

Gas chromatographic identification of *Clostridium difficile* and detection of cytotoxin from a modified selective medium

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SUMMARY A modification of an existing selective medium for *Clostridium difficile* is described. Inclusion in the medium of DL *nor*-leucine and *p*-hydroxyphenylacetic acid enables identification of *C difficile* to be made directly from primary isolation plates by gas chromatographic detection of caproic acid and *p*-cresol. Plugs of agar withdrawn from the selective medium also allow the detection of cytotoxin production in vitro.

The isolation of *Clostridium difficile* from faeces has been greatly facilitated by the evolution of efficient selective media and procedures.¹⁻⁴ These media are not absolutely selective,⁵ however, and thus all presumptive isolates of *C difficile* require definitive identification. The time taken to obtain pure cultures and perform biochemical tests may delay identification by as much as five days, which is clearly undesirable in the diagnostic laboratory. In addition, it may be necessary to determine the ability of an isolate to produce cytotoxin in vitro, since both cytotoxigenic and non-cytotoxigenic strains of *C difficile* may be present in faecal specimens.⁶ In this paper we describe the use of two simple tests carried out on primary isolation plates to confirm the presence of *C difficile* and its cytotoxigenic status.

Material and methods

BACTERIAL STRAINS

Fresh clinical isolates of the following species were studied: five *C bifementans*, two *C butyricum*, 19 *C difficile*, two *C glycolicum*, two *C innocuum*, one *C paraputrificum*, five *C sordellii*, and 15 *C sporogenes*. *C difficile* NCTC 11209 and *C scatologenes* NCTC 9800 were also studied.

STOOLS

One hundred and ninety stool specimens were received from hospitals throughout the United Kingdom. All samples examined were from patients

with diarrhoea which was thought to be due to *C difficile* and were processed immediately after receipt.

MEDIA

Modified cycloserine cefoxitin fructose agar (CCFA medium) was prepared containing the following: *C difficile* agar base (Oxoid CM601) 69 g/l; 50% fresh egg yolk emulsion 36 ml/l; freeze dried cycloserine and cefoxitin (Oxoid SR96), 250 mg/l and 8 mg/l, respectively; 2% (wt/vol) neutral red solution 1.4 ml/l; *p*-hydroxyphenylacetic acid (Sigma Chemical Co) 1 g/l; DL *nor*-leucine (BDH Ltd) 2 g/l. A similar medium was prepared without the addition of antibiotics. Five percent horse blood agar plates were obtained commercially (Tissue Culture Services, Slough, Berks).

CULTURE

All specimens were subjected to alcohol shock⁴ to select for spores, followed by plating on to modified CCFA medium and blood agar plates. All plates were incubated in an anaerobic cabinet at 37°C for 48 h. Enrichment cultures in cooked meat broth were performed as described previously.⁷

IDENTIFICATION

All colony types with morphologies typical of *C difficile* were subcultured on to blood agar plates and identified using the criteria of Holdeman *et al.*⁸

CYTOTOXIN ASSAY

Faecal cytotoxin was assayed as described previously.⁷ Production of cytotoxin in vitro was detected on CCFA plates⁹; a plastic drinking straw was used

to withdraw two plugs from an area of the plate adjacent to the growth of suspected *C difficile*. One plug was placed in a tube containing a monolayer of MRC 5 cells; the second plug was placed in another tube with the addition of five drops of *C sordellii* antitoxin (Wellcome Diagnostics Ltd). The tubes were incubated at 37°C and examined after 24 h and 48 h for a characteristic cytopathic effect neutralised by *C sordellii* antitoxin. A conventional broth technique for the in vitro production of cytotoxin was used in parallel with the plate method.⁹

GAS-LIQUID CHROMATOGRAPHY

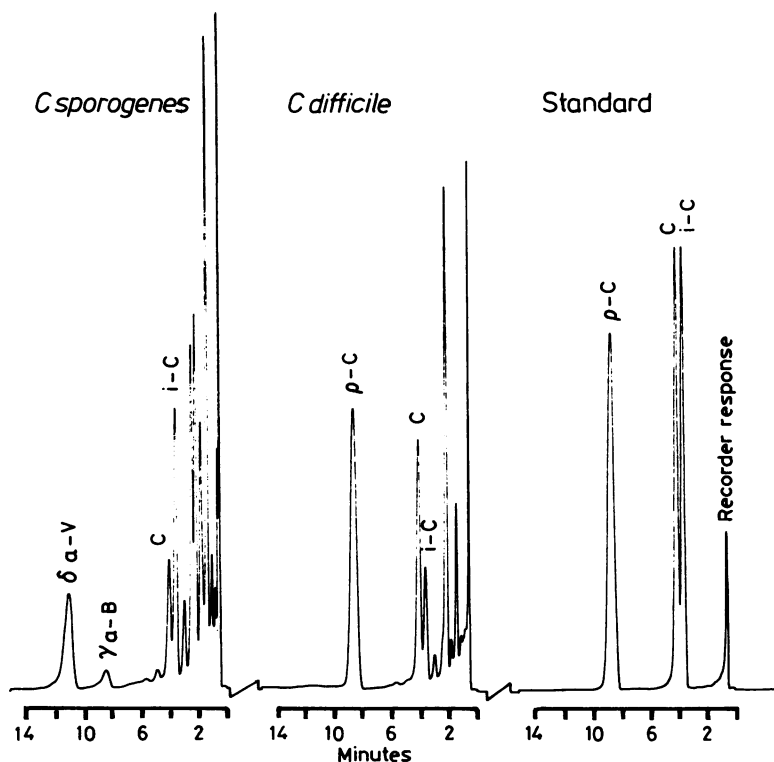
A plug of agar was withdrawn from an area of each CCFA plate underlying growth of suspected *C difficile*.³ One drop of sterile distilled water was placed on the plug and left for 10 min at room temperature. Samples (1 µl) of the aqueous extract were assayed by gas-liquid chromatography for the presence of volatile fatty acids and *p*-cresol. A Pye Unicam Series 204 gas chromatograph was equipped with a flame ionisation detector, employing a 1.5 m × 4 mm internal diameter glass column

packed with 10% FFAP on diatomite CLQ (JJ's Chromatography Ltd). The oven temperature was 200°C and the carrier gas was nitrogen at a flow rate of 50 ml/min. Attenuation of the instrument was routinely set at 2×10^2 (2×10^{-10} Amps/full scale deflection). The recorder chart speed was 0.5 cm/min.

Results

Pure cultures of *C difficile* on the modified CCFA medium without antibiotics produced iso-caproic and caproic acids and *p*-cresol (Figure). *C bifermensans*, *C sordellii*, and *C sporogenes* produced iso-caproic, caproic, γ -amino butyric, and δ -amino valeric acids. Iso-caproic acid, caproic acid, and *p*-cresol were not detected in uninoculated CCFA medium nor in cultures of *C butyricum*, *C glycolicum*, *C innocuum*, or *C paraputrificum*. *C scatologenes* NCTC 9800 produced iso-caproic acid and *p*-cresol but not caproic acid.

C difficile was isolated from 66/190 stools (35%). All plates were examined by gas-liquid chromato-



Gas chromatograms of a standard solution of iso-caproic acid (i-C), caproic acid (C) and *p*-cresol (*p*-C), and cultures of *C difficile* and *C sporogenes*. $\delta \alpha-V$ = δ -amino valeric acid; $\gamma \alpha-B$ = γ -aminobutyric acid.

graphy, and 66 contained a characteristic pattern of peaks of iso-caproic acid, caproic acid, and *p*-cresol. No other inoculated plates contained all of these metabolites. All of the 66 specimens positive by gas-liquid chromatography yielded a growth of *C difficile* ranging from four colonies to a pure, confluent growth; two further specimens produced a light growth of *C sporogenes*, which was easily distinguishable from *C difficile* by its lipase reaction¹⁰ and its characteristic gas-liquid chromatography pattern (Figure).

Faecal cytotoxin was found in 58 specimens, from 50 of which *C difficile* was isolated. Three of the eight faecal specimens which were cytotoxin positive and culture negative were from one patient who was originally culture and cytotoxin positive. Additionally, 16 faecal specimens yielded *C difficile* on culture but did not contain detectable cytotoxin.

Culture plates from all cytotoxin positive stools were tested for in vitro production of cytotoxin. All *C difficile* positive cultures from these stools contained cytotoxin; no cytotoxin was detected in those cultures which did not yield *C difficile*. Of the 16 *C difficile* cultures isolated from cytotoxin negative stools, 13 produced cytotoxin in vitro and three did not, which suggests that excretion of non-cytotoxigenic strains in adults may be rare. There was 100% correlation between the results of the agar plug cytotoxin assay and the conventional broth technique.

Discussion

Production of *p*-cresol by clostridia has been described previously,^{11,12} but conflicting observations were recorded. Elsdén *et al*¹¹ found that *C difficile* and *C scatologenes*, but not *C bifermentans*, *C sordellii*, or *C sporogenes*, produced *p*-cresol from L-tyrosine, whereas Bone *et al*¹² reported *p*-cresol production by *C butyricum*, *C paraputrificum*, *C septicum*, and *C sporogenes*. None of the strains of *C bifermentans*, *C sordellii*, or *C sporogenes* examined in this study produced *p*-cresol; however, all produced γ -amino butyric acid and δ -amino valeric acid, as described by Mead.¹³

Nunez-Montiel *et al*¹⁴ reported the production of caproic acid from *nor*-leucine by *C difficile*. We have found that caproic acid is also produced by *C bifermentans*, *C sordellii*, and *C sporogenes* in addition to *C difficile* in media supplemented with *nor*-leucine. *C difficile*, *C bifermentans*, and *C sordellii* are related antigenically,^{15,16} and these findings provide further evidence of their taxonomic proximity. Makin¹⁷ suggested that *C difficile* could be identified by the detection of volatile fatty acids produced in primary culture plates, but since *C bifermentans*, *C*

sordellii, *C sporogenes*, and *C difficile* produce closely similar volatile acid profiles this method is quite clearly unsatisfactory if used as the sole criterion for identification.

The use of plugs of agar for the detection of cytotoxin was described by Chang and Gorbach.⁹ These authors reported the detection of cytotoxin from cultures containing as few as two colonies of *C difficile*. Our results confirm this observation; detection of iso-caproic and caproic acids and *p*-cresol was possible with a similar number of colonies. Plugs of agar have been used successfully to test for the production of *p*-cresol by *C difficile*³ and more recently for the production of volatile fatty acids by *C difficile*.¹⁷

The incorporation of egg yolk in the modified CCFA medium described here permits the differentiation of *C difficile* from other commonly occurring clostridia which have similar metabolic profiles. Reliance on the detection of volatile fatty acids in the series acetic to iso-caproic¹⁷ for the identification of *C difficile* is unsatisfactory. With the exception of *C difficile*, no lecithinase or lipase negative clostridia have been found to produce iso-caproic acid, caproic acid, and *p*-cresol. The modified CCFA medium described above facilitates definitive identification of *C difficile* within 24–48 h.

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