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Evaluation of Cycloserine-Cefoxitin Fructose Agar (CCFA), CCFA with Horse Blood and Taurocholate, and Cycloserine-Cefoxitin Mannitol Broth with Taurocholate and Lysozyme for Recovery of *Clostridium difficile* Isolates from Fecal Samples

Kerin L. Tyrrell,[a] Diane M. Citron,[a] Eliza S. Leoncio,[a] C. Vreni Merriam,[a] Ellie J. C. Goldstein[a,b]

R. M. Alden Research Laboratory, Culver City, California, USA[a]; UCLA School of Medicine, Los Angeles, California, USA[b]

Cycloserine-cefoxitin fructose agar (CCFA), CCFA with horse blood and taurocholate (CCFA-HT), and cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (CCMB-TAL) were compared for recovery of *Clostridium difficile* from 120 stool specimens. Compared to CCFA, CCFA-HT enhanced *C. difficile* growth and improved recovery by 4%. In a separate study, 9% (8/91) of stool samples previously *C. difficile* negative on plate medium were *C. difficile* positive when cultured in CCMB-TAL.

Culture of fecal specimens for toxin-producing *Clostridium difficile* is an important epidemiological tool to determine the frequency of various genotypes and is a gold standard for comparing newer detection methods. Cycloserine-cefoxitin fructose agar (CCFA) was developed (1) as both a selective and differential medium, but while this medium has become a standard for the isolation of *C. difficile*, occasional strains have been inhibited by this medium due to the cycloserine concentration (2).

This study first compared CCFA with two novel media that, while selective, also offer enrichment for *C. difficile*. CCFA-HT has added horse blood for enrichment and taurocholate to stimulate spore germination (3, 4) and uses half the concentration of cycloserine used by CCFA. CCMB-TAL is cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme, which also stimulate spore germination (5). Next, the enrichment potential of CCMB-TAL was tested against previously toxin-positive but plate culture-negative fecal specimens.

A simple and time-saving method of direct enzyme immunoassay (EIA) toxin testing using colonies from plate medium was described by Delmee et al. (6) using CCFA containing egg yolk. This study compared nine different *C. difficile* restriction endonuclease analysis (REA) types to determine whether direct EIA toxin testing could be suitable with CCFA-HT.

Media evaluated were CCFA, CCFA-based CCFA-HT plates, and CCMB-TAL (Anaerobe Systems, Morgan Hill, CA). See Table 1 for complete descriptions. The nutritive base of these media consists of animal peptones and fructose or mannitol and is supplemented with cefoxitin and cycloserine at concentrations that inhibit the growth of most normal fecal flora. Neutral red is added as a pH indicator (pH range of 6.8 to 8.0) in CCFA and CCMB-TAL. Breakdown of peptones by *C. difficile* increases the pH and turns the medium from pink-orange to yellow. The media are prepared, dispensed, and stored at 2 to 8°C in aluminum packets under oxygen-free conditions.

All specimens used in the following studies were pretreatment, toxin-positive fecal samples from patients enrolled in an ongoing clinical trial of a new drug for *C. difficile* infection. Per protocol, patients from both arms of the study could receive one dose of either vancomycin or metronidazole within 24 h of specimen collection. All fecal samples were frozen on-site and transported frozen to the testing laboratory, where they were stored at −70°C until culture. REA typing was performed at another laboratory as described elsewhere (7).

C. difficile colony appearance on CCFA-HT agar is nonhemolytic, 2 to 6 mm in diameter from 24 to 72 h, creamy yellow to gray-white in color, and irregular. Colonies have a coarsely mottled to mosaic internal structure, a matte or dull surface, green fluorescence under long-wave UV light, and a para-cresol odor. From experience, breakthrough colonies on CCFA and CCFA-HT look much different and are generally much smaller than *C. difficile* colonies. Final identification was performed by current methods (8, 9), including the use of special potency disks and proline, determination of the absence of aerobic growth, and Gram stain. If needed, further confirmation utilized API 20A kits (bioMérieux, St. Louis, MO) or 16S rRNA gene sequencing (10).

CCFA/CCFA-HT/CCMB-TAL comparison study. A total of 120 sequential fecal samples were studied. On the day of culture, samples were transferred from the freezer into an anaerobic chamber. After samples were thawed, one drop of sample was added directly to CCMB-TAL. Another 0.25 ml of the sample was ethanol shocked by mixing it with an equal volume of ethanol and allowing it to stand at room temperature for 10 to 20 min. Two drops of the ethanol-shocked sample was plated onto CCFA and CCFA-HT. Plates were streaked for isolation into 4 equal quadrants. All media were incubated at 37°C. Relative colony size on the two plate media and growth (0 to 4+ according to quadrant growth) were recorded at 20 to 24, 48, and 72 h. CCMB-TAL was examined at 24 to 72 h for yellow color predicting the presence of *C. difficile*. Yellow tubes were

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Address correspondence to Diane M. Citron, diane@rmaldenresearch.com.

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TABLE 1 Ingredients of CCFA, CCFA-HT, and CCMB-TAL

Ingredient[a]	Amt of ingredient in:		
	CCFA	CCFA-HT	CCMB-TAL
Agar (g)	15.0	15.0	
Cycloserine (mg)	500.0	250.0	500.0
Cysteine (g)			0.5
Fructose (g)	6.0	6.0	
Horse blood (ml)		70.0	
Lysozyme (mg)			5.0
Mannitol (g)			6.0
Neutral red (g)	0.03		0.03
Sodium taurocholate (g)		1.0	1.0

[a] All media contain cefoxitin (15.5 mg), magnesium sulfate (0.1 g), potassium phosphate (1.0 g), proteose peptone no. 2 (40.0 g), sodium chloride (2.0 g), sodium phosphate (5.0 g), and distilled water (1,000 ml).

TABLE 3 Summary of *C. difficile* growth versus contamination breakthrough for 93 sample-paired CCFA-HT and CCFA plates at 24 and 48 h

Growth of media[a]	No. of specimens positive (%)[b]			
	C. difficile		Breakthrough	
	24 h	48 h	24 h	48 h
HT > CCFA	**61 (66)**	**58 (62)**	8 (9)	11 (12)
HT = CCFA	27 (29)	28 (30)	**69 (74)**	**58 (62)**
HT < CCFA	5 (5)	7 (8)	16 (17)	24 (26)

[a] CCFA-HT > CCFA, CCFA-HT growth greater than CCFA; CCFA-HT = CCFA, CCFA-HT growth equal to CCFA; CCFA-HT > CCFA, CCFA-HT growth less than CCFA.

[b] Boldfaced values highlight the lack of change between 24 and 48 h that might be expected.

subcultured onto CCFA, which was the standard comparator medium for the study.

In all, 78% (93/120) of the fecal samples were *C. difficile* positive in at least one of the three media (Table 2). Of the 93 *C. difficile*-positive cultures, 99% (92/93) were positive at 24 h on CCFA-HT and 100% at 48 h. However, on CCFA, only 90% (84/93) were positive at 24 h, and 96% (89/93) were positive at 48 to 72 h. At 24 h, of the nine specimens that were *C. difficile* negative on CCFA, five were *C. difficile* positive in CCMB-TAL. At 48 to 72 h, four specimens were *C. difficile* negative on CCFA; of these, two were *C. difficile* positive in CCMB-TAL at 48 h and one more at 72 h, demonstrating enhanced recovery of *C. difficile* in CCMB-TAL for these samples. To determine if taurocholate may have increased growth results from CCFA-HT, 24- and 48-h cultures were examined when the CCFA-HT growth was greater than or equal to 2+ more growth than CCFA and vice versa. In each set, the REA types and their proportions were consistent with those of the clinical trial as a whole.

CCMB-TAL cultures were *C. difficile* positive in 86% (80/93) of the samples at 24 h and 90% (84/93) and 92% (86/93) at 48 h at 72 h, respectively. Of the seven samples that remained *C. difficile* negative in CCMB-TAL at 72 h, two were also negative on CCFA at 72 h; however, all were *C. difficile* positive on CCFA-HT at 24 h. Isolates that grew on plate media from the seven samples that were CCMB-TAL negative included three isolates from the REA BI group and four with nonspecific REA types. Although these findings represent too small a sample for statistical analysis, the BI group was found in a proportion similar to that in the clinical trial as a whole.

CCFA-HT colony size was 1.7 times larger on average than CCFA at 24 and 48 h. *C. difficile* growth and contamination breakthrough for sample-paired CCFA-HT and CCFA plates were similar at 24 and 48 h (Table 3). Quantity of growth on CCFA-HT was ≥1 quadrant more than that on CCFA for 66 and 62% of the cultures at 24 and 48 h, respectively. The proportion of REA types from cultures where CCFA-HT growth was ≥1 quadrant more than CCFA did not vary significantly from that of this study as a whole.

The amount of breakthrough for the majority of cultures on CCFA-HT and CCFA was consistent at 24 and 48 h: 74% and 62%, respectively. All subcultures from the selective plates resulted in good growth, unlike other reports of selective formulations for *C. difficile* that demonstrated some inhibition (11).

CCMB-TAL enrichment study. To examine the enrichment value of CCMB-TAL, 91 previously plate culture-negative samples were inoculated into CCMB-TAL directly without ethanol shock, incubated at 37°C, and examined at 24, 48, and 72 h for yellow color and turbidity. All yellow or turbid cultures were plated onto CCFA-HT and incubated for up to 72 h before being discarded as negative for *C. difficile*.

Of the 91 CCMB-TAL cultures from previously negative plate culture specimens, 41 (45%) of the tubes appeared pink and clear and were discarded as negative. Two (2%) were pink and turbid but grew non-*C. difficile* breakthrough organisms upon subculture to CCFA-HT. Forty-eight (53%) of the CCMB-TAL tubes appeared yellow and cloudy, and of these, eight grew *C. difficile* and the remainder grew breakthrough organisms upon subculture to CCFA-HT. Six of the eight *C. difficile*-positive cultures were turbid and changed the indicator to yellow after 24 h, with the remainder turning yellow at 48 h. The eight *C. difficile*-positive cultures reflect 9% of the total specimens cultured and 17% of the CCMB-TAL tubes that changed the indicator to yellow. Four isolates were of the REA-type BI group, which was similar to the BI group percentage (45%) of the clinical trial as a whole. The remaining isolates included one Y group, and three isolates were nonspecific REA groups.

Of these eight CCMB-TAL-positive specimens, five patients

TABLE 2 CCFA/CCFA-HT/CCMB-TAL comparison study and results of 93 *C. difficile* culture-positive specimens

Incubation time (h)	CCFA-HT		CCFA		
	No. of positive specimens (%)	No. of negative specimens (no. TAL+)	No. of positive specimens (%)	No. of negative specimens (no. TAL+)	CCMB-TAL, no. of positive specimens (%)
24	92 (99)	1 (1)	84 (90)	9 (5)	80 (86)
48	93 (100)		89 (96)	4 (2)	84 (90)
72	93 (100)		89 (96)	4 (3)	86 (92)

had had no CDI therapy 24 h prior to the start of the study, while the other three patients were treated with metronidazole on the day of specimen collection, suggesting possible enhanced enrichment of *C. difficile* in the latter three samples.

Toxin studies. The suitability of *C. difficile* direct toxin testing using 24- and 48-h colonies taken from CCFT-HT-grown colonies was tested similarly to the method of Delmee et al. (6). Seven known toxin-producing strains of *C. difficile* representing REA groups BI, K, G, BK, J, Y, and CF were cultured on CCFA-HT. Three colonies of each were transferred into 200 μl diluent and tested using the *C. difficile* TOX A/B II kit (TechLab, Princeton, NJ) per the manufacturer's directions for liquid fecal samples. Two of seven toxin-positive strains, REA types BI and J, gave trace positive results at 24 h when tested directly from CCFA-HT colonies. Colonies from all seven REA type strains tested strongly toxin positive at 48 h.

Improved recovery of *C. difficile* at 24 h can be accomplished by use of CCFA-HT. For those cultures with significantly more growth on CCFA-HT than on CCFA, there was no correlation with REA type, even with those *C. difficile* strains that are known to produce greater amounts of spores. CCMB-TAL offers an effective culture enrichment medium for specimens that contain fewer organisms and potentially for environmental sampling. Direct toxin testing of colonies from CCFA-HT cultures provides a rapid toxin-testing tool for patients whose fecal samples have been toxin negative previously.

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