JOURNAL OF CLINICAL MICROBIOLOGY, July 1984, p. 74–76
0095-1137/84/070074-03$02.00/0

Vol. 20, No. 1

Selective Enrichment Broth Culture for Detection of *Clostridium difficile* and Associated Cytotoxin

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Received 4 November 1983/Accepted 9 April 1984

A procedure was devised for routine examination of feces for *Clostridium difficile* with selective enrichment broth culture containing increased levels of carbohydrates and antibiotics to detect cytotoxin and volatile acids in broths inoculated with fecal samples. *C. difficile* was detected and identified with a rapidity comparable to that of conventional culture on selective cycloserine-cefoxitin fructose agar. Detection rates for *C. difficile* in inoculated broths (111/401 or 27%) were significantly higher than for culture on cycloserine-cefoxitin fructose agar (47/401 or 11%, $P > 0.001$). All fecal samples containing *C. difficile* and cytotoxin were correctly identified by the procedure. Isocaproic acid peak heights >2 mm in selective enrichment broths inoculated with fecal samples indicated that *C. difficile* was present in the fecal sample examined. Of the positive specimens examined, 58% (64/111) produced peak heights >10 mm. Peak heights <2 mm were not associated with *C. difficile* in the fecal sample. The investigated procedure provided a reliable alternative to the routine processing of feces for detecting *C. difficile* and associated cytotoxin in feces. Inoculated broths with isocaproic acid peak heights >2 mm, after 24 to 48 h of incubation, and in which cytotoxin was detected, were subcultured to blood agar to obtain isolates of the organism as required. Broths which showed isocaproic acid peak heights <2 mm, and in which cytotoxin was not detected, were discarded as negative for *C. difficile*. The procedure was deemed potentially useful for epidemiological surveys of *C. difficile*.

Clostridium difficile, the causative agent of antibiotic-associated pseudomembranous colitis and some cases of antimicrobial-associated diarrhea lacking pseudomembranes, is routinely identified in clinical laboratories by culture on a selective agar medium (3). Tissue culture assay of *C. difficile* cytotoxin is a recommended diagnostic test (1). *C. difficile* cytotoxin is detectable in broth cultures (2), and caproic acid production in norleucine-tyrosine supplemented broth has been reported (4). A relatively simple selective enrichment broth is described for detecting *C. difficile* cytotoxin and isocaproic acid as an alternative to routine culture on selective agar medium. This selective broth medium, which combined enrichment and antibiotics to facilitate detection of relatively small numbers of *C. difficile* in feces, should prove useful for epidemiological studies, to determine carriage rates in asymptomatic individuals, and to detect organisms present in low numbers from environmental sources.

MATERIALS AND METHODS

Media. Cooked-meat carbohydrate-selective (CM+S) broth was made by adding cycloserine and cefoxitin to cooked meat base (Difco Laboratories, Detroit, Mich.) to give final concentrations of 500 and 16 μg/ml, respectively (3). The cooked meat base was supplemented with 3 g of glucose, 1 g of cellobiose, 1 g of maltose, and 1 g of soluble starch per liter of medium (4). Cycloserine and cefoxitin were added to cooled, autoclaved 5-ml samples of the broth medium in screw-capped tubes. Broths were kept at 4°C and used within 2 weeks of preparation. Cooked meat (CMC) broth was supplied by Scott Laboratories, Inc., Richmond, Calif. (R.I. 02823). Cycloserine-cefoxitin fructose agar (CCFA) was prepared as described previously (3).

Examination of feces for *C. difficile*. Feces received in the Clinical Microbiology Laboratory during an 8-month period were routinely examined for *C. difficile* by inoculating feces to CCFA, CM+S broth, and blood agar (BA) plates. Sterile cotton-tipped swabs were used to transfer ca. 0.2 g of feces to CCFA, BA plates, and CM+S broths in screw-capped tubes. Feces were initially spread over a corner of the CCFA and BA plates with the swab. The CCFA and BA plates were subsequently streaked with an inoculating loop to produce isolated colonies. Swabs were left in the CM+S broths until these broths were examined. Inoculated media were incubated anaerobically at 37°C in an anaerobic chamber. Volatile acids were ether extracted from broths and identified by gas-liquid chromatography (4), by the CAPCO anaerobe identification system (Sunnyvale, Calif.; model no. 94086), consisting of a gas chromatograph fitted with a thermal conductivity detector and two stainless steel columns (19 in. by 6 ft. [48.26 by 182.88 cm]) packed with 20% Resoflex on 30/60 mesh Chromosorb WAW. Column temperature was set at 140°C, and the detector current was 110 mA. Helium at a flow rate of 120 cm^3/min was used as the carrier gas. Attenuation was set at 1. A 14-μl sample of the ether extract was injected for each run. Volatile acid standards were prepared (4) and run daily. *C. difficile* on CCFA and BA plates was identified by characteristic features in culture, gram stain, fluorescence, and volatile acids profile of acetic, isobutyric, butyric, isovaleric, valeric, and isocaproic acids (4). *C. difficile* organisms present in CM+S broths showing acetic, isobutyric, butyric, valeric, and isocaproic acids was confirmed by subculture of the broth to BA and detection of volatile acids produced by isolates in CMC broth. The API-20A (Analytab Products, Montreal, Canada) anaerobic system was also used to confirm the identity of all isolates as *C. difficile*. Reports sent out by the Clinical Microbiological Laboratory were reviewed to compare routine isolation of *C. difficile* from feces on CCFA with chromatographic detection of volatile acids in CM+S broth.

Cytotoxin assay. *C. difficile* toxin was detected by rounding of McCoy cells (A. C. Maniar, T. W. Williams, T. J. Louie, H. Bell, W. Forsythe, and J. C. Wilt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C200, p. 345) cultured in

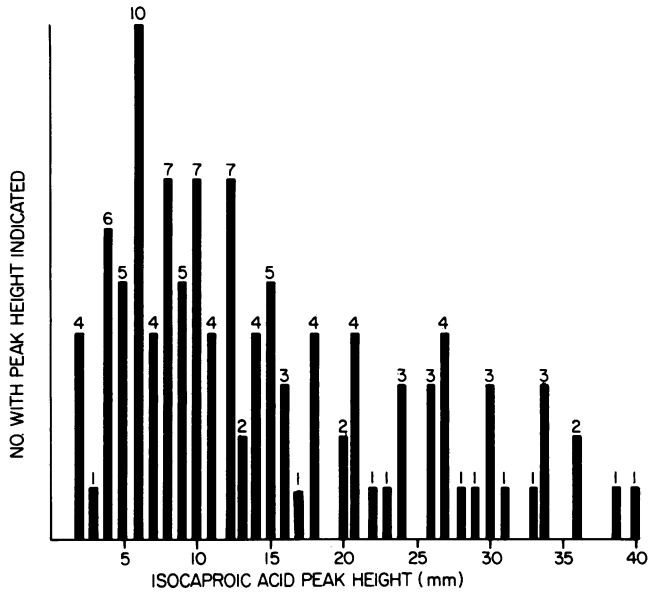

FIG. 1. Distribution of isocaproic acid peak heights in CMC+S broths inoculated with feces. Isocaproic acid detected in ether extracts of CMC+S broths.

minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and 10 μg of gentamicin per liter (7). The cytotoxin assay procedure was used essentially as described by Tracy D. Wilkins (Virginia Polytechnic Institute and State University, Blacksburg, Va.) but with McCoy cells to detect cytotoxicity. *C. difficile* cytotoxin was confirmed in feces or broths showing cytotoxicity by neutralizing the dilution corresponding to the titer with specific antitoxin to *C. difficile* toxins (Virginia Polytechnic Institute and State University, Blacksburg, Va.). Cytotoxic effects were confirmed by neutralization on the same day as screening without freezing the filtrates. Cytotoxin production by *C. difficile* isolates was confirmed by examination of CMC broth cultures inoculated with the isolates and incubated anaerobically at 37°C for 24 to 48 h. All feces examined in this study were tested directly for *C. difficile* cytotoxin within 24 h of receipt into the Clinical Microbiological Laboratory. Samples not immediately tested were stored at 4°C until examined.

RESULTS

***C. difficile* isolation.** Stool samples (401) from several wards in a 450-bed hospital were examined for *C. difficile* toxin and isocaproic and other volatile acids produced in CM+S broths. All 401 stool samples were inoculated directly to CCFA plates; 47 of 401 (11%) of these contained *C. difficile*, whereas 111 of 401 (27%) of the stool samples inoculated to CM+S broths contained *C. difficile*, as demonstrated by gas chromatographic examination and confirmed by subsequent isolation on BA plates ($P > 0.001$ by the chi-square test). All CM+S broth cultures were subcultured to BA plates regardless of whether isocaproic and other volatile acids or cytotoxin was detected. *C. difficile* was not subsequently isolated from any CM+S broth deemed negative by gas chromatography, and all broths in which acetic, isobutyric, butyric, isovaleric, valeric, and isocaproic acids were detected were shown to have *C. difficile*. All six volatile acids associated with *C. difficile* (acetic, isobutyric, butyric, isovaleric, valeric, and isocaproic) were detected in CM+S broths. Daily reports sent out by the Clinical Microbiological Laboratory from culture of stool samples on CCFA were in agreement with those reported by this study.

Cytotoxin assays. *C. difficile* toxin neutralized by specific antibody was detected in 92 of 401 (23%) of the stool samples inoculated to CM+S broths. All 401 fecal samples were directly examined for cytotoxin; 28 (7%) of these were cytotoxin positive. Nineteen (4%) other inoculated CM+S broths and direct stool examinations were toxin negative. These contained nontoxigenic strains of *C. difficile*, as confirmed by assaying CMC broth cultures of isolates. All toxigenic *C. difficile* organisms present in the stool samples were detected by examination of inoculated CM+S broths. All specimens proven to have *C. difficile* toxin were detected within 24 to 48 h after inoculation. No other stool samples were shown to have *C. difficile* cytotoxin after incubation for >48 h. Filtrates were more easily prepared from inoculated CM+S broths than from feces, and it was not necessary to centrifuge these broths before filtration as it was with fecal samples. CM+S filtrates producing cytotoxicity were easier to read than corresponding fecal filtrates, presumably associated with fewer nonspecific cytopathogenic effects in the former. Cytotoxin was not detected in any CM+S broth inoculated with feces which showed an isocaproic acid peak height <2 mm.

Isocaproic acid detection. Isocaproic acid detection has been suggested as a screening test for *C. difficile* in stools (6); thus, gas chromatographic results were evaluated with respect to isocaproic acid production. All inoculated broths with isocaproic acid peak heights of 2 mm or greater were found to contain *C. difficile*. Most of the inoculated broths yielded peak heights >2 mm after 24 to 48 h of incubation; 64 of 111 (58%) yielded peak heights of 10 mm or greater (Fig. 1). Peak heights generally increased with prolonged anaerobic incubation at 37°C. *C. difficile* was not isolated from inoculated broths with isocaproic acid peak heights <2 mm. An isocaproic acid peak height of 20 mm was equivalent to 10 μmol of isocaproic acid per ml under the conditions used for gas chromatography.

Serial dilutions of isolates of *C. difficile* inoculated to CM+S broths showed that ca. 100 CFU/ml was required to give detectable isocaproic acid peaks in 24 to 48 h.

DISCUSSION

Routine laboratory detection and confirmation of isolates of *C. difficile* on CCFA usually requires 3 to 5 days; even though the organism may be detected in 24 h on CCFA (3), isolates have to be confirmed as *C. difficile* by biochemical tests and gas chromatography.

The norleucine-tyrosine broth previously reported (5) lacks the selective advantage of suppressing the growth of other bacteria that is conferred by the CM+S broth procedure described. The selective CM+S broth detected *C. difficile* from fecal samples by gas chromatography of volatile acids, including isocaproic acid, more reliably than did culture on CCFA, significantly increasing overall detection rates of *C. difficile* in fecal samples. *C. difficile* in feces was detected and presumptively identified in 1 to 2 days by gas chromatography, results similar to those with routine culture on CCFA. Isocaproic acid peak heights of 2 mm or greater indicated that *C. difficile* was present in the stool samples examined. Cytotoxin was reliably detected in filtrates of the selective broth medium inoculated with stool samples at a rate considerably higher (23%) than that of direct examination of feces (7%). This selective enrichment broth favored

the proliferation of relatively few *C. difficile* organisms present in the fecal samples, conferring some advantage in comparison with the solid, selective CCFA. Acetic, isobutyric, butyric, isovaleric, valeric, and isocaproic acids associated with active growth of the organism were easily detected in the selective enrichment broth media, and isocaproic acid production specifically indicated the presence of *C. difficile*. Advantages conferred by the selective broth media were supported by the low numbers of organisms detected by the broth (100 CFU/ml). The increased sensitivity of the selective broth procedure in detecting small quantities of *C. difficile* suggests that this procedure be used in epidemiological studies referring to carriage rates and distribution of the organism. Low numbers of *C. difficile* which may have been missed by direct inoculation to CCFA can be detected by inoculation of the fecal sample to the selective enrichment broth described. Nontoxigenic and toxigenic strains of the organism can both be identified by appropriate examination of inoculated broths. The procedure should prove useful for epidemiological surveys of *C. difficile*.

The selective broth medium described by this report expedited detection and presumptive identification of *C. difficile*. *C. difficile* may now be reliably presumptively identified by inoculating a single selective enrichment broth followed by gas chromatographic detection of acetic, isobutyric, butyric, isovaleric, valeric, and isocaproic acids. Filtrates prepared from these broths can be examined for cytotoxin. Stool samples which do not show isocaproic acid or cytotoxin when inoculated into CM+S broths may be discarded as negative; samples with isocaproic acid, cytotoxin, or both may then be subcultured to blood agar for isolation as required.

The procedure of inoculating feces into CM+S broths and examining filtrates prepared from these broths for cytotoxin is more time consuming than the customary direct examination of feces for toxin, but some advantage may be associated with the proliferation of small numbers of toxigenic *C. difficile* that are detectable in broths but not by direct examination of feces. The application of the procedure described by this report for clinical diagnosis of antibiotic-associated colitis has yet to be determined, since detection of low numbers of *C. difficile* may not be clinically relevant. The procedure should prove useful as an epidemiological tool and as a possible screen of clinical material to detect small numbers of organisms in feces.

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