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C.difficile (including epidemiology)

Investigating the effect of supplementation on *Clostridium difficile* spore recovery in two solid agars

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ABSTRACT

Background: A variety of supplemented solid media are used within *Clostridium difficile* research to optimally recover spores. Our study sought to investigate different media and additives, providing a method of optimised *C. difficile* spore recovery. Additionally, due to the results observed in the initial experiments, the inhibitory effects of three amino acids (glycine, L-histidine & L-phenylalanine) on *C. difficile* spore outgrowth were investigated.

Methods: Spores of five *C. difficile* strains (PCR ribotypes 001,015,020,027,078) were recovered on two commonly used solid media (BHI & CCEY, or cycloserine-cefoxitin egg yolk) supplemented with various concentrations of germinants (taurocholate, glycine & lysozyme). Agar-incorporation minimum inhibitory concentration (MIC) testing was carried out for glycine and taurocholate on vegetative cells and spores of all five strains. Additionally a BHI broth microassay method was utilised to test the growth of *C. difficile* in the presence of increasing concentrations (0,1,2,3,4%) of three amino acids (glycine,L-histidine,L-phenylalanine).

Results: CCEY agar alone and BHI supplemented with taurocholate (0.1/1%) provided optimal recovery for *C. difficile* spores. Glycine was inhibitory to spore recovery at higher concentrations, although these varied between the two media used. In agar-incorporated MIC testing, glycine concentrations higher than 2% (20 g/L) were inhibitory to both *C. difficile* spore and vegetative cell growth versus the control (mean absorbance = 0.33 ± 0.02 vs 0.12 ± 0.01) ($P < 0.001$). This indicates a potential mechanism whereby glycine interferes with vegetative cell growth. Further microbroth testing provided evidence of inhibition by two amino acids other than glycine, L-histidine and L-phenylalanine.

Conclusions: We provide two media for optimal recovery of *C. difficile* spores (CCEY alone and BHI supplemented with 0.1/1% taurocholate). CCEY is preferred for isolation from faecal samples. For pure cultures, either CCEY or supplemented BHI agar are appropriate. The inhibitory nature of three amino acids (glycine,L-histidine,L-phenylalanine) to *C. difficile* vegetative cell proliferation is also highlighted.

1. Introduction

Clostridium difficile is a Gram-positive anaerobe responsible for *C. difficile* infection (CDI). CDI can vary in severity; mild diarrhoea is the most common presentation but some patients may develop pseudomembranous colitis [1] and subsequent toxic megacolon, a surgical emergency with a high mortality rate (~30-80%) [2]. Although cases of CDI have declined considerably in the UK, from 55,498 cases in 2007/2008 to 13,361 in 2013/2014 [3], it remains an infection of concern and the subject of considerable research efforts.

Different solid media are used for *C. difficile* recovery according to particular requirements. However the design of all *C. difficile* isolation media must optimise vegetative outgrowth from *C. difficile* spores while providing a selective environment to suppress other bacteria present in the sample. CCEY (cycloserine-cefoxitin egg yolk) agar is a selective medium previously shown to be the most sensitive and cost-efficient medium for isolating *C. difficile* from stool samples when compared to cycloserine-cefoxitin fructose agar (CCFA), ChromID *C. difficile* and tryptone soy agar (TSA) with 5% sheep's blood [4]. CCEYL (cycloserine-cefoxitin and 5%

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lysozyme) is used by the *C. difficile* Ribotyping Network (CDRN) for the isolation of *C. difficile* from faecal samples [5] on the basis of evidence suggesting the increased recovery of environmental spores treated with lysozyme [6]. CCEYL is suitable for faecal *C. difficile* isolation due to the antibacterial actions against the normal microflora of cycloserine-cefoxitine. Brain Heart Infusion (BHI) agar with incorporation of taurocholate (a primary bile acid) and glycine in combination has been widely used to recover spores from pure culture. BHI is not suitable for the isolation of *C. difficile* from faecal samples; it is non-selective media allowing the growth of multiple organisms [7]. The effect of bile acids on *C. difficile* spore germination has been recognised since 1983, and these interactions are still the focus of much research [8–12]. Subsequently, Sorg et al. have further investigated glycine and taurocholate as stimulatory cogerminants [11] and have recorded the inhibitory nature of secondary bile acids on *C. difficile* spore germination [13,14]. More recently, Buffie et al. (2015) have demonstrated the protective effect of *C. scindens* against *C. difficile* [15], due to the conversion of primary to secondary bile acids by 7α-hydroxylation. The inhibitory nature of secondary bile acids has subsequently been supported by in vitro work [16].

In summary, this study seeks to optimise the recovery of *C. difficile* spores on solid media.

2. Methods

2.1. Spore production

Spores of five PCR ribotypes (RT 001,015,020, 027 & 078) of *C. difficile* were prepared as previously described [17]. Spore stocks were enumerated by serial dilution in phosphate-buffered saline (PBS) in a 96-well plate and growth of 20 μl aliquots of each dilution on to CCEYL agar.

All experiments were carried out in triplicate unless otherwise stated. All spores were fresh (<30 days old) unless otherwise stated. In all experiments agar plates were incubated anaerobically at 37 °C and counts of colony forming units (CFU) were carried out at 48 h post-inoculation. Spore suspensions were vortexed vigorously and homogenised for 20 s prior to use.

2.2. Recovery of C. difficile spores on solid media

A range of *C. difficile* test recovery agars were prepared. BHI and CCEY were used as the agar bases and prepared according to the manufacturer's instructions with additions as detailed in Table 1. Taurocholate and glycine additions were made prior to autoclaving, but lysozyme was added subsequently.

Spore suspensions of the five *C. difficile* PCR ribotypes were serially diluted (10-fold) in PBS to 10^{-9} and 20 μl of each dilution were spread on to a range of solid agar plates (Table 1).

Table 1
Solid agar plates utilised in *C. difficile* spore recovery experiments. Media types and additives are shown.

Media	Additional Additives
BHI/CCEY	Nil
BHI/CCEY	5% lysozyme
BHI/CCEY	0.1% taurocholate
BHI/CCEY	1% taurocholate
BHI/CCEY	0.1% taurocholate, 0.4% glycine
BHI/CCEY	1% taurocholate, 0.8% glycine
BHI/CCEY	1% taurocholate, 4% glycine

2.3. Minimum inhibitory concentration (MIC) testing

Glycine and taurocholate were tested both independently and in combination (4:1 ratio glycine:taurocholate) using an agar-incorporation minimum-inhibitory concentration method, as previously described [18]. Briefly, test compounds were weighed out in doubling concentrations and added to individual aliquots of Wilkins-Chalgren anaerobe agar or CCEY agar. CCEY agar was supplemented with 2% lysed, defibrinated horse blood. Both spore and vegetative (1:10 dilution of 24 h Schaedler's broth culture) populations of the five *C. difficile* strains were inoculated (~10^4 cells) on to glycine/taurocholate incorporated agar. Inhibition of growth was assessed after anaerobic incubation at 37 °C for 48 h, where the lowest concentration at which visible *C. difficile* growth was inhibited was recorded as the MIC.

2.4. Broth microassay of C. difficile inhibition by L-amino acids

BHI broths with increasing concentrations (1, 2, 3, 4%) glycine, L-histidine or L-phenylalanine) were prepared and 180 μl aliquots distributed into a 96-well plate. Twenty microlitres of spore suspension (~5×10^5 CFU/ml concentration) were aliquoted in to each well at time zero. Five strains of different ribotypes were utilised (001, 015, 020, 027, 078). Plates were incubated anaerobically at 37 °C for 48 h. At 0, 24 and 48 h absorbance readings at 595 nm were determined (Tecan Infinite 200 Pro reader).-Reads were carried out at 25 °C under 1 atm of pressure. Negative controls were prepared for each concentration, and the absorbance for the blanks was subtracted from the absorbance of the inoculated wells to determine an accurate absorbance reading based on growth alone. All wells were prepared in triplicate.

2.5. Data analysis

Statistical analysis was carried out on IBM SPSS Statistics 22. Data normality was assessed using histograms and Kolmogorov-Smirnov tests. Levene's test was used for calculating homogeneity of variance. In both experiments, the variance between groups was significantly different ($P < 0.001$), hence Welch's ANOVA was utilised. Group means were compared using Welch's ANOVA with Games-Howell multiple comparisons. $\bar{x}$ represents the mean spore recovery of all five ribotypes used. All means are reported with standard error of the mean (SEM). P values < 0.05 were considered significant, <0.01 highly significant and $P < 0.001$ extremely highly significant.

3. Results

3.1. Recovery of C. difficile spores on solid media

Considerable variation in spore recovery was observed between the solid media used (Fig. 1). In the absence of any additives, spore recovery was on average 1log CFU/mL greater for CCEY *vs* BHI (range = 0.1–2.4$\log_{10}$ CFU/ml) and was significantly different for all but the 078 strain ($P > 0.05$). Greatest spore recovery was observed for CCEY ($\bar{x} = 8.2 \pm 0.03\log_{10}$ CFU/ml) and taurocholate supplemented BHI ($\bar{x} = 8.3 \pm 0.06\log_{10}$ CFU/ml). The addition of lysozyme to either media appeared to have no substantial effect on the recovery of spores (CCEY $\bar{x} = 8.2 \pm 0.03$ vs $8.3 \pm 0.04\log_{10}$ CFU/ml, BHI $\bar{x} = 7.3 \pm 0.18\log_{10}$ CFU/ml vs $7.1 \pm 0.20\log_{10}$ CFU/ml) ($P > 0.05$). Spore recovery increased on average by 1logCFU/mL when BHI was supplemented with 0.1 or 1% taurocholate (range = 0–2.7$\log_{10}$ CFU/mL), but no equivalent increase was