Identification of *Bacillus anthracis* Using Gas Chromatographic Analysis of Cellular Fatty Acids and a Commercially Available Database

Application

Homeland Security

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Abstract

The U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID, Fort Detrick, MD), the Department of Defense's lead laboratory for medical aspects of biological warfare defense, has established a database entry for *Bacillus anthracis* using the Sherlock® Microbial Identification System (MIS) from MIDI, Inc., Newark, DE. The Sherlock MIS uses gas chromatographic analysis of fatty acid methyl esters (FAMEs) to identify over 1,500 species of bacteria, including six bacteria highly considered to be potential agents of biological terrorism. Identification is made upon comparison of unknown chromatographic patterns to the Sherlock libraries (databases) using precise peak naming, sophisticated pattern recognition algorithms and extensive microbial databases. The new *B. anthracis* data set entry in the Sherlock® Bioterrorism (BIOTER1.0) Library allowed for accurate identification of *B. anthracis* isolates, both previously seen and never before seen. The system could estimate the relatedness of strains, which has potential in forensic analyses to determine attribution to the use of biological threat agents.

Introduction

The rapid and accurate identification of etiologic agents, whether from natural sources or terrorist acts, is a vital and continuing necessity. Prior to this study, *Bacillus anthracis*, the etiologic agent anthrax, lacked solid database entries to support its identification. Through the use of *B. anthracis* strain sets that have been well characterized with respect to their plasmid profiles, phenotypic traits, and bacteriophage susceptibility, USAMRIID evaluated the Sherlock® MIS, based on the chromatographic analysis of FAMEs derived from bacterial membranes [1].

Strengths of the Sherlock MIS include its ability to create custom libraries and its history of application in regulated clinical environments.

The system was recently used in diagnosis of an anthrax infection in a 94-year-old Connecticut woman. Identification of the clinical isolate as *B. anthracis* bacterium was accomplished at the Connecticut Department of Public Health Laboratory, even though there was no reason to suspect an anthrax infection at the time. This case demonstrates the usefulness of the Sherlock MIS system as a screening tool for *B. anthracis* [2].

In the clinical laboratory, the microorganism can be easily isolated on 5% sheep blood agar (SBA), as nonhemolytic colonies with a ground glass appearance. Typical Gram-positive stained rods appear 1–1.5 µm × 3–5 µm, with cells aligned to form long chains. Spores, free or within vegetative cells, are often seen in older cultures, and

when properly prepared, are the most threatening form of the agent. Fully virulent strains carry two plasmids designated as pXO1 and pXO2, both of which are required for virulence. The pXO1 plasmid encodes two protein factors (Lethal Factor and Edema Factor) which combine with multiple copies of a third pXO1 encoded protein, Protective antigen to form Lethal Toxin and Edema Factor respectively. The pXO2 plasmid encodes enzymes required for the poly-D-glutamic acid capsule present during infection.

Experimental

A total of 73 isolates of *Bacillus anthracis* were selected for this study. These isolates represented wide geographical areas and were associated with human or animal disease, and avirulent strains cured of either or both plasmids. In order to represent "real world" variation in the handling of cultures, library entries were established using:

- Different lots of the same medium.
- Different culture incubation times for each lot of medium.
- Replicate cultures prepared on the same day or different days
- Cultures prepared and extracted by different microbiologists

For the Sherlock BIOTER1.0 Library, each isolate was examined using 5% SBA plates along with the CLIN4.0 method.

The CLIN4.0 method is used for the identification of all clinical aerobic bacteria and represents the growth conditions and GC parameters required for these isolates. The chromatographic output is then compared to the CLIN4.0 and BIOTER1.0 Libraries for identification.

Initial Screening

Each isolate was initially screened using the gamma phage assay, a method in use at USAMRIID since the 1980s [3]. Inoculation of the phage specific for *B. anthracis* leaves a clear zone of lysis on a plate culture of the target bacillus. All selected isolates tested positive.

Additional identity confirmation was obtained using the polymerase chain reaction (PCR). Genomic DNA was extracted from the study strains with the QIAamp Tissue Kit (Qiagen, Inc., Valencia, CA). DNA from each strain was tested using PCR primers and probes specific for the *B. cereus* group (chromosomal), the protective antigen (PA) gene on the pX01 plasmid, and one of the genes on the plasmid encoding one of three enzymes required for capsule synthesis.

Creation of the BIOTER1.0 anthrax library entry proceeded after both gamma phage and PCR independently confirmed strain identities.

B. anthracis strains were grown on 5% SBA plates at 35 ±1 °C for 24 ±2 h and extracted according to the recommended five-step manual extraction procedure described below. Extracts were then run on an Agilent 6890 Series Gas Chromatograph and the resulting chromatograms analyzed with the Sherlock MIS software.

The actual database library was generated from the same software. Once established, the "*B. anthracis* library entry" was further evaluated by rerunning the isolates used to create the database, as well as assessing *B. anthracis* isolates previously not seen by the Sherlock MIS. This "anthrax library entry" demonstrated the ability to correctly identify *B. anthracis*. Using the Sherlock BIOTER1.0 Library, *B. anthracis* could be distinguished from other species in the *Bacillus* genus.

Isolate Preparation Procedure for the Sherlock MIS

Prepare reagents A through D. No proprietary reagents are used.

A. 45 g sodium hydroxide, 150 mL methanol, and 150 mL water

B. 625 mL certified 6.0 N hydrochloric acid and 275 mL methanol

C. 200 mL hexane and 200 mL methyl-tert-butyl ether

D. 18.8 g sodium hydroxide dissolved in 900 mL water

Follow Steps 1 through 5.

1. **Harvesting:** 40 mg cells, from culture on 5% SBA, 35 °C, 24 h, was transferred to a glass culture tube.
2. **Saponification:** To tube, 1 mL reagent A is added and heated at 100 °C for 30 min.
3. **Methylation:** 2 mL reagent B is added and heated at 80 °C for 10 min.
4. **Extraction:** 1.25 mL reagent C is added, mixed for 10 min, and the bottom phase discarded.
5. **Base wash:** Reagent D is added to top phase, mixed 10 min, transferred 2/3 of top phase to a 2-mL glass vial and loaded into the GC autosampler.

Analysis

The Sherlock MIS uses a flame ionization detector (FID) on an Agilent Technologies 6850 or 6890 series gas chromatograph.

Column:	25 m × 0.2 mm, phenyl methyl silicone fused silica
Temperature program:	170 to 270 °C at 5 °C/min.
GC run time:	22 min.
Post-incubation:	3 h (batched samples)

Results

An example of a methylated cellular fatty acid (CFA) chromatogram for *B. anthracis* is shown in Figure 1. The corresponding Sherlock report is shown in the Appendix.

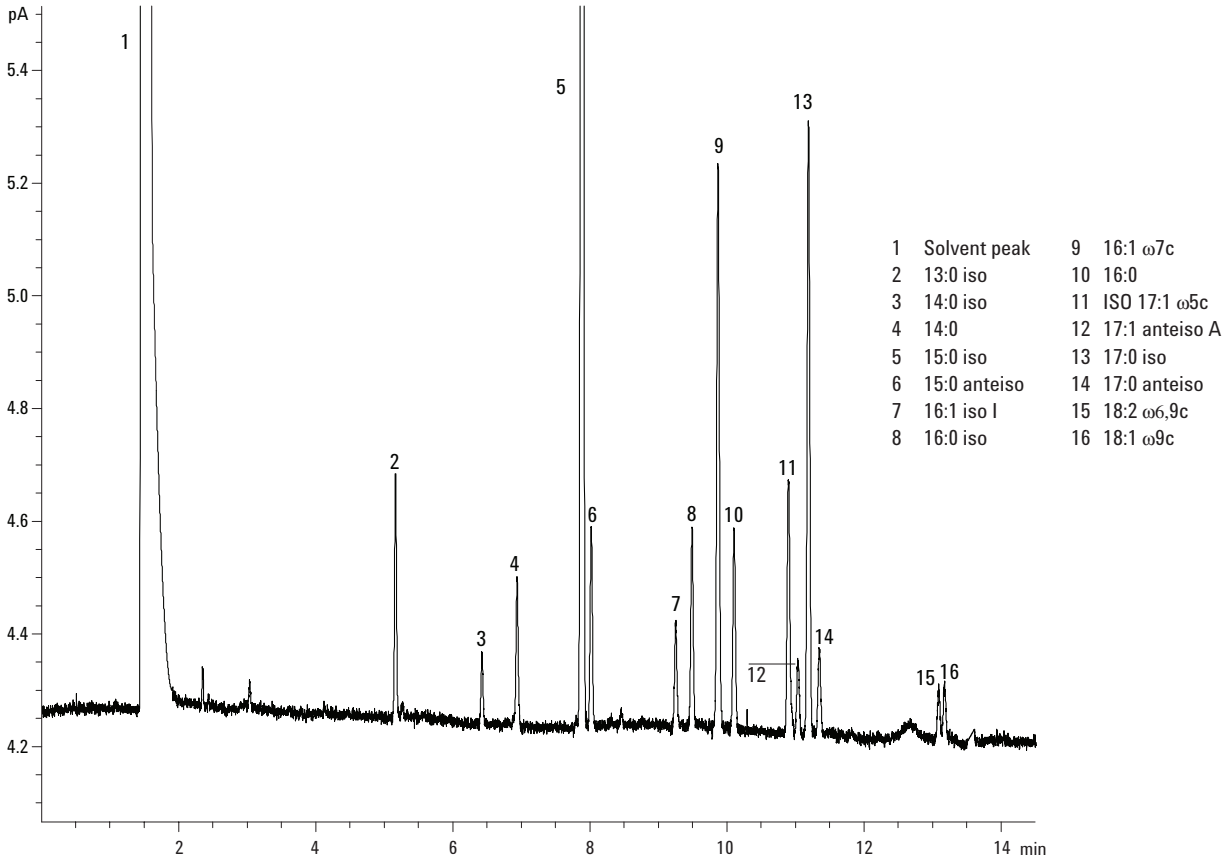

Figure 1. This chromatogram helped confirm *B. anthracis* in the Connecticut anthrax case (November, 2001). Courtesy of the Connecticut Department of Public Health Laboratory.

Sherlock MIS uses an external calibration standard, a mixture of the straight chain saturated fatty acids from 9 to 20 carbons in length and five hydroxy acids.

The hydroxy compounds are especially sensitive to changes in pressure/temperature relationships and to contamination of the injection port liner. As a result, these compounds function as quality control checks for the system. Retention time data obtained injecting the calibration standard is converted to Equivalent Chain Length (ECL) data for bacterial fatty acid naming. The ECL value for each fatty acid is derived as a function of its elution time in relation to the elution time of a known series of straight chain fatty acids. The GC and column allow windows to be set at 0.010 ECL unit while giving exceptional precision in resolution of isomers. Sherlock compares the ECL values for the most stable series (for example, saturated straight chain or branched chain acids) to the peak naming table's theoretically perfect values and may recalibrate internally if sufficient differences are detected [4].

Table 1 shows the typical fatty acid features present in each of four *Bacillus* species. Summed Features are groups of fatty acids that cannot be separated and are treated as one feature for library comparisons. As shown in Table 1, ISO 17:1 ω10c does not occur in *B. anthracis*, and 17:1 anteiso A only occurs in *B. anthracis* and *B. thuringiensis*. The two species can be distinguished with the two fatty acids and with other subtleties (qualitative and quantitative) in the fatty acid profiles that are used to differentiate bacterial species. The Sherlock MIS software incorporates sophisticated mathematical tools to recognize these subtleties and help with the identification of over 1,500 bacterial species. The Sherlock MIS software also has several tools for strain tracking purposes.

Table 1. CFA Profiles for Four Closely Related Bacilli. *B. anthracis* Data Courtesy of USAMRIID

	B. anthracis **N=73**		***B. cereus**** **N=29**		***B. subtilis**** **N=17**		***B. thuringiensis**** **N=6**	
CFA Name	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**
12:0 iso							0.69	0.05
13:0 iso	2.39	0.84	7.26	0.62			10.10	0.39
13:0 anteiso							1.22	0.14
14:0 iso	1.67	0.50	2.48	0.43	0.90	0.19	4.56	0.32
14:0	2.23	0.41	2.76	0.19			4.20	0.25
15:0 iso	45.37	3.25	41.94	2.16	23.67	4.18	34.88	1.76
15:0 anteiso	3.92	0.89	3.54	0.78	35.88	3.12	4.61	0.12
16:1 ω7c alcohol							1.13	0.05
16:0 iso	6.41	1.53	4.03	0.74	2.70	0.27	5.42	0.24
16:1 ω11c					0.57	0.35		
16:0	3.98	0.62	4.28	1.22	3.63	1.17	3.76	0.50
15:0 2OH							0.91	0.13
ISO 17:1 ω10c			1.94	0.50	1.53	0.53	3.08	0.14
ISO 17:1 ω5c	4.44	1.77	4.85	2.86			3.77	0.20
17:1 anteiso A	1.47	0.36					0.94	0.04
17:0 iso	11.33	2.13	9.38	1.25	14.46	3.28	4.57	0.22
17:0 anteiso	2.66	0.75	1.01	0.95	11.07	2.54	0.66	0.33
18:1 ω9c	1.18	0.37	4.47	1.31	1.13	2.07		
18:0	1.05	0.30	1.67	0.48	1.16	0.88		
Summed Feature 2	2.01	0.70	1.40	0.99			2.94	0.11
Summed Feature 3	7.34	1.42	7.18	2.45			12.30	0.25
Summed Feature 4					0.48	0.39		
Summed Feature 5	1.12	0.29	1.51	0.97	1.71	3.12		

*Comparison data provided by Dr. Ralph Paisley, MIDI, Inc.

Conclusions

The customer-proven Sherlock MIS is a complete system that includes standardized sample preparation and analytical procedures. It performs complete data processing by using extensive libraries of CFA profiles to perform bacterial screening and confirmation of identity. In addition, the Sherlock MIS has several epidemiology tools, including the new Sherlock® Tracker, which allows a sample profile to be compared to thousands of other profiles in real-time for the closest matches.

In the current study, a *B. anthracis* library entry was created and validated by USAMRIID for the Sherlock BIOTER1.0 Library. Consequently, this system can be used to identify and confirm the presence of *B. anthracis* from isolates, as demonstrated in the aforementioned Connecticut case.

Since the CFA profile of *B. anthracis* is sufficiently unique, the profile can be used to both distinguish it from other related *Bacillus* species and to assist in the tracking of individual strains. Tracking the origin of strains is further facilitated by the Sherlock MIS software through the use of dendrograms or 2-D plots [5]

The Sherlock BIOTER1.0 Library includes entries for other potential biological warfare agents, including the bacterial agents of brucellosis, glanders, melioidosis, plague, and tularemia. These, in addition to over 1,500 other bacterial species catalogued in Sherlock's standard microbial libraries, significantly expand the usefulness of the system for both routine microbial identification and the identification and confirmation of bacterial biological warfare agents.

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Appendix

Identifying Anthrax in the Connecticut Case– The Sherlock Bioterrorism Library was used to identify the anthrax bacterium from the 94-year-old woman in the Connecticut anthrax case in November 2001. The following report confirmed Bacillus anthracis in this case. The data is courtesy of the Connecticut Department of Public Health Laboratory.

Created: 11/20/2001 9:49:11 AM
Sample ID: K-BACI-ANTHR A (Connecticut case)
Calc. method: BIOTER

RT	Response	ECL	Peak name	Percent
1.482	4.08E+8	7.040	Solvent peak	
5.155	1608	12.614	13:0 iso	3.18
6.417	569	13.619	14:0 iso	1.08
6.928	1196	14.000	14:0	2.24
7.875	29458	14.624	15:0 iso	54.07
8.011	1684	14.713	15:0 anteiso	3.08
9.242	917	15.482	16:1 iso I	1.64
9.482	1711	15.627	16:0 iso	3.05
9.861	5292	15.857	16:1 ω7c	9.38
10.096	1814	16.000	16:0	3.20
10.888	2626	16.460	ISO 17:1 ω5c	4.58
11.028	786	16.541	17:1 anteiso A	1.37
11.181	5759	16.630	17:0 iso	10.01
11.340	825	16.723	17:0 anteiso	1.43
13.083	479	17.721	18:2 ω6,9c	0.81
13.167	505	17.769	18:1 ω9c	0.86

Matches:

Library	Sim index	Entry name
BIOTER 1.00	0.713	*Bacillus-anthracis*-GC subgroup A

Courtesy of the Connecticut Department of Public Health Laboratory

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