Germination efficiency of clinical Clostridium difficile spores and correlation with ribotype, disease severity and therapy failure

P. Moore,1,3 L. Kyne,1,2 A. Martin2 and K. Solomon1

Correspondence
K. Solomon
katie.solomon@ucd.ie

1UCD School of Medicine and Medical Science, University College Dublin, Dublin 4, Ireland
2Department of Medicine for the Older Person, Mater Misericordiae University Hospital, Eccles St., Dublin 7, Ireland
3Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Dublin 4, Ireland

Spore germination is an important part of the pathogenesis of Clostridium difficile infection (CDI). Spores are resistant to antibiotics, including those therapeutically administered for CDI and strains with a high germination rate are significantly more likely to be implicated in recurrent CDI. The role of germination efficiency in cases of refractory CDI where first-line therapy fails remains unclear. We investigated spore germination efficiencies of clinical C. difficile isolates by measuring drop in OD600 and colony forming efficiency. Ribotype 027 isolates exhibited significantly higher germination efficiencies in the presence of 0.1 % (w/v) sodium taurocholate (51.66 ± 8.75 %; 95 % confidence interval (CI) 47.37–55.95 %) than ribotype 106 (41.91 ± 8.35 %; 95 % CI 37.82–46 %) (P < 0.05) and ribotype 078 (42.07 ± 8.57 %, 95 % CI 37.22–46.92 %) (P < 0.05). Spore outgrowth rates were comparable between the ribotype groups but the exponential phase occurred approximately 4 h later in the absence of sodium taurocholate. Spore germination efficiencies for isolates implicated in severe CDI were significantly higher (49.68 ± 10.00 %, 95 % CI 47.06–52.30 %) than non-severe CDI (40.92 ± 9.29 %, 95 % CI 37.48–44.36 %); P < 0.01. Germination efficiencies were also significantly higher in recurrent CDI or when metronidazole therapy failed than when therapy was successful [(49.00 ± 10.49 %, 95 % CI 46.25–51.75 %) versus (41.42 ± 9.43 %, 95 % CI 37.93–44.91 %); P < 0.01]. This study suggests an important link between C. difficile spore germination, CDI pathogenesis and response to treatment; however, further work is warranted before the complex interplay between germination dynamics and CDI outcome can be fully understood.

INTRODUCTION

Clostridium difficile is an anaerobic spore-forming bacterium and a major cause of hospital-acquired diarrhoea. Spores play a pivotal role in transmission of C. difficile infection (CDI), as they are resistant to a wide range of chemical and physical stressors and can persist in a contaminated environment for several weeks to months (Gerding et al., 2008; Riggs et al., 2007). Spore germination in the gastrointestinal tract is an essential precursor to CDI, by enabling vegetative cell growth, metabolism and production of toxins A and B that mediate intestinal epithelium injury, inflammation and colitis (Kelly et al., 1994; Voth & Ballard, 2005).

The recommended therapy for an initial episode of mild to moderate CDI is metronidazole (500 mg three times per day for 10–14 days), with vancomycin therapy (125 mg four times per day for 10–14 days) indicated for severe CDI (Cohen et al., 2010). If symptoms persist at the end of initial therapy, then vancomycin is usually indicated for secondary therapy where first-line therapy with metronidazole has failed (Cohen et al., 2010). In approximately 10–35 % of cases, CDI symptoms can recur after symptoms have resolved and therapy has ceased (Barbut et al., 2000; McFarland et al., 2002). Approximately half of recurrent CDI cases are thought to be due to reinfection rather than relapses of infection with the original persistent strain (Barbut et al., 2000; Norén et al., 2004; Wilcox et al., 1998).

Mechanisms underlying recurrent and refractory CDI remain unclear. Vancomycin and metronidazole are highly
active bacteriostatic agents against vegetative bacteria, but have no anti-spore activity, allowing persistence of a residual spore population after therapy that can undergo germination and outgrowth once antimicrobial agents reach sub-inhibitory levels in the gut (Baines et al., 2009; Freeman et al., 2005; Warren et al., 2013).

The mechanism of spore germination and outgrowth in C. difficile is not yet fully understood and is thought to follow a three-stage process as seen in Bacillus subtilis: (i) binding of germinants to specific receptors, (ii) release of monovalent cations and (iii) hydrolysis of the spore peptidoglycan cortex by cortex-lytic enzymes, core rehydration and initiation of metabolic activity and replication (Paidhungat & Setlow, 2000; Paredes-Sabja et al., 2009; Setlow, 2003). C. difficile germinants and associated receptors are not well characterized; however, a role for specific bile salts, including sodium taurocholate, and the amino acid glycine has been shown (Heeg et al., 2012; Howerton et al., 2011; Sorg & Sonenshein, 2008; Wilson, 1983).

Interstrain variation in C. difficile spore germination rate and proportion of spores completing germination has been shown in a gut model of CDI (Baines et al., 2009; Freeman et al., 2007) and in vitro (Burns et al., 2010a; Heeg et al., 2012; Paredes-Sabja et al., 2008). Although germination rates in reference strains 630 and VP10463 have been shown to be similar (Ramirez et al., 2010), the germination frequency of an epidemic BI/NAP-1/ribotype 027 ‘hyper-virulent’ strain was shown to be lower compared to non-epidemic C. difficile 630Δerm (Burns et al., 2010b). More recent studies have found that C. difficile spore germination in response to sodium taurocholate varied significantly even within the BI/NAP-1/027 group, suggesting that ‘hypervirulence’ with regard to germination may be isolate specific rather than strain type specific (Heeg et al., 2012). A link between high germination rate and CDI recurrence has been previously identified (Oka et al., 2012); however, a link between germination rate, strain type and refractory or relapsing CDI has yet to be established.

In this study we measured in vitro spore germination efficiency and outgrowth rates of clinical C. difficile isolates characterized by PCR ribotyping. We also examined patient clinical data from each CDI case to determine whether spore germination efficiency correlated with ribotype, disease severity or outcome, including therapy failure and recurrence.

METHODS

Bacterial strains. Bacterial isolates under investigation in this study were selected from an archived collection of 86 C. difficile isolates from toxin-positive fecal samples provided at diagnosis of first infection episode by patients that met the CDI case definition in a previously studied prospective cohort in the Republic of Ireland between 2009 and 2010 (Solomon et al., 2011). Isolates of ribotypes 027, 106 and 078 were selected for this study on the basis of the high prevalence of these three types in the Republic of Ireland during the collection period: ribotype 027 (19 %), ribotype 106 (13 %) and ribotype 078 (10 %) (Burns et al., 2010c). Four isolates from each ribotype were selected that were genetically diverse, i.e. categorized into separate rep-PCR subtypes or exhibited different antimicrobial susceptibility phenotypes, as previously reported (Solomon et al., 2011), to reduce the risk of selecting clonal isolates (Table 1).

Isolates selected for further study had been fully characterized as part of a previous study (Solomon et al., 2011). In brief, isolates were assigned a PCR ribotype by PCR amplification of the 16-23S intergenic-spacer region by the method described by Stubbs et al. (1999). Isolates were assigned a toxinotype by comparison of their RFLP banding patterns to those obtained by the method described by Rupnik et al. (1998). Isolates were assessed for the presence of the binary toxin gene (cdtB) and the 18 bp/39 bp deletion in the regulatory gene tcdC by the methods described by Cohen et al. (2000). Antimicrobial susceptibility testing was performed by the E-test method. Breakpoints of resistance for moxifloxacin, clindamycin and erythromycin (MIC $\geq 8$ mg l$^{-1}$) and metronidazole and vancomycin (MIC $\geq 4$ mg l$^{-1}$) were defined by reference to the Clinical and Laboratory Standards Institute (CLSI, 2007). Genotypic and phenotype characteristics of the isolates selected for further study are presented in Table 1.

Patient clinical data. Patient clinical and demographic data were collected at diagnosis and faecal sample collection and for the following 120 days as part of a previous prospective cohort study (Solomon et al., 2011). The episode of CDI was defined as ‘severe’ if the patient met the criteria outlined by Cohen et al. (2010) as white cell count $>15 \times 10^9$ l$^{-1}$ or a serum creatinine rise $\geq 1.5$ times the baseline level. The treatment regimens and success or failure of treatment were recorded. Failure of metronidazole therapy was defined as persistence of diarrhea for more than 10 days after starting metronidazole or a clinical decision by the subject’s physician to start vancomycin prior to the 10 day end point on the basis of clinical markers of severe disease (Hu et al., 2008). Recurrence was defined as recurrence of C. difficile toxin positive diarrhea within 60 days after resolution of the initial episode for at least 48 h and after discontinuation of therapy with metronidazole or vancomycin (Kyne et al., 2001). Where C. difficile or CDI was mentioned on the death certificate, death was considered attributable to CDI. This study was approved by the local research ethics committees and consent was obtained from all participants.

C. difficile spore preparation. C. difficile isolates were resuscitated from frozen stocks (passage 0–1) by cultivation on brain heart infusion (BHIS) agar supplemented with yeast extract (5 mg ml$^{-1}$, Oxoid) and L-cysteine (0.1 % w/v, Sigma Aldrich) for 48 h at 37 °C in an anaerobic workstation (Don Whitley).

A single colony was then inoculated into BHIS broth and cultured overnight at 37 °C under anaerobic conditions. A 100 μl aliquot of overnight culture was spread onto Columbia blood agar (Oxoid) supplemented with 5 % sheep blood (Cruinn Diagnostics) and cultured anaerobically for 5 days at 37 °C to allow for sporulation. After 5 days, all spores and vegetative cells were harvested from the plates and resuspended in sterile distilled water. Spore suspensions were then heat treated at 60 °C for 25 min to kill any vegetative cells and washed at least ten times in dH2O by centrifuging at 16 000 g for 5 min. Spore suspensions were checked for purity and absence of vegetative cells and cell debris by phase-contrast microscopy before storage at OD$_{600}$ $\sim$6 in sterile distilled water at $-20$ °C prior to use.

On thawing, spore suspensions were brought to room temperature, checked for excessive clumping and were vortexed vigorously to ensure a homogeneous suspension prior to use.

Measurement of C. difficile spore germination efficiency by loss of optical density. To assess the germination efficiency of C.
difficile isolates, spore suspensions were adjusted with sterile distilled water so that a two-fold dilution of suspension would result in an OD
600 of 1. A 100 μl aliquot of spore suspension was added to 8 wells of a 96-well plate and under anaerobic conditions, 100 μl of pre-warmed, pre-reduced double-strength (2 ×) BHIS was added to four wells and 100 μl of pre-warmed, pre-reduced double-strength (2 ×) BHIS supplemented with sodium taurocholate (to obtain a final concentration of 0.1 % (w/v) Sigma Aldrich) was added to the remaining four wells. This was repeated for all spore preparations. To obtain a negative control, spore suspensions or culture media were replaced with dH2O. Germination was measured as the loss of OD
600 by using a MultiSkan FC microplate reader (Fisher Scientific) under anaerobic conditions at 37 °C over a 2 h time period with readings taken at 2 min intervals.

The germination efficiency or percentage germination was defined following the method outlined in Paredes-Sabja et al. (2008). The extent of germination was determined by measuring the decrease in OD
600 of the spore suspension within 120 min (by which time a steady state was obtained) and expressed as a percentage of initial OD
600. Paredes-Sabja et al. (2008) determined that a decrease in OD
600 of ~65 % corresponded to ≥99 % spore germination by phase-contrast microscopy, therefore the percentage decrease in OD
600 was converted to germination efficiency by taking a decrease in OD
600 of 65 % as 100 % germination efficiency.

The germination efficiency was calculated for each of four replicates of each of the 12 clinical isolates.

Measurement of C. difficile spore germination efficiency by colony forming efficiency. To further assess the germination efficiency of C. difficile isolates, the colony-forming efficiency of spores was determined. Spore suspensions were serially diluted and plated on either BHIS agar or BHIS agar supplemented with 0.1 % (w/v) sodium taurocholate. Plates were incubated anaerobically at 37 °C for 24 h and colony counts of the starting suspension were determined. The number of phase-bright spores per millilitre of starting suspension was counted directly by phase-contrast microscopy using a haemocytometer counting chamber (Improved Neubauer). The germination efficiency was defined as the percentage of total spores that gave rise to exponential vegetative cell growth, spore suspensions were standardized to obtain 1 × 103 ‘viable’ spores in 200 μl medium at the start of the experiment. Determination of ‘viable’ spore counts was obtained after calculation of the germination efficiency for each isolate by assessment of colony formation as previously described. The appropriate volume of spore suspension to obtain 1 × 103 ‘viable’ spores in a final volume of 200 μl was added to each of eight wells of a 96-well plate and made up to 100 μl with sterile dH2O. Under anaerobic conditions, 100 μl of pre-warmed, pre-reduced double-strength (2 ×) BHIS supplemented with sodium taurocholate (to obtain a final concentration of 0.1 %, w/v) was added to five wells and 100 μl of pre-warmed, pre-reduced double-strength (2 ×) BHIS was added to the remaining three wells. This was repeated for all spore preparations. To obtain a negative control, spore suspensions or culture media were replaced with sterile dH2O.

Measurement of spore outgrowth and return to vegetative cells. To assess the time taken for germinated spores to return to exponential vegetative cell growth, spore suspensions were standardized to obtain 1 × 103 ‘viable’ spores in 200 μl medium at the start of the experiment. Determination of ‘viable’ spore counts was obtained after calculation of the germination efficiency for each isolate by assessment of colony formation as previously described. The appropriate volume of spore suspension to obtain 1 × 103 ‘viable’ spores in a final volume of 200 μl was added to each of eight wells of a 96-well plate and made up to 100 μl with sterile dH2O. Under anaerobic conditions, 100 μl of pre-warmed, pre-reduced double-strength (2 ×) BHIS supplemented with sodium taurocholate (to obtain a final concentration of 0.1 %, w/v) was added to five wells and 100 μl of pre-warmed, pre-reduced double-strength (2 ×) BHIS was added to the remaining three wells. This was repeated for all spore preparations. To obtain a negative control, spore suspensions or culture media were replaced with sterile dH2O. Cell growth was measured at OD
600 under anaerobic conditions at 37 °C over a 24 h time period with readings taken at 12 min intervals.

Table 1. C. difficile isolates used in this study

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Ribotype</th>
<th>tcdC</th>
<th>cdtB</th>
<th>Toxinotype</th>
<th>Antimicrobial susceptibility*</th>
<th>rep-PCR subtype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>117</td>
<td>078</td>
<td>39 bp</td>
<td>neg</td>
<td>V</td>
<td>VAN, MET, CLI, MXF, ERY</td>
<td>078-7</td>
<td>Solomon et al. (2011)</td>
</tr>
<tr>
<td>122</td>
<td></td>
<td>39 bp</td>
<td>pos</td>
<td>V</td>
<td>VAN, MET, CLI, MXF, ERY</td>
<td>078-15</td>
<td>Solomon et al. (2011)</td>
</tr>
<tr>
<td>144</td>
<td></td>
<td>39 bp</td>
<td>neg</td>
<td>V</td>
<td>VAN, MET, CLI, MXF, ERY</td>
<td>078-5</td>
<td>Solomon et al. (2011)</td>
</tr>
<tr>
<td>158</td>
<td></td>
<td>39 bp</td>
<td>pos</td>
<td>V</td>
<td>VAN, MET, CLI, ERY, MXF</td>
<td>078-12</td>
<td>Solomon et al. (2011)</td>
</tr>
<tr>
<td>121</td>
<td>106</td>
<td>WT</td>
<td>neg</td>
<td>0</td>
<td>VAN, MET, CLI, MXF, ERY</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>128</td>
<td></td>
<td>WT</td>
<td>neg</td>
<td>0</td>
<td>VAN, MET, CLI, ERY, MXF</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>145</td>
<td></td>
<td>WT</td>
<td>neg</td>
<td>0</td>
<td>VAN, MET, CLI, ERY, MXF</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>162</td>
<td></td>
<td>WT</td>
<td>neg</td>
<td>0</td>
<td>VAN, MET, CLI, MXF, ERY</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>191</td>
<td>027</td>
<td>18 bp</td>
<td>pos</td>
<td>IIIa</td>
<td>VAN, MET, CLI, ERY, MXF</td>
<td>027-1</td>
<td>Solomon et al. (2011)</td>
</tr>
<tr>
<td>203</td>
<td></td>
<td>18 bp</td>
<td>pos</td>
<td>IIIa</td>
<td>VAN, MET, CLI, ERY, MXF</td>
<td>027-1</td>
<td>Solomon et al. (2011)</td>
</tr>
<tr>
<td>209</td>
<td></td>
<td>18 bp</td>
<td>pos</td>
<td>IIIb</td>
<td>VAN, MET, CLI, ERY, MXF</td>
<td>027-6</td>
<td>Solomon et al. (2011)</td>
</tr>
<tr>
<td>216</td>
<td></td>
<td>18 bp</td>
<td>pos</td>
<td>IIIa</td>
<td>VAN, MET, CLI, ERY, MXF</td>
<td>027-8</td>
<td>Solomon et al. (2011)</td>
</tr>
</tbody>
</table>

*VAN, vancomycin; MET, metronidazole; CLI, clindamycin; MXF, moxifloxacin, ERY, erythromycin; ND, not defined.
Growth curves were generated by plotting the natural logarithm of OD₆₀₀ values against time.

The time taken for the bacterial population to reach a log optical density of −0.9 within the culture was defined for each replicate by extrapolation from each growth curve. This value was set as the initiation of detectable exponential phase growth (by reference to all growth curves).

Statistical analysis. Statistical analyses were carried out in SPSS (IBM) using one-way analysis of variance with Tukey’s post hoc compensation for multiple comparisons of the germination efficiency of the ribotyped groups. The Students unpaired t-test was used to assess the degree of significance between the germination efficiencies of isolates grouped according to severe or non-severe CDI and according to success or failure of initial CDI therapy.

RESULTS

Determination of germination efficiency of C. difficile spores in response to 0.1 % taurocholate by loss of OD₆₀₀ and by colony forming efficiency

Analysis of the loss of OD₆₀₀ of heat-treated spores from 12 different C. difficile isolates grouped according to ribotype during incubation with 0.1 % (w/v) taurocholate showed a variation in the germination response across isolates. The extent of germination was shown to reach a maximal level within 30 min for all isolates, but this level varied between isolates and ribotype groups (Fig. 1). The drop in OD₆₀₀ from baseline adjusted level (t=0) to maximal level (t=120 min) was used to calculate the percentage germination efficiency for each replicate and the mean (±SEM) calculated for each isolate (Table 2). The mean germination efficiency across all isolates studied was 45.43 ± 9.93 % with the lowest germination efficiency exhibited by strain 128 (21.59 ± 1.78 %) and the highest by strain 191 (57.72 ± 6.74 %). There was a minor increase in mean OD₆₀₀ within the first 20 min, most likely due to microscopic clumping of some spores; however, there was no significant drop in OD₆₀₀ within 120 min observed in response to incubation in BHIS without the addition of sodium taurocholate for any isolate (mean overall germination efficiency 3.92 ± 4.19 %) (Fig. 1).

The germination efficiency of C. difficile spores was also assessed by colony forming efficiency after growth for 24 h on BHIS agar with 0.1 % (w/v) sodium taurocholate and comparison to direct spore counts obtained by phase-contrast microscopy. This method also demonstrated that there was a variation in the mean germination efficiency across isolates (Table 2). The mean germination efficiency across all isolates studied by this method was 48.06 ± 10.83 % with the lowest germination efficiency exhibited by strain 128 (32.08 ± 4.53 %) and the highest by strain 203 (59.91 ± 15.98 %).

The combined mean germination efficiency for each isolate was calculated by averaging the germination efficiencies for all replicates (n=7) for each isolate obtained by both methods.

Ribotype 027 spores exhibited significantly higher germination efficiency in response to 0.1 % taurocholate

The mean germination efficiency as determined by either drop in OD₆₀₀ or colony forming efficiency was calculated for each ribotype group (Table 2). Isolates of ribotype 027 were shown to exhibit significantly higher mean germination efficiency than isolates of ribotype 106 by both drop in OD₆₀₀ method [(51.66 ± 8.75 %; 95 % CI 47.37–55.95 %) versus (41.91 ± 8.35 %; 95 % CI 37.82–46 %); P<0.05] and by colony formation method [(54.95 ± 10.33 %; 95 % CI 49.11–60.79 %) versus (41.10 ± 11.62 %; 95 % CI 34.53–47.67 %); P<0.05]. Ribotype 027 isolates exhibited a significantly higher mean germination efficiency than isolates of ribotype 078 by the drop in OD₆₀₀ method only [(51.66 ± 8.75 %; 95 % CI 47.37–55.95 %) versus (42.07 ± 8.57 %; 95 % CI 37.22–46.92 %); P<0.05].

Outgrowth of C. difficile spores in rich medium

An assessment of variation in the time taken for spore germination, outgrowth and return to exponential vegetative cell growth was carried out with spore suspensions standardized at the start of the experiment with 1 × 10⁶ ‘viable’ spores in 200 µl medium. All isolates were capable of germination and cell outgrowth, generating characteristic growth curves (Fig. 2). The time taken for each bacterial culture to reach a log optical density of −0.9 (defined here as initiation of exponential growth) was calculated by reference to each growth curve and the mean time calculated for each isolate. In the presence of 0.1 % sodium taurocholate, there was no difference in the time taken for initiation of exponential growth between isolates belonging to ribotype 027 (3.64 ± 0.10 h), ribotype 106 (4.06 ± 0.58 h) and ribotype 078 (3.60 ± 0.25 h). In the absence of sodium taurocholate, there was no difference in the time taken for initiation of exponential growth between isolates belonging to ribotype 027 (7.72 ± 0.55 h), ribotype 106 (7.90 ± 0.20 h) with the exclusion of isolate 128 and ribotype 078 (8.19 ± 0.28 h). Spore outgrowth and initiation of detectable exponential growth occurred approximately 4 h earlier for all isolates (with the exclusion of isolate 128) incubated in BHIS supplemented with 0.1 % sodium taurocholate when compared to incubation in BHIS alone.

Isolate 128 (ribotype 106) was slower to return to vegetative growth in the absence of sodium taurocholate than all other isolates, reaching exponential growth within 13.69 ± 0.72 h, approximately 9.5 h later than it took in the presence of 0.1 % sodium taurocholate.

High spore germination efficiency is significantly associated with severe disease and treatment failure

We assessed the relationship between germination efficiency of individual isolates and the likelihood of severe
disease, failure of initial CDI antimicrobial therapy or recurrence of disease, by comparing clinical laboratory data and outcomes for CDI patients, for whom the infecting isolate had been studied.

Of the 12 patients studied, 8 exhibited severe disease and these patients were infected with \textit{C. difficile} isolates representative of each of the ribotype groups (ribotype 078, \(n=3\); ribotype 106, \(n=2\); ribotype 027, \(n=3\)). The mean combined germination efficiency was calculated for all seven replicates of eight isolates where the host had experienced severe CDI (\(n=56\)) and compared with isolates where the host had experienced non-severe CDI.

### Table 2. Percentage spore germination efficiencies of clinical \textit{C. difficile} isolates in response to 0.1 % sodium taurocholate

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>Isolate ID</th>
<th>Mean (± SEM) % germination efficiency by loss of OD(_{600}) nm</th>
<th>Mean (± SEM) % germination efficiency by colony formation</th>
<th>Combined mean (± SEM) % germination efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(n=4)</td>
<td>(n=3)</td>
<td>(n=7)</td>
</tr>
<tr>
<td>078</td>
<td>117</td>
<td>45.92 ± 4.48</td>
<td>47.69 ± 8.08</td>
<td>46.51 ± 5.84</td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>34.26 ± 4.32</td>
<td>42.84 ± 7.49</td>
<td>40.69 ± 6.92</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>32.38 ± 3.19</td>
<td>55.76 ± 7.33</td>
<td>39.39 ± 10.04</td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>52.57 ± 10.52</td>
<td>43.83 ± 7.85</td>
<td>49.30 ± 9.79</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>42.07 ± 8.58</td>
<td>47.53 ± 8.67</td>
<td>44.18 ± 9.05</td>
</tr>
<tr>
<td>106</td>
<td>121</td>
<td>41.78 ± 4.04</td>
<td>53.59 ± 4.78</td>
<td>46.84 ± 6.77</td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>21.59 ± 1.78</td>
<td>32.08 ± 4.53</td>
<td>26.46 ± 4.50</td>
</tr>
<tr>
<td></td>
<td>145</td>
<td>47.59 ± 4.55</td>
<td>40.01 ± 18.33</td>
<td>43.80 ± 12.32</td>
</tr>
<tr>
<td></td>
<td>162</td>
<td>46.47 ± 7.18</td>
<td>35.9 ± 2.71</td>
<td>43.78 ± 7.25</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>41.91 ± 8.35</td>
<td>41.10 ± 11.62</td>
<td>41.61 ± 9.63</td>
</tr>
<tr>
<td>027</td>
<td>191</td>
<td>57.72 ± 6.74</td>
<td>57.06 ± 11.07</td>
<td>57.47 ± 8.38</td>
</tr>
<tr>
<td></td>
<td>203</td>
<td>54.71 ± 2.24</td>
<td>59.91 ± 15.98</td>
<td>56.44 ± 6.59</td>
</tr>
<tr>
<td></td>
<td>209</td>
<td>49.20 ± 4.23</td>
<td>44.88 ± 5.02</td>
<td>47.58 ± 4.91</td>
</tr>
<tr>
<td></td>
<td>216</td>
<td>44.42 ± 19.11</td>
<td>57.99 ± 7.36</td>
<td>49.51 ± 15.76</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>51.66 ± 8.75</td>
<td>54.9 ± 10.33</td>
<td>52.86 ± 9.05</td>
</tr>
</tbody>
</table>
The mean combined spore germination efficiency for isolates implicated in severe CDI was significantly higher than that for isolates implicated in non-severe CDI \([49.68 \pm 10.00\%\), 95\% CI 47.06–52.3\%] versus \([40.92 \pm 9.29\%\), 95\% CI 37.48–44.36\%]; \(P<0.01\). Of the 12 patients studied, metronidazole therapy (500 mg three times per day for 10–14 days) was the first choice of treatment regimen employed in 11 cases and vancomycin therapy (125 mg four times per day for 10–14 days) in one case (Table 3). First-line metronidazole therapy for non-severe CDI was successful for three out of four cases. In the one case where it was unsuccessful (Patient 162), the patient died within 12 days of diagnosis and the death was recorded as attributable to \text{C. difficile}. Of the eight patients exhibiting severe CDI, one patient was successfully treated with metronidazole therapy (Patient 117) and a second patient was treated with vancomycin first-line therapy (Patient 122) but this patient died within 12 days of diagnosis and the death was recorded as attributable to \text{C. difficile}. Failure of metronidazole therapy was recorded for six out of the eight patients with severe CDI and a clinical decision was made by the subject’s physician to start vancomycin prior to the 10 day end point. Second-line treatment with either vancomycin or metronidazole and vancomycin combined was successful in all cases where first-line treatment had failed (except in the two cases where the patients had died within 12 days of diagnosis). There was only one recurrence observed within the patient group (Patient 216) and this occurred within 16 days of resolution of symptoms following vancomycin therapy.

The mean combined spore germination efficiency was calculated for all seven replicates of the eight isolates where the host had experienced initial therapy failure, recurrence or death occurred \((n=56)\) and compared with all replicates of the four isolates where the host had experienced successful therapy \((n=28)\). The mean combined spore germination efficiency for isolates implicated in CDI where therapy failed, recurrence or death occurred was significantly higher than that for isolates implicated in CDI where therapy was successful \([49.00 \pm 10.49\%\), 95\% CI 46.25–51.75\%] versus \([41.42 \pm 9.43\%\), 95\% CI 37.93–44.91\%]; \(P<0.01\).

**DISCUSSION**

Spore germination is one of the most important factors in the pathogenicity of \text{C. difficile} and although in vitro germination responses have been studied in some detail (Baines et al., 2009; Burns et al., 2010b; Heeg et al., 2012; Paredes-Sabja et al., 2008; Sorg & Sonenshein, 2009),
In this study we found that germination efficiencies in response to 0.1% sodium taurocholate when measured by two separate methods were comparable although spore germination efficiency calculated by drop in OD$_{600}$ was more sensitive for observing the time frame for maximal germination. We also demonstrated that although the time taken to reach maximal germination was the same across all isolates regardless of ribotype (approx. 30 min), the percentage germination efficiency was significantly higher for isolates of ribotype 027. Isolates selected for this study were not clonal, but representative of different rep-PCR subtypes and antimicrobial susceptibility profiles suggesting that high germination efficiency was ribotype 027-specific and not just isolate-specific in our study. Whilst some studies have not demonstrated a ribotype-specific germination response in vitro (Heeg et al., 2012; Oka et al., 2012), others have identified that PCR ribotype 027 exhibits greater germination capacity than other PCR ribotypes (Baines et al., 2009; Freeman et al., 2007). Whilst the data presented here are representative of only four ribotype 027 isolates, these were isolated directly from stool samples by our laboratory. Germination characteristics of isolates from reference laboratories may have inadvertently become attenuated during serial passages. This may in part explain the difference between results from previous studies and those observed here. Alterations to genes important for germination may also explain the greatly reduced germination and outgrowth ability of strain 128, when compared to other strains of the same ribotype; however, until the genetic basis of C. difficile spore germination is defined, this hypothesis remains unproven.

All clinical isolates exhibited similar outgrowth rates, reaching late exponential phase within the same time frame when the starting inoculum was standardized. This suggests that variation in spore germination efficiency plays an even greater role than vegetative growth rate in determining the length of time for particular strains to colonize the gut and initiate disease. We have also shown that all clinical isolates were able to germinate and outgrow in BHIS alone, albeit at a slower rate. Germination and outgrowth in the absence of sodium taurocholate as shown previously (Heeg et al., 2012; Paredes-Sabja et al., 2008) may be an important virulence determinant by increasing the number of niches in the gut that are favourable for spore outgrowth. It has been suggested that strains exhibiting higher germination efficiencies may consequently elicit prolonged inflammation, due to an extended vegetative phase of growth and therefore increased cytotoxin production within the gut (Baines et al., 2009; Freeman et al., 2007). This may explain the observation that strains with higher germination efficiencies were significantly more likely to be implicated in severe disease. This observation was independent of the ribotype of the isolate. We also identified a link between high germination efficiency and likelihood of first-line therapy failure.

### Table 3: Comparison of combined mean percentage germination efficiency for each clinical C. difficile isolate compared with CDI patient clinical outcome

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Combined mean % (±SEM)</th>
<th>1st therapy failure</th>
<th>1st therapy</th>
<th>2nd therapy</th>
<th>2nd therapy failure</th>
<th>Recurrence (within no. of days)</th>
<th>Severe CDI*</th>
<th>1st therapy</th>
<th>2nd therapy</th>
<th>2nd therapy failure</th>
<th>Recurrence (within no. of days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>117</td>
<td>46.51 ± 5.84</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>122</td>
<td>40.69 ± 6.32</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>144</td>
<td>49.39 ± 10.04</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>128</td>
<td>46.84 ± 6.77</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>145</td>
<td>48.46 ± 4.30</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>146</td>
<td>43.80 ± 12.32</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>162</td>
<td>57.47 ± 6.38</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>191</td>
<td>47.58 ± 4.91</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>209</td>
<td>49.51 ± 15.76</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Severe CDI defined as white cell count >15 x 10$^9$ l$^{-1}$ or a serum creatinine rise ≥ 1.5 times the baseline level.
†First therapy failure defined as persistence of diarrhoea >10 days after starting metronidazole or a transfer to vancomycin prior to end point due to severe disease.
‡Patient died within 12 days of diagnosis, death attributable to CDI.
Failure of metronidazole therapy may be due to sub-inhibitory antimicrobial concentrations in the gut lumen, as has been demonstrated in vitro (Freeman et al., 2007). The strains that are more efficient at germination and outgrowth would be more likely to precipitate symptomatic CDI during sub-inhibitory therapy in these cases. An assessment of the gut concentration of metronidazole would have confirmed whether this was the case in our study. Second-line therapy with vancomycin was successful in all six cases where initial metronidazole therapy had failed, confirming that a high germination efficiency may conversely increase the numbers of vegetative cells in the gut that are susceptible to vancomycin treatment as has been demonstrated (Baines et al., 2009).

The germination ability of C. difficile has been suggested to be a potential risk factor for recurrent CDI (Oka et al., 2012). Spores from strains that are more efficient at germination and outgrowth would be more likely to repopulate the gut once the antimicrobial concentration has dropped below inhibitory levels. There was only one case of recurrence in our study population, but the infecting isolate exhibited a high germination efficiency and the recurrence occurred within 16 days, suggesting a higher likelihood of recurrence of a persisting strain, although reinfection from a heavily contaminated environment cannot be ruled out. The response of CDI patients to particular therapeutic regimens may therefore be related in part to the germination efficiency of the infecting strain and may also help to explain why first-line vancomycin treatment is more effective for severe CDI than metronidazole.

Investigations into the correlation between microbiological characteristics of clinical C. difficile and relationship to severity and outcome of CDI are of considerable importance, but are uncommon due to the extensive patient clinical data required and time-consuming laboratory analysis of isolated strains. We have attempted to investigate the relationship between germination characteristics of C. difficile strains and associated CDI patient outcome. We identified that strains with higher germination efficiencies were significantly more likely to be associated with severe CDI and metronidazole treatment failure and that strains with the highest germination efficiency were predominantly ribotype 027. Whilst this study is limited by the small sample size, we aimed to reduce bias by choosing representatives of each ribotype that were genetically unrelated and without prior knowledge of patient clinical outcome.

This study has identified an important link between spore germination and pathogenesis of C. difficile disease; however, further work is warranted before the complex interplay between germination dynamics and CDI outcome can be fully understood.

ACKNOWLEDGEMENTS

This work was supported by the Health Research Board, Ireland (to K.S, A.M and L.K). We thank Dr E. Kuiper and colleagues in Leiden University Medical Centre, The Netherlands for ribotyped library strains.

REFERENCES


