JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1982, p. 465–467
0095-1137/82/030465-03$02.00/0

Vol. 15, No. 3

Rapid Identification of *Clostridium difficile* by Toxin Detection

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Rapid identification of *Clostridium difficile* in a stool specimen could be accomplished within 24 h by detection of toxin elaborated in an agar or broth culture containing cycloserine and cefoxitin. Broth culture seemed to give a more rapid and sensitive result than the agar plate culture. For cultivation of *C. difficile* in stool, we recommend the use of chopped meat broth and blood agar plate, the former for toxin detection in 1 to 2 days and the latter for colonial morphology and isolation of a pure culture.

Isolation of *Clostridium difficile* from stool specimens has become feasible since a selective medium was formulated (3). Compared with toxin detection by tissue culture assay, isolation of the organism has yielded a higher incidence of positivity for *C. difficile* from stools of patients with antimicrobial agent-associated diarrhea (1). However, the procedure involved in identification of *C. difficile* is still cumbersome and time consuming. In this communication, we describe a one-step, rapid identification method and present data on its application to 404 stools from patients with antimicrobial agent-associated diarrhea. This method involves the detection of *C. difficile* toxin either in broth culture supernatant or in an agar plate directly inoculated with the stool specimen.

MATERIALS AND METHODS

Media. Chopped meat broth was purchased from Carr-Searborough Microbiologicals, Decatur, Ga., or prepared freshly from dehydrated cooked meat medium (Difco Laboratories, Detroit, Mich.). Before use, cycloserine (500 μg/ml, final concentration) and cefoxitin (15 μg/ml) were added. Thioglycolate broth was freshly prepared from dehydrated medium (Difco). Blood agar plates were prepared freshly in our laboratory; the constituents, per 1,000 ml, were brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.), 52 g; yeast extract (BBL), 5 g; hemin (Difco), 5 mg; vitamin K (Sigma Chemical Co., St. Louis, Mo.), 10,000 μg; sheep blood, 50 ml; cycloserine (Sigma), 500 mg; and cefoxitin, 15 mg. The sheep blood, vitamin K, hemin, and antibiotics were added separately. Ten milliliters of medium was distributed to each small plastic petri dish (60 by 15 mm; Falcon Plastics, Oxnard, Calif.).

Tissue culture. Cells grown on microplates were purchased from Flow Laboratories, McLean, Va. Either WI-38 or embryonic skin-muscle cells were used. The cultures were grown in 10% calf serum in Eagle medium. Microplates were purchased from Costar, Cambridge, Mass. Incubation was carried out at 37°C, with the microplates placed in a candle jar (vacuum desiccator).

Toxin detection. Toxin assay of serially diluted stool samples was carried out according to the method described earlier (2), with the modification that the test was done in microplates instead of test tubes. Detection of toxin in the agar plate was done similarly, except that no dilutions were made. By means of a sterile Pasteur pipette, a piece of agar (0.5 to 1.0 cm) was bored out into the tip of the pipette through rotatory motion. The agar piece was selected from a site adjacent to a suspicious colony in order to be free of gross contamination by bacteria. The agar piece was blown gently into a tissue culture well. A similar piece of agar from the same site was placed into a second well containing *C. sordellii* antitoxin. From broth cultures, a 1-ml portion was spun at 12,000 rpm for 10 min before inoculation to two wells, one with and the other without *C. sordelli* antitoxin.

Toxin production in relation to size of inoculum. A strain of *C. difficile* which has been used for toxin purification studies was diluted serially 10-fold. Then 0.05 ml of each dilution was inoculated to each of three agar plates and three chopped meat broths. A sterile glass rod was used to spread the inoculum evenly over the agar surface. The plates were incubated in an anaerobic chamber at 37°C. Assay for toxin was done daily, in the manner described above.

Appearance of toxin in agar from plates inoculated with toxin-positive stools. Forty stools which had been stored at −20°C during the past year were thawed and inoculated heavily onto the agar plates. The plates were assayed for the presence of toxin on days 2, 4, and 6.

Demonstration of *C. difficile* toxin in broth culture inoculated with stool samples. Fresh stool specimens from patients with antimicrobial agent-associated diarrhea were processed for toxin assay and inoculated into chopped meat broth and thioglycolate broth. Equal portions were removed daily for toxin detection.

Comparison between broth and agar media for cultivation of toxigenic *C. difficile*. Fresh stool specimens submitted for *C. difficile* toxin assay were inoculated simultaneously to chopped meat broth and blood agar plates. A total of 68 stools were examined. Detection

TABLE 1. Toxin production in relation to size of inoculum

Dilution inoculum	No. of colonies/plate	Toxin in agar[a]	Toxin in broth
10^{-1}	Numerous	+ + + (day 2)	+ + + (day 1)
10^{-2}	Numerous	+ + + (day 2)	+ + + (day 1)
10^{-3}	Numerous	+ + + (day 2)	+ + + (day 1)
10^{-4}	164,140,172	+ + + (day 2)	+ + + (day 1)
10^{-5}	24,26,45	+ + + (day 3)	+ + + (day 2)
10^{-6}	3,5,2	+ + + (day 3)	+ + + (day 2)
10^{-7}	0,0,0	− − −	− − −
10^{-8}	0,0,0	− − −	− − −

[a] At 24 h (day 1), only three of six heavily inoculated *C. difficile* cultures yielded toxin in the adjacent agar. At 48 h (day 2), all six cultures showed toxin in agar. Each + (positive) or − (negative) represents one culture.

of toxin was done on day 3 (broth cultures) and day 6 (plate cultures).

Stool collection. Stools were submitted to our laboratory for *C. difficile* toxin assay. They were collected from patients with antimicrobial agent-associated diarrhea. "Toxin-positive" or "toxin-negative" stools refer to result of direct toxin assay in tissue culture systems (2).

RESULTS

Toxin production was studied in relation to inoculum size. At 24 h after inoculation, only three of six heavily inoculated plates (10^{-1} and 10^{-2}) yielded toxin (Table 1). At 48 h, all plates containing 140 or more colonies yielded toxin. At 72 h, all plates showing evidence of growth of *C. difficile* excreted toxin in the adjacent agar, including a plate with only two colonies. Toxin production in the chopped meat broth was detected at 24 h (day 1) in those with heavy inocula and at 48 h in those with light inocula, when the broth became cloudy.

Agar plates were inoculated directly with stools that were positive for toxin by tissue culture assay. By day 2 of incubation, 32 of 40 (80%) toxin-positive stools had toxin demonstrable in the agar plate. Further incubation increased the incidence of positivity to 90% on day 4 and 95% on day 6. The remaining two plates which failed to show toxin in the agar did not show colonies on the plate that bore any resemblance to *C. difficile*.

TABLE 2. Demonstration of *C. difficile* toxin in broth culture inoculated with stool samples

Stool Toxin	No. positive[a]	No. negative[a]
No. tested	8	31
Toxin present		
24 h	5[b]	0
48 h	8	4
72 h		6
96 h		6

[a] Original direct toxin assay of stool.
[b] Cumulative totals.

TABLE 3. Comparison between broth and agar media for cultivation of toxigenic *C. difficile*

Stool toxin[a]	No. tested	No. positive	
		Broth	Agar
Positive	15	15	14
Negative	53	11	9
Total	68	26 (38%)	23 (34%)

[a] "Positive" and "negative" refer to original direct toxin assay of stool.

Toxin production in broth culture inoculated with toxin-positive stools was demonstrated in five of eight specimens within 24 h (Table 2) and in all eight samples in 48 h. On the other hand, toxin-negative stools did not show evidence of toxin production until 48 h. Further incubation (48 to 72 h) increased the yield of toxin-positive cultures. Thioglycolate broth did not support the growth of *C. difficile* consistently, and therefore was not used thereafter. Broth culture yielded slightly higher percentages of toxin-positive culture than agar plate culture (Table 3). This was seen with toxin-positive as well as toxin-negative stools.

Table 4 summarizes a study of 364 stools submitted for *C. difficile* toxin assay. Of these, 78 (21%) yielded toxin; 72 (92%) of toxin-positive stools also yielded positive cultures on agar plate. Of toxin-negative stools, 42 (15%) yielded toxin on plate culture, an additional yield of 10%. Thus, of 364 stools examined, 114 (31%) demonstrated toxin on culture.

DISCUSSION

Identification of clostridial species other than *C. perfringens* is a complicated and time-consuming procedure that requires a series of biochemical reactions and gas-liquid chromatographic analysis. Taking advantage of production of a cytotoxin by *C. difficile* and not by other clostridia, we have developed a rapid, presumptive identification technique. This procedure is similar to that for the demonstration of alpha-toxin in *C. perfringens*.

TABLE 4. Demonstration of *C. difficile* toxin in agar plates inoculated with stool samples

Stool toxin[a]	No. tested	No. positive	%
Positive	78	72	92
Negative	286	42	15
Total	364	114	31

[a] See Table 3.

When a pure culture of *C. difficile* was used to test the feasibility of this procedure, toxin production was regularly detected in both agar and broth. The presence of two colonies on an agar plate was sufficient to give a positive toxin test; as long as there was visible growth in broth medium, the toxin was detected. This identification procedure appears to be as sensitive as lethecinase detection for identifying *C. perfringens*.

The appearance of toxin in agar from plates inoculated with toxin-positive stools (Table 4), in contrast to the virtual complete sensitivity with pure culture, was detected in 92 to 95%. Two of six toxin-negative plates did not contain colonies resembling *C. difficile*. The time of incubation required for toxin detection from plates inoculated with stool samples was longer than that required in pure culture, indicating either the presence of inhibitors in the stool or relatively slow growth rate in primary cultures.

The use of chopped meat broth seems to be advantageous over the plate culture. The time required for toxin detection is reduced by 50%, and the degree of sensitivity is higher (Tables 3 and 4). One disadvantage of broth medium is the inability to study the colonial morphology.

By using plate cultures for toxin detection, 114 stools were positive of a total of 364 stools submitted from patients with antimicrobial agent-associated diarrhea, a recovery rate of 31% (Table 4). This represents a 10% increase over direct toxin detection from stools using the tissue culture assay for toxin. Certainly, the incidence of toxin-positive stools from patients with antimicrobial agent-associated diarrhea varies from time to time and from place to place. Our 2-year accumulated experience indicates an average of 13.5% positive stools in such patients by direct toxin assay (570 positive of 4,218 stools examined).

Thus far in our surveys, we have not encountered toxin-negative *C. difficile*, nor have we detected cytopathic toxin from other clostridial species except *C. botulinum*. It appears that the demonstration in the stool of cytotoxic toxin which is neutralized by *C. sordelli* antitoxin is a unique property of *C. difficile*. However, it should be noted that when only API 20 is used for biochemical testing or when gas-liquid chromatographic analysis of diethyl ether extracts of broth cultures is used for identification, as recommended by the Virginia Polytechnic Institute (4), *C. difficile* cannot be differentiated from *C. sporogenes* (5). As shown by Larsson et al. (5), more complete biochemical testing is needed to separate these two species. This may explain the controversial reports on the recovery of "nontoxigenic" strains of *C. difficile*.

In our previous studies, stools from patients with pseudomembranous colitis almost invariably yielded both a positive toxin assay and a positive culture for *C. difficile*. On the other hand, patients with uncomplicated, antibiotic-associated diarrhea sometimes had a negative toxin assay in the stool, but a positive culture for *C. difficile* (1). The present findings are consistent with this earlier experience. When we have calculated the geometric mean titer of *C. difficile* toxin in stools of patients with antibiotic-associated diarrhea, it appears that watery stools contain five times higher toxin levels than soft or formed stools, suggesting a relationship between stool toxin levels and the severity of the diarrheal disorder. In addition, patients with *C. difficile* diarrhea treated with vancomycin show rapid disappearance of stool toxin, but they may continue to excrete the organism, as shown by positive cultures of the same stool. These observations support the concept that stool culture is more sensitive than stool toxin assay in making an etiological diagnosis of antibiotic-associated diarrhea, especially in milder cases.

Based on these studies, we propose the use of culture medium in addition to direct toxin detection in stool from patients with antibiotic-associated diarrhea.

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