of toxin had higher C. albicans levels than those with non-detectable toxin levels (ANOVA, P=0.10). Outpatients had significantly higher average levels of C. albicans than inpatients (KW test, P=0.01). This relationship was maintained even after controlling by age, gender, stool consistency and C. difficile ribotype in a multivariate linear regression model. An increase of 4.76 c.f.u. log of C. albicans was observed between the outpatient and inpatient categories (LR, P=0.02).

High levels of C. albicans (‘overgrowth’, defined as five or more logs of C. albicans) were detected in 13 % (n=22). C. albicans overgrowth did not vary by age (χ², P=0.61) or stool consistency (χ², P=0.33). Although outpatients were 2.4 times more likely to have C. albicans overgrowth than inpatients (95 % CI 1.0–5.8), this association was explained by C. difficile ribotype. Stool samples with ribotypes 027–078 were 2.91 (OR 95 %, CI: 1.05–8.10) times more likely than other ribotypes (excluding 014–020) to have C. albicans overgrowth, and this association remained after adjustment by host variables [OR 4.05 (95 %, CI: 1.33–12.33)]. There were no significant differences in the distributions of toxin (χ², P=0.12) or spore levels (KW test, P=0.21) between patients with C. albicans overgrowth compared to those without it. The positive association between spore and toxin levels was not affected by C. albicans overgrowth, and remained significant among samples with C. albicans overgrowth (ANOVA, P<0.001) and among those without it (ANOVA, P=0.04).

DISCUSSION

We observed a strong positive association between in vivo levels of C. difficile toxin and spore production among 200 C. difficile-positive stool samples from in- and

Fig. 4. Distribution of log-transformed (using a base 10 logarithm) spore levels by ribotype across 200 CDI-positive stool samples. The top panel includes ribotypes 027–078 (n=40), the middle panel includes ribotype 014–020 (n=26) and the bottom panel includes the rest of the ribotypes in our population (n=120) (ANOVA, P=0.006).
outpatients. This association remained even after stratifying by ribotype. Contrary to a previous in vitro finding [6], our results do not support a trade-off between spore and toxin production within the human CDI host. As increased toxin production has been associated with more severe symptoms [6, 21], it is likely that CDI patients with more severe symptoms will produce more spores, enhancing C. difficile transmission. Similarly, as the number of spores increases, it is possible that more will germinate within the host, increasing toxin production and thus disease severity. While results from a mouse model showed a positive association between C. difficile toxin and spore levels [9], we found no similar prior reports in humans.

In addition, we observed a significant difference in spore levels between toxin-positive and toxin-negative/PCR-positive samples. However, the distribution of spore levels among toxin-positive and toxin-negative/PCR-positive samples overlapped (Fig. 3). Furthermore, the distribution of spores overlapped across ribotypes (Fig. 4). This confirms previous reports of inter-strain variation of sporulation and germination patterns across ribotypes [22–24]. This observed overlap and the positive toxin/spore correlation we described raise the question of whether the same cell population produces both toxin and spores, or if the cell population is separated into toxin and spore producers. Saujet et al. described the alternative sigma factor SigH in C. difficile that has an inverse correlation with toxin production, but a direct one with spore regulation [25]. This supports the hypothesis of separate populations [26]. However, in a related species, Clostridium perfringes, spores and enterotoxin (CPE) are released simultaneously [27]. Further studies are required to better elucidate how spore and toxin production might be linked in C. difficile and to identify the bacterial and host signals regulating their productions in vivo.

In spite of the previously mentioned inter-strain variation, our results also support the hypervirulence previously described for ribotypes 027 and 078–126 [6, 19, 20]. Our results are also consistent with the increased competitive fitness reported for ribotype 027 in comparison to other strains [28]. Ribotypes 027 and 078–126 were strongly associated with higher levels of faecal toxin even after controlling for age, gender, location and stool consistency. Although our study was unable to link these ribotypes to clinical severity, a point still under debate [18, 29], we were able to associate them with a mucoid stool consistency, which one may expect to be correlated with more severe CDI clinical symptoms.

In order to reduce CDI overdiagnosis, the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) guidelines recommend against CDI testing of formed stools or test of cure [30], yet our sample contained 8% (n=16) formed/semi-formed stool samples. This higher than expected percentage of formed/semi-formed stools among the collected clinical samples suggests that semi-formed stools (soft but still formed) may be under-recognized in clinical settings and thus accepted for CDI testing even though it is not appropriate. Interestingly, we found clinically relevant levels of toxin and spore production in these samples. These findings underscore the importance of considering the full clinical picture before testing for C. difficile, as once found it is often difficult to ignore. Notably, antibiotic therapy is a primary risk factor for CDI and treating asymptomatic carriage may lead to CDI and transmission to others [31, 32]. Our results also highlight that patients with no or milder symptoms (i.e. formed/semi-formed stools) may still merit contact precautions, as they may be shedding spores at significant levels. Likewise, these results support the use of PCR testing for CDI infection prevention and control purposes, even if its diagnostic applicability is still controversial [21, 33–35].

Although our definition of C. albicans was quite sensitive (two positives out of six replicates was considered positive), the proportion of samples with C. albicans observed in the tested subset (18%) is similar to that in previous reports among CDI patients. In a double-blinded, randomized, phase III clinical trial in multiple hospitals in the USA and Canada, Nerandzic et al. reported 16% Candida spp. positivity among 301 CDI patients before the initiation of treatment [36]. Manian et al. reported 17% Candida spp. overgrowth among 60 American CDI cases [14]. Both negative and positive correlations between C. albicans and CDI have been reported in the literature [13, 14]. However, among our CDI-positive population, we found no statistically significant difference between the distribution of toxin and spore levels among patients with C. albicans overgrowth and those without it. Interestingly, Nerandzic et al. [36] reported that vancomycin treatment can favour Candida spp. acquisition; however, our samples were collected from the laboratory before CDI treatment was usually given for the current CDI episode. Thus, future research should investigate whether Candida spp. affects the in vivo spore/toxin correlation during or after CDI treatment, where overgrowth of Candida spp. may be more prevalent. Additionally, similar to Raponi et al. [13], we found a high prevalence of C. albicans overgrowth among cases infected with ribotype 027. This association merits further research using longitudinal studies.

Our study was somewhat limited by the moderate sensitivity of our toxin test (95%, CI: 87–95 according to the manufacturer [16]). This test was chosen because our purpose was to identify the total toxin A and B produced in the stool, and other, more sensitive, tests, such as a cytotoxicity assay, only detect active toxin. However, our sample size was large and the association between spore and toxin production was strong – even after controlling for confounders. Recent debate about the effectiveness of heat treatment to eliminate vegetative cells may also limit our results somewhat. Edwards et al. recently reported that heat treatment can significantly reduce vegetative cell counts – by up to three logs [37] – which would limit the effectiveness of this method in terms of the initial faecal C. difficile burden. Unfortunately,
we are unable to adjust for this variable, as we did not quantify the initial *C. difficile* faecal load as part of this study. Furthermore, the methodology used for spore quantification counted only spores that germinated under study conditions. This methodology may have underestimated the total number of spores on stool, as some spores may be unable to germinate. Additionally, our study is also somewhat limited by its design. CDI patients potentially have different faecal levels of toxins and spores at different points during their infection process. Due to our cross-sectional design, we were only able to capture spore and toxin levels at one point in time, which may vary across our study population. Longitudinal studies are needed to better elucidate the *in vivo* association between *C. difficile* spores and toxin.

In conclusion, *in vivo* toxin and spore production were positively and significantly correlated among clinical CDI samples, even after stratifying by ribotype. Therefore – as is already recommended – contact precautions are in order for all individuals with CDI during their entire hospital stay. Nearly all of our patients with no detectable toxin production shed spores, sometimes at significant levels. This highlights the complexity of diagnosis and prevention of a condition with an asymptomatic state and underscores the need to identify alternative therapies and control measures that better address not only the health of an infected individual, but the risk of transmission to others.

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### Conflicts of interest
The authors declare that there are no conflicts of interest.

### Ethical statement
The Institutional Review Boards at Saint Joseph Mercy Health System and the University of Michigan (IRB Health Sciences and Behavioral Sciences) approved the study protocol. The study was exempt and informed consent was waived.

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