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Improved recovery of *Clostridium difficile* spores with the incorporation of synthetic taurocholate in cycloserine-cefoxitin-fructose agar (CCFA)

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Summary

Aim: Culture remains important for the detection and typing of *Clostridium difficile*. Culture of *C. difficile* spores can be enhanced on media supplemented with a germinant. Despite this, unsupplemented media continues to be used in some laboratories. The aim of this study was to quantify the effect of the known germinant sodium taurocholate on recovery of *C. difficile* spores and to determine if the supplement impacts on the recovery of vegetative *C. difficile*.

Methods: The recovery on cycloserine-cefoxitin-fructose agar (CCFA) with and without taurocholate, of spore, vegetative, and total cell fractions of broth cultures of eight *C. difficile* isolates was compared.

Results: Taurocholate in CCFA did not inhibit growth of vegetative *C. difficile* and significantly increased recovery of spores ($p = 0.04$).

Conclusions: The routine incorporation of taurocholate in CCFA is recommended for improved sensitivity in *C. difficile* culture from specimens.

Keywords: Bacterial, clostridium difficile, culture media, spores, taurocholate.

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INTRODUCTION

Cycloserine-cefoxitin-fructose agar (CCFA) for the isolation of *Clostridium difficile* was first described in 1979[1] and became one of the mainstays for the laboratory diagnosis of *C. difficile* infection (CDI).[2] Following the introduction of enzyme immunoassays (EIAs) the need for culture declined despite the occasional call for its re-introduction.[3] With the advent of more severe disease caused by *C. difficile*[4] and the recent realisation that EIAs were not particularly sensitive,[5–7] laboratories are returning to culture for either diagnosis of CDI, epidemiological investigations or evaluation of new tests.[6] Laboratories that have taken this approach have also moved to either heat or alcohol enrichment of *C. difficile* spores in faecal samples prior to plating on CCFA.[8,9] However, it is apparent from the literature that some laboratories do not realise that maximum recovery of *C. difficile* spores on CCFA requires the addition of a spore germination enhancer.[10–12]

Taurocholate is known to enhance recovery of *C. difficile* spores, while not reducing (or improving) growth of vegetative cells.[13,14] However, some crude preparations of taurocholate inhibit the growth of vegetative *C. difficile* and outgrowth of germinated spores is also affected. Reports show lower recovery of *C. difficile* spores when poorer grades of taurocholate are used.[15,16] Some deoxycholate salts that are inhibitory to the growth of vegetative *C. difficile*[17] may be present in these crude extracts. Pure preparations of taurocholate, preferably synthetic preparations, are recommended for optimal recovery of *C. difficile* spores with no inhibition of vegetative cells.[18] With one exception,[17] the above studies were conducted in the 1980s. In this study we re-examine these issues in order to provide contemporary data on the impact of spore germinants on *C. difficile* culture.

MATERIALS AND METHODS

Media

CCFA was prepared by the media section of PathWest Laboratory Medicine (WA), Nedlands, Australia, as described in the original publication,[1] except with reduced agar content (15 g/L), additional parahydroxyphenylacetic acid at 1 g/L, increased 50% egg yolk content (60 mL/L) and halved concentrations of antimicrobials (250 μg/mL cycloserine and 8 μg/mL cefoxitin). CCFA containing taurocholate (TCCFA) also included 0.1% w/v taurocholate added directly to the medium prior to autoclaving. Taurocholate was purchased as synthetic taurocholic acid sodium salt hydrate (Cat. #T4009; Sigma, USA).

Bacterial culture

Eight *C. difficile* isolates were examined (Table 1). To test the media for recovery of vegetative *C. difficile*, 18 h cultures in 10 mL brain heart infusion broth (BHIB) [prereduced ≥4 h in a Whitley A35 Anaerobic Workstation (80% N_2, 10% CO_2, 10% H_2, 75% relative humidity)] were prepared from 1 μL loops of 48 h blood agar (BA) cultures. BHIB was incubated statically under anaerobic conditions. Dilutions prepared in pre-reduced trypticase soy broth were plated in duplicate on pre-reduced (≥2 h) CCFA and TCCFA to quantitate total culturable cells.

To determine the proportion of cells that were spores, 1 mL of each BHIB culture was heated at 56°C for 10 min to kill vegetative cells, then diluted and plated in duplicate on prereduced CCFA and TCCFA. The remaining proportion of total culturable cells was considered vegetative *C. difficile*.

Dilutions and inoculation of agar plates were performed outside of the anaerobic chamber but were completed and the plates returned to the chamber within 15 min to minimise the effects of oxygen exposure. Only one broth culture was removed from the anaerobic chamber at any time, and the heat treatment of aliquots was performed after inoculation of agar media for quantitation of total culturable cells. Colony counts were performed after 48 h incubation.

The experiment was performed on two separate occasions. To control for any effect of oxygen exposure during the inoculation of agar plates, CCFA plates were inoculated first on the initial occasion, and TCCFA plates were inoculated first when the experiment was repeated. There was no statistically significant difference between experiments for each medium, irrespective of the culture fraction, so the matched data from the two experiments were averaged.

Statistical methods

Data were analysed using GraphPad Instat 3. Matched analyses were performed as paired *t*-tests for parametric data and the Wilcoxon matched pairs test for

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Table 1 Characteristics of the isolates used in this study

Strain	Ribotype	Toxin profile	Characteristics
ES 214	001	$A^+B^+CDT^-$	Common UK ribotype
R11446*	014	$A^+B^+CDT^-$	Common Australian ribotype
ATCC 43598	017	$A^-B^+CDT^-$	Outbreak-associated
SSCC 28297†	027	$A^+B^+CDT^+$	'Hypervirulent', outbreak-associated
R10725*	078	$A^+B^+CDT^+$	Common international animal ribotype, becoming common in humans
AI 35		$A^-B^+CDT^+$	Australian novel pig ribotype
ATCC 43255		$A^+B^+CDT^-$	Reference strain
Strain 630 (ATCC BAA-1382)	012	$A^+B^+CDT^-$	Reference strain

* From Anaerobe Reference Laboratory, Cardiff, Wales.
† From Dr Luis Arroyo, Guelph, Canada.

non-parametric data. One-tailed *p*-values were determined unless otherwise stated. *p* values that were ≤ 0.05 were considered statistically significant.

RESULTS

The concentrations of vegetative *C. difficile* recovered on TCCFA and CCFA are shown in Table 2. Taking into account the eight test strains, there was no significant reduction in the average concentration of vegetative *C. difficile* in an 18 h culture when determined on TCCFA (TCCFA and CCFA, 6.2×10^7 CFU/mL; paired *t*-test, $p = 0.41$). Furthermore, while concentrations were lower on TCCFA for half of the test strains, these were not significantly lower than on CCFA (Wilcoxon matched-pairs test, $p = 0.06$). This suggests that 0.1% taurocholate is not inhibitory to vegetative *C. difficile*.

The concentration of spores determined was consistently higher on TCCFA than CCFA (Table 3). The increase in average spore concentrations between the media was statistically significant (8.2×10^5 CFU/mL and 1.43×10^5 CFU/mL, respectively; paired *t*-test, $p = 0.04$). This did not influence the concentration of total culturable *C. difficile* which did not significantly differ between the media (6.3×10^7 CFU/mL and 6.1×10^7 CFU/mL, respectively; paired *t*-test, two-tailed $p = 0.70$).

Interestingly, despite ensuring minimum oxygen exposure during the inoculation of media, the medium inoculated last in each experiment often performed significantly better for the recovery of vegetative and therefore total *C. difficile* [paired t-test of TCCFA versus CCFA; Experiment 1 (TCCFA inoculated last), $p = 0.01$ for both; Experiment 2 (CCFA inoculated last), $p = 0.02$ and $p = 0.03$, respectively]. This effect was not seen during spore recovery.

DISCUSSION

When quantitative recovery of *C. difficile* is important or low numbers of *C. difficile* are expected from a clinical specimen or environmental sample, a sensitive culture medium is required. The bile salt taurocholate is a known germination factor for *C. difficile* spores.[14,17] As such, it is commonly incorporated into culture media, improving recovery of *C. difficile*.[13,14,19,20] Although Buggy *et al.*[13] found slightly lower vegetative *C. difficile* counts on media containing synthetic taurocholate compared to media without taurocholate, this difference was not significant. Bliss *et al.*[20] reported poorer recovery of *C. difficile* on TCCFA in one of eight patient weekly surveillance time-points and *C. difficile* was isolated later on this medium in one of 36 patients, while Wilson *et al.*[14] reported no recovery of *C. difficile* on TCCFA in one of 60 culture positive (CCFA or TCCFA) human faecal specimens. Later, Buggy *et al.*[19] could not culture *C. difficile* on TCCFA in three of 67 culture-positive (CCFA or TCCFA) environmental samples. In general, however, vegetative *C. difficile* grew equally well on either medium in these studies and *C. difficile* detection was better on TCCFA because of enhanced recovery of *C. difficile* spores. While in the present study the most efficient medium for vegetative *C. difficile* recovery differed between strains, there was no significant difference in the average concentration on TCCFA compared to CCFA in matched analyses. The average count on TCCFA was higher than on CCFA. Overall, synthetic taurocholate did not significantly reduce recovery of vegetative *C. difficile*.

Clostridium difficile spores and vegetative cells were shed by infected patients in their faeces at concentrations of approximately 10^4–10^7 CFU per gram.[21,22] While vegetative

Table 2 Recovery of vegetative *C. difficile* (CFUs $\times 10^6$/mL) from an 18 h culture, using TCCFA and CCFA

	Experiment 1		Experiment 2		Average (SD)	
Strain	TCCFA	CCFA	TCCFA	CCFA	TCCFA	CCFA
ES 214	**70.0**	61.3	48.8	**89.3**	59.4 (15.0)	**75.3** (19.8)
R11446	**60.2**	47.5	19.7	**20.0**	**39.9** (28.7)	33.7 (19.4)
ATCC 43598	**29.4**	12.2	12.8	**15.0**	**21.1** (11.7)	13.6 (2.0)
SSCC 28297	**22.0**	17.4	80.9	**119.3**	51.4 (41.7)	**68.3** (72.0)
R10725	**80.8**	64.0	**110.6**	109.9	**95.7** (21.1)	86.9 (32.5)
AI 35	**98.1**	45.9	77.9	**87.8**	**88.0** (14.3)	66.9 (29.7)
ATCC 43255	**151.7**	130.8	125.1	**149.1**	138.4 (18.8)	**140.0** (12.9)
630	**4.4**	1.8	6.4	**9.4**	5.4 (1.4)	**5.6** (5.4)

Bold indicates the medium with the highest colony count.

Table 3 Recovery of *C. difficile* spores (CFUs $\times 10^3$/mL) from an 18 h culture, using TCCFA and CCFA

	Experiment 1		Experiment 2		Average (SD)	
Strain	TCCFA	CCFA	TCCFA	CCFA	TCCFA	CCFA
ES 214	**1980.0**	225.0	**725.0**	160.0	**1352.5** (887.4)	192.5 (46.0)
R11446	**260.5**	26.5	**291.0**	29.0	**275.8** (21.6)	27.8 (1.77)
ATCC 43598	**140.0**	41.5	**160.0**	39.5	**150.0** (14.1)	40.5 (1.41)
SSCC 28297	**535.0**	100.0	**605.0**	220.0	**570.0** (49.5)	160.0 (84.9)
R10725	**4240.0**	18.0	**1425.0**	100.0	**2832.5** (1990.5)	59.0 (58.0)
AI 35	**915.0**	145.0	**625.0**	155.0	**770.0** (205.1)	150.0 (7.07)
ATCC 43255	**780.0**	655.0	**395.0**	375.0	**587.5** (272.2)	515.0 (198.0)
630	**2.6**	0.5	**2.6**	0.3	**2.6** (0.1)	0.4 (0.1)

Bold indicates the medium with the highest colony count.

C. difficile survived for up to 3 h in room air, dependent on moist conditions,[22] it is *C. difficile* spores that allow the organism to remain viable for months in an aerobic environment, resisting desiccation and disinfection.[23] Therefore, one is more likely to encounter viable *C. difficile* spores rather than vegetative cells in environmental samples, and in much lower numbers,[24] supporting the use of TCCFA.

The present study recovered *C. difficile* from 18 h cultures to preferentially test the effect of taurocholate on vegetative *C. difficile*. While TCCFA did not significantly improve recovery of total *C. difficile* from an 18 h culture rich in vegetative *C. difficile*, the enhanced recovery of *C. difficile* spores from the culture infers that total *C. difficile* recovery from environmental samples would be better on this medium. How relevant is this to the examination of clinical specimens? Vegetative cells were more common in faecal samples of patients prior to treatment for *C. difficile* infection, while spores were more common after treatment had commenced.[22] Other than this study, little is known about the *C. difficile* vegetative cell versus spore ratio in human faecal specimens. Nevertheless, with the presence of competing background organisms in clinical specimens potentially limiting *C. difficile* detection by culture, the popular use of heat or ethanol shock to kill background vegetative cells (including *C. difficile*), and the findings of our study and previous studies that there is no reduction in vegetative cell recovery on TCCFA compared to CCFA, the potential for better *C. difficile* enumeration through the improved recovery of spores supports the universal use of TCCFA over CCFA despite the (approximately 50%) increase in cost of the medium as a result of this change.

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