Comparison of toxin and spore production in clinically relevant strains of *Clostridium difficile*

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Clostridium difficile is a major cause of nosocomial diarrhoea. The toxins that it produces (TcdA and TcdB) are responsible for the characteristic pathology of *C. difficile* infection (CDI), while its spores persist in the environment, causing its widespread transmission. Many different strains of *C. difficile* exist worldwide and the epidemiology of the strains is ever-changing: in Scotland, PCR ribotype 012 was once prevalent, but currently ribotypes 106, 001 and 027 are endemic. This study aimed to identify the differences among these ribotypes with respect to their growth, and toxin and spore production *in vitro*. It was observed that the hypervirulent ribotype 027 produces significantly more toxin than the other ribotypes in the exponential and stationary phases of growth. Further, the endemic strains produce significantly more toxins and spores than ribotype 012. Of note was the observation that *tcdC* expression did not decrease into the stationary phase of growth, implying that it may have a modulatory rather than repressive effect on toxin production. Further, the increased expression of *tcdE* in ribotype 027 suggests its importance in the release of the toxins. It can thus be concluded that several genotypic and phenotypic traits might synergistically contribute to the hypervirulence of ribotype 027. These observations might suggest a changing trend towards increased virulence in the strains currently responsible for CDI.

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INTRODUCTION

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacillus that was first identified as the cause of antibiotic-associated pseudomembranous colitis in 1978 (Bartlett *et al.*, 1978; George *et al.*, 1978; Larson *et al.*, 1978). Today, it is the most common cause of nosocomial diarrhoea. Once mainly associated with the use of antibiotics and being elderly, it is now also found in young, previously healthy adults with no history of antibiotic usage (McFarland *et al.*, 2007). *C. difficile* is acquired from the environment in the form of spores and transmitted by the faecal–oral route. The organism is able to colonize the gut when the normal protective flora is disrupted by the use of broad-spectrum antibiotics. Once the infection is established, the bacterium produces two large Rho glucosylating exotoxins, toxin A (TcdA), an enterotoxin, and toxin B (TcdB), a cytotoxin, which result in the characteristic pathology of *Clostridium difficile* infection (CDI). Though asymptomatic carriage of *C. difficile* is common (Riggs *et al.*, 2007), the presentation of CDI can vary from mild self-limiting diarrhoea to severe diarrhoea, which can progress to pseudomembrane formation, toxic megacolon, perforation, shock and even

death. During carriage or an infection, patients have the potential to release large amounts of spores into the environment (Jump *et al.*, 2007). In this way, the toxins and spores of *C. difficile* ensure a continual presence and spread in the human population.

TcdA and TcdB are encoded on the 19.6 kb pathogenicity locus (PaLoc), along with the positive regulator TcdR, the negative regulator TcdC and a putative holin, TcdE. Study of the five genes of the PaLoc has shown an increased transcription of *tcdA*, *tcdB*, *tcdR* and *tcdE* and a decreased transcription of *tcdC* during the progression of *C. difficile* from the exponential to the stationary phase of growth (Hundsberger *et al.*, 1997).

Since the early 2000s, ribotype 027 (BI/NAP1) has emerged as the cause of several outbreaks and disease of increased severity, morbidity and mortality (Loo *et al.*, 2005; Pépin *et al.*, 2005). It has been dubbed the hypervirulent strain, following the observation of excessive toxin production by this ribotype: up to 16 times more toxin A and 23 times more toxin B (Warny *et al.*, 2005). An explanation of this increased toxin production has been found in the deletions observed in the *tcdC* gene of most, but not all, ribotype 027 strains (MacCannell *et al.*, 2006; Spigaglia & Mastrantonio, 2002). The $\Delta 117$ frame-shift mutation results in a truncated protein lacking in

Abbreviations: CDI, *Clostridium difficile* infection; PaLoc, pathogenicity locus.

function. Strains with both $\Delta 117$ and an 18 bp deletion have also been found to produce more toxin in the exponential phase of growth and to cause more severe disease (Curry *et al.*, 2007). However, it has also been shown that this ribotype does not produce more toxin than others, although the duration of the toxin production is increased (Freeman *et al.*, 2007). Interestingly, it has been observed that 027 strains not only produce more toxins but also have increased sporulation rates, giving them an added advantage in dissemination (Akerlund *et al.*, 2008).

The changing epidemiology of *C. difficile* in Scotland has been studied extensively and has revealed changes in the ribotypes implicated in CDI over the years (Taori *et al.*, 2009). Ribotype 012, to which the reference and the first sequenced strain 630 belongs, represented 5% of the *C. difficile* isolates collected between 1979 and 2004, but is no longer reported in the infected population. The incidence of ribotype 001 has increased over the years from 1.5 to 75.8% (Mutlu *et al.*, 2007). Ribotype 106, not identified in Scottish isolates till 2004, represented 8.1% of the isolates collected in 2005. In 2006, the prevalence of ribotype 001 started declining and that of ribotype 106 increased steadily. The same year saw the first case of ribotype 027 infection in Scotland, and its incidence has since been on the rise. The most common causes of CDI in Scotland today are ribotypes 106, 001 and 027 (Health Protection Scotland, 2008, 2010).

All the ribotypes mentioned above are multidrug-resistant and have been isolated from CDI patients. Though only ribotype 027 is considered to be hypervirulent, strains 106 and 001 have also been shown to cause severe disease (Arvand *et al.*, 2009; Sundram *et al.*, 2009). Strain 630 was isolated from a patient with pseudomembranous colitis during a diarrhoeal outbreak in a Zurich hospital (Wüst *et al.*, 1982). These observations led to the hypothesis that not only excessive toxin production but also enhanced growth and increased sporulation might explain the severity of disease caused by these strains, as well as their ability to spread in the environment. To test this, the growth of these strains over a 24 h period was studied, along with toxin production and sporulation. For a more detailed understanding, the transcription of the genes of the PaLoc and the sporulation initiator *spo0A* were investigated over the same time period. Strain VPI 10463 was used as the reference strain, as it is known to be a high toxin and low spore producer (Akerlund *et al.*, 2006).

METHODS

Bacterial strains. Three ribotypes of *C. difficile* were used in this study: 027 (a clinical isolate obtained from E. J. Kuijper, Leiden University Medical Center), 001 and 106 (isolates from toxin-positive faecal samples from CDI cases in south-east Scotland; Mutlu *et al.*, 2007). Strain 630 (ribotype 012, obtained from P.

Mullany, UCL Eastman Dental Institute, London, UK) was used as a reference strain, representing a historic isolate. VPI 10463 (obtained from Unipath, Bedford, UK), a known high toxin producer, was the other reference strain. The strains were purified and maintained as spore suspensions in Robertson's cooked meat medium.

Growth measurement. A starter culture was prepared by inoculating 0.5 ml of the spore suspension into 3 ml pre-reduced anaerobic incubation medium (AIM) (Brown *et al.*, 1996). This was incubated anaerobically (80% N₂, 10% H₂, 10% CO₂) for 16 h at 37 °C in a Mark III workstation (Don Whitley Scientific) till OD₆₀₀ 1.0 (± 0.05) was achieved. The starter culture (3 ml) was inoculated into 300 ml pre-reduced AIM. Growth was determined by measuring OD₆₀₀ at 4, 8, 12, 16, 20 and 24 h. Cultures were checked for purity by Gram-staining and aerobic and anaerobic subculture on blood agar. All growth curves and related studies were performed in triplicate.

Total toxin production. Total toxin (A+B) production was measured using the *C. difficile* TOX A/B II kit (TechLab) according to the manufacturer's instructions. Culture supernatants were collected every 4 h by centrifugation at 13 000 g for 1 min and stored at -80 °C. The supernatants were diluted 1:5 in the supplied diluent and run in duplicate. Total toxin levels were determined by measuring A₄₅₀/OD₆₀₀.

ELISA for toxin A. A sandwich ELISA was developed for the quantification of toxin A. ELISA plates were coated with 50 μ l of 1.5 μ g ml⁻¹ rabbit polyclonal antibody to *C. difficile* toxin A (Meridian Life Science) in 0.1 M carbonate/bicarbonate buffer (pH 9.6). The plates were incubated at 4 °C overnight. They were then washed with PBS (pH 7.4) containing 0.05% Tween 20 and blocked with 100 μ l PBS containing 3% gelatin. Culture supernatants (50 μ l) diluted in PBS with 1% gelatin were then added to the plate in duplicate and incubated. To generate standard curves, twofold dilutions of toxin A (Calbiochem, Merck) from 250 to 0.25 ng ml⁻¹ were added to each plate. The plates were washed as above and 50 μ l of 0.5 μ g ml⁻¹ mouse monoclonal antibody to toxin A (Novus Biologicals) was added to the plate and incubated. After washing, 50 μ l 1:1000 anti-mouse IgG (whole molecule)-peroxidase antibody produced in rabbits (Sigma) was added to the plates. All the above incubations were performed for 1 h at 37 °C. Finally, the plates were washed and 100 μ l substrate, 3,3',5,5'-tetramethylbenzidine (TMB), was added for 5 min at room temperature. The reaction was stopped with 100 μ l 0.2 M H₂SO₄ and A₄₅₀ was measured.

Quantitative cytotoxicity assay for toxin B. It proved impossible to develop an ELISA for toxin B with commercially available reagents, and thus a modified cytotoxicity assay was performed for its quantification. Vero cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% non-essential amino acids, 100 units penicillin ml⁻¹ and 100 μ g streptomycin ml⁻¹ at 37 °C in 5% CO₂. The monolayers were trypsinized at 37 °C for 5 min, washed with medium and resuspended at 5 $\times 10^5$ cells ml⁻¹. The cells were added to a 96-well tissue culture plate in 50 μ l volumes and incubated overnight. The medium was replaced with 50 μ l of suitable dilutions of culture supernatant prepared in medium, in duplicate. To generate standard curves, twofold dilutions of toxin B (Calbiochem, Merck) from 250 to 0.25 ng ml⁻¹ were added to each plate. The plates were incubated for 48 h, washed with pre-warmed PBS and then 20 μ l of 5 μ g ml⁻¹ thiazolyl blue tetrazolium bromide (Sigma) was added to the wells, followed by incubation at 37 °C for 4 h. Any formazan produced by the cells was dissolved in 100 μ l DMSO (Sigma) and A₅₇₀ was measured.

Real-time RT-PCR. Transcriptional analysis of the PaLoc genes and *spo0A* was performed. Culture was collected every 4 h corresponding to approximately 5×10^8 cells (50 ml at 4 h to 5 ml at 24 h) by centrifuging at 4000 g for 10 min. The pellets obtained were immediately treated with 500 µl RNAwiz (Ambion) or TRIzol (Invitrogen), vortexed vigorously in a Mini-BeadBeater (Biospec Products) and stored at -80°C for a maximum of 7 days before use. RNA was extracted according to the manufacturer's instructions and treated with DNase I (Ambion) at 37°C for 1 h, followed by deactivation of the DNase with DNase Inactivation Reagent (Ambion) for 2 min at room temperature. The quantity and quality of the RNA were assessed using a NanoDrop spectrophotometer, and aliquots were stored at -80°C . The SuperScript VILO cDNA Synthesis kit (Invitrogen) was used to convert 2 µg RNA into cDNA according to the manufacturer's instructions. The cDNA was aliquoted and stored at -20°C . Primers for *tcdA*, *tcdB*, *tcdC*, *tcdR*, *tcdE*, *spo0A* and *rrn* (16S rRNA gene) were designed using Primer3 software (Rozen & Skaletsky, 2000) based on the genome of strain 630 (Table 1). These were first tested by conventional PCR with genomic DNA to confirm specificity and product size. The real-time RT-PCRs were performed in duplicate in 20 µl volumes using 50 ng cDNA, primers at 200 nM for *tcdA*–*R*, 500 nM for *tcdE* and 100 nM for *spo0A* and *rrn*, and 10 µl SYBR Green JumpStart Taq ReadyMix (Sigma) in an Mx3000P quantitative PCR system (Stratagene). Standard curves were generated using fourfold dilutions of cDNA pools on each plate for each gene to determine the efficiency of the reactions. RNA and diethylpyrocarbonate (DEPC) water controls were also maintained. The thermal profile used was: initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 20 s, and extension at 72°C for 20 s. This was followed by a dissociation curve to check the product specificity. The expression of the test genes was normalized to that of *rrn* and calculated by the Pfaffl method (Pfaffl, 2001) using the amplification efficiencies determined in each run. The 4 h value was used as the calibrator for expression at the successive time points.

Spore production. Spore production was assessed using 10 ml of the culture every 4 h from the same culture as above. Pellets were obtained by centrifugation at 4000 g for 10 min, washed twice in distilled water and treated with 50 % ethanol for 1 h. After washing twice, the pellets were suspended in 1 ml distilled water and 10-fold

serial dilutions were plated onto blood agar. After anaerobic incubation for 48 h, the colonies were counted and the number of spores per millilitre of culture was determined.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 4.0 software. Strain comparison at individual time points was performed by one-way ANOVA. To assess the overall trends of growth and the corresponding phenotypic traits over the time-course, area under curve (AUC) analysis was performed for each strain, and strains were then compared by one-way ANOVA.

RESULTS AND DISCUSSION

The hypervirulence of ribotype 027 has been of increasing interest since the emergence of this strain and its apparent ability to cause severe disease and be responsible for many outbreaks (Kuijper *et al.*, 2007; Pépin *et al.*, 2004; Smith, 2005). The hypervirulence has been directly associated with the excessive toxin production observed in this ribotype. Here we show that ribotype 027 produces much greater amounts of toxin than other strains in this study. Also, ribotypes 106 and 001 produce more toxins than strain 630, and ribotype 106 produces markedly more spores than the other strains.

All the *C. difficile* strains show similar patterns of growth

The growth curves obtained for all the *C. difficile* strains were similar (Fig. 1). Ribotypes 001 and 106 showed slightly but significantly increased growth at 4 h when compared with strains 630 and VPI 10463 ($P < 0.001$), but not when compared with ribotype 027. Throughout the 24 h, the growth of all the strains was

Table 1. Primer pairs used to amplify the genes studied by real-time RT-PCR

Gene	Primer sequence
<i>tcdA</i>	5'-GCTATTGCTGCAGTCGGATT-3' 3'-TACCATTAAACAGTCTGCCAACCC-5'
<i>tcdB</i>	5'-TGGTGAAGATGGTGTTCATGC-3' 3'-TTCTCCCTCAAAATTCTCATCC-5'
<i>tcdC</i>	5'-TTAAGAGCACAAAGGGTATTGC-3' 3'-TGACCTCCTCATGGTCTTCAG-5'
<i>tcdR</i>	5'-AACTCAGTAGATGATTTGCAAGAA-3' 3'-TTAAATCTGTTTCTCCCTCTTCA-5'
<i>tcdE</i>	5'-AAATATGTGCTTATGTGGATTACCAG-3' 3'-TTCATCCTTAGCATTTCATTCATC-5'
<i>spo0A</i>	5'-TGTTGAGCTTTTAGGTGCAG-3' 3'-TGATGTCTTATTGCTCTTTCAA-5'
<i>rrn</i>	5'-AGTGAAAGGCTACGGCTCAA-3' 3'-CTACGCATTTACCGCTACA-5'

Fig. 1. Growth curves of five *C. difficile* strains. The growth of strain 630 (■), VPI 10463 (▲), ribotype 027 (▼), ribotype 001 (◆) and ribotype 106 (●) was measured by OD₆₀₀ over 24 h. The patterns of growth were similar for all the strains. Error bars, SEM of 12 growth curves (performed in triplicate on four different occasions).

Fig. 2. Toxin production in five *C. difficile* strains. Toxin production in strain 630 (■), VPI 10463 (▲), ribotype 027 (▼), ribotype 001 (◆) and ribotype 106 (●) was measured over the 24 h period studied. (a) Total toxin production (A+B) measured using a combined kit showed significantly higher toxin production in ribotype 027 and VPI 10463. Error bars, SEM of three experiments. (b) Toxin A was quantified by a newly developed in-house ELISA. In all the strains, extracellular levels of toxin A increased over 24 h. Ribotype 027 produced significantly greater amounts of toxin A from 8 h. By the stationary phase, ribotype 027 and VPI 10463 produced markedly more toxin than ribotypes 106 and 001. Strain 630 produced the least toxin A. Error bars, SEM of six experiments. Lower y axis, 0–100 ng ml⁻¹; upper y axis, 100–2000 ng ml⁻¹. (c) Toxin B was quantified by a modified cytotoxicity assay. Ribotype 027 produced significantly greater amounts of toxin B from 8 h, while that in VPI 10463 increased steadily over time. In ribotypes 106 and 001, the amounts of toxin

B increased gradually till 20 h and then showed a sudden increase at 24 h. In strain 630, toxin B levels decreased over time. Error bars, SEM of six experiments. Lower y axis, 0–250 ng ml⁻¹; upper y axis, 250–2000 ng ml⁻¹.

significantly higher than that of strain 630 ($P<0.001$). Thus, varying growth rates do not appear to explain the degrees of virulence observed in different strains of *C. difficile*.

***C. difficile* ribotype 027 produces significantly more toxin than other strains**

Total toxin (A+B) production as measured by the combined ELISA kit varied significantly between the strains studied (Fig. 2a). In ribotype 027, total toxin production increased significantly between 8 and 12 h. By 12 h, ribotype 027 and VPI 10463 produced significantly more toxin than the other strains ($P<0.001$), which showed a gradual increase in toxin production over time. Ribotype 106 produced more toxin than 001, which in turn was greater than strain 630. Over the 24 h, ribotype 027 and VPI 10463 produced significantly higher amounts of total toxin ($P<0.001$). Beyond 12 h, the levels of toxin production in ribotype 027 and VPI 10463 could not be determined due to saturation of the assay. Thus, to investigate further the amounts of individual toxins produced by the different strains, quantitative methods were developed to detect each of the toxins.

Toxin A was detected in the cultures of all strains by ELISA (Fig. 2b). Ribotype 027 produced the most toxin A up to 12 h ($P<0.001$) and the amounts increased till 24 h. All the other strains produced low levels of toxin A till 12 h. Beyond 12 h, large amounts of toxin A were detected in cultures of VPI 10463, as expected, and the amounts were similar to those of ribotype 027. Ribotypes 106 and 001 produced toxin A at similar levels, which were significantly lower than those of VPI 10463 and ribotype 027 ($P<0.001$) and slightly higher than those of strain 630. Toxin B production was assessed using a modified cytotoxicity assay, and levels were marginally higher than those of toxin A in all strains over time (Fig. 2c). VPI 10463 showed a steady increase in toxin B production that reached high levels at 24 h. Ribotype 027 produced significantly more toxin B than the other strains at 8 h ($P<0.01$) and this level remained almost constant till 24 h. In ribotypes 106 and 001, toxin B production increased gradually up to 20 h and then increased sharply at 24 h, possibly due to accumulation in the culture medium. Interestingly, in strain 630, toxin B production decreased over time. Overall, toxin B production in the hypervirulent ribotype 027 was significantly higher than that in the other test strains ($P<0.01$).

From these results, it is evident that ribotype 027 produces significantly more toxin than the other strains. As

observed by others, our data also suggested that ribotype 027 is capable of producing up to 20 times more toxin (A or B) than other strains (Warny *et al.*, 2005), except VPI 10463. This was also true for seven isolates of ribotype 027 from Scotland and five from the Netherlands (data not shown) (Vohra & Poxton, 2010). This appears to be a phenotypic advantage for this strain, enabling it to cause severe disease.

Another observation was the detection of moderately more toxin A than toxin B at 24 h in ribotypes 027 and 106 (Fig. 4). Toxin A, an enterotoxin with the ability to bind to epithelial cells in the gut via receptors (Krivan *et al.*, 1986; Tucker & Wilkins, 1991), causes initial damage by glucosylation of Rho proteins (Aktories *et al.*, 2000). In animal studies, it has been observed that in the absence of toxin A, toxin B is unable to induce the pathology characteristic of CDI (Lyerly *et al.*, 1985). Even in cell cultures with Caco2 cells, it has been demonstrated that when toxin A challenge is removed, no damage is caused to the epithelial barrier (Sutton *et al.*, 2008). Thus, large amounts of toxin A may contribute to increased disease severity and perhaps the increased potential of ribotypes 027, 106 and 001 to induce CDI in healthy humans: the greater the initial damage to the gut by toxin A, the greater the chance of toxin B causing extensive cytotoxicity. However, outbreaks caused by A⁻B⁺ strains have been reported (Drudy *et al.*, 2007), questioning the clinical importance of toxin A in disease. Toxin B is 1000-fold more cytotoxic than toxin A; however, its role in the development of the characteristic pathology of CDI is debated, though A⁺B⁻ strains have not yet been isolated. It has also been shown that toxin B knockout strains are ineffective at causing fatal disease in hamsters (Lyras *et al.*, 2009). However, more recently it has been suggested that both toxins are important in CDI (Kuehne *et al.*, 2010). Whatever the importance of toxin B in disease, it is clear that the current epidemic strains produce large quantities of it. This, coupled with the production of large amounts of toxin A, could explain the severity of disease associated with ribotypes 027, 106 and 001.

High toxin producers show increased transcription of the PaLoc genes

Transcriptional analysis of the PaLoc genes was performed by real-time RT-PCR (Fig. 3). In VPI 10463, the transcription of *tcdA*, *tcdB* and *tcdR* increased over 24 h, while that of *tcdC* decreased after 8 h, as shown by Hundsberger *et al.* (1997). *tcdE* levels were found to increase till 12 h, after which they decreased, with a transient increase at 24 h. This served as a basis for transcription studies in the other strains using the methodology developed. However, varying patterns of PaLoc gene expression were observed in the other strains.

tcdA expression increased till 12 h in ribotype 027, ribotype 106 and strain 630, and then decreased, whilst

remaining almost constant in ribotype 001 over time. A similar trend was observed for *tcdB* in ribotype 106 and strain 630, though in the latter, the levels were below the 4 h value. In ribotype 001, an increase in *tcdB* transcription was observed at 20 h. Interestingly, ribotype 027 was the only strain to show constantly increasing *tcdB* expression over the 24 h period studied, though it was less than that of *tcdA*. The pattern of *tcdA* expression suggests that transcription peaks at 12 h and the toxin levels detected in culture thereafter are the result of accumulation. This was observed less markedly and from 8 h for toxin B. This observed correlation between gene expression and toxin detection (Fig. 4) was analysed by expressing the results as the ratio of toxin production to toxin gene transcription. Though the transcription of *tcdB* was lower than that of *tcdA* in all the strains, the levels of toxin B detected were always higher than those of toxin A, suggesting more efficient production of TcdB or perhaps greater degradation of TcdA in culture.

tcdR transcription increased steadily over time in strain 630, but in ribotypes 027, 001 and 106, peak expression was observed at 12 h, followed by a decline. A transient increase in expression was observed at 24 h in ribotypes 027 and 106. Notably, *tcdC* transcription showed a similar pattern and at similar levels, contrary to the observations in VPI 10463.

tcdC is the negative regulator of toxin production (Dupuy *et al.*, 2008; Matamouros *et al.*, 2007), and it has been shown that its transcription decreases as cultures enter stationary phase (Hundsberger *et al.*, 1997). The levels of the protein itself have also been shown to diminish over time (Govind *et al.*, 2006). Further, the deletions found in *tcdC* in ribotype 027 have been used to explain the excessive toxin production in this strain, as the truncated TcdC protein would be ineffective in preventing the complexing of TcdR with RNA polymerase (Curry *et al.*, 2007). Contrary to the gene and protein studies, our data showed that *tcdC* transcription increased over time, at least till 12 h, and then decreased. Though there was an evident decrease in *tcdC* expression, it was not considered to fit into the pattern described elsewhere (Hundsberger *et al.*, 1997), as the *tcdR* expression observed showed a similar pattern and was at similar levels. This suggests that *tcdC* might have a modulatory effect on toxin production, rather than a strictly inhibitory one. Also, both toxins, A and B, were detected in the exponential and stationary phases of growth, despite increasing *tcdC* expression. Others have also shown this expression of *tcdC* in both the phases of growth, though slightly diminished in the stationary phase (Dineen *et al.*, 2007; Karlsson *et al.*, 2008). It is possible that TcdC, being a membrane-associated protein, has an effect on the release of toxins. These hypotheses could be tested by gene knockout and protein interaction studies, and would greatly add to the understanding of toxin production and release in *C. difficile*.

Fig. 3. Transcription of the PaLoc in five *C. difficile* strains. Transcription of the PaLoc genes was studied by real-time RT-PCR in (a) strain 630, (b) VPI 10463, (c) ribotype 027, (d) ribotype 001 and (e) ribotype 106. The expression of all the genes was normalized to that of *rrn*, using the 4 h value as the baseline of expression. (a) In strain 630, *tcdA* expression increased till 12 h and then decreased, similar to *tcdB*, though that of *tcdB* was below the 4 h value. *tcdR* and *tcdC* expression increased over time, while that of *tcdE* remained constant. (b) In VPI 10463, the expression of *tcdR*, *tcdA*, *tcdB* and *tcdE* increased over the 24 h, while that of *tcdC* decreased. (c) In ribotype 027, the transcription of *tcdR*, *tcdE*, *tcdA* and *tcdC* peaked at 12 h and then decreased. *tcdB* transcription increased over 24 h. (d) In ribotype 001, *tcdR* and *tcdC* transcription was similar, while that of *tcdB*, *tcdE* and *tcdA* did not vary considerably over time. (e) In ribotype 106, *tcdR*, *tcdB*, *tcdA* and *tcdC* transcription peaked at 12 h, while that of *tcdE* increased till 16 h. Thereafter, expression of all the genes decreased. Error bars, SEM of six experiments for *tcdA* and *tcdB* expression, and four experiments for *tcdR*, *tcdE* and *tcdC* expression.

Fig. 4. *tcdA* and *tcdB* transcription and the corresponding toxin A and toxin B production. The transcription of the toxin genes *tcdA* and *tcdB* and the corresponding production of toxin A and toxin B was studied in (a) strain 630, (b) VPI 10463, (c) ribotype 027, (d) ribotype 001 and (e) ribotype 106. The extracellular levels of both toxins increased over time in all the strains, even when gene expression decreased. Ribotype 027 produced greater amounts of both toxins and also showed greater gene expression. The correlation of toxin production to toxin gene transcription was assessed by analysing ratios of toxin A value:*tcdA* expression and toxin B value:*tcdB* expression at the different time points. This is represented by the line graphs above the toxin values for each toxin (log₁₀ scale ranging from 10⁻¹ to 10⁵). In all the strains, the release of toxin A at 12 h is evident. For toxin B, earlier release is indicated. Error bars, SEM of six experiments.

Fig. 5. *spoOA* transcription and spore production in five *C. difficile* strains. *spoOA* transcription, normalized to that of *rrn*, in (a) strain 630, (b) VPI 10463, (c) ribotype 027, (d) ribotype 001 and (e) ribotype 106 decreased over time, except for the slight increase in ribotype 027 at 8 and 12 h. The number of spores detected in the cultures of all the strains increased over the 24 h, with ribotype 106 producing the highest number of spores, followed by ribotypes 027 and 001 and strain 630. VPI 10463 produced the lowest number of spores. Error bars, SEM of six experiments for the transcription of *spoOA* and three experiments for spore production.

tcdE transcription was also studied, though not in a very efficient reaction. However, the results obtained showed that there was a major increase, approximately 15-fold, in *tcdE* expression till 12 h in ribotype 027 and VPI 10463, the highest toxin producers, which corresponds to the time beyond which large amounts of toxin are detected in the cultures of these strains. The almost steady expression of *tcdE* in the other strains corresponded to the slower release of toxins seen in those strains. Thus, it is likely that TcdE plays an important role in the release of the *C. difficile* toxins, given its holin-like properties (Tan *et al.*, 2001). The increased *tcdE* transcription in ribotype 027 may also contribute to hypervirulence.

Currently common *C. difficile* ribotypes produce more spores than a previously dominant strain

Sporulation is the other key trait of *C. difficile* that enables it to survive and spread in the environment. That sporulation and toxin production are alternate mechanisms for survival has been debated (Kamiya *et al.*, 1992). VPI 10463 fits this idea, showing high toxin production and low sporulation. Contrary to this, ribotype 027 has been shown to produce high levels of both toxins and spores, and this has been observed in 12 different isolates of ribotype 027 (data not shown) (Vohra & Poxton, 2010).

All the strains studied here produced alcohol-resistant spores, and their numbers in culture increased over time (Fig. 5). Ribotype 106 produced significantly more spores ($P < 0.001$) than the other strains, though this significance was less when compared with spore production by ribotype 027 ($P < 0.05$). At 24 h, a final count of 443 spores (ml culture)⁻¹ was obtained for ribotype 106, which was significantly higher than that of the other strains. VPI 10463 produced the fewest spores. The number of spores obtained was very low, although this was possibly due to the absence of bile salts, which enhance spore germination (Wilson, 1983), in the medium used.

spoOA, the master regulator of sporulation, was selected as the indicator of the magnitude of the spore-producing capacity of the *C. difficile* strains studied. *spoOA*

transcription in all the strains decreased over time (Fig. 5). In the epidemic strains, however, there was a slight difference in the pattern of expression: in ribotype 027, *spo0A* expression increased at 8 h before decreasing and then spiked at 24 h, whilst in ribotypes 106 and 001, the levels of expression increased marginally at 12 h before falling. These differences, however, were not significant.

Despite the expected decrease in gene expression, the trends over the first few hours suggested subtle inter-strain differences. Perhaps the increased duration of transcription of *spo0A* in the early stages of growth enhances the expression of the subsequent genes involved in the process, resulting in a greater number of spores being produced and released into the environment, irrespective of environmental stresses. The link between toxin and spore production has recently been described: *spo0A* mutants show decreased sporulation and toxin production (Underwood *et al.*, 2009). Thus, the observed increase in *spo0A* transcription in ribotype 027 might also increase its toxin production and add to its hypervirulence.

During the preparation of this manuscript, a similar study was published comparing hypervirulent ribotype 027 (BI) strains with non-hypervirulent strains (Merrigan *et al.*, 2010). The increase in the transcription of the PaLoc genes, including *tcdC*, was also observed in that study, and those authors too hypothesize a modulatory function for TcdC. However, unlike their results, we detected toxin production in all our strains in the exponential phase of growth as well as the stationary phase. It is possible that this difference was due to the medium used. We used anaerobic incubation medium (AIM) in our experiments, a medium that does not contain any glucose, which inhibits toxin production (Dupuy & Sonenshein, 1998; Karlsson *et al.*, 1999). It does contain cysteine, though at subinhibitory levels. This might explain the earlier detection of toxins in our study and the greater levels detected in the stationary phase of growth. This might also address the significantly higher *tcdA* transcription in ribotype 027 observed in our study.

Although a single isolate was tested for each ribotype, from the data presented here, we can conclude that ribotype 027 has the ability to produce large amounts of toxins and spores, both key phenotypic advantages that are likely to have aided its emergence. Further, ribotype 106 possesses traits that may directly address its presence as the strain most commonly associated with CDI in Scotland. The increased toxin and spore production in the current epidemic strains corresponds directly with the severity of disease and extent of spread associated with them, and gives further insight into the evolving bacterial factors that affect the epidemiology of CDI.

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