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Comparison of culture based methods for the isolation of *Clostridium difficile* from stool samples in a research setting

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ABSTRACT

Effective isolation of *Clostridium difficile* from stool samples is important in the research setting, especially where low numbers of spores/vegetative cells may be present within a sample. In this study, three protocols for stool culture were investigated to find a sensitive, cost effective and timely method of *C. difficile* isolation. For the initial enrichment step, the effectiveness of two different rich media, cycloserine-cefoxitin fructose broth (CCFB) and cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (CCMB-TAL) were compared. For the comparison of four different, selective solid media; Cycloserine-cefoxitin fructose agar (CCFA), Cycloserine-cefoxitin egg yolk agar (CCEY), ChromID *C. difficile* and tryptone soy agar (TSA) with 5% sheep's blood with and without preceding broth enrichment were used. As a means to enable differentiation between *C. difficile* and other fecal flora, the effectiveness of the inclusion of a pH indictor (1% Neutral Red), was also evaluated. The data derived indicated that CCFB is more sensitive than CCMB-TAL, however, the latter had an improved recovery rate. A broth enrichment step had a reduced sensitivity over direct plating. ChromID *C. difficile* showed the best recovery rate whereas CCEY egg yolk agar was the most sensitive of the four. The addition of 1% Neutral Red did not show sufficient colour change when added to CCEY egg yolk agar to be used as a differential medium. For a low cost, timely and sensitive method of isolating *C. difficile* from stool samples we recommend direct plating onto CCEY egg yolk agar after heat shock.

1. Introduction

Clostridium difficile is a major nosocomial pathogen that can cause fatal diarrhoeal disease, placing a huge financial burden on health care systems worldwide [1]. To study and diagnose infection, suitable methods for isolation of this bacterium from stool samples are required. Sensitivity and efficient recovery is crucial to isolate *C. difficile* from clinical specimens where low numbers of spores/vegetative cells may be present, especially after antibiotic treatment. There have been recent studies into the isolation of *C. difficile* from clinical stools which have focused on a small range of different media [2–5] or culture from environmental surfaces [6]. Many of these studies have focused on expensive pre-made agars which may not be affordable in the research setting [3–5]. Also their main focus is on the recovery of *C. difficile* from clinical samples and not the semi-quantification of a known concentration of viable spores [2–5].

The purpose of this study was to compare pre-made agar against media prepared in-house to identify a method of isolating *C. difficile* from stool samples. The method would ideally be cost effective and reasonably rapid but also be sensitive enough to recover low numbers of spores/vegetative cells. To achieve this, three aspects of the process of culturing *C. difficile* were examined. Firstly, two commonly used broths were compared in an enrichment step to ascertain which of these provided the best recovery of *C. difficile* spores. These were cycloserine-cefoxitin fructose broth (CCFB), a medium commonly used in environmental sampling [7] and cycloserine-cefoxitin mannitol broth supplemented with taurocholate and lysozyme, (CCMB-TAL) [8]. The latter has recently been reported to increase sensitivity and allows the recovery of as little as 10 CFU/mL *C. difficile* [9]. Each broth was supplemented with 1% Neutral Red (MP Biomedicals) as a pH indicator. Secondly, the ability of four well known solid media, with and without a pre-broth enrichment step, to semi-quantify a known concentration of spores were compared. These media were Cycloserine-cefoxitin

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fructose agar (CCFA) [7], Cycloserine-cefoxitin egg yolk agar (CCEY) [10], ChromID *C. difficile* (bioMérieux) and tryptone soy agar (TSA) with 5% sheep's blood (VWR). Thirdly, the best performing agars were supplemented with an indicator to assess the ease with which *C. difficile* could be identified from mixed cultures on the solid media.

2. Materials and methods

2.1. Media

Liquid media prepared in-house: CCFB and CCMB-TAL, both supplemented with 1% (wt/vol) Neutral Red. Solid media prepared in-house: CCFA supplemented with Amphotericin B 250 μg/mL (Sigma) and 0.1% (wt/vol) taurocholate (Sigma); CCEY (LabM, United Kingdom) supplemented with 4% (wt/vol) egg yolk emulsion (Oxoid) sterilised by autoclaving at 121 °C, 15 psi for 15 min. All media prepared in-house were supplemented with cefoxitin (8 μg/mL), cycloserine (250 μg/mL). Sterile supplements were added after sterilisation of the respective media. Pre-poured solid media: ChromID *C. difficile* and TSA with 5% sheep's blood. Where indicated 1% Neutral Red was used as a pH indicator (pH range 6.8–8.0), as fermentation of a carbon source by *C. difficile* increases the pH, resulting in a colour change from red to yellow, within the medium. Media were reduced for at least 4 h for solid media and 8 h for liquid media before inoculation.

2.2. Broth comparison study

Four fecal samples from golden Syrian hamsters infected with *C. difficile* were added to 1 mL PBS, homogenised and heat shocked (10 min at 80 °C). Fecal samples contained two strains of *C. difficile*; either CD630 [11] or R20291 [12] (one strain per sample). Samples were centrifuged (4000× *g* for 1 min) and 100 μL supernatant was added to 5 mL CCFB and CCMB-TAL and incubated in an anaerobic workstation (Don Whitley, Yorkshire, UK) at 37 °C with the atmospheric conditions CO_2:H_2:N_2 (80:10:10 vol:vol:vol) at 37 °C for 24–96 h. A positive broth was indicated by both turbidity and a pH change altering the colour of the broth from red to yellow. Once positive, 50 μL of broth was subcultured onto CCFA using a four quadrant streak method. Plates were further incubated for 24–48 h. Recovery of *C. difficile* was enumerated semi-quantitatively (0 = no growth. 1 = growth in the 1st quadrant, 2 = growth in the 2nd quadrant, 3 = growth in the 3rd quadrant, 4 = growth in the 4th quadrant). Each sample was examined in triplicate.

All procedures involving research animals were performed in compliance with the United Kingdom Home Office and local ethics review board requirements to reduce animal suffering.

2.3. Solid media comparison study

Spore stocks of *C. difficile* 630Δ*erm* [13] were produced by preparing an overnight culture in BHIS broth (Brain Heart Infusion [Oxoid] supplemented with 5 mg/mL yeast extract [Oxoid], 0.1% [wt/vol] cysteine [Sigma]). 100 μL volumes were then plated onto BHIS agar and incubated for 5 days anaerobically at 37 °C. Cells from three plates were harvested into 1 mL dH_2O and incubated overnight at 4 °C. The spore/cell suspension was washed 10× in ice cold water, centrifuging at 12,000× *g* for 2 min in between. Spores were then enumerated using phase contrast microscopy. Non-infected mouse fecal samples were homogenised in PBS (0.5 g in 5 mL). Serial dilutions of spore stocks were prepared in the fecal/PBS mixture to give final concentrations of 10^1–10^4 spores/mL. Samples were heat shocked and centrifuged as before. For each dilution, 50 μL of heat shocked supernatant was directly plated, using a four quadrant streak, onto CCFA, CCEY, ChromID *C. difficile* and TSA with 5% sheep's blood. An aliquot of 100 μL of heat shocked supernatant was added to CCFB, the enrichment broth chosen from the broth comparison study. After a positive indication from the broth culture, 50 μL was plated onto CCFA, CCEY, ChromID *C. difficile* and TSA with 5% sheep's blood using a four quadrant streak. Broths were incubated anaerobically for up to 120 h and solid media up to 72 h. Negative broth cultures were plated onto CCFA to confirm a true negative result. Recovery of *C. difficile* was enumerated semi-quantitatively, using the four quadrant streak method described in the broth comparison study. Each sample was examined in triplicate.

2.4. Presumptive identification of C. difficile

After obtaining, four clinical samples were stored at −80 °C before use. Samples were mixed in a 1:1 ratio with PBS, homogenised and heat shocked (10 min at 80 °C). Samples were centrifuged (4000× *g* for 1 min) and 50 μL supernatant was plated, using a four quadrant streak, onto CCEY, CCEY supplemented with 1% Neutral Red and ChromID *C. difficile*. Plates were compared after 24 and 48 h.

3. Results and discussion

3.1. Broth comparison study

The first aspect of this study was to determine from two previously described broths (CCFB and CCMB-TAL) which was the most effective in an enrichment step for the isolation of *C. difficile* from stool samples. Four different fecal samples from hamsters infected with *C. difficile* were used. These samples contained two different strains of *C. difficile* (CD630; Samples 1069 and 1076, R20291; Samples 1547 and 1557). Growth was detected in all CCFB samples. In four of the CCMB-TAL samples, containing feces with *C. difficile* CD630, no growth was detected after 120 h, suggesting that fructose is a preferable carbon source for *C. difficile*. When growth was detected in CCMB-TAL it was later than 24 h after it was detected in CCFB in all cases except one (Table 1). However, semi-quantification of positive broths showed that CCMB-TAL gave an improved recovery rate over CCFB (Table 1). This observation seems to confirm previous studies finding that the presence of lysozyme increases the recovery rate of *C. difficile* [14]. Subsequently, lysozyme was added to CCFB to see if its addition improved recovery in this medium. However, lysozyme addition did not affect recovery in this medium (data not shown). Due to the lack of recovery in four of the CCMB-TAL broth cultures, CCFB was chosen for the enrichment step in the solid media comparison study.

Table 1
Recovery of *C. difficile* from two different broth compositions.

	CCFB		CCMB-TAL	
Sample	Time (h)	Recovery[a]	Time (h)	Recovery[a]
1069	36	1	48[b]	3
1076	36	1	48[b]	3
1547	24	1/3[c]	36	1
1557	24	1	24/36[d]	1/3[e]

[a] Recovery semi-quantified as 0 = no growth. 1 = 1st quadrant, 2 = 2nd quadrant, 3 = 3rd quadrant, 4 = 4th quadrant.
[b] Growth was only detected in one of the three broths tested.
[c] Recovery was semi-quantified as one in two broths and three in one broth.
[d] Positive broth recorded as 1 at 24 h and two at 36 h.
[e] Recovery was semi-quantified as three in two broths and one in one broth.

Table 2
Recovery of *C. difficile* from spiked non-infected mouse faeces using a direct plating method.

	CCFA		CCEY		ChromID *C. difficile*		TSA (5% sheep blood)	
No. of sample	Time (h)	Recovery[a]	Time (h)	Recovery[a]	Time (h)	Recovery[a]	Time (h)	Recovery[a]
10^4	72	0/1[b]	48	1	48	1/2[d]	72	0
10^3	72	0	72	0/1[c]	72	0	72	0
10^2	72	0	72	0/1[b]	72	0	72	0
10^1	72	0	72	0	72	0	72	0

[a] Recovery semi-quantified as 0 = no growth. 1 = 1st quadrant, 2 = 2nd quadrant, 3 = 3rd quadrant, 4 = 4th quadrant.
[b] Recovery was semi-quantified as 0 on two plates and 1 on one plate.
[c] Recovery was semi-quantified as 1 on two plates and 0 on one plate.
[d] Recovery was semi-quantified as 1 on two plates and 2 on one plate.

3.2. Solid media comparison study

When samples were subjected to a broth enrichment step in CCFB, broths containing 10^1–10^3 spore/fecal mixtures did not produce the expected colour change or turbidity indicative of growth. These samples were plated, as described in materials and methods, after 120 h to ensure a false negative had not occurred; no growth was observed. Only those cultures that contained 10^4 spores/fecal mixtures gave a positive reaction. Growth was detected up to and including the 4th quadrant on CCEY and ChromID *C. difficile* after 24 h, on CCFA and TSA (5% sheep blood) after 48 h. This observation seems to be contradictory to a recently published study [9] in which the authors suggested that up to 10 CFU/mL *C. difficile* can be recovered after broth enrichment using CCMB-TAL. Hink *et al.*, however, did not take into account the number of spores within the spiked stool sample. When calculating the number of CFU/mL used for spiking, the medium utilised did not contain a germinant, therefore, only vegetative cells and not spores would be enumerated. However, spiked stools used in the experimental procedure were then cultured in medium containing taurocholate, allowing spores to germinate and altering the CFU/mL in each sample by an undetermined amount. Therefore, the 10 CFU/mL value may be overestimated.

When direct plating was employed, TSA with 5% sheep blood performed the poorest of the four solid media with no spores being recovered after 72 h incubation (Table 2). CCFA was able to recover 10^4 spores, after 72 h incubation, in one of the replicates with growth being detected in the first quadrant (Table 2). CCEY agar supplemented with egg yolk proved the most sensitive of the media trialled, with 10^2 spores being detected in one of the replicates in the first quadrant after 72 h incubation (Table 2). There were also colonies large enough for manipulation in the 1st quadrant for all three replicates after 48 h in the sample containing 10^4 spores. ChromID *C. difficile* was not as sensitive as CCEY agar being able to recover 10^4 spores. It did, however, provide the best recovery rate of the four agars in this study with growth being detected in the 2nd quadrant in one replicate.

For the purposes of recovering *C. difficile* from stool samples, sensitivity is more important than recovery rate, especially in samples from patients where antibiotic treatment for *C. difficile* associated disease may have commenced. Overall, we found that a broth enrichment step reduced sensitivity of isolation and of the solid media tested CCEY was the most sensitive while ChromID *C. difficile* had an improved recovery rate. From our findings, therefore, CCEY media without broth enrichment would be the recommended medium.

3.3. Identification of C. difficile from best performing media

Being able to easily identify *C. difficile* in stool samples where mixed fecal flora is present will save time by preventing unnecessary identification steps. ChromID *C. difficile* and CCEY agar, with and without 1% Neutral Red (to act as a pH indicator) were compared using four different clinical samples. CCEY, with and without 1% Neutral Red, gave the characteristic flat grey colonies (Fig. 1A) although plates containing 1% Neutral Red had a slight yellow tinge (Fig. 1B). Both types of CCEY did not cultivate other fecal flora in our experiments. The addition of the 1% neutral red did not give a sufficient colour change to be recognized as an indicator of *C. difficile*. On ChromID agar, *C. difficile* produced the expected black colonies (Fig. 1C) due to the chromogen incorporated into the media. No other fecal flora was detected in the samples. The black pigment produced by ChromID *C. difficile* makes the identification of *C. difficile* very easy, however, it has been reported that not all *C. difficile* strains produce this pigment. Perry et al. [4] found that 3% and 1% of samples did not produce the expected black pigment after 24 h and 48 h, respectively. This should be taken into account if using ChromID to identify *C. difficile* from fecal samples.

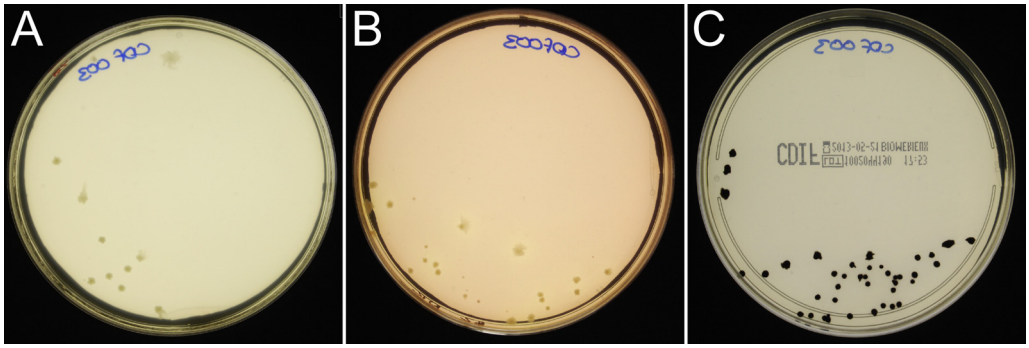

Fig. 1. An example of comparison plates when identifying *C. difficile* from the best performing media. Each plate was inoculated with an identical clinical sample. 1a: CCEY; 1b: CCEY with 1% Neutral Red; 1c: ChromID *C. difficile*.

4. Conclusion

The isolation of *C. difficile* from stool samples requires a high sensitivity and efficient recovery where low numbers of spores/vegetative cells may be present. It has been suggested that using a broth enrichment step can increase the sensitivity of isolation for *C. difficile* [9,15]. In contrast, here we find that broth enrichment was less sensitive. They were, however, able to improve recovery rates. It should also be noted that if a mixed population of *C. difficile* is present at diagnosis of infection, broth enrichment may allow one strain/ribotype to outcompete another and a true representation of the population may not be seen. We found direct plating onto CCEY to be the most sensitive method even though the recovery of vegetative cells was not as high as with ChromID *C. difficile*. The method suggested in this paper has been employed to culture *C. difficile* from 106 clinical stool samples (in our laboratory), as part of a different study, recovering a wide range of ribotypes, including 027, 078, 015, 002, 005 which are prevalent within the United Kingdom. Of these 106 samples there were only five (4.7%) from which *C. difficile* were not recoverable.

Thus, for reasons such as relative low cost and high sensitivity we suggest direct plating of fecal samples after heat shock onto CCEY supplemented with cefoxitin and cycloserine.

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