

Rapid Detection of Bacteremia in Mice by Gas Chromatography

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Gas chromatography of the serum of mice inoculated 6 hr earlier with *Staphylococcus aureus*, *Escherichia coli* K-12, and *Clostridium chauvoei* revealed the formation of products associated with the infections. Longer periods were required before compounds resulting from infecting the animals with *Salmonella typhimurium* and other *Clostridium* species were detected by gas chromatographic means. The technique is sensitive to the presence of metabolites elaborated in vivo as a result of the presence of 100 to 35,000 cells. Each bacterial species caused the formation of distinctive compounds in infected mice, and substances chromatographically identical to these products were generated by the microorganisms in vitro as well.

Many species of bacteria may produce bacteremia in man and animals. Frequently, an exact and rapid identification of the offending microorganism is necessary to select the appropriate antibiotic for treatment, but conventional methods for detection and identification of microorganisms in clinical specimens often require a 48-hr to 2-week period. Furthermore, these methods are not sensitive enough to detect the low number of organisms in body fluids, and hence a period for bacterial replication is necessary before their presence can be established by the laboratory techniques employed. Hence, a rapid and sensitive method for detecting and identifying the causal agents of bacteremia is urgently needed.

Gas chromatography has been used for differentiation of microorganisms growing in vitro (3, 4, 12, 13). In previous studies, we have reported on the application of gas chromatographic techniques for the rapid and sensitive detection of viable bacteria and viruses (8, 9). The electron capture detector, in particular, was found to be extremely sensitive for the detection of several microbial metabolites (7). Although several investigators have employed gas chromatographic methods for the analysis of chemical components in biological and clinical specimens (1, 2, 5, 10, 11), few studies have dealt with a systematic examination of pathogens with a view towards diagnostic application. This investigation was designed to determine the feasibility of employing ultrasensitive gas chromatographic techniques for rapid detection and identification of bacteremia in laboratory animals.

MATERIALS AND METHODS

Salmonella typhimurium, *Staphylococcus aureus*, *Escherichia coli* strains K-12 and ATCC 4157, *Clostridium chauvoei*, *C. septicum*, *C. perfringens*, and *C. pasteurianum* were grown for 24 hr at 37 C either in Brain Heart Infusion medium or, with the clostridia, in thioglycolate medium. A 24-hr culture was injected into mice, and the colonies isolated from blood cultures were then grown for 16 hr in Brain Heart Infusion medium. The cells were collected by centrifugation, the bacteria were washed twice with distilled water, and the population density was adjusted to 10^8 to 10^9 cells per ml. A 0.5-ml amount of this cell suspension was injected into mice weighing 25 to 30 g, and blood samples were collected at 0, 6, 16, 24, 48, and 72 hr by heart puncture.

Sera from four mice were pooled at each sampling time, and 2-ml serum samples were introduced into test tubes stoppered with serum caps and stored frozen until the time of extraction. Serum from infected and untreated animals and both uninoculated and inoculated culture media were handled in a similar manner. Immediately before extraction, the sample was thawed, and 0.2 ml of 5 N HCl and 1.0 ml of 0.2 M HCl-KCl buffer (pH 2.0) were added. The liquid was mixed well and centrifuged at $3,000 \times g$ for 5 min. A 0.3-ml portion of the supernatant fluid was dried with anhydrous sodium sulfate and extracted for 2 min with 3.0 ml of ether.

A 3- μ liter sample of the ether layer was used for the initial gas chromatographic screening of the sample for characteristic microbial metabolites. The remaining portion of the supernatant was evaporated to dryness, and excess HCl was removed by adding methanol three times and evaporating to dryness. The solution was then adjusted to pH 7.5 with NH_4OH , and the solutions were evaporated to dryness. The dry

sample was then transferred into a glass-stoppered tube, dissolved in 1.0 ml of dry pyridine, and incubated at 70 C for 20 min. The clear solution was then mixed thoroughly with 0.2 ml of trimethylchlorosilane and 0.4 ml of hexamethyldisilazane. After 30 min at room temperature, the reaction mixture was centrifuged at $3,000 \times g$ for 5 min. The supernatant solution was dried with sodium sulfate, and a 3- μ liter sample was injected into a gas chromatograph (Aerograph model 204) fitted with an electron capture detector (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.). The chromatograph was fitted with a 50:50 splitter so that compounds in a 1.5- μ liter sample were recorded by the detector.

Gas chromatographic conditions were essentially the same as described previously by Mitruka and Alexander (8). The operating temperatures were 110, 175, and 190 C for the column, detector, and injector, respectively.

RESULTS

A suspension containing 10^6 *C. chauvoei* cells injected intraperitoneally into mice caused acute bacteremia, and all of the animals died with bacteremia within 72 hr. Injections of *S. typhimurium* brought about the death of 50% of the mice within 48 hr, whereas 95% of the animals died within 72 hr after infection with *C. septicum* or *S. typhimurium*. The other bacterial species tested were not lethal to mice, although a small number of certain of the bacteria could be isolated from blood cultures of the mice 72 hr after infection. Patterns of induced bacteremia after injection with pathogenic and nonpathogenic bacteria are shown in Fig. 1. The population density of viable cells of some species in the blood of mice decreased initially, but after 16 hr their numbers rose. On the other hand, *E. coli*, *S. aureus*, and several *Clostridium* species were eliminated rapidly from the blood of mice.

Gas chromatography of the serum 6 hr after inoculation revealed the presence of previously undetected compounds generated as a consequence of infection by *S. aureus*, *E. coli* K-12, and *C. chauvoei*. By contrast, products formed in the animal as a result of inoculation with *S. typhimurium*, *E. coli* 4157, *C. perfringens*, *C. pasteurianum*, and *C. septicum* were not observed until 16 to 24 hr (Table 1). Although 5×10^3 to 4×10^5 cells per ml of blood were present when the first new peaks were noted in the serum samples, the sensitivity for bacterial detection ranged from 100 (*C. chauvoei*) to 35,000 (*S. typhimurium*) cells per 10 mm^2 peak. These values for sensitivity were calculated from the number of bacteria in the 1.5- μ liter sample taken at the time of detection and the area of the first peak found in the serum sample from infected

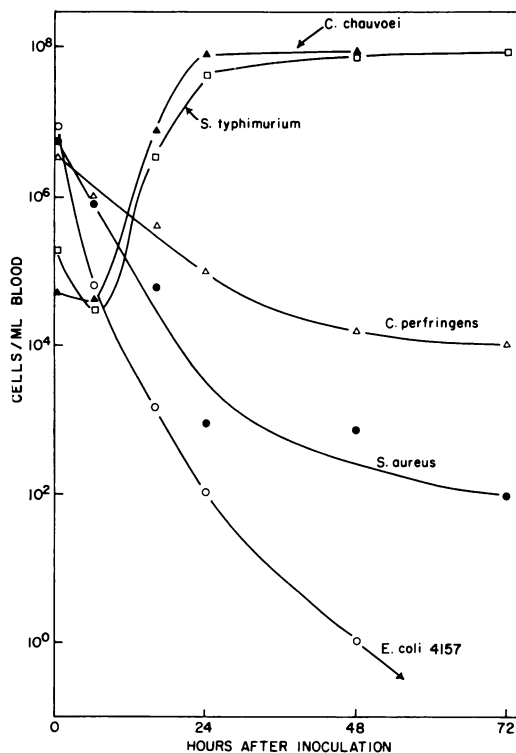


FIG. 1. Induced bacteremia in mice. Bacteria were inoculated intraperitoneally, and samples were drawn at various time intervals and plated for viable counts. Each point represents an average of samples from at least eight mice.

animals which was not present in the serum of uninoculated mice.

Gas chromatography revealed the generation of a variety of metabolites in the serum of mice inoculated with various bacteria. Extracts of the serum from all of the experimental animals contained compounds with retention times of 15, 20, 30, 35, 45, 80, 90, and 205 sec as sensed by the electron capture detector (components present in the extracts of blood samples taken both before and after infection). Substances with retention times of 25, 40, 145, 170, 220, 230, 290, 330, 360, 435, 485, 510, 530, and 870 sec were present in the serum of uninfected mice and also in the serum of animals inoculated with one or more different bacteria. In some instances, one or several of these peaks were absent at the time of inoculation but appeared in one or more types of bacterial infection. These compounds were likewise occasionally present in certain bacterial cultures in Brain Heart Infusion medium. Most of these compounds probably have little or no diagnostic value and

TABLE 1. Detection of bacteremia in experimental mice

Organism	Bacteria/ml $\times 10^8$		Detection time (hr)	Sensitivity ^a
	Inoculum	At time of detection in blood		
<i>Salmonella typhimurium</i>	20,000	400	16	35,000
<i>Staphylococcus aureus</i>	420,000	60	6	840
<i>Escherichia coli</i> K-12.....	480,000	7.2	6	240
<i>E. coli</i> 4157.....	400,000	8.0	16	270
<i>Clostridium chauvoei</i>	10,000	5.0	6	100
<i>C. septicum</i>	20,000	10	24	400
<i>C. perfringens</i>	100,000	300	16	2,000
<i>C. pasteurianum</i>	70,000	200	16	650

^a Bacteria/10 mm² peak.

TABLE 2. Detection of activity of bacteria in mouse blood

Bacteremia	Retention time of peak in vivo	Time after inoculation					
		0 hr	6 hr	16 hr	24 hr	48 hr	72 hr
	<i>sec</i>						
<i>Salmonella typhimurium</i>	245 ^a	0 ^b	<10 ^b	240 ^b	400 ^b	320 ^b	380 ^b
	395 ^a	0	0	170	1,040	6,800	8,000
	590 ^a	<10	0	8,250	8,990	8,400	6,000
	700 ^a	0	40	270	380	75	30
	1,200	0	0	0	506	600	710
<i>Staphylococcus aureus</i>	65	10	256	400	380	450	500
	405 ^a	0	900	600	400	210	64
	1,120 ^a	0	1,200	2,980	490	400	282
<i>Escherichia coli</i> K-12	615 ^a	0	0	510	780	360	296
	930 ^a	0	0	234	210	256	300
<i>E. coli</i> 4157	545 ^a	0	450	300	280	240	200
<i>Clostridium chauvoei</i>	55	1,200	800	400	560	600	— ^c
	70 ^a	0	40	200	290	400	— ^c
	100 ^a	<10	2,800	2,000	2,200	2,150	— ^c
	185 ^a	0	60	100	180	160	— ^c
	1,070 ^a	0	750	7,000	8,400	8,600	— ^c
<i>C. perfringens</i>	950 ^a	0	0	200	100	160	210
	1,005 ^a	0	0	2,250	2,000	2,120	2,160
<i>C. septicum</i>	660	0	0	5,600	400	36	<10
	820 ^a	0	0	0	380	410	400
<i>C. pasteurianum</i>	275 ^a	0	<10	400	450	258	298
	1,685 ^a	0	0	4,610	3,050	2,120	2,200

^a Peaks also observed in in vitro cultures.

^b Peak area (mm²).

^c All animals dead.

presumably reflect variability in bacterial behavior or host responses. On the other hand, one to five characteristic peaks appeared in gas chromatograms prepared from sera of animals

infected with these bacteria (Table 2). Such peaks were specific for the infection produced by a particular species of bacteria, and the compounds were not present in any other infection

TABLE 3. *Distinctive products formed by Salmonella typhimurium in culture medium and in mice 16 hr after inoculation*

Peak retention time (sec)	
<i>in vitro</i>	<i>in vivo</i>
25	165
95	205
245	245
290	395
395	435
590	530
630	590
700	700
	830
	1,200

studied. Furthermore, most of these peaks were consistently present in Brain Heart Infusion cultures of the organisms. The characteristic peaks were distinct and occupied areas ranging from less than 10 to almost 9,000 mm².

Chromatograms of samples prepared from Brain Heart Infusion cultures of each bacterial strain contained several different peaks. Some compounds were identical to those observed in uninoculated broth sampled at 0, 6, 16, 24, 48, and 72 hr, identical at least on the basis of their retention times. However, as shown in Table 3, in which only representative data obtained for *S. typhimurium* are presented, compounds with retention times of 245, 395, 590, and 700 sec were generated both *in vitro* and *in vivo*. These compounds were not present in the uninfected animals or in animals from which serum was taken before the time of inoculation except for traces of a constituent with a retention time of 590 sec. With other bacteria too, chromatographically identical compounds were present in gas chromatograms prepared from cultures grown *in vitro* and from the serum of infected animals, and these metabolites were different

from those associated with *S. typhimurium* (Table 2).

Chromatographic signatures reflecting the metabolic changes occurring in animals infected with each of the bacteria were obtained by assigning letters to the peaks in order of their retention times, as described by Henis et al. (4). The signatures in each of these bacteremias were different (Table 4). Each infection had associated with it at least one peak which was absent from all other infections studied.

DISCUSSION

The results show that highly sensitive gas chromatographic techniques can be employed for the detection of either bacterial activity *in vivo* or host responses to these infectious agents. Such methods may prove useful for the rapid detection of bacteremia and the identification of the causal organism. Identification would be facilitated if the early bacterial product detected was a unique metabolite. The present findings with bacteria, namely that each type of bacteremia had associated with it the formation of at least one distinctive metabolite and that these distinctive products were elaborated by the bacteria when grown *in vitro* as well, are in agreement with previous studies of viruses (9) and of bacterial infections of man (Mitruka and Jonas, unpublished data).

Although these investigations of bacterial and viral infections suggest that gas chromatography may be quite useful as an aid in clinical pathology, more work is needed before such techniques can be employed in the diagnostic microbiology laboratory. Nevertheless, if a sufficient number of microbial infections can be characterized by these means, then the signatures or microbial fingerprints obtained from a clinical specimen might serve as a rapid diagnostic tool for communicable diseases. Moreover, essentially the same procedures might be applied to dis-

TABLE 4. *Signatures of serum samples taken from mice with induced bacteremia*

Organism	Signatures ^a
<i>Salmonella typhimurium</i>	A B D E G L M Q S V Y C ₁ E ₁ J ₁ N ₁ P ₁ W ₁
<i>Staphylococcus aureus</i>	A B D E F G H J L M B ₁ D ₁ E ₁ L ₁ V ₁
<i>Escherichia coli</i> K-12.....	A B D E F G H L M N S F ₁ H ₁ K ₁ Q ₁ R ₁
<i>E. coli</i> 4157.....	A B D E F G L M N F ₁ H ₁ I ₁ L ₁ Q ₁
<i>Clostridium chauvoei</i>	A B C D E F G I K L M O P Q R T U B ₁ F ₁ L ₁ U ₁
<i>C. perfringens</i>	A B C D E F G L M E ₁ F ₁ L ₁ O ₁ S ₁ T ₁
<i>C. septicum</i>	A B C D E F G L M N U Y B ₁ H ₁ M ₁ P ₁
<i>C. pasteurianum</i>	A B C D E F G L M Z E ₁ F ₁ L ₁ O ₁ Y ₁

^a The respective retention times for peaks A through Y₁ are: 15, 20, 25, 30, 35, 40, 45, 50, 55, 65, 70, 80, 90, 95, 100, 140, 170, 185, 205 to 210, 220, 230, 240, 250, 275, 295, 315, 330, 360, 395, 405, 435, 485, 510, 530, 545, 590, 615, 630, 660, 700, 740, 820, 870, 930, 950, 1,005, 1,070, 1,120, 1,200, 1,275, and 1,685 sec. The first 26 peaks are designated A to Z, and the remaining ones are designated A₁ to Y₁.

orders of nonmicrobial origin. Caution must be exercised, however, because not only has a mere handful of strains been examined to date but also there has been no concern with the effects of drugs, problems of mixed or multiple infections, difficulties arising because of idiosyncracies of the host, and limitations of the gas chromatographic procedure itself, as discussed by Marmion (6).

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