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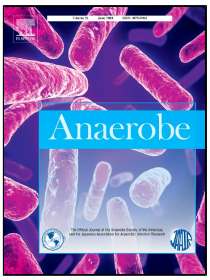

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Development of an optimized broth enrichment culture medium for the isolation of *Clostridium difficile*

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Abstract

Clostridium difficile is a spore forming bacterium and the leading cause of colitis and antibiotic associated diarrhoea in the developed world. Effective recovery of spores, particularly in low numbers, is imperative to obtain accurate prevalence data, due to the low number of spores found within non-clinical samples (<20/ml). Through comparison of *C. difficile* enrichment media, this study showed the importance of selecting an effective enrichment media. Commonly used broths, such as Cooked Meat broth, promote significantly less growth than other available broths such as Brain Heart Infusion broth, BHI. The optimization of BHI using selective antibiotics, moxalactam and norfloxacin, and sodium taurocholate at a concentration of 0.4%, allowed for high growth rate (0.465 $hour^{-1}$), short lag times (<14 hours) and recovery

of spores at low concentrations. The optimized broth, designated BHIMN-T, outperformed other commonly used broths so can be recommended for future studies.

Keywords: *Clostridium difficile*; Culture media; Growth; Taurocholate; Spores

1. Introduction

Clostridium difficile is a Gram positive, spore forming, anaerobic bacterium and a major cause of diarrhoea and pseudomembranous colitis (1, 2). Transmission and spread of *C. difficile* is facilitated by the resistance of *C. difficile* spores to harsh environmental conditions, for example, heat, cleaning and disinfectant agents along with alcohol based hand sanitizers (3–6). Spores of *C. difficile* can persist on surfaces for many months, providing a reservoir of infection in hospitals and community-care settings. It is increasingly evident that *C. difficile* inhabits a wider spectrum of environments, with toxigenic strains having been isolated from animals, foodstuffs, soil, water and wastewater treatment facilities (7–19). Spore burden in such non-clinical samples is often low: <20 spores/ml have been reported in food samples (20), while the infectious dose required to cause *C. difficile* infection (CDI) in mice from controlled surface contamination is estimated at <7 spores/cm^2 (21). Given the public health concern, *C. difficile* monitoring in non-clinical reservoirs is important to further our understanding of community-acquired CDI (22, 23).

Central to such surveillance is the development of an effective isolation media. Based on Google Scholar citations (March 2018) the four most widely used formulations for *C. difficile* broth enrichment and growth are; Brain Heart Infusion broth (BHI: 5130 cites), Cooked Meat broth (CM: 4860 cites), Cycloserine Cefoxitin Fructose broth

(CCFB: 964 cites) and *C. difficile* Moxalactam Norfloxacin broth (CDMN: 294 cites). These, along with a relatively new broth - *Clostridium difficile* Brucella Broth with L-cystine and thioglycolic acid (CDBB-TC), referenced in one study – were selected as the basis of this investigation. Use of these basal media preparations is further complicated by the addition of various supplements. These include: a) antibiotics, added as a combination of either cefoxitin and cycloserine, or norfloxacin and moxalactam (24–26); b) sodium taurocholate and/or horse blood (26–29); and c) the use of thioglycolic acid and L-cystine *in lieu* of incubation within an anaerobic cabinet (30).

We report an evaluation of these media formulations for the recovery and growth of *C. difficile*, and development of an optimized enrichment media based on BHI broth supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml) and 0.4% (w/v) sodium taurocholate (designated BHIMN-T). Use of BHIMN-T for the recovery of *C. difficile* M120 spores resulted in a 10 hour decrease in the lag phase and a 55% increase in growth rate when compared to other published media preparations.

2. Materials and methods

2.1 Preparation of spore suspension

Spore suspensions of representative isolates from *C. difficile* clades 1-5 were prepared: - TL178 (Clade 1, Ribotype 002); R20291 (Clade 2, Ribotype 027); CD305 (Clade 3, Ribotype 023); CF5 (Clade 4, Ribotype 017) and M120 (Clade 5, Ribotype 078), from isolates obtained from the archives at the Kelvin Laboratories, Royal Victoria Hospital, Belfast, Northern Ireland.

Fastidious anaerobe blood agar (FAABL) (Oxoid, Basingstoke, UK) was inoculated with *C. difficile* M120 and incubated anaerobically (N_2 /CO_2 /H_2) at 37°C for 72 h in an

anaerobic chamber (Don Whitley Scientific, Shipley, UK). To inactivate vegetative cells, colonies were suspended in 5 ml sterile Phosphate Buffered Saline (PBS) and subjected to alcohol shock (5 ml of absolute ethanol added to 5 ml suspension) for 1 hour to kill vegetative cells, in accordance with the spore culture method of UK Standards for Microbiology Investigations (31). Alcohol shocked suspensions were centrifuged at 3,000 xg for 4 min and washed once in 5 ml sterile PBS with the resultant pellet resuspended in 70% (v/v) ethanol. Spore stock concentrations were determined by viable spore counts (32). Spore stock was stored in 50 µl aliquots at -20ºC.

2.2 Preparation of enrichment media

Enrichment media were prepared as follows. All products were supplied by Sigma Aldrich, UK, unless otherwise stated.

Brain Heart Infusion broth (BHI) (Oxoid, Basingstoke, UK). Lyophilised media (3.7% [w/v]) which contained, per litre; brain infusion solids (12.5 g), beef heart infusion solids (5.0 g), proteose peptone (10.0 g), glucose (2.0 g), sodium chloride (5.0 g) and disodium phosphate (2.5 g).

C. difficile broth supplemented with Moxalactam and Norfloxacin (CDMN) was prepared in accordance with Xu *et al* (17) and contained, per litre; proteose peptone (40.0 g), disodium hydrogen phosphate (5.0 g), potassium dihydrogen phosphate (1.0 g), magnesium sulphate (0.1 g), sodium chloride (2.0 g), fructose (6.0 g) and CDMN supplement (Oxoid, Basingstoke , UK); cysteine hydrochloride (500.0mg), norfloxacin (12.0 mg), moxalactam (32.0 mg) was added post sterilisation.

Cycloserine Cefoxitin Fructose Broth (CCFB) was prepared as described by Arroyo *et al*. (25) and contained, per litre; proteose peptone (40.0 g), disodium hydrogen phosphate (5.0 g), potassium dihydrogen phosphate (1.0 g), magnesium sulphate (0.1 g), sodium chloride (2.0 g) and fructose (6.0 g). D-cycloserine (250.0 mg) (Oxoid, Basingstoke, UK), cefoxitin (8.0 mg) (Oxoid, Basingstoke, UK) and defibrinated horse blood (70.0 ml) (Lab Supplies Ltd, Antrim, UK) were added post sterilisation.

C. difficile Brucella Broth with thioglycolic acid and L-cysteine (CDBB-TC) was prepared according to Cadnum *et al.* (30) and contained, per litre; Brucella broth (28.0 g), vitamin K1 solution (1.0 mg), hemin solution (5.0 mg) (Fisher Scientific, Loughborough, UK), sodium bicarbonate (0.1 g), D-mannitol (6.0 g). Neutral red solution (1% [v/v]) (5.0 ml), sodium taurocholate (0.5 g), lysozyme (5.0 mg) (Fisher Scientific, Loughborough, UK), D-cycloserine (500.0 mg), cefoxitin (16.0 mg), agar (1.0 g), thioglycolic acid (1.0 g) and L-cystine (1.0 g) (Fisher Scientific, Loughborough, UK) were added post sterilisation.

Cooked meat broth (CM) (Oxoid, Basingstoke, UK). CM was purchased as a pre-prepared media, which contained, per litre; heart muscle (454.0 g), peptone (10.0 g), Lab-Lemco powder (10.0 g), sodium chloride (5.0 g) and glucose (2.0 g).

2.3 Growth of *C. difficile* M120

Broths (5 ml) were aliquoted into sterile 30 ml polystyrene screw cap universals (Medline Scientific, Chalgrove, UK) and pre-reduced for 5 hours in an anaerobic chamber prior to inoculation. The pre-reduced broths were inoculated to a concentration of 2% (v/v) spore inoculum, under anaerobic conditions, by centrifuging

100 µl of spore suspension at 14,000 xg for 5 min. Inocula were subsequently prepared by resuspension of the spore pellet in 100 µl of the appropriate enrichment broth. Triplicate growth assays were carried out for each broth.

Determination of growth rate via qPCR

Growth rate of *C. difficile* M120 in each media variant was determined via real-time quantitative PCR using a method modified from Nadkarni *et al*. (33). For media which did not contain defibrinated horse blood, DNA was extracted according to Stubbs *et al.* whereby 100 µl of culture was boiled in 300 µl of sterile water containing 5% (w/v) Chelex-100 (Sigma-Aldrich, Irvine, UK) for 5 min (34). For media which did contain defibrinated horse blood, DNA was extracted and purified using the DNA/Viral Kit on the MagNA Pure 96 extraction platform (Roche Applied Science, Penzberg, Germany) in accordance with the manufacturer's instructions. A previously described method to monitor bacterial load (growth) using real-time PCR (33) was adapted for this study using an assay targeting the *C. difficile* toxin B gene, *tcdB*. Assays were performed on a LightCycler 480 (Roche Applied Science, Penzberg, Germany) using the TaqMan® primer and probe set of Luna *et al.* (35) tcdB_F - GAAAGTCCAAGTTTACGCTCAAT; tcdB_R - GCTGCACCTAAACTTACACCA; tcdB_TM- FAM - ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA**.** Unless otherwise specified all reagents were supplied by Invitrogen Ltd (Paisley, UK). Each reaction contained; 1x Platinum® Quantitative PCR SuperMix-UDG, Nuclease Free Water (Sigma Aldrich, Irvine, UK), Bovine Serum Albumin (0.2 µg/ml) (Sigma Aldrich, Irvine, UK), $MgCl_2$ (3 mM), tcdB_F (0.4 µM), tcdB_R (0.4 µM) and tcdB_TM (0.2 µM).

For each assay, 2 µl of template DNA was added to 8 µl of Mastermix, giving a final reaction volume of 10 µl. Reactions were cycled using the following conditions: 50°C for 2 min, 1 cycle of 95°C for 5 min, 45 cycles of 95°C for 10 min, 57°C for 20 sec, and 72°C for 10 sec.

Purified and quantified *C. difficile* M120 DNA was used for calibration of qPCR, using duplicate 10-fold serial dilutions of DNA in 0.1x Tris EDTA with yeast tRNA (10 µg/ml) (Thermo Scientific, Leicestershire, UK). The *tcdB* qPCR was run for each dilution in triplicate over a 6-log range (calibration R^2= 9.78). Calibrators were tested alongside extracts from growth assay samples and specific growth rates calculated from qPCR copy numbers.

2.4 Optimization of BHI

Antibiotic supplementation of BHI

The effect of supplementing BHI media with moxalactam (32 µg/ml) and norfloxacin (12 µg/ml), designated BHIMN, was assessed by comparison of *C. difficile* M120 growth in BHI without antibiotic addition. Moxalactam and norfloxacin have been reported to provide a higher degree of selectivity than the other commonly used antibiotic supplements, cycloserine and cefoxitin (26). BHIMN media (n=3) was prepared by addition of moxalactam and norfloxacin selective supplement containing cysteine hydrochloride (500.0 µg/ml), norfloxacin (12.0 µg/ml), and moxalactam (32.0 µg/ml) (Oxoid, Basingstoke, U.K) to BHI (n=3). All media was pre-reduced for 5 hours prior to growth assays.

168

169 *Effect of sodium taurocholate*

170 The effect of sodium taurocholate concentration on growth of *C. difficile* M120 in
171 BHIMN was investigated. It is known that the secondary bile salt deoxycholate, formed
172 through metabolism of sodium taurocholate, inhibits vegetative cell growth, therefore
173 a range of sodium taurocholate concentrations (0 – 1% [w/v]) were investigated with
174 regard to optimal growth of *C. difficile* M120.

175 Sodium taurocholate was added to final concentrations (w/v) of 0.1%, 0.2%, 0.3%,
176 0.4%, 0.5% or 1%. Replicate growth assays (n=12) of *C. difficile* M120 in pre-reduced
177 BHIMN at each sodium taurocholate concentration were carried out under anaerobic
178 conditions in microtitre plates at 37°C with growth compared to that of a sodium
179 taurocholate free control. Broths, with a total volume of 200 µl per well, were inoculated
180 to a concentration of 2% (v/v) inoculum. Growth was assessed by determining optical
181 density at 600nm using a FLUOstar Omega Microplate reader (BMG Labtech,
182 Ortenberg, Germany).

183

184 *Effect of horse blood*

185 BHIMN was augmented with defibrinated horse blood to final concentrations (v/v) of
186 1%, 2%, 3%. 5% or 7%. Broths (5ml) were inoculated to a concentration of 2% (v/v)
187 inoculum. Growth assays of *C. difficile* M120 in BHIMN at each horse blood
188 concentration were carried out, in triplicate, under anaerobic conditions at 37°C with
189 growth compared to that of a horse blood free control. As broth containing horse blood
190 is opaque, growth could not be measured using optical density therefore growth
191 assays used real-time PCR as described.

Addition of blood to growth media may hinder bacterial growth due to the bactericidal components naturally occurring within blood, such as complement (36). Heat treatment inactivates the complement system without affecting the nutritional value of the blood (36). A 10 ml aliquot of horse blood was heated at 56°C for a total of 30 min, with mixing every 5 min, and then transferred to ice to cool immediately (37). Once cooled, it was added to BHIMN and reduced prior growth assay. Pre-reduced BHIMN-HB supplemented with untreated horse blood from the same batch was used as a control.

2.5 Comparison of commonly used media

The optimized media (designated BHIMN-T) was compared to CCFB and CDMN using the supplements described in Table 1. Pre-reduced broths (5 ml) were inoculated to a concentration of 2% (v/v) inoculum. Growth assays in each broth were completed in triplicate of *C. difficile* M120 in each broth variant were carried out under anaerobic conditions at 37°C and growth was determined using real-time PCR as described.

2.6 Evaluation of spore recovery in BHIMN-T

In order to confirm BHIMN-T was effective for recovery across genetically diverse *C. difficile* isolates, spore recovery using the optimal BHIMN-T broth was assessed over a range of spore concentrations from representative isolates from clades 1-5 of *C. difficile*. Serial dilutions of spore suspension were prepared in sterile PBS. Pre-reduced BHIMN-T was aliquoted into a microtitre plate, 200 µl/well, within an anaerobic chamber. Replicate wells (n=14) were inoculated with appropriate dilution

to achieve target spore number (**10^{-4}:** 130 spores ± 3; **10^{-5}:** 13 spores ± 0.3; **10^{-6}:** 1.3 ± 0.03) and incubated anaerobically for 24 hours. Positive growth was determined by visible growth in the microtitre plate and the results were evaluated using the Most Probable Number (MPN) method (38), to estimate the MPN/ml and assess ability of BHIMN-T to recover spores across a range of inoculum concentrations.

2.7 Statistical Analysis

All statistical analyses were carried out using GraphPad Prism v5 (GraphPad Software Inc.). A One-Way Analysis of Variance (ANOVA) was employed to determine significant differences in growth rate obtained by *C. difficile* M120 in each broth. Statistical significance was determined at $p < 0.05$.

MPN/ml and corresponding 95% confidence limits were estimated using the standard MPN method, approved by the US Food and Drug Administration (FDA) (38). Each dilution was subject to 14 replicates.

3. Results and Discussion

3.1 Evaluation of enrichment media

Growth of *C. difficile* M120 was assessed in five different broth enrichment culture media. The results show highest growth rate in non-selective BHI broth (0.202 ± 0.045 $hour^{-1}$) compared to CCFB, CDMN, CDBB-TC and CM (Figure 1). CCFB and CDMN are similar selective media, both consisting of a fructose base (17, 25). CCFB is however augmented with the antibiotics cycloserine (250 µg/ml) and cefoxitin (8 µg/ml), and defibrinated horse blood (7% [v/v]); CDMN is supplemented with the

antibiotics moxalactam (32 µg/ml) and norfloxacin (12 µg/ml). Both CCFB and CDMN are equally effective for growth of *C. difficile* M120. No significant difference in *C. difficile* M120 growth rate was observed between these media, with growth rates of 0.10 ± 0.030 $hour^{-1}$ and 0.08 ± 0.021 $hour^{-1}$, respectively.

The growth rates observed in CM and CDBB-TC were significantly less than that of the other broths (p<0.001). CDBB-TC is a recently described culture medium for the growth of *C. difficile* without the need for strict anaerobic incubation (30). No growth was observed in CDBB-TC, with or without 0.1% [w/v] sodium taurocholate addition, under either anaerobic or aerobic conditions. Cadnum *et al.* have however reported the successful growth of *C. difficile* from a range of environmental samples using this formulation (30). CM provided a growth rate of 0.017 ± 0.003 $hour^{-1}$, which was significantly lower than CCFB (0.10 ± 0.030 $hour^{-}$), CDMN (0.08 ± 0.021 $hour^{-1}$) and BHI (0.202 ± 0.045 $hour^{-1}$). The CM and CDBB-TC media combinations were not considered further in this study. Studies using these media might be at risk from false negatives (Figure 1) (30, 39).

3.2 Optimization of BHI

Addition of antibiotics: moxalactam and norfloxacin

In initial studies BHI outperformed the other media (Figure 1) although this medium is non-selective for *C. difficile*. Moxalactam (32 µg/ml) and norfloxacin (12 µg/ml) have been shown to provide higher selectivity when isolating *C. difficile* when compared to cycloserine (250 µg/ml) and cefoxitin (8 µg/ml) (26, 40). BHI medium was therefore supplemented with moxalactam (32 µg/ml) and norfloxacin (12 µg/ml), and designated

as BHIMN. No significant difference was found in *C. difficile* M120 growth rate between BHI and BHIMN media (Figure 2) so BHIMN replaced BHI in subsequent studies.

Addition of sodium taurocholate and defibrinated horse blood to BHIMN broth

To determine if sodium taurocholate enhanced growth of *C. difficile* in BHIMN, growth assays were carried out in the presence of varying concentrations of sodium taurocholate (0 - 1% [w/v]: Figure 3). Previous studies have suggested that sodium taurocholate should be supplemented to a concentration of 0.1% (w/v) in *C. difficile* growth media (17, 25, 30, 41). In this study, a concentration of 0.4% was found to be most effective for promoting the growth of *C. difficile* M120 in BHIMN (Figure 3): 0.4% sodium taurocholate provided significantly faster growth than 0.1% ($p< 0.001$), 0.2% ($p< 0.001$), 0.3% ($p< 0.05$), 0.5% ($p< 0.001$) and 1% ($p< 0.0001$), respectively (Figure 3).

Additionally, BHIMN broth supplemented with horse blood (0 – 7% [v/v]) showed no significant increase in the growth of *C. difficile* M120 (Figure 3; $p> 0.05$): In other publications, horse blood supplementation is recommended at 7% (v/v) (25, 26). Although horse blood does appear to enhance growth of *C. difficile* in certain circumstances, e.g. growth of *C. difficile* on CCFB (26). It has also been suggested that the addition of blood may hinder bacterial growth due to the bactericidal components, such as complement, naturally occurring within blood (36). In this study, no significant difference was observed in *C. difficile* M120 growth rate between BHIMN supplemented with defibrinated horse blood and complement inactivated BHIMN horse blood broth, Figure 4 (E) and Figure 4 (F). Inactivation of the complement

system within the blood did not improve growth of *C. difficile* M120 in BHIMN (Figure 4).

This study thus found that BHI supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml) and sodium taurocholate (0.4% [w/v]), designated as BHIMN-T, provided optimal growth of *C. difficile* M120 from spores.

3.3 Comparison of commonly used media and BHIMN-T

Growth in BHIMN-T was compared with CCFB and CDMN. A number of variations of CCFB and CDMN have been reported in the literature, including the addition of defibrinated horse blood (HB) to CDMN (CCFB contains horse blood as prepared) and sodium taurocholate (T) to both (17, 25, 26, 45). BHIMN-T was thus not only compared to CCFB and CDMN but also to their defibrinated horse blood (HB) and sodium taurocholate (T) supplemented variants: These are designated as CCFB-T, CDMN-T, CDMN-HB and CDMN-THB respectively (Table 1). The most effective broth for growth of *C. difficile* M120 was BHIMN-T, with a growth rate of 0.4645 $hour^{-1}$ and a lag phase of less than 14 hours (Table 2). Notably, including this optimal concentration of sodium taurocholate more than doubled the observed growth rate. Growth in BHIMN-T out-performed both CCFB and CDMN, with and without their recommended horse blood and sodium taurocholate supplementation (Figure 5); CCFB-T ($p< 0.05$), CDMN-HB ($p< 0.01$), CDMNT ($p< 0.01$), CDMN-THB ($p< 0.001$), CCFB ($p< 0.001$) and CDMN ($p< 0.001$) (Figure 5). All broths containing sodium taurocholate provided shorter lag times (Table 2), most likely due to promotion of spore germination (27, 28, 41). Dharmasena and Jiang (2018) found BHI broth supplemented with 0.5% yeast extract, 0.1% L-cysteine, 0.1% sodium taurocholate, moxalactam (32 mg/l) and norfloxacin (12

mg/l) proved to be the most effective broth in their study for recovery of *C. difficile* from spiked compost (40). Their study did not however evaluate media containing sodium taurocholate concentrations above 0.1% (w/v), well below the 0.4% (w/v) level shown to be optimal in this study. They also found moxalactam and norfloxacin were a more effective selective supplement than cycloserine and cefoxitin (40).

The addition of horse blood to *C. difficile* growth media is common practice (46, 47). Horse blood improved the growth of *C. difficile* M120 in CDMN (Figure 5), however it had no effect on growth rate across the varying concentrations tested in BHIMN (Figure 3). The reason for this is unknown. BHI base may already provide optimal growth nutrients for *C. difficile* growth. We note that broths containing horse blood are less attractive due to their expense, short shelf life and ease of contamination associated with blood products (48).

3.4 Evaluation of spore recovery using BHIMN-T

BHIMN-T was assessed for both its effectiveness and efficiency in promoting spore recovery across clades 1-5 of *C. difficile*, particularly from samples containing low spore numbers (<20 spores/ml)]. Using spore suspensions prepared from representative isolates across a range of spore concentrations [where each $1x10^{-5}$ dilution is representative of 13 ± 0.3 spores/ml: Table 3), spores were recovered from each clade tested, and at each dilution (Table 3). No growth was however observed in BHIMN without sodium taurocholate supplementation at spore concentrations less than 130 ± 0.3 spores/ml (Table 3). In spore suspensions ≥130 spores/ml BHIMN-T recovered a median of 12.5 times more spores across the isolates tested (range 9.5

– 13.1 times: Table 3) than BHIMN alone (estimated using the Most Probable Number (MPN) method (38)] .

Lister *et al.* (2014) have previously reported that direct plating on Cycloserine-Cefoxitin Egg Yolk agar, rather than broth enrichment, increases *C. difficile* spore recovery. Using a semi-quantitative plate streaking approach Cycloserine-Cefoxitin Egg Yolk agar recovered $1x10^2$ spores: no recovery was observed at $1x10^1$ spores / ml (49) cf. BHIMN-T (Table 3).

4. Conclusion

Given the increasing evidence that *C. difficile* spores reside in a much wider spectrum of environments than hospitals and community care settings (17, 24, 50–53), the monitoring of potential infection reservoirs is essential to understand its spread. A number of different media formulations have been described for *C. difficile* enrichment yet few studies compare their efficacy.

To develop an optimal broth medium for recovering *C. difficile* from clinical and non-clinical samples, a number of factors must be considered. Firstly, high growth rate and short lag times are desirable. Secondly, ease of preparation, shelf-life and complexity must be considered. A number of published media, such as CDBB-TC, are complex to prepare and contain ingredients which may not be readily available, whilst others, based on for example horse blood, have short shelf-lives.

In this paper we describe the development of an improved media, designated BHIMN-T, for the growth of *C. difficile* which combines ease of preparation and longer shelf life (due to absence of horse blood), with high growth rates (0.465 $hour^{-1}$), short lag

355 times (<14 hours). Furthermore, BHIMN-T proved effective for the recovery of spores
356 at low concentrations, across a number of genetically diverse *C. difficile* isolates (Table
357 3).
358
359 This study highlights the need for optimal methods for isolation of *C. difficile*, especially
360 from environmental and other non-clinical samples. Use of suboptimal culture methods
361 can result in false negatives leading to skewed prevalence data (54), which may hinder
362 attempts to track and prevent spread of *C. difficile* and its ubiquitous spores.
363

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368

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555 3099(14)70991-0

556 **Table 1:** Recommended supplementation of CCFB and CDMN

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Media	Sodium taurocholate concentration (T) (%)	Horse blood concentration (HB) (%)	References
CCFB	NA	NA*	(26)
CCFB-T	0.1	NA*	(25)
CDMN	0.1	7	(45)
CDMN-T	0.1	NA	(17)
CDMN-HB	NA	7	(26)
CDMN-THB	0.1	7	(17, 26)

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559 *CCFB contains 7% (v/v) horse blood as prepared.

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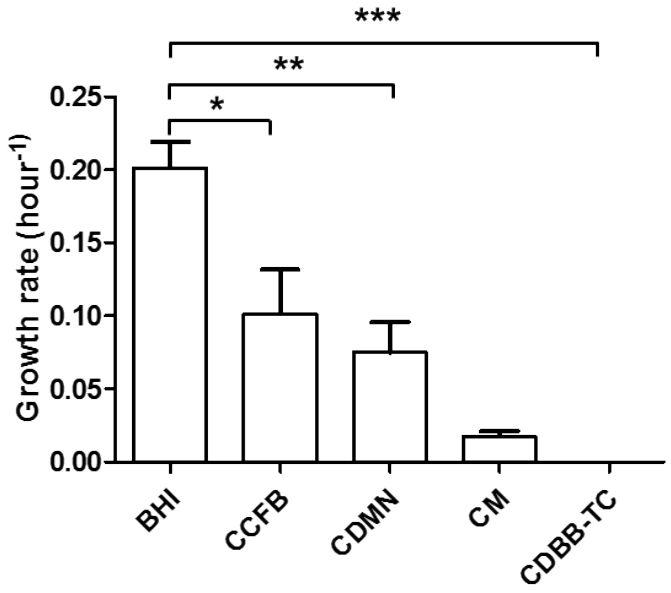

Figure 1: Growth rate of *C. difficile* M120 in each BHI, CCFB, CDMN, CM and CDBB-TC incubated anaerobically at 37°C for 33 h; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

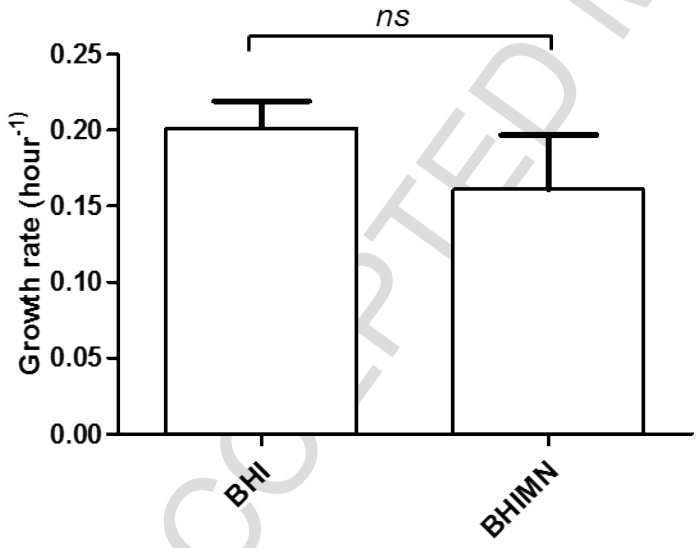

Figure 2: Growth of *C. difficile* M120 in BHI and BHIMN incubated anaerobically at 37°C for 33 h.

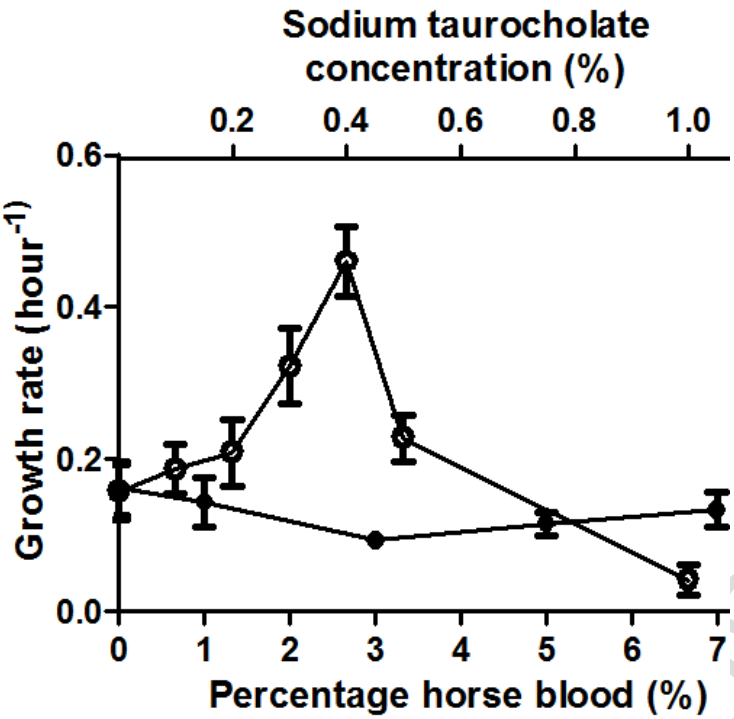

Figure 3: Growth of *C. difficile* M120 with varying concentrations of sodium taurocholate, 0-1% (**o**), or defibrinated horse blood in BHIMN, 0-7% (●).

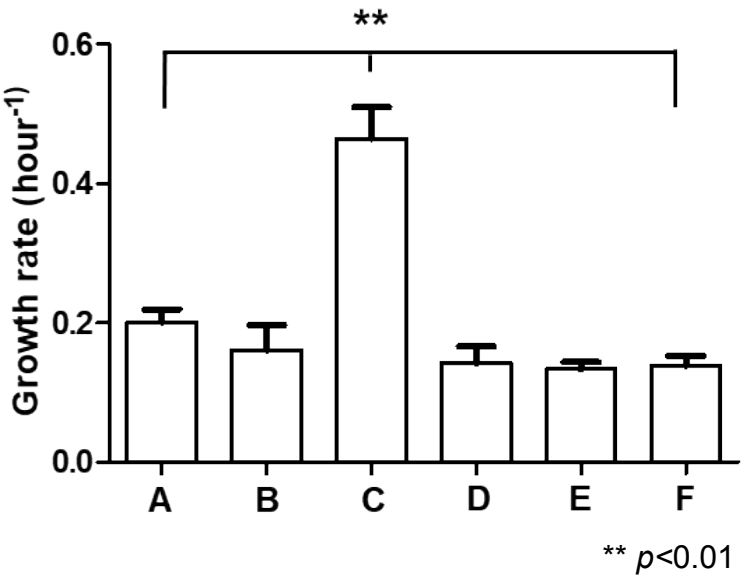

Figure 4: Growth rates of *C. difficile* M120 in **A) BHI** – without supplementation, **B) BHIMN** – BHI supplemented with antibiotics, moxalactam (32 µg/ml) and norfloxacin (12 µg/ml), **C) BHIMN-T** – BHI supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml) and 0.4% (w/v) sodium taurocholate, **D) BHIMN-HB** – BHI supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml) and 7% (v/v) defibrinated horse blood. **E) BHIMN-HB (heat)** – BHI supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml) and 7% (v/v) defibrinated horse blood which has been heat treated to inactivate complement, **F) BHIMN-THB** – BHI supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml), and 0.1% (w/v) sodium taurocholate and 7% (v/v) defibrinated horse blood.

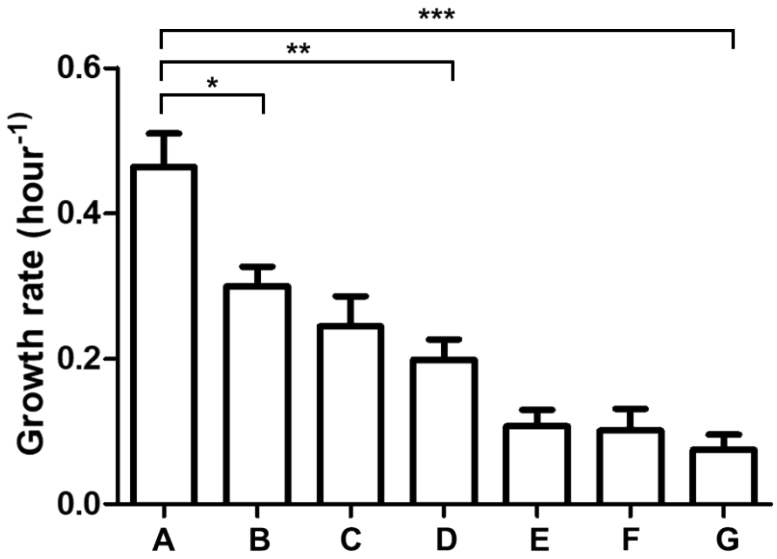

Figure 5: Specific growth rate for *C. difficile* M120 in **A)** BHIMN-T, **B)** CCFB-T- as prepared by Arroyo *et al*. (25) **C)** CDMN-HB as prepared by Aspinall and Hutchinson(26), **D)** CDMNT - as prepared by Xu *et al.*(17), **E)** CDMN-THB (17, 26), **F)** CCFB - as prepared by Aspinall and Hutchinson (26), **G)** CDMN - as prepared by Esfandiari *et al.* (45); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

695 **Table 2:** Summary of specific growth rate of *C. difficile* M120 and corresponding lag
696 phase in each medium
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Media	Lag phase (hours)	Growth rate ($hour^{-1}$)	SEM
BHIMNT	<14 hours	0.465	0.045
CCFB-T	~24 hours	0.300	0.027
CDMN-HB	~18 hours	0.245	0.041
CDMN-T	~15 hours	0.199	0.028
CDMN-THB	~14 hours	0.107	0.022
CCFB	~27 hours	0.102	0.030
CDMN	~33 hours	0.075	0.021

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704 **Table 3:** Evaluation of the optimum concentration of sodium taurocholate in *C. difficile* spore recovery across representative
705 isolates from clades 1-5, using estimation of MPN/ml
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		Number of positives at each dilution factor					Confidence limits	
Isolate (Clade)		**-4[a]**	**-5[b]**	**-6[c]**	**Reported positives**	**MPN/ml**	**Lower**	**Upper**
TL178 (1)	**0.4% sodium taurocholate**	12/14	6/14	1/14	12-6-1	2.83×10^4	1.7×10^4	4.9×10^4
	No sodium taurocholate	4/14	0/14	0/14	0-0-0	2.98×10^3	1.1×10^3	8.0×10^3
R20291 (2)	**0.4% sodium taurocholate**	11/14	9/14	5/14	11-9-5	3.72×10^4	2.3×10^4	5.9×10^4
	No sodium taurocholate	4/14	0/14	0/14	4-0-0	2.97×10^3	1.1×10^3	8.0×10^3
CD305 (3)	**0.4% sodium taurocholate**	14/14	3/14	1/14	14-3-1	3.80×10^4	2.0×10^4	7.4×10^4
	No sodium taurocholate	4/14	0/14	0/14	4-0-0	2.98×10^3	1.1×10^3	8.0×10^3
CF5 (4)	**0.4% sodium taurocholate**	12/14	5/14	2/14	12-5-2	2.80×10^4	1.6×10^4	4.8×10^4
	No sodium taurocholate	3/14	0/14	0/14	3-0-0	2.14×10^3	6.9×10^2	6.7×10^3
M120 (5)	**0.4% sodium taurocholate**	13/14	8/14	2/14	13-8-2	5.0×10^4	2.2×10^4	6.1×10^4
	No sodium taurocholate	6/14	0/14	0/14	6-0-0	4.9×10^3	2.2×10^3	1.1×10^4

707 [a] 1×10^{-4} dilution is representative of 130 ± 3 spores/ml
708 [b] 1×10^{-5} dilution is representative of 13 ± 0.3 spores/ml
709 [c] 1×10^{-6} dilution is representative of 1.3 ± 0.03 spores/ml

Highlights

i. Comparison of enrichment media using qPCR to monitor growth of *C. difficile.*
ii. 0.4% sodium taurocholate provided optimum growth in Brain Heart Infusion (BHI) supplemented with moxalactam and norfloxacin (BHIMN-T) when compared to other media with recommended supplementation.
iii. BHIMN-T provided effective for the recovery of spores at low concentrations.