Gas-liquid chromatography in routine processing of blood cultures for detecting anaerobic bacteraemia

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SUMMARY Gas-liquid chromatography was performed on 233 positive blood cultures and findings were compared with culture results. Obligate anaerobic bacteria were recovered from 78 out of 79 blood cultures containing butyric or iso-valeric acids, or both; from 28 out of 69 blood cultures containing succinic acid; and from only one out of 41 blood cultures containing succinic but not butyric or iso-valeric acid. Good correlations (88%) were found for the recovery of anaerobic bacteria and the detection of butyric and/or iso-valeric acids. Detecting volatile fatty acids by gas-liquid chromatography performed on blood cultures at the first signs of growth can therefore provide an early and reliable indication of the presence of anaerobic bacteria.

Short-chain fatty acids (SCFA) are so regularly produced by obligate anaerobic bacteria that they are used as a taxonomic tool. Gas-liquid chromatography (GLC) has been used to detect SCFA in clinical specimens or in blood cultures as a means of diagnosing anaerobic bacterial infection.¹⁻⁵ The aim of our study was to discover (1) which of these acids are the most specific anaerobe markers, and (2) whether significant amounts of these specific acids correlated well with the presence of anaerobes in blood cultures. We hoped that by limiting the GLC analysis to the detection of volatile fatty acids (VFA) the technique could become part of the routine processing of blood cultures in clinical laboratories where large numbers have to be examined.

Material and methods

BLOOD CULTURE PROCEDURES

Blood was inoculated (10%, v/v) into one bottle each of brain-heart infusion (BHI) broth and thioglycollate broth under vacuum with CO₂ and with 0.025% sodium polyanethol sulphonate. On arrival at the laboratory the BHI broth bottles were transiently vented with a sterile cotton-plugged needle. All bottles were then incubated at 37°C for seven days and examined macroscopically daily. Blind aerobic subcultures were performed after the cultures had been incubated for at least 24 hours. Macroscopically positive cultures were immediately Gram-stained,

and aerobic and anaerobic subcultures were made, as follows. Samples of each bottle were withdrawn with a sterile needle and syringe and inoculated on to three different media: (a) Columbia agar base supplemented with 5% sheep blood; (b) chocolate blood agar; and (c) freshly prepared brucella agar supplemented with 5% sheep blood, hemin (5 µg/ml), and vitamin K₁ (10 µg/ml). Columbia agar plates were incubated in normal atmosphere at 37°C for 24 hours. Chocolate blood agar plates were incubated in air with 5-10% CO₂ at 37°C for 48 hours, and brucella agar plates were incubated in anaerobic atmosphere (GasPak anaerobic system BBL) at 37°C for 48 hours. Aerobic and facultatively anaerobic bacteria were identified by standard procedures.⁶

Anaerobes were identified according to the VPI *Anaerobe Laboratory Manual*⁷ and the CDC manual.⁸ Biochemical reactivity was measured by the modified Minitek system⁹ for carbohydrate fermentation, aesculin hydrolysis, and nitrate reduction. The ability to grow in 20% bile and the indol and enzyme production (catalase, gelatinase, lipase, lecithinase) were tested by conventional methods, and metabolic end products were analysed by gas-liquid chromatography.

GAS-LIQUID CHROMATOGRAPHY

Cultures were analysed by GLC at the first signs of growth (14 cases) or after 24-48 hours of additional incubation (219 cases). Cultures without any macroscopic evidence of growth were never analysed. A 5-ml sample was taken and centrifuged for the analysis.

Volatile fatty-acid extraction To 1 ml of the supernatant were added 0.2 ml 50% H_2SO_4 , 0.4 g NaCl, and 1 ml ether. The complete specimen was inverted 20 times and centrifuged to break the emulsion. One microlitre of the ether layer was injected into the chromatographic column with a 10 μl syringe (Hamilton 701-N).

Non-volatile fatty acid methylation and extraction For the non-volatile lactic and succinic acids 0.4 ml 50% H_2SO_4 and 2 ml methanol were added to 1 ml of the supernatant. The mixture was heated to 60°C for 30 min. Then 1 ml water and 1 ml chloroform were added and mixed by gentle inversion of the tube 20 times. After centrifugation 1 μg of the chloroform phase was injected into the column.

Chromatography instrumentation A Hewlett-Packard 5831-A dual-column gas chromatograph equipped with flame ionisation detectors and a Hewlett-Packard 18850 AGC terminal were used. The glass columns, 1.8 m long and 4 mm internal diameter, contained 10% diethylene glycol succinate (DEGS) and 2% H_3PO_4 on Cromosorb W AW 80-100 mesh. Operating conditions were: injection and detector blocks temperature 200°C, column oven temperature 125°C, nitrogen carrier gas flow 60 ml/min, chart speed 0.5 cm/min, attenuation 64.

Chromatograms evaluation The approximate concentration values of acids ($\mu\text{mol/ml}$) in the test samples were calculated by comparing the peak heights of the test sample with those of the appropriate acid standards. Blood cultures without growth were analysed to serve as controls. Significant amounts of acetic and lactic acids were found and traces of butyric, iso-valeric, and succinic acids. According to these findings and for the purpose of study propionic, butyric, iso-valeric, and succinic acids were the SCFA considered. Their amounts were termed as significant when propionic and iso-valeric acids were 0.1 $\mu\text{mol/ml}$, butyric acid 0.2 $\mu\text{mol/ml}$, and succinic acid 0.5 $\mu\text{mol/ml}$. Iso-butyric acid was not considered because this peak was not well resolved from propionic acid by our column.

Table 1 SCFA in positive blood cultures

Isolated bacteria	No. of blood cultures:						Total
	Without succinic acid			With succinic acid			
	None or acetic	Propionic	Butyric and/or iso-valeric*	None or acetic	Propionic	Butyric and/or iso-valeric*	
Anaerobes	7	3	50	1	0	28	89
Aerobes or facultatives	93	10	0	30	10	1	144

*With or without propionic or acetic acid.

MICRO-ORGANISMS RECOVERED FROM CULTURES Gas-liquid chromatography was performed on 233 positive blood cultures: 144 of them contained only aerobic or facultatively anaerobic bacteria and 89 contained obligate anaerobes—15 of the 89 containing also aerobes or facultative anaerobes.

Strains isolated from those cultures were: *Staphylococcus epidermidis* (26), *Staphylococcus aureus* (5), *Streptococcus* spp (17), *Corynebacterium* spp (1), *Candida* spp (1), *Escherichia coli* (36), *Klebsiella pneumoniae* (23), *Serratia marcescens* (16), *Hafnia alvei* (2), *Proteus* spp (9), *Yersinia enterocolitica* (3), *Enterobacter cloacae* (4), *Salmonella typhi* (11), *Pseudomonas aeruginosa* (3), *Acinetobacter calcoaceticus* (1), *Bacillus* spp (1), *Bacteroides fragilis* (28), *Bacteroides* spp (5), *Bacteroides melaninogenicus* (2), *Clostridium perfringens* (21), *Clostridium sphenoides* (1), *Clostridium* spp (1), *Propionibacterium* spp (28), *Peptostreptococcus micros* (1), *Peptostreptococcus parvulus* (1), Gram-positive anaerobic cocci (1), Gram-negative anaerobic rod (1). The last two strains died before identification was completed.

In 15 of the 89 blood cultures containing obligate anaerobes only VFA were analysed.

Results

Results are summarised in Tables 1 and 2. Out of the 89 blood cultures containing obligate anaerobes 82 (92%) had significant concentrations of either propionic, butyric, iso-valeric, or succinic acid. Only 51 (35%) of the 144 blood cultures without anaerobes had significant amounts of at least one of these SCFA. When only butyric and iso-valeric acids were

Table 2 Positive blood cultures with significant amounts of SCFA

Isolated bacteria	No. of blood cultures	No. (%) of blood cultures with at least one of the stated acids*		
		P/B/iV/S	P/B/iV	B/iV
Anaerobes	89	82 (92)	81 (91)	78 (88)
Aerobes or facultatives	144	51 (35)	21 (14)	1 (0.7)

*Propionic (P); butyric (B); iso-valeric (iV); succinic (S).

Table 3 Recovery of anaerobes from blood cultures with significant amounts of SCFA

Fatty acids detected*	No. of cultures	No. (%) of cultures:	
		With anaerobes	Without anaerobes
B and/or iV	79	78 (98.7)	1 (1.3)
P without B or iV	23	3 (13.0)	20 (87.0)
S with or without B or iV	70†	29 (41.5)	41 (58.5)
S without B or iV	41	1 (2.5)	40 (97.5)

*Propionic (P); butyric (B); iso-valeric (iV); succinic (S).

†Including 29 of the 79 with B and/or iV and 10 of the 25 with P.

considered 88% of the blood cultures yielding anaerobes still had significant amounts of at least one of these two acids. With only one exception, none of these SCFA could be detected in any blood culture containing only anaerobes. A *Corynebacterium* spp was obtained from the only blood culture with iso-valeric acid and that failed to grow anaerobes

Seventy-eight out of 79 blood cultures that had butyric or iso-valeric acid, or both, yielded anaerobes

(Table 3; Figs 1, 2), including the 14 blood cultures in which chromatography was performed as soon as there were macroscopic signs of growth in the medium. Blood cultures with propionic acid but without butyric or iso-valeric acids failed to grow anaerobic bacteria in 20 out of 23 cases. Succinic acid was detected in 70 of the 233 blood cultures

Fig. 1 Chart records of chromatographic analysis of one blood culture containing *B. fragilis*. (a) VFA, (b) methylated fatty acids. (A) acetic, (P) propionic, (iV) iso-valeric, (L) lactic, (S) succinic.

Fig. 2 Chart records of chromatographic analysis of one blood culture containing *C. perfringens*. (a) VFA, (b) methylated fatty acids. (A) acetic, (B) butyric, (L) lactic, (S) succinic.

Fig. 3 Chart records of chromatographic analysis of one blood culture containing *S. typhi*. (a) VFA, (b) methylated fatty acids. (A) acetic, (S) succinic.

studied but only 29 of them yielded anaerobes, and of these all but one had also significant amounts of butyric or iso-valeric acids, or both. There were 41 cultures that contained succinic acid without significant amounts of butyric or iso-valeric acids and, with only one exception, all of them contained only aerobic or facultatively anaerobic bacteria (Fig. 3).

When blood cultures yielding corynebacteria or propionibacteria as the only isolate (one case and

28 cases respectively) were not considered (Table 4) good correlations for the recovery of anaerobes and the presence of butyric and/or iso-valeric acids were maintained and the only false-positive results disappeared—that is, none of the blood cultures with butyric and/or iso-valeric acids failed to grow anaerobes.

Discussion

In previous reports SCFA such as propionic, iso-butyric, butyric, iso-valeric, and succinic acids have been considered as metabolic fingerprints of the presence of anaerobes in clinical specimens. Gorbach *et al.*¹ found a good correlation between the recovery of anaerobic Gram-negative bacilli and the presence of iso-butyric, butyric, and succinic acids in clinical specimens. Phillips *et al.*² were always able to isolate anaerobes from samples in which volatile acids other than acetic were detected. Wüst³ found that 24 out of 26 blood cultures yielding obligate anaerobic bacteria had significant amounts of propionic, iso-butyric, butyric, iso-valeric, and/or succinic acids.

Our results suggest that butyric and iso-valeric acids are the metabolic products that most reliably indicate the presence of anaerobes in blood cultures. We agree with Wüst³ that the presence of succinic acid has to be interpreted with caution for it could be produced in fairly large amounts by aerobic or facultative anaerobic bacteria. The same applies to propionic acid. Therefore, although the detection of succinic and propionic acids is invaluable for the identification of isolated anaerobic bacteria, none of them can be used as a reliable indicator for the presumptive differentiation of anaerobic from aerobic infection.⁵

If succinic acid is not looked for the technique is simplified without decreasing its efficiency. In fact, the sensitivity is almost the same (in our study only one out of 41 blood cultures with succinic acid and without butyric or iso-valeric acids yielded anaerobes) and the specificity is increased (40 out of 41 blood cultures with succinic acid and without butyric or iso-valeric had only aerobes).

In our experience, taking butyric and iso-valeric acids as the anaerobe markers, the method would have a sensitivity of 87-89% (blood cultures with anaerobes that could be detected by the presence of metabolic markers) and a specificity of 89-100% (blood cultures with SCFA considered as anaerobe markers that grow anaerobes). Since the SCFA of taxonomic relevance were regularly produced in the blood culture medium a presumptive identification to genus level could be made in most cases when, as usual, direct GLC analysis and direct Gram stain were performed simultaneously. That is particularly

Table 4 Positive blood cultures with significant amounts of SCFA

Isolated bacteria	No. of blood cultures*	No. (%) of blood cultures with at least one of the stated acids:†		
		P/B/iV/S	P/B/iV	B/iV
Anaerobes	61	54 (88)	53 (87)	53 (87)
Aerobes or facultatives	143	50 (35)	20 (14)	0 (0)

*Other than those yielding corynebacteria or propionibacteria as the only isolate.

†Propionic (P); butyric (B); iso-valeric (iV); succinic (S).

true for the Gram-negative rods *Bacteroides* and *Fusobacterium*.

When the effect of time on SCFA production was evaluated for five strains of *B. fragilis*¹⁰ it was found that the maximum rates of glucose metabolism, SCFA production, and bacterial growth all occurred within the first 24 hours of incubation. Therefore we may reasonably assume that when GLC is performed either on the day when the first signs of bacterial growth appear or after further incubation for 24-48 hours the SCFA detected will be the same. This assumption is supported by the results of Wüst,³ who performed GLC analysis on blood cultures when bacterial growth was first observed macroscopically and had only two strains of *Bacteroides thetaiotaomicron* that did not produce significant amounts of SCFA to suggest anaerobic bacteraemia.

When GLC analysis and anaerobic subculture were simultaneously performed by us (14 cases) significant amounts of butyric acid (11 cases) and iso-valeric acid (3 cases) were found, suggesting the presence of anaerobes. That was then confirmed by the growth of *Clostridium perfringens* and *Bacteroides fragilis* respectively. We did not attempt either the GLC analysis of blood cultures without macroscopic signs of growth or the routine anaerobic subculture of them, because it has become increasingly clear that routine anaerobic subculture of macroscopically negative blood cultures does not significantly improve the detection or the early identification of anaerobic bacteria.¹¹⁻¹³ This would mean that soon after anaerobes start growing macroscopic signs of growth in the medium appear. In our view, we cannot expect to find metabolic products of anaerobes when they are not yet actively growing.

We conclude that direct GLC of blood cultures for VFA at the first signs of growth provides reliable information on the same day for the clinician to start antimicrobial therapy. In suggesting an anaerobic bacteraemia it can prevent the clinician from giving an aminoglycoside as the single antimicrobial drug, and in allowing a presumptive identification of the bacterial genus it can justify the initiation of the specific antimicrobial chemotherapy.

Furthermore, routine GLC analysis of blood cultures may improve the overall recovery of anaerobes by alerting the microbiologist of their presence in cultures that also contain some facultative anaerobic

bacteria, which could mask the obligate anaerobes in the anaerobic subcultures.

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