Chromatographic Analysis in Bacteriologic Diagnostics of Blood Cultures, Exudates, and Bronchoalveolar Lavages

Julák J.
Institute of Immunology and Microbiology of the First Faculty of Medicine, Charles University in Prague, Czech Republic

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Abstract: This article summarizes our previously achieved and published results. The method for the determination of bacterial volatile fatty acid patterns (VFA) in clinical samples was elaborated. It employs gas chromatography (GC), solvent extraction or head-space solid phase microextraction (SPME). This method was validated by analyses of reference bacterial strains. After cultivation in defined media, aerobic and facultative anaerobic bacteria provided profiles with a low or none acid content, while anaerobic bacteria provided characteristic but medium-dependent profiles with a higher acid content. This method was used for the analyses of clinical samples of total 375 blood cultures, 205 suppurative and apyogenous exudates, and 210 bronchoalveolar lavages (BALs). These analyses enabled within 30 minutes the detection of microbes, probably non-sporulating anaerobes not found by false-negative cultivation, in 11.2% of blood cultures, in 20.0% of exudates, and in 9.0 to 20.0% of BALs. Using the mass spectrometry (MS) methods, a number of other components with unclear diagnostic importance were found in BAL samples, in particular hydrogen cyanide, methanol, ethanol, hexanol, acetone, cyclohexanone, acetonitrile, formaldehyde, acetaldehyde, ethyl acetate, and other esters. Cyclohexanone, occurring mainly in BALs of patients with pneumonia, undergoing intensive care, may originate as a residual solvent from the plastic parts of the ventilation apparatus.

Key words: Anaerobic infections – Blood culture – Exudate – Pneumonia – Selected ion flow tube mass spectrometry – Solid phase microextraction-gas chromatography-mass spectrometry

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Mailing Address: Assoc. Prof. RNDr. Jaroslav Julák, PhD., Institute of Immunology and Microbiology of the First Faculty of Medicine, Charles University, Studničkova 7, 128 08 Prague 2, Czech Republic, Phone: +420 224 968 461, Fax: +420 224 968 496; e-mail: jaroslav.julak@lf1.cuni.cz

Introduction

The idea of using the chemical analysis for the characterization, or identification of bacteria is far from being the new. To realize this idea, a number of determinations of various cellular bacteria components or products of their metabolism were utilized, by the use of many different analytical methods summarized in [1]. Among numerous characteristic products of metabolism, the determination of carboxylic acids as products of bacterial fermentation was the most frequently used for this purpose. The gas chromatography (GC) method was usually used, even though high-performance liquid chromatography, or capillary electrophoresis, has many advantages too. The GC method requires the separate determination of volatile fatty acids (VFA) and non-volatile fatty acids (NVFA). The determination of VFA and NVFA profiles is deemed necessary in particular for the characterization of fermenting anaerobes in pure cultures [2, 3]. This method has also been applied to aerobic or facultative anaerobic microbes, where produced amounts of VFA and NVFA are lower, and their profiles are less characteristic [4, 5]. Composition of VFA and NVFA depends on culture conditions, in particular on media composition; therefore, the interpretation of results from clinical materials is rather problematic. In spite of this fact, a number of authors have attempted to do it: for blood cultures, partial success is reported for example in [6–10], for microbiological diagnostics of peritoneal dialysis fluid also in [11]. This article summarizes our results published in detail in [12–17].

Due to the technology progress, possibilities of chromatographic determination have been considerably improved, as compared with previous studies, particularly using capillary columns. In the area of sample preparation, separation from a sample matrix by means of solid phase microextraction (SPME) replaces the classical extraction by organic solvents. The SPME method consists of interception of substances from solutions or vapours to a silica fibre covered with a chromatographic phase or a solid sorbent, and their desorption or elution to a chromatograph. In this manner, the extraction step is accelerated, and volatile, often toxic organic solvents, are eliminated. In addition to VFA analysis [19], a number of described SPME applications [18] include also microbiological applications, such as [20].

In the initial stages of our studies, self-made manual systems were used for the screening of blood cultures for the diagnostics of bacteremia or, as the case may be, fungemia. At present, commercial systems based on various detection ways of bacteria growth are commonly used. Their evaluation is well described in the international [21] and Czech [22, 23, 24] reference materials which, among others, point out the importance of detection of relatively frequent anaerobic bacteremia [25, 26]. In addition to blood, these systems may also be utilised for the examination of other fluids [11, 27].

Pneumonia represents a serious medical problem, particularly as the life-threatening nosocomial infection in patients on mechanical ventilation. From

the number of papers dealing with this topic, the comprehensive reviews [28] and [29] should be mentioned. The microbiological diagnosis of these diseases presents many real problems, starting from the collection of samples, when it is difficult to differentiate between etiologic agents and physiological colonisation of the upper respiratory tract. This is important particularly in endotracheal aspirates, but also in bronchoalveolar lavages; the solution seems to be the use of high-tech and expensive protected specimen brush technique (PSB). Nevertheless, the adequacy and relevance of the findings are controversial, because no "gold standard" for the analyses is available. As referred to by [28] and [30], the bacteriological findings itself also seem to be questionable. Although the Gram-negative rods including *Pseudomonas aeruginosa*, staphylococci and streptococci are markedly the main causative agent of pneumonia, the importance of anaerobic bacteria remains uncertain. Only the study [31], carefully conducted under special conditions that ensured the survival of anaerobes during transport of samples and during the microbiological procedures, reported significant anaerobic flora: anaerobes were found in 23% of cases, consisting mainly with the species *Prevotella melaninogenica*, *Fusobacterium nucleatum* and *Veillonella parvula*. Other studies indicated significantly lower percentages: the review [28] quotes the incidence of anaerobes in only 0.9% out of 1689 cases; the studies [32, 33] and [34] reported only solitary findings. As concluded by [28], the anaerobes and other agents may be more common, and are potentially under-reported due to the poor diagnostic techniques.

Material and methods

Reference strains

The methodology was verified by analyses of standard VFA mixtures (Supelco, Praha) and reference bacterial strains (CCM, Brno), cultivated anaerobically at a temperature of 37 °C, in parallel in Vf and VL media (Imuna, Šarišské Michalany). To verify the growth in blood cultures, reference strain suspensions were inoculated to various blood culture bottles (see below), and cultivated aerobically and anaerobically at a temperature of 37 °C. Samples from cultures were collected for analyses after 6, 24, and 48 hours, and after 5 days.

Clinical samples

Sets of samples consisted of randomly selected clinical materials taken from patients hospitalised at various departments of the General Teaching Hospital in Prague, and at the Vinohrady Hospital in Prague. All samples were bacteriologically examined using standard cultivation procedures in microbiological laboratories of the particular hospitals. If an unexpected VFA profile was ascertained, additional microscopic and cultural aerobic anaerobic examinations were performed.

Blood cultures
A sample set consisted of clinical blood cultures taken from patients with suspicious bacteremia, and they were cultivated in one of the below-mentioned systems. A total number of 375 blood cultures have been analysed, out of that 288 was found positive by cultivation, and 87 negative by cultivation. Cultivation of these samples was terminated at first signs of growth, or when a positive signal of the BacT/Alert system appeared.

Home-made media: Blood cultures were cultivated in media prepared in microbiological laboratories on the basis of Brain Heart Infusion (Oxoid), with an addition of saccharose, and 0.03% of Liqoid, or with an addition of glucose, citric acid, and sodium citrate. Prior to use, the media were filled to 100 ml transfusion bottles (NTS).

BacT/Alert: Blood culture bottles BacT/Alert (Organon Teknika) are filled with casein hydrolysate and soya hydrolysate based media, with an addition of 0.035% of polyanethol sodium sulphonate, amino acids, and carbohydrates. In addition, the bottles for aerobic cultivation contain 0.001% of pyridoxine, and the bottles for anaerobic cultivation contain 0.00005% of menadione, 0.0005% of hemine, and reduction agents.

BacT/Alert FAN: Blood culture bottles BacT/Alert FAN are filled with 2.8% BHI medium with 8.5% of Ecosorb, with an addition of 0.05% of polyanethol sodium sulphonate, 0.001% of pyridoxine, 0.00005% of menadione, 0.0005% of hemine, and a complex of amino acids and carbohydrates. Bacterial growth in the BacT/Alert system is detected by photometric monitoring of colour change in the indication disk, induced by produced carbon dioxide, and partially also by produced acids [35].

Exudates
A sample set consisted of clinical exudates of various origin and consistence, i.e. the samples labelled pus, empyema, ascites, effusion, puncture, aspirate, abscess content, drainage content, haematoma, or amniotic fluid. To compare extractions using *tert*-butyl methyl ether (MTBE), another 73 exudates were analysed (30 positive and 43 negative by cultivation).

Bronchoalveolar lavages (BALs)
Samples (210 in total) were selected from BALs, obtained using sterile physiological saline from patients with pneumonia. It seems sensible to separate the BAL samples into two groups: a) those from patients receiving intensive care including mechanical ventilation, hospitalised at the departments of anaesthesiology and resuscitation, cardiovascular surgery, and intensive care units of both hospitals (138 samples); b) those from patients not in intensive care, hospitalised at the other departments, namely of internal medicine, haematology and tuberculosis and respiratory diseases (72 samples).

Sample preparation

Blood cultures: 2 ml was taken in a sterile manner from blood culture bottles into the screw cap test tubes, and 100 mg of NaCl and 100 μl of 5 mM 2-methylhexanoic acid solution (i.e. 6.5 μg 100 ml^{-1}) in 20% H_3PO_4 was added as an internal standard (I.S.). A concurrent addition of phosphoric acid ensured the acid environment. Free VFAs were extracted with an addition of 0.5 ml of *tert*-butyl methyl ether (MTBE), and by shaking. Following the centrifugation of test tubes for 5 minutes at 3000 rpm, the upper organic layer was separated, and from this layer, a dose of 2 μl (1 or 0.5 μl at a very high content of VFA) was injected into the chromatograph. If not analysed immediately, the samples were stored at a temperature of 4 °C.

Exudates: For SPME extraction, NaCl and internal standard (I.S.) were added as in the previous case to approx. 1 ml of sample (reference culture, exudate, or a swab eluted in 1 ml of water) in a 4 ml vial with septum. SPME was performed for 15 minutes at a temperature of 50 °C without stirring from headspace vapours using a 85 μm Polyacrylate Fibre (Supelco, Praha). MTBE extractions were performed in the same way as with blood cultures. If not analysed immediately, the samples were stored at a temperature of –20 °C.

BAL: During the microbiological examination, 1 ml of the lavage was taken in a sterile manner into a 4 ml vial. NaCl and 100 μl of 0.77 mM 2-methylhexanoic acid (i.e. 0.01%, 10 mg 100 ml^{-1}) in 20% H_3PO_4 as an internal standard (I.S.) were added. SPME was performed as in the previous case, except using a 50/30 μm DVB/Carboxen/PDMS Stable Flex Fibre (Supelco, Praha). If not analysed immediately, the samples were stored at a temperature of –20 °C.

Gas chromatography (GC)

Gas chromatography was performed using a Chrom 5 gas chromatograph (Laboratorní přístroje, Praha) on a capillary column 30 m × 0.32 mm × 0.25 μm FSOT NUKOL (Supelco), with a pre-column 1 m × 0.32 mm Polar (Supelco), under the following conditions:

MTBE extracts: temperature: 160 °C, carrier gas: nitrogen, split 1:25, column flow rate 0.6 ml min^{-1}, injector 250 °C, detector 160 °C, flame ionisation detector (FID): 160 °C, makeup nitrogen 25 ml/min.

SPME: The analytes were desorbed at 230 °C into a modified injection port of a Chrom 5 gas chromatograph (Laboratorní přístroje, Praha), and separated on the same column held at 140 °C, carrier gas nitrogen 1.2 ml min^{-1} splitless, FID at 160 °C, makeup 25 ml N_2 min^{-1}.

In the absence of higher chain length VFA, analyses were stopped after elution of the I.S. at the adjusted retention time t'_R = 10.9 min.

Evaluation

VFAs were identified according to their adjusted retention times t'_R and compared with standard substances (Volatile Acid Standard Mix, Supelco). The VFAs are

denoted as follows: acetic **a**, propionic **p**, isobutyric **ib**, butyric **b**, isovaleric **iv**, valeric **v**, isocaproic **ic**, caproic **c**, heptanoic **h**. Additional small peaks, appearing at t_M and at shorter retention times than **a**, were identified by other methods (see below). The amount of each component was expressed as a ratio of their corresponding peak height to that of the I.S., values of 0.0 below indicate trace amounts with ratios less than 0.1.

Mass spectrometry (MS)
Selected representative samples of BALs were analysed using the following methods:

Solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS)
Samples were extracted by SPME under the conditions as above and analysed using TRACE 2000 (Thermo Quest, U.S.A.) gas chromatograph, equipped with columns HP-VOC (60 m × 0.2 mm × 1.1 μm) or HP-INNOWAX (30 m × 0.25 mm × 0.25 μm). Samples were analysed on both columns under the following conditions: injector 250 °C, desorption splitless for 3 min, column temperature 45 °C for 3 min, then programmed increase in temperature at 7 °C min^{-1} up to 200 °C and at 15 °C min^{-1} up to 275 °C. Separated analytes were transferred at 275 °C into the ion trap mass spectrometric detector Polaris Q (Finnigan, U.S.A.). The mass spectra were registered in the segment scan mode from m/z 28 to 220 and evaluated using XCALIBUR 1.1.2 (Finnigan, U.S.A.) software.

Solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) of silylated derivatives
The fatty acids in the samples were converted to the tert-butyldimethylsilyl derivatives using the method described by [36]. Briefly, the samples were treated and extracted as above, except a 85 μm Carboxen/PDMS Fibre (Supelco) was used. The fibre was then introduced into the headspace of a 2 ml vial containing 50 μl of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, Fluka); hence, the adsorbed analytes were silylated *in situ* for 15 min at ambient temperature. The derivatives were desorbed into the injector of the Varian 3400 gas chromatograph at 285 °C for 0.2 min, introduced splitless onto the 60 m × 0.25 mm × 0,25 μm DB-5MS column (J&W) and analysed at a temperature of 60 °C for 5 min, then programmed 10 °C min^{-1} to 300 °C. Components were identified using attached Varian Saturn II mass spectrometer in EI-modus.

Selected ion flow tube mass spectrometry (SIFT-MS)
Detailed descriptions of the SIFT-MS method of headspace analysis are given in [37] and [38]. Clean glass bottles 150 ml were flushed with dry clean cylinder air, each charged with 2 ml of the representative BAL samples and sealed by septa. The charged bottles were placed in a water bath at 40 °C for 10 minutes and the headspace sampled directly to the input port of the SIFT-MS instrument. H_3O^+

and NO^+ mass selected precursor ions were injected into the flow tube of the SIFT-MS instrument and the product ion mass spectra were recorded in the full-scan mode in the range m/z from 10 to 150. The important volatile compounds present in the headspace were identified by the characteristic product ions of their reactions with the precursor ion, and their concentrations were calculated as in [39, 40] and [41].

Results

Comprehensive results both from the analysis of reference cultures, or clinical samples, are not specified here in detail, and the following text only summarizes them along with relevant references.

Methodical aspects

The analyses of reference strain cultures (for more detailed information see [14, 15]) have found that the methodology used provided accessible VFA profiles in some cases already after 6 hours, and reliable profiles after 24 hours of cultivation. Contents of individual VFAs increased usually within 48 hours, and these contents stagnated after longer cultivation. In cultures of aerobic and facultative anaerobic bacteria, VFA contents only exceptionally exceeded the triple of the I.S., while in anaerobes, these contents were usually higher (up to 100 times in dominant acids). Analyses of swabs soaked in a culture provided the same profiles as at 1 ml of sample and only with approximately 10 times lower quantity of VFA. It has also been verified that the storage of cultures at a temperature of 4 °C does not change the contents and VFA profiles in the course of one month. As expected, VFA profiles were quantitatively, sometimes also qualitatively, significantly different after cultivation in Vf and VL media, particularly at anaerobes. The selected results are specified in Table 1.

After cultivation in various blood culture media (see [12, 13]), reference cultures of individual species usually showed similar profiles, and the dependence of the VFA profile on medium composition was expressed mainly in staphylococci and in some anaerobes. No differences were observed between cultivation in the aerobic and anaerobic atmosphere; anaerobic species showed VFA production comparable with anaerobic cultivation in the aerobic home-made media system. The background of pure media, subtracted from individual VFA peaks, was 0.1–0.5 I.S.; BacT/Alert FAN bottles showed a higher background, 0.2–1.6 I.S. For exudates and BAL, it is not possible to determine the background pertaining to a pure medium.

Effectiveness of the following three types of fibres has been tested: fibre 65 μm Carbowax-Divinylbenzene provided the same VFA yields as Polyacrylate fibre, and fibre 50/30 μm DVB-Carboxen-PDMS StableFlex provided approximately double yields. Preliminary tests were carried out in the preparation of VFA derivatives by means of *in situ* reaction with 1-pyrenyldiazomethane (PDAM), which would,

according to [42], enable the concurrent determination of volatile and non-volatile acids. The results are not specified because the derivatization provided only small yields of relevant PDAM derivatives, and appeared to be unsuitable for practical use.

Blood cultures

For more detailed information see [12, 13]. Among blood cultures positive by cultivation, the samples containing *Acinetobacter baumannii*; *Candida* sp.; *Corynebacterium jeikeium*; *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were found to be chromatographically negative (i.e. not containing VFA). Among chromatographically positive blood cultures, acetic acid (**a**) was found as the sole product in samples containing *Acinetobacter baumannii* and *A. calcoaceticus*; *Corynebacterium pseudodiphtheriticum* and *C. xerosis*; *Enterococcus faecium* and *E. faecalis*; *Pseudomonas* sp.; *Serratia marcescens*; *Streptococcus agalactiae*, *S. millerii*, *S. mitis,* and viridans streptococci. In addition to generally dominant **a,** propionic acid (**p**) was present in *Citrobacter freundii*; *Escherichia coli*; *Klebsiella oxytoca*, *K. ozenae* and *K. pneumoniae*; and *Salmonella enteritidis*. Acids **a**, **p** and **iv** were present in *Enterobacter aerogenes* and *E. cloacae*. The same profile displayed also the most frequently found staphylococci, however, in BacT/Alert media, **iv** was sometimes missing; the species *Staphylococcus aureus, S. epidermidis* and *S. haemolyticus* were not differentiated. Dominant **p** (40 I.S.) and **iv** (20 I.S.), along with **a,** was present in *Propionibacterium* sp., dominant **b** (over 100 I.S.), as well as **a**, **p**, **ib**, **iv** and **v,** were found in *Peptostreptococcus* sp.

Table 1 – VFA patterns of some reference strains on different media

	Peak height / I.S.								
Species (medium)	**a**	**p**	**ib**	**b**	**iv**	**v**	**ic**	**c**	**h**
Bacteroides fragilis (Vf)	3.0	17.4	1.2		21.5				0.1
Bacteroides fragilis (VL)	3.8	2.1			0.3			0.2	
Clostridium bifermentans (Vf)	3.5	2.5	6.0	3.0	28.5		102.0		12.0
Clostridium bifermentans (VL)	1.4	0.3		0.0	0.1				
Clostridium difficile (Vf)	3.0	4.5	5.5		23.0		90.0		11.0
Clostridium difficile (VL)	4.8	0.5		0.0		0.1	5.5		
Clostridium perfringens (Vf)	13.5	0.7	4.8	9.9	25.5		24.9		
Clostridium perfringens (VL)	2.2	0.3	1.4	6.9	6.5	0.3	12.7		
Escherichia coli (Vf)	0.2	0.2							
Escherichia coli (VL)	1.6	0.1							
Klebsiella pneumoniae (Vf)	2.3	1.5			0.3				
Klebsiella pneumoniae (VL)	2.1	0.3							
Propionibacterium acnes (Vf)	0.2	2.6			2.1				
Propionibacterium acnes (VL)		1.1			0.0				
Propionibacterium propionicum (Vf)	5.0	23.3			1.5				
Propionibacterium propionicum (VL)	11.1	34.5			0.7				

Empty cells: not detected; 0.0: trace amounts.
The unexpected values are printed in bold and underlined. G+, G-: Gram-positive, Gram-negative.

Table 2 – Selected blood cultures with unusual volatile fatty acid profiles

Bacterial species found by routine examination	Corrected peak height / I.S.								Other bacteria found by additional examination
	a	p	ib	b	iv	v	ic	c	
Acinetobacter baumannii	**2.1**	**10.9**		**3.5**	**2.6**				anaerobic G-coccobacilli (*Bacteroides*?), anaer. G+ cocci, *Propionibacterium* sp.
Candida sp.	**0.2**	**0.1**	**0.1**	**0.7**	**0.2**				solitary anaerobic G+ cocci
Corynebacterium jeikeium	**2.8**	**34.3**			**14.7**				*Propionibacterium* sp.
Corynebact. pseudodiptheriticum	1.0	**15.2**			**0.3**				*Propionibacterium* sp.
Enterococcus faecalis	3.7		**0.1**		**2.9**				Other anaerobic. G+ cocci
Klebsiella pneumoniae	3.7	**12.0**		**14.4**					anaerobic G- rods, *Peptostreptococcus* sp.
negative by cultivation	**0.5**			**0.2**	**0.1**				fusiform G- rods, *Bacteroides* sp.
negative by cultivation	**0.4**				**0.1**				*Staphylococcus aureus*
negative by cultivation	**0.1**	**6.6**							Propionibacterium sp.
negative by cultivation	**3.3**	**1.1**	**0.8**		**5.3**				anaerobic G+ rods
negative by cultivation		**0.1**	**0.3**	**1.4**	**1.2**				anaerobic G+ cocci (*Peptostreptococcus*?)
negative by cultivation	**4.3**	**33.4**	**0.4**						polymorph anaerobic G+ rods and cocci
Propionibacterium sp.	**0.0**	**0.0**			**0**				negative by cultivation and microscopy
Propionibacterium sp.	2.6	39.3		**0.9**	19.6				anaerobic G+ cocci (*Peptostreptococcus*?)
Pseudomonas sp.	0.1	0.1		**0.4**	**0.3**				anaerobic G+ rods, G+ cocci
Pseudomonas sp.	**4.6**	**16.0**	**0.1**	**0.7**	**11.9**				*Propionibacterium* sp.
Pseudomonas sp	**1.9**	**0.6**		**0.1**	**0.7**				chains of anaerobic G+ cocci
Staphylococcus aureus	**0.0**			**0.3**					no *Staphylococcus*, anaerobic G-rods by microscopy
Staphylococcus eidermidis	2.4	**1.1**							*Klebsiella oxytoca*
Staphylococcus epidermidis	1.0	**0.8**			0.1				anaerobic G+ rods
Staphylococcus epidermidis	0.6	**0.5**			0.3				*Propionibacterium* sp.
Staphylococcus epidermidis	2.0	**1.7**	**0.2**	**44.3**	1.2	**0.2**		**0.2**	unidentified anaerobe
Staphylococcus haemolyticus	1.8	**7.6**			0.2				*Propionibacterium* sp.
Staphylococcus haemolyticus	0.9	**0.6**		**0.1**	0.3				anaerobic G-cocci, G+ rods
Streptococcus mitis	*1.9*				**0.3**				*Staphylococcus epidermidis*
Streptococcus mitis	4.0	**14.0**			0.1				unidentified anaerobe

Empty cells: not detected; 0.0: trace amounts.
The unexpected values are printed in bold. G+, G-: Gram-positive, Gram-negative.

Table 3 – Clinical exudates with unusual volatile fatty acid profiles

Material	Found by cultivation	Peak height / I.S. ratio									Additional finding
		a	**p**	**ib**	**b**	**iv**	**v**	**ic**	**c**	**h**	by microscopy
Abdominal empyema	*Propionibacterium* sp.	10.8	2.2	1.8	6.4	12.0	1.0	0.1	3.5	0.1	G+ cocci in chains, fusiforms
Pus from decubitus	*Proteus mirabilis*	9.7	4.8	2.5	38.8	4.4	7.1	7.0	2.8		G+ cocci in chains, fusiforms
Cannula content	*Klebsiella pneumoniae*	17.1	3.6	20.6	53.1	34.4	6.1	59.4	0.6	0.3	G+ cocci in chains, fusiforms
Pus from decubitus	*Streptococcus pyogenes*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*	7.9	4.1	0.4	21.7	2.3	2.5	3.0	0.7		polymorph mixture
Peritonsillar abscess puncture	viridans streptococci	41.7	67.3		31.7						polymorph mixture (bacteroids, coryneforms, G- rods)
Pus	*Propionibacterium* sp., *Escherichia coli*	44.0	54.0	55.0	159.0	122.0	15.0	115.0	1.0	1.0	G+ cocci, large G- rods
Abdominal empyema	*Propionibacterium* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*	6.5	0.5	9.5	40.0	41.0	0.5	1.5	7.5		thin G- rods, tiny G+ cocci in chains
Pus	*Escherichia coli*	3.6	2.4	0.7	0.7	7.0					bacilli, G+ cocci, fusiforms
Lesion puncture	*Streptococcus agalactiae*	3.6	0.0	1.0	2.7	4.9	0.0	18.4			bacteroids
Pus	*Propionibacterium* sp., *Escherichia coli*	19.0	5.0	43.0	161.0	91.0	20.0	125.0	1.0		G+ cocci
Pus from abscess	*Propionibacterium acnes*, *Escherichia coli*	6.1	0.4	2.9	4.4	5.9	0.2	16.0	0.0	0.6	G+ a G- cocci, fusiforms
Abdominal empyema	*Propionibacterium acnes*, *Escherichia coli*	0.1	0.1	0.0	0.1	0.2	0.1	0.0			G+ cocci
Amniotic fluid	negative	0.6				0.0					G+ cocci
Pleural puncture	negative	0.1	0.1			0.1					G+ cocci
Pleural puncture	Streptococcus agalactiae	3.1	0.5			0.2					irregular G+ cocci
Lesser pelvis empyema	*Escherichia coli*	1.1	0.8	1.3	2.3	6.2	0.5		0.2		fusiforms
Pleural drainage	*Bacteroides* sp.	1.9	1.7	2.1	3.7	6.1	0.7	6.3	1.4	0.1	numerous fusiform
Abdominal exudate	*Proteus mirabilis*, *Klebsiella pneumoniae*	9.0	0.7	4.8	36.8	1.5	0.1	0.8			G+ cocci in chains
Abdominal empyema	*Escherichia coli*, *sporadic Clostridium* sp.	0.3	0.2								G- coccobacilli, no Clostridium

Material	Found by cultivation	Peak height / I.S. ratio									Additional finding by microscopy
		a	**p**	**ib**	**b**	**iv**	**v**	**ic**	**c**	**h**	
Cannula content	*Candida* sp.	0.3				0.0					G- cocci, sporadic G- polymorph
Pleural puncture	negative	0.1	0.6			0.0					G+ cocci
Haematoma	negative	1.2	0.1			0.0					large G+ rods
Pus	Candida albicans	0.5	0.0	0.0	40.0	0.0	0.5	0.0	62.0		G+ cocci in chains
Pus	*Proteus mirabilis*	0.8	0.1	1.2	0.0	0.3	0.0	0.0	1.1		fusiforms
Puncture	negative	0.5	0.7	0.2	0.4	0.5	0.0	0.0	0.6	0.0	uncertain – cellular debris
Pus from abscess	*Propionibacterium acnes*, *Klebsiella oxytoca*	13.3	2.5	17.0	54.0	53.5	2.0	62.5	0.3	0.3	numerous G+ cocci in chains
Gall bladder empyema	negative	0.0	0.3			0.1					G+ cocci
Pus	*Pseudomonas aeruginosa*, *Escherichia coli*, *Propionibacterium* sp.	2.0	1.8	1.5	5.9	4.1	1.5	4.5	1.6	0.0	numerous tiny G+ cocci, fusiforms
Pus from lesion	negative	0.8	1.0	0.0	0.0	0.3	0.4	0.0	0.0	0.0	bacteroids
Liver puncture	*Bacteroides fragilis*	0.2	0.2	0.0	0.1	0.0	0.1	0.0	0.4	0.0	non-sporulating G+ rods in chains, no Bacteroides
Cannula content	*Enterococcus faecalis*, *Acinetobacter sp.*, *Proteus mirabilis*	0.6	0.6	1.0	0.1	0.8	0.2	0.0	0.1	0.1	fusiforms, G+ cocci
Abscess	*Pseudomonas aeruginosa*	0.4	0.3	0.2	0.1	0.1	0.1	0.0	0.6	0.0	large and tiny G+ cocci, fusiforms, bacteroids, coryneforms
Pus from liver	*Streptococcus agalactiae*, *Pseudomonas aeruginosa*	2.3	0.2	0.5	6.6	1.7	0.0	1.1	0.0	0.1	fusiforms
Pus	*Klebsiella pneumoniae*, *Acinetobacter baumannii*	4.0	3.8	1.6	4.0	4.4	0.4	4.8	0.2	0.0	tiny G+ cocci
Pus	*Escherichia coli*	1.0	0.3	0.1	0.3	0.3	0.1	0.0	0.0	0.0	G+ cocci in clumps
Pus	*Bacteroides* sp.	0.9	0.6	0.7	0.5	4.2	0.2	0.0	0.0	0.0	bacteroids and G+ rods, G+ cocci
Pus	negative	0.1	0.3	0.0	0.1	0.1	0.2	0.0	0.0	0.0	tiny G+ cocci
Pericardial exudate	negative	0.0	0.6	0.0	0.2	0.0	0.3	0.0	0.0	0.0	tiny G+ cocci, fusiforms
Abscess content	negative	0.4	0.0	0.4	0.5	0.9	0.1	1.1	0.1	0.0	fuisiforms, sporadic G+ cocci
Pleural exudate	negative	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	G+ cocci

Empty cells: not detected; 0.0: trace amounts.

Mixed cultures of two or more species with superposed profiles of present species were found in 17 samples. 13 samples detected the positivity in the BacT/Alert system without the cultivation and chromatographic findings.

In 42 blood cultures, microbiological findings did not correspond to the above-mentioned VFA profiles because they were negative by cultivation, or they contained VFAs at the concurrent finding of chromatographically negative species, or they showed the high content of **p**, **ib**, **b**, **iv**, **v** and **c** at samples where only aerobes were found. Their additional examination usually ascertained other, mostly anaerobic minor bacterial flora, especially staphylococci, propionibacteria, peptostreptococci, and other unidentified anaerobes, often in mixed cultures. The original finding of propionibacteria was not validated in case of one chromatographically negative culture. The selection of these findings is specified in Table 2.

Exudates

For more detailed information see [14, 15]. In clinical samples negative by cultivation, trace amounts of **a** and **p** occasionally appeared. VFA profiles of the majority of positive samples roughly corresponded to the profiles of reference strains and cultivation findings. However, the total quantities of VFA in these samples, even with the same cultivation findings, varied within a very wide range from single- up to the hundred-fold of I.S., because they depend on the amount of bacteria in the exudate and their metabolic activity, as well as on the consistency of sample matrix, ranging from liquid up to a solid gel.

In the samples provided as a swab, only small amounts of VFA were found in all cases, and these did not provide assessable profiles. This sample transfer, well-proven on reference cultures, is obviously not suitable for the analysis of clinical samples. Approximately 0.2 ml represented the lowest successfully analysed amount of a liquid sample.

Other compounds than VFA, eluted at low retention times and incident irregularly, were not taken into consideration during evaluation.

41 samples analysed using SPME, and 7 samples extracted by MTBE, which were negative by cultivation or provided only aerobic flora, displayed different profiles than the above-mentioned, often with a very high content of VFA higher than **p**. Microscopic examination of these samples usually found other microbial flora, characterized as G+ cocci (often in chains), irregular G+ cocci, fusiforms, bacteroids, coryneforms, fine G- rods, or polymorphous mixture of these shapes. The samples, which provided these aberrant results, are specified in Table 3.

Bronchoalveolar lavages

For more detailed information, see [16, 17]. Using SPME-GC with FID detection, almost all samples were seen to contain considerable amounts of **a** (from 0.1 to 5.1 of I.S., average 0.4 of I.S.). Many samples contained also VFA higher in the series than **a**, in amounts only exceptionally exceeding the concentration of added I.S.

Table 4 – BAL samples with unequivocal (upper part) and suspected (lower part) content of higher volatile fatty acids

Unequivocal	Pek height/ I.S. ratio									
Microbiological findings	**6ol**	**c6on**	**a**	**p**	**ib**	**b**	**iv**	**v**	**ic**	**c**
Candida albicans	0.0		2.9			1.2	0.3			
Streptococcus sp., *Neisseria* sp.	0.0		0.6	0.1	0.1	0.1	0.5	0.0	0.3	
negative	0.2	0.0	0.0		0.1	0.5				
Streptococcus sp.	0.3		3.4	2.5		0.1	0.8			
negative, G– filaments	0.4		0.7	0.8	0.3		0.6		0.5	
Streptococcus sp.	1.0		0.6	0.5	0.2	0.1	0.1			
Streptococcus sp.	0.7		0.6	0.6	0.2	0.1	0.3		0.0	
Streptococcus sp., G– filaments	0.9	0.4	0.7	1.7	0.3	0.2	0.2		0.2	
Staphylococcus aureus, Klebsiella oxytoca	0.6	0.6	0.4	0.2	0.2		0.3		0.1	
Candida sp., G+ filaments	0.3		0.1	0.2	0.3	0.0	0.4		0.1	
Staphylococcus epidermidis, Candida sp.	1.3		0.7	0.1		0.1	0.5	0.2		0.1
Staphylococcus epidermidis, Enterococcus faecalis	5.4	1.5	1.1	1.2		0.1		0.2		
Acinetobacter sp., *Klebsiella pneumonie,*	1.7	0.4	1.5		0.3	0.2	1.0	0.2		
Pseudomonas aeruginosa, Proteus sp. *Staphylococcus epidermidis*	0.2	0.1	0.1	0.0				0.6		
Streptococcus sp., *Klebsiella oxytoca, Acinetobacter* sp.	0.6	0.6	0.2	0.6	0.2	0.9	2.3	1.1		0.6
negative, G– rods	4.4	2.7	1.5			0.1		0.5		
Neisseria sp., *Pseudomonas aeruginosa*	0.7	2.8	1.3		0.1	0.3	0.2			
Citrobacter freundii	0.9	1.1	1.0			0.4				
Staphylococcus epidermidis	2.1	2.9	3.1			0.2		0.3		
Suspect	Pek height/ I.S. ratio									
Microbiological findings	**6ol**	**c6on**	**a**	**p**	**ib**	**b**	**iv**	**v**	**ic**	**c**
Pseudomonas aeruginosa	0.2	2.7	0.7	0.1						
Streptococcus sp., *Neisseria* sp.	0.2		0.1	0.1						
Pseudomonas aeruginosa	0.1	3.1	1.4				0.1	0.0		
negative	0.4		1.6			1.5				
Streptococcus sp.	0.7	1.4	0.5			0.0				
negative	0.3	0.7	0.3	0.2						
Escherichia coli	0.2		0.1				0.2			
Staphylococcus haemolyticus	0.1	0.3	0.5			0.0				
Streptococcus sp., *Candida* sp.	0.2		1.6			0.4				
Streptococcus sp., *Candida* sp., *Neisseria* sp.			0.3	0.2						
Streptococcus sp., *Neisseria* sp., *Escherichia coli, Leptotrichia buccalis*			0.1	0.0						
Klebsiella pneumoniae	1.3	1.1	0.4				0.0			
Enterobacter cloacae, Pseudomonas aeruginosa	2.9	0.3	0.0				0.0			
negative	2.2	1.8	1.6			0.4				
Staphylococcus epidermidis, Enterococcus faecalis, Candida sp.	2.5	1.1	0.7			0.1		0.1		
Pseudomonas aeruginosa	2.6	0.8	0.8			0.1		0.1		
Enterococcus faecalis, *Alcaligenes odorans*			0.1			0.1				

Table 4 (continued)

Suspect	Pek height/ I.S. ratio									
Microbiological findings	**6ol**	**c6on**	**a**	**p**	**ib**	**b**	**iv**	**v**	**ic**	**c**
Enterobacter cloacae, Candida albicans, Corynebacterium pseudodiphteriticum	0.0		0.5	0.2	0.1					
Streptococcus sp.	0.1		0.5	0.1			0.0			
Klebsiella oxytoca	0.2	0.2	0.2			0.0	0.1			
negative	0.8	1.0	1.7			0.2				
Staphylococcus epidermidis	0.2	0.1	0.4			0.0				
Streptococcus sp., *Neisseria* sp., *Acinetobacter* sp.	0.2		0.0					0.1		

6ol: hexanol; c6on: cyclohexanone.
Peak height / I.S. 0.0: less than 0.1; empty cells: not detected.

These acids were found either as mixtures of various amounts of **p**, **ib**, **b**, **iv**, **v** and **ic** (rarely also **c** or traces of **h)**, or in low or trace amounts of one VFA (in decreasing order of occurrence, **b**, **p**, **iv**, **v** and **ib**). The samples containing higher VFA may thus be divided into two groups: (a) markedly VFA-positive, containing two or more VFAs in amount greater than 0.2 of the I.S.; (b) dubiously VFA-positive, containing one VFA only, or two VFAs in amounts less than 0.2 of the I.S. Into the first group fall 19 samples (9.0% out of 210), the second group contains 23 samples (11.0%). The total number of these samples was 42, i.e. 20.0% of the total of 210 samples. Amongst the 7 samples with the additional microscopical findings, 4 were markedly VFA-positive, whereas 3 of them contained no higher VFAs. The samples containing higher VFAs are summarised in Table 4.

Numerous additional peaks appeared in almost all of the samples at t_M and at shorter retention times than **a**. All these components were dismissed as impurities in blood cultures and exudates, but in BALs they have been tentatively identified by mass spectrometric methods (see below). They were present in small quantities and were not evaluated by SPME-GC with FID, except for the peaks at t'_R = 0.7 and 1.1 min, which appeared in considerable quantities, reaching or exceeding the I.S. signal. These two components, previously tentatively labelled as "alcohol" and "acetoin", have now been identified by MS as an isomer of hexanol and the cyclohexanone, respectively. As

Table 5 – Occurrence of cyclohexanone (c6on) in BAL from patients receiving intensive care as compared with other patients

	Intensive care	Others
Total No of samples	138	72
No of samples containing c6on	135	6
No of samples without c6on	3	66
Highest content of c6on (related to I.S.)	7.0	0.4
Average content of c6on (related to I.S.)	1.4	0.0
Standard deviation (STD)	1.25	0.07

0.0: trace amounts < 0.1 I.S.

documented in Table 5, the cyclohexanone occurred almost exclusively in samples from patients receiving intensive care (in amounts up to 7.0 I.S.; 0.4 I.S. on average). Only 6 out of 135 samples from other patients contained detectable amounts of cyclohexanone (up to 0.4 I.S.; 0.0 I.S. on average). This effect was less markedly expressed also for the hexanol, which was found in amounts up to 6.1 I.S. (0.8 I.S. on average) in patients receiving intensive care, whereas in others its content did not exceed 1.3 I.S. (0.3 I.S. on average) but was completely absent only in 12 out of 72 samples.

The SPME-GC-MS analysis revealed the presence of numerous components in all analysed samples, namely acetone, ethyl acetate and 10 species of various esters (methyl- and butyl esters of butyric to octanoic acids); these were not subjected to detailed analysis. The unknown compound, previously labelled as "alcohol", was identified as a C6-alcohol, possibly the structural isomer 1-hexanol, or the isomers 4- or 3-methyl-1-pentanol. The second unknown, considered previously as "acetoin", was determined to be cyclohexanone. The VFAs were not identified nor quantitatively evaluated because of poor shape (tailing) of free acid peaks on the used GC columns.

The SPME-GC-MS of the silylated derivatives confirmed the identities of the VFAs. The structural isomers of **iv** acid (i.e., 2-methylbutyric and 3-methylbutyric acid) were distinguished by this method; these isomers were present in nearly equimolar amounts. The abundances of VFAs were in general agreement with the results for the same samples obtained using the SPME-GC with FID. In the samples containing "alcohol" and "acetoin", two species of alcohols (not subjected to detailed analysis) and cyclohexanone were detected.

The SIFT-MS analyses revealed the presence of a number of low-molecular weight compounds in the headspace of the BAL samples. Some of these compounds were present in the headspace of all samples, including acetaldehyde (30 to 550 parts per billion, ppb, of the headspace volume), acetone (1800 to 7200 ppb), methanol (170 to 1000 ppb), ethanol (340 to 5100 ppb) and acetonitrile (60 to 290 ppb). Small amounts of formaldehyde (~100 ppb) were found in two samples. The SIFT-MS spectra also confirmed the presence of hexanol and cyclohexanone. Concentrations of the VFAs in the headspace were determined and confirmed their SPME-GC identification. Quantitative differences in the ratios of the concentrations of VFAs between the two methods were observed in most cases, as the internal standard I.S. (isoheptanoic acid) was not analysed by SIFT-MS. One sample, yielding *Pseudomonas aeruginosa* by cultivation, contained in its headspace a readily measurable amount (300 ppb) of hydrogen cyanide, HCN.

Discussion

The head-space SPME-GC method has been shown as suitable for the analysis of clinical materials, even though, especially in the case of exudates, these regards to very diverse matrixes with the consistency disabling sample stirring, providing

different yields of analytes, and requiring the use of I.S. Unlike examination by cultivation lasting at least one day, these determinations enable rapid detection of mainly anaerobic bacteria during 30 minutes, whence approximately 15 minutes is intended for the sample preparation, and approximately 15 minutes is intended for the analysis. The selected methodology is based on the determination of volatile acids (VFA) and neglects information about the presence of diagnostically significant non-volatile acids. This limitation has been defined by the pursuit of speed and simplicity of the method, because the determination of NVFA requires their esterification and further analysis. Concurrent determination of VFA and NVFA, which is possible, for example, using the analysis of butylesters [43], is methodically problematic, labour- and time-consuming, therefore, less suitable for a rapid detection of bacteria. The use of other methods, such as capillary electrophoresis, is more expensive. However, the determination of VFA, feasible at negligible non-investment material costs, is obviously suitable for the demonstration of presence of fermenting anaerobic and some aerobic or facultative anaerobic bacteria.

There was confirmed the anticipated dependence of composition (profile) of produced acids on media composition, which was significantly different in various bacteria, but it does not exclude the profile comparison of clinical materials. The identification value of individual VFA profiles, i.e. the possibility of their allocation to a certain taxon, is only preliminary because profiles are depended, as expected, on the nature of media; this independence is significant particularly in exudates with an undefined composition. Mixed cultures with overlapping profiles of individual bacteria are also frequent.

From the results of blood culture analyses, we can see that the chromatographic detection of aerobic bacteria is possible only in selected bacterial species. Unexpected chromatographic profiles may be regarded as an indicator of presence of other, usually anaerobic bacteria, not detected using the routine diagnostics. These omissions need not mean a negligent examination: For example, in blood cultures, a faster growing aerobic flora may indicate the positivity of culture, cultivation is stopped, and slower growing anaerobes do not need to be entrapped. The support to this presumption is provided by conclusions specified in the study [11], which used the methodology similar to our procedures: During the inspection of 168 samples of fluid from peritoneal dialyses, performed by the blood culture system, the considerable number of 15 (i.e. approx. 9%) "chromatologically positive" samples with a higher content of acids was found; their further cultivation provided anaerobic bacteria in 14 cases. In our study, 42 from a total number of 375 blood culture samples, i.e. 11.2%, was enlisted as false negative by cultivation, even though all samples have not been validated. The match of both studies may thus be regarded as good.

In the exudate set, the discrepancy of cultivation findings and VFA profiles was found in 41 samples (20.0%) analysed using SPME, and in 7 samples (9.6%)

extracted by *tert*-butyl methyl ether. These samples, usually negative by cultivation or providing only aerobic flora, often contained considerable amounts of VFA higher in the series than **p**, and they may be regarded as false negative by cultivation. Microscopic examination of these samples usually found other microbial flora, characterized as G+ cocci (often in chains), irregular G+ cocci, fusiforms, bacterioids, coryneforms, fine G- rods, or polymorphous mixture of these shapes. In these samples it is possible to anticipate the presence of non-sporulating anaerobes, probably of the genus *Fusobacterium*, *Propionibacterium*, *Peptococcus* or *Peptostreptococcus,* and the like. Higher frequency of these findings by means of SPME, rather than using solvent extractions, in this and in the previous study [11] may be attributable to the higher incidence of non-sporulating anaerobes in exudates, or higher efficiency of SPME.

As compared with blood cultures and exudates, where the quantities of VFAs exceeded sometimes the hundred-fold of I.S., their concentrations in BALs only rarely exceeded the amount of the I.S. This may be due to the dilution of bronchoalveolar content by rinsing with extensive amounts of physiological saline, or due to the presence of small number of anaerobes, or their low metabolic activity. Nevertheless, even small amounts of higher VFAs may be regarded as end products of bacterial anaerobic fermentation, and thus as an indicator of the presence of anaerobes. Using conventional cultivation methods, the presence of anaerobes in clinical samples may be easily overlooked for various reasons, and only revealed by careful purposely-directed examination, as mentioned in the Introduction. In this study, we considered 9% of cultivation examinations to be unambiguously false negative with respect to anaerobes. Using less strict criteria, the additional 11% may be included, making a total of 20% of BALs containing anaerobes, which is quite consistent with the value of 23% found by [31]. These results alone, however, say nothing of the complicity of anaerobes on the aetiology of particular cases of pneumonia.

One of the **iv** isomers, the 2-methylbutyric or "pre-isovaleric" acid, was previously reported as a characteristic metabolite of *Clostridium difficile* [44, 45]. Our findings of considerable amounts of 2-methylbutyric acid in BALs make this conclusion dubious, since the presence of *Clostridium difficile* in BAL seems to be highly improbable.

At this time, we have no comments to make concerning the origin or diagnostic significance of the other low-molecular compounds detected in BALs (methanol, ethanol, acetone, acetonitrile, formaldehyde, acetaldehyde, ethyl acetate and other esters). Except for the previously described connection of HCN with *Pseudomonas aeruginosa* [46, 47], there has been no observed relation between their occurrence and corresponding bacteriological findings, so that their bacterial origin remains unclear. This must be the subject of further extensive studies to establish these relationships.

Concerning the presence of the hexanol and cyclohexanone, they were previously observed in some samples of purulent exudates and tentatively labelled as "alcohol" and "acetoin", but their occurrence was random and irregular and they were thus considered as impurities [15]. By the more thorough examination, their prevalence in BALs from patients receiving intensive care including mechanical ventilation has been positively established. The hexanol was detected in almost all samples and its prevalence in the intensive care cases was manifested rather by the quantitative difference from patients not undergoing intensive care whose BAL samples contained lower amounts of this compound. Statistical analysis [16] showed that the difference between the two sets of results is not significant, with probability $p = 0.086$ only. On the other hand, the selective occurrence of cyclohexanone seems to be very significant: it was present in the BAL of almost all patients in intensive care and absent or present in trace amounts only in almost all patients not undergoing intensive care; the statistical analysis in this case revealed that $p = 0.0001$. As the bacterial or metabolic origin of cyclohexanone is highly improbable, it may originate as a residual solvent from the plastic parts of the ventilation apparatus, which is released in low concentrations and accumulates in the lungs. Although its toxicity is not high, its prolonged exposure to patients' respiratory tract may cause severe irritation. This possibility should stimulate further toxicological studies.

Conclusion

Chromatographic determination of profiles of volatile carboxylic acids, extracted by organic solvent, or by means of SPME, provides the possibility of rapid detection of anaerobic and some aerobic or facultative anaerobic etiologic agents in blood cultures, exudates, and bronchoalveolar lavages. Determination, pursuable without cultivation during 30 minutes, enables the selection of samples for anaerobic cultivation. The fatty acid profiles may become in selected cases an identification marker of present bacteria. Cyclohexanone, found in bronchoalveolar lavages of patients undergoing mechanical ventilation, may originate as a residual solvent from the plastic parts of the ventilation apparatus.

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