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Cultures for *Clostridium difficile* in Stools Containing a Cytotoxin Neutralized by *Clostridium sordellii* Antitoxin

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Stools from patients with antibiotic-associated diarrhea or colitis were cultured to detect the presence of *Clostridium difficile*. All specimens contained a cytotoxin which was neutralized by *Clostridium sordellii* antitoxin. Initial testing employed several methods with comparative merits in recovering this organism. These included the use of nonselective media, antibiotic-incorporated media, alcohol shock, and paracresol-containing broth. Optimal results were achieved with primary plating of serial dilutions onto a selective agar containing cycloserine and cefoxitin. This technique was then employed in a large number of specimens. The overall results showed that *C. difficile* was recovered in specimens from 71 of 73 patients. All isolates of *C. difficile* produced a cytotoxin which was neutralized by *C. sordellii* antitoxin in vitro. These results verify the utility of this medium and support the concept that *C. difficile* accounts for the cytotoxin found in stools in nearly all cases.

Recent studies indicate that stools from the vast majority of patients with antibiotic-associated pseudomembranous colitis contain a cytopathic toxin which is neutralized by *Clostridium sordellii* antitoxin (4, 6, 15). The source of the toxin is believed to be *Clostridium difficile* since this organism produces a similar or identical cytotoxin in vitro (5, 16). These observations have led to the use of a variety of bacteriological methods to recover *C. difficile* from the fecal flora. The present report is a two-part study in which the initial work compared several culture techniques for relative merits in detecting *C. difficile* in stool specimens. The second part of the study utilized the optimal technique in a large sample size to determine the recovery rate of *C. difficile* in specimens which contained the cytotoxin.

METHODS

Source of specimens. Stools selected for culture had been shown with previous testing to have a cytotoxin that was neutralized by *C. sordellii* antitoxin. All specimens were obtained from patients with antibiotic-associated diarrhea or colitis and were stored at −40°C before processing. Controls consisted of frozen stools from healthy adults who had no gastrointestinal symptoms and had not received antimicrobial agents during the previous 4 weeks. At the time of culturing, the specimens were thawed at room temperature, a 0.1-g amount was weighed, and this was then passed into an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). Serial 100-fold dilutions were prepared in the chamber with VPI dilution salts (11), and subsequent plating was performed with media which had been stored in the chamber for 18 to 24 h before use.

Comparison of culture techniques. Initial studies examined four methods to recover *C. difficile* from stool, as follows.

(i) Brucella base blood agar. Samples of 0.1 ml of each serial dilution (10^{-1} to 10^{-7}) were plated onto brucella base blood agar. These plates were obtained from a commercial source (Scott Laboratories, Fiskeville, R.I.) and were used within 3 weeks of reception.

(ii) Selective media containing cycloserine and cefoxitin (C&C). Samples of 0.1 ml of the serial dilutions (10^{-1} to 10^{-7}) were plated onto brain heart infusion medium containing 250 μg of cycloserine per ml and 10 μg of cefoxitin per ml. One liter of brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.) was prepared according to the manufacturer's recommendations. After cooling to 45°C, 50 ml of Alsever sheep blood (Scott Laboratories) was added. Sterile solutions of cycloserine (Eli Lilly & Co., Indianapolis, Ind.) and cefoxitin (Merck Sharp & Dohme, West Point, Pa.) were added to give desired concentrations. These plates were stored at 4°C and used within 1 week of preparation.

(iii) Reinforced clostridial broth containing 0.2% paracresol. Reinforced clostridial medium (Oxoid Ltd., London, England) was prepared according to the manufacturer's recommendations. After steaming for 30 min, the medium was cooled to room temperature, and 10% paracresol (ICN Pharmaceuticals, Inc., Plainview, N.Y.) was added to give a final concentration of 0.2%. The pH of the broth was adjusted to 7.2, 20-ml aliquots were dispensed in screw-capped tubes,

and the tubes were autoclaved. The broth medium was inoculated with 0.1 ml of the first dilution of stool before and after alcohol shock (see below). After incubation in the anaerobic chamber for 5 days, serial 100-fold dilutions (10^{-1} to 10^{-7}) were prepared, and 0.1-ml aliquots were subcultured onto the previously described selective medium containing C&C.

(iv) Ethanol shock. After the previously described techniques, the remainder of the first dilution was subjected to alcohol shock by using an equal volume of 100% ethanol for 1 h. Serial 100-fold dilutions (10^{-1} to 10^{-7}) were prepared, and 0.1-ml samples were plated onto brucella base blood agar and the antibiotic-incorporated selective agar; a 0.1-ml amount of the first dilution was also inoculated into the paracresol broth for processing as described above.

Interpretation. All plates and the paracresol broth were retained in the anaerobic chamber, which was maintained at 35°C. Plate media were interpreted after 48 to 72 h of incubation. Organisms which had the colonial morphology of *C. difficile* were enumerated, isolated, and identified with established techniques (11). No attempt was made to identify organisms which did not have this colonial morphology, although a number of distinctive colony types were recorded from plates which did not show confluent growth. Total bacterial counts were determined from the brucella base blood agar plates. Counts of *C. difficile* were determined with all plate media except subcultures of the paracresol broth cultures which, by the nature of the processing technique, were not quantitative.

Cultures for *C. difficile* based on results of screening tests. The previously noted studies were evaluated for comparative merits in terms of yield, simplicity, and rapidity in recovering *C. difficile*. The results indicated superiority of the antibiotic-incorporated selective medium. This medium was then used to culture specimens which contained the cytotoxin from 55 additional patients by using the methods described above.

Tissue cultures. Toxin assays were performed on stools and 5-day cultures of *C. difficile* isolates in chopped-meat-glucose broth by previously described methods (1-3, 5). WI-38 cells (HEM Research Inc., Rockville, Md.) were grown in flat-bottomed microtiter wells (Limbro Scientific Co., Hamden, Conn.) containing 50 μl of medium 199 modified with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and $NaHCO_3$ (Microbiological Associates, Bethesda, Md.) and supplemented with 10% fetal calf serum, 2 mM L-glutamine, streptomycin (100 U/ml), and penicillin (100 U/ml). Samples were prepared by centrifugation at 10,000 × *g* for 10 min and filtration of the supernatant through a 0.45-μm membrane filter. The cell-free supernatant was mixed with an equal volume of phosphate-buffered saline, 50 μl was added to the wells, and results were read at 24 h. Neutralization was tested by adding an equal volume of *C. sordellii* antitoxin (Bureau of Biologics, Rockville, Md., lot no. 40067-3647) to the cell-free supernatant in place of phosphate-buffered saline. The criteria for a positive response were cytopathic changes affecting at least 50% of the monolayer and complete neutralization by the antitoxin.

RESULTS

Stool specimens from 73 patients were selected for culture. All patients had antibiotic-associated diarrhea in which prior analysis of stool had shown a cytotoxin which was neutralized by *C. sordellii* antitoxin as previously described (1-3, 5, 16). Titers of the toxin varied from 10^{-1} to 10^{-5} dilutions, and the median titer was 10^{-3} dilutions. Clinical studies indicated that 53 patients had endoscopic evidence of pseudomembranous colitis (PMC), 3 had colitis without pseudomembranes, 2 had a normal colonic mucosa, and 15 failed to undergo endoscopy. In each instance, the specimen selected for culture was obtained during symptoms of diarrhea and before any antimicrobial therapy directed against *C. difficile* (vancomycin). The specimens were collected and stored at room temperature or under refrigeration for periods of 6 h to 4 days before reception in our laboratory. An exception was stools from three patients who were reported in a study of PMC 5 years previously (17); these specimens were stored in ill-defined conditions for 4 years before submission. Once received in this laboratory, all specimens were stored at −40°C for periods of 2 weeks to 31 months. No specimens were included in which culture results have been previously reported (2, 3).

Comparison of primary isolation techniques. The results of comparison testing with different methods of primary isolation with 18 randomly selected specimens are summarized in Table 1. Brucella base blood agar proved quite

TABLE 1. *Comparison of methods to recover C. difficile from stool*

Method	No. tested	No. with *C. difficile* as the predominant organism (%)
Direct plating		
BMB[a]	18	6 (33)
C&C[b]	18	18 (100)
Ethanol shock to		
BMB[a]	18	7 (39)
C&C[b]	18	18 (100)
Paracresol broth subcultured to C&C[b]		
Without ethanol shock	18	15 (83)
After ethanol shock	18	13 (72)

[a] Brucella base blood agar with 10 μg of vitamin K_1 per ml.

[b] Brain heart infusion blood agar with 250 μg of cycloserine per ml and 10 μg of cefoxitin per ml.

nonselective, as would be anticipated. All specimens showed multiple types of bacterial colonies, and there was an average of six colony types on plates which did not show confluent growth. The distinctive colonial morphology of *C. difficile* was detected after 48 h of incubation in just 6 of the 18 specimens. At this time, the colonies of *C. difficile* were 3 to 5 mm, flat, and irregular with an undulate edge. The mean concentration of all bacterial isolates on brucella base blood agar media was $10^{7.5}$/g (wet weight) with a range of $10^{4.6}$ to $10^{9.1}$; the mean concentration of *C. difficile*, when detected, was $10^{5.6}$/g (wet weight).

The antibiotic-incorporated selective agar (C&C) proved superior in terms of both the yield of *C. difficile* and ease of technical performance. All 18 specimens yielded strains of *C. difficile* which were readily detected by their unique colonial morphology on the primary isolation plates after 48 to 72 h of incubation. The colonies at this time were 5 to 7 mm, flat, and filamentous and had a greenish tinge. *C. difficile* was the only organism detected at 48 h with 11 of these 18 specimens. The mean concentration of *C. difficile* in these 18 specimens was $10^{5.8}$/g (wet weight) with a range of $10^{3.1}$ to $10^{7.7}$/g.

Alcohol shock was used to selected for sporulating bacteria. Subculture to brucella base blood agar offered little advantage over direct plating on this medium since *C. difficile* was readily detected in just 7 of the 18 specimens. All specimens showed organisms other than *C. difficile* after alcohol shock, with an average of three distinctive colony types per specimen. The mean concentration of all bacteria recovered on brucella base blood agar after alcohol shock was $10^{6.2}$/g. Subculture of specimens after alcohol shock to C&C medium yielded *C. difficile* in all specimens; with 14, this was the only organism isolated. The mean concentrations of *C. difficile* on C&C medium after alcohol shock was $10^{5.2}$/g (wet weight).

We plated paracresol broth onto C&C medium, anticipating that this method would prove highly selective (9). Twelve of 18 specimens yielded *C. difficile* in mixed culture, and three yielded *C. difficile* in pure culture. The paracresol broth medium seeded with inocula after alcohol shock gave comparable results. In comparison to direct plating onto C&C, the paracresol broth proved less sensitive and no more selective.

Culture results with C&C medium. The conclusion from the previous study was that the antibiotic-incorporated medium (C&C) was optimal for the expeditious recovery of *C. difficile*. This medium was then employed with specimens from additional patients to determine recovery rates with a larger sample size. The results showed *C. difficile* was recovered in 53 of 55 specimens with a median concentration of $10^{5.8}$/g (wet weight). All three specimens which were obtained 6 years previously yielded *C. difficile* in concentrations ranging from $10^{5.5}$ to $10^{7.3}$/g. Review of the two specimens in which *C. difficile* was not recovered showed no obvious problems in methods for transport or storage. Repeat studies of these two specimens showed that both contained the cytotoxin, and neither yielded *C. difficile*. Thus, in the entire series, *C. difficile* was recovered in 71 of 73 stools which contained a cytotoxin that was neutralized by *C. sordellii* antitoxin. The use of this medium to culture stools from 60 healthy adults failed to yield *C. difficile*.

Tissue culture assays. Tissue culture assays of the 71 isolates of *C. difficile* showed that each produced a cytotoxin which was neutralized by *C. sordellii* antitoxin.

DISCUSSION

Clostridium difficile is now a widely recognized pathogen responsible for antibiotic-associated PMC. This observation followed three decades of study in which repeated flora analyses failed to uncover an etiological agent. In retrospect, the delay in detection is not surprising, due to the complexity of the fecal flora and the difficulty encountered in recovering *C. difficile* from mixed bacterial populations. The latter observation, in fact, accounts for the original appellation assigned to this microbe, "*Bacillus difficilis*" (10).

Two separate lines of investigation led to the pursuit of *C. difficile* as the agent of PMC. The first was the hamster model of antibiotic-induced cecitis, which permitted experimental manipulation under controlled conditions. Studies with this model showed that *C. difficile* was the only stool isolate that reproduced the disease noted with antibiotic exposure (4). The second major clue was the detection of a cytotoxin in stools from patients with PMC (13) and the subsequent observation that the cytotoxin was neutralized by *C. sordellii* antitoxin (1, 3, 5, 14). This cytotoxin was consistently found in stools of both animals and patients with antibiotic-associated colitis (1). Ensuing work indicated that *C. difficile* produces a cytotoxin in vitro which is neutralized by *C. sordellii* antitoxin, thus establishing antigenic cross-reactivity (3, 5). These observations implicated *C. difficile* as the source of the cytotoxin in stools and led to culture efforts with a focus of attention on *C. difficile*.

Initial studies of PMC from this laboratory employed serial dilutions of stool plated onto brucella base blood agar, clostrisel agar, and clostrisel containing 10 μg of clindamycin hydrochloride per ml (2–4). It should be noted that these media were not employed in a specific attempt to detect *C. difficile* because the work was done before the previously noted studies were completed. Nevertheless, *C. difficile* was recovered from each of 25 hamsters with clindamycin-induced cecitis and from four of five patients with antibiotic-associated PMC. A review of our experience showed that extensive subculturing was often necessary and no individual agar provided a sufficiently high yield for reliable use as a single medium.

Several methods have been employed by other investigators in an attempt to recover *C. difficile* from the complex fecal flora. One of the first was by Hafiz in studies which actually antedated the work linking *C. difficile* to PMC. This investigator noted that *C. difficile* had a rather unique property of making paracresol from phenylalanine, leading to the use of paracresol in broth cultures as a selective medium. With this broth, Hafiz was able to recover *C. difficile* in stool from animals and infants with such ease that he somewhat facetiously proposed the term "facile" to supplant "difficile" (8, 9). Subsequent work in our laboratory has verified that the organisms recoyered by Hafiz were, in fact, *C. difficile* by current taxonomic schema (11). George et al. reported difficulty in recovering *C. difficile* in agar containing 0.2% paracresol, but failed to employ the broth that was considered preferable by Hafiz (7).

In more recent studies, Burdon and his colleagues of the General Hospital in Birmingham, England, showed extraordinary success with relatively nonselective media consisting of laked blood agar containing kanamycin, menadione, and hemin. These investigators recovered *C. difficile* from 27 of 30 stools which contained the cytotoxin (6, 12). This high recovery rate may have been facilitated by a hospital epidemic of PMC which allowed ample access to fresh samples. Larson et al. employed heat shock (75°C for 20 min or 80°C for 10 min) to select sporulating bacteria in frozen samples before subculture onto a number of selective and enriched media which were not further defined. These investigators recovered *C. difficile* from the stools of four of five patients with PMC and the cytotoxin (15).

Recently, George et al. reported the use of a selective agar containing 500 μg of cycloserine per ml and 16 μg of cefoxitin per ml based on in vitro susceptibility tests showing isolates of *C. difficile* were consistently resistant to these agents at the concentrations employed (7). Quantitative cultures of stock strains showed that counts on the antibiotic-incorporated media were comparable to the counts on brucella base blood agar. Further testing showed *C. difficile* was recovered from 14 of 28 specimens, including 8 of 10 from patients with PMC. Results of tissue culture assays were not reported so that, in contrast to the other studies cited, the number of specimens containing the cytotoxin is not known.

The initial portion of the present study was designed to compare methods which were considered somewhat analogous to those used by various investigators as summarized above. It is necessary to acknowledge that no pretense is made to have reproduced their techniques. Specifically, extensive subculturing was not done because an effort was made to find a method in which colonies of *C. difficile* were readily apparent with the primary isolation procedure. The C&C-incorporated medium proved superior in terms of technical ease and rapidity of recovery. The selective agar used here was somewhat different from that reported by George et al. in that the basal agar was brain heart infusion instead of egg yolk-fructose, and the concentrations of antibiotics employed were somewhat lower. This is because our study was initiated before the report of George et al., although we had general information regarding the potential utility of these media by personal communication with the authors (7). In both studies, *C. difficile* grew rapidly, colonies could be readily detected after 48 to 72 h of incubation, and the rather unique colonial morphology permitted presumptive identification which proved to be nearly infallible. The same type of colonial morphology was also readily detected with brucella base blood agar when the organism was not overgrown by other components of the fecal flora. However, the advantage of antibiotic-incorporated medium was its selectivity: *C. difficile* was the only organism recovered after 48 to 72 h in 11 of 18 specimens. Alcohol shock to select for sporulating bacteria proved to be an advantage over direct plating onto nonselective media, since the number of bacterial strains other than *C. difficile* was reduced. However, this was considerably less selective than C&C medium. Subcultures of specimens after alcohol shock gave a high yield of *C. difficile*, but this introduced an additional step in processing which appeared superfluous when compared to results obtained with direct plating onto C&C. Paracresol broth suffered the disadvantage that quantitation was not possible, results were de-

layed, and the recovery rate of *C. difficile* was somewhat less.

The concentrations of *C. difficile* and storage conditions of the specimens before culture deserve comment since all specimens had been retained at −40°C for at least 2 weeks before culture. Preliminary studies were conducted on stools from four hamsters with clindamycin-induced cecitis to determine the change in concentrations of *C. difficile* with different methods of storing specimens before culture onto C&C medium. Tissue cultures of all specimens showed cytotoxin levels of 10^{-6} or 10^{-7} dilutions, and cultures of fresh specimens yielded *C. difficile* in concentrations of $10^{6.9}$ to $10^{7.7}$/g (wet weight). Refrigeration for 48 h resulted in decreased viable counts which never exceeded $10^{0.3}$ organisms per g, whereas freezing at −40°C for 5 weeks resulted in losses of $10^{-0.6}$ to $10^{2.1}$/g. This observation indicates that changes in concentrations obviously occurred before culture in the studies reported here, and that fresh or refrigerated specimens would be preferable for accurate quantitation. The storage and culture methods employed were obviously satisfactory for detecting *C. difficile* in the vast majority, although the accuracy of quantitation may have been compromised by our methods of handling specimens.

A final comment concerns the overall results with bacterial cultures to detect *C. difficile*. Tissue culture assays have shown that stools from 76 of 77 patients with antibiotic-associated PMC contain a cytotoxin which is neutralized by *C. sordellii* antitoxin (1). It is presumed that *C. difficile* is the source of this toxin based on the observation that it produces a similar cytotoxin in vitro. The number of reported patients with culture evidence of *C. difficile* in stools which contain the cytotoxin is relatively modest, and it is possible that other bacteria could also produce similar cytotoxins which are antigenically related. The largest experience with cultures has been reported from the General Hospital in Birmingham, England (6, 12). However, this hospital has an ongoing epidemic which may reflect an endemic strain. The source of stools in the present report was patients at 38 different hospitals during a study period of 6 years. *C. difficile* was recovered in stools from 71 of 73 patients, and all isolates produced a cytotoxin in vitro. These findings support the concept that this organism accounts for the cytotoxin in stools in the vast majority of cases regardless of the epidemiological setting.

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