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NOTES

Presumptive Identification of *Clostridium difficile* by Detection of *p*-Cresol in Prepared Peptone Yeast Glucose Broth Supplemented with *p*-Hydroxyphenylacetic Acid

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Prereduced, anaerobically sterilized peptone yeast glucose broth was supplemented with *p*-hydroxyphenylacetic acid and used for the presumptive identification of *Clostridium difficile*. Two hundred eighty-two organisms were grown in this medium for 18 h and tested for *p*-cresol production by gas-liquid chromatography. All 49 stock and reference strains of *C. difficile* and 19 organisms confirmed as *C. difficile* produced *p*-cresol. *p*-Cresol was not produced by 53 negative control or 161 test organisms. The system was convenient and effective.

Clostridium difficile-related enteric disease (15) is diagnosed through a combination of clinical and laboratory procedures, including isolation of the pathogen (9; E. J. Baron, Clin. Microbiol. Newsl. **11**:118–120, 1989). George et al. (4) developed cycloserine-cefoxitin-fructose-egg yolk agar (CCFA) for the isolation of *C. difficile*, and the organism was observed to grow on this medium with characteristic morphological and fluorescence properties. Such characteristics are used by several laboratories as criteria for the presumptive identification of the organism on CCFA. However, cost and time-related factors may dictate the use of other media, which are often less selective. This makes it unsafe to rely on visual characteristics alone. Consequently, an additional test is sometimes needed, as in our laboratory, where anaerobic brain heart infusion agar (6) containing 5% sheep blood (BHIA) supplemented with 16 μg of cefoxitin (Merck Sharp & Dohme, West Point, Pa.) per ml is used instead of CCFA. *C. difficile* and other organisms produce raised, creamy colonies on primary plates of BHIA-cefoxitin.

Elsden et al. (3) have found that *C. difficile* produces *p*-cresol through the breakdown of tyrosine and have suggested that *p*-hydroxyphenylacetic acid (PHPA) is the intermediary. D'Ari and Barker (2) have shown that the organism decarboxylates PHPA to *p*-cresol. This characteristic has been exploited to design agar media (CCFA with PHPA) for the rapid presumptive identification of *C. difficile*, using *p*-cresol as the marker (8, 13). However, these media take time to prepare, and it can take more than 48 h for adequate amounts of *p*-cresol to build up around *C. difficile* colonies (our observation). Nunez-Montiel et al. (11) describe norleucine-tyrosine broth for the rapid identification of *C. difficile*. They do not, however, indicate whether the medium allows for *p*-cresol detection in less than 24 h. Navarro-Alonso et al. (10) have described a liquid medium which requires 48 h of incubation before detectable amounts of *p*-cresol appear. Both these media must be prepared in the laboratory. In this report we describe how the previously described (6) prereduced, anaerobically sterilized (PRAS) peptone yeast glucose broth (PYG) can be supplemented with PHPA (PYG + PHPA) and used for a one-step presumptive identification of *C. difficile* through *p*-cresol detection in 18 h.

A total of 282 organisms were used in this study. They included 47 stock strains of *C. difficile*, 180 test organisms recently isolated on BHIA-cefoxitin from 80 patients, *C. difficile* ATCC 43593 and ATCC 43594, and 53 negative control species. The negative control species consisted of seven *Bacteroides fragilis*, one *Bacteroides ovatus*, one *Bacteroides thetaiotaomicron*, one *Bacteroides uniformis*, three *Clostridium sporogenes*, two *Clostridium paraputrificum*, two *Clostridium septicum*, one *Clostridium bifermentans*, one *Clostridium butyricum*, one *Clostridium glycolicum*, one *Clostridium histolyticum*, one *Clostridium innocuum*, one *Clostridium oceanicum*, one *Clostridium perfringens*, one *Clostridium sordellii*, one *Clostridium subterminale*, one *Clostridium tertium*, one *Fusobacterium necrophorum*, one *Fusobacterium* sp., two *Peptostreptococcus anaerobicus*, seven *Enterobacter* spp., three *Escherichia coli*, one *Citrobacter freundii*, one *Klebsiella pneumoniae*, one *Proteus mirabilis*, one *Salmonella typhimurium*, one *Serratia marcescens*, one *Shigella dysenteriae*, one *Shigella sonnei*, one *Yersinia enterocolitica*, two *Enterococcus* spp., one *Aeromonas* sp., and one facultative *Bacillus* sp. Facultative aerobes were identified by routine procedures, and anaerobes were identified as described by Holdeman et al. (6) by using PRAS II (Scott Laboratories, Fiskeville, R.I.) biochemicals. Base-line tests used for confirming the identity of *C. difficile* were the following: barnyard odor; Gram stain; spore formation; glucose, fructose, lactose, maltose, mannitol, mannose, sucrose, and xylose fermentations; esculin hydrolysis; starch pH; gelatin liquefaction; lecithinase and indole production; nitrate reduction; and the volatile fatty acid profile.

PYG + PHPA containing 0.48 mg of PHPA (Sigma Chemical Co., St. Louis, Mo.) per ml was prepared by adding 0.15 ml of a 16-mg/ml, filter-sterilized (pore size, 0.45 μm) stock

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solution of PHPA in double-distilled H_2O to 5-ml quantities of PYG (PRAS II; Scott Laboratories). Care was taken not to introduce air while piercing through the alcohol-flamed septum of the PRAS II tube. PYG + PHPA was stored at 4°C for up to 3 weeks. However, subsequent observations indicated that the medium could be left at room temperature for the same period without affecting its quality. The PHPA stock solution was stored in 2-ml aliquots at −20°C for up to 5 months.

For *p*-cresol screening, organisms were subcultured on plain BHIA and checked for purity, and two to four colonies were inoculated anaerobically into 5 ml of PYG + PHPA. Tubes were incubated at 37°C for 18 h in a water bath. *p*-Cresol was extracted by adding 1.0 ml of diethyl ether (Fisher Scientific, Nepean, Ontario, Canada) to the entire 5 ml of the 18-h broth culture, vortexing the culture for 15 s, and centrifuging the culture at 2,800 × *g* for 15 min at 4°C. The diethyl ether layer (0.8 μl) was subjected to gas-liquid chromatography. A 0.4-μl amount (2.75 nmol) of a 6.9 mM solution of *p*-cresol (Sigma) in diethyl ether was used to mark the *p*-cresol peak. A gas chromatograph (Sigma 2000; The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a flame ionization detector (FID) and a stainless steel column (0.32 cm [inner diameter] by 1.83 m) packed with 8% SP-1000 and 1% H_3PO_4 on chromosorb W/AW 80/100 mesh was used for analysis. An oven temperature of 185°C for 3.9 min, with injector and detector temperatures maintained at 235°C, was used. Helium was the carrier gas and was used at a rate of 30 ml/min. An integrator (LCI 100; Perkin-Elmer) was used to record peaks; chart speed and attenuation were set at 100 mm/min and 256 mV full-scale deflection, respectively.

All 282 organisms were screened for *p*-cresol. *C. difficile* and *C. butyricum* were used as positive and negative controls, respectively. Test organisms were screened once each; the remaining organisms were tested twice. *p*-Cresol was produced by all 47 stock strains of *C. difficile*, *C. difficile* ATCC 43593 and ATCC 43594, and 19 test organisms confirmed as *C. difficile* (Fig. 1A). Fifty-three negative control strains and 161 test organisms did not produce *p*-cresol (Fig. 1B). Thirty-four *p*-cresol-negative test organisms, which were selected randomly, were identified as bacteroides, coliforms, clostridia other than *C. difficile*, and enterococci.

p-Cresol production by mixed cultures containing *C. difficile* was evaluated. Individual tubes of PYG + PHPA containing two colonies of *C. difficile* were set up. Each was then inoculated with two to four colonies of one of each of the following organisms and tested after 18 h: *Bacteroides fragilis*, *C. butyricum*, *C. sporogenes*, *Fusobacterium* sp., *P. anaerobicus*, *Escherichia coli*, *Enterobacter* spp., and *Enterococcus* spp. *C. difficile* was also tested with each of the following pairs of organisms: *B. fragilis* and *C. butyricum*, *C. butyricum* and *Escherichia coli*, *Escherichia coli* and *Enterobacter* spp., and *Escherichia coli* and *Enterococcus* spp. In each case, *p*-cresol production was not inhibited, and peaks resembling that in Fig. 1A were obtained.

C. difficile did not produce *p*-cresol in PYG without PHPA, nor was *p*-cresol detected in uninoculated PYG or PYG + PHPA.

Elsden et al. (3) have found that *Clostridium scatologenes* and *C. difficile* were the only clostridia, of those they studied, that produced *p*-cresol from tyrosine. *p*-Cresol production by these two organisms has been confirmed by others (8, 13). *C. scatologenes* has never been encountered in our laboratory. In this investigation, *C. difficile* was the only organism that produced *p*-cresol, and results of the study show how commercially prepared PYG can be conveniently modified and used for its presumptive identification. The *p*-cresol extraction procedure and the gas-liquid chromatography run described here are shorter than those described by other workers (8, 10, 11, 13). Interpretation of chromatograms involved a search for a single component, *p*-cresol. Variation in the size of the *p*-cresol peak among strains of *C. difficile* did not affect interpretation.

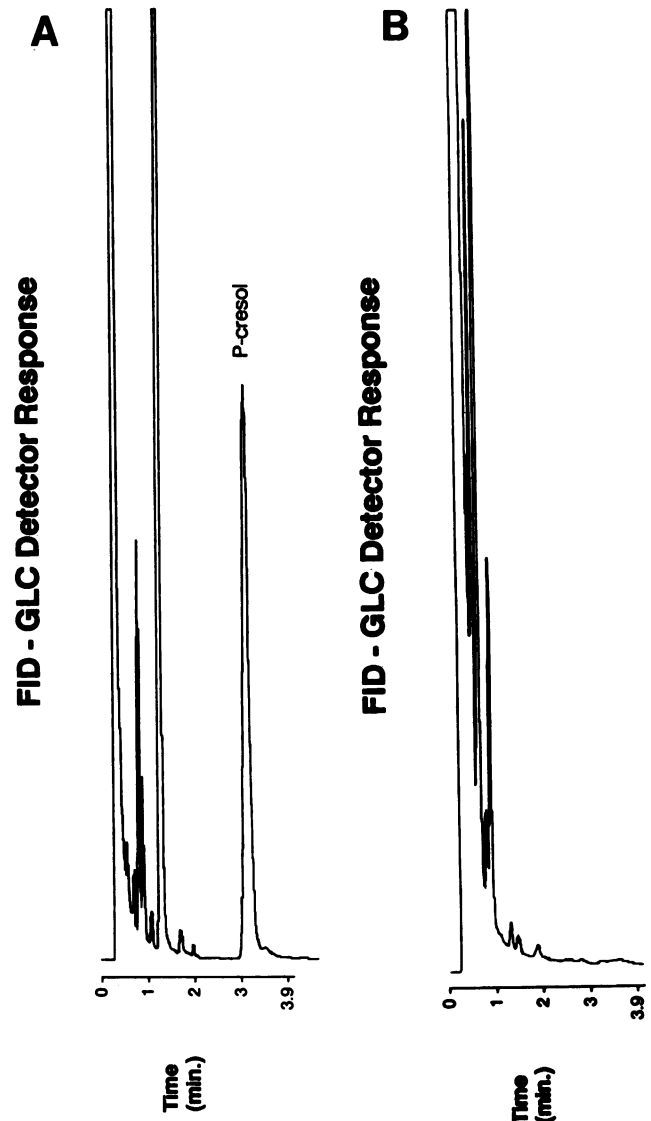

FIG. 1. Chromatograms following extraction for *p*-cresol. Organisms were grown in PYG + PHPA for 18 h. (A) Growth of *C. difficile*. (B) Growth of *C. butyricum*.

Microsystems are available for the rapid identification of *C. difficile* (1). In our experience, none is without flaws. We agree with Lyerly et al. (9) that these microsystems are in need of critical evaluation. *p*-Cresol detection, as described in this report, could be used as an adjunct to any one of the microsystems.

Several investigators (5, 7, 12, 14) have attempted to predict the presence of *C. difficile* in stool specimens by screening such specimens for *p*-cresol and fatty acids. Their results seem to indicate that such products, when detected in

stool specimens, are not specific indicators of the presence of *C. difficile*. Lyerly et al. (9) felt that these systems were not specific enough for use in clinical diagnosis.

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LITERATURE CITED

1. **Bate, G.** 1986. Comparison of Minitek Anaerobe II®, API AnIdent®, and RapID ANA® Systems for identification of *Clostridium difficile*. Am. J. Clin. Pathol. **85**:716–718.
2. **D'Ari, L., and H. A. Barker.** 1985. p-Cresol formation by cell-free extracts of *Clostridium difficile*. Arch. Microbiol. **143**: 311–312.
3. **Elsden, S. R., M. G. Hilton, and J. M. Waller.** 1976. The end products of metabolism of aromatic amino acids by clostridia. Arch. Microbiol. **107**:283–288.
4. **George, W. L., V. L. Sutter, D. Citron, and S. M. Finegold.** 1979. Selective and differential medium for isolation of *Clostridium difficile*. J. Clin. Microbiol. **9**:214–219.
5. **Gianfrilli, P., A. Pantosti, and I. Luzzi.** 1985. Evaluation of gas-liquid chromatography for the rapid diagnosis of *Clostridium difficile* associated disease. J. Clin. Pathol. **38**:690–693.
6. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
7. **Levett, P. N.** 1984. Detection of *Clostridium difficile* in faeces by direct gas liquid chromatography. J. Clin. Pathol. **37**:117–119.
8. **Levett, P. N., and K. D. Phillips.** 1985. Gas chromatographic identification of *Clostridium difficile* and detection of cytotoxin from a modified selective medium. J. Clin. Pathol. **38**:82–85.
9. **Lyerly, D. M., H. C. Krivan, and T. D. Wilkins.** 1988. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev. **1**:1–18.
10. **Navarro-Alonso, T., A. Bernabeu-Esclapez, P. L. Valero-Guillen, and F. Martin-Luengo.** 1985. Identification of *Clostridium difficile* by detection of p-cresol in a cooked meat medium. Eur. J. Clin. Microbiol. **4**:438–439.
11. **Nunez-Montiel, O. L., F. S. Thompson, and V. R. Dowell, Jr.** 1983. Norleucine-tyrosine broth for rapid identification of *Clostridium difficile* by gas-liquid chromatography. J. Clin. Microbiol. **17**:382–385.
12. **Pepersack, F., M. Labbe, C. Nonhoff, and E. Schoutens.** 1983. Use of gas-liquid chromatography as a screening test for toxigenic *Clostridium difficile* in diarrheal stools. J. Clin. Pathol. **36**:1233–1236.
13. **Phillips, K. D., and P. A. Rogers.** 1981. Rapid detection and presumptive identification of *Clostridium difficile* by p-cresol production on a selective medium. J. Clin. Pathol. **34**:642–644.
14. **Potvliege, C., M. Labbe, and E. Yourassowsky.** 1981. Gas liquid chromatography as screening test for *Clostridium difficile*. Lancet **ii**:1105.
15. **Walker, R. C., P. J. Ruane, J. E. Rosenblatt, D. M. Lyerly, C. A. Gleeves, T. F. Smith, P. F. Pierce, Jr., and T. D. Wilkins.** 1986. Comparison of culture, cytotoxicity assays, and enzyme-linked immunosorbent assay for toxin A and toxin B in the diagnosis of *Clostridium difficile*-related enteric disease. Diagn. Microbiol. Infect. Dis. **5**:61–69.