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Variation in germination of *Clostridium difficile* clinical isolates correlates to disease severity

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Abstract

Over the past two decades, *Clostridium difficile* infections have been increasing in both number and severity throughout the world. As with other spore forming bacteria, germination is a vital step in the life cycle of this pathogen. Studies have examined differences in sporulation and toxin production among a number of *C. difficile* clinical isolates; however, few have examined differences in germination and the relationship between this phenotype and disease severity. Here, over 100 *C. difficile* isolates from the University of Michigan Health System were examined for overall germination in response to various combinations of known germinants (taurocholate) and co-germinants (glycine and histidine). Significant variation was observed among isolates under all conditions tested. Isolates representing ribotype 014-020, which was the most frequently isolated ribotype at our hospital, exhibited increased germination in the presence of taurocholate and glycine when compared to isolates representing other ribotypes. Interestingly, isolates that caused severe disease exhibited significantly lower germination in response to minimal germination conditions (taurocholate only), indicating increased control over germination in these isolates. These data provide a broad picture of *C. difficile* isolate germination and indicate a role for precise control of germination in disease severity.

Keywords

Clostridium difficile; spore; ribotype; NAP1/027; germination

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1.0 Introduction

Clostridium difficile, a Gram-positive, spore-forming anaerobic bacterium, is currently a leading cause of nosocomial infection world-wide [1, 2]. *C. difficile* infection (CDI) often manifests as the bacterium colonizes the gastrointestinal tract following antibiotic treatment, which increases patient susceptibility by altering the population structure of the commensal bacteria that typically provide colonization resistance [1–4]. The prevalence and severity of CDI have escalated over the last decade, with over a 250,000 cases resulting in 14,000 deaths occurring annually in the United States alone [1, 5, 6].

Spores play a crucial role in the transmission of *C. difficile*. These spores are metabolically dormant and resistant to a wide range of physical and chemical stresses, including environmental oxygen and the acidic environment of the stomach [7]. Upon entry into the small intestine, spores germinate to become the metabolically active vegetative cells [8, 9]. Germination occurs in response to specific compounds, germinants, found in the intestinal environment. The bile salt, taurocholate, is the primary germinant for *C. difficile* spores, with amino acids including glycine and histidine serving as co-germinants, which enhance germination in the presence of taurocholate [10, 11]. Spore germination is an early-event prerequisite for CDI and the process is essential for both pathogenesis and the infectious cycle.

Differences in germination rates of *C. difficile* clinical isolates have been examined in previous studies, with differing conclusions drawn. In one study, isolates of the epidemic ribotype, 027, were shown to exhibit higher germination efficiency than isolates of other ribotypes [12]. In contrast, others reported decreased germination levels in 027 ribotype spores as compared to other ribotypes [11]. In the most comprehensive study to date, Heeg, et al. examined 29 *C. difficile* isolates and found significant diversity among isolates regardless of ribotype [13].

The current study builds on, and clarifies, these earlier studies through the examination of 106 clinical isolates and comparison with clinical data from the original cases of CDI from which these were isolated. To determine if a correlation existed between *C. difficile* disease severity and spore germination *in vitro*, we measured the germination of 106 recent *C. difficile* isolates under multiple laboratory conditions. As clinical data were available for the individuals originally infected with each isolate, these germination data then were examined to determine if there was a correlation between germination efficiency and disease severity as well as if there were any correlations between germination and ribotype.

2.0 Methods and Materials

2.1 Strain isolation and selection

Clinical cases of *C. difficile* [14, 15] were identified from potential cases identified by the clinical microbiology laboratory at the University of Michigan as previously described. *C. difficile* isolates were cultured from patient stool samples using taurocholate-cefoxitin-cycloserine-fructose agar (TCCFA) plates using a published protocol [16]. All isolates examined were previously characterized by our laboratory [17]. All isolates studied were

toxigenic based on initial diagnostic testing (ELISA and/or PCR for the toxin B gene, *tcdB*) and a confirmatory Vero cell toxicity assay. Isolates were selected for this study using three criteria. First, a broad selection of isolates was chosen to represent the diversity of ribotypes in the University of Michigan hospital between January 2010 and February 2011, including multiple representatives of 12 distinct ribotypes as well as 27 single isolates representing other ribotypes. Second, increased numbers of the epidemic ribotype 027 (n=27) and the predominant ribotype in our hospital at the time, 014-020 (n=21) were included to assess differences in these highly studied ribotypes. Finally, isolates from every case of severe CDI identified at our hospital during the sampling time were included in the study (n=34). Cases exhibiting an infection that either: i) required admission to an ICU, ii) required an interventional surgery (e.g. colectomy), or iii) resulted in death, within 30 days of diagnosis were defined as severe, according to CDC recommendations [18]. These presentations had to be attributable to CDI in order for the case to be considered severe CDI.

2.2 Bacterial growth conditions and spore stocks

C. difficile isolates were cultured in an anaerobic chamber (Coy Laboratory Products, MI). For *in vitro* experiments, *C. difficile* isolates were cultured in BHIS (brain-heart infusion broth supplemented with 0.5% yeast extract and 0.1% cysteine; no glucose or iron were added to this medium) unless otherwise indicated. Spore stocks for all isolates were produced as follows. Freezer stocks of clinical isolates (single passage) were plated on BHIS. An isolated colony was used to inoculate an overnight culture. Four plates were then inoculated with 100µl of overnight culture and incubated for seven days at 37°C under anaerobic conditions before being moved to normal oxygen for one day to kill vegetative bacteria. Bacterial lawns were then resuspended in 4°C water and washed at least four times to remove vegetative cell debris [16]. Spore suspensions were also heated at 65°C for 30 minutes to ensure killing any remaining vegetative bacteria. Spore stocks were stored in water at 4°C. The number of viable spores produced in this method did vary between isolates, as would be expected from previous studies [17]. To confirm consistency across preparations, a small number of isolates were subjected to multiple preparations using both the method above and by growth in liquid broth. These different spore preparations resulted in nearly identical performance germination assays (data not shown). Also, the purity of spore stocks, as observed by phase contrast microscopy, did not have an effect on germination totals (data not shown).

2.3 Germination assays

Germination was measured as a function of the ability of a germinated spore to outgrow in the absence of taurocholate. Spore stocks incubated at 65°C for 30 minutes a second time immediately prior to use in germination assays to remove any spores that had undergone spontaneous germination during storage. Spores were incubated anaerobically with either 0.1% taurocholate (Tc) or 0.1% Tc/0.4% glycine in PBS for 30 minutes. Following incubation, germination was stopped by immediately performing a 1:10 dilution to lower Tc well below optimal levels. Germinated spores were enumerated by plating for colony-forming units (CFU) on BHIS plates without Tc. Colonies that grew on these plates were considered to be germinated vegetative bacteria and were compared to total CFU/ml of the sample determined by plating on BHIS + 0.1% Tc. Percent germination of a given sample

was calculated as follows: (CFU/ml BHIS only plate)/(CFU/ml BHIS+Tc plate) *100). Assays were performed in triplicate for each isolate. Follow up studies were performed using the same assay with PBS only, PBS/glycine/histidine (0.4%)/Tc, PBS/histidine/Tc, or BHIS/Tc as the germination mixture.

2.4 Statistical analyses

All statistical methods were performed using GraphPad Prism version 6 (GraphPad Software, San Diego California USA). For comparison of two groups, the nonparametric Mann-Whitney test was used. For three group comparisons, the nonparametric Kruskal-Wallis test was used.

2.9 Human Subjects Approval

The University of Michigan Institutional Review Board approved all sample and clinical data collection protocols used in this study. Where applicable, written, informed consent was received from all patients prior to inclusion in this study.

3.0 Results

3.1 Clinical isolate selection criteria

Clinical isolates of *C. difficile* exhibit equal levels of germination in response to rich conditions *in vitro* [17], however, the ability of these isolates to germinate under more stringent conditions has not been examined. We hypothesized that a correlation may exist between the ability of an individual isolate to germinate under stringent conditions with the severity of disease caused by that isolate. To test this, clinical isolates of *C. difficile* were examined for the ability to germinate under such conditions. Isolates were chosen to include a broad range of ribotypes including an over-representation of ribotypes 027 and 014-020, the two most common ribotypes found in our hospital system. In order to identify a correlation between disease severity and germination, isolates from all 34 cases of severe CDI that had been identified during the collection period were included in the study.

3.2 Germination of *C. difficile* clinical isolates in Taurocholate only

Although significant differences in germination rates were not observed in rich media [17], the possibility remained that these isolates would exhibit differences when germination was induced under more restricted/stringent conditions. It is likely that the conditions within the gut are not as nutrient rich as bacterial culture media; so testing germination under these more restricted conditions could be more relevant than examining germination in BHIS. To test this, clinical isolates of *C. difficile* were incubated in PBS with 0.1% taurocholate only (no co-germinant) for 30 minutes and germination totals during this incubation were assessed. Under these restricted/stringent germination conditions, a wide range of germination levels was observed (0.001 – 29.4%), with the majority of the clinical isolates tested exhibited very low levels of germination (less than 6%) during this time frame (Figure 1A). A few isolates did exhibit higher levels of germination under these conditions (Figure 1A). When isolates were grouped based on ribotype, no significant differences were observed in the ability of one group to germinate in response to taurocholate alone (Figure 1B). Interestingly, when isolates were grouped based on disease severity, a significant

difference was observed (Figure 1A, black bars; Figure 1C). Isolates that caused severe disease were more likely to germinate poorly in response to taurocholate alone (median germination 0.355% vs. 1.85% for non-severe isolates; Figure 1C). This low level of germination for severe disease-associated isolates implies an important role for the precise control of germination in the pathogenesis of *C. difficile*.

One possible explanation for the higher levels of germination observed for some isolates in response to taurocholate alone is that these isolates are undergoing spontaneous germination, as has been observed previously for clinical isolates of *C. difficile* [13]. If this were the case, these isolates would exhibit the same level of germination in the absence of taurocholate. To exclude this possibility, all isolates that exhibited germination greater than 5% in taurocholate alone (n = 19) were assessed for germination in PBS alone (Figure 2). Only two of the isolates tested exhibited significant levels of spontaneous germination (Figure 2, gray bars). In fact, the majority (15/19) of isolates tested did not exhibit any spontaneous germination after 30 minutes (Figure 2). These results indicate that, for the majority of isolates tested, the observed germination in response to taurocholate alone is specific to this germinant and not due to spontaneous germination events.

3.2 Germination of clinical isolates with co-germinant

While taurocholate has been identified as the primary germinant for *C. difficile* and it is required for germination of spores ([10], Figure 2), germination is much more efficient in the presence of the co-germinant glycine ([10, 11]). To assess the role of glycine on the germination of *C. difficile* clinical isolates, each isolate was incubated in PBS with 0.1% taurocholate and 0.4% glycine for 30 minutes and then assessed for total germination. Again, these isolates exhibited a wide range of germination levels (6.2 –100 %), however 86.7% of isolates tested (92/106) exhibited greater than 60% germination under these conditions (Figure 3A). When the isolates were grouped based on ribotype, a clear pattern emerged. Isolates of ribotype 014-020 exhibited significantly higher levels of germination (median = 93%) compared to the other ribotypes tested (027 = 82%, “other” = 85%) (Figure 3B). These isolates also exhibited the least variation in germination levels between isolates (Figure 3B). Unlike for germination in taurocholate alone, when the isolates were grouped based on disease severity, no significant difference was observed for germination in the presence of glycine and taurocholate, with the two groups exhibited almost identical median germination values (Figure 3C).

Some of the isolates tested exhibited low germination totals even in the presence of glycine. In order to assess any influence of as yet unknown co-germinants in these isolates, each isolate exhibiting germination of less than 60% in the presence of taurocholate and glycine was incubated in taurocholate plus either glycine, histidine, glycine and histidine, or BHIS broth (Figure 4). Taurocholate/glycine mixture induced germination as discussed above (Figure 3; Figure 4, black bars). The addition of histidine, another known *C. difficile* co-germinant [11], led to a significant increase in total germination, with most isolates reaching more than 80% germination in 30 minutes (Figure 4, dark gray bars). Although histidine can enhance germination in the presence of glycine and taurocholate, the combination of histidine and taurocholate (without glycine) did not induce more than 15% germination in

any single isolate and, in many cases, resulted in less than 1% germination (Figure 4, light gray bars). As expected, incubation in rich media with taurocholate led to nearly complete germination in the all but one isolate tested (Figure 4, white bars), suggesting the presence of either additional co-germinants or higher levels of glycine/histidine in this medium. One isolate (Cd004) exhibited poor germination under all conditions tested, although germination was enhanced by the presence of glycine + histidine or BHIS broth in addition to taurocholate. Data for individual isolates are provided in the supplementary information (Table S1).

4.0 Discussion

In recent years, hospitals have seen a significant increase in cases of *C. difficile* infection. Many studies have examined clinical isolates, and specific common ribotypes, in attempts to account for the increases in both cases and the severity of the cases being seen [19, 20]. Here we show evidence linking the ability to respond to germinants in a specific, controlled manner to an increase in case severity. Also, isolates of the most common ribotype observed in the University of Michigan hospitals, 014-020, exhibit a greater level of germination than those of other ribotypes tested, including the highly studied ribotype 027.

The increased incidence of *C. difficile* infections over the past decade has led to an ever growing body of research examining individual isolates, specific ribotypes, and individual cases in an attempt to find correlates of disease severity [21–26]. In this study, specific focus was placed on two ribotypes, 027 and 014-020. For years, ribotype 027 has been studied as a hypervirulent and/or epidemic ribotype and has often been found to be responsible for greater numbers of CDI than other ribotypes [27, 28]. Interestingly, while multiple studies have found greater levels of toxin for isolates of ribotype 027 [17, 21, 29], this phenotype has not been consistently correlated to disease severity in humans in all studies [15, 17]. The epidemic ribotype, 014-020, has been seen in increasing numbers in recent years and has become the most common ribotype found in some hospitals throughout the world [30–32]. This was also the most commonly isolated ribotype at the University of Michigan hospitals during our collection period [15, 17].

We recently reported a correlation between sporulation levels and disease severity, with isolates that caused severe disease producing higher levels of spores than those that caused more mild cases of CDI [17]. In that study, no difference in isolate germination was identified when incubated under rich conditions (BHIS broth plus taurocholate) [17]. Here, in contrast, significant differences were observed in germination levels of the same group of isolates when more stringent conditions were examined. When isolates were incubated in taurocholate alone, significant differences in germination rates were observed. While there were general differences across all isolates, when these were grouped based on ribotype alone, no significant patterns emerged, indicating a lack of correlation between ribotype and germination under these conditions. These results are similar to previous studies that have failed to identify phenotypic differences between isolates based on ribotype groups, with the exception of toxin production, which is increased among isolates representing ribotype 027 [17].

Other studies have reported differences in germination efficiencies for isolates of ribotype 027, one observing increased germination and another decreased germination among these isolates [11, 12]. There are multiple possible explanations for the discrepancy between these three studies. First, the methods of measuring germination differ with Moore et. al. using drop in optical density [12] while Wheeldon et al. and our study used loss of heat resistance and inability to outgrow in the absence of germinant to identify germination percentages ([11], Methods). A more likely possibility is the relatively low number of isolates tested these previous studies. In the current study, 27 isolates representing ribotype 027 were examined. No significant differences were found in germination for these isolates under any conditions tested (Figure 1, 3). A similar situation occurred previously when laboratories began examining sporulation of ribotype 027 isolates. Early studies looking at low numbers of isolates showed increased sporulation for this ribotype [21, 33], while studies examining high numbers of isolates showed no significant difference between this ribotype and others [17, 34].

Interestingly, when these isolates were grouped based on disease severity, a clear pattern emerged. Isolates that caused severe disease were actually impaired for germination under this minimal condition. This result was surprising as it was hypothesized that isolates that caused severe disease would exhibit higher levels of germination. One possible explanation for this result is that having tighter control over the specific location of germination could provide a selective advantage for one isolate over another. By not germinating until entering optimal conditions, represented by the presence of both taurocholate and a co-germinant, the entire infecting dose of spores may germinate and proliferate with only minimal loss of viability. This would essentially lead to a high infectious dose, which could, in turn, produce a more severe case of CDI. The relationship between infectious dose and disease severity has been described in murine models of CDI [35].

With a few exceptions, isolates of all ribotypes germinated very well in response to the combination of taurocholate and glycine. These results are similar to what was observed previously for a subset of these isolates incubated in BHIS with 0.1% taurocholate, where no differences were observed between 30 isolates [17]. This high rate of germination indicates that, for the majority of isolates tested, the combination of taurocholate and glycine is sufficient to induce nearly complete germination of viable spores. Interestingly, isolates representing ribotype 014-020 exhibited significantly greater levels of germination in response to a combination of glycine and taurocholate (Figure 3). It is possible that the success of this ribotype in spread through hospitals is specifically related to this increased germination efficiency. More efficient germination could result in a lower infectious dose for a given strain.

The data presented here show a clear correlation between both an epidemic ribotype (014-020) and disease severity of clinical isolates and the ability to recognize and respond to specific combinations of germinants. These data suggest a connection between the ability to germinate in the appropriate environment and the ability of a given isolate to cause severe disease. Further study will be needed to identify differences inherent to these isolates that lead to this increased control over germination and to elucidate the role of this control in the pathogenesis of *C. difficile*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Decreased *in vitro* germination in response to taurocholate alone was associated with severe human CDI.
- We have identified an additional correlate of disease severity for this pathogen.
- Isolates representing epidemic Ribotype 014 isolates exhibit higher levels of germination in response to taurocholate plus glycine.
- No significant germination differences could be associated with the prevalent ribotype 027.

Figure 1. Germination of *C. difficile* clinical isolates in taurocholate alone

(A) Total germination was examined for spores of each clinical isolate following incubation in PBS+0.1% taurocholate for 30 minutes. All measurements were performed in triplicate for each isolate and presented as the percent germinated spores = $((\text{vegetative bacteria})/(\text{total spores})) \times 100$. Data are presented as % germination and represent mean $\pm$ SD of triplicate measurements. Black bars represent isolates that caused severe disease, while gray bars represent those that did not. (B) Comparison of median values with interquartile range for isolates representing ribotypes 027, 014, or “other.” Groups were compared for statistical

significance using Kruskal-Wallis test ($p = 0.1429$) followed by Dunn's test for column comparisons (no significant differences). (C) Comparison of median values with interquartile range for isolates that caused severe disease were compared to other using unpaired Mann-Whitney t -test ($p = 0.0001$).

Figure 2. Increased levels of germination in taurocholate alone are germinant specific

Isolates that exhibited germination greater than 5% were tested for spontaneous germination by incubation in PBS only for 30 minutes. Germination levels for each isolate in either PBS/0.1% taurocholate (black bars) or PBS only (white bars) are shown. All measurements were performed in triplicate for each isolate and presented as the percent germinated spores = $((\text{vegetative bacteria})/(\text{total spores})) \times 100$. Data are presented as % germination and represent mean $\pm$ SD of triplicate measurements.

Figure 3. Germination of *C. difficile* clinical isolates in the presence of glycine and taurocholate
 (A) Total germination was examined for spores of each clinical isolate following 30 minute incubation in PBS/0.4% glycine/0.1% taurocholate. All measurements were performed in triplicate for each isolate and presented as the percent germinated spores = ((vegetative bacteria)/(total spores)) $\times$ 100. Data are presented as % germination and represent mean $\pm$ SD of triplicate measurements. Black bars represent isolates that caused severe disease, while gray bars represent those that did not. (B) Comparison of median values with interquartile range for isolates representing ribotypes 027, 014, or “other.” Groups were

compared for statistical significance using Kruskal-Wallis test ($p = 0.0009$) followed by Dunn's test for column comparisons ($* = p < 0.05$). (C) Comparison of median values with interquartile range for isolates that caused severe disease were compared to other using unpaired Mann-Whitney t -test ($p = 0.0001$).

Figure 4. Additional co-germinants are necessary for efficient germination of some *C. difficile* clinical isolates

Isolates that exhibited germination less than 60% in PBS/glycine/taurocholate were tested for germination in the presence of additional germinants for 30 minutes. Germination levels for each isolate in either PBS/glycine/taurocholate (black bars), BHIS/taurocholate (white bars), PBS/glycine/histidine/taurocholate (dark gray bars), or PBS/histidine/taurocholate (light gray bars) are shown. All measurements were performed in triplicate for each isolate and presented as the percent germinated spores = ((vegetative bacteria)/(total spores)) $\times$ 100. Data are presented as % germination and represent mean $\pm$ SD of triplicate measurements.