

Use of Cycloserine-Cefoxitin-Fructose Agar and L-Proline–Aminopeptidase (PRO Discs) in the Rapid Identification of *Clostridium difficile*

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The PRO Disc (Carr-Scarborough Microbiologicals, Inc., Decatur, Ga.) can be used to screen for L-proline-aminopeptidase produced by *Clostridium difficile* grown on cycloserine-cefoxitin-fructose agar (CCFA). Fifty stored isolates of *C. difficile* (48 toxin-positive and 2 toxin-negative isolates) and 47 fresh *C. difficile* isolates (39 toxin-positive and 8 toxin-negative isolates) were all PRO Disc positive. Other *Clostridium* species that were PRO Disc positive could be differentiated from *C. difficile* by failure to grow on CCFA, different colonial morphology on CCFA, or morphology upon Gram staining.

Most laboratories use assays to detect the presence of *Clostridium difficile* toxin(s) in stool rather than culture to diagnose *C. difficile*-induced diarrhea or pseudomembranous colitis. Although culture is the most sensitive method for the detection of *C. difficile* in stool specimens, not all isolates of *C. difficile* produce toxins; therefore, all isolates must be tested for their ability to produce toxin before the diagnosis of *C. difficile*-associated diarrhea or colitis can be made (6). Culture for *C. difficile* can be easily performed with cycloserine-cefoxitin-fructose agar (CCFA). Cycloserine and cefoxitin make the medium selective, and the presence of fructose with neutral red as a pH indicator allows the medium to be differential because *C. difficile* can ferment this sugar.

Culture for *C. difficile* from stool specimens may be useful under certain circumstances. For patients with *C. difficile*-associated diarrhea treated with vancomycin, toxin will rapidly disappear from the stool, but the patients may continue to excrete the organism (4). Asymptomatic carriage of *C. difficile* is common in areas of outbreaks of *C. difficile*-associated diarrhea (10). For epidemiological studies, culture can be used to identify asymptomatic carriers. In addition, culture is required to obtain organisms for serological or molecular typing in nosocomial outbreaks of *C. difficile*-associated diarrhea.

The production of the enzyme L-proline-aminopeptidase is a useful characteristic for the identification of *C. difficile* because it is one of the few biochemical tests for which the organism is positive in the clinical microbiology laboratory. This enzyme can be detected in a rapid disc test which uses L-proline-β-naphthylamide as the substrate for the enzyme and *p*-dimethylaminocinnamaldehyde to detect the product of the enzymatic activity. We tested the ability of the PRO Disc (Carr-Scarborough Microbiologicals, Decatur, Ga.) to detect L-proline-aminopeptidase produced by stock and fresh *Clostridium* isolates. In addition, we present recommendations for the use of colonial morphology on CCFA, the use of morphology upon Gram staining, and the detection of L-proline-aminopeptidase to identify *C. difficile* in stool specimens.

Stored *Clostridium* isolates were evaluated for their ability to

grow on CCFA and for their ability to produce L-proline-aminopeptidase with the PRO Disc (Carr-Scarborough Microbiologicals, Inc.). Fifty isolates of *C. difficile* and 15 different non-*C. difficile* isolates (a total of 32 strains) were grown in an anaerobic chamber (model 1024 anaerobe chamber; Forma Scientific, Marietta, Ohio) on prerduced, anaerobically sterilized (PRAS) modified CCFA (PRAS-CCFA) containing 500 μg of cycloserine per ml and 16 μg of cefoxitin (Anaerobe Systems, San Jose, Calif.) per ml and Centers for Disease Control and Prevention Agar (CDCA) plates (Remel, Lenexa, Kans.) at 35°C. The 50 *C. difficile* isolates included 48 cytotoxin-positive and 2 cytotoxin-negative isolates. One or two colonies of each isolate were taken from a culture plate and were rubbed into a water-moistened PRO Disc. The bacteria were allowed to react on the disc for 2 min. One drop of *p*-dimethylaminocinnamaldehyde was added to the disc and was allowed to react for 1 min before the results were read. The development of a dark pink to red color on the disc in which the inoculum was placed indicated a positive reaction. Spot indole tests and tests for lipase and lecithinase production on egg yolk agar (Remel) were performed with all 15 non-*C. difficile* species as described previously (5).

Culture for *C. difficile* was performed with 468 consecutive stool specimens received in the laboratory with a request for *C. difficile* toxin testing. Spore selection was performed by mixing 1.0 ml of the stool specimen with 1.0 ml of 95% ethanol (2, 3). After the suspension was incubated at room temperature for 1 h, 50 μl of the suspension was inoculated onto PRAS-CCFA and CDCA media, and the media were incubated in an anaerobic chamber at 35°C for 48 h. Colonies which resembled *C. difficile* (large, flat colonies with the appearance of ground glass) were Gram stained, tested for the production of L-proline-aminopeptidase by using the PRO Disc, and identified by the An-Ident system and the API 20A system (bio-Merieux, Hazelwood, Mo.) by following the manufacturer's instructions. Isolates of *C. difficile* were tested for their ability to produce cytotoxin by using a Pasteur pipet to remove an agar plug adjacent to a 48-hour-old isolated colony and substituting the agar plug for a stool specimen in a cell culture cytotoxin assay (Cytotoxi Test; Advanced Clinical Diagnostics, Toledo, Ohio) by following the manufacturer's instructions (4).

The results from the testing of stored clinical isolates are presented in Table 1. All 50 *C. difficile* isolates grew on CCFA

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TABLE 1. Results from testing stored clinical *Clostridium* isolates

<i>Clostridium</i> organism (no. of strains)	Test results ^a		
	PRO Disc	Indole	Other tests ^b
<i>C. bifementans</i> (2)	Pos	Pos	Lecithinase Pos, CCFA Pos ^c
<i>C. butyricum</i> (1)	Neg	Neg	
<i>C. cadaveris</i> (1)	Neg	Pos	
<i>C. clostridiforme</i> (1)	Neg	Neg	
<i>C. difficile</i> (50)	Pos	Neg	CCFA Pos
<i>C. glycolicum</i> (2)	Pos	Neg	CCFA Pos ^c
<i>C. histolyticum</i> (2)	Neg	Neg	
<i>C. innocuum</i> (2)	Neg	Neg	CCFA Pos ^d
<i>C. novyii</i> (1)	Neg	Neg	
<i>C. paraputrificum</i> (2)	Neg	Neg	
<i>C. perfringens</i> (3)	Neg	Neg	
<i>C. ramosum</i> (2)	Neg	Neg	
<i>C. septicum</i> (7)	Neg	Neg	
<i>C. sordellii</i> (2)	Pos	Pos	Lecithinase Pos
<i>C. sporogenes</i> (2)	Pos	Neg	Lipase Pos
<i>C. tertium</i> (2)	Neg	Neg	

^a Pos, positive result; Neg, negative result.

^b The other tests column lists positive reactions that may be useful for the further differentiation of the *Clostridium* species listed.

^c Colonial morphology on CCFA is different from that of *C. difficile*.

^d Colonial morphology on CCFA resembles that of *C. difficile*.

and CDCA and displayed the typical *C. difficile* colonial morphology of large, flat colonies with the appearance of ground glass and with yellowing of the medium on CCFA. In addition, all *C. difficile* isolates had the typical Gram staining morphology of gram-positive straight rods (0.5 to 1.9 by 3.0-6.9 μ m) with oval subterminal spores. All of the *C. difficile* isolates were PRO Disc positive. *Clostridium sordellii* and *Clostridium sporogenes* were PRO Disc positive but did not grow on CCFA. *Clostridium innocuum* was PRO Disc negative, grew on CCFA, and had a colonial morphology which resembled that of *C. difficile*. *Clostridium bifementans* and *Clostridium glycolicum* were PRO Disc positive, grew on CCFA, and had colonial morphologies different from that of *C. difficile*. All non-*C. difficile* clostridial species that grew on CCFA had Gram staining morphologies different from that of *C. difficile*.

Culture of 468 consecutive stool specimens yielded 47 isolates (39 cytotoxin-positive and 8 cytotoxin negative isolates) of *C. difficile* from 47 different patients. All of these isolates grew on both CCFA and CDCA plates with typical *C. difficile* colonial morphology, and all were PRO Disc positive. One isolate of *C. innocuum* and one isolate of *Clostridium clostridiforme* grew from stool specimens on CCFA. The *C. innocuum* isolate had a colonial morphology similar to that of *C. difficile* and was PRO Disc negative. The *C. clostridiforme* isolate did not display a colonial morphology typical of that of *C. difficile* and was considered weakly PRO Disc positive because a light pink color was observed on the PRO Disc where the inoculum was placed. Except for the isolate of *C. clostridiforme*, all positive reactions on the PRO Disc were clearly dark pink to red, and the negative reactions displayed no color development.

Culture of *C. difficile* from stool specimens is needed for epidemiological tracking of the nosocomial spread of infection and can be a valuable tool for the laboratory diagnosis of *C.*

difficile-associated diarrhea (7). Up to 30% more patients with *C. difficile*-associated diarrhea can be identified with the combination of culture and toxin testing of stool specimens (8). Peterson et al. (9) have demonstrated the utility of culture and continue to promote toxin testing plus culture isolation as the "gold standard" for the laboratory confirmation of *C. difficile*-associated diarrheal disease.

For the rapid identification of *C. difficile*, the PRO Disc can be used in conjunction with a primary culture medium that is both selective and differential. A variety of culture media have been described for the isolation of *C. difficile* from stool specimens. If the medium is not differential, a long-wave UV lamp (Wood's lamp) can be used to detect *C. difficile* colonies by observation of their characteristic yellow-green fluorescence (2). We have found that the combination of alcohol treatment and inoculation of a prereduced CCFA plate provides optimal recovery of *C. difficile* from stool specimens. Some reports indicate that some isolates of *C. difficile* do not grow well on CCFA on primary inoculation (1, 2). The data from the current study suggest that this is an uncommon occurrence in our laboratory.

We tested 87 toxigenic and 10 nontoxigenic isolates of *C. difficile* for the production of the enzyme L-proline-aminopeptidase by using the PRO Disc. All 97 isolates of *C. difficile* were PRO Disc positive. The colonial morphology of *C. innocuum* growing on CCFA is similar to that of *C. difficile*, and like *C. difficile*, *C. innocuum* can be isolated from stool specimens. Therefore, we recommend confirming *C. difficile* isolates by using colonial morphology, appearance on Gram staining, and the PRO Disc. The list price for PRO Discs is \$12.00 per vial of 25 discs, or \$0.48 per disc. The PRO Disc is a sensitive, specific, and inexpensive method for confirmation of isolates of *C. difficile* from media that are both selective and differential.

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