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Comparison of Five Cultural Procedures for Isolation of *Clostridium difficile* from Stools

LINDA M. MARLER,* JEAN A. SIDERS, LINDA C. WOLTERS, YOLANDA PETTIGREW, BECKY L. SKITT, AND STEPHEN D. ALLEN

Department of Pathology, Indiana University Medical Center, Indianapolis, Indiana 46202-5113

Received 12 August 1991/Accepted 13 November 1991

Several procedures have been described for the culture of *Clostridium difficile* from stool specimens. The goal of this study was to determine the effectiveness of five of these methods for the isolation of *C. difficile* from feces of patients suspected of having *C. difficile*-associated illness. A total of 564 stool specimens were cultured by using heat shock, ethanol treatment (ET), and direct plating on Carr-Scarborough cycloserine-cefoxitin-fructose agar (CCFA) with horse blood (C/S medium), BBL CCFA medium, and Remel *C. difficile* agar. Cytotoxin assays were performed on all specimens. A total of 113 specimens (20%) were positive for *C. difficile* by one or more methods. The numbers of positive cultures by using heat shock, ET, and direct plating on C/S medium, BBL CCFA medium, and Remel *C. difficile* agar were 79 (70%), 89 (79%), 91 (81%), 79 (70%), and 52 (46%), respectively. We concluded that ET and direct plating on C/S medium were the most effective procedures for isolating *C. difficile* from stool specimens and found significant variation in the performance of modified CCFA from different manufacturers.

Clostridium difficile is widely recognized as the causative agent of a wide spectrum of conditions, ranging from asymptomatic carriage of the organism to antibiotic-associated diarrhea to pseudomembranous colitis. The diagnosis of pseudomembranous colitis is ideally based upon the findings of pseudomembranes or plaques during colonoscopy. However, pseudomembranous colitis is not specific for *C. difficile*-associated disease and can be seen in conditions caused by other organisms (1). The diagnosis of *C. difficile*-associated disease generally depends upon the demonstration of a toxin(s) produced by *C. difficile* and isolation of the organism from stool specimens, coupled with clinical findings (1). The original tissue culture assay described by Bartlett in 1978 is still considered the "gold standard" assay for detecting *C. difficile* toxin in stools (3). Despite the fact that newer methods (enzyme-linked immunosorbent assay, enzyme immunoassay, latex agglutination, and counterimmunoelectrophoresis) for detecting toxin have been marketed or described, the tissue culture assay remains the method preferred by many authorities (1, 2, 4–6, 17). The tissue culture kit procedure used in this study was Bartels *C. difficile* cytotoxin assay kit (Bartels Immunodiagnostic Supplies, Inc., Bellevue, Wash.) The kit can be easily adapted to laboratories previously unable to set up tissue culture assays. Antitoxin, diluent, and controls are provided with the easily inoculated tissue culture plates. Incubation of these kits is in ambient air at 37°C for 24 and 48 h.

Sporulation by *C. difficile* may play a major role in patients on antimicrobial therapy. *C. difficile* can be highly susceptible to the antibiotics implicated in an antibiotic-associated illness (e.g., ampicillin), yet may survive by sporulation with regeneration of vegetative forms occurring as antimicrobial levels are reduced (2, 8). Effective isolation of *C. difficile* from stools requires the use of selective media, spore selection techniques, or both. A variety of media and methods have been described. The use of heat shock (HS) and the use of ethanol treatment (ET) of stools have been effective for the selection and detection of spores present in specimens (1, 4, 7, 13, 14).

A variety of culture media for the isolation of *C. difficile* are currently marketed. Cycloserine-cefoxitin-fructose agar (CCFA) has long been the recognized first choice of isolation media (10). This medium, however, has been modified for commercial distribution and is currently marketed in modified form or in accordance with the original formulation by different manufacturers. It has been recognized that modifications, as slight as they might appear on paper, have produced major variations in the abilities of selective media to isolate *C. difficile* from stool specimens (4).

The purpose of this study was to determine the effectiveness of five methods for the primary isolation of *C. difficile* from feces of patients suspected of having *C. difficile*-associated illness. A total of 564 stool specimens from 421 patients were evaluated in this study. All stool specimens accompanied by requests for *C. difficile* studies that were submitted to the Anaerobe Laboratory at the Indiana University Medical Center during the 6-month study were included. Upon receipt of the specimen in the laboratory, each of the following five methods was performed.

Cytotoxin assay. Cytotoxin assays were performed on all specimens by using Bartels *C. difficile* cytotoxin assay kit. The manufacturer's directions were followed.

HS. For the HS method, aliquots (2 to 4 ml) of stool were heated in an 80°C water bath for 10 min. One drop (≈0.10 ml) of the heated specimen was subsequently plated on CDC anaerobe blood agar (Carr-Scarborough). All plates were incubated in an anaerobic glove box for 48 h. Colonies with characteristics typical of *C. difficile* (large, irregular, gray to gray-white to yellow, coarsely mottled) were picked for identification.

ET. In the ET method, equal amounts of stool and absolute ethanol were mixed for 30 min. One drop (≈0.10 ml) of the treated specimen was plated on CDC anaerobe blood agar. All plates were incubated in an anaerobic glove box for 48 h. Colonies with colonial features consistent with those of *C. difficile* were picked for identification.

Direct plating. In the direct plating method, formed stools

* Corresponding author.

TABLE 1. Comparison of five procedures for the isolation of *C. difficile* from stools

Isolation procedure	No. of specimens		Total no. (%) positive (n = 113)
	Culture positive, cytotoxin positive (n = 55)	Culture positive, cytotoxin negative (n = 58)	
Spore selection techniques			
HS	42	37	79 (70)
ET	45	44	89 (79)
Selective media for *C. difficile*			
C/S	51	40	91 (81)
BBL	44 (4 NG)[a]	35 (4 NG)	79 (70)
REM	30 (8 NG)	22 (7 NG)	52 (46)

[a] NG, no growth on subculture (i.e., of 44 culture-positive isolates; 4 apparent *C. difficile* colony types failed to grow on subculture plates).

were diluted 1:1 with buffered gelatin as a diluent. Liquid stools were not diluted. Specimens (liquid stools and diluted, formed stools) were plated, by using a 0.1-ml inoculum size, onto Carr-Scarborough CCFA with horse blood (C/S medium) (Carr-Scarborough Microbiologicals, Inc., Stone Mountain, Ga.), CCFA from BBL Microbiology Systems (Cockeysville, Md.) (BBL medium), and Remel *C. difficile* agar (REM medium) (Remel, Lenexa, Kans.). Plates were incubated for 48 h at 37°C in an anaerobic glove box. Colonies with characteristics consistent with those of *C. difficile* were picked for identification.

Identification. Identifications were made by using conventional media and biochemical methods (1, 11). Differential characteristics included relationship to oxygen, Gram reaction, cellular morphology, colonial characteristics, short-chain acid metabolic products determined by using gas-liquid chromatography, reactions in prereduced anaerobically sterilized biochemical test media, and reactions on egg-yolk agar.

A total of 113 specimens (20%) were positive by one or more culture methods. Table 1 illustrates the number of positive results obtained by each method. Statistical analysis of the data, by using the G-test for correlated proportions, was used to evaluate the individual patient culture results obtained by the five different methods (16). Of the direct plating methods, significant differences were found between C/S and BBL media ($P < 0.001$) and between C/S and REM media ($P < 0.001$). A comparison of the two spore selection techniques demonstrated a significant difference between the ET and HS methods ($P < 0.025$). No statistically significant difference could be discerned between direct plating on C/S medium and the ET method ($P > 0.20$).

Several studies have been published evaluating the performance of a new medium or method for detecting *C. difficile* in stool specimens. It was our goal to compare the more widely utilized methods to determine the difference, if any, in their abilities to isolate *C. difficile*. This study indicated that considerable variation does exist in the effectiveness of these methods for the isolation of *C. difficile*. Considerable variation was apparent in the performance of CCFA or modified CCFA from different manufacturers. No single culture method resulted in 100% recovery of *C. difficile* from our study specimens. Our recommendation for the isolation of *C. difficile* from stools would include direct plating of the specimen to C/S medium and/or a spore selection technique

TABLE 2. Formulations of C/S, BBL, and REM media

Components of media	Amt of component
C/S	
Proteose peptone	40.0 g
Disodium hydrogen phosphate	5.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulfate	0.1 g
Sodium chloride	2.0 g
Fructose	6.0 g
Agar	15.0 g
D-Cycloserine	500.0 mg
Cefoxitin	16.0 mg
Horse blood	7.0 %
BBL	
Peptic digest of animal tissue	32.0 g
Fructose	6.0 g
Monopotassium phosphate	1.0 g
Disodium phosphate	5.0 g
Sodium chloride	2.0 g
Magnesium sulfate	0.1 g
Agar	20.0 g
Neutral red	30.0 mg
Cycloserine	250.0 mg
Cefoxitin	16.0 mg
REM	
Proteose peptone no. 2	40.0 g
Disodium hydrogen phosphate	5.0 g
Potassium dihydrogen phosphate	1.0 g
Sodium chloride	2.0 g
Magnesium sulfate	0.1 g
Deionized water	1000.0 ml
Fructose	6.0 g
Agar	20.0 g
Neutral red 1%	3.0 ml
Cycloserine	500.0 mg
Cefoxitin	16.0 mg
Egg-yolk saline 50%	50.0 ml

such as ET with subsequent plating to CDC anaerobe blood agar. The use of both methods would have increased the recovery of *C. difficile* to 96% (109 of 113 specimens positive). It may be impractical, however, to use both methods as part of a routine laboratory protocol. The variations in the medium formulations between the three agar media used appear to be minimal (Table 2). It is possible that the horse blood in C/S medium provides extra enrichment, allowing for better growth of *C. difficile*, than was seen for the other selective media. The concentrations of cycloserine in the BBL medium (250 mg) and the REM medium (500 mg) and the addition of egg-yolk saline in the REM formulation appear to be the only differences between these two media. It has been proposed previously (4, 6, 12) that the concentration of cefoxitin in CCFA medium is critical, with higher concentrations possibly inhibiting some strains of *C. difficile*. This may also be true for cycloserine concentrations. The formulation of the REM medium closely resembles that of the original CCFA medium developed by George et al. (10). Edelstein et al. (9) more recently recognized that egg yolk in media is not an advantage for the isolation of *C. difficile*. Of special note is the high number of specimens, after incubation on REM and BBL media, that demonstrated colonies typical of *C. difficile*, but the colonies were nonviable when a transfer for identification was attempted. This phenomenon was not encountered with the C/S medium, probably because of the horse blood supplement in that medium. The length of this study (6 months) precluded the

use of a single lot of any medium. No lot variability was noted. A controlled lot variability study may perhaps be warranted.

Toxin studies have also been long recommended for the diagnosis of *C. difficile*-associated illness. The significance of the toxin-negative, culture-positive specimens is currently under investigation. Many of these isolates could be from patients who are carriers of *C. difficile* with no significant related disease. Evidence suggests, however, that *C. difficile* can be a significant cause of diarrhea in some patients, without demonstration of toxin production in the stool (15). One specimen in our study was toxin positive and culture negative by all five methods. The significance of this has not yet been determined. A prospective evaluation of all of these cases will be presented in a later publication.

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