Technical methods

Improved gas-liquid chromatography method for the identification of *Clostridium difficile*

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The identification of *Clostridium difficile* may be aided by gas-liquid chromatography. This has included the analysis of short chain fatty acids^{1,2} from agar³ and broth cultures,⁴ from extracts of faeces,⁵ and also analysis of agar plugs beneath suspected colonies of the organism.⁶ Often only a semi-quantitative assessment has been used; however, it is important that any limitations of the chosen extraction technique are known so that metabolite profiles are correctly interpreted.

The production of p-cresol by *C. difficile* was first shown by Elsdon *et al.*⁷ Phillips and Rogers³ suggested that the presence of p-cresol might be a useful marker of *C. difficile*, and Nunez-Montiel *et al.*⁴ indicated that p-cresol together with caproic acid provided rapid and simple identification of this organism.

This work presents data on the efficiency of different extraction procedures for a simple one stage extraction technique to remove short chain fatty acids and p-cresol from culture broth. A gas-liquid chromatography technique has been developed which enables estimation of p-cresol and short chain fatty acids with greater certainty and in a much shorter time than in the previously published method.⁴

Material and methods

Isocaproic acid, acetic acid, chloroform, diethyl ether, and ethyl acetate were purchased from BDH Chemicals Limited, Poole, England. Propionic, isobutyric, butyric, isovaleric, valeric, and caproic acids together with p-cresol were purchased from Sigma, Poole, England.

A standard solution was prepared by the addition of 100 µl of each short chain fatty acid and p-cresol to 25 ml of water and methanol (80/20), giving a concentration range between 32 and 70 mmol/l for the short chain fatty acids and 38 mmol/l for p-cresol. The internal standard used to assess the extraction technique was propionic acid; when the extraction of propionic acid was being assessed, however, isobutyric acid was used as an internal standard. A gas-liquid chromatography broth (Lab M) with cooked meat granules (Lab M) and a norleucine-tyrosine broth as used previously,⁴ except with proteose (Difco) replacing trypticase, were prepared. To these media were added the short chain fatty acids and p-cresol (100 µl of each in 25 ml of broth supernatant), with the omission of the short chain fatty acid to be used as the internal standard.

EQUIPMENT

The gas-liquid chromatograph was a Pye 104 fitted with dual flame ionisation detectors and linked to a Hewlett Packard 3390A computing integrator. Two 3 m × 2 mm columns were packed with 15% SP1220, 1% H₃PO₄ on Chromasorb W 100-120 mesh (Supelco Inc, Bellefonte, USA). The columns were conditioned for 72 h at 190°C to reduce column bleed. The nitrogen carrier gas flow rate was 30 ml/min. Samples (1 µl) were injected using a syringe with a 7 cm needle. The column oven temperature was programmed from 140°C to 180°C at a ramp of 6°C/min, and then remained at 180°C for 8 min. The detector oven was set at 230°C.

EXTRACTION PROCEDURE

The selected organic solvent (2 ml; chloroform, diethyl ether, or ethyl acetate) was added to the broth (2 ml) in a glass screw-top tube, which was then sealed and vortex mixed for 1 min. Acidification of the broth to pH 1 was accomplished by the addition of concentrated hydrochloric acid. After separation of solvent and aqueous phases, 100 µl of solvent was sampled and added to 100 µl of internal standard. The peak area ratio of each extracted analyte to the internal standard was compared with the same ratio for 100 µl of the aqueous standard added to the internal standard. Using this procedure it was possible to quantitate the amount of each analyte extracted.

Results

The analysis of extracts from norleucine-tyrosine broth and cooked meat broth without the addition of p-cresol and short chain fatty acids showed no gas-liquid chromatography peaks. The recovery of

Percentage extraction of short chain fatty acids and p-cresol added to norleucine-tyrosine broth

	Chloroform				Diethyl ether				Ethyl acetate			
	Unacidified		pH 1		Unacidified		pH 1		Unacidified		pH 1	
	Mean*	CV%†	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
Acetic	13	7.6	14	7.8	33	2.3	41	2.9	39	2.4	47	4.4
Propionic	15	8.7	19	5.0	60	4.1	69	4.3	65	1.9	70	2.3
Isobutyric	75	2.8	75	1.7	88	1.3	90	4.8	85	3.0	82	2.6
Butyric	73	3.3	74	1.4	90	1.1	98	2.0	86	8.9	82	7.6
Isovaleric	86	4.1	85	1.8	101	0.9	95	3.2	91	6.6	84	3.2
Valeric	85	3.0	87	1.3	101	1.8	92	7.9	91	5.7	83	4.8
Isocaproic	87	4.0	87	2.3	111	2.0	88	5.1	88	7.1	76	4.4
Caproic	90	4.1	90	2.6	106	1.9	77	6.2	90	6.6	73	5.1
p-Cresol	96	4.9	85	4.2	101	1.9	88	2.1	95	3.2	92	2.2

*Mean of five extractions.

†CV% = Standard deviation mean $\times$ 100.

p-cresol and short chain fatty acids from norleucine-tyrosine broth using different solvents and the effect of acidification are given in the Table. Similar results were obtained for extraction from cooked meat broth.

A gas-liquid chromatography trace from an extract of a growth of *C. difficile* in norleucine-tyrosine broth is presented in the Figure. Each peak is well separated and in particular p-cresol formed a discrete peak.

Gas-liquid chromatography trace of an ethyl acetate extraction of p-cresol and short chain fatty acids from a 48 hour incubation of *Clostridium difficile* in norleucine-tyrosine broth. A = acetic acid; P = propionic acid; i-B = isobutyric acid; B = butyric acid; i-V = isovaleric acid; V = valeric acid; i-C = isocaproic acid; C = caproic acid; p-C = p-cresol.

Discussion

The results obtained for the extraction of p-cresol and short chain fatty acids indicate that acidification of bacterial broth is of little value to the extraction. Overall, ethyl acetate and diethyl ether showed better extraction characteristics than chloroform. The extraction procedure described is rapid and simple to perform and is acceptable for an analysis where only semi-quantitative results are needed. Compensation for variation in extraction efficiency to different analytes may be obtained by taking a standard through the extraction procedure.

The use of a small injection volume (1 μ l) and a simple temperature programme gives good separation of the short chain fatty acids and p-cresol. The overall time for the gas-liquid chromatography method is less than 15 min, compared with over 30 min for the previously published method of Nunez-Montiel *et al.*⁴ Furthermore, in their isothermal run the p-cresol peak was extremely broad, increasing the possibility that it could be misidentified or, if present in a small amount, not distinguished from the baseline.

Further work is still required to establish the best microbiological medium for the production of caproic acid and p-cresol by *C. difficile* and also to verify whether measurement of these analytes is as specific as has been claimed⁴ for the detection of this organism.

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Demonstration of *Mycobacterium tuberculosis* in epoxy resin sections by the Ziehl-Neelsen technique

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The Ziehl-Neelsen technique is the fundamental staining procedure for demonstrating acid fast bacilli. But the results obtained with this technique on semithin sections of tissue embedded in epoxy resin have not been satisfactory. Adequate staining of epoxy embedded tissue has required either oxidation of the section with periodic acid¹ or hydrogen peroxide,² without the removal of resin, or extraction of the resin with sodium ethoxide³ or bromine vapour.⁴ These two approaches have been combined, the latter approach being essential because of the resin's affinity for basic fuchsin.

Material and methods

TISSUE FIXATION AND EMBEDDING

Human tissue previously fixed in 10% neutral buffered formalin and known to contain *Mycobacterium tuberculosis*, *M leprae*, and bacteria was cleared in acetone, embedded in epoxy resin (TAAB, Polybed, Polaron, Epon), and cut at 4 µm.

RESIN SOLVENTS

Two agents were used: saturated alcoholic sodium hydroxide for 15 min followed by washes in alcohol and water, and bromine vapour. A 2 ml ampoule of bromine was scored, and broken by a glass rod in a Coplin jar under a fumehood. The Coplin jar was closed. When the bromine vapour reached maximum concentration a slide was quickly inserted and the jar closed again. After 30-60 s the slide was removed and rapidly washed in two changes of

acetone and then placed in water. Maximum concentration of bromine vapour is an essential factor for success with this technique.

OXIDISING AGENTS

The oxidising agents evaluated were 5% chromic acid, 0.5% and 1% potassium permanganate, 3% hydrogen peroxide, and both 1% and 10% periodic acid. Tissue sections were exposed to the above oxidising agents for periods of 5, 10, 15 and 20 min. Chromic acid was also applied for 1 h and 10% periodic acid for 4 h. All reactions took place at 22°C. The 1% and 10% periodic acid were also applied at 60°C.

STAINING

After oxidation the sections were washed well in running water. Ziehl-Neelsen's carbol fuchsin was applied, heated until steaming, and allowed to stain for 10 min. The sections were decolorised with acid alcohol and counterstained with 0.1% malachite green, dried, and coverslipped.

Results

Only the bromine vapour together with 10% periodic acid oxidation for 5-10 min at 22°C gave a strong acid alcohol fast reaction. The result was independent of the brand of epoxy resin used. Saturated ethanolic sodium hydroxide with the same oxidation was essentially negative. If the sections were allowed to remain in the bromine vapour for more than 60 s, the tubercle bacilli rapidly lost colour.

Five per cent chromic acid, 0.5% and 1% potassium permanganate, 3% hydrogen peroxide, and 1% periodic acid (also at 60°C) failed to intensify the Ziehl-Neelsen stain. The 10% periodic acid at 22°C was positive after 15 min but the background was pink. This colour intensified with time and made the detection of the bacilli impossible. The 10% periodic acid at 60°C for 5 min produced a positive result but the bacilli and tissue showed structural disruption.

M leprae and bacteria were used as controls and