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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.

1. 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

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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. 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 100 μl 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 min 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 min 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 min. 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.

2.5. 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.. 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 overrepresentation 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.