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Research Article

Clostridium (Now *Clostridioides*) *difficile* Spore Formation Is Higher in Epidemic Isolates When Treated With Vancomycin *in Vivo* and *in Vitro*

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Abstract

Between 2000-2007, *Clostridium* (now *Closteroides*) *difficile* infections have increased by a factor of 400% and have been associated with greater disease severity. These increases are associated to the increased prevalence of the NAP1/BI/027 ribotype (ribotype-027). This ribotype was characterized as hypervirulent, and one reason was the ability to produce greater numbers of spores *in vitro*. However, it is unclear whether the epidemic ribotype-027 are able to produce greater numbers of spores *in vivo*, and if this plays a role during clinically relevant treatments. To determine if epidemic strains are able to produce more spores during clinically relevant treatments, the growth and *in vivo* production of spores of four *C. difficile* isolates (2 non-epidemic and 2-epidemic) were determined in the hamster model of *C. difficile* infection (CDI). By using this model, the epidemic isolates of *C. difficile* were found to produce more spores than the non-epidemic isolates during treatment with vancomycin. The difference in spore numbers in response to the presence of vancomycin also occurred in *in vitro* cultures. These differences between the epidemic and non-epidemic isolates were consistent despite there being no difference to sensitivity to vancomycin *in vitro*. Thus, antibiotic treatment promoted higher levels of spores of epidemic isolates *in vivo* and *in vitro* than found

in non-epidemic isolates, suggesting this difference in response to clinically relevant antibiotics is a factor that contributes to the ribotype-027 being more frequently diagnosed in *C. difficile* cases.

Keywords: *Clostridium*; *Clostridioides difficile*; animal models; virulence; *in vitro*; phenotype; ribotype; epidemic; vancomycin

List of Abbreviations

ABSL-2 - Animal biosafety level 2

ANOVA – Analysis of Variance

CDI - *C. difficile* infections

CFU – Colony forming units

DMEM - Dulbecco's Modified Eagle's Medium

EMEM - Eagles Minimal Essential Medium

FBS - Fetal bovine serum

PBS – Phosphate-buffered saline

PCR - Polymerase chain reaction

SM - Sporulation medium

ST-80 - Surfactant tween 80

TGY-veg - Tryptone glucose yeast abstract vegetative

TSA – Tryptic soy agar

TSB - Tryptic soy broth

Background

Clostridium difficile (now *Clostridioides*), a spore forming bacillus, is the cause of *C. difficile*-associated disease. In the United States of America, the occurrence of *C. difficile* infections (CDI) increased by a factor of 400% between 2000-2007 [12]. *C. difficile* is estimated to cause 500,000 infections in the US each year that results in 29,000 deaths and associated annual healthcare costs of approximately $3 billion [9, 21]. This increase in the frequency and severity of CDI in the United States is partially attributed to the emergence of the epidemic *C. difficile* clinical isolates, e.g. BI/NAP1/027 (ribotype-027) [10, 15]. Ribotype-027 is commonly associated with healthcare-associated CDI and is responsible for 19 to 22.5% of hospital-acquired CDI [13].

The increased frequency of diagnosis, associated with the epidemic ribotype-027, is potentially linked to increased numbers of virulence determinates compared to other ribotypes. One such virulence determinate are the ability to form spores [25]. Clostridial endospores are essential for the environmental transmittance of *C. difficile* in humans and are resistant to a broad variety of physical and chemical treatments [8, 22]. Within the host, *C. difficile* spores germinate into vegetative cells, which enable colonization of the intestinal tract, toxin production, and eventual disease [3, 11]. Stages of disease progression include intestinal inflammation, perforation, toxic megacolon, pseudo-

membranous colitis, and death [1, 3]. Typically, the antibiotic vancomycin is utilized for the treatment of CDI, and when treated with an antibiotic *C. difficile* produces endospores [20]. These spores are not only essential for transmission of the bacteria, but they are also essential for episodes of recurrence to occur in the host.

There are multiple *in vitro* studies that characterize ribotype-027's spore production, but these studies have produced conflicting results. Some *in vitro* studies have concluded that ribotype-027 isolates produce a significantly greater number of spores compared to non-epidemic isolates, while other studies suggest this may not be the case [2, 6, 18, 23, 24]. There are also a smaller number of *in vivo* studies, and these have observed that the ribotype-027 does not produce significantly more spores that non-epidemic ribotypes. Thus, it is unclear whether epidemic ribotype's spore production could be a potential factor that contributes to the increase of CDI cases associated with ribotype-027.

To examine spore production, we determined the *in vivo* spore production of four *C. difficile* isolates [2 non-epidemic and 2 epidemic (ribotype-027)] in the hamster model of *C. difficile* when the hamsters were untreated or treated with vancomycin. The hamster model is very sensitive to *C. difficile* and, though there are differences, closely parallels the characteristics of clinical *C. difficile* associated-disease [4]. By using this approach, there was no difference in the recovery of spores from untreated animals; however, higher numbers of spores were recovered from vancomycin-treated hamsters infected with epidemic isolates in comparison to the non-epidemic isolates. Thus, the epidemic isolates produced greater number of spores when animals were treated with vancomycin, and that this could be a potential factor contributing to the spread or relapse of disease associated with the epidemic ribotype of *C. difficile.*

Materials and Methods

Ethics statement

Animal studies were conducted in accordance with protocols 2016-0015 approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Texas Health Science Center (UNTHSC). IACUC established guidelines ensuring that approved protocols are in compliance with federal and state laws regarding animal care and use activity at UNTHSC. The UNTHSC animal program in USDA registered (74-R0081) and fully AAALAC accredited.

Bacterial strains and Ribotype Confirmation

All *C. difficile* isolates used in this study are listed in Table 1. *C. difficile* UNT 101-1, UNT-103-1, UNT 107-1, and UNT 108-1 were kindly provided by Dr. Curtis Donskey (Cleveland VA). The source of relevant characteristics of each isolate can be found in Table 1. Ribotypes were confirmed by running polymerase chain reaction (PCR) ribotyping with primers found in Bidet *et al.* [5]. PCR fragments were analyzed in a Hitachi 3500xL genetic analyzer with a 36 cm capillary loaded with a POP4 gel (Applied Biosystems). The size of each peak was determined using Peak Scanner software (Applied Biosystems). A database was generated from the results of the

capillary gel electrophoresis-based PCR ribotyping result of each strain (http://webribo.ages.at). An error margin of ±4 bp was incorporated into the analysis algorithm of the database [14].

***Clostridium difficile* Isolates and Sources**			
UNT Strain #	**Species**	**Source**	**Relevant Characteristics**
UNT 101-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	Non-epidemic, Other Designation VA1
UNT 103-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	REA J-type strain, binary toxin negative, non-epidemic, Other Designation VA 11
UNT 107-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation VA17
UNT 108-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation VA20

Table 1: *Clostridium difficile* Strain Designation, Sources, and Characteristics. This table denotes the source of the individual isolates, other designations for each isolate, and some of the major characteristics associated with each of the isolates.

Media

Sporulation medium (SM) contained 90 g Trypticase Peptone, 5 g Proteose Peptone no. 3, 1 g Ammonium Sulfate, and 1.5 g of Tris in 1 liter of distilled water. The pH was adjusted to 7.4 at 37^{o} with 1 M NaOH. SM is a broth medium made according to what has been previously described [26].

TSA with 5% blood agar was made with 1L of distilled water (DI), 30 grams of TSB, and 15 grams of granulated agar with constant mixing over low heat. Once the granulated agar was dissolved, the mixture was autoclaved (20 minutes, 121 °C, 15 psi). Once cooled to approximately 50 °C, 50 mL of the medium was removed, and 50 mL of sterile defibrinated sheep blood (Remel, Lenexa, KS) was added and mixed into the medium. Approximately 12 mL of medium was then poured into petri dishes and cooled overnight to solidify and stored in a 4 °C refrigerator until used.

TGY-vegetative medium contained 5 g Tryptone, 5 g Yeast extract, 1 g Glucose, 1 g Potassium Phosphate, 15 g agar, and 1 liter of distilled water. This liquid-based medium was made according to what has been previously published [19].

CCFA and CCFA with Taurocholate contained 40g Proteose Peptone #2, 5g Sodium Phosphate, 1.0g of Potassium Phosphate, 2.0g Sodium Chloride, 0.1g Magnesium Sulfate, 6.0g Fructose, 3.0mL of 1% stock Natural Red solution, 15g of granulated agar, and 1.0g sodium taurocholate (Only in CCFA with Taurocholate). The media was heated until just before boiling and autoclaved (20 minutes, 121 C, 15 psi). The media was then cooled to 50 C before the volume was adjusted to 990 mL with autoclaved DI water, followed by the addition of heat sensitive components: 10 mL of a 50 mg/mL stock of cycloserine and 1.0 ml of a 15.5 mg/ml stock of cefoxitin. Plates were then poured, cooled overnight to solidify, and refrigerated at 4 C until used.

Preparation of *C. difficile* spore stocks

Spore stocks of each *C. Difficile* strain was generated for use in the cellular adherence assay and the experimental animal models of CDI. These stocks were generated by growing each strain on 5% TSAb plates incubated at 37^{o}C in anaerobic conditions for 7 days. Plate growth was collected in a 1X PBS solution containing 1% (V/V) Tween-80 (ST-80), and suspensions were washed 3 times in equal volumes of ST-80. Suspensions were incubated for 1 hour at 65 ± 2°C, washed with ST-80, and re-suspended in 4 mL of sterile nanopore water. Suspensions were then stored overnight at 4°C in order to promote the maturation of endospores for each strain. Spores were separated from vegetative cells and residual debris by density gradient centrifugation (10 minutes at 4,500 x *g*) with a 25% (W/V) HistoDenz solution. Spore pellets were washed 3 times with ST-80 and suspended in sterile nanopore water to a final volume of 2 mL. Spore stocks for each strain were stored at -80°C until used in *in vitro* or *in vivo* studies

Minimum inhibitory concentrations (MIC) assay

In vitro susceptibility to metronidazole, vancomycin, rifampin, clindamycin, and moxifloxacin were performed using a broth dilution method. Wells with doubling concentrations of 0.03–64 µg/mL of clindamycin, 0.12–32 µg/mL of metronidazole, 0.03–16 µg/mL of moxifloxacin, 0.004–8 µg/mL of rifaximin, and 0.12–64 µg/mL of vancomycin was made and allowed to reduce for 3 hours prior to the assay. Frozen *C. difficile* isolates were subcultured on TGY-Veg plates twice prior to dilution in Brucella broth. The organism suspension was measured and diluted to an optical density of 1.00, the suspension was then diluted again 1:20. The organism suspension and the antimicrobial solution was then added to each well in a 1:1 equivalent into a new 96-well plate. Once inoculated, the plates were placed in an incubator at 37 °C for 48-hours. After 48-hours growth or lack of was checked for each antimicrobial and the MIC was determined.

Hamster *C. difficile* associated disease models

Male Golden Syrian hamsters that were 6 to 7 weeks old were purchased from Envigo RMS Inc., and individually housed in sterile cages. 30 hamsters were used in each study with 15 animals in each group that were orally

inoculated with either UNT 101-1, 103-1, 107-1, 108-1. The animals were inoculated with 0.5 mL of *C. difficile* spores from a spore preparation culture though oral gavage. The inoculation dose for all strains ranged from $5x10^5$ – $1x10^6$ spores/mL, and the exact titers chosen for each strain were based on previously conducted studies. Clindamycin was administered subcutaneously to each animal at 10 mg/kg per body weight approximately 24 hours after infection. At day 3 of the infection, animals were treated with 20 mg/kg of Vancomycin and this treatment continued throughout the rest of the study. On days 3, 4, 7, and 11, three hamsters were culled to take cecum samples. Starting the day of infection, and each day after, approximately 0.1 to 0.2 g of feces was collected from each cage to determine *C. difficile* counts. Bedding was changed daily to ensure fresh feces were collected for analysis, and census of survivors were recorded daily for 11 days after infection. Sterile 1x PBS was added to the recovered feces, this solution was then homogenized and 1 mL was separated for each total CFU and spore recovery. Viable cell and spore counts were quantified as described in the Material and Methods. The homogenized solution separated for spore quantification was heated to 65 ± 2°C for 1 hour to facilitate the isolation of only spores.

In vitro growth of *C. difficile* vegetative cells and spore formation

Plate growth of each *C. difficile* isolate was transferred into TGY-veg broth and anaerobically incubated at 37°C for 24 hours. TGY-veg associated growth for each strain was adjusted to an optical density of 0.1 (600nm) in either SM or TGY-veg broth, which were anaerobically incubated at 37 °C. Samples from each broth culture were collected in triplicate every 24 hours through 72 hours of total incubation, and these samples were 10-fold serially diluted and plated onto Columbia horse blood agar. Additionally, a second sample from each culture were possessed for spore counts by incubating each sample in an equal volume of 200 proof ethanol for 30 minutes, and then incubating the samples at 65 ± 2 °C for 1 hour. The ethanol and heat-treated samples were centrifuged, washed with PBS, and the spore-containing pellets were suspended in a volume of PBS equal to the original volume of the sample. Ethanol and heat-treatment at 65 ± 2 °C were tested and sufficient to remove all viable vegetative cells during this stage. The spore suspension of each sample was 10-fold serially diluted and plated on Columbia horse blood agar supplemented with 0.1% sodium taurocholate. Both sets of plates were anaerobically incubated at 37 °C for 48 hours and colony counts were used to calculate the vegetative CFU or spore counts per mL at each time point.

Statistical analyses

Data were evaluated by Two-way ANOVA with Tukey's post-hoc test. A p value ≤ 0.05 was considered statistically significant. Representation of survival rate against Log10 [daily dose]. Analyses were performed using Prism 8 software (Graphpad Software).

Results

Epidemic isolates of *C. difficile* produce greater numbers of spores and CFU when treated with vancomycin in the hamster model of CDI compared to non-epidemic isolates

A hamster model of CDI was used to compare the spore and CFU production of the non-epidemic and epidemic *C. difficile* isolates *in vivo* when vancomycin was administered. This is a commonly used model to study shedding and

disease progression than closely mirrors the disease in humans. In this model, the intestinal microbiome was disrupted with clindamycin and then the hamsters were orally inoculated with approximately 5 x 10^4 *C. difficile* spores. On day 3, the remaining hamsters were then treated with 20 mg/kg of vancomycin, and treatment was continued daily until the end of the study. Hamsters were culled on days 1, 2, 3, 4, 7, and 11 to obtain cecum samples, and feces were also collected on those days to determine CFU and spore counts. In previous studies, all animals would have died that were untreated with vancomycin, while most survived when treated with this dose of antibiotic.

The epidemic isolates (UNT 107-1 and UNT 108-1) produced greater amounts of CFU and spores recovered from hamsters after treatment with vancomycin than non-epidemic isolates (UNT 101-1 and UNT 103-1) (Figure 1). This was true for both the feces recovered and in the cecum samples that were taken. CFU numbers were higher on days 7 and 11 after infection (4 and 8 days after treatment began). Increased spore production in the epidemic isolates was also observed after vancomycin was administered to the animals. Interestingly, untreated animals prior to treatment produced similar amounts of CFU and spores regardless of the isolate they were infected with. The differences in spore numbers were present beginning one day after vancomycin treatment (day 4) with about 8- to 9-fold higher spore numbers from animals infected with epidemic isolates than non-epidemic isolates. When the spore numbers were normalized to the number of CFU recovered was compared between the isolates, the epidemic isolates produced spores in higher quantities compared to the non-epidemic isolates during treatment with vancomycin. On days 4 and 7 after infection (after 1 and 4 days of vancomycin treatment), epidemic isolates were found to produce 2.1 to 2.6 more spores per CFU recovered than non-epidemic isolates, indicating that there was higher spore formation relative to vegetative cells (CFU).

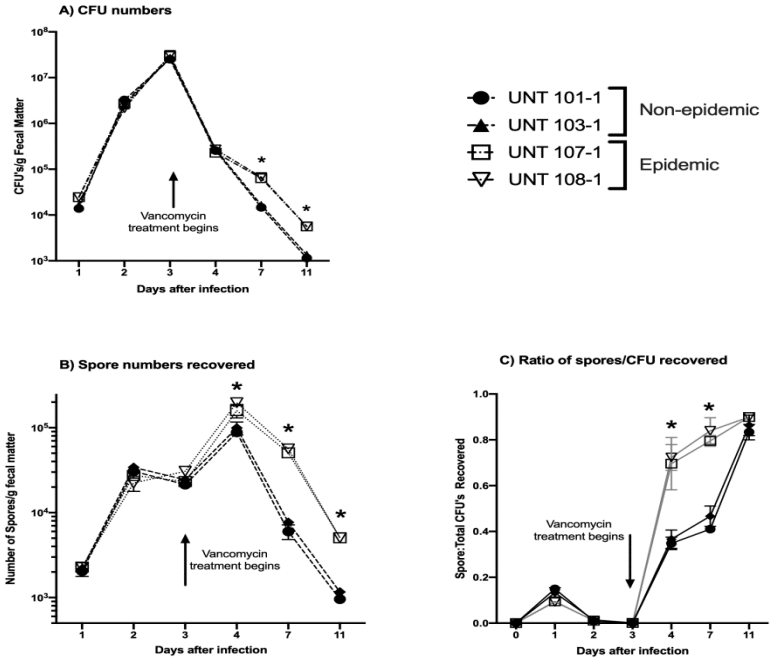

Figure 1: Epidemic isolate infected hamsters had significantly more fecal-associated spores than hamsters infected with non-epidemic isolates of *C. difficile* when treated with vancomycin. For each isolate, 15 hamsters

were split into 5 groups, housed individually, and inoculated with approximately $1x10^5 – 5x10^5$ *C. difficile* spores. Fecal pellets were then collected, weighed, and processed to measure for total CFU's and spores recovered. **(A)** Total CFU's recovered per gram of feces that was collected from epidemic or non-epidemic infected hamsters on days 0 to 11 of the studies. **(B)** Total spores recovered per gram of feces that was collected from epidemic or non-epidemic infected hamsters on days 0 to 11 of the studies. **(C)** Ratio of the total spores:total CFU's that were collected from epidemic or non-epidemic infected hamsters on days 0 to 11 of the studies. These data represent the average of three independent groups, and error bars indicate the standard errors of the means. An asterisk denotes significant difference at $p \leq 0.05$ (Two-way ANOVA with Tukey's post-hoc test).

In vitro growth and spore production are higher in epidemic isolates of *C. difficile* in the presence of vancomycin

To confirm that there are no inherent differences in growth and spore production of the isolates, *in vitro* growth and spore formation of the four *C. difficile* isolates were determined over a 72-hour period, and, it was found, that non-epidemic and epidemic isolates have similar *in vitro* growth patterns. Also, when places in sporulation medium, there was no difference over a 72-hour period between the epidemic and non-epidemic isolates in spore formation or the number of remaining vegetative cells.

In the presence of vancomycin, however, the epidemic isolates did produce higher numbers of spores *in vitro*, while the number of vegetative (CFU) cells dropped due to the antibiotic (Figure 2). By 48 hours in culture, the epidemic isolates produced 2.7- to 4.2-fold more spores than the epidemic isolates when exposed to vancomycin *in vitro*, indicating an inherent difference between the two groups of isolates.

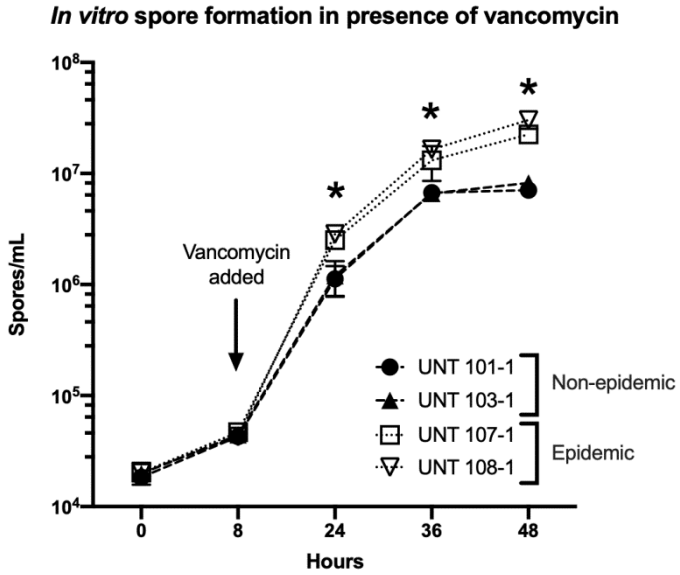

Figure 2: Mean spore recovery from *in vitro* cultures differed between non-epidemic and epidemic isolates when exposed to vancomycin. The 4 isolates (2 non-epidemic and 2 epidemic) were incubated in TGY-veg broth

over a 72-hour period, and vancomycin was administered at hour 8 of the studies. A representative sample was then taken from each culture and plated on an agar medium ± 0.1% taurocholate to measure the number of spores/mL at each timepoint. The non-epidemic isolates are represented by the black symbols, and the epidemic isolates are represented by the open symbols. This data represents the average of three independent experiments and error bars indicate the standard errors of the means. An asterisk denotes significant difference at $p \leq 0.05$ (Two-way ANOVA with Tukey's post-hoc test).

To determine if there was a difference in resistance to vancomycin between the isolates, *in vitro* minimum inhibitory concentrations (MIC) were determined. All isolates were susceptible to vancomycin. The epidemic isolates, UNT 107-1 and UNT 108-1, both had MIC values of 2 mg/L vancomycin, while the non-epidemic isolates, UNT 101-1 and 103-1, had MIC values of 1 and 4 mg/L, respectively. Thus, differences in susceptibility to vancomycin does not account for the higher recovery of CFU and spores from animals infected with the epidemic isolates and treated with vancomycin.

Discussion

With the identification and labeling of the NAP/BI/027 ribotype as epidemic, there is an ongoing debate if this ribotype is epidemic due to virulence, or if the increased frequency in diagnosis is due to other factors [10, 16, 23]. One key virulence factor may be linked to the ability to produce spores [7, 20]. Some papers found the ribotype-027 isolates can produce greater amounts of spores *in vitro* that non-epidemic ribotypes [2, 18]. In contrast, other papers have stated there is little differences between the ribotype-027 and other non-027 ribotype's spore production *in vitro* [6, 23]. Therefore, we undertook a set of *in vitro* and *in vivo* studies of four clinically relevant *C. difficile* isolates (2 non-epidemic and 2 epidemic) isolates and compared their ability to grow and produce spores. To do this, the isolates were not only characterized *in vitro* and *in vivo* using a hamster model of CDI, but also used a unique approach of characterizing the impact of the same isolates' *in vivo* growth and spore production during treatment with a clinically relevant antibiotic. With this approach, we were able to further our understanding about *C. difficile*'s growth and spore production for the epidemic ribotype in comparison to other non-epidemic ribotypes prior to and after antibiotic therapy.

In the hamster model of *C. difficile*, epidemic isolates produced greater amounts of spores than non-epidemic isolates when exposed to vancomycin. The hamster model is very sensitive to *C. difficile* and closely parallels the characteristics of clinical *C. difficile* associated disease [4]. Epidemic and non-epidemic isolates had similar *in vivo* growth patterns and numbers of spores recovered in hamsters prior to beginning treatment with vancomycin. However, once treatment with vancomycin started on day 3, there was a significant difference in spores and CFU recovered from hamsters infected with epidemic and non-epidemic isolates. Spore and CFU numbers from hamsters infected with the epidemic isolates and treated with vancomycin averaged approximately half a $\log_{10}$ greater than spores recovered from similarly treated hamsters infected with the non-epidemic isolates. During treatment, the epidemic isolates also had a significantly greater ratio of spores:total CFU recovered than non-epidemic isolates,

indicating that after normalization of results to vegetative organisms there was a higher production of spores. The difference in spore numbers in response to the presence of vancomycin also occurred in *in vitro* cultures. These differences between the epidemic and non-epidemic isolates were consistent despite there being no difference to sensitivity to vancomycin *in vitro*. Thus, antibiotic treatment promoted higher levels of spores *in vivo* and *in vitro* than found in other non-epidemic ribotypes, suggesting this difference in response to clinically relevant antibiotics is a factor that contributes to the ribotype-027 being more frequently diagnosed in *C. difficile* cases.

Within the last decade, *C. difficile* has become an ever-increasing threat, and a major reason for this is the rise of the NAP/BI/027 ribotype [15, 17]. Previous studies debated whether the current epidemic ribotype is able to produce greater amounts of spores, and if this could be a potential factor in its ability to persist as epidemic over this period of time [2, 15, 18, 23, 24]. The results presented here helps to bridge the gap between the organism's microbiology and the public health and disease outcome. Although there were no differences in spore production between the epidemic and non-epidemic isolates in the absence of antibiotic, the epidemic isolates produced more spores when exposed to vancomycin. This increased spore production could contribute to an increased environmental shedding of epidemic *C. difficile* spores, and therefore, increase exposure to epidemic ribotypes. Another possible outcome is that infection with the epidemic isolates may more frequently result in relapse disease after treatment with antibiotics. The higher numbers of vegetative cells due to infection epidemic isolates after treatment may contribute to more severe and prolonged disease. However, further studies are needed to determine the impact of this response to antibiotic therapy on the spread and disease outcome in patients. Thus, this characterization will contribute to our understanding of this pathogen and its role in healthcare associated infectious diseases.

Conclusion

Epidemic isolates of *C. difficile* produced greater number of spores during *in vivo* treatment or in the presence *in vitro* of vancomycin, a clinically relevant antibiotic. This increased spore production could be a potential factor contributing to the more frequent appearance and relapse of disease associated with the epidemic ribotype of *C. difficile*, as compared to the other non-epidemic ribotypes.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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