

Evaluation of gas-liquid chromatography for the rapid diagnosis of *Clostridium difficile* associated disease

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SUMMARY Direct gas-liquid chromatography of faecal specimens with isocaproic acid as a marker was used for the rapid diagnosis of *Clostridium difficile* associated diarrhoeal diseases. Ninety stools were examined and results were compared with conventional culture on selective medium and cytotoxin assay in tissue culture. Using a combined analysis of isocaproic acid and butyric acid peak heights we defined three categories: positive, negative, and indeterminate. When the indeterminate group was excluded, the positive and negative predictive values of gas-liquid chromatography analysis were 86.9% and 85% respectively compared with culture and 71.4% and 95% respectively compared with cytotoxin assay.

Gas-liquid chromatography has been extensively used for the rapid diagnosis of anaerobic infections by detection of short chain fatty acids in clinical specimens.¹ *Clostridium difficile* is a toxin producing anaerobe, which in recent years has been shown to be responsible for diarrhoea or colitis following antibiotic treatment.² Its metabolic end products consist of several volatile fatty acids, including isocaproic acid. This is a good marker for the identification of *C difficile* strains in pure culture; it is produced by only a few other anaerobes (*C bifermentans*, *C sordelli*, *C sporogenes*, *Ps anaerobius*).³ The detection of this acid directly in faeces might be useful to predict the presence of *C difficile*, and make an early provisional diagnosis, before results of conventional culture⁴ and cytotoxin assay are available.⁵ This approach has been used in previous studies, but the present results and conclusions are not in agreement with those.⁶⁻⁸

This paper reports our experience in the use of gas-liquid chromatography as a rapid test for the provisional diagnosis of *C difficile* associated disease and the evaluation of the usefulness of semiquantitative analysis of the volatile fatty acids present in faeces.

Material and methods

STOOLS

We examined 90 stool specimens from patients in hospital suspected of suffering from *C difficile* associated diarrhoea. The specimens were processed immediately or stored at -20°C.

CULTURE

Stools were plated on to a selective medium, cycloserine-cefoxitin-fructose agar (Oxoid),⁴ and incubated in an anaerobic cabinet (PACE Lab-Line Instruments Inc, Melrose Park, Illinois) for up to five days. Colonies resembling *C difficile* by their morphological appearances were further identified by conventional methods.^{3,9}

CYTOTOXIN ASSAY

Stool specimens were processed as indicated by Chang *et al*¹⁰ and assayed in HEP-2 tissue culture monolayers using a micromethod described elsewhere.¹¹ A characteristic cytopathic effect neutralised by *C difficile* antitoxin (T Wilkins, Virginia Polytechnic Institute, Blacksburg) was considered positive.

GAS-LIQUID CHROMATOGRAPHY

Volatile fatty acids were extracted from faecal specimens by adding 0.2 ml of H₂SO₄ (50%) and 1 ml of ether to 1 ml of liquid faeces or by adding 4 ml of phosphate buffer (0.01M, pH 7), 0.4 ml of

Table 1 Comparison between results obtained by detection of isocaproic acid by gas-liquid chromatography (GLC) and culture or cytotoxin assay

	Culture			Cytotoxin assay		
	Positive	Negative	Total	Positive	Negative	Total
GLC						
Positive	21	6	27	16	9	25
Negative	15	48	63	6	56	62
Total	36	54	90	22	65	87*

*Three samples not tested.

H₂SO₄ (50%), and 2 ml of ether to 1 g of solid faeces. The equipment used was a gas chromatograph Varian 3700 with a 15% FFAP on 100/120 Chromosorb 6 ft × 2 mm TD glass column and a Varian 9176 recorder.

The working conditions were as follows: column temperature 140°C, inlet temperature 200°C, detector temperature 250°C, flow rate 60 ml/min. N₂, detector FID, sensitivity 8 × 10⁻¹⁰ AFS, chart speed 1 cm/min, sample size 5 µl.

Standard solutions of volatile fatty acids were used to identify peaks in the chromatogram by comparing the retention time. Semiquantitative analysis was performed by measuring the peak heights. Under our conditions 0.1 mEq/100 ml of butyric acid represented a peak height of 5 cm; 0.1 mEq/100 ml of isocaproic acid represented a peak height of 2.5 cm.

Results

Gas-liquid chromatography was performed on 90 faecal specimens to detect the presence of isocaproic acid, and the results were compared with culture for *C. difficile* and with the cytotoxin assay in tissue culture. Thirty six faecal specimens yielded *C. difficile* in culture; 22 were also positive for cytotoxin. Results of gas-liquid chromatography analyses are summarised in Table 1.

Twenty seven samples were positive for isocaproic acid: when the chromatography results were compared with the findings on culture, the sensitivity and the specificity of the method were 58.3% and

88.8% respectively, and the positive and negative predictive values were 77.7% and 76.2% respectively. When the results of gas-liquid chromatography were compared with the results of the cytotoxin assay the sensitivity and the specificity were 72.7% and 86.1% respectively, and the positive and negative predictive values 64% and 90.3% respectively.

To improve the sensitivity of the method a semiquantitative analysis was tentatively applied. As the amount of the volatile fatty acids can be extremely variable in different faecal specimens—it was usually less in watery samples—we compared the amount of isocaproic acid with the amount of other volatile fatty acids. We arbitrarily chose butyric acid as being representative of the volatile fatty acids in faeces because it was present in almost all specimens. A combined analysis of isocaproic and butyric acid was used to establish different criteria. We defined as positive all samples with isocaproic acid with a peak height of > 0.5 cm; samples with isocaproic acid with a peak height of ≤ 0.5 cm were considered positive providing that the height of the butyric acid peak was ≤ 5 cm. We defined as negative all samples with isocaproic acid not detectable or ≤ 0.5 cm and butyric acid > 5 cm. All samples with undetectable peaks of isocaproic acid and peaks of butyric acid ≤ 5 cm were considered indeterminate. Accordingly, 27 samples examined (30%) were defined as indeterminate and therefore excluded from further analysis. Results are shown in Table 2.

The sensitivity and the specificity of gas-liquid chromatography were 82% and 91% respectively

Table 2 Comparison between results obtained by semiquantitative gas-liquid chromatography (GLC) analysis and culture or cytotoxin assay (indeterminate samples are excluded)

	Culture			Cytotoxin assay		
	Positive	Negative	Total	Positive	Negative	Total
GLC						
Positive	20	3	23	15	6	21
Negative	6	34	40	2	38	40
Total	26	37	63	17	44	61*

*Two samples not tested.

when compared with culture and 88.2% and 86.3% when compared with cytotoxin assay. The positive and negative predictive values were 86.9% and 85% respectively when compared with culture and 71.4% and 95% when compared with toxin.

Discussion

Laboratory diagnosis of antibiotic associated diarrhoea or pseudomembranous colitis requires the isolation of *C difficile* and the detection of its cytotoxin in the faecal specimen.^{2,11} The demonstration of a cytopathic effect in tissue culture and the neutralisation by *C difficile* antitoxin is the method of choice in the diagnosis.

Laboratory facilities and technical expertise for cell culture are needed, however, and so most laboratories rely on the more time consuming culture methods. The search for a more rapid and simpler technique has progressed in the last few years.^{12,13}

Gas-liquid chromatography is a widely used technique in bacteriology laboratories to identify anaerobes³ and to detect their metabolic end products directly in clinical specimens.¹ Some authors have suggested the use of gas-liquid chromatography for the detection of *C difficile* by its metabolites, such as p-cresol or isocaproic acid, directly from the selective plates^{14,15} or, even more rapidly, from faecal extracts.⁶⁻⁸

We used isocaproic acid as a marker of the presence of *C difficile* in faeces of patients with diarrhoea and compared our results with culture and cytotoxin assay. They show that this technique is more specific than sensitive. The percentage of false positives was 11% and the percentage of false negatives was large (41%) when compared with culture; when compared with cytotoxin assay, however, only six of 22 samples (27%) gave false negative results.

Papersack and coworkers⁷ in a similar study found a large number of false positive but no false negative results; however, they examined only a small number of stools which were positive for both *C difficile* and toxin. Potvliege,⁶ adopting different working conditions, obtained 39% false negative results, which is an unacceptable rate as pointed out by Borriello.¹⁶ Levett⁸ found isocaproic acid in only 41% of samples positive for *C difficile* and therefore considered that gas-liquid chromatography was not a satisfactory screening test.

With our method gas-liquid chromatography was less sensitive than the analysis reported by Papersack and coworkers;⁷ however, we had a smaller number of false positive results. The sensitivity of our method was the same as in previous studies^{6,8} when compared with culture; however, the sensitiv-

ity was greatly increased when chromatography results were compared with the presence of cytotoxin, which is the most reliable marker for *C difficile* associated disease.

In an attempt to improve the sensitivity of our method we adopted a semiquantitative analysis. After several samples were examined we found that the amount of isocaproic acid detectable by our method was often related to the amount of the total volatile fatty acids: samples positive for *C difficile* that showed small amounts of isocaproic acid also showed small amounts of the other volatile fatty acids. As butyric acid was present in all but eight samples examined we chose a peak height breakpoint for butyric acid so that different quantitative criteria for isocaproic acid were established. Samples with small amounts of isocaproic acid were considered positive if the peak height of butyric acid was lower than the breakpoint, and negative if higher. Samples with small amounts of all the volatile fatty acids, a butyric acid peak lower than breakpoint, and undetectable isocaproic acid were considered indeterminate. On the basis of these criteria 30% of the samples were indeterminate and gas-liquid chromatography could not be used for a provisional diagnosis.

Excluding this group we obtained a small number of false negative results, the specificity and the sensitivity were improved if compared with both culture and cytotoxin assay, and the positive and negative predictive values increased.

We think that, especially for laboratories already equipped with gas-liquid chromatography, isocaproic acid detection with a semiquantitative analysis can provide a rapid provisional diagnosis with a good predictivity in at least two thirds of cases. The results of gas-liquid chromatography could be helpful to decide whether to start promptly an appropriate treatment, which is sometimes expensive, as in the case of oral vancomycin. Cytotoxin assay is necessary, however, for the definitive diagnosis of *C difficile* associated disease and further decisions about treatment. Furthermore, gas-liquid chromatography may give additional information in understanding the finding of *C difficile* but not of cytotoxin in stools. In nine out of 14 specimens positive for *C difficile* and negative for cytotoxin, isocaproic acid was not present. Most *C difficile* strains isolated from these specimens produced cytotoxin when cultured in vitro (seven of nine). It could be that these strains were not metabolically active in vivo because of the absence of two different metabolites: isocaproic acid and cytotoxin. Alternatively, there may have been only a few organisms present in the stools; unfortunately, quantitative data on our samples are not available. In both instances *C*

difficile does not appear to have a role in the aetiology of these cases of diarrhoea.

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