Rapid detection and presumptive identification of *Clostridium difficile* by *p*-cresol production on a selective medium

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SUMMARY A modification of a selective medium for *Clostridium difficile* is described. The ability of *Cl difficile* to produce *p*-cresol from *p*-hydroxy phenyl acetic acid provides a means for the rapid, sensitive detection and presumptive identification of this species in faecal cultures.

The role of *Clostridium difficile* and its toxin in the aetiology of pseudomembranous colitis is now firmly established.¹⁻⁷ Diagnosis of this disease may be difficult⁸ and is achieved by a combination of clinical and laboratory procedures including sigmoidoscopy and biopsy, screening of faeces for specific toxin, and cultural methods aimed at isolation of the pathogen. A significant innovation in this field was the development by George *et al.*⁹ of an effective selective medium for *Cl difficile*.

Elsden *et al.*¹⁰ reported that *Cl difficile* produced *p*-cresol as an end-product of tyrosine metabolism and suggested that *p*-hydroxy phenyl acetic acid was the precursor of *p*-cresol in the metabolic pathway. This paper describes a modification of the selective medium of George *et al.*,⁹ designed to exploit *p*-cresol production for the rapid, sensitive detection and presumptive identification of *Cl difficile* in faecal cultures.

Material and methods

MEDIUM

The medium of George *et al.*⁹ (known as CCFA) comprises a buffered proteose peptone agar base incorporating fructose, neutral red indicator, egg yolk suspension and solutions of cycloserine and cefoxitin. In this study the medium was prepared according to the original specifications but with the following modifications:

The antibiotic concentrations were reduced by half, giving a final concentration in the medium of 250 µg/ml of cycloserine and 8 µg/ml of cefoxitin.

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A filter-sterilised solution of *p*-hydroxyphenyl acetic acid was added to the molten medium with the antibiotic solutions to give a final concentration of 0.1% wt/vol.

ORGANISMS

Seven stock strains of *Cl difficile* were employed in this study. These strains had been isolated from faeces and were identified according to the criteria of Holdeman *et al.*¹¹ In addition, two reference strains of *Cl difficile* (NCTC 11223 and NCIB 10666) and a reference strain of *Cl scatologenes* (NCTC 9800) were included for comparison in all investigations. All strains were initially screened for production of *p*-cresol from tyrosine in an aromatic amino acid broth similar to that of Elsdén *et al.*¹⁰ and subsequently for growth and *p*-cresol production from *p*-hydroxy phenyl acetic acid on modified CCFA medium; *p*-cresol was detected by gas chromatography as described below.

FAECAL SPECIMENS

One hundred randomly-selected specimens of faeces obtained from patients under investigation for other bacterial pathogens were cultured directly on the modified medium. In addition, two hundred samples of faeces were obtained from healthy adults (food handlers undergoing statutory screening for carriage of food-poisoning organisms). These specimens were sampled with dry swabs and transferred to peptone water for enrichment. The peptone water cultures were subsequently plated on the modified medium after 48 h incubation.

CULTURAL CONDITIONS

All cultures were incubated at 37°C for up to 48 h in anaerobic jars; anaerobiosis was achieved by means

of the evacuation-replacement technique of Willis,¹² using a gas mixture of 90% H₂ and 10% CO₂.

GAS CHROMATOGRAPHY

A Pye Unicam 204 Series gas chromatograph equipped with a flame ionisation detector was used for *p*-cresol analysis. A glass analytical column 1.5 m × 4 mm (internal diameter), was packed with 5% OV17 on Diatomite CQ (JJ's Chromatography Ltd, Kings Lynn, Norfolk) and was operated isothermally at 110°C. The injection port and the detector temperatures were maintained at 250°C. Nitrogen was employed as the carrier gas at a flow rate of 50 ml/min. Attenuation of the instrument was routinely set at 2×10^2 (2×10^{-10} A fsd). The recorder chart speed was 0.5 cm/min.

A *p*-cresol standard was prepared in aqueous solution at a concentration of 10 µmol/ml. Qualitative analysis was performed by extraction of *p*-cresol from 0.1 ml of the standard solution by 0.5 ml of diethyl ether followed by injection of 1 µl of the ether extract into the chromatograph (peak A on Figure).

was then withdrawn by microsyringe for gas chromatographic analysis of *p*-cresol.

Results

GROWTH OF STOCK STRAINS

All stock and reference strains of *Cl difficile* produced small, but visible colonies after overnight incubation on the modified medium. After further incubation these colonies developed the flat, ground-glass morphology distinctive of this species. In addition all strains of *Cl difficile* produced detectable amounts of *p*-cresol from *p*-hydroxy phenyl acetic acid usually within 18 h on the modified medium (peak C on Figure): the concentration of *p*-cresol in the medium increased to approximately 10 µmol/ml after 48 h incubation (peak D on Figure).

The only other species of *Clostridium* reported to produce *p*-cresol is *Cl scatologenes*.¹⁰ The only strain of this organism available failed to grow on this selective medium.

ISOLATION OF *CL DIFFICILE* FROM FAECAL SPECIMENS

The number of strains of *Cl difficile* isolated from patients suspected of harbouring intestinal pathogens is shown in Table 1. None of these patients was diagnosed as having pseudomembranous colitis. All clinical specimens were directly cultured on the modified medium and were incubated for periods varying between 18 and 48 h. In most instances, specimens containing *Cl difficile* yielded a moderate to heavy growth of the organism. Some loss of selectivity was apparent as a consequence of the lowered antibiotic concentrations, particularly in heavily inoculated areas; colonies of *Cl difficile* nevertheless were usually apparent on other areas of the medium and could be presumptively identified by detection of *p*-cresol. Culture of three specimens yielded only one or two colonies of the organism. These colonies were apparent only after 48 h incubation and although they were largely obscured by unsuppressed intestinal flora, they could be readily detected and identified by *p*-cresol production. All strains of *Cl difficile* isolated were subsequently identified by the criteria of Holdeman *et al.*¹¹ No other species of *Clostridium* was isolated from these faecal cultures. Under standard conditions of test

Gas chromatograms of: A *p*-cresol standard (10 µmol/ml); B uninoculated medium; C *p*-cresol production by *Cl difficile* at 24 h; D *p*-cresol production by *Cl difficile* at 48 h.

Production of *p*-cresol from *p*-hydroxy phenyl acetic acid by cultures of *Cl difficile* on the modified medium was detected as follows: five plugs of agar (total volume approximately 0.1 ml) were removed by means of a hollow tyne, either from an area of confluent growth or from beneath five discrete colonies and transferred to a test tube. Diethyl ether (0.5 ml) was added and the contents were vortex-mixed for 30 s. One microlitre of the clear ether layer

Table 1 Isolation rates of *Cl difficile* from patients' faeces

	No of specimens	No of isolates	% isolation rate
Infants (<1 year)	16	7	43.6
Others (>1 year)	84	4	4.8

there were no false-positive or false-negative results, nor was *p*-cresol produced in the uninoculated medium (peak B on Figure). The isolation rate of *Cl difficile* from normal adult faeces is shown in Table 2.

Table 2 Isolation rate of *Cl difficile* from normal adult faeces

No of specimens	No of isolates	% isolation rate
200	4	2

Discussion

In a comprehensive survey of the amino acid fermenting clostridia, Elsdén *et al.*¹⁰ found that *Cl difficile* possessed the ability to produce *p*-cresol from tyrosine. This characteristic appears to be shared only with *Cl scatologenes*, an observation which was confirmed in this study.

In a preliminary investigation, 0.2% wt/vol of L-tyrosine was incorporated in the selective medium. Stock strains of *Cl difficile* however failed to produce detectable amounts of *p*-cresol within 48 h. Accordingly *p*-hydroxy phenyl acetic acid was substituted for L-tyrosine in an attempt to obtain positive results. After overnight incubation of primary cultures of *Cl difficile*, *p*-cresol was usually detectable in the modified selective medium by gas chromatography. Moreover, the distinctive odour of *p*-cresol was often discernible on examination of culture plates, making gas chromatography essentially confirmatory.

The antibiotic concentrations as specified by George *et al.*⁹ (500 µg/ml of cycloserine and 16 µg/ml of cefoxitin) were halved in this study because the unmodified medium reduced qualitatively and quantitatively the growth of stock strains of *Cl difficile*. As a result of using lowered concentrations of antibiotics, some growth of extraneous faecal flora was observed. The loss of selectivity is inevitable, but was acceptable in the light of the positive results obtained.

The isolation of *Cl difficile* from patients with diarrhoea but without obvious pseudomembranous colitis is of special interest, since Larson¹³ has suggested that these patients may represent an extension of the clinical spectrum of disease in which *Cl difficile* is implicated. From the results of the present investigation it appears that in healthy adults there is a carriage rate of *Cl difficile* of approximately 2%. The high carriage rate among infant patients suggests this may be a normal phenomenon.¹⁴

Presumptive identification of *Cl difficile* is currently based on distinctive microscopic and colonial

morphology, which may require 48 h incubation. Gas chromatographic detection of *p*-cresol in the new medium provides a specific and sensitive chemical marker for this species in the absence of mature colony development.

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