

## Modification of Cycloserine Cefoxitin Fructose Agar to Suppress Growth of Yeasts from Stool Specimens

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Cycloserine cefoxitin fructose agar is a widely used selective isolation medium for *Clostridium difficile* from stool specimens. Yeasts often colonize in the intestine of *C. difficile* disease patients and, if colonized heavily, pure culture of *C. difficile* can be delayed. The aim of this study was to modify cycloserine cefoxitin fructose agar to suppress the growth of yeasts. Antimicrobial activities of three commonly available antifungal agents were tested against recent clinical isolates of *Candida* species. Amphotericin B was most active in inhibiting all isolates by  $\leq 0.5$  mg/L concentration. Cycloserine cefoxitin fructose agar was modified by adding 2 mg/L of amphotericin B. Serial ten-fold dilution of stool specimens from 126 suspected *C. difficile*-associated diarrhea patients were cultured both on cycloserine cefoxitin fructose agar plates and modified agar plates. Yeasts grew from 60 specimens on cycloserine cefoxitin fructose agar, but none grew on the modified medium. Growth of *C. difficile* was detected from 37 and 39 of 126 specimens on cycloserine cefoxitin fructose agar and modified medium, respectively. The number of *C. difficile* colonies was similar on both media. In conclusion, 2 mg/L of amphotericin B supplementation to cycloserine cefoxitin fructose agar can facilitate the isolation of *C. difficile* from stool specimens which are densely colonized with yeasts.

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### Introduction

*Clostridium difficile* causes 10–20% of all cases of antibiotic-associated diarrhea and nearly all cases of antibiotic associated pseudomembranous colitis [1].

*C. difficile* is a significant nosocomial pathogen. One study estimated the cost of treating a single *C. difficile*-associated diarrhea (CDAD) infection to be in excess of £4000 [2].

Culture of *C. difficile* from stool specimens is the most useful tool for the epidemiological studies [3,4]. Also, as the toxin is not always detected in stools of some CDAD patients, culture and subsequent toxin test is useful for the diagnosis of additional patients [5]. In a Canadian survey, 32.1% of hospitals used *C. difficile* culture either as the only test or together

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with other tests [6]. Quantitative culture [7] may be useful to differentiate a normal carrier with small number of *C. difficile* in their stool.

Various media and their modifications are used for the isolation of *C. difficile* from stool specimens. Cycloserine cefoxitin fructose agar (CCFA) has been widely used because it efficiently suppressed most of the bacterial species present in stool specimens [8]. However, with the increase of antimicrobial-resistant bacteria, we have been experiencing frequent growth of intestinal bacterial flora on CCFA plates. The growth of intestinal bacteria did not hinder the isolation and identification of *C. difficile* because of their distinct morphology and smaller size. Most of the stool specimens for *C. difficile* cultures are from inpatients who are treated with broad-spectrum antimicrobial agents. Therefore, we have been increasingly experiencing stool specimens which contain a large number of yeasts that make it difficult to determine colony-forming units of *C. difficile*. Also subculture is often required to obtain pure cultures. For the selective isolation of certain fastidious bacteria, various antifungal agents are also incorporated to suppress yeasts. However, CCFA does not contain any antifungal agents.

In this study, we compared antifungal activities of amphotericin B, fluconazole and 5-fluorocytosine against recent clinical isolates of *Candida* spp. Based on the test, we selected amphotericin B as a candidate to supplement CCFA. We evaluated the effects of adding amphotericin B to CCFA for the isolation and enumeration of *C. difficile* and the suppression of yeast from stool culture.

## Materials and Methods

*Candida* spp. were isolated from various clinical specimens. Minimum inhibitory concentrations (MICs) of amphotericin B (Bristol-Meyers Squibb Co., Princeton, NJ, U.S.A.), fluconazole (Pfizer Inc., New York, NY, U.S.A.), and 5-fluorocytosine (Sigma Chemical, St. Louis, MO, U.S.A.) were determined by the broth microdilution test [9] using RPMI medium 1640 (Gibco Laboratories, Grand Island, NY, U.S.A.)

CCFA plates were prepared by adding 5% of egg yolk emulsion and Clostridium-selective supplement (Oxoid, Basingstoke, U.K.) to autoclave-sterilized Clostridium difficile agar base (Oxoid). Final concentrations of cycloserine and cefoxitin were 500 mg/L and 16 mg/L, respectively. Amphotericin B-supplemented CCFA (CCFA-AMB) were prepared by adding amphotericin B to CCFA to a final concentration of 2 mg/L.

Soft or fluid stool specimens were collected from suspected CDAD patients in a tertiary-care hospital.

Serial ten-fold dilutions of stool specimens were prepared according to the method of Summanen *et al.* [7] with modification; thioglycollate medium without dextrose or indicator (Difco, Detroit, MI, U.S.A.) was used instead of 0.05% yeast extract solution. Then, 0.1 mL amounts of  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  dilutions of specimens were spread onto both CCFA and CCFA-AMB plates. The plates were incubated in an anaerobic chamber (Forma Scientific Inc., Marietta, OH, U.S.A.) for 48 h and the number of colonies of suspected *C. difficile* and yeasts were counted. Suspected *C. difficile* isolates were identified by ATB rapid ID 32A (bioMerieux sa, Marcy-l'Etoile, France). Gram-stained smears of suspected yeast colonies were observed microscopically, but species were not identified.

## Results and Discussion

Laboratory diagnosis is required for the proper management of CDAD patients. For the laboratory diagnosis of CDAD, cytotoxin (toxin B) detection is still considered the gold standard, but as many as 15% to 38% of patients with confirmed CDAD were not detected by direct stool cytotoxin neutralization assay [10]. Stool cultures alone are not sufficient for the diagnosis of CDAD, as some strains are non-toxigenic and hence non-pathogenic. However, isolation of a toxigenic strain can detect additional patients and it is also necessary for epidemiological study.

In this study, the growth of all *C. parapsilosis* isolates was inhibited by 2 mg/L or less of fluconazole or 5-fluorocytosine, but some isolates of other species were inhibited only by a much higher concentration. Amphotericin B 0.5 mg/L or less inhibited all of the *Candida* strains tested (Table 1). Amphotericin B was selected for the modification of CCFA because it not only has strong antifungal activity, but it is also easily available in hospitals.

Among the 126 stool specimens cultured, 47.7% yielded yeasts on CCFA. The number of yeast colonies developed on CCFA ranged from  $10^3$  to  $\geq 10^6$  CFU/mL, and the count was  $\geq 10^6$  in 33.3% of specimens.

**Table 1.** Antifungal activities of amphotericin B, fluconazole and 5-fluorocytosine against clinical isolates of *Candida* spp.

<i>Candida</i> spp. (No. tested)	MIC ranges (mg/L) of:		
	Amphotericin B	Fluconazole	5-fluorocytosine
<i>C. albicans</i> (60)	0.06–0.25	0.12–>64	≤0.12–>64
<i>C. tropicalis</i> (14)	0.12–0.25	2–>64	≤0.12–0.5
<i>C. parapsilosis</i> (10)	0.06–0.12	0.5–2	≤0.12
<i>C. lusitanae</i> (4)	0.12–0.25	2–>64	≤0.12–0.25
<i>C. famata</i> (2)	0.12–0.5	1–>64	≤0.12
<i>C. krusei</i> (2)	0.25–0.5	≥64	1–8

**Table 2.** Comparison of yeast growth on CCFA and CCFA-AMB from stool specimens

Yeast growth (CFU/mL)	No. (%) of specimens with yeast growth on:	
	CCFA	CCFA-AMB <sup>a</sup>
<10 <sup>2b</sup>	66 (52.3)	126 (100)
10 <sup>3</sup>	1 (0.7)	0 (0)
10 <sup>4</sup>	7 (5.5)	0 (0)
10 <sup>5</sup>	10 (7.9)	0 (0)
≥10 <sup>6</sup>	42 (33.3)	0 (0)
Total	126 (100)	126 (100)

<sup>a</sup>Amphotericin B-supplemented CCFA.<sup>b</sup>No growth.

CCFA-AMB completely inhibited the growth of yeasts (Table 2).

A small number of *C. difficile* in a stool should be less significant than a large number organisms. To determine the number of *C. difficile* in stool specimens, the quantitative culture method was used [7]. The lowest detection limit of *C. difficile* in this study was 10<sup>2</sup> CFU/mL. *C. difficile* was isolated in 37 of 126 stool specimens inoculated on CCFA, while the number was 39 on CCFA-AMB (Table 3). Among the *C. difficile*-positive specimens, the number of *C. difficile* colonies was similar and mostly ≥10<sup>6</sup> CFU/mL on both media. It should be natural to see that the addition of an antifungal agent did not adversely affect the growth of *C. difficile* (Table 3). From two samples, growth of *C. difficile* was detected on CCFA-AMB, but not on CCFA: one sample was with 10<sup>6</sup> CFU/mL of yeasts and another sample was with heavy growth of bacteria. From another sample with 10<sup>5</sup> CFU/mL of yeasts, ten-fold more CFU/mL of *C. difficile* were detected on CCFA-AMB. These differences may be due to the presence of yeasts or bacteria.

Aerobic and anaerobic bacteria grew from some specimens on both CCFA and CCFA-AMB. The species were mostly *Enterococcus*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Serratia marcescens* and *Pseudomonas aeruginosa*, which are the species most often resistant to antimicrobial agents. Further improvement of selectivity of CCFA-AMB may facilitate the pure culture of *C. difficile*. However, we considered that this is not necessary because colonies of these organisms can be differentiated from that of *C. difficile* by their distinct colony size and morphology.

In conclusion, amphotericin B can be added to a final concentration of 2 mg/L to CCFA to inhibit the growth of yeasts, thereby to facilitate the isolation of a

**Table 3.** Comparison of growth of *C. difficile* on CCFA and CCFA-AMB from stool specimens

Growth (CFU/mL) on CCFA (No. of specimens)	No. of specimens with growth (CFU/mL) on CCFA-AMB <sup>a</sup>				
	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	≥10 <sup>6</sup>
<10 <sup>2</sup> <sup>b</sup> (2)	1 <sup>c</sup>	1			
10 <sup>2</sup> (1)	1				
10 <sup>3</sup> (2)		1	1 <sup>c</sup>		
10 <sup>4</sup> (2)			2		
10 <sup>5</sup> (3)				3	
≥10 <sup>6</sup> (29)					29
Total (39)	2	2	3	3	29

<sup>a</sup>Amphotericin B-supplemented CCFA.<sup>b</sup>No growth.<sup>c</sup>≥10<sup>5</sup> CFU/mL of yeast grew together.

pure culture and the determination of colony forming units of *C. difficile* from stool specimens.

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