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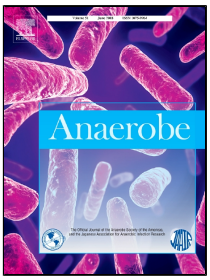

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Development of an optimized broth enrichment culture medium for the isolation of *Clostridium difficile*

Mairéad C. Connor*[a], John W. McGrath[a], Geoff McMullan[a], Nikki Marks[a] and Derek J. Fairley[b]

a. School of Biological Sciences and the Institute for Global Food Security, Medical Biology Centre, Queens University Belfast, Northern Ireland.
b. Department of Microbiology, Belfast Health & Social Care Trust, Belfast, Northern Ireland.

*Corresponding author: School of Biological Sciences and the Institute for Global Food Security, Medical Biology Centre, Queens University Belfast, 97 Lisburn Road, Belfast, Northern Ireland, BT9 7BL; m.connor@qub.ac.uk.

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, out-performed 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

49 (CCFB: 964 cites) and *C. difficile* Moxalactam Norfloxacin broth (CDMN: 294 cites).
50 These, along with a relatively new broth - *Clostridium difficile* Brucella Broth with L-
51 cystine and thioglycolic acid (CDBB-TC), referenced in one study – were selected as
52 the basis of this investigation. Use of these basal media preparations is further
53 complicated by the addition of various supplements. These include: a) antibiotics,
54 added as a combination of either cefoxitin and cycloserine, or norfloxacin and
55 moxalactam (24–26); b) sodium taurocholate and/or horse blood (26–29); and c) the
56 use of thioglycolic acid and L-cystine *in lieu* of incubation within an anaerobic cabinet
57 (30).

58 We report an evaluation of these media formulations for the recovery and growth of *C.*
59 *difficile*, and development of an optimized enrichment media based on BHI broth
60 supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml) and 0.4% (w/v)
61 sodium taurocholate (designated BHIMN-T). Use of BHIMN-T for the recovery of *C.*
62 *difficile* M120 spores resulted in a 10 hour decrease in the lag phase and a 55%
63 increase in growth rate when compared to other published media preparations.
64

65 ## 2. Materials and methods

66 ### 2.1 Preparation of spore suspension

67 Spore suspensions of representative isolates from *C. difficile* clades 1-5 were
68 prepared: - TL178 (Clade 1, Ribotype 002); R20291 (Clade 2, Ribotype 027); CD305
69 (Clade 3, Ribotype 023); CF5 (Clade 4, Ribotype 017) and M120 (Clade 5, Ribotype
70 078), from isolates obtained from the archives at the Kelvin Laboratories, Royal
71 Victoria Hospital, Belfast, Northern Ireland.
72 Fastidious anaerobe blood agar (FAABL) (Oxoid, Basingstoke, UK) was inoculated
73 with *C. difficile* M120 and incubated anaerobically (N_2 /CO_2 /H_2) at 37°C for 72 h in an

74 anaerobic chamber (Don Whitley Scientific, Shipley, UK). To inactivate vegetative
75 cells, colonies were suspended in 5 ml sterile Phosphate Buffered Saline (PBS) and
76 subjected to alcohol shock (5 ml of absolute ethanol added to 5 ml suspension) for
77 1 hour to kill vegetative cells, in accordance with the spore culture method of UK
78 Standards for Microbiology Investigations (31). Alcohol shocked suspensions were
79 centrifuged at 3,000 xg for 4 min and washed once in 5 ml sterile PBS with the resultant
80 pellet resuspended in 70% (v/v) ethanol. Spore stock concentrations were determined
81 by viable spore counts (32). Spore stock was stored in 50 µl aliquots at -20ºC.

82

83 **2.2 Preparation of enrichment media**

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

86 Brain Heart Infusion broth (BHI) (Oxoid, Basingstoke, UK). Lyophilised media (3.7%
87 [w/v]) which contained, per litre; brain infusion solids (12.5 g), beef heart infusion solids
88 (5.0 g), proteose peptone (10.0 g), glucose (2.0 g), sodium chloride (5.0 g) and
89 disodium phosphate (2.5 g).

90

91 *C. difficile* broth supplemented with Moxalactam and Norfloxacin (CDMN) was
92 prepared in accordance with Xu *et al* (17) and contained, per litre; proteose peptone
93 (40.0 g), disodium hydrogen phosphate (5.0 g), potassium dihydrogen phosphate (1.0
94 g), magnesium sulphate (0.1 g), sodium chloride (2.0 g), fructose (6.0 g) and CDMN
95 supplement (Oxoid, Basingstoke , UK); cysteine hydrochloride (500.0mg), norfloxacin
96 (12.0 mg), moxalactam (32.0 mg) was added post sterilisation.

97

98 Cycloserine Cefoxitin Fructose Broth (CCFB) was prepared as described by Arroyo *et*
99 *al*. (25) and contained, per litre; proteose peptone (40.0 g), disodium hydrogen
100 phosphate (5.0 g), potassium dihydrogen phosphate (1.0 g), magnesium sulphate (0.1
101 g), sodium chloride (2.0 g) and fructose (6.0 g). D-cycloserine (250.0 mg) (Oxoid,
102 Basingstoke, UK), cefoxitin (8.0 mg) (Oxoid, Basingstoke, UK) and defibrinated horse
103 blood (70.0 ml) (Lab Supplies Ltd, Antrim, UK) were added post sterilisation.
104
105 *C. difficile* Brucella Broth with thioglycolic acid and L-cysteine (CDBB-TC) was
106 prepared according to Cadnum *et al.* (30) and contained, per litre; Brucella broth (28.0
107 g), vitamin K1 solution (1.0 mg), hemin solution (5.0 mg) (Fisher Scientific,
108 Loughborough, UK), sodium bicarbonate (0.1 g), D-mannitol (6.0 g). Neutral red
109 solution (1% [v/v]) (5.0 ml), sodium taurocholate (0.5 g), lysozyme (5.0 mg) (Fisher
110 Scientific, Loughborough, UK), D-cycloserine (500.0 mg), cefoxitin (16.0 mg), agar
111 (1.0 g), thioglycolic acid (1.0 g) and L-cystine (1.0 g) (Fisher Scientific, Loughborough,
112 UK) were added post sterilisation.
113
114 Cooked meat broth (CM) (Oxoid, Basingstoke, UK). CM was purchased as a pre-
115 prepared media, which contained, per litre; heart muscle (454.0 g), peptone (10.0 g),
116 Lab-Lemco powder (10.0 g), sodium chloride (5.0 g) and glucose (2.0 g).
117
118 **2.3 Growth of *C. difficile* M120**
119 Broths (5 ml) were aliquoted into sterile 30 ml polystyrene screw cap universals
120 (Medline Scientific, Chalgrove, UK) and pre-reduced for 5 hours in an anaerobic
121 chamber prior to inoculation. The pre-reduced broths were inoculated to a
122 concentration of 2% (v/v) spore inoculum, under anaerobic conditions, by centrifuging

123 100 µl of spore suspension at 14,000 xg for 5 min. Inocula were subsequently
124 prepared by resuspension of the spore pellet in 100 µl of the appropriate enrichment
125 broth. Triplicate growth assays were carried out for each broth.
126
127 *Determination of growth rate via qPCR*

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

146 For each assay, 2 µl of template DNA was added to 8 µl of Mastermix, giving a final
147 reaction volume of 10 µl. Reactions were cycled using the following conditions: 50°C
148 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
149 72°C for 10 sec.

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

156

157 **2.4 Optimization of BHI**

158 *Antibiotic supplementation of BHI*

159 The effect of supplementing BHI media with moxalactam (32 µg/ml) and norfloxacin
160 (12 µg/ml), designated BHIMN, was assessed by comparison of *C. difficile* M120
161 growth in BHI without antibiotic addition. Moxalactam and norfloxacin have been
162 reported to provide a higher degree of selectivity than the other commonly used
163 antibiotic supplements, cycloserine and cefoxitin (26). BHIMN media (n=3) was
164 prepared by addition of moxalactam and norfloxacin selective supplement containing
165 cysteine hydrochloride (500.0 µg/ml), norfloxacin (12.0 µg/ml), and moxalactam (32.0
166 µg/ml) (Oxoid, Basingstoke, U.K) to BHI (n=3). All media was pre-reduced for 5 hours
167 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.

192

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

201

202 **2.5 Comparison of commonly used media**

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

208

209 **2.6 Evaluation of spore recovery in BHIMN-T**

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

216 to achieve target spore number (**10^{-4}:** 130 spores ± 3; **10^{-5}:** 13 spores ± 0.3; **10^{-6}:** 1.3
217 ± 0.03) and incubated anaerobically for 24 hours. Positive growth was determined by
218 visible growth in the microtitre plate and the results were evaluated using the Most
219 Probable Number (MPN) method (38), to estimate the MPN/ml and assess ability of
220 BHIMN-T to recover spores across a range of inoculum concentrations.

221

222 **2.7 Statistical Analysis**

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

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

230

231 **3. <u>Results and Discussion</u>**

232 **3.1 Evaluation of enrichment media**

233 Growth of *C. difficile* M120 was assessed in five different broth enrichment culture
234 media. The results show highest growth rate in non-selective BHI broth (0.202 ± 0.045
235 $hour^{-1}$) compared to CCFB, CDMN, CDBB-TC and CM (Figure 1). CCFB and CDMN
236 are similar selective media, both consisting of a fructose base (17, 25). CCFB is
237 however augmented with the antibiotics cycloserine (250 µg/ml) and cefoxitin (8
238 µg/ml), and defibrinated horse blood (7% [v/v]); CDMN is supplemented with the

239 antibiotics moxalactam (32 µg/ml) and norfloxacin (12 µg/ml). Both CCFB and CDMN
240 are equally effective for growth of *C. difficile* M120. No significant difference in *C.*
241 *difficile* M120 growth rate was observed between these media, with growth rates of
242 0.10 ± 0.030 $hour^{-1}$ and 0.08 ± 0.021 $hour^{-1}$, respectively.

243 The growth rates observed in CM and CDBB-TC were significantly less than that of
244 the other broths (p<0.001). CDBB-TC is a recently described culture medium for the
245 growth of *C. difficile* without the need for strict anaerobic incubation (30). No growth
246 was observed in CDBB-TC, with or without 0.1% [w/v] sodium taurocholate addition,
247 under either anaerobic or aerobic conditions. Cadnum *et al.* have however reported
248 the successful growth of *C. difficile* from a range of environmental samples using this
249 formulation (30). CM provided a growth rate of 0.017 ± 0.003 $hour^{-1}$, which was
250 significantly lower than CCFB (0.10 ± 0.030 $hour^{-}$), CDMN (0.08 ± 0.021 $hour^{-1}$) and
251 BHI (0.202 ± 0.045 $hour^{-1}$). The CM and CDBB-TC media combinations were not
252 considered further in this study. Studies using these media might be at risk from false
253 negatives (Figure 1) (30, 39).
254

255 **3.2 Optimization of BHI**

256 *Addition of antibiotics: moxalactam and norfloxacin*

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

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

264

265 *Addition of sodium taurocholate and defibrinated horse blood to BHIMN broth*

266 To determine if sodium taurocholate enhanced growth of *C. difficile* in BHIMN, growth
267 assays were carried out in the presence of varying concentrations of sodium
268 taurocholate (0 - 1% [w/v]: Figure 3). Previous studies have suggested that sodium
269 taurocholate should be supplemented to a concentration of 0.1% (w/v) in *C. difficile*
270 growth media (17, 25, 30, 41). In this study, a concentration of 0.4% was found to be
271 most effective for promoting the growth of *C. difficile* M120 in BHIMN (Figure 3): 0.4%
272 sodium taurocholate provided significantly faster growth than 0.1% ($p < 0.001$), 0.2%
273 ($p < 0.001$), 0.3% ($p < 0.05$), 0.5% ($p < 0.001$) and 1% ($p < 0.0001$), respectively (Figure
274 3).

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

285 system within the blood did not improve growth of *C. difficile* M120 in BHIMN (Figure
286 4).

287 This study thus found that BHI supplemented with moxalactam (32 µg/ml), norfloxacin
288 (12 µg/ml) and sodium taurocholate (0.4% [w/v]), designated as BHIMN-T, provided
289 optimal growth of *C. difficile* M120 from spores.

290

291 **3.3 Comparison of commonly used media and BHIMN-T**

292 Growth in BHIMN-T was compared with CCFB and CDMN. A number of variations of
293 CCFB and CDMN have been reported in the literature, including the addition of
294 defibrinated horse blood (HB) to CDMN (CCFB contains horse blood as prepared) and
295 sodium taurocholate (T) to both (17, 25, 26, 45). BHIMN-T was thus not only compared
296 to CCFB and CDMN but also to their defibrinated horse blood (HB) and sodium
297 taurocholate (T) supplemented variants: These are designated as CCFB-T, CDMN-T,
298 CDMN-HB and CDMN-THB respectively (Table 1). The most effective broth for growth
299 of *C. difficile* M120 was BHIMN-T, with a growth rate of 0.4645 $hour^{-1}$ and a lag phase
300 of less than 14 hours (Table 2). Notably, including this optimal concentration of sodium
301 taurocholate more than doubled the observed growth rate. Growth in BHIMN-T out-
302 performed both CCFB and CDMN, with and without their recommended horse blood
303 and sodium taurocholate supplementation (Figure 5); CCFB-T ($p< 0.05$), CDMN-HB
304 ($p< 0.01$), CDMNT ($p< 0.01$), CDMN-THB ($p< 0.001$), CCFB ($p< 0.001$) and CDMN
305 ($p< 0.001$) (Figure 5). All broths containing sodium taurocholate provided shorter lag
306 times (Table 2), most likely due to promotion of spore germination (27, 28, 41).
307 Dharmasena and Jiang (2018) found BHI broth supplemented with 0.5% yeast extract,
308 0.1% L-cysteine, 0.1% sodium taurocholate, moxalactam (32 mg/l) and norfloxacin (12

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

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

321

322 **3.4 Evaluation of spore recovery using BHIMN-T**

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

332 – 13.1 times: Table 3) than BHIMN alone (estimated using the Most Probable Number
333 (MPN) method (38)] .

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

339

340 **4. Conclusion**

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

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

352 In this paper we describe the development of an improved media, designated BHIMN-
353 T, for the growth of *C. difficile* which combines ease of preparation and longer shelf
354 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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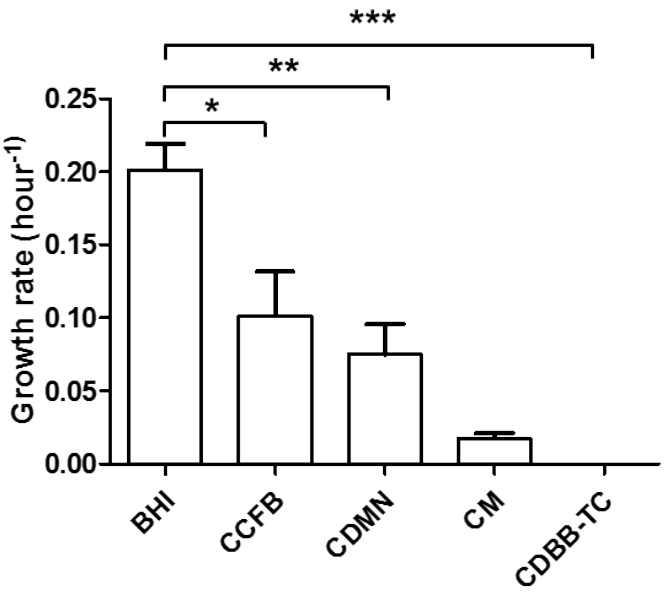

Figure 1: Growth rate of *C. difficile* M120 in each BHI, CCFB, CDMN, CM and CDBB-TC incubated anaerobically at 37°C for 33 h; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

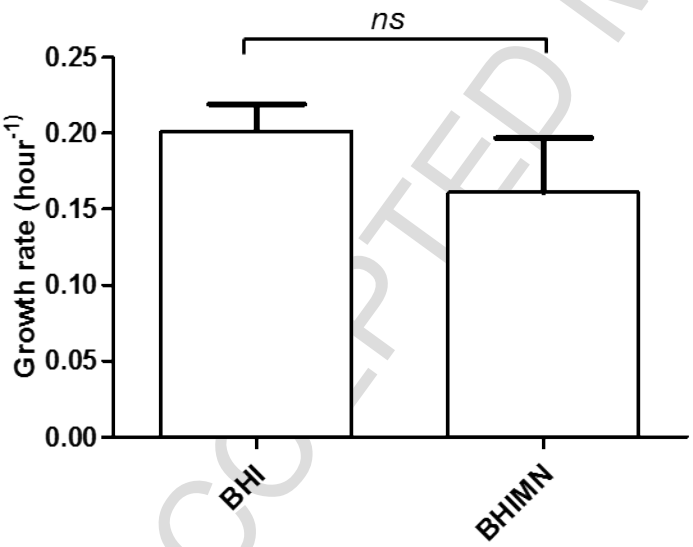

Figure 2: Growth of *C. difficile* M120 in BHI and BHIMN incubated anaerobically at 37°C for 33 h.

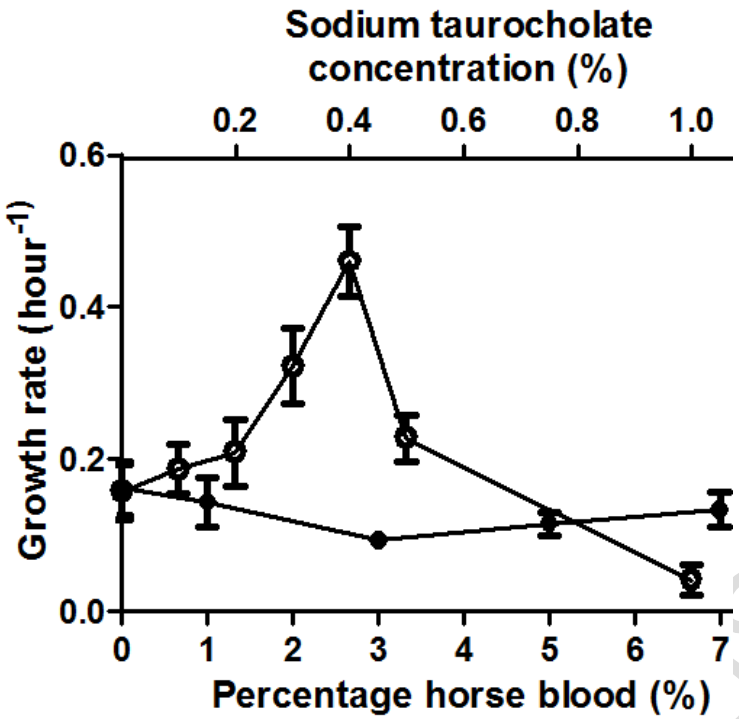

Figure 3: Growth of *C. difficile* M120 with varying concentrations of sodium taurocholate, 0-1% (**o**), or defibrinated horse blood in BHIMN, 0-7% (●).

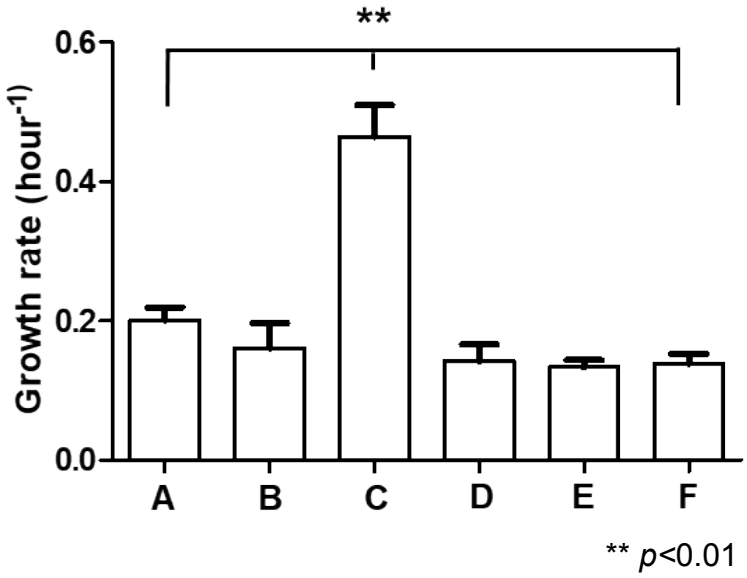

Figure 4: Growth rates of *C. difficile* M120 in **A) BHI** – without supplementation, **B) BHIMN** – BHI supplemented with antibiotics, moxalactam (32 µg/ml) and norfloxacin (12 µg/ml), **C) BHIMN-T** – BHI supplemented with moxalactam (32 µg/ml), norfloxacin (12 µg/ml) and 0.4% (w/v) sodium taurocholate, **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.

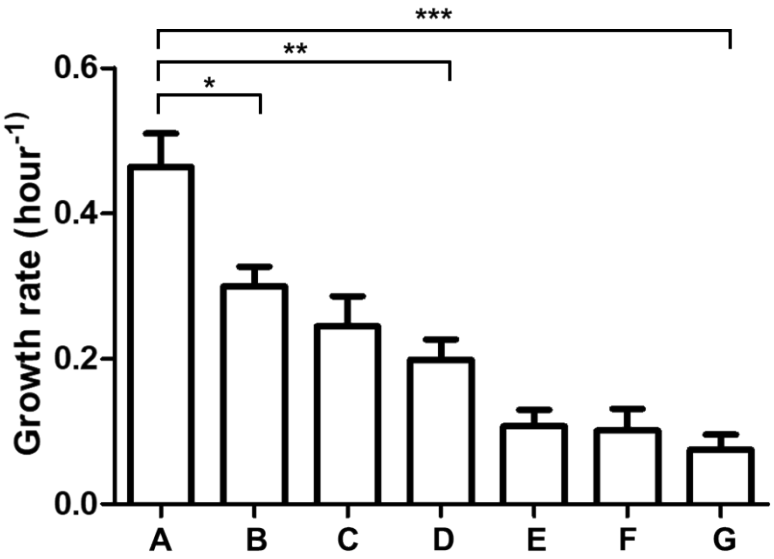

Figure 5: Specific growth rate for *C. difficile* M120 in **A)** BHIMN-T, **B)** CCFB-T- as prepared by Arroyo *et al*. (25) **C)** CDMN-HB as prepared by Aspinall and Hutchinson(26), **D)** CDMNT - as prepared by Xu *et al.*(17), **E)** CDMN-THB (17, 26), **F)** CCFB - as prepared by Aspinall and Hutchinson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

1 **Highlights**

2 i. Comparison of enrichment media using qPCR to monitor growth of *C. difficile.*
3 ii. 0.4% sodium taurocholate provided optimum growth in Brain Heart Infusion
4 (BHI) supplemented with moxalactam and norfloxacin (BHIMN-T) when
5 compared to other media with recommended supplementation.
6 iii. BHIMN-T provided effective for the recovery of spores at low concentrations.
7