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Laboratory Aspects of Bioterrorism-related Anthrax – from Identification to Molecular Subtyping to Microbial Forensics

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During the bioterrorism-associated anthrax investigation of 2001 in the United States, 11 patients were diagnosed with inhalational anthrax and 11 more with the cutaneous forms of the disease. Over 125,000 specimens were processed at laboratories of the Laboratory Response Network including those at the Centers for Disease Control and Prevention. Although the 2001 anthrax investigation initially began as a public health investigation, the forensic aspect quickly became a preeminent component of the investigation. Whereas a public health investigation aims primarily to identify the causative agent and its source, so that appropriate and timely control and preventative measures can be implemented, a forensic investigation goes further to associate the source of the causative agent with a specific individual or group. In addition to identification and molecular characterization of the causative agents, which are the crucial components of forensic microbiology, there are many other requirements and activities that need to be in place for investigators to successfully complete a forensic investigation. These activities include establishment of quality assurance/quality control criteria and regular proficiency testing for all laboratories where evidence is analyzed; additional and/or specialized training in handling and processing samples in accordance with forensic microbiology criteria, not only for first responders but also for laboratory and other public health scientists; and establishing and maintaining repositories and databases containing isolates of diverse temporal and geographic origins to provide a comparative and diverse background for investigators to identify and track the origin and source of such agents.

Key words: anthrax; Bacillus anthracis; bacterial typing techniques; bioterrorism; forensic medicine; laboratories; polymerase chain reaction

Prior to the events of October 2001, the last case of inhalational anthrax in the United States was reported in 1976 in a home craftsman from California who died after being infected by contaminated, imported yarn containing goat hair (1). Since then, only a few cases of cutaneous anthrax have been reported, the last case occurring in the summer of 2001 in a Texas farm worker who contracted the disease during the disposal of infected animal carcasses (2).

Public Health Efforts in the United States in Bioterrorism Preparedness and Response

Starting in the late 1990s, emergency funding and a renewed commitment for improving the public health response to possible acts of bioterrorism achieved an admirable state of readiness at the Centers for Disease Control and Prevention (CDC) for possible bioterrorism threats. Within the Meningitis and Special Pathogens Branch, a Biosafety Level-3 laboratory was developed and established, with expertise in isolation, identification, and molecular subtyping of *Bacillus anthracis*.

The CDC also took the lead in establishing a nationwide public health Laboratory Response Network designed to aid in the rapid detection of a bioterrorism event and in identification of the agents used (ref. 3; *www.bt.cdc.gov, www.lrnb.cdc.gov*). The Laboratory Response Network is a multi-level system designed to link state and local public health laboratories with advanced capacity specialty (clinical, military, veterinary, agricultural, water-, and food-testing) laboratories. It operates as a network of laboratories (laboratory levels designated A through D), with progressively stringent levels of safety requirements, containment, and technical proficiency necessary to perform the essential rule-out, rule-in, and referral functions required for identification of agents that could potentially be used as biological weapons. Currently, the Laboratory Response Network has over 120 level B and C laboratories throughout the country. This network has also been crucial in allowing public health workers to develop and validate specific assays for identification of *B. anthracis* and other threat agents. One of CDC's, and especially the Laboratory Response Network's, major initiatives in support of a

rapid and appropriate public health response has been to train level B laboratorians in the isolation and identification of threat agents likely to be used in a bioterrorism event. In the fall of 2000, the CDC's Bioterrorism Preparedness and Response Program sponsored four training sessions for public health laboratorians, focusing on four agents: *B. anthracis*, *Brucella* spp., *Yersinia pestis,* and *Francisella tularensis*. Staff from the National Center for Infectious Diseases and the Public Health Practice Program Office, CDC, and the National Laboratory Training Network conducted these one-week-long sessions. Sixty-four laboratorians representing all 50 U.S. states and the Federal Bureau of Investigations (FBI) attended the workshops. Using specific, recently developed criteria for the presumptive and confirmatory identification of *B. anthracis* by standardized and validated methods, level B laboratorians were trained to rapidly confirm the presence of *B. anthracis* in both clinical and environmental samples (4,5). This investment in training U.S. public health laboratorians has proven to be invaluable as Laboratory Response Network laboratorians played key roles during the 2001 bioterrorism-associated anthrax investigation by processing over 125,000 specimens for the isolation of *B. anthracis*.

B. anthracis is a member of the *Bacillus cereus* complex, which includes *B. anthracis*, *B. cereus*, *B. mycoides*, and *B. thuringiensis*. Evidence from DNA-DNA hybridization studies, 16S rRNA sequences, and other genetic analyses suggests that these very closely related bacilli could be considered one species (6-8). While identification of *B. anthracis* has traditionally been determined by using phenotypic differences between *B. anthracis* and the rest of the *B. cereus* group, such as lack of motility, lack of hemolysis, susceptibility to penicillin, typical colony morphology, and susceptibility to lysis by gamma phage, these methods require at least 24 h for completion. The response to the recent bioterrorism-associated outbreak and an ongoing threat of further use of *B. anthracis* as a biological warfare weapon emphasize the importance of rapid microbiological diagnosis for the timely and adequate implementation of control and preventative measures.

From Public Health Investigation to Forensic Investigation and Microbial Forensics

The major characteristic of the 2001 anthrax investigation was that, although it began as a public health investigation, soon it became a forensic investigation when the evidence suggested that the cause of the outbreak was an intentional release of *B. anthracis* spores. At that point, the FBI became a crucial component of this investigation. A public health investigation aims primarily to identify the causative agent and its source so that public health workers and clinicians can implement appropriate and timely control and preventative measures. A forensic investigation goes one step further to associate the source of the causative agent with a specific individual or group. Physical, scientific, and other evidence has to be handled in a manner that allows verification of all transfers and accounts for the possession of this evidence at all times; this verification is accomplished by extensive chain of custody documentation. Consequently, establishing an unbroken chain of evidence is paramount and requires that the experts presenting the evidence can successfully withstand the scrutiny of both the criminal investigation and the defense during a trial. The importance of microbial forensics has been repeatedly emphasized following the events of 2001, but microbial forensics is neither new nor limited to biothreat events. Recently, a colloquium was convened by the American Academy of Microbiology to consider issues specifically relating to microbial forensics, such as the detailed identification of a microorganism used in a bioterrorist event and analysis of such a microorganism to identify its source and the perpetrators of the event (9). We will briefly discuss here some of the major points and recommendations developed by the participants of the colloquium in the context of our own experience during the 2001 investigations of the bioterrorism-associated anthrax. In addition, we will provide an overview of the diagnostic capabilities that were used at the CDC, from the very basic procedures to the state-of-the-art molecular techniques.

Collection of Specimens and Identification of the Causative Agent

The anthrax investigation of 2001 resulted in 11 patients diagnosed with inhalation anthrax and additional 11 with the cutaneous forms of the disease (10). For the CDC laboratory scientists, the investigation began on October 3, 2001, with the report of a suspect *B. anthracis* isolate in Florida and immediate phone conferences with public health laboratory scientists in Florida. On October 4, 2001, the first CDC team consisting of epidemiologists, laboratory scientists, and support staff was deployed to Florida.

The initial clinical suspicion of inhalation anthrax quickly led to the isolation and identification of *B. anthracis* in cerebrospinal fluid of the index case-patient (10-12). Immediately, the investigation was expanded to focus on the source of this organism. Hundreds of environmental specimens were collected from the index patient's home, workplace, and places that he frequented. Receiving, documenting, distributing, and processing of specimens associated with the 2001 anthrax investigation occurred under emergency conditions. Consequently, it was important to have well-established protocols for both managing and accurately tracking each specimen. The procedures employed may be substantially influenced by investigators' previous knowledge or assumptions of an event being associated with a particular biological agent. Whereas no agent is initially excluded, special steps might be taken to focus on the agent most likely to be associated with this event. As pointed out in the scientific assessment (9), the first responders will not only have to collect the materials for evidence, but also need to maintain safety, avoid dissemination of materials beyond the scene, and avoid introduction of contaminating materials into the scene. The initial collection of samples in Florida was conducted under the guidance of both CDC and

Florida state epidemiologists and laboratory scientists who used standard procedures designed specifically for such events in their investigation.

Two days after the deployment of the first CDC team, over 200 clinical and environmental specimens arrived at CDC. The number and types of samples received over the following few days prompted us to rapidly expand our laboratory capacities and assign many staff to work around the clock in three 8-hour shifts, seven days a week. One of the many challenges was to ensure accurate tracking of all samples and the testing done on them.

Realizing that a large number of specimens would continue to arrive, we rapidly developed a logistical and organizational set-up at the CDC. All specimens were received in the Rapid Response and Advanced Technology Laboratory, where they were screened by using rapid bio-detection assays: real-time polymerase chain reaction (PCR) and time-resolved fluorescence. Specimens were then forwarded to the CDC Anthrax Laboratory for isolation, confirmatory testing, and molecular characterization. Biopsy materials and tissues were sent to a pathology laboratory where recently developed immuno-histochemistry assays (13) specific for *B. anthracis* were used. This basic structure was quickly supplemented by one more specialized laboratory, where hundreds of sera were tested by anti-protective antigen (PA) IgG enzyme-linked immunosorbent assay (ELISA) (14), and finally specimens were sent to the CDC Surge Capacity Laboratory where rapid initial processing and screening of environmental specimens was performed before forwarding all suspect isolates to the CDC Anthrax Laboratory.

In the CDC Anthrax Laboratory, specimens were processed by direct inoculation on and into microbiological media and by extraction of DNA for molecular testing. Once the organisms were growing, we screened for colony morphology and lack of hemolysis typical for *B. anthracis*. Finally, non-motility of *B. anthracis* was a very helpful characteristic in differential diagnosis and establishing presumptive diagnosis of an isolate as *B. anthracis* (Fig. 1). The full spectrum of laboratory diagnostic procedures used at the CDC laboratories is presented in Figure 2. Confirmatory identification was carried out by using standard microbiological procedures according to the Laboratory

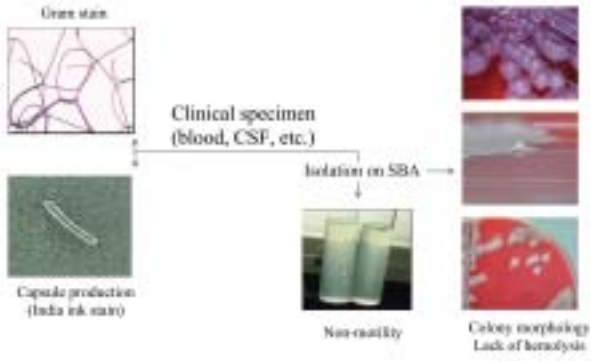

Figure 1. Laboratory tests used for presumptive identification of *Bacillus anthracis* in Level A laboratories of the Laboratory Response Network. CSF – cerebrospinal fluid.

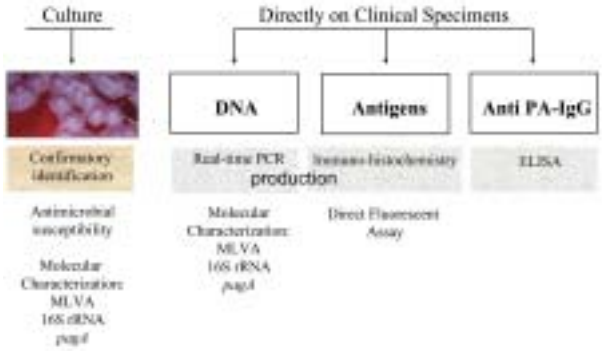

Figure 2. Spectrum of laboratory tests used at the laboratories of the Centers for Disease Control and Prevention for identification and characterization of *Bacillus anthracis* and for laboratory confirmation of anthrax. PA – protective antigen; PCR – polymerase chain reaction; ELISA – enzyme-linked immunosorbent assay; MLVA – multi-locus variable-number-of-tandem-repeat analysis; 16S rRNA – sequencing of entire 16S rRNA gene; *pagA* – sequencing of the entire *pagA* gene, gene coding for protective antigen.

Response Network algorithm (3,13). Most frequently, this approach included lysis by gamma phage in conjunction with demonstration of a capsule. Capsule production could have been induced in either of two ways: by growth on bicarbonate-supplemented medium in an enhanced CO_2 atmosphere or by incubation in horse blood. The capsule was visualized with the M'Fadyean or India ink stain. Alternative confirmatory approach was to detect both cell wall and capsule antigens by the direct fluorescence assay (15).

We gained many valuable pieces of information during this investigation that consequently led to modifications of some of the protocols. An example is the modification of protocols for collecting surface samples by starting with swabbing the surface, then using wipes, and finally using standardized vacuum systems with filters. Based on the insight provided by the events of 2001, protocols have been further modified for collecting and evaluating environmental specimens suspected to contain *B. anthracis* spores (16). Standard procedures were developed to assure that all investigators at the geographically diverse sites collected the samples appropriately. From the very beginning of the investigation, chain of custody forms were used to verify a specimen's location at all times. A 10-digit unique identifier was used to label all samples and to identify all subsequent aliquots and tests conducted on them as part of the original sample. Upon arrival at CDC, all samples were verified against the accompanying chain of custody documentation and were photographed before they were processed.

Laboratorians carried out identification of *B. anthracis* during the investigation using the standard microbiological procedures and the Laboratory Response Network algorithm (3,13). As already stated, many laboratorians within the Laboratory Response Network had been trained in the basic microbiological procedures for isolation and identification of *B. anthracis*, and detailed protocols were placed on a secure web page in the fall of 2000. Presently, numer-

ous efforts are underway to develop rapid field tests and kits that could be used for screening either as a bedside test or in an environmental setting.

Molecular Characterization as a Tool to Track the Source of the Organism

Once the organism has been identified, patient treatment, prophylaxis, and intervention decisions can be made or modified. The next step is to molecularly subtype the isolated agent for tracking the source and possibly the perpetrator. The 2001 anthrax outbreak clearly demonstrated the importance of molecular subtyping of *B. anthracis* isolates. However, molecular subtyping of *B. anthracis* has proven to be more challenging than the subtyping of many other bacterial agents. Different methods, such as multiple-locus enzyme electrophoresis and multiple-locus sequence typing, revealed the lack of genetic diversity of *B. anthracis* (6,17-19). However, amplified fragment length polymorphism revealed differences between *B. anthracis* isolates and was also used to examine phylogenetic relationships between *B. anthracis* and its close relatives, *B. cereus* and *B. thuringiensis* (19,20). Multilocus variable-number-of-tandem-repeats analysis, unlike amplified fragment length polymorphism, was specifically designed to subtype *B. anthracis* by focusing on a number of specific targets in the *B. anthracis* chromosome and its two plasmids. This method determines the copy number of variable number of tandem repeats at eight genetic loci (six chromosomal and one on each of the two plasmids) (21). Consequently, it allows for association of particular patterns (genotypes) with geographic, temporal, and other designations of a given strain (21,22). In several studies that took place before the 2001 bioterrorism-related anthrax outbreak, e.g., Keim et al (21,23) used this approach to study the ecology of anthrax and differentiated 426 *B. anthracis* isolates into 89 distinct genotypes. In addition to providing the highest level of discrimination among *B. anthracis* isolates, multilocus variable-number-of-tandem-repeats analysis is relatively simple, reproducible, and allows for rapid (<8 h) testing of multiple strains on a single gel.

Multilocus variable-number-of-tandem-repeats analysis was indeed the molecular subtyping method used during the 2001 investigation. Following the confirmatory identification, we used multilocus variable-number-of-tandem-repeats analysis to subtype 135 *B. anthracis* isolates associated with the 2001 anthrax outbreak and determined that all were genotype 62, the same genotype as the Ames strain widely distributed in laboratories worldwide (22). This information was crucial for linking anthrax to environmental samples and envelopes containing powders that were mailed to the major media organizations and government leadership. We also analyzed the DNA sequence of the PA gene (*pagA*), one of the three anthrax toxin proteins. Previously, the sequencing of *pagA* had been used to subtype 26 diverse *B. anthracis* isolates into six PA genotypes (24). Whereas sequencing of *pagA* yields only a limited number of subtypes, it still offers the advantage of assessing if the *pagA* gene has been altered in any way. All *B. anthracis* strains from the outbreak that were sequenced had a *pagA* sequence indistinguishable from that seen in the Ames strain (PA genotype I) (22). We used multilocus variable-number-of-tandem-repeats analysis and *pagA* sequencing for direct testing of clinical specimens, making it possible to molecularly characterize *B. anthracis* without culturing the isolate. This method was especially useful in instances when no isolate was cultured because of prior antimicrobial treatment. All outbreak isolates were indistinguishable from each other when these methods were used, suggesting their probable origin from a single source.

Current technological developments, such as microarrays, may play a major role in future biothreat events, to enhance rapid testing and identification of large numbers of samples and markers. Subsequent to the 2001 investigation, Dr. P. Keim's laboratory at the Northern Arizona University expanded multilocus variable-number-of-tandem-repeats analysis from the original eight to currently used 15 markers (P. Kleim, personal communication). Recent whole-genome sequencing of the isolate from the 2001 index case showed very little variability from the Sterne and Pasteur strains (25).

Developing a database that contains extensive molecular characterization data on a diverse collection of strains, including closely related species that can pose a differential diagnostic challenge, is a prerequisite for successful microbial forensics activities. An important question that will have to be rapidly answered is whether the causative agent had been bio-engineered in any way. Comparison with the genetic information in such databases should be able to provide a rapid answer. Even with state-of-the-art technologies, it would have been impossible to provide detailed analyses of the causative agent, were it not for the existence of a molecular subtyping database established and maintained by Dr. P. Keim; that database contains data obtained by multilocus variable-number-of-tandem-repeats analysis for over a thousand *B. anthracis* isolates collected worldwide.

The Investigation

While identification and molecular characterization of a suspect agent are the key steps, there are many additional requirements and activities that need to be in place to allow for the successful completion of a forensic investigation, ie, identification of the source of the causative agent and the perpetrator. Some of the most important activities are the establishment of quality assurance/quality control criteria and regular proficiency testing for all laboratories where evidence is analyzed. Working under the stringent requirements of the criminal investigation, laboratories used many diagnostic approaches to allow for maximum speed and reliability of the laboratory information. Successful implementation of alternative laboratory assays during the 2001 investigation permitted expansion of the definition of a confirmed case of anthrax beyond the previously established criteria, which was a clinically compatible case of cutaneous or inhalation illness that is laboratory-confirmed by isolation of *B. anthracis* from an affected tissue or site. Because of new information gained in the investiga-

tion of the 2001 anthrax outbreak, the definition of a confirmed case was expanded to a clinically compatible case accompanied with laboratory evidence of *B. anthracis* infection based on at least two supportive laboratory tests: (a) evidence of *B. anthracis* DNA by the three-target Laboratory Response Network PCR from specimens from an affected tissue or site, (b) demonstration of *B. anthracis* in a clinical specimen by immunohistochemical staining, or (c) 4-fold rise in serum anti-PA IgG levels. Overall, over 7,500 specimens were processed at CDC laboratories during this investigation and over 400 *B. anthracis* isolates were identified and molecularly characterized. Overall, more than 125,000 specimens were analyzed at Laboratory Response Network laboratories throughout the country.

Education, Training, and Communication

Because of the complexity of microbial forensics, the involvement of experts from many diverse scientific areas and the critical role played by the first responders, education, and cross training of all involved in a forensic investigation are clearly key components necessary for its overall success. These activities include establishing certification programs in microbial forensics, developing first responders training programs, and preparing public education programs. During the 2001 investigation, an enormous level of interest, both from the general public and from the media, followed every step of the investigation. At the same time, the education of primary health care workers and laboratorians establishing the laboratory diagnoses could not be overlooked. Videoconferences focusing on clinical and laboratory aspects of diagnosing anthrax were launched within weeks from the onset of the investigation (October and November 2001). The CDC's Morbidity and Mortality Weekly Reports provided continued updates on all aspects of the investigation. Daily press releases, health alerts, and tele-conferences were held with key CDC staff members (*www.bt.cdc.gov*).

An international team was established to assist epidemiologists, microbiologists, and clinicians around the world who were responding to widespread political and public concerns. From October 12 to December 12, 2001, 128 requests for assistance from 68 countries and two territories were received (26). The team also played an active role in disseminating documents on anthrax and bioterrorism preparedness. A special issue of the journal Emerging Infectious Diseases was devoted to the events of 2001, which provided an overview of clinical, microbiological, epidemiological, and overall public health activities during this bioterrorism-associated anthrax investigation (27).

Summary

The bioterrorism attack in the fall of 2001 that resulted in 22 anthrax cases in the United States, including five deaths, reemphasized the importance and value of microbial forensics. As we have demonstrated from our own experience, a wide range of disciplines are involved in this complex scientific and legal activity. The following paragraph from a scientific assessment of a colloquium recently convened by the American Academy of Microbiology clearly outlines similarities and differences between microbial forensics and epidemiology:

While epidemiology and forensics are similar sciences with similar goals when applied to biocrimes, forensics has additional and more stringent requirements. Maintaining a chain of custody on evidentiary samples is one example of an extra requirement imposed on an investigation of a biocrime. Another issue is the intent in microbial forensics to identify a bioattack organism in greater detail. If possible, forensic investigations will strive to identify the precise strain and substrain, rather than just to the species level, which might be sufficient in an epidemiological investigation. Some pathogen attributes that are unimportant to protecting public health may provide clues in a forensic investigation (9).

Herein lays the crucial component of the definition of microbial forensics: its ability to detect the molecular variations between related microbial strains and then use that information to identify the origin of a particular isolate (28). Numerous challenges are still ahead, such as establishment of and strict adherence to quality assurance/quality control, and proficiency testing programs. The development of programs for cross-training of first responders, laboratory, and other public health scientists in handling and processing samples in accordance to forensic microbiological criteria is also necessary. Just as important as these considerations is the training of the law enforcement staff in the scientific basis of identification and molecular characterization of microbes, and the establishment and maintenance of repositories and databases containing isolates of diverse temporal and geographic origins. Microbial forensics will also be augmented by the development of new assays and modifications and validations of existing state-of-the-art molecular approaches for characterization of microbes. Tremendous support by the public, government, and the private sector worldwide has made it possible for many of these activities to be well under way, assuring that the accumulation and exchange of scientific data progress rapidly in an effort to most efficiently fight bioterrorism, by both preventing it and, if need be, by responding in an unprecedented way.

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