Presumptive Diagnosis of Anaerobic Bacteremia by Gas-Liquid Chromatography of Blood Cultures

JÜRGEN WÜST

Institute of Medical Microbiology, University of Zurich, CH-8028 Zurich, Switzerland

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By quantitative gas-liquid chromatography of glucose-containing blood cultures at the moment of first signs of growth, a presumptive diagnosis of anaerobic bacteremia could be made in 24 out of 26 cultures yielding obligate anaerobes upon subsequent culture. With *Bacteroides* sp. (20 strains isolated), elevated levels of isovaleric acid ($\geq 0.1 \mu\text{mol/ml}$) and/or succinic acid ($\geq 5 \mu\text{mol/ml}$) were detected in the medium used. An exception to this were two strains of *Bacteroides thetaiotaomicron* that did not produce sufficient quantities of these acids. In the case of butyrate-producing gram-positive cocci (four strains), butyric acid in amounts of $\geq 0.8 \mu\text{mol/ml}$ was detected. Propionibacteria (five strains) produced propionic acid in amounts of $\geq 3.9 \mu\text{mol/ml}$. No false positive results were found in 103 blood cultures with growth of aerobic or facultatively anaerobic bacteria only.

The rapid and accurate isolation and identification of microorganisms in blood is one of the most important procedures performed in the clinical laboratory. This importance is reflected by numerous reports on the evaluation of media and methods, as reviewed by Bartlett et al. (2) and Washington (19), as well as by the introduction of radiometric methods (15) and by experimental trials with measurement of electrical impedance (9) for rapid detection of growth.

The mortality rate in anaerobic bacteremia can be high, especially in infections caused by the *Bacteroidaceae* (4, 20). The *Bacteroides fragilis* group (3), the most frequently encountered obligate anaerobes in human infections, is resistant to the combination of β -lactam antibiotics and aminoglycosides frequently used for initial therapy of bacteremia before culture results are available (7, 17). Since isolation and identification of obligately anaerobic bacteria are usually time consuming, methods for rapid presumptive diagnosis may be helpful to the clinician. As most obligate anaerobes produce fatty acids in glucose-containing media (10), a study was undertaken to determine whether quantitative gas-liquid chromatography (GLC) can be used as an indicator for the presence of obligately anaerobic bacteria.

MATERIALS AND METHODS

Blood culture medium. The medium used (16) consisted of Trypticase soy broth (BBL), 30.0 g; glucose, 2.5 g; agar, 0.5 g; and sodium polyanetholsulfonate (Hoffmann-La Roche & Co. AG, Basel, Switzerland),

0.5 g in 1,000 ml of distilled water. Medium (75 ml) was dispensed into 100-ml glass bottles with a rubber-diaphragmed screw cap. A small piece of calcium carbonate was added to each bottle prior to autoclaving to neutralize acids produced during bacterial growth. The uninoculated medium contained acetic acid (27 to 65 $\mu\text{mol/ml}$), butyric acid (0.01 to 0.04 $\mu\text{mol/ml}$), isovaleric acid (0.02 to 0.08 $\mu\text{mol/ml}$), lactic acid (2 to 12 $\mu\text{mol/ml}$), and succinic acid (0.09 to 0.15 $\mu\text{mol/ml}$) (five bottles from different batches were tested).

Culture procedure. Five milliliters of blood were added to the blood culture bottles. Usually cultures were submitted to the laboratory on the same day. On some occasions, they were incubated in the ward for up to 2 days at 37°C. Upon arrival in the laboratory, routine subcultures were done. The cultures were incubated at 37°C and examined daily for a minimum of 7 days. Final routine subcultures were performed on day 6. Upon any visible evidence of turbidity, hemolysis, gas production, or colonies, a Gram stain and aerobic and anaerobic subcultures were made in accordance with the methods outlined by Washington (19); nonselective and selective media such as kanamycin-vancomycin blood agar and phenylethyl alcohol blood agar (18) were used. At the same time, a 5-ml sample was taken for quantitative GLC. Identification of obligate anaerobes was performed according to the methods described by Holdeman and Moore (10). Aerobic and facultatively anaerobic bacteria were identified by standard procedures (1, 5, 12).

Volatile fatty acid extraction. To 1 ml of the supernatant of the centrifuged sample from the blood culture, 0.2 ml of 50% H_2SO_4 , 0.4 g of NaCl, and 1 ml of ether were added in an ice bath. The complete specimen was inverted 20 times and centrifuged to break the emulsion, and the ether layer was withdrawn for GLC (10).

Nonvolatile fatty acid methylation. To 1 ml of the supernatant described above, 0.4 ml of 50% H_2SO_4 and 2 ml of methanol were added. The mixture was heated to 55°C for 30 min. One milliliter of water and 1 ml of chloroform were added, and the tubes were inverted 20 times. After centrifugation, the chloroform phase was removed for GLC (10).

Chromatography instrumentation. A Perkin Elmer 3920 dual-column gas chromatograph equipped with flame ionization detectors was used. Samples (2.0 μ l) were injected using a 10- μ l syringe (Scientific Glass Engineering, Inc., Austin, Tex.) and later a 5- μ l syringe (Hamilton Co., Inc., Whittier, Ca.) into glass columns (1.8-m length, by 2-mm I D) containing 5% FFAP on Chromosorb G (acid washed) treated with DMSC, 70/80 mesh (Varian Aerograph, Walnut Creek, Calif.). Standard analysis conditions included: injection and detector block, 180°C; oven isothermal, 140°C; nitrogen, hydrogen, and air flows at 30, 30, and 300 ml/min, respectively.

Quantitation. A Spectra-Physics "Minigrator" was used for the integration of peak areas. With each series, a standard aqueous solution containing 1 μ mol of the fatty acids investigated per ml was extracted strictly in parallel with the blood culture samples. The ether and chloroform contained 1 μ mol of malonyl dimethyl ester per ml as internal standard for determination of retention times as well as for quantitative evaluation (11). No difference could be detected between standard solutions prepared in water and in the blood culture medium. To ascertain the accuracy of the method, blood culture medium containing 1 μ mol of the acids per ml was chromatographed five times. The relative standard deviations for acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, and caproic acids were between 16.0 and 17.8%; for lactic acid, 21.1%; and for succinic acid, 28.6%. Using solutions containing 0.1 to 100 μ mol of the acids per ml, good linearity was observed between concentration and peak areas.

RESULTS

Of the 147 blood cultures investigated by quantitative GLC, 103 contained only aerobic or facultatively anaerobic bacteria. The 120 strains isolated from those cultures consisted of the following: 24 *Escherichia coli*, 9 *Klebsiella pneumoniae*, 3 *Proteus mirabilis*, 3 *P. vulgaris*, 2 *Salmonella enteritidis*, 3 *Serratia marcescens*, 3 *Haemophilus influenzae*, 3 *Pseudomonas aeruginosa*, 5 other *Pseudomonas* sp., 1 *Acinetobacter anitratus*, 1 *Flavobacterium* sp., 2 *Neisseria meningitidis*, 8 *Staphylococcus aureus*, 22 *S. epidermidis*, 2 *Micrococcus* sp., 9 β -hemolytic streptococci, 1 α -hemolytic streptococcus group B, 8 enterococci, 4 pneumococci, 1 *Listeria monocytogenes*, 2 *Corynebacterium* sp., 1 *Bacillus* sp., and 3 *Candida albicans*.

There were 26 blood cultures that contained obligate anaerobes (see Table 1); two of these also contained an aerobe or a facultative anaerobe species (blood cultures no. 11 and 12).

The results are summarized in Table 1. Eighteen blood cultures without growth had appreciable amounts of acetic acid (14 to 76 μ mol/ml), lactic acid (2 to 21 μ mol/ml), traces of butyric acid (0.01 to 0.04 μ mol/ml), isovaleric acid (0.02 to 0.08 μ mol/ml), and succinic acid (0.06 to 0.15 μ mol/ml). Most of the 103 cultures containing only aerobic or facultatively anaerobic organisms produced some succinic acid: *Enterobacteriaceae*, up to 4.5 μ mol/ml; *H. influenzae*, ≤ 1.8 μ mol/ml; *Pseudomonas* sp., ≤ 0.7 μ mol/ml; meningococci, ≤ 0.3 μ mol/ml; staphylococci, ≤ 0.4 μ mol/ml; streptococci, ≤ 0.5 μ mol/ml; and *Corynebacterium* sp., ≤ 4.6 μ mol/ml. Two of nine cultures of *K. pneumoniae* contained propionic acid in amounts of 0.65 and 1.20 μ mol/ml; one of these strains also produced 0.17 μ mol of butyrate per ml. Some cultures also had higher amounts of acetic (up to 167 μ mol/ml) and lactic (up to 98 μ mol/ml) acids than the blood cultures without growth. Otherwise, blood cultures with aerobic and facultatively anaerobic bacteria did not contain higher amounts of propionic, isobutyric, butyric, and isovaleric acids than the blood cultures without growth.

With only two exceptions, all blood cultures containing obligately anaerobic bacteria had elevated amounts of propionic, isobutyric, butyric, isovaleric, and/or succinic acids. For the *B. fragilis* group, presence of isovaleric acid in amounts of ≥ 0.1 μ mol/ml and/or succinic acid in amounts of ≥ 5 μ mol/ml suggested anaerobic bacteremia. There were two strains of *B. thetaiotaomicron* (blood cultures no. 13 and 14) that did not produce amounts of acids indicative for anaerobic bacteremia when the bacterial growth was first observed in the medium. The two cultures with *B. melaninogenicus* ss. *asaccharolyticus* (no. 16 and 17) produced mainly isobutyric, butyric, and isovaleric acids. *Peptococcus asaccharolyticus* and *P. prevotii* produced primarily butyric acid (no. 20 and 21). In the mixed culture with *P. prevotii* and *B. rumincola* subsp. *brevis* (no. 18), the acids probably originated mainly from the *P. prevotii*, whereas in the mixture of *P. asaccharolyticus* and an unidentified *Bacteroides* strain (no. 19), elevated amounts of isovaleric and succinic acids could be found indicating the presence of the gram-negative obligately anaerobic rod; the *Bacteroides* strain resembled *B. disiens* (L. V. Holdeman and W. E. C. Moore, personal communication), but it died before final identification was completed. The propionibacteria produced mainly propionic acid. In most cultures with gram-negative obligately anaerobic bacteria, small amounts of isobutyric acid could be

Table 1. Fatty acids detected in blood cultures yielding anaerobic bacteria.^a

Blood Culture Number	Fatty Acid (μ Mole/ml)					Organisms Isolated
	Propionic	Isobutyric	Butyric	Isovaleric	Succinic	
1		.04	.03	.34	26.84	<i>B. fragilis</i>
2		.03	.04	.27	16.90	<i>B. fragilis</i>
3		.04	.05	.40	23.66	<i>B. fragilis</i>
4		.03	.04	.32	18.80	<i>B. fragilis</i>
5		.02	.04	.26	9.03	<i>B. fragilis</i>
6		.06	.04	.21	1.87	<i>B. fragilis</i>
7				.20	24.80	<i>B. fragilis</i>
8		.06	.04	.33	4.47	<i>B. fragilis</i>
9		.11	.04	.42	11.50	<i>B. fragilis</i>
10		.11	.02	.56	12.95	<i>B. fragilis</i>
11	.26	.06	.01	.11	17.50	<i>B. fragilis</i> , <i>B. distasonis</i> , <i>P. aeruginosa</i>
12		.10		.10	12.07	<i>B. thetaiotaomicron</i> , <i>S. epidermidis</i>
13	.17		.07	.04	3.61	<i>B. thetaiotaomicron</i>
14			.04	.04	.10	<i>B. thetaiotaomicron</i> <i>P. magnus</i>
15			.05	.08	16.45	<i>B. ovatus</i> , <i>P. magnus</i>
16	.26	.66	2.16	1.37	.47	<i>B. melaninogenicus</i> <i>ss. asaccharolyticus</i>
17	.26	.45	1.02	.63	.44	<i>B. melaninogenicus</i> <i>ss. asaccharolyticus</i>
18		.02	.77	.14	2.03	<i>B. ruminicola</i> <i>ss. brevis</i> , <i>P. prevotii</i>
19		.11	2.72	.79	16.70	<i>Bacteroides sp.</i> , <i>P. asaccharolyticus</i>
20	.18		28.60		.39	<i>P. asaccharolyticus</i>
21	.43		7.04		.08	<i>P. prevotii</i>
22	14.70		.10	.05	.44	<i>P. acnes</i>
23	5.97		.04	.10	.70	<i>P. acnes</i>
24	3.86		.02	.04	.55	<i>P. acnes</i>
25	15.50		.05	.26	.55	<i>P. acnes</i>
26	12.56		.04	.15	1.03	<i>P. acnes</i>
			$\leq .04$	$\leq .08$	$\leq .15$	Blood cultures without growth
	≤ 1.20		$\leq .17$	$\leq .08$	≤ 4.60	Aerobic and facultative bacteria ^b

^a Lowest reliably detectable amounts, micromoles per milliliter: propionic acid, 0.2; isobutyric acid, 0.02; butyric acid, 0.02; isovaleric acid, 0.02; succinic acid, 0.1. The acids have been tentatively identified according to retention times.

^b Blood cultures yielding aerobic or facultative bacteria did not contain higher amounts of propionic, isobutyric, butyric, and isovaleric acids than the ones without growth with the exception of two cultures of *K. pneumoniae* (1.2 μ mol of propionic acid and 0.17 μ mol of butyric acid per ml, and 0.65 μ mol of propionic acid per ml, respectively). Succinic acid was often elevated (see text).

detected; however, they were often only slightly above the detectable level, making evaluation difficult.

DISCUSSION

Direct GLC of clinical specimens has been found to provide a rapid presumptive diagnosis of anaerobic infections (8, 14), although some false positive and negative results have been reported (6). Gorbach et al. (8) have found that the presence of isobutyric, butyric, and/or succinic acids in amounts ≥ 0.1 μ mol/ml are suggestive of infection with gram-negative obligately anaerobic rods, i.e., *Bacteroidaceae*. In contrast to Gorbach et al. (8), I have found similar and higher amounts of succinic acid in purulent spec-

imens from pure infections by *S. aureus* (five cases with 0.1 to 0.95 μ mol of succinic acid per ml), β -hemolytic streptococci group A (one case, 0.13 μ mol/ml), *P. aeruginosa* (one case, 0.18 μ mol/ml), *Nocardia asteroides* (one case, 0.13 μ mol/ml), and in a case of multiple amebic liver abscesses (0.46 μ mol/ml; amebiasis diagnosed by serology) (unpublished data). Although direct GLC of clinical specimens is a useful tool in establishing a presumptive and rapid diagnosis of anaerobic infection, the presence of succinic acid has to be interpreted with caution. The same applies to some extent to the quantitative GLC of blood cultures, as facultatively anaerobic and aerobic bacteria also may produce some succinic acid.

The present study indicates that quantitative GLC is useful for rapid presumptive detection of obligately anaerobic bacteria in blood cultures at the time when first signs of bacterial growth in the medium appear, such as turbidity, hemolysis, gas, and colony formation. In 24 of 26 blood cultures yielding obligately anaerobic bacteria in subsequent culture, a presumptive diagnosis could be made on the basis of Gram stain and quantitative GLC. For *Bacteroides* sp., isovaleric and succinic acids were the most indicative metabolic products; for gram-positive cocci, it was butyric acid, and for the propionibacteria, it was propionic acid. No false positive results were encountered in 103 blood cultures containing aerobic or facultatively anaerobic organisms. One *K. pneumoniae* strain produced propionic acid; another produced propionic acid and traces of butyric acid. The latter result could have led to a false positive presumptive diagnosis "*Fusobacterium* sp.," but as fusobacteria produce mainly butyric acid, a false result could be avoided with careful evaluation. Unfortunately, no fusobacteria have been found during this investigation; therefore, no limits can be set. According to Wilson et al. (20), fusobacteria are relatively rare in bacteremia; they have found only three strains among 71 gram-negative obligately anaerobic rods in their series.

In general, the acids produced by the organisms when the blood cultures were chromatographed were the same as described by Holdeman and Moore (10). There will be problems with obligate anaerobes that do not produce fatty acids, such as *Eubacterium lentum* and some anaerobic gram-positive cocci. However, these organisms are relatively rare in blood cultures, especially from patients with clinically significant bacteremia (20). In a study performed at the Mayo Clinic, *Bacteroidaceae*, mainly the *B. fragilis* group, were the etiological agents in 78% of the patients with clinically significant bacteremia (20).

As there are different media used in culturing blood, the amounts of fatty acids produced by aerobic, facultatively anaerobic, and obligately anaerobic bacteria have to be evaluated for each medium, and values for a presumptive diagnosis of anaerobic bacteremia have to be set. Most blood culture media, such as Trypticase soy broth, thioglycolate broth, and brain heart infusion broth, contain various amounts of glucose. Glucose favors growth of many obligate anaerobes as well as production of larger quantities of fatty acids at a faster rate than glucose-free media (10, 13).

Quantitative GLC of blood cultures allows a presumptive diagnosis of anaerobic bacteremia 1 day earlier than cultural methods, and this

can be especially important in infections with organisms of the *B. fragilis* group. Although GLC is not absolutely diagnostic of the *B. fragilis* group, the severity of many infections with these bacteria justify, in my view, an initiation of specific antimicrobial chemotherapy on the basis of a presumptive diagnosis. Therapy should then be modified according to the results of culture. Subculture and identification cannot be replaced by GLC; GLC is only meant as an additional help for establishing a preliminary diagnosis.

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