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Effective and Reduced-Cost Modified Selective Medium for Isolation of *Clostridium difficile*▽

Michelle M. Nerandzic[1] and Curtis J. Donskey[1,2]*

Research Service[1] and Geriatric Research, Education and Clinical Center,[2] Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, Ohio

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Both for epidemiologic studies and for diagnostic testing, there is a need for effective, economical, and readily available selective media for the culture of *Clostridium difficile*. We have developed a reduced-cost substitute for cycloserine-cefoxitin-fructose agar (CCFA), which is an effective but expensive selective medium for *C. difficile*. The modified medium, called *C. difficile* brucella agar (CDBA), includes an enriched brucella base as a substitute for proteose peptone no. 2, and the concentration of sodium taurocholate has been reduced from 0.1% to 0.05%. To compare the sensitivities and selectivities of CDBA and CCFA, cultures for *C. difficile* were performed using stool samples from patients with C. *difficile*-associated disease. CDBA was as sensitive as CCFA for the recovery of *C. difficile*, with a similar frequency of breakthrough growth of stool microflora (25% versus 31%, respectively). A liquid formulation of the modified medium, termed *C. difficile* brucella broth (CDBB), stimulated rapid germination and outgrowth of *C. difficile* spores, at a rate comparable to that in cycloserine-cefoxitin-fructose broth. Our results suggest that CDBA and CDBB are sensitive, selective, and reduced-cost media for the recovery of *C. difficile* from stool samples.

Clostridium difficile is the most common infectious cause of health care-associated diarrhea in developed countries (2, 6, 18). In recent years, large outbreaks of *C. difficile*-associated disease (CDAD) in North America and Europe have been attributed to the emergence of an epidemic strain termed North American pulsed-field gel electrophoresis type 1, or NAP1 (13, 15). The emergence of this epidemic strain has been associated with reports of increased proportions of severe cases of CDAD and infections in populations previously considered to be at low risk, including peripartum women and healthy persons living in the community (5, 11). However, nearly all clinical microbiology laboratories diagnose CDAD on the basis of toxin testing by an enzyme-linked immunosorbent assay, which is much less sensitive than cell culture and does not provide isolates for molecular typing (10, 16). Therefore, limited information is available to confirm that the changes in the epidemiology of CDAD are attributable to specific strains (10). Both for epidemiologic studies and for diagnostic testing, there is a need for effective, economical, and readily available selective media for the culture of *C. difficile*.

Cycloserine-cefoxitin-fructose agar (CCFA) supplemented with 0.1% taurocholic acid is a commonly used selective medium long recognized as the first choice for the recovery and isolation of *C. difficile* (4, 9, 23). However, premade CCFA selective medium is expensive, costing $6.00 per agar plate (Remel, Lenexa, KS), and no broth formulation of CCFA is available for purchase. Broth enrichment cultures have increased sensitivity for the culture of *C. difficile* from contaminated environmental surfaces (19). Laboratories have the option of making their own CCFA medium (20). However, some of the ingredients of CCFA medium are expensive, particularly proteose peptone no. 2 (~$22.00 per liter of medium; Becton Dickinson [BD], Franklin Lakes, NJ), taurocholic acid (~$14.00 per liter of medium; Sigma-Aldrich, St. Louis, MO), and D-cycloserine (~$10.00 per liter of medium; Acros Organics, Geel, Belgium). In addition, in our experience, proteose peptone no. 2 (BD, Franklin Lakes, NJ) has often been unavailable due to lengthy back orders.

Previous studies have demonstrated that changes in the ingredients of selective media can cause significant differences in the sensitivity of recovery of *C. difficile* from both stool and environmental samples (3, 14, 16). The purpose of the current study was to develop an effective but less expensive and widely available substitute for CCFA. We examined the use of alternatives to proteose peptone no. 2 and D-cycloserine. A new modified selective medium, termed CDBA (*Clostridium difficile* brucella agar), was compared with CCFA and CDSA (*Clostridium difficile* selective agar; BD, Franklin Lakes, NJ) for the recovery of *C. difficile* from toxin-positive stool samples. In addition, we tested whether a reduced concentration of sodium taurocholate could be effective for the recovery of *C. difficile* using CDBA. To examine the efficacy of a liquid form of the modified selective medium, spore germination and outgrowth in cycloserine-cefoxitin-fructose broth (CCFB) and *Clostridium difficile* brucella broth (CDBB) were compared by monitoring the optical density at 600 nm (OD_{600}) along with quantitative analysis of CFU by plating.

MATERIALS AND METHODS

Evaluation of alternatives to proteose peptone no. 2 and D-cycloserine. Proteose peptone, proteose peptone no. 3, and enriched brucella broth base were tested as alternatives to proteose peptone no. 2 for the growth of *C. difficile* in an anaerobic chamber (Coy Laboratories, Grass Lake, MI). Proteose peptone and proteose peptone no. 3 did not support high levels of growth, whereas *C. difficile* grew rapidly and to high concentrations in enriched brucella broth base (data not

* Corresponding author. Mailing address: Research Service, Louis Stokes Cleveland Veterans Affairs Medical Center, 10701 East Blvd., Cleveland, OH 44106. Phone: (216) 791-3800, ext. 5103. Fax: (216) 229-8509. E-mail: curtisd123@yahoo.com.

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TABLE 1. Formulations of CCFA, CDBA, and CDSA

Medium and component	Amt
CCFA	
Proteose peptone no. 2	40.0 g
Disodium phosphate	5.0 g
Monopotassium phosphate	1.0 g
Magnesium sulfate	0.1 g
Sodium chloride	2.0 g
Fructose	6.0 g
Agar	20.0 g
Neutral red solution (1%)	3.0 ml
Sodium taurocholate	1.0 g
Lysozyme	5.0 mg
Cycloserine	500.0 mg
Cefoxitin	16.0 mg
CDBA	
Brucella broth powder	28.0 g
Vitamin K_1 solution (1 mg/ml)	1.0 ml
Hemin solution (5 mg/ml)	1.0 ml
Sodium bicarbonate solution (20 mg/ml)	5.0 ml
Agar	20.0 g
Mannitol	6.0 g
Neutral red solution (1%)	3.0 ml
Sodium taurocholate	0.5 g[a]
Lysozyme	5.0 mg
Cycloserine	500.0 mg
Cefoxitin	16.0 mg
CDSA	
Peptic digest of animal tissue	32.0 g
Disodium phosphate	5.0 g
Monopotassium phosphate	1.0 g
Magnesium sulfate	0.1 g
Sodium chloride	2.0 g
Mannitol	6.0 g
Growth factors	3.3 g
Cycloserine	250.0 mg
Cefoxitin	16.0 mg

[a] Initial experiments were performed with 1.0 g sodium taurocholate per liter, but 0.5 g was shown to be equally sensitive for the recovery of *C. difficile* from stool samples (see the text).

shown). Enriched brucella broth base is available from multiple vendors and is also the least expensive nutrient supplement of those tested. Therefore, brucella broth base was incorporated into the modified medium as a substitute for proteose peptone no. 2.

Norfloxacin at 12 or 32 μg/ml was evaluated as an alternative to D-cycloserine because it has been reported previously to be effective, when used in combination with moxalactam (latamoxef), for selective isolation of *C. difficile* (1). However, high levels of breakthrough growth of multiple organisms other than *C. difficile* from 30 toxin-positive stool samples were observed on CCFA plates in which norfloxacin had been substituted for D-cycloserine. Less breakthrough growth was observed with the higher concentration of norfloxacin, but at this concentration, too, some strains of *C. difficile* in stool samples were inhibited. In addition, decreasing the concentration of D-cycloserine to 250 μg/ml substantially reduced the selectivity of CCFA plates (data not shown).

Media. The ingredients for the three selective media tested are shown in Table 1. CCFA was prepared as previously described by George et al. (9), the only exception being the substitution of sodium taurocholate and lysozyme (both from Sigma-Aldrich) for egg yolk solution (4, 21, 23). The agar base consisted of proteose peptone no. 2 (BD, Franklin Lakes, NJ), disodium phosphate, monopotassium phosphate, sodium chloride, fructose, magnesium sulfate, neutral red solution prepared in absolute ethanol, and agar in 1,000 ml of distilled water. The base was adjusted to pH 7.6 with hydrochloric acid, autoclaved at 121°C for 15 min, and cooled to 50°C in a water bath. Sterile solutions of cycloserine (Sigma-Aldrich), cefoxitin (Sigma-Aldrich), sodium taurocholate, and lysozyme were prepared in distilled water and added to the cooled agar base after being filtered through a 0.2-μm-pore-size membrane (Corning, Corning, NY). The final concentration of sodium taurocholate was 0.1% (wt/vol). Agar was poured into petri dishes, cooled at room temperature, and prereduced in the anaerobic chamber for a minimum of 4 h before use.

CDSA was purchased from Becton Dickinson as a ready-made medium in petri dishes. The ingredients of CDSA are similar to those of CCFA, with the substitution of a peptic digest of animal tissue for proteose peptone no. 2, mannitol for fructose, growth factors (proprietary) for sodium taurocholate and lysozyme, and only 250 μg/ml of cycloserine. Plates were placed in the anaerobic chamber in order to prereduce the medium for a minimum of 4 h before use.

The medium that we developed based on our preliminary studies was termed CDBA. CDBA was prepared by adding selective supplements to an enriched brucella agar base. The enriched brucella base was prepared by methods outlined in *Wadsworth Anaerobic Bacteriology Manual* (20). The base consisted of brucella broth powder (Sigma-Aldrich), vitamin K_1, hemin, sodium bicarbonate, and agar in 1,000 ml of distilled water. Mannitol and neutral red prepared in absolute ethanol were added in order to distinguish *C. difficile* colonies by the typical yellow color change. The base was prepared, additional ingredients were added, and plates were made as described for CCFA.

CCFB and CDBB were prepared as described above, with the omission of agar from the ingredients. For studies involving optical density measurements, CCFB and CDBB were prepared without neutral red. Liquid medium was stored in 50-ml tubes and prereduced for 24 h before use.

Comparison of the sensitivities and selectivities of the *C. difficile* media. To compare the sensitivities and selectivities of the three *C. difficile* selective media, we assessed the recovery of *C. difficile* from 55 stool samples from patients with CDAD. The samples were obtained from the clinical microbiology laboratory and frozen at −80°C prior to analysis. The presence of toxin in the stool samples was confirmed using the *C. difficile* Tox A/B II test (Wampole Laboratories, Princeton, NJ). The samples were transferred to the anaerobic chamber, and 10-μl aliquots were streaked onto CCFA, CDBA, and CDSA plates. The plates were incubated at 37°C for 48 h. Yellow colonies with the typical appearance were streaked for isolation on blood plates and were confirmed to be *C. difficile* on the basis of the typical odor and appearance of colonies and by a positive reaction using *C. difficile* latex agglutination (Microgen Bioproducts, Camberley, United Kingdom). Colonies with a nontypical color or morphology were also transferred to blood plates and identified by using the RapID ANA II system (Remel) for obligate anaerobes or the Vitek 2 system (bioMerieux, Durham, NC) for facultatively anaerobic organisms. Pure cultures of *Clostridium clostridioforme*, *Clostridium innocuum*, and *Clostridium perfringens* were streaked heavily and then diluted into four quadrants on CDBA plates to confirm the selectivity of the medium upon challenge with *Clostridium* species other than *C. difficile*.

Assessment of *C. difficile* recovery using reduced concentrations of sodium taurocholate. After confirming that the modifications in the new selective medium were effective for the selective recovery of *C. difficile*, we tested whether reduced concentrations of sodium taurocholate would yield a similar level of recovery of *C. difficile* from stool samples. CDBA was prepared as described above except that the final concentrations of sodium taurocholate were reduced to 0.05%, 0.025%, 0.01%, and 0%. Because some studies suggest that lysozyme may stimulate spore germination and increase the recovery of *C. difficile* spores (18), the effectiveness of lysozyme as a substitute for taurocholate was also assessed. Because the price of lysozyme is negligible, we did not evaluate whether taurocholate alone was as effective as taurocholate plus lysozyme. Stool samples of 102 patients with CDAD were processed as described previously. Following 48 h of incubation, colonies of *C. difficile* were counted on all plates, and the numbers of colonies recovered were compared.

Measurement of spore germination and outgrowth. To examine the efficacy of a liquid form of the modified selective medium, spore germination and outgrowth in CCFB and CDBB were compared. The strain tested was VA 17, an epidemic NAP1 binary-toxin-positive isolate from our institution. Pulsed-field gel electrophoresis and analysis for the binary toxin gene were performed as previously described by Riggs et al. (19). Spore germination was monitored by measuring the decrease in the OD_{600}. Complete spore germination in liquid nutrient medium is noted by a 50% drop in absorbance, while the absorbance gradually increases during spore outgrowth, as described by Nicholson et al. (17). Spore germination and outgrowth were monitored for 4.5 h in anaerobic cuvettes (Starna, Atascadero, CA) at 37°C, with spores at an initial OD_{600} of 1. Samples were gently mixed by inversion between readings to assure that the suspension was homogeneous.

Aliquots were taken every 30 min and diluted 1:1 in either phosphate-buffered saline (PBS) or absolute ethanol; then they were serially diluted (1:10 through 1:10,000) in PBS and plated onto CCFA plates for the enumeration of CFU. The ethanol shock method provides a measurement of the spore concentration, whereas the samples diluted in PBS provide a measurement of the total *C. difficile* count (i.e., spores and vegetative cells).

TABLE 2. Comparison of the sensitivities and selectivities of the three selective media for culture of *Clostridium difficile* from stool samples of patients with *C. difficile*-associated disease

Medium	No. (%) of plates:	
	Positive for *C. difficile* ($n = 55$)	With non-*C. difficile* breakthrough ($n = 55$)
CDBA	55 (100)	14 (25)
CCFA	55 (100)	17 (31)
CDSA	34 (62)	38 (69)

Preparation of spores. Spores were prepared by growth on Duncan and Strong agar medium. Duncan and Strong agar medium was prepared as described previously by Duncan and Strong (8) and prereduced in an anaerobic chamber for a minimum of 4 h before use. Prereduced Duncan-Strong plates were spread with 100 μl of a 4-h culture (a 0.8 McFarland standard) of *C. difficile* grown in enriched brucella broth (20). The plates were incubated for 1 week at 37°C in an anaerobic chamber and then for 1 week at room temperature on the bench top. Spores were harvested from the plates by using sterile swabs and 2 ml of sterile distilled water and absolute ethanol (final concentration, 50%). The spore suspension was placed at 4°C overnight. The spores were centrifuged at 3,000 × *g* for 10 min; the supernatant was removed; and the spores were resuspended in 20 ml of sterile distilled water. Sonication was performed using a microsonicator tip at the maximum setting for 4 min, with incubation in an ice bath every minute, to break up the remaining vegetative debris. The spores were washed twice by centrifuging at 3,000 × *g* for 10 min and resuspending in 20 ml of sterile distilled water. The spores were centrifuged a final time at 3,000 × *g* for 10 min and resuspended in 1 ml of sterile distilled water.

A Percoll gradient was prepared by mixing 18 ml of Percoll (Sigma-Aldrich, St. Louis, MO) with 2 ml of 2.5 M sucrose and centrifuging at 25,000 × *g* for 15 min. Then 1 ml of spores was layered on the top of the gradient and centrifuged first at 1,000 × *g* for 20 min and then at 3,000 × *g* for 15 min. The top layers of the gradient were removed, and the spore pellet was collected in approximately 1 ml of the remaining Percoll solution. Spores were washed four times by centrifuging at 3,000 × *g* for 5 min and resuspending in 1 ml of sterile distilled water. Spores were stored at 4°C in sterile distilled water until use. Spores were confirmed by phase-contrast microscopy and malachite green staining to be >98% free of vegetative cells or cell debris.

Data analysis. The chi-square test was used to examine differences in the proportions of plates positive for *C. difficile* and non-*C. difficile* breakthrough growth. Student's *t* test was used to compare the numbers of CFU recovered using different media.

RESULTS

Comparison of recovery and selectivity. The results of the comparison of recovery and selectivity among media are presented in Table 2. Both CDBA and CCFA yielded 100% recovery of *C. difficile* from toxin-positive stool samples ($P = 1$). CDSA resulted in considerably reduced recovery, with only 34 of 55 (62%) plates yielding *C. difficile* ($P < 0.001$ for comparison to CDBA and CCFA). Colonies of *C. difficile* appeared similar on all media, but colonies on CDBA had a more filamentous edge and a slightly deeper yellow hue. The numbers of colonies recovered on positive CDBA and CCFA plates were not significantly different (mean ± standard error [SE], 84.31 ± 10.19 versus 80.47 ± 9.42 CFU; $P = 0.783$).

The frequencies of breakthrough growth of microorganisms other than *C. difficile* on CCFA and CDBA plates were similar (31% versus 25% breakthrough growth, respectively; $P = 0.525$) (Table 2). CCFA and CDBA plates had significantly less breakthrough growth of non-*C. difficile* organisms than CDSA plates ($P < 0.001$). The most common breakthrough organisms recovered on all three of the selective media were *Staphylococcus epidermidis* (round, yellow colonies), *Candida* spp. (small, round, pink colonies), and *C. perfringens*. *C. perfringens* appeared as yellow, ground-glass colonies on CCFA and CDSA, nearly indistinguishable from *C. difficile* colonies. However, on CDBA plates, the *C. perfringens* colonies remained pink, making them clearly distinguishable from the *C. difficile* colonies. Pure cultures of *C. clostridioforme*, *C. innocuum*, and *C. perfringens* did not grow on CDBA within 24 h. After 48 to 72 h, a heavy inoculum of *C. clostridioforme* and *C. perfringens* showed minimal breakthrough growth. *C. innocuum* did not grow on CDBA.

Recovery of *C. difficile* with reduced concentrations of sodium taurocholate. The presence of 0.1% to 0.01% sodium taurocholate was associated with significantly higher recovery of *C. difficile* from stool samples than that on plates with no sodium taurocholate ($P < 0.003$). When 0.05% (wt/vol) sodium taurocholate was used, a greater mean number of *C. difficile* colonies was recovered (mean ± SE, 137.49 ± 12.72 versus 134.62 ± 12.44 CFU) and a higher percentage of plates were *C. difficile* positive (100% versus 96%) than when 0.1% (wt/vol) sodium taurocholate was used, but the differences were not statistically significant ($P > 0.874$). Although 0.025% and 0.01% sodium taurocholate resulted in the same percentage of positive plates (i.e., 96%), the total number of CFU recovered was significantly lower than that with 0.05% sodium taurocholate ($P < 0.041$). Therefore, 0.05% taurocholate supplementation was selected as the optimal concentration for the CDBA medium. The addition of lysozyme to CDBA in the absence of taurocholate did not significantly increase the number of positive plates (89% positive with or without lysozyme) or the number of colonies recovered (mean ± SE, 38.21 ± 5.24 versus 29.65 ± 4.45 CFU; $P = 0.215$).

Spore germination and outgrowth in liquid media. Analysis of *C. difficile* spores by the OD_{600} showed that full germination of spores, as indicated by a 50% decrease in absorbance, occurred in both CDBB and CCFB within 1 h, followed by outgrowth within 4.5 h. At 4.5 h, the absorbance was approximately 15% higher in CDBB than in CCFB.

The findings upon examination of the broth cultures by plating correlated with the OD_{600} results. There was a 92% decrease in the number of spores within 30 min in both liquid media, and <1% of spores remained after 2 h. Following germination, outgrowth began, with a 200% increase in total CFU at approximately 2 h in both liquid media. At 4.5 h, the total *C. difficile* count in CDBB was 100% higher than that in CCFB.

DISCUSSION

Our results demonstrate that the modified selective medium that we developed (CDBB) is as effective and as selective as CCFA for the recovery of *C. difficile* from the stool samples of patients with CDAD. The major advantage of CDBB is the decrease in cost in comparison to CCFA. In our laboratory, the total price per liter for reagents to make *C. difficile* medium has been reduced from $62.00 for CCFA to $39.00 for CDBA (~$1.55 versus $0.99 per plate, respectively). This reduction in cost is due to the reduction of the concentration of sodium taurocholate from 0.1% to 0.05% (wt/vol) (~$14.00 versus $7.00 per liter) and the replacement of proteose peptone no. 2

with enriched brucella broth (~$22.00 versus $6.00 per liter). A secondary advantage of CDBB is that enriched brucella broth is a common laboratory staple, and brucella broth powder can be purchased from several vendors. As noted previously, in our experience proteose peptone no. 2 has often been unavailable due to lengthy back orders.

Our findings are consistent with those of previous studies that demonstrated that the addition of bile salts to *C. difficile* selective medium enhances the recovery of spores (22–23). When sodium taurocholate was eliminated from CDBA, the recovery of *C. difficile* from stool samples was reduced significantly. Although the percentages of recovery of *C. difficile* from stool samples were equivalent for 0.05%, 0.025%, and 0.01% taurocholate, we selected 0.05% for the CDBA medium because the number of colonies recovered was significantly decreased at lower concentrations of taurocholate. In addition to taurocholate, we included lysozyme in CDBA on the basis of a previous study that demonstrated a significant increase in the recovery of *C. difficile* spores from environmental surfaces when lysozyme (5 mg/liter) was added to bile salts (21). However, it is noteworthy that the addition of lysozyme without taurocholate to the CDBA base agar resulted in only a minimal and nonsignificant increase in the recovery of *C. difficile* over that with the base agar alone.

Cefoxitin and cycloserine are added to *C. difficile* selective media to suppress breakthrough growth of stool microflora that may obscure the detection of *C. difficile*. Our observation that plates with a reduced cycloserine concentration of 250 μg/ml resulted in increased breakthrough growth of other organisms is consistent with those of previous studies (1, 16). Aspinall and Hutchinson (1) found that norfloxacin at 12 μg/ml in combination with moxalactam was as effective as cycloserine and cefoxitin for suppressing breakthrough growth of stool microflora. In contrast, we found that breakthrough of stool microflora was common when norfloxacin was substituted for cycloserine, a finding probably attributable to the high prevalence of norfloxacin-resistant microorganisms in the intestinal microflora of patients in our institution (7).

Our study has some limitations. First, we did not assess the effect of adding an ethanol shock treatment prior to plating on the recovery of *C. difficile* from, and the selectivity of, CDBA. Levett (12) has demonstrated that a prior ethanol shock treatment might allow a reduction in the cycloserine concentration to 250 μg/ml, which would further decrease the cost of the selective medium. Second, we did not compare the sensitivity of CDBA or CDBB to that of CCFA or CCFB for the recovery of spores from environmental surfaces. However, in our experience, the use of CCFB broth enrichment cultures provides excellent sensitivity for the recovery of spores from the environment (our unpublished data). Third, for the comparison of spore germination and outgrowth in CDBB and CCFB, only one strain was tested. We cannot exclude the possibility of differences among strains. Finally, we did not characterize the *C. difficile* isolates recovered from stool samples in this study. However, previous studies in our institution have demonstrated that about half of *C. difficile* infection cases are currently due to epidemic NAP1 strains and the remainder are due to multiple distinct nonepidemic strains (our unpublished data). Therefore, the epidemiology of *C. difficile* in our institution is consistent with recent findings from other centers in North America (13, 15).

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