Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Development of a novel method for detection of *Clostridium difficile* using HS-SPME-GC-MS

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Keywords
Clostridium difficile, HS-SPME-GC-MS, *p*-hydroxyphenylacetate decarboxylase enzyme, specificity, stool samples.

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2014/2133: received 22 October 2013, revised 29 November 2013 and accepted 3 December 2013

doi:10.1111/jam.12418

Abstract

Aims: A novel method has been developed that allows successful differentiation between *Clostridium difficile* culture-positive and culture-negative stool samples based on volatile organic compound (VOC) evolution and detection by headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME-GC-MS).

Methods and Results: The method is based on the activation of *p*-hydroxyphenylacetate decarboxylase produced by *Cl. difficile* and the detection of a specific VOC, that is 2-fluoro-4-methylphenol from an enzyme substrate. In addition, other VOCs were good indicators for *Cl. difficile*, that is isocaproic acid and *p*-cresol, although they could not be used alone for identification purposes. One hundred stool samples were tested, of which 77 were positive by culture. Detection using HS-SPME-GC-MS allowed confirmation of the presence of *Cl. difficile* within 18 h with a sensitivity and specificity of 83·1 and 100%, respectively.

Conclusions: It is recommended that this new approach could be used alongside conventional methods for *Cl. difficile* detection, including toxin detection methods, which would allow any false-negative results to be eliminated.

Significance and Impact of the Study: The ability to identify *Cl. difficile*-positive stool samples by the analysis of VOCs could allow the development of a VOC detection device which could allow rapid diagnosis of disease and hence prompt treatment with appropriate antibiotics.

Introduction

Clostridium difficile is a Gram-positive sporulating anaerobe and a major cause of nosocomial infections, commonly occurring after the ingestion of spores by hospital patients whose intestinal flora has been disturbed by use of antibiotics (Dawson *et al.* 2009). Proliferation of *Cl. difficile* can lead to severe diarrhoea, pseudomembranous colitis and even death. Pathogenic *Cl. difficile* releases two types of toxins: enterotoxin A and cytotoxin B, and these toxins cause the symptoms associated with *Cl. difficile*-associated diarrhoea (CDAD) (Voth and Ballard 2005; Belyi and Aktories 2010). Current approaches for diagnosis of *Cl. difficile* include isolation of the organism by culture, PCR for detection of toxin genes, detection of glutamate dehydrogenase antigen using immunoassay or toxin detection using enzyme immunoassay (EIA) and toxin B detection using a cell cytotoxicity assay (Bartlett 2010). The most commonly used culture medium employed to detect *Cl. difficile* is a selective agar medium incorporating the antibiotics D-cycloserine (500 mg l^{-1}) and cefoxitin (16 mg l^{-1}), which suppress commensal bacteria; the selective agar cycloserine cefoxitin fructose agar (CCFA) is based on the formulation by George *et al.* (1979). Subsequent modifications of CCFA contain reduced concentrations of the antibiotics; it has been reported by Levett (1985), however, that a reduced antibiotic concentration reduces the selectivity of the medium. Isolation of *Cl. difficile* via culture is sensitive but can take up to 3–5 days to obtain results, and there

may be a heavy growth of other faecal bacteria when stool samples are cultured directly onto agar (Bloedt *et al.* 2009). Techniques employed to reduce faecal flora, and therefore improve the selectivity and sensitivity of methods, include alcohol shock (Clabots *et al.* 1989). Sodium taurocholate can aid germination of spores; inclusion of sodium taurocholate into culture media has led to improved sensitivity of *Cl. difficile* detection from stool samples (Wilson *et al.* 1982). A chromogenic medium for *Cl. difficile* has recently been marketed and in a recent study showed a sensitivity of 87% after 48 h of incubation and superiority over conventional nonchromogenic media (Eckert *et al.* 2013).

It has long been recognized that evolution of certain volatile organic compounds (VOCs) such as short-chain fatty acids and *p*-cresol are strongly associated with growth of *Cl. difficile* (Phillips and Rogers 1981; Gianfrilli *et al.* 1985), although other anaerobic bacteria including *Cl. bifermentans*, *Cl. sordellii* and *Cl. sporogenes* yield fatty acid profiles comparable to *Cl. difficile* (Levett and Phillips 1985). In addition, short and branched-chain fatty acids are also products of carbohydrate and amino acid fermentation by common gut microflora (Cummings and Macfarlane 1997).

p-Cresol is formed in *Cl. difficile* by the decarboxylation of *p*-hydroxyphenylacetic acid (*p*-HPAA), which is an intermediate in the metabolism of L-tyrosine to *p*-cresol (Hafiz and Oakley 1976; D'Ari and Barker 1985). *Clostridium difficile* is almost unique in its ability to form *p*-cresol using this pathway, with the exception of a *Lactobacillus* strain (Yokoyama and Carlson 1981). Other species have been shown to produce *p*-HPAA from L-tyrosine but not to decarboxylate *p*-HPAA to *p*-cresol (Elsden *et al.* 1976) in addition to species which cannot produce *p*-cresol from L-tyrosine but can decarboxylate *p*-HPAA to *p*-cresol (Ward *et al.* 1987). The enzyme responsible for decarboxylation of *p*-HPAA is *p*-hydroxyphenylacetate decarboxylase. The exact mechanism of this reaction is unclear, but the hydroxyl group in the *para* position on the phenyl ring is an essential requirement for the reaction to proceed (Selmer and Andrei 2001); therefore, any alternative substrates to be used in place of *p*-HPAA would also require a *para*-hydroxyl group.

Previous attempts to identify *Cl. difficile* using the analysis of VOCs coupled with GC have suffered from a lack of specificity, particularly when monitoring VOCs directly from stool samples. Levett (1984) concluded that GC was not a suitable method for *Cl. difficile* testing due to the high number of false positives obtained with the detection of *p*-cresol and isocaproic acid in *Cl. difficile* culture-negative stool samples. More recent attempts to use the detection of bacterial VOCs as a tool to identify *Cl. difficile* have included solid-phase microextraction coupled with gas chromatography mass spectrometry (Garner *et al.* 2007).

This paper assesses an improved approach for detection of VOCs via headspace solid-phase microextraction coupled with GC-MS (HS-SPME-GC-MS) in comparison with culture for detection of *Cl. difficile* in stool samples. A new and novel approach for specific and selective VOC generation is proposed based on the incorporation of enzyme substrates. The success of this new approach is evaluated by its application to 100 stool samples and its ability to differentiate between *Cl. difficile* culture-positive and culture-negative stool samples.

Materials and methods

Chemicals/Reagents

Standards of isobutyric acid (99%), butyric acid (≥99%), isocaproic acid (99%), caproic acid (99%), *p*-cresol (99%), 3-fluoro-4-hydroxyphenylacetic acid (98%), D-cycloserine, amphotericin and cefoxitin sodium salt were purchased from Sigma-Aldrich (Poole, UK), while 2-fluoro-4-methylphenol (98%) was obtained from Alfa Aesar (Morecambe, UK). All organic solvents (e.g. acetone) were of analytical reagent grade and were obtained from Fisher Scientific (Loughborough, UK). Cooked meat granules were obtained from Bioconnections (Knypersley, UK); brain heart infusion broth and Columbia blood agar were purchased from Oxoid (Basingstoke, UK); and taurocholic acid and sodium salt were purchased from Calbiochem (Nottingham, UK). Brazier's CCEY agar (BC2160) supplemented with cycloserine and cefoxitin according to manufacturer's instructions (Bioconnections) was used as the reference culture method.

Instrumentation

Bacterial VOCs were extracted and concentrated using an 85-μm polyacrylate (PA) SPME fibre with a manual solid-phase microextraction holder obtained from Supelco (Bellefonte, PA). The GC-MS instrument used to separate and detect bacterial VOCs was a Trace GC Ultra fitted with a Polaris Q ion-trap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) with Xcalibur 1.4 SR1 software. Separation of VOCs was carried out using a 30 m × 0·25 mm ID × 0·25 μm VF-WAX-ms capillary column (Agilent Technologies, Stockport, UK). The S/SL injector was set at 230°C and operated in split mode with a flow rate of 10 ml min^{-1} and a split ratio of 10. The temperature program used was as follows: 50°C held for 2 minutes then increased at a rate of 10°C min^{-1} to 220°C with a final 2-min hold. The

carrier gas was helium with a flow rate of 1·0 ml min^{-1}. Electron ionization (EI) was used (70 eV), and the mass spectrometer was operated in total scan mode over an m/z range of 50–650 amu. The transfer line was held at 250°C, while the ion source temperature was maintained at 260°C.

Microbiology

Clostridium difficile ribotype 106 was obtained from the Microbiology Department, Freeman Hospital, Newcastle upon Tyne. All bacteria were subcultured on Columbia blood agar with 5% defibrinated horse blood and incubated at 37°C under anaerobic conditions. Stool samples were obtained from the Microbiology Department at the Freeman Hospital where they were cultured for *Cl. difficile* as follows: *c.* 0·5 g or 0·5 ml was emulsified in an equal volume of 96% ethanol and left at room temperature for 30 min. Then, 50 μl of this suspension was inoculated onto CCEY, and plates were incubated anaerobically at 37°C for 48 h. Growth of *Cl. difficile* was identified by colonial appearance, fluorescence under UV light and matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker, Coventry, UK). To test whether the isolated strains were toxigenic, they were tested using the VIDAS toxin A/B detection system (bioMerieux, Marcy-l'Étoile, France). This involved subculturing *Cl. difficile* into a cooked meat broth and incubating for 48 h at 37°C. An aliquot of this broth was then centrifuged at 13 000 ***g*** for 5 min. The supernatant was then tested with the VIDAS toxin A/B detection system. All stool samples were also tested for GDH (glutamate dehydrogenase) antigen using a standard immunoassay (TechLab) obtained from Alere (Stockport, UK).

Bacteria media

Our standard medium for testing for evolution of VOCs from stool samples was Robertson's cooked meat broth. This was prepared by addition of 1·5 g ± 0·1 g cooked meat granules to 10 ml brain heart infusion (BHI) broth followed by sterilization by autoclaving at 116°C for 20 min.

Preliminary investigations

For preliminary VOC screening of stool samples, *c.* 0·5 ml of stool sample was added to a 20-ml clear vial with PTFE septum and screw cap. Ten *Cl. difficile* culture-positive and eight culture-negative samples were prepared. Stool samples were incubated for 18 hours at 37°C without any culture medium to characterize the volatile compounds present in the samples.

Evaluation of parameters on VOC profiles

Stool samples (0·5 ml) were added to the broth and incubated overnight at 37°C before generation of a VOC profile. Using four known culture-positive stool samples and four known culture-negative stool samples, the VOC profiles were examined under a variety of conditions. To all samples, 100 μg ml^{-1} 3-fluoro-4-hydroxyphenylacetic acid (FHPAA) was added. An antibiotic cocktail of D-cycloserine and cefoxitin (250 and 8 μg ml^{-1}, respectively) was added to two culture-positive stool samples and two culture-negative stool samples. An antibiotic cocktail of D-cycloserine, cefoxitin and amphotericin (250, 8 and 4 μg ml^{-1}, respectively) was added to the remaining two culture-positive stool samples and two culture-negative stool samples. The effect of sodium taurocholate on VOC profiles was also investigated. Each stool sample was split into 0·5 ml aliquots with one 0·5 ml aliquot added to broth plus 2·5 g l^{-1} sodium taurocholate, the other 0·5 ml aliquot was added to broth without sodium taurocholate. VOC evolution was evaluated under all conditions, and the effect of these various parameters was assessed.

Influence of alcohol shock on VOC profiles

The inclusion of an alcohol shock step, that is the addition of 0·5 ml of 95% ethanol to 0·5 ml stool sample followed by vigorously shaking and then letting it to stand at room temperature for 30 min (Borriello and Honour 1981), was evaluated by dividing three stool samples into six separate parts of 0·5 ml each; the subsamples were then subjected to different treatments, that is method A, B or C. Method A did not include alcohol shock; stool samples were pipetted directly into media. Method B included alcohol shock followed by centrifuging at 13 000 ***g***, removal of ethanol and transferring the solid residue to media. Method C was identical to method B but included the final additional step of transferring tube plus solid residue to a heating block after solvent removal and heating the sample for 20 min at 40°C, after which the solid residue was transferred to media.

Time study

A time study was also conducted by the preparation of 0·5 McFarland suspensions (0·132 absorbance units at OD_{600nm}) of *Cl. difficile* ribotype 106 in 10 ml cooked meat broth. A 100 μl aliquot of the suspension was added to 10 ml cooked meat broth with added antibiotics, sodium taurocholate and FHPAA. Samples were incubated at 37°C and subjected to volatile profiling via HS-SPME-GC-MS after 1-h incubation at 37°C and then

every subsequent 2 h up until 23 h total incubation time. This was carried out in duplicate.

Evaluation of method sensitivity

The sensitivity of the method in cooked meat broth was also evaluated; *Cl. difficile* ribotype 106 was subcultured on Columbia blood agar, cells were harvested and subjected to alcohol shock. The samples were centrifuged at 13 000 ***g*** for 5 min, and a suspension of 0·5 McFarland units (0·132 absorbance units at OD_{600nm}) was made up of the resulting pellet in sterile water (Neat (N) sample). Samples were serially diluted using decimal dilutions, and 10 μl of each dilution added to 10 ml cooked meat broth. The broth contained FHPAA, antibiotics and sodium taurocholate. Samples were incubated overnight at 37°C. In addition, 10 μl of each dilution was transferred to a selective CCEY agar plate and incubated overnight at 37°C. The number of CFU (colony-forming units) was determined from the agar plate, and VOCs were monitored the following day.

Application of the developed method to the identification of *Clostridium difficile* in stool samples

The developed method for identification of *Cl. difficile* in stool samples was as follows: 0·5 ml stool sample was subjected to alcohol shock; this was followed by centrifugation at 13 000 ***g*** for 5 min. The ethanol was then removed, and the remaining solid residue was inoculated into 10 ml cooked meat broth. The broth contained 250 μg ml^{-1} D-cycloserine, 8 μg ml^{-1} cefoxitin, 4 μg ml^{-1} amphotericin, 100 μg ml^{-1} FHPAA and 2·5 g l^{-1} sodium taurocholate. All samples were incubated overnight at 37°C. This developed method was applied to 100 stool samples, confirmed as 77 *Cl. difficile* culture positive and 23 *Cl. difficile* culture negative. Quantification of evolved VOCs was done using HS-SPME-GC-MS. In addition, a 10 ml blank cooked meat broth was analysed during every day of sampling. VOC profiles were analysed and subjected to statistical analysis including the pattern recognition method, principal component analysis (PCA) (SPSS version 19, IBM, New York, NY).

Sample analysis using HS-SPME-GC-MS

Bacterial VOCs were extracted using a polyacrylate (PA) SPME fibre for 10 min at 37°C prior to 2-min desorption at 230°C in the injection port of the GC-MS prior to separation and identification. VOCs were identified by both their retention times and mass spectra with confirmation of VOCs achieved using known standards and a customized mass spectral library. The VOCs *p*-cresol, 2-fluoro-4-methylphenol and the fatty acids isobutyric acid, butyric acid, isocaproic acid and caproic acid were quantified using external calibration. Calibration graphs were prepared by spiking VOC standards into 10 ml cooked meat broth to which 100 μg ml^{-1} FHPAA, 2·5 g l^{-1} sodium taurocholate, 250 μg ml^{-1} D-cycloserine, 8 μg ml^{-1} cefoxitin and 4 μg ml^{-1} amphotericin were added. Limits of detection (LOD) and limits of quantification (LOQ) were determined as the peak area of three times the signal-to-noise ratio and ten times the signal-to-noise ratio, respectively. Samples were considered to be negative for VOCs if VOC signal size was below that of the LOQ. Blanks were analysed on every day of sampling.

Results

Preliminary investigations and evaluation of parameters on VOC profiles

The VOCs *p*-cresol, isobutyric acid, butyric acid, isocaproic acid, caproic acid and 2-fluoro-4-methylphenol were separated and identified by both mass spectra and retention times (Table S1).

Volatile organic compound profiles detected in stool samples were diverse (Table S2) with fatty acids and *p*-cresol produced by multiple *Cl. difficile* culture-positive stool samples; but these VOCs were also generated by several *Cl. difficile* culture-negative stool samples.

2-Fluoro-4-methylphenol was liberated by 2/2 *Cl. difficile* culture-positive stool samples in broth spiked with the antibiotics D-cycloserine and cefoxitin; 2-fluoro-4-methylphenol was also produced by 2/2 *Cl. difficile* culture-positive stool samples with three added antibiotics (D-cycloserine, cefoxitin and amphotericin) (Table 1). 2-Fluoro-4-methylphenol was liberated by 1/2 *Cl. difficile* culture-negative samples in broth spiked with two antibiotics and by 1/2 *Cl. difficile* culture-negative samples with three added antibiotics. Sodium taurocholate had a variable effect on the amount of 2-fluoro-4-methylphenol and isocaproic acid produced by all stool samples.

Influence of alcohol shock on VOC profiles

The effect of alcohol shock on VOC liberation was evaluated using three stool samples (one *Cl. difficile*-positive and two *Cl. difficile*-negative stool samples). 2-Fluoro-4-methylphenol was liberated as expected with methods A, B and C by 1/1 *Cl. difficile*-positive sample. Alcohol shock methods B and C were comparable for 2-fluoro-4-methylphenol liberation, as well as *p*-cresol and fatty acid production. 2-Fluoro-4-methylphenol was produced by 1/2 *Cl. difficile*-negative samples using method A (no alcohol shock step) and was liberated by 0/2 *Cl. difficile*-negative stool samples with methods B and C (both

Table 1 Influence of enzyme substrate, D-cycloserine, cefoxitin, amphotericin and sodium taurocholate on volatile organic compound (VOC) profiles of stool samples

	Clostridium difficile culture-positive stool samples*								*Clostridium difficile* culture-negative stool samples*							
	A†		B†		C‡		D‡		E†		F†		G‡		H‡	
VOC (μg ml^{-1})	ST	No ST	ST	No ST	ST	No ST	ST	No ST	ST	No ST	ST	No ST	ST	No ST	ST	No ST
2-Fluoro-4-methylphenol	2·6	2·7	12·2	2·2	35·0	29·5	39·1	39·5	33·2	45·6	ND	ND	43·9	38·6	ND	ND
Isobutyric acid	ND	ND	34·9	12·1	47·8	52·1	205	230	ND	ND	ND	ND	50·6	31·7	ND	ND
Butyric acid	315	138	170	135	190	321	>400	272	>400	>400	ND	ND	>400	>400	ND	ND
Isocaproic acid	ND	ND	236	43·0	311	127	>400	>400	ND	ND	ND	ND	ND	ND	ND	ND
p-Cresol	0·30	0·32	3·89	2·85	4·63	4·91	>10	>10	>10	>10	1·35	1·43	>10	>10	ND	ND

ST: 2·5 g l^{-1} sodium taurocholate, no ST: no added sodium taurocholate, >10 μg ml^{-1}: VOC detected outside of upper calibration range, >400 μg ml^{-1}: VOC detected outside of upper calibration range, ND: not detected.
*Stool samples A–D were *Cl. difficile* positive, and stool samples E–H were *Cl. difficile* negative (All samples had FHPAA added at 100 μg ml^{-1}).
†250 μg ml^{-1} D-cycloserine and 8 μg ml^{-1} cefoxitin.
‡250 μg ml^{-1} D-cycloserine, 8 μg ml^{-1} cefoxitin and 4 μg ml^{-1} amphotericin.

Table 2 Influence of alcohol shock on stool samples*

		VOC (μg ml^{-1})				
Sample	Method	2-Fluoro-4-methylphenol	Isobutyric acid	Butyric acid	Isocaproic acid	*p*-Cresol
Clostridium difficile culture-positive stool sample	A	52·1	75·7	303	253	>10
	A	44·7	89·2	264	328	>10
	B	24·1	ND	108	268	3·3
	B	27·9	ND	112	204	4·7
	C	25·5	ND	79·3	162	2·6
	C	31·1	ND	127	242	>10
Clostridium difficile culture-negative stool sample	A	ND	ND	ND	ND	1·2
	A	ND	ND	ND	ND	0·98
	B	ND	ND	ND	ND	ND
	B	ND	ND	ND	ND	ND
	C	ND	ND	ND	ND	ND
	C	ND	ND	ND	ND	ND
Clostridium difficile culture-negative stool sample	A	37·2	ND	356	ND	>10
	A	43·5	ND	270	ND	>10
	B	ND	ND	ND	ND	0·19
	B	ND	ND	ND	ND	0·34
	C	ND	ND	ND	ND	0·29
	C	ND	ND	ND	ND	0·15

>10 μg ml^{-1}: VOC detected outside of upper calibration range, Method A: no alcohol shock (stool samples were pipetted directly into media), Method B: with alcohol shock followed by centrifuging at 13 000 ***g***, removal of ethanol and transferring the solid residue to media, Method C: identical to method B but included the final additional step of transferring tube plus solid residue to a heating block after solvent removal and heating the sample for 20 min at 40°C after which the solid residue was transferred to media, ND: not detected.
*Duplicate samples run for each method.

method included an alcohol shock step) (Table 2). Method B was selected for further work due to its simpler experimental procedure.

Time study

Two *Cl. difficile* samples in broth with FHPAA were monitored over a 24-hour period. The time study (Table 3) indicated that 2-fluoro-4-methylphenol was liberated by *Cl. difficile* in 2/2 samples after 11 h of incubation at 37°C. Isocaproic acid and *p*-cresol were generated by both samples. Both VOCs were produced after 17 h for sample 1. Isocaproic acid was produced after 19 h and *p*-cresol after 21 h for sample 2. VOCs were extracted from blank broths periodically over the 24 h. 2-Fluoro-4-methylphenol, *p*-cresol and the fatty acids

Table 3 Time study data for the production of 2-fluoro-4-methylphenol in broth

	2-Fluoro-4-methylphenol concentration (μg ml^{-1})	
Time (h)	Sample 1	Sample 2
1	ND	ND
3	ND	ND
5	ND	ND
7	ND	ND
9	2·1	Trace*
11	3·3	2·0
13	5·9	2·0
15	15·2	2·3
17	19·6	3·8
19	22·4	4·8
21	27·0	6·2
23	28·1	8·5

ND: not detected.
*2-Fluoro-4-methylphenol was detected at trace levels but not enough to be quantifiable.

isobutyric, butyric, isocaproic and caproic acid were not emitted by uninoculated cooked meat broth.

Evaluation of method sensitivity

The sensitivity of the developed method was assessed in terms of the number of CFU per ml of broth required for the detection of 2-fluoro-4-methylphenol using the HS-SPME-GC-MS method. The detection of *Cl. difficile* in broth required 150 CFU per 10 ml of cooked meat broth to liberate quantifiable amounts of 2-fluoro-4-methylphenol after overnight incubation when a pure inoculum of *Cl. difficile* ribotype 106 was used.

Application of developed method to the identification of *Clostridium difficile* in stool samples

The evolved VOCs (2-fluoro-4-methylphenol, isobutyric acid, butyric acid, isocaproic acid, caproic acid and *p*-cresol) were quantified. Results are shown in Table 4 indicating the specific VOCs liberated and their mean concentration for the 100 stool samples. Figure 1 shows representative HS-SPME-GC-MS chromatograms of (A) *Cl. difficile* culture-positive and (B) *Cl. difficile* culture-negative samples. 2-Fluoro-4-methylphenol was detected in 83·1% of *Cl. difficile* culture-positive samples and was not detected in any *Cl. difficile* culture-negative samples. *p*-Cresol was detected in the majority of *Cl. difficile*-positive and *Cl. difficile*-negative samples (81·8 and 69·6%, respectively), although *Cl. difficile*-positive samples generated, on average, a significantly higher *p*-cresol concentration than negative samples (0·59 and 0·13 μg ml^{-1}, respectively; $t = 4{\cdot}741$, $P = 0{\cdot}000$). In addition, isocaproic acid was detected in 62·3% of *Cl. difficile*-positive samples and was rarely detected in *Cl. difficile*-negative samples (8·7%).

Multivariate analysis of the data using principal component analysis (PCA) was carried out using the VOC profiles generated by all stool samples. It was shown that a combination of isocaproic acid, *p*-cresol and 2-fluoro-4-methylphenol could distinguish between *Cl. difficile*-positive and culture-negative samples. Principal components (PC) 1 and 2 accounted for 93·8% of variation in the data set and allowed separation of *Cl. difficile*-positive and *Cl. difficile*-negative samples (Fig. 2). *Cl. difficile*-negative samples had PC1 scores of $<-0{\cdot}41$ and PC2 scores of $<-0{\cdot}26$, whereas *Cl. difficile*-positive samples had PC1 scores of above $-0{\cdot}73$ and PC2 scores of above $-3{\cdot}25$. Detection of isocaproic acid and/or *p*-cresol in addition to 2-fluoro-4-methylphenol was further confirmation of a *Cl. difficile* culture-positive sample. However, the detection of isocaproic acid and *p*-cresol without 2-fluoro-4-methylphenol was not sufficient to identify a sample as *Cl. difficile* positive. (Note: PCA was carried out including all VOCs (2-fluoro-4-methylphenol, *p*-cresol and fatty acids); however, using all VOCs did not allow discrimination between *Cl. difficile* culture-positive and culture-negative samples).

Table 4 Volatile organic compounds (VOCs) detected from 100 stool samples

	Clostridium difficile culture-positive stool samples		*Clostridium difficile* culture-negative stool samples	
VOC	*n* (%)*	Mean ± 1SD (μg ml^{-1})	*n* (%)	Mean ± 1SD (μg ml^{-1})
2-Fluoro-4-methylphenol	64 (83·2)	10·0 ± 9·4	0 (0)	N/A
Isobutyric acid	3 (3·9)	56·0 ± 37·2	0 (0)	N/A
Butyric acid	17 (22·1)	110 ± 123	2 (8·7)	N/A
Isocaproic acid	48 (62·3)	87·0 ± 86·1	2 (8·7)	N/A
Caproic acid	10 (13·0)	16·6 ± 9·3	0 (0)	N/A
p-Cresol	63 (81·8)	0·59 ± 0·75	16 (69·6)	0·13 ± 0·10

N/A: not applicable (insufficient samples to calculate mean VOC concentration), SD: standard deviation.
*Percentage of samples positive for VOCs.

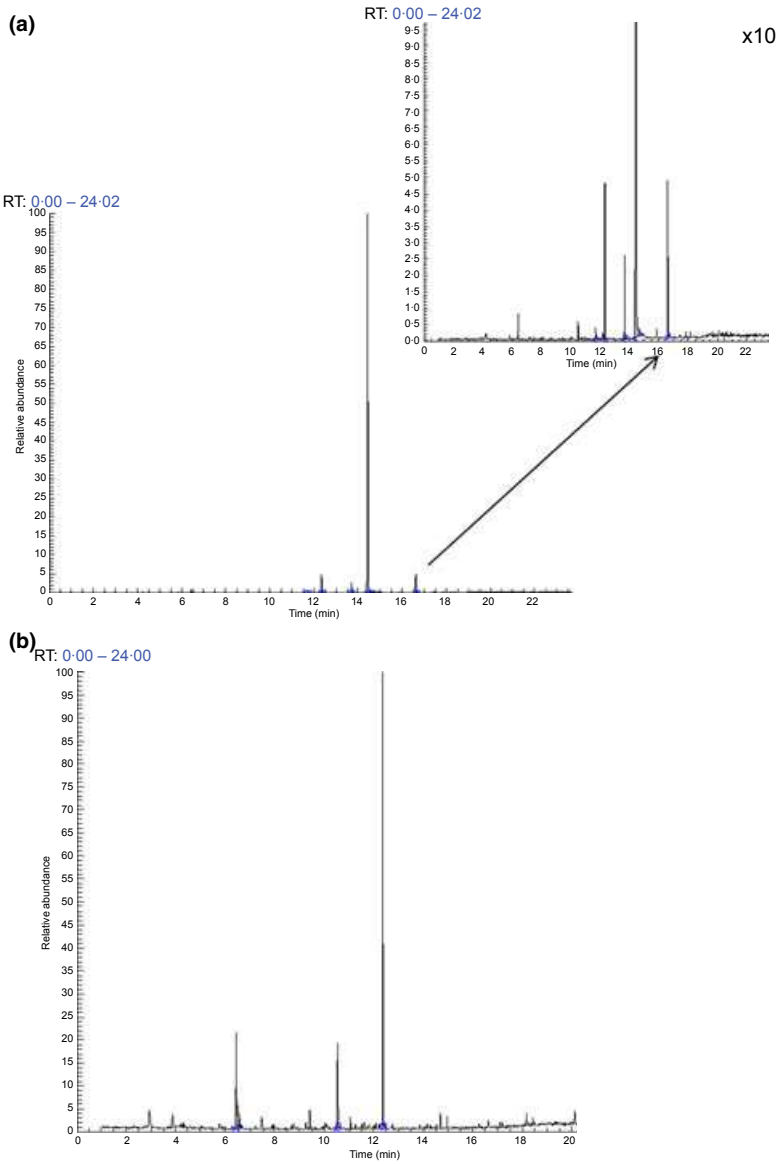

Figure 1 HS-SPME GC/MS chromatogram of (a) *Clostridium difficile*-positive and (b) *Clostridium difficile*-negative stool samples. Main peaks: butyric acid (t_R 11·7 min), isocaproic acid (t_R 13·7 min), 2-fluoro-4-methylphenol (t_R 14·4 min), *p*-cresol (t_R 16·6 min). (peak at t_R 12·3 min is N-methyl-pyrrolidone – solvent used to dissolve amphotericin). Peak at 6·4 min and other smaller peaks are either unknown compounds evolving from the broth or background noise from the SPME fibre.

Further work was therefore undertaken to investigate whether it was possible to differentiate between *Cl. difficile* culture-positive (toxin-positive) and *Cl. difficile* culture-positive (toxin-negative) stool samples. Of the 77 *Cl. difficile* culture-positive stool samples, 60 yielded toxigenic isolates and 17 yielded nontoxigenic *Cl. difficile*. *p*-Cresol was detected in 83·3 and 76·5% of culture-positive (toxin-positive) and culture-positive (toxin-negative) specimens, respectively. However, mean *p*-cresol concentration generated by toxigenic and nontoxigenic samples was comparable, 0·54 ± 0·59 and 0·79 ± 1·19 μg ml^{-1}, respectively. Isocaproic acid was produced by 68·3% of culture-positive (toxin-positive) and 41·2% of culture-positive (toxin-negative) specimens; however, while mean concentration of isocaproic acid was higher for nontoxigenic than toxigenic specimens, there was still a large amount of overlap in isocaproic acid concentration between toxin-positive and toxin-negative samples (78·8 ± 71·6 and 134·8 ± 144·1 μg ml^{-1}, respectively). 2-Fluoro-4-methylphenol was generated by 86·7% of culture-positive (toxin-positive) and 70·6% of culture-positive (toxin-negative) specimens. Although toxigenic

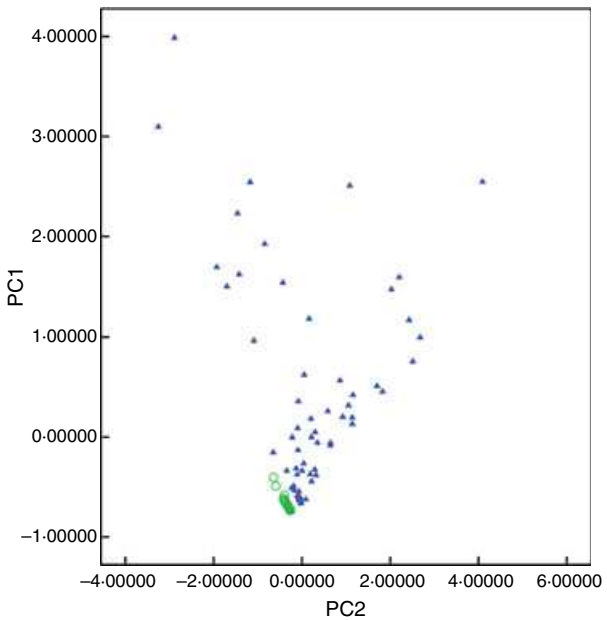

Figure 2 Discrimination between *Clostridium difficile* culture-positive and culture-negative samples using the VOCs 2-fluoro-4-methylphenol, isocaproic acid and p-cresol concentrations (μg ml^{-1}) using principal component analysis. (▲) *Clostridium difficile* culture-positive stool sample; (○) *Clostridium difficile* culture-negative stool sample.

specimens liberated a higher mean 2-fluoro-4-methylphenol concentration (11·1 ± 9·9 μg ml^{-1}) than those that were nontoxigenic (5·6 ± 4·8 μg ml^{-1}), there was a significant degree of overlap in amount of 2-fluoro-4-methylphenol generated, therefore, these samples could not be differentiated. PCA was carried out with all VOCs and with the VOCs 2-fluoro-4-methylphenol, isocaproic acid and *p*-cresol; it was confirmed that no discrimination between *Cl. difficile* culture-positive (toxin-positive) and *Cl. difficile* culture-positive (toxin-negative) samples was possible in either case.

Discussion

The possible identification of *Cl. difficile* using one or a combination of the VOCs *p*-cresol and the fatty acids isobutyric acid, butyric acid and isocaproic acid has been previously reported (Phillips and Rogers 1981; Gianfrilli *et al.* 1985). In addition, the detection of these VOCs, particularly *p*-cresol, in *Cl. difficile* culture-negative stool samples has previously been reported (Pepersack *et al.* 1983; Levett 1984) and is a key reason for the lack of specificity of VOC analysis when previously applied to stool samples. The presence of *p*-cresol and fatty acids in *Cl. difficile* culture-positive and *Cl. difficile* culture-negative stool samples could also be explained by the presence of other faecal bacteria, which are known to ferment a range of amino acids forming short and branched-chain fatty acids and phenolic compounds (Smith and Macfarlane 1997; Macfarlane and Macfarlane 2003).

The enzyme substrate 3-fluoro-4-hydroxyphenylacetic acid (FHPAA) was used as a diagnostic tool to enhance specificity of the HS-SPME-GC-MS method. This specificity was possible as FHPAA is decarboxylated by *p*-hydroxyphenylacetate decarboxylase enzymes present in *Cl. difficile* to release 2-fluoro-4-methylphenol; 2-fluoro-4-methylphenol was detected as a VOC using HS-SPME-GC-MS. Addition of culture medium to stool samples provided the growth conditions required for decarboxylation of FHPAA to 2-fluoro-4-methylphenol, but also allowed decarboxylation of the substrate by faecal bacteria in *Cl. difficile*-negative samples. This occurred with the inclusion of the antibiotics D-cycloserine, cefoxitin and amphotericin in the culture medium. *Lactobacillus* species are known to produce *p*-cresol by decarboxylation of *p*-hydroxyphenylacetic acid in the same manner as *Cl. difficile* (Yokoyama and Carlson 1981) and can grow in the presence of cefoxitin and D-cycloserine (George *et al.* 1979). The inclusion of three antibiotics was not sufficient to inhibit all faecal bacteria therefore allowing the decarboxylation of FHPAA by a species of bacteria other than *Cl. difficile*. Sodium taurocholate appeared to have a slight variable effect on VOC generation. There were significant changes to the signal size of 2-fluoro-4-methylphenol and isocaproic acid in one stool sample which indicated that sodium taurocholate could be useful in some cases by its ability to facilitate germination of spores, particularly as *Cl. difficile* is likely to be in spore form in stool samples.

The alcohol shock step allowed clear differentiation between *Cl. difficile* culture-positive and culture-negative stool samples with regard to the detection of 2-fluoro-4-methylphenol. It was also shown to eliminate previous false-positive results with *Cl. difficile* culture-negative stool samples. There was a slight loss of sensitivity in the amount of 2-fluoro-4-methylphenol produced, but this did not lead to any lack of clarity in results as the amount of 2-fluoro-4-methylphenol detected was still significant. The fatty acids and *p*-cresol also suffered a loss in sensitivity. Previous reports have indicated that alcohol shock followed by inoculation in broth increases the isolation rate of *Cl. difficile* from stool samples when compared with direct culturing and has been recommended to be used alongside culture methods (Clabots *et al.* 1989; Marler *et al.* 1992).

2-Fluoro-4-methylphenol was liberated by *Cl. difficile* after 11 h of incubation at 37°C; this indicated that the decarboxylation of FHPAA requires growth. It also

demonstrated that it was possible to identify *Cl. difficile* in broth by the presence of 2-fluoro-4-methylphenol after 11 h which is significantly quicker than current methods. However, the initial inoculum used in the time study was fairly high (100 μl of a 0·5 McFarland suspension of *Cl. difficile*); the generation of a detectable amount of 2-fluoro-4-methylphenol would depend on the amount of *Cl. difficile* in specimens; this would vary considerably between each sample.

To detect *Cl. difficile* in cooked meat broth via the production of 2-fluoro-4-methylphenol, an initial inoculum of 150 CFU per 10 ml cooked meat broth is required.

Differentiating between *Clostridium difficile*-positive and *Clostridium difficile*-negative stool samples

2-Fluoro-4-methylphenol was not detected in any *Cl. difficile* culture-negative stool sample which suggests that the alcohol shock step was successful in killing other bacteria with *p*-hydroxyphenylacetate decarboxylase activity. However, as 2-fluoro-4-methylphenol was not generated by 13 *Cl. difficile* culture-positive specimens, using the production of this VOC as a marker for *Cl. difficile*-positive samples gives the method a sensitivity of 83·1% as well as a specificity of 100%. The generation of 2-fluoro-4-methylphenol was therefore indicative of *Cl. difficile*-positive specimens. *p*-Cresol and isocaproic acid were detected in both *Cl. difficile*-positive and *Cl. difficile*-negative samples, and there was a degree of overlap between the amount of *p*-cresol and isocaproic acid generated between samples. This indicates that *p*-cresol and isocaproic acid production themselves are not sufficient to allow discrimination between *Cl. difficile*-positive and *Cl. difficile*-negative specimens. Isobutyric acid, butyric acid and caproic acid were infrequently generated and were therefore not of use in discriminating between *Cl. difficile*-positive and *Cl. difficile*-negative samples.

In summary, we have developed a novel approach for the detection of *Cl. difficile* which involves the incubation of stool samples in the presence of a novel enzyme substrate targeting *p*-hydroxyphenylacetate decarboxylase. The method allows confirmation of the presence of *Cl. difficile* with very high specificity (100%) after 18 h. The limitations of the method include a modest sensitivity (83·1%) which requires improvement. As with culture, the method targets all strains of *Cl. difficile,* and further testing would be required (e.g. using immunoassay or PCR) to distinguish whether positive samples contain toxigenic strains. The innovative approach of exploiting novel enzyme substrates that release unusual VOCs that are not normally found in bacterial cultures may find application in the detection of other bacterial pathogens in clinical or food microbiology.

A novel method has been developed that allows identification of *Cl. difficile* culture-positive stool samples based on the evolution of the specific volatile organic compound 2-fluoro-4-methylphenol. The developed protocol has been evaluated against 100 stool samples.

Acknowledgements

The financial support of bioMérieux S.A. is gratefully acknowledged. High-resolution mass spectrometry was acquired at the EPSRC UK National Mass Spectrometry Facility at Swansea University. In addition, technical support from Mr. E. Ludkin and Mr. Gary Noble is also gratefully acknowledged.

Conflict of interest

The authors have no conflict of interest to declare other than that reported.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Quantitative data for *Clostridium difficile* VOCs.

Table S2 VOCs detected in ten *Clostridium difficile* culture-positive and eight culture-negative samples.