

# Detection of *Clostridium difficile* in faeces by direct gas liquid chromatography

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**SUMMARY** Stool specimens examined for the presence of *Clostridium difficile* and its cytotoxin were screened by gas liquid chromatography for the presence of volatile fatty acids and *p*-cresol. Twenty seven of 110 (25%) stools yielded *C difficile* or cytotoxin; iso-valeric acid was detected in 63/110 (57%) and iso-caproic acid in 18/110 (16%) stools. Para-cresol was found in 24/71 (34%) stools examined. Iso-valeric acid was detected in 85% of stools positive for *C difficile*, whereas iso-caproic acid (41%) and *p*-cresol (52%) were found in much lower numbers of *C difficile*-positive stools.

It is concluded that gas chromatographic detection of volatile fatty acids or *p*-cresol in faeces are not satisfactory screening tests for the presence of *C difficile*.

The development of efficient selective media<sup>1-3</sup> and isolation procedures<sup>4</sup> have greatly simplified the detection of *Clostridium difficile* in stool specimens, enabling many laboratories lacking the facilities for cytotoxin assay to screen specimens for the organism.

Isolation of *C difficile*, however, generally requires at least 48 h incubation. For those laboratories without access to tissue culture facilities a rapid method for the detection of *C difficile* in faeces would be of considerable value. The use of counter-immunoelectrophoresis to detect the cytotoxin of *C difficile* has been reported,<sup>5-7</sup> but the value of this test is reduced by the use of impure antitoxin preparations.<sup>8,9</sup> A fluorescent antibody test applied to stool smears detected 81% of *C difficile* positive stools from patients with antibiotic associated diarrhoea,<sup>10</sup> but many cross reactions were noted between antisera raised against *C difficile* and other clostridia. In addition, a high proportion of false positive results was observed in stools from normal adults and patients with diarrhoea not associated with antibiotics. Examination of Gram stained films of faeces was found to be of little value in the diagnosis of *C difficile* infection.<sup>11</sup>

Gas liquid chromatography of stool specimens has been employed,<sup>12</sup> but doubts have been expressed about the predictive value of this test.<sup>13</sup> This paper reports the results of an attempt to predict the pres-

ence of *C difficile* in stools on the basis of gas chromatographic detection of volatile fatty acids and of *p*-cresol.

## Material and methods

### STOOLS

One hundred and ten stool specimens were received from hospitals throughout the United Kingdom. All samples examined were from patients suspected of suffering from *C difficile*-associated diarrhoea.

### MEDIA

Modified cycloserine cefoxitin fructose agar (CCFA medium)<sup>3</sup> was prepared containing *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 µg/ml and 8 µg/ml, respectively; 2% (wt/vol) neutral red solution 1.4 ml/l; and *p*-hydroxy phenyl acetic acid 1 g/l.

Cooked meat broths containing dried meat granules (Lab M Lab 24) 37.5 g/l, brain heart infusion (Lab M Lab 49) 17.5 g/l, yeast extract (Oxoid L21) 6 g/l, and haemin/menadione solution (Gibco) 10 ml/l were dispensed into universal containers and autoclaved at 121°C for 15 min.

Plates containing 5% horse blood agar were obtained commercially (Lab M).

### CULTURE

All specimens were cultured directly onto modified CCFA selective medium<sup>3</sup> and were also subjected to

alcohol treatment<sup>4</sup> followed by plating onto blood agar and CCFA medium. In addition, two cooked meat broths were inoculated with each specimen before and after alcohol treatment. After incubation at 37°C for five days these broths were sub-cultured onto blood agar and CCFA medium. All plates were incubated in an anaerobic cabinet at 37°C for 48 h.

#### IDENTIFICATION

All colony types with typical morphology were sub-cultured onto blood agar plates and identified using the criteria of Holdeman *et al.*<sup>14</sup>

#### CYTOTOXIN ASSAY

A suspension of faecal material was made in sterile phosphate buffered saline (PBS) containing penicillin (1000 µg/ml), streptomycin (1000 µg/ml), and metronidazole (100 µg/ml). After centrifugation at 2000g for 20 min, cytotoxicity was assayed on MRC V tissue culture monolayers. A characteristic cytopathic effect neutralised by *C difficile* antitoxin was considered positive.

#### GAS LIQUID CHROMATOGRAPHY

A 50% (wt/vol) suspension of faecal material was prepared in 1 ml PBS (without antibiotics). This was acidified with 0.2 ml 50% H<sub>2</sub>SO<sub>4</sub> and extracted in 1 ml diethyl ether. Samples (1 µl) were assayed by gas chromatography for the presence of volatile fatty acids and *p*-cresol.

Volatile fatty acids were analysed using a Pye Unicam Series 104 gas chromatograph equipped with a flame ionisation detector, employing a 1.5 m × 4 mm id glass column packed with 10% FFAP on diatomite CLQ (JJ's Chromatography). The oven temperature was 150°C and the carrier gas was nitrogen at a flow rate of 50 ml/min.

*p*-Cresol was detected in a similar fashion, using a column packing of 3% OV1 on diatomite CLQ (JJ's Chromatography) and an oven temperature of 110°C.

#### Results

Of 110 stools investigated, 27 (25%) contained *C difficile* or cytotoxin. Eighteen stools (16%) were

positive for cytotoxin; only one stool was cytotoxin positive and culture negative. The distribution of fatty acids in the series acetic to caproic was acetic (92/110), propionic (75), iso-butyric (61), butyric (68), valeric (29), iso-caproic (18), and caproic (0). No association was found between the presence of *C difficile* or cytotoxin and acetic, propionic, iso-butyric, butyric, or valeric acids.

Significant associations were apparent between *C difficile* and iso-valeric acid ( $\chi^2 = 9.83$  with Yates' correction,  $p < 0.01$ ) and between *C difficile* and iso-caproic ( $\chi^2 = 13.375$  with Yates' correction,  $p < 0.001$ ). Iso-valeric acid was detected in 23/27 (85%) stools positive for *C difficile* (Table), but only 11/27 (41%) stools positive for *C difficile* contained iso-caproic acid.

*Para*-cresol was found in 24/71 (34%) stools. Of these 71 stools, 21 (30%) were positive for *C difficile* or cytotoxin; *p*-cresol was found in 11/21 (52%) stools containing *C difficile* (Table).

No combination of iso-valeric and iso-caproic acids and *p*-cresol produced a better correlation with the presence of *C difficile* or cytotoxin than iso-valeric acid alone. Moreover, when multiple specimens from the same patient were examined over six weeks the presence of iso-valeric acid, iso-caproic acid, and *p*-cresol did not correlate well with the detection of *C difficile*.

#### Discussion

A previous study indicated that gas chromatographic detection of iso-caproic acid might be a useful screening test for the presence of *C difficile* in stools. The authors' interpretation of their data was questionable, however<sup>13 15</sup>; the results of the present study essentially confirm those of Potvliege *et al.*<sup>12</sup> but the interpretation placed on them is quite different.

A significant association was found between the presence of iso-caproic acid and the detection of *C difficile*. This was not unexpected since *C difficile* is one of relatively few common gut organisms that produce iso-caproic acid *in vitro*.<sup>14</sup> Caution must be exercised before any reliance is placed on this association, however, since there is no guarantee

Association of *Clostridium difficile* with iso-valeric acid, iso-caproic acid, and *p*-cresol in stools

Metabolite	No of stools examined	No of stools positive for metabolite and <i>C difficile</i>	No of stools positive for metabolite but not <i>C difficile</i>	No of stools positive for <i>C difficile</i> but not metabolite
Iso-valeric acid	110	23	40	4
Iso-caproic acid	110	11	7	16
<i>p</i> -cresol	71	11	13	10

that metabolic end products in vitro are the same as those elaborated in vivo. In addition, other organisms that produce iso-caproic acid in vitro, such as *C bifermentans*, *C sordellii*, and *C sporogenes*, are commonly isolated from stools.<sup>16</sup> Of paramount importance, however, is the finding that only 41% of *C difficile*-positive stools contained iso-caproic acid, implying a false negative rate of 59%. Even when iso-caproic acid and *C difficile* are found in the same stool, this correlation may be only intermittent.

Of the volatile acids studied, iso-valeric acid showed the greatest predictive value, being found in 85% of stools positive for *C difficile*. The high proportion of false positive results with iso-valeric acid may reflect the much wider range of intestinal organisms which produce this compound in vitro.

Production of *p*-cresol has been used as an aid to the presumptive identification of *C difficile*.<sup>3</sup> A high rate of false negatives (48%) is again quite unacceptable. In addition, most stools containing *p*-cresol did not yield *C difficile*. Since a combination of isolation methods was employed this finding suggests that other organisms in the intestines may produce *p*-cresol, although previous reports have been contradictory.<sup>17 18</sup>

It is concluded that detection of volatile fatty acids or *p*-cresol in stools are not satisfactory screening tests for *C difficile*.

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