C-159

Evaluation of CCFA, CCFA-HT agars and CCMB-TAL broth for recovery of *Clostridium difficile* from fecal samples

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Abstract (updated)

Background: Stool culture for *C. difficile* (CD) is increasingly used as the 'gold standard' in studies evaluating new methods for detection of toxigenic CD. We compared three different selective and differential media for their ability to recover CD from fecal samples.
Methods: Media used were cycloserine-cefoxitin fructose agar (CCFA) and new CCFA-based CCFA-HT (HT) plates and CCMB-TAL (TAL) broth (Anaerobe Systems). HT also contains horse blood, taurocholate but no neutral red. TAL broth also contains lysozyme, taurocholate and mannitol instead of fructose. Toxin-positive fecal samples from patients enrolled in a CD clinical trial were stored at –70º C. After thawing, one drop was added directly to TAL for an enrichment culture. A 0.25ml sample was ethanol-shocked for 10–20 minutes prior to inoculating 2–3 drops onto CCFA and HT media. All work was conducted inside an anaerobic chamber. After incubation at 37º C for 20–24h, 48 and 72h, relative colony size and plate growth (0–4+ scale) were recorded. Typical colonies were large and flat with irregular edges. TAL was examined at 24–72h for yellow color predicting the presence of CD. Yellow tubes were subcultured to CCFA. Seventeen TAL fecal cultures were tested for toxin using *C. difficile* TOX A/B II™ (TechLab).
Results: 77% (76/99) of specimens were culture-positive for CD (CdPOS) on any media. On HT, 99% (75/76) were CdPOS in 24h; 100% in 48h. On CCFA, 91% (69/76) were CdPOS in 24h, 96% (73/76) in 48h but 4% of CdPOS specimens were negative on CCFA. HT colony size was 1.7 times larger on average than CCFA at 24 and 48h. Growth was 1+ greater on CCFA-HT than on CCFA. While the difference in growth of breakthrough organisms was similar on the two media, the 1+ greater quantity of growth and larger colony size of CD made HT plates better for isolation of CD TAL cultures were CdPOS in 2/3 CCFA CD-negative cultures. Only 5 of 17 culture positive samples were toxin positive at 24h in TAL.
Conclusion: CCFA-HT was superior to CCFA for 24h recovery of CD. CCMB-TAL enhanced recovery of CD compared to CCFA and CCFA in some samples. Direct testing for toxin in TAL broth cultures of feces at 24h is not sensitive.

Introduction

Culture of feces for toxin-producing CD is an important epidemiological tool to determine the frequency of various genotypes including the serious outbreak REA type BI (= PFGE NAP1, = ribotype 027) strain. Isolation of CD from the mixed flora environment of feces is, however, problematic. Cycloserine-Cefoxitin Fructose Agar (CCFA) medium was developed (3) as both a selective and differential medium to resolve this dilemma. Cycloserine inhibits gram-negative bacteria, while cefoxitin inhibits both gram-positive and gram-negative organisms. Neutral red is added as a pH indicator that will turn yellow (pH 6.8-8.0) from the breakdown of peptones in the media. Ethanol-shock of the specimen to kill vegetative cells before plating increases the isolation of CD. Although this medium has become the standard for the isolation of CD, some strains of CD were observed to be inhibited by this medium due primarily to the cycloserine concentration (5).

This study compares CCFA with two novel media that while selective, also offer enrichment of CD. CCFA-HT, CCFA with Horse blood and Taurocholate (HT), offers enrichment with the added horse blood and stimulation of spore germination by taurocholate (8). It also decreases possible inhibition of CD by using half the amount of cycloserine as CCFA. CCMB-TAL, Cycloserine-Cefoxitin Mannitol Broth with Taurocholate And Lysozyme (TAL), is an amalgam of CCFA and HT in a broth enrichment form. Breakdown of peptones changes the indicator to yellow and taurocholate and lysozyme stimulate spore germination. The broth medium enhances the selective growth and recovery of CD as compared to the difficulty of the detection of rare colonies on a directly inoculated plate.

Detection of toxin in diarrheal stool filtrates using tissue-culture cytotoxicity assay is regarded as the 'gold standard' for diagnosis of *Clostridium difficile* associated-diarrhea (CDAD) and is thought to be the most sensitive test, however, the results may take up to 2 days. Less sensitive but more rapid tests are enzyme immunoassays (EIAs) (6), therefore a broth method for culture enrichment of CD that would allow the detection of toxin by EIA in previously toxin-negative specimens would be useful. We tested broth culture of CD in CCMB-TAL in previously CdPOS cultures by direct EIA testing.

Materials & Methods

MATERIALS

Media evaluated were Cycloserine-Cefoxitin Fructose Agar (CCFA), new CCFA-based CCFA-HT (HT) plates and CCMB-TAL (TAL) broth (Anaerobe Systems, Morgan Hill, CA). See Table 2. These media are selective, differential and/or enrichment media for the isolation and presumptive identification of CD. The media are prepared, dispensed and stored under oxygen-free conditions to prevent the formation of oxidized products prior to use. Storage is at 2–8º C. Most strains of CD are not inhibited by these media (1,2,5).

CCFA (Cycloserine-Cefoxitin Fructose Agar)
The basic nutritive base consists of animal peptones and fructose, supplemented with cefoxitin and cycloserine at concentrations that inhibit the growth of most normal fecal flora. Cycloserine inhibits gram-negative bacteria, while cefoxitin inhibits both gram-positive and gram-negative organisms. Neutral red is added as a pH indicator. Breakdown of peptones by CD increases the pH and turns the medium around the CD colonies from pink/orange to yellow. See Figures 1b and 2b. Colonies exhibit a characteristic yellow, ground-glass colony morphology on this media (4).

CCFA-HT (Cycloserine-Cefoxitin Fructose Agar with Horse blood and Taurocholate)
Horse blood is added to CCFA base as an enriched nutrient source. Taurocholic acid stimulates spore germination (8). Colonies exhibit characteristic colony morphology, fluorescence, and odor (4). See Figures 1a and 2a.

CCMB-TAL (Cycloserine-Cefoxitin Mannitol Broth with Taurocholate And Lysozyme)
The basic nutritive base consists of animal peptones and mannitol instead of fructose, supplemented with cefoxitin and cycloserine at concentrations that inhibit the growth of most normal fecal flora. Taurocholate and lysozyme are added to stimulate spore germination (7,8). Neutral red is added as a pH indicator. Breakdown of peptones by CD increases the pH of the media and turns it from pink/orange to yellow. See Figure 3.

METHODS

CCFA/CCFA-HT/CCMB-TAL Comparison Study
Samples were stored at –70º C until culture. On the day of culture, samples were transferred inside an anaerobic chamber. After thawing, one drop of sample was added directly to TAL broth for an enrichment culture. A 0.25ml sample was then ethanol-shocked by adding it to an equal volume of ethanol and allowing it to stand at room temperature for 10–20 minutes. Two to three 2–3 drops of the ethanol-shocked sample were plated onto CCFA & CCFA-HT. All media were incubated at 37º C for 20–24, 48 and 72h. Relative colony size and plate growth (0–4+ scale) were recorded. Typical colonies were large and flat with irregular edges. TAL was examined at 24–72h for yellow color predicting the presence of CD. Yellow tubes were subcultured to CCFA.

CCMB-TAL Enrichment Study
In a separate study, in order to examine the enrichment value of TAL, 78 samples of pre-therapy patients whose primary culture with ethanol-shock on HT alone was CD-negative were re-cultured in TAL. All work was done in the anaerobic chamber. After thawing in the chamber, 0.25 ml of feces was transferred directly into TAL *without ethanol-shock*. TAL was incubated at 37º C for 72h and examined at 24, 48 and 72h for yellow color, predicting the presence of CD, as well as turbidity. All yellow or turbid cultures were plated onto HT and incubated for up to 72h before discarding as negative for CD.

TAL Toxin Enrichment Study
Seventeen known CD culture-positive TAL media were tested for toxin at 24 and 48h using *C. difficile* TOX A/B II™ (TechLab, Princeton, NJ). TAL media was tested directly and by diluting 1:5 as per the manufacturer's directions.

Discussion

Fecal samples were obtained from patients enrolled in an ongoing double-blind clinical trial for CD. *All samples were obtained pre-therapy for the study drug; however, per protocol, some patients may have had a same-day dose of vancomycin, therefore, some of the samples may have had high concentrations of vancomycin and so 100% recovery of CD was not expected.* Because we could not reliably interpret culture-negative data in this study, we compared the performance of each media against the others in terms of specimens that were culture-positive as follows...

CCFA/CCFA-HT/CCMB-TAL Comparison Study
See Table 1. In all, 77% (76/99) of samples were culture-positive for CD (CdPOS). Of those samples that were positive, 99% (75/76) were CdPOS in 24h on HT and 100% in 48h. However, on CCFA, only 91% (69/76) were CdPOS in 24h, 96% (73/76) in 48h and 4% of CdPOS specimens were culture-negative on CCFA. HT colony size was 1.7 times larger on average than CCFA at 24 and 48h. Quantity of growth was 1+ greater on CCFA-HT than on CCFA. See Figures 1 and 2. While the difference in growth of breakthrough organisms was similar on the two media, the 1+ greater quantity of growth and larger colony size of CD made HT plates better for isolation of CD. TAL cultures were CdPOS in 2 of 3 CCFA CD-negative cultures.

CCMB-TAL Enrichment Study
In this subsequent study, 78 other specimens that were negative on primary HT-only culture were recultured in TAL without ethanol-shock, of these, 36 appeared clear and pink and were culture-negative upon subculture to HT. However, 6 of the 42 cultures that appeared yellow and cloudy were CdPOS (14.3%) upon subculture to HT. Four of the six TAL CdPOS cultures changed the indicator to yellow in 24h and two in 48h.

TAL Toxin Enrichment Study
Only 5 of 17 CD culture-positive samples were toxin-positive using a 1:5 dilution of 24h TAL culture by TOX A/B II™. Two additional samples became positive after 48h. Testing samples directly by TOX A/B II™ did not increase the number of positive results.

Conclusion

- **CCFA-HT was superior to CCFA for rapid, 24h recovery of CD.**
- **CCMB-Tal offers an alternative culture enrichment medium for specimens that test toxin-positive but are culture-negative, when recovery of the isolate for further study is desired.**
- **CCMB-TAL enhanced recovery of CD compared to CCFA and CCFA-HT in some cases.**
- **Direct testing for toxin in CCMB-TAL broth cultures of feces at 24h is not sensitive.**

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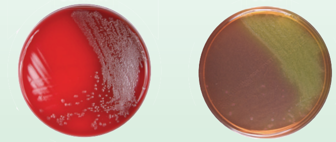
Results

Table 1. Media Comparison. Stool samples cultured, 99; Total CdPOS cultures by all media, 76

	CCFA-HT		CCFA	
	24h	48h	24h	48h
Percent of all CdPOS (no.)	99% (75)	100% (76)	91% (69)	96% (73)

CCFA-HT | CCFA

Figures 1a, 1b: 24hr incubation



Figures 2a, 2b: 48hr incubation

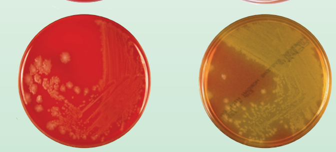


Table 2. Media ingredients.

Ingredients	CCFA	CCFA-HT	CCMB-TAL
Agar	15.0 gm	15.0 gm	
Cefoxitin	15.5 mg	15.5 mg	15.5 mg
Cycloserine	500.0 mg	250.0 gm	500.0 mg
Cysteine			0.5 gm
Distilled Water	1000.0 ml	1000.0 ml	1000.0 ml
Fructose	6.0 gm	6.0 gm	
Horse Blood		70.0 ml	
Lysozyme			5.0 mg
Magnesium Sulfate	0.1 gm	0.1 gm	0.1 gm
Mannitol			6.0 gm
Neutral Red	0.03 gm		0.03 gm
Potassium Phosphate	1.0 gm	1.0 gm	1.0 gm
Proteose Peptone no. 2	40.0 gm	40.0 gm	40.0 gm
Sodium Chloride	2.0 gm	2.0 gm	2.0 gm
Sodium Phosphate	5.0 gm	5.0 gm	5.0 gm
Sodium Taurocholate		1.0 gm	1.0 gm

Figure 3. CCMB-TAL after 24hr.