LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

UCRL-CONF-209735

Physical and Chemical Analytical Analysis: A key component of Bioforensics

S. P. Velsko

February 15, 2005

AAAS Annual Conference
Washington, DC, United States
February 17, 2005 through February 20, 2005

Physical and Chemical Analytical Analysis: A key component of Bioforensics

Stephan P. Velsko
Lawrence Livermore National Laboratory
UCRL-CONF-209735

Introduction

The anthrax letters event of 2001 has raised our awareness of the potential importance of non-biological measurements on samples of biological agents used in a terrorism incident. Such measurements include a variety of mass spectral, spectroscopic, and other instrumental techniques that are part of the current armamentarium of the modern materials analysis or analytical chemistry laboratory. They can provide morphological, trace element, isotopic, and other molecular "fingerprints" of the agent that may be key pieces of evidence, supplementing that obtained from genetic analysis or other biological properties. The generation and interpretation of such data represents a new domain of forensic science, closely aligned with other areas of "microbial forensics.[1]" This paper describes some major elements of the R&D agenda that will define this sub-field in the immediate future and provide the foundations for a coherent national capability.

Data from chemical and physical analysis of BW materials can be useful to an investigation of a bio-terror event in two ways. First, it can be used to compare evidence samples collected at different locations where such incidents have occurred (e.g. between the powders in the New York and Washington letters in the Amerithrax investigation[2]) or between the attack samples and those seized during the investigation of sites where it is suspected the material was manufactured (if such samples exist). Matching of sample properties can help establish the relatedness of disparate incidents, and mis-matches might exclude certain scenarios, or signify a more complex etiology of the events under investigation. Chemical and morphological analysis for sample matching has a long history in forensics, and is likely to be acceptable *in principle* in court, assuming that match criteria are well defined and derived from known limits of precision of the measurement techniques in question. Thus, apart from certain operational issues (such as how to prioritize such measurements in the face of limited sample availability, or how to render samples safe for handling in the analytical laboratory,) instrumental analysis of biological agents for purposes of sample matching alone is unlikely to present fundamental problems that require extensive research and development investments.

The second way that the data generated by instrumental analysis can be useful to an investigation is through inferences that can be drawn regarding the processes used to grow and "weaponize" the agent. In contrast to the case of sample matching, there are significant R&D challenges associated with developing a robust capability that will reliably permit such inferential uses of instrumental data. Elaborating these challenges occupies the major portion of this paper.

In addition to the technical issues we will discuss, it is important to note that, as with other areas of microbial forensics, instrumental analysis faces a significant challenge in the realm of the perceptions and expectations of the prosecutors or other decision makers who are the ultimate consumers of the data generated in an bio-terror or bio-criminal investigation. It is unlikely that scientific data alone will directly and uniquely identify the perpetrator of a bio-terror incident or bio-crime, or even permit investigators to infer the geographic or temporal locus of manufacturing with high precision. Nonetheless, inferences about manufacturing processes deduced from the chemical and physical properties of an agent can provide limited but important information that clearly bears on attribution. For example, analysis may

- help decide if a putative agent is artificial or natural in origin,
- indicate if the agent was "weaponized" by a crude or a sophisticated method, or by a method that lends itself to making large quantities;
- show that certain unique or unusual materials were used in the process;
- provide an estimate of how recently the material was made.

Such information may shape the response to an extortion demand or influence the scope and locus of an investigation. In some cases, the choice of manufacturing method *may* point to a unique source of knowledge, e.g. from a foreign BW program, but the significance of this must be interpreted very carefully in the light of all other available information. In this regard, the technical community bears a significant responsibility for managing the expectations of the national security and law enforcement communities regarding both the power and limitations of scientific analysis for "attribution" of a biological terror incident.

R&D challenges related to inferences about the manufacture of BW agents

In principle, the morphological and chemical properties of an agent contain information about the methods and materials used to generate it (Figure 1). Thus, from instrumental analysis, one or more plausible "recipes" by which the material was made may be deduced. The validity of these deductions must then be confirmed by showing that new material made using a putative method matches the evidence sample. This type of information is of value to an investigation to the extent that it constrains the pool of suspects to those that have access to the equipment, materials, and information necessary to carry out specific "recipes". In practice, however, outside of the very specific information that was gathered in the Amerithrax investigation, the knowledge base from which such deductions may be drawn is currently quite sparse, and what information exists is not easily accessible to investigators - or is of questionable reliability.

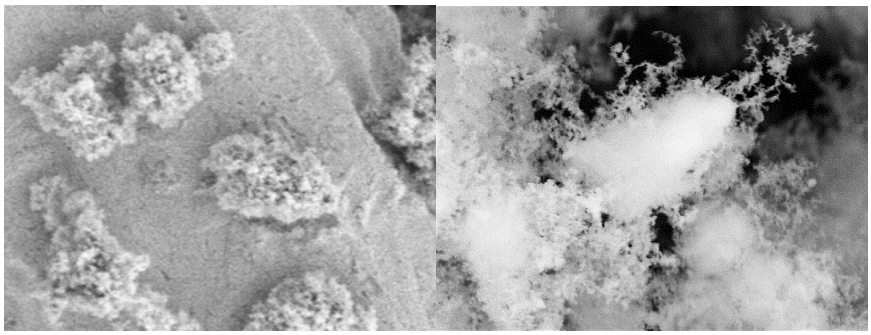

Figure 1. Spores coated with silica using two different processing methods.

The knowledge base that is required to deduce process associations from measurement data consists of two basic components. The first is a systematic understanding of the many different possible "recipes" for generating agents. While much current expertise in this area centers around archival knowledge generated by the historical U.S. biological weapons program (and to a lesser extent, knowledge about foreign BW programs) it is important to recognize that would-be bio-terrorists are likely to utilize information from a broader range of sources, including open scientific literature, the internet, underground "cookbooks", and information that has, unfortunately, been divulged to the news media in recent years. There is no necessary presumption that this information is always accurate or leads to an effective biological weapon. But only by collecting and organizing this information (and keeping it up-to-date) can we hope to recognize the recipe used to make an agent in the widest variety of possible incidents.

To organize such information, it is useful to adopt a principle from chemical engineering, and break complex recipes down into their basic steps, called "unit processes." A generic recipe for making a biological weapon might consist of the following unit processes: growth of the organism, separation and concentration of the organism from the growth medium, drying it to powdered form, grinding it to a fine grade, and mixing it with additives might enhance its delivery or effect (Table 1). For each unit process in the recipe, the terrorist or proliferator usually has a choice among many specific techniques and materials. Thus, the goal of analysis is to identify the specific sequence of steps that the perpetrator has chosen from this matrix of unit process options. Of course, in many cases, agents used in bio-crimes are likely to be crudely processed, and in some cases no "processing" as such may be used.

Table 1. Examples of generic unit process variations for production of biological agents.

Growth	Separation	Washing	Drying	Grinding	Additives
Plate culture	Filtration	Water	Freeze drying	Ball mill	Flow enhancers
Liquid culture	Centrifugation	Solvents	Spray drying	Jet mill	Stabilizers
Choice of medium			Air drying	Mortar&pestle	Encapsulants

The second necessary (and currently inadequate) knowledge component is a database of the specific chemical and physical signatures that each unit process variant will imprint on a finished agent. To acquire such a database will require the systematic analysis of reference samples produced by various paths through the unit process matrix. Experiments should be designed to establish statistically validated signatures for a given unit process variant – i.e. demonstrating that a putative chemical or morphological signature of that step is present at statistically significant levels in samples produced by recipes that include that step, but *not* in samples produced by alternative methods. This issue of "false positives" and "false negatives" is distinct from, but dependent on the precision of the techniques used to acquire the signatures, and is thus connected with the establishment of criteria for declaring matches among samples.

Experience has shown that there are two sources of variability that must be understood and carefully controlled when generating reference samples for determining chemical and physical signatures of growth and processing methods. The first is the natural variation shown by microorganisms even when they are grown under nominally identical conditions. The other is inhomogeneity in the characteristics of powder samples produced by a single method. Figure 2 shows the different degree of purity exhibited by spore samples taken from two different volumes of the same batch of material processed into a dry powder. Thus, quality assurance and control measures must also extend to the procedures used to generate such reference samples, as well as to those for handling and analyzing the materials.

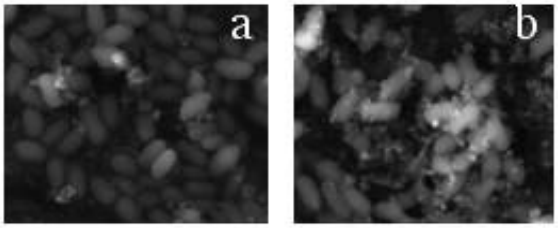

Figure 2. Two samples drawn from a single batch of dried spore reference material.

Another, somewhat unique challenge in this technical area is the need to balance the requirement for classification in some matters with the necessity to publish as widely as possible to achieve the criteria for scientific acceptability in the courtroom. This is especially true with respect to the publication of complete detailed "recipes" for producing agents, and with revealing specific unit process variants that may be derived from intelligence on foreign programs or terrorist efforts. However, it is unlikely that the basic acceptability of instrumental analysis will be seriously compromised in practice by the requirement to protect this kind of information.

Methods for analyzing trace samples

There are many standard analytical techniques that can be applied to bulk quantities of pure agent, when it is available. In this sense, it was fortunate that several letters containing substantial quantities of powder were secured during the Amerithrax investigation. To aid the FBI, the scientific community was able to offer a number of reasonable methods for addressing important questions about this material. However, even here some studies were constrained by available sample size. Moreover, at some Amerithrax crime scenes bulk powder evidence was not available, so these methods could not be applied. In many imaginable attack scenarios, the only agent samples that would be available for analysis are those recovered from contaminated surfaces, ductwork or filters from building air conditioning systems, or material on the filter units used in urban air samplers, e.g. the BioWatch program[3]. Such samples may be heavily mixed with other materials, requiring the analyst to identify and isolate the agent particles from the mixture. In the future, it will clearly be important to extend the reach of more kinds of chemical and physical analysis to such situations.

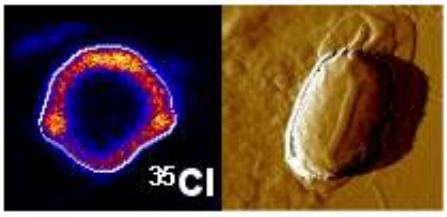

Figure 4. (a) Distribution of chlorine within a spore, determined by Secondary Ion Mass Spectrometry (SIMS); (b) Image of spore surface generated by Atomic Force Microscopy (AFM).

There are a number of instruments that can measure the chemical and physical properties of trace quantities of agent, even single agent particles. Most important are those that provide high-resolution structural imagery such as electron microscopy (EM) or atomic force microscopy (AFM), and those that can "map" chemical composition within a single particle, such as secondary ion mass spectrometry (SIMS). Examples of SIMS and AFM images of single spores are shown in Figure 4. Techniques like these provide information about subtle structural characteristics of the agent particles that can be related to the growth conditions or other unit process steps. However, the task of identifying these kinds of signatures and establishing their statistical validity is at a very early stage. Generalized techniques for mounting and handling samples to permit different instruments to analyze the same particle remain to be developed.

Determining date and place of manufacture

Establishing the approximate date that an agent was manufactured could be an important piece of evidence in an investigation. Currently, our capabilities for dating such samples are limited. Radioisotope dating, particularly ^{14}C determinations using Accelerator Mass Spectrometry (AMS) is capable of moderate (± 1 year) time resolution under many

circumstances. However, in many cases, bio-terror incidents can be expected to involve materials that are made only weeks or months prior to release. Even if time resolution were adequate, the accuracy of ^{14}C AMS dating is affected by a number of well-known factors that complicate the interpretation of such results. Strictly speaking, the method only establishes the date that the nutrient components of the growth medium were manufactured. Thus, the inferred date may reflect more the history of the distribution and storage of the nutrients rather than the date the organism was grown. Moreover, growth media are often the mixture of more than one nutrient from different sources and manufacturing dates. Finally, in some post-growth process steps, materials that contain fossil fuel derived carbon can be added to the agent, thus affecting the date derived from ^{14}C measurements.

There are several possible rate processes that might be exploited to achieve sample dating that goes beyond the limitations of ^{14}C AMS. For example, chemical changes like oxidation of particular molecular species on the agent particle surfaces might be detectable by certain spectroscopic or mass analytical techniques. Relaxation of the microstructure associated with certain surface components of the agent may be detectable with electron microscopy or AFM. The diffusive relaxation of certain internal ionic or molecular gradients that are fixed within agent particles (especially spores) by growth or drying processes can be measured with high spatial resolution mass analysis techniques like SIMS. Many of these concepts are similar to those that are exploited for dating other materials of forensic interest, such as inks or paints. A concerted R&D campaign would be needed to understand the phenomenology and validate protocols for dating using these methods.

The success of stable isotope and trace element "fingerprinting" for locating the geographical origins of drugs and other organic substances has raised hopes that similar approaches might work for biological agents. Certainly, if an agent contains additives (for example the putative added silica that received so much media attention in the Amerithrax investigation) the trace element and isotopic profiles of the additive would provide a possible basis for locating the origin of its manufacture. However, the notion of "geo-locating" the origin of an agent through its isotopic fingerprint should be regarded with caution. *In general*, the isotopic and trace element profiles of organisms arise from a complex mixture of substances used to grow them. R&D is needed to answer two basic questions: First, can we obtain enough independent stable isotope and trace element information from a sample to deconvolve the various contributions to the fingerprint; and second, even if this is successful, will the geographical resolution that this information provides be useful? Answering these questions may require several years of focused experimental study. In addition, a representative archive of manufacturing materials (growth media, additives, etc.) and a database of their isotope and trace element signatures must be developed.

Other R&D challenges

There are several other issues that must be addressed by R&D early in the development of this field:

Surrogates. In many cases it is desirable to use non-pathogenic surrogates for instrumental analysis R&D. However, the transferability of conclusions drawn from studies on surrogates to the actual agent may not be straightforward. Therefore, it will be necessary to understand the limitations of surrogates and how to choose the best surrogate for a given study.

Irradiation and other decontamination protocols. In cases where dedicated instrumentation that can be used in a biosafety level 3 or 4 environment is not available, suitable inactivation protocols must be used to render samples safe for analysis. The effect of these treatments on the relevant properties of the agent must be understood

Sample collection and preservation. While a number of collection methods have been validated for DNA analysis or organism culture, little systematic development for instrumental analysis of agent samples has occurred. It will be important to establish collection and storage methods that do not inadvertently distort or contaminate subtle structural or chemical clues, and minimize their degradation with time.

Optimizing the choice and priority of analytical methods. When only limited or trace samples are available, a systematic procedure for choosing the most informative analytical tests becomes crucial. It will be important to formalize such considerations to expedite the development of the best analytical plan when an incident occurs.

Information management

Given the wide variety of agents and processing methods we can expect that a large body of knowledge can be generated in this area. It is important that this information is accessible to those who will require it during the planning and execution of a bio-terror investigation, and during the preparation of critical briefs for forensic or national security purposes. A robust information management system would link information on processes and signatures as well as auxiliary data derived from component materials and relevant information on pathogen growth and physiology. To provide a useful tool for attribution analysis, the information management system must provide a transparent and flexible query structure for asking how the properties of a new "unknown" sample compare to those of archived data sets. Protocols for storing images and spectra in standard formats, and metrics for ranking matches must be developed. Commercial IM software systems probably provide a sufficient base on which to build such a system. Eventually, this body of knowledge may be incorporated into larger information management systems such DHS's Biodefense Knowledge Center (BKC).

Concluding remarks

A knowledge base that allows law enforcement agencies to extract manufacturing signatures from a BW material and assess the level of sophistication and possible sources of information used in its manufacture is as important as the genetic fingerprinting methods that are emerging from the microbial forensics effort. At the same time, the forensic utility of the data that can be obtained from instrumental analysis of agents is less developed than that of genetic fingerprinting, and rests critically on establishing rigorous and defensible scientific underpinnings. With sufficient targeted investment the R&D challenges outlined here can be met, and the national expertise in this area will move from its present ad-hoc form to an established capability.

Acknowledgements

I would like to acknowledge several colleagues at Lawrence Livermore National Laboratory who generously provided the illustrative images used in this paper. Figure 1 was provided by Bruce Woods and James Ferriera. Susan Martin and Joanne Horn provided Figures 2a and b. Alexander Malkin and Peter Weber provided Figures 3a and b respectively.

References

1. Budowle, B, Schutzer SE, Einseln A, Kelly, LC, Walsh AC, Smith JA, Marrone, BL, Robertson J, Campos J, "Public Health: Building microbial forensics as a response to bioterrorism", Science **301**, 1852, (2003).

2. Jernigan DB, et. al. "Investigation of Bioterrorism-Related Anthrax, United States, 2001: Epidemiological Findings", Emerging Infectious Diseases **8**(10), 1019, (2002).

3. Fitch JP, Raber E, Imbro D, "Technology Challenges in Responding to Biological or Chemical Attacks in the Civilian Sector", Science **302**, 1350, (2003).

Auspices Statement

This work was performed under the auspices of the U.S. Department of Energy by University of California, Lawrence Livermore National Laboratory under Contract W-7405-Eng-48.