Journal of
Medical Microbiology & Diagnosis

Worthington and Hilton, J Med Microb Diagn 2016, 5:2
DOI: 10.4172/2161-0703.1000230

Research Article Open Access

# Sporulation of *Clostridium difficile* in Aerobic conditions is Significantly Protracted when Exposed to Sodium Taurocholate

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**Rec Date:** Apr 28 2016; **Acc Date:** May 24 2016; **Pub Date:** May 31 2016

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## Abstract

Elimination of *Clostridium difficile* spores from the clinical setting requires stringent application of infection control procedures including the use of hard-surface disinfectants. A unique combination of sodium taurocholate together with amino acids has been reported as an alternative approach to potentially eliminating spores of *C. difficile* by increasing their sensitivity to common disinfectants. In this study, the efficacy of this spore germination solution was investigated to explore its effect on the sporulation process under aerobic conditions. Vegetative cells of *C. difficile* NCTC 11204 (Ribotype 001) and R20291 (Ribotype 027) were exposed to the germination solution comprising 6.9 mM sodium taurocholate and 50 mM of the following amino acids: histidine, glycine, arginine, aspartic acid, valine in TRIS buffer, and a control solution. Total viable counts, the rate and extent of sporulation, and percentage recovery of vegetative cells in both ribotypes were assessed by culture. At 24 hours, sporulation was protracted in ribotypes 001 and 027 and there were significantly more (p=<0.01) vegetative cells following exposure to the germination solution compared to those exposed to the control. No vegetative cells of either ribotype exposed to the control solution were detected at 24 hours. At 48 and 72 hours, vegetative cells of ribotype 027 were not detected however a significantly higher (p<0.001) percentage (43%) of viable vegetative cells of *C. difficile* 001 were recovered by culture. Exposing vegetative cells of *C. difficile* to a germination solution protracts the sporulation process in aerobic conditions. In previous studies, the application this solution to spores of *C. difficile* has been shown to initiate germination thus rendering them more sensitive to common disinfectants. In this investigation, the findings demonstrate that sodium taurocholate protracts the sporulation process and may provide an additional adjunct to future *C. difficile* infection control strategies.

**Keywords:** *Clostridium difficile*; Sporulation; Germination; Sodium taurocholate; Infection control

## Background

*Clostridium difficile* is a strictly anaerobic, spore-forming, Gram-positive bacterium and *C. difficile* infection (CDI) is one of the leading healthcare-associated infections in the UK [1]. In susceptible patients, CDI is caused by ingestion of the spores of *C. difficile* which germinate and proliferate in the small intestine following exposure to sodium taurocholate and other co-germinants [2].

In vulnerable patients, for example immunocompromised and those treated with broad-spectrum antibiotics (e.g. cephalosporins and the beta-lactams, clindamycin), the elimination and disruption of the commensal flora creates an ideal non-competitive environment for CDI to become established [3]. It is now recognised that between 20-35% of all cases of antibiotic-associated diarrhoea is caused by *C. difficile* [4]. The resistant spores of *C. difficile* which are ubiquitous and contaminate the clinical environment are the principle vector by which CDI is transmitted to patients. Therefore, stringent infection control strategies including effective cleaning and disinfection of the clinical environment is paramount in limiting the transmission of CDI. Commonly used disinfectants in the healthcare setting have little effect on *C. difficile* spores; indeed, quaternary ammonium compound-based solutions and 70% (v/v) industrial methylated spirit are common hard-surface disinfectants that have no effect against this reservoir of infection and as a consequence the environment may harbour spores for extended periods of time [5]. Harsh and unfavourable sporicidal agents such as sodium hypochlorite and peracetic acid are currently required to effectively eliminate spores [6]. Whilst the resilient spores are the principle component in the *C. difficile* chain of infection, patients with CDI can excrete up to 10-fold more metabolically active vegetative cells of *C. difficile* in their faeces compared to spores; these vegetative cells are significantly more susceptible to standard hard surface disinfectants and are therefore easily eliminated from the environment [7]. Vegetative cells of *C. difficile* cannot survive for extended periods of time within the aerobic environment and rapidly undergo sporulation which renders elimination more difficult.

Whist there are several approaches to potentially limiting the spread of infection within the healthcare setting, the application of a specific germinant to bacterial spores has been considered as a strategy for reducing their resistance and increasing their susceptibility to environmental stressors [8]. In recent years, the focus within our research group has been the development of a sodium taurocholate-based germination solution for elimination of *C. difficile* spores pioneering the concept of 'germinate to exterminate'. The germination solution, which is now patented, has been shown to increase significantly the sensitivity of *C. difficile* spores to antimicrobial agents, including 70% (v/v) ethanol and copper surfaces [6]. The unique combination of germinants and co-germinants within the solution actively promotes the irreversible germination process and provides a nutritionally favourable environment for metabolically active vegetative cells to develop. However, the potential of the germination solution to prevent or protract the sporulation process; a concept that

is plausible given that its unique formulation promotes germination of dormant spores into metabolically active cells, has not been investigated. Preventing or protracting the sporulation process in the healthcare setting would potentially result in a reduction in the number of dormant, resistant spores of *C. difficile* thus facilitating breaking the chain of CDI. The aim of this current study was to therefore explore the effect of applying the germination solution to metabolically active vegetative cells of *C. difficile* in aerobic conditions and to assess the rate of sporulation and survival of vegetative cells in its presence.

## Methods

### Strains of *Clostridium difficile*

*C. difficile* NCTC 11204 (Ribotype 001) and *C. difficile* R20291 (Ribotype 027) (supplied by the Anaerobic Reference Laboratory, Cardiff, UK) were used in all experiments.

### Preparation of *C. difficile* spore suspensions

*C. difficile* isolates were stored on beads at -70°C. Spore suspensions of each strain of *C. difficile* were prepared from the method described in Shetty et al. [9]. Firstly, vegetative cells of *C. difficile* were cultured on Wilkens Chalgren agar (Oxoid Ltd, UK) following incubation in anaerobic conditions (DG 250 anaerobic workstation, Don Whitely Scientific, UK) at 37˚C for 48 hours. To encourage sporulation in vegetative cells, the agar plates were removed from anaerobic conditions and placed in aerobic conditions at room temperature for 5 days. All visible colonies were harvested using a sterile loop and inoculated into 10 mL 50% (v/v) methylated spirit (Sigma, UK) in sterile saline and then filtered through glass wool (Sigma-Aldrich, UK) to eliminate any remaining vegetative cells. A total spore count using a haemocytometer (Camlab, UK) and a viable count were performed to ascertain spore numbers in the suspensions. Total viable spore counts were achieved through serial dilution of the spore suspension and culture onto Wilkens Chalgren agar containing 0.1% (w/v) sodium taurocholate (Sigma-Aldrich, UK).

### Preparation of vegetative *C. difficile* suspensions

Briefly, vegetative *C. difficile* suspensions of each strain of were prepared by inoculating Wilkins Chalgren Broth (Oxoid, Ltd., UK) with a single colony of *C. difficile* from a Wilkens Chalgren agar plate and incubating the broth at 37°C in anaerobic conditions for 48 hours.

To confirm that a pure vegetative cell suspension (with the absence of spores) had been achieved, the suspension was heat-shocked by exposing a 1ml sample to 70°C in a waterbath for 20 minutes followed by inoculation on Wilkens Chalgren agar supplemented with 0.1% (w/v) sodium taurocholate (for maximal recovery of spores) and subsequent incubation at 37°C in anaerobic conditions for 48 hours. Absence of colonies following this procedure indicated that the cell suspension contained 100% vegetative (heat-sensitive) cells.

### Preparation of a buffered *C. difficile* germination solution comprising sodium taurocholate and amino acids

A germination solution comprising 6.9 mM sodium taurocholate and 50 mM of the following: histidine, valine, glycine, arginine aspartic acid (Sigma-Aldrich, UK) in TRIS buffer (pH 7) 50 mM (Sigma-Aldrich, UK) was prepared as outlined by Wheeldon et al. The germination solution was prepared in sterile distilled water, adjusted to pH 7.0, and filter sterilised by passing through a 0.45 μm membrane syringe filter (Merck, Millipore, Germany).

### Assessment of sporulation rate in vegetative cells of *C. difficile* in aerobic conditions following exposure to the germination solution

Briefly, 500 μl of each vegetative *C. difficile* suspension (106 CFU/mL) was placed in a microcentrifuge tube and 500 μl of germination solution added at room temperature in aerobic conditions. The suspension was mixed by vortexing for 10 seconds. After 6, 12, 24, 48 and 72 hours, 100 μl of the suspension was removed and serial dilutions performed before inoculating Wilkens Chalgren agar and Wilkens Chalgren agar containing 0.1% (w/v) sodium taurocholate (to maximise recovery of vegetative cells and spores respectively). Agar plates were then incubated at 37°C in anaerobic conditions for 48 hours and the total number of vegetative cells and spores determined. The difference in colony counts obtained on Wilkens Chalgren agar and Wilkens Chalgren agar with 0.1% (w/v) sodium taurocholate gave a measure of the percentage sporulation occurring within the vegetative cell suspensions of *C. difficile*.

Control assays were undertaken following the same experimental protocol but exposing each *C. difficile* suspension to a solution of TRIS buffer and amino acids in the absence of sodium taurocholate. All assays were performed simultaneously and in triplicate on five independent occasions.

## Statistical Analysis

Data were analysed using a Four-Factor ANOVA (Statistica, ver. 6.0; StatSoft Inc., Oklahoma, USA).

## Results

Sporulation rate of vegetative cells of C. difficile NCTC 11204 (Ribotype 001) and R20291 (Ribotype 027) in aerobic conditions following exposure to the control solution comprising glycine, histidine, valine, arginine, aspartic acid and TRIS buffer.

A 1.88 log reduction in total viable count of *C. difficile* NCTC 11204 (Ribotype 001) was observed following exposure to the control solution in aerobic conditions after 6 hours. In addition, a 4 log reduction in viable vegetative cells was demonstrated. The 2.12 log disparity between the total viable count and the viable vegetative count was due to sporulation within a proportion of the cells. At 6 hours, 99.3% of the total viable count comprised spores of *C. difficile*. After 12 hours, no vegetative cells were recovered. This observation remained consistent for 72 hours (Table 1).

After 6 hours exposure, *C. difficile* R20291 (Ribotype 027) demonstrated a 1.2 log reduction in total viable count and a 1.9 log reduction in viable vegetative cells, showing significantly less sensitivity to the aerobic conditions than NCTC 11204 (Ribotype 001) ($P=<0.01$). Consistent with *C. difficile* NCTC 11204 (Ribotype 001), no vegetative cells of R20291 (Ribotype 027) were recovered after 24 hours. *C. difficile* R20291 (Ribotype 027) demonstrated an overall total viable count reduction of 1.3 log over the course of the study (Table 1).

Sporulation rate in vegetative cells of *C. difficile* NCTC 11204 (Ribotype 001) and R20291 (Ribotype 027) in aerobic conditions following exposure to the germination solution comprising: sodium

taurocholate, glycine, histidine, valine, arginine, aspartic acid and TRIS buffer.

| Time (h) | NCTC 11204 (Ribotype 001) | | R20291 (Ribotype 027) | |
|---|---|---|---|---|
| | Mean WCAST Count | Mean WCA Count | Mean WCAST Count | Mean WCA Count |
| | (Log CFU ml$^{-1}$) | (Log CFU ml$^{-1}$) | (Log CFU ml$^{-1}$) | (Log CFU ml$^{-1}$) |
| 0 | 6.15 (0.02) | 6.14 (0.02) | 6.24 (0.02) | 6.26 (0.02) |
| 6 | 4.26 (0.03) | 2.11 (0.06) | 4.98 (0.02) | 4.36 (0.02) |
| 12 | 4.30 (0.40) | 0 (0.00) | 4.88 (0.02) | 4.21 (0.05) |
| 24 | 4.22 (0.03) | 0 (0.00) | 4.93 (0.02) | 0 (0.00) |
| 48 | 4.22 (0.01) | 0 (0.00) | 4.94 (0.01) | 0 (0.00) |
| 72 | 4.15 (0.02) | 0 (0.00) | 4.94 (0.01) | 0 (0.00) |

WCAST: Wilkens Chalgren supplemented with 0.1% (w/v) sodium taurocholate, WCA: Wilkens Chalgren agar ( ) = standard deviation of the mean

**Table 1:** Sporulation rate in vegetative cells of *C. difficile* NCTC 11204 (Ribotype 001) and R20291 (Ribotype 027) in aerobic conditions following exposure to the control solution.

*C. difficile* NCTC 11204 (Ribotype 001) exposed to the germination solution in aerobic conditions demonstrated a 2.3 log total viable count reduction after 6 hours. A 2.2 log reduction in viable vegetative cells was also observed. The total viable count comprised entirely vegetative cells (Table 2). A further 2.3 log reduction in total viable count and a 2.5 log reduction in viable vegetative cells was observed between up to the conclusion of the investigation. Vegetative cells remained recoverable for 72 hours and comprised 45.8% of the total viable count after this period of time. Following 72 hours' exposure to germination solution, an overall 4.6 log reduction in total viable count was shown.

| Time (h) | NCTC 11204 (Ribotype 001) | | R20291 (Ribotype 027) | |
|---|---|---|---|---|
| | Mean WCAST Count | Mean WCA Count | Mean WCAST Count | Mean WCA Count |
| | (Log CFU ml$^{-1}$) | (Log CFU ml$^{-1}$) | (Log CFU ml$^{-1}$) | (Log CFU ml$^{-1}$) |
| 0 | 6.25 (0.02) | 6.25 (0.01) | 6.40 (0.01) | 6.39 (0.02) |
| 6 | 4.00 (0.05) | 4.0 (0.03) | 5.03 (0.02) | 5.10 (0.02) |
| 12 | 3.66 (0.01) | 3.62 (0.01) | 4.87 (0.03) | 4.82 (0.00) |
| 24 | 3.42 (0.01) | 3.36 (0.01) | 4.58 (0.01) | 4.41(0.01) |
| 48 | 1.42 (0.07) | 1.37 (0.04) | 3.47 (0.01) | 0.72 (0.25) |
| 72 | 1.41 (0.09) | 1.56 (0.05) | 3.43 (0.01) | 0.26 (0.26) |

WCAST: Wilkens Chalgren supplemented with 0.1% (w/v) sodium taurocholate, WCA: Wilkens Chalgren agar, ( ) = standard deviation of the mean

**Table 2:** Sporulation rate in vegetative cells of *C. difficile* NCTC 11204 (Ribotype 001) and R20291 (Ribotype 027) in aerobic conditions following exposure to a germination solution comprising 6.9 mm sodium taurocholate and 50 mM glycine, histidine, valine, aspartic acid, arginine and TRIS buffer.

After 6 hours exposure to germination solution, a 1.4 log reduction in total viable count of *C. difficile* R20291 (Ribotype 027) was observed. Additionally, a 1.3 log reduction in viable vegetative cells was also shown. At this observation time, the total viable count was comprised entirely of viable vegetative cells (Table 2). Between 6 and 24 hours samples, a further 0.45 log and 0.7 log reduction was observed in R20291 (Ribotype 027) total viable count and viable vegetative counts, respectively. The 48 hour sample demonstrated a further 1.1 log reduction in total viable count and no vegetative cells were recoverable. No further notable reduction in total viable count was observed up to 72 hours. Following 72 hours' exposure of *C. difficile* R20291 (Ribotype 027) to germination solution, an overall >2.9 log reduction in total viable count was shown.

Percentage of viable vegetative cells of *C. difficile* (Ribotypes 001 and 027) following exposure to sodium taurocholate and co-germinants of glycine, histidine, valine, arginine and aspartic acid, and the control solution.

After 24 hours there was a significant difference ($p<0.001$) in the percentage of viable vegetative cells of *C. difficile* (ribotypes 001 and 027) following exposure to the germination solution and SDW with the germination solution protracting sporulation. This significant difference was maintained within both ribotypes for a period of 72 hours.

## Discussion

In this study, the potential of a previously-described *C. difficile* spore germination solution comprising sodium taurocholate as the principle germinant and glycine, histidine, valine, arginine and aspartic acid as co-germinants, was assessed for its ability to inhibit or protract sporulation in metabolically active vegetative cells of two major ribotypes of *C. difficile*. As the formulation is known to germinate spores into metabolically active cells sensitive to antimicrobials, it may also prolong cells in this sensitive vegetative state.

The results of this current investigation demonstrated that exposing vegetative cells of *C. difficile* to the germination solution maintained the cells in a vegetative state and protracted sporulation for extended periods of time compared to exposure to the control solution from which sodium taurocholate was removed. Whilst previous research within the field of spore germination has focused upon the ability of specific germinants to encourage germination of bacterial spores to render them susceptible to common antimicrobials [6,10], this investigation is the first to demonstrate that sodium taurocholate, the key germinant of *C. difficile* spores, also protracted the sporulation process in vegetative cells of *C. difficile*.

In a previous study by Jump et al. [7] it was suggested that vegetative *C. difficile* can remain viable and survive on moist surfaces in aerobic conditions for up to 6 hours, whilst desiccation promotes rapid cell death. The vegetative cells of *C. difficile* in this study were kept hydrated throughout the investigation by continued exposure to either the control solution or the germination solution. The recovery of NCTC 11204 (Ribotype 001) vegetative cells exposed to the control solution after 6 hours supports previous findings by Jump et al. suggesting that moisture creates a barrier between the air and the vegetative bacterial cells and prevents desiccation.

Interestingly, the data also showed that a greater proportion of *C. difficile* R20291 (Ribotype 027) was maintained in a vegetative state

than NCTC 11204 (Ribotype 001) after 6-12 hours exposure to germination and control solutions. This finding is particularly interesting as R20291 (Ribotype 027) has often been considered a prolific spore-former and this characteristic has been linked to its hyper virulent nature [11,12]. The findings of this current study suggest that presence of a favourable environment including sodium taurocholate and amino acids may therefore protract sporulation more readily in strains of *C. difficle* that are abundant spore producers, however further work including a wider panel of clinical isolates recovered from infected patients is necessary to explore this hypothesis. The key finding within this investigation is that sporulation in two different strains of *C. difficile* was significantly protracted (P<0.01) when exposed to a solution comprising specific *C. difficile* a germination solution containing sodium taurocholate.

It is the resistant nature of *C. difficile* spores to disinfection and standard cleaning protocols that is the major factor in the transmission and spread of the disease in the healthcare setting. The findings of this *in vitro* study, coupled with previously published research [2,6,10], suggest that application of a *C. difficile* germination solution to spores of *C. difficile* in aerobic conditions promoted rapid germination, whilst germination in metabolically active vegetative cells was significantly protracted. These collective findings suggest that a 'germinate to exterminate' approach stimulates *C. difficile* to exist in its less antimicrobial resistant form (either through germination of spores or protraction of sporulation in vegetative cells) in aerobic conditions; thus potentially rendering the organism more susceptible to common hard surface disinfectants that do not exhibit direct sporicidal activity. Research findings within this field support the incorporation of a *C. difficile* germination solution into the clinical setting; potentially as an adjunct to existing infection control strategies. Further studies are warranted to explore the specific mechanism by which sodium taurocholate interacts with vegetative cells in protracting the sporulation process.

## Conclusions

The results from this study demonstrate that sporulation is protracted in two different ribotypes of *C. difficile* when metabolically active vegetative cells are exposed to sodium taurocholate. In addition, the findings also highlight the potential application of a specific germination solution when applied to contaminated surfaces not only in encouraging spores to germinate, which can then be eliminated with conventional hard surfaces disinfectants and self-disinfecting surfaces (e.g. copper), but preventing viable antimicrobial-sensitive vegetative cells from sporulating for extended periods of time.

## Authors' contributions

TW and ACH contributed equally to the entirety of the study.

## Acknowledgments

The authors would like to thank Insight Health Ltd (Wembley, UK) and the EPSRC for funding this study.

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