

Use of gas-liquid chromatography as a screening test for toxigenic *Clostridium difficile* in diarrhoeal stools

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SUMMARY In order to determine if gas-liquid chromatography (GLC) on concentrated stool extracts could be substituted to cell culture assay for cytotoxicity, we prospectively studied 154 diarrhoeal stools submitted for detection of *Clostridium difficile* toxin. Isocaproic-positive samples were cultured on egg yolk agar supplemented with cycloserine, cefoxitin and fructose for isolation of *C difficile*, and on egg yolk agar plus kanamycin for isolation of other clostridium species. Of the 154 samples, 129 were GLC-negative (height of the isocaproic peak less than 1.2 cm) and were toxin-negative. Twenty-five stools yielded isocaproic acid; *C difficile* isolated from 13 of them, six of which were also toxin-positive. Four other isocaproic-positive samples yielded *C bifermentans* and *C sordellii*; all were toxin-negative. These results indicate that a negative GLC is an excellent screening test for excluding *C difficile* infection; positive results must be checked by toxin testing and culture since they are not necessarily associated with the presence of *C difficile* or its toxin.

Toxigenic strains of *Clostridium difficile* are the major cause of antibiotic-associated colitis (AAC);¹ they have also been implicated in severe relapses of inflammatory bowel disease.² Classically, the microbiological diagnosis of these colitis is based on the detection of the toxin by a tissue culture assay,³ a method which is not within the range of all laboratories. For this reason, other methods, principally counterimmunoelectrophoresis⁴ and immunofluorescence⁵ have been proposed as toxin detection technique. An association between the detection of isocaproic acid by gas-liquid chromatography (GLC) and the presence of *C difficile*, or its toxin in the stools, has already been mentioned.⁶ The present study was undertaken in view of evaluating GLC as screening test for toxigenic *C difficile* in diarrhoeal stools.

Material and methods

SPECIMENS

Between February 1982 and February 1983, 154 unselected diarrhoeal stools (obtained from 131 different patients), sent to the laboratory for the detection of *C difficile* toxin, were submitted to the following tests: GLC, detection of *C difficile* by culture

and cytotoxicity assay. Any sample showing an isocaproic acid peak by GLC was also cultured for detection of other clostridial species producing isocaproic acid.

GAS-LIQUID CHROMATOGRAPHY

A portion of the sample (1.5 ml) was added to 7 ml of phosphate-buffered saline, (PBS) then mixed by shaking and centrifuged at 2500 rpm for 5 min. 0.4 ml 50% H₂SO₄ and ether (2 ml) were added to 4 ml of supernatant. The mixture was shaken vigorously and centrifuged at 2500 rpm for 5 min. The ether layer was withdrawn and concentrated three times in a 56°C waterbath. Two microlitres of this was injected into the column. Glass columns (6 ft long; 4 mm internal diameter) were used, packed with 15% SP-1220/1% H₃PO₄ on 100/120 mesh Chromasorb WAW (Supelco). Chromatography was performed on a Hewlett-Packard 5710A/30A fitted with a flame ionisation detector. Operating conditions were as follows: carrier gas (oxygen-free nitrogen): 60 ml/min. Oven temperatures: detector 200°C, column 145°C, injector 200°C. Attenuation 64 × 10.

Fatty acids were identified by comparing the retention times of peaks in the test samples with those of known standard solutions which were

examined each day diarrhoeal stools were tested. The concentration of isocaproic acid was assessed semiquantitatively by the peak height of the isocaproic acid in the test sample. In our operating conditions, the peak height of the isocaproic acid in a freshly prepared standard solution titrating 1 $\mu\text{mol/ml}$ averaged 14 cm.

CYTOTOXIN DETECTION

Faecal specimens were tested for a cytotoxin neutralised by *C. sordellii* antitoxin (Wellcome Research Laboratory) using the technique described by George.⁷ The cells utilised were HEp 2 cells.

CULTURE

Undiluted stools were swabbed on a selective agar medium, cycloserine, cefoxitin, fructose egg yolk agar (CCFA Oxoid) for the isolation of *C. difficile*.⁸ After 48 h of anaerobic incubation (Forma Anaerobic System model 1024), colonies resembling *C. difficile* by their morphological appearance and green fluorescence under longwave ultraviolet light were further identified by classical methods.⁹ Moreover, all stools presenting an isocaproic peak by GLC were swabbed on egg yolk agar + kanamycin (75 $\mu\text{g/ml}$) for detection of other clostridial species. Identification of strict anaerobic Gram-positive bacilli was confirmed by the VPI methods.⁹

CYTOTOXIN PRODUCTION BY *C. DIFFICILE* STRAINS

In a few cases, the toxigenic property of the isolated strains was checked. Supernatant from 48-hour Modified Lombard Dowell¹⁰ broth cultures of clostridial species (either *C. difficile* or others) were tested at an initial dilution of 1/8 for cytotoxin in a manner similar to that used for the faecal specimens.

CONTROLS

Ten samples of stools of 10 healthy adults were submitted to GLC, cytotoxin assay, and culture of CCFA medium and on EYA + kanamycin. As these specimens were not diarrhoeic, they were processed in the following way: approximately 1.5 g of stool were introduced in a test tube containing a few glass balls. After the addition of 4 ml of PBS, the sample was emulsified by shaking (Vortex) for one minute. Then, 3 ml of PBS were added to the suspension which was centrifuged at 2500 rpm for 5 min. Fatty acids were extracted as described above.

Results

Results are summarised in the Table. No isocaproic peak could be detected in 93 samples which all were culture- and cytotoxin-negative. Sixty-one samples

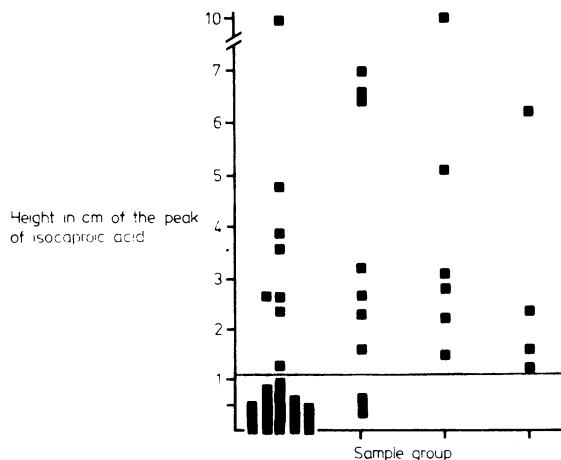
Table 1 Comparison of GLC and other methods for detection of *C. difficile* in diarrhoeal stools.

GLC	No of samples	No of samples showing	
		Growth of <i>C. difficile</i>	Toxin production
Negative*	93	0	0
Positive	61	16	6

*No peak of isocaproic acid.

collected from 52 different patients contained isocaproic acid. *C. difficile* was isolated by culture in 16 of these samples, six of which were also toxin-positive.

The Figure shows the distribution of the values of the isocaproic peaks in function of sample grouping based on results of cultures and of cytotoxin assay. On the whole, culture- and toxin-negative samples had peaks which did not exceed one centimetre in height. Eight samples however showed peaks with heights ranging from 1.3 to more than 10 cm. They all came from patients where the diagnosis of AAC was not retained: one patient suffered from acute gastroenteritis due to a Salmonella group G strain after consuming undrinkable water which also contained the strain, another patient presented an acute diarrhoeal episode after abdominal surgery for neoplastic disease; the other patients were eventually discharged with the diagnosis of relapse of acute inflammatory bowel disease based on endoscopic



Toxin production	0	0	+	0
Growth of <i>C. difficile</i>	0	+	+	0
Growth of other clostridial species	0	0	0	+*

**C. bifermentans* 3 *C. sordellii* 1

Distribution of the values of the isocaproic peaks in function of sample grouping based on results of cultures and of cytotoxin assay.

examination, barium enema and intestinal biopsy. The samples in which *C difficile* alone, or associated with a cytotoxin, was present, usually showed isocaproic peaks higher than 1.2 cm; however, *C difficile* was isolated by culture in 3 samples, all toxin-negative, where the height of the isocaproic peaks did not reach 1 cm. These three strains were cytotoxin-producing in vitro. One of these samples came from a patient treated for 24 h by oral vancomycin for AAC proved by endoscopic examination and confirmed by GLC, culture and cytotoxin assay in a former stool sample; the two other samples were collected at 48 h intervals from one patient admitted 10 days earlier for her first episode of Crohn's disease. In four samples, the presence of an isocaproic peak could be correlated with the presence of another isocaproic-producing clostridial species: *C bifermentans* in three cases, and *C sordellii* in one case. None of these strains was cytotoxin-producing. Although the highest isocaproic peak was observed with carriage of a toxigenic strain of *C difficile*, there seems to be no correlation between the height of the isocaproic peak and the toxigenic character of the isolated strains of *C difficile*, or the species of isocaproic-producing clostridial strains recovered.

If we define a positive GLC by the presence of an isocaproic peak at least 1.2 cm high, this technique allowed us to detect 13 of the 16 culture-positive samples (including the 6 toxin-positive samples); in four cases, the peak was linked to the presence of another clostridial species; in 8 cases, no isocaproic producing clostridial strain could be isolated. Furthermore, the adoption of this threshold of positivity allowed us to discard 129 of the 154 samples studied; among the rejected samples, only three were culture-positive but toxin-negative. All 10 samples obtained from healthy adults were negative by GLC, culture and cytotoxin assay.

Discussion

The presence of *C difficile* toxin is classically demonstrated in faecal supernatants by the development of a cytopathic effect which is neutralisable by *C sordellii* or *C difficile* antitoxin. This simple technique requires, however, the disposal of fresh uncontaminated cell cultures and of a specific antitoxin. The handling and maintenance of cell cultures necessitates a specialised team which laboratories who do no virology, do not always have available. Some authors seeking a more simple rapid method, that is as equally sensitive as tissue culture, have suggested the use of counterimmunoelectrophoresis (CIE) to detect the presence of a *C difficile* toxin.⁴ It would appear that the precipitin bands obtained by this

method are not due to the toxin itself—West *et al*¹¹ suspect that the amount present in most faeces is probably insufficient for detection by CIE—but to other antigens of *C difficile* present in the partially purified toxin preparation used to prepare the *C difficile* antitoxin.¹² In these conditions, West and Wilkins¹¹ observe that positive results do not necessarily confirm the presence of *C difficile* toxins in the sample. More recently, Wilson *et al*¹⁵ have shown the value of a fluorescent antibody (FA) test for detection of *C difficile* in stools specimens from patients with AAC. In these patients, the FA test agreed with culture and toxin testing in 93% of the specimens. These authors have however observed an excessively high rate of positive FA tests in a normal population rendering their test inapplicable for stool specimens received at a routine microbiological laboratory.

The detection of short chain volatile fatty acids by GLC is commonly utilised in bacteriological laboratories to identify anaerobes⁹ and to diagnose quickly the infections that they generate.¹³ In vitro, *C difficile* produces notably isocaproic acid. But a small number of other anaerobes isolated in man produce this compound such as certain clostridial species (*C bifermentans*, *C sordellii*, *C sporogenes*, *C botulinum*, *C ghoni*, *C hastiforme*) and *Peptostreptococcus anaerobius*.⁹ On this basis, Potvliege and her co-workers, have shown a correlation between the presence in stools of isocaproic acid and that of *C difficile*.⁶

However, with the type of chromatograph used by these authors—equipped with a thermoconductivity detector less sensitive than a flame ionisation detector—the method they proposed had only a 60.7% sensitivity. Moreover, the 39% of falsely negative results were a major obstacle to the utilisation of this test as a screening procedure.¹⁴

In our working conditions, and assuming the adoption of a threshold of positivity fixed at a height of 1.2 cm (which corresponds approximately to 0.8 $\mu\text{mol/ml}$), we found GLC of great value in this diagnosis; a negative GLC was never associated with the presence of toxin in stools. It did not forcibly exclude the presence of *C difficile* (in our experience, a *C difficile* strain was isolated in 3 of the 129 GLC-negative samples), but this organism may occasionally be found in stools of healthy individuals;¹⁵ in the absence of in vivo detectable cytotoxin, the mere demonstration of a strain of *C difficile* even if it is capable of in vitro toxin production is insufficient to establish the diagnosis of AAC.³ A positive GLC (presence of an isocaproic peak at least 1.2 cm high) may not however be substituted for the classical cytotoxicity test to determine the microbiological diagnosis of AAC. Indeed, in our

experience, such a result coincides in 50% of the cases with the presence of *C. difficile* in stools; in only 25% of the GLC-positive samples a toxin may be detected simultaneously. Furthermore, 50% of the positive GLC results do not correspond to the presence either of *C. difficile* or of its toxin; in some cases, the detection of another clostridial species in the stools might justify the isocaproic peak; other cases remain unclear. The isocaproic peak corresponds perhaps to the presence of other isocaproic-producing anaerobes (such as *Peptostreptococcus anaerobius* which we did not search for). It is also possible that a spore selection technique might have more often demonstrated an isocaproic-producing clostridial species than the selective egg yolk agar + kanamycin actually used.

Much more probable is the hypothesis put forward by Brooks (personal communication) that the flame ionisation detector makes it impossible to differentiate the isocaproic acid peak from other metabolites having the same retention time and that the use of a frequency pulsed electron capture detector would considerably reduce the ratio of false positive GLC.

In conclusion, GLC performed on diarrhoeal stools appears to us as an excellent rapid screening test to select the samples to be tested for *C. difficile* toxin by the classical cytotoxin assay. The procedure is simple and performed in less than an hour. Only those samples showing the presence of an isocaproic peak higher than 1.2 cm should be submitted to the cytotoxin assay. Indeed, in our experience, a negative GLC was never associated with the presence of a *C. difficile* toxin in the stools and thus renders the hypothesis of an AAC most improbable.

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References

- ¹ Bartlett JG, Taylor NS, Chang TW, *et al.* Clinical and laboratory observations in *Clostridium difficile* colitis. *Am J Clin Nutr* 1980;**33**:2521-6.
- ² La Mont JT, Trnka YM. Therapeutic implications of *Clostridium difficile* toxin during relapse of chronic inflammatory bowel disease. *Lancet* 1980;*i*:381-4.
- ³ Bartlett, JG. Antibiotic-associated diarrhea. In: Remington JS, Swartz MN, eds. *Current clinical topics in infectious diseases*. New York: Mc Graw-Hill Book Co, 1980:240-64.
- ⁴ Ryan RW, Kwasnik I, Tilton RC. Rapid detection of *Clostridium difficile* toxin in human faeces. *J Clin Microbiol* 1980;**12**:776-9.
- ⁵ Wilson KH, Silva J, Fekety FR. Fluorescent-antibody test for detection of *Clostridium difficile* in stool specimens. *J Clin Microbiol* 1982;**16**:464-8.
- ⁶ Potvliege C, Labbe M, Yourassowsky E. Gas-liquid chromatography as screening test for *Clostridium difficile*. *Lancet* 1981;*ii*:1105.
- ⁷ George RH. A micro-method for detecting toxins in pseudomembranous colitis. *J Clin Pathol* 1979;**32**:303-4.
- ⁸ George WL, Sutter VL, Citron D, *et al.* Selective and differential medium for isolation of *Clostridium difficile*. *J Clin Microbiol* 1979;**9**:214-9.
- ⁹ Holdeman LV, Cato EP, Moore WEC. *Anaerobic laboratory manual*. 4th ed. Blacksburg, Virginia: Virginia Polytechnic Institute, 1977:79-106.
- ¹⁰ Jessee MT, Robinson PJ. Modified Lombard-Dowell broth as a general growth medium. *J Clin Microbiol* 1977;**6**:538-42.
- ¹¹ West SEH, Wilkins TD. Problems associated with counter-immunoelectrophoresis assays for detecting *Clostridium difficile* toxin. *J Clin Microbiol* 1982;**15**:347-9.
- ¹² Poxton IR, Byrne MD. Detection of *Clostridium difficile* toxin by counterimmuno-electrophoresis: a note of caution. *J Clin Microbiol* 1981;**14**:349.
- ¹³ Watt B, Geddes PA, Greenan OA, *et al.* Gas-liquid chromatography in the diagnosis of anaerobic infections: a three year experience. *J Clin Pathol* 1982;**35**:709-14.
- ¹⁴ Borriello SP. Gas-liquid chromatography and *Clostridium difficile*. *Lancet* 1981;*ii*:1283.
- ¹⁵ George WL, Rolfe RD, Sutter VL, *et al.* Diarrhea and colitis associated with antimicrobial therapy in man and animals. *Am J Clin Nutr* 1979;**32**:251-7.

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