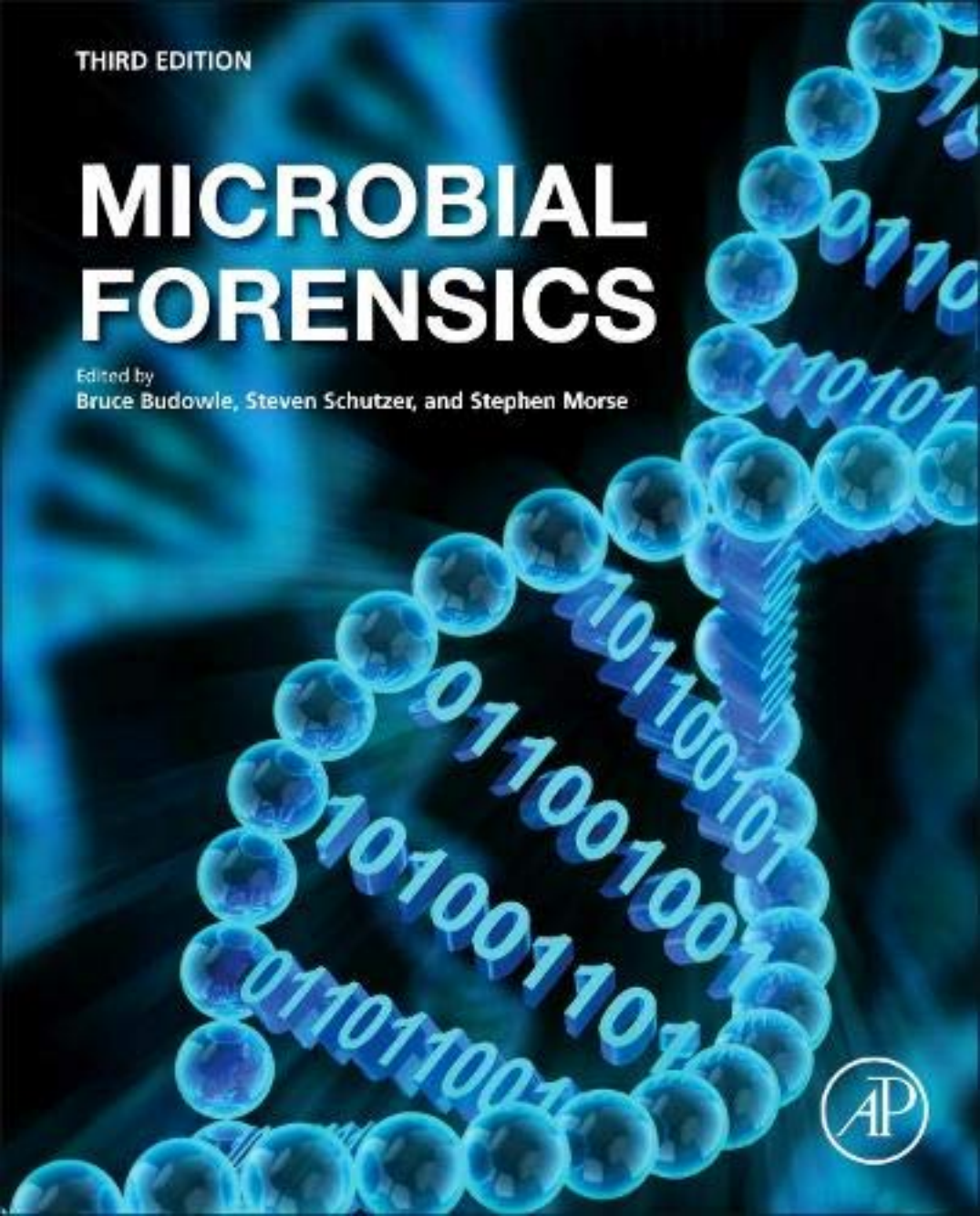
THIRD EDITION

MICROBIAL FORENSICS

Edited by
Bruce Budowle, Steven Schutzer, and Stephen Morse



MICROBIAL FORENSICS

THIRD EDITION

Edited by

Bruce Budowle

Steven Schutzer

Stephen Morse

ACADEMIC PRESS

An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

Notices

Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

Practitioners and researchers must always rely on their own experience and knowledge in evaluating and using any information, methods, compounds, or experiments described herein. In using such information or methods they should be mindful of their own safety and the safety of others, including parties for whom they have a professional responsibility.

To the fullest extent of the law, neither the Publisher nor the authors, contributors, or editors, assume any liability for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein.

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

ISBN: 978-0-12-815379-6

Publisher: Stacy Masucci
Acquisition Editor: Elizabeth Brown
Editorial Project Manager: Pat Gonzalez
Production Project Manager: Surya Narayanan Jayachandran
Cover Designer: Mark Rogers
Typeset by TNQ Technologies

Contributors

M.W. Allard Division of Microbiology, Office of Regulatory Science, CFSAN, US FDA, College Park, MD, United States

Jonathan Allen Lawrence Livermore National Laboratory, Livermore, CA, United States

Philip S. Amara College of Public Health, University of Nebraska Medical Center, Omaha, NE, United States

Neel G. Barnaby FBI Laboratory, Quantico, VA, United States

John R. Barr Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, GA, United States

Aeriel Belk Animal Sciences, Colorado State University, Fort Collins, CO, United States

Nicholas H. Bergman National Bioforensic and Analysis Center, Frederick, MD, United States

Christopher A. Bidwell Federation of American Scientists, Washington, DC, United States

Dawn Birdsell The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Thomas A. Blake Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, GA, United States

E.W. Brown Division of Microbiology, Office of Regulatory Science, CFSAN, US FDA, College Park, MD, United States

Sibyl Bucheli Department of Biological Sciences, Sam Houston State University, Huntsville, TX, United States

Bruce Budowle Center for Human Identification, Department of Microbiology, Immunology and Genetics, University of North Texas Health Science Center, Fort Worth, TX, United States

Robert L. Bull FBI Laboratory, Quantico, VA, United States

James Burans National Bioforensic and Analysis Center, Frederick, MD, United States

David O. Carter Laboratory of Forensic Taphonomy, Forensic Sciences Unit, Division of Natural Sciences and Mathematics, Chaminade University of Honolulu, Honolulu, HI, United States

Angela Choi Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, United States

Brooke L. Deatherage Kaiser Pacific Northwest National Laboratory, Richland, WA, United States

R. Scott Decker Bio-Logic Security, LLC, Las Vegas, NV, United States

Heather Deel Animal Sciences, Colorado State University, Fort Collins, CO, United States

David M. Engelthaler TGen North, Translational Genomics Research Institute, Flagstaff, AZ, United States

Hector F. Espitia-Navarro School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, United States; PanAmerican Bioinformatics Institute, Cali, Valle del Cauca, Colombia

Jacqueline Fletcher Department of Entomology & Plant Pathology, National Institute for Microbial Forensics & Food and Agricultural Biosecurity, Oklahoma State University, Stillwater, OK, United States

Jeffrey T. Foster The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Adolfo García-Sastre Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, United States; Department of Medicine, Icahn School of Medicine at Mount Sinai, New York,

NY, United States; Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY, United States; The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, United States

Jennifer S. Goodrich National Bioforensic and Analysis Center, Frederick, MD, United States

Bradley Hart Lawrence Livermore National Laboratory, Livermore, CA, United States

David R. Hodge Department of Homeland Security, Washington, DC, United States

Paul J. Jackson Adjunct Professor, Nonproliferation and Terrorism Studies, James Martin Center for Nonproliferation Studies, Middlebury Institute of International Studies at Monterey, Monterey, California, United States

Crystal Jaing Lawrence Livermore National Laboratory, Livermore, CA, United States

Rudolph C. Johnson Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, GA, United States

I. King Jordan School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, United States; PanAmerican Bioinformatics Institute, Cali, Valle del Cauca, Colombia; Applied Bioinformatics Laboratory, Atlanta, GA, United States

Suzanne R. Kalb Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, GA, United States

Arnold F. Kaufmann United States Public Health Service, Stone Mountain, GA, United States

Paul Keim The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Terry L. Kerns Bio-Logic Security, LLC, Las Vegas, NV, United States

Ali S. Khan College of Public Health, University of Nebraska Medical Center, Omaha, NE, United States

Rachel E. Kieser Center for Human Identification, Graduate School of Biomedical Sciences, University of North Texas Health Science Center, Fort Worth, TX, United States

Rob Knight Department of Pediatrics, Department of Computer Science and Engineering, Microbiome Innovation Center, University of California, San Diego, CA, United States

Asja Korajkic U.S. Environmental Protection Agency, Office of Research and Development, Cincinnati, OH, United States

Steven B. Lee International Forensic Research Institute, Florida International University, Miami, FL, United States; Forensic Science Program, Department of Justice Studies, San Jose State University, San Jose, CA, United States

Anastasia P. Litvintseva Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA, United States

Douglas G. Luster USDA ARS, Foreign Disease - Weed Science Research Unit, Fort Detrick, MD, United States

Aaron Lynne Department of Biological Sciences, Sam Houston State University, Huntsville, TX, United States

Leonard W. Mayer PanAmerican Bioinformatics Institute, Cali, Valle del Cauca, Colombia; Applied Bioinformatics Laboratory, Atlanta, GA, United States

Ulrich Melcher Oklahoma State University, Department of Biochemistry & Molecular Biology, Stillwater, OK, United States

Eric D. Merkley Pacific Northwest National Laboratory, Richland, WA, United States

Jessica L. Metcalf Animal Sciences, Colorado State University, Fort Collins, CO, United States

DeEtta K. Mills International Forensic Research Institute, Florida International University, Miami, FL, United States

Stephen A. Morse IHRC, Inc., Atlanta, GA, United States

Ted D. Mullins American Type Culture Collection (ATCC), Manassas, VA, United States

Randall Murch Virginia Polytechnic Institute and State University, Arlington, VA, United States

Forrest W. Nutter, Jr. Iowa State University, Department of Plant Pathology and Microbiology, Ames, IA, United States

Francisco M. Ochoa Corona Department of Entomology & Plant Pathology, National Institute for Microbial Forensics & Food and Agricultural Biosecurity, Oklahoma State University, Stillwater, OK, United States

Sam Ogden Animal Sciences, Colorado State University, Fort Collins, CO, United States

Richard T. Okinaka The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Talima Pearson The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Bernard R. Quigley IHRC, Inc., Atlanta, GA, United States

Lavanya Rishishwar School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, United States; PanAmerican Bioinformatics Institute, Cali, Valle del Cauca, Colombia; Applied Bioinformatics Laboratory, Atlanta, GA, United States

Jason W. Sahl The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Stephen A. Saltzburg Wallace and Beverley University Professor, The George Washington University Law School, United States

Harald Scherm University of Georgia, Department of Plant Pathology, Athens, GA, United States

David G. Schmale, III Virginia Tech University, School of Plant and Environmental Sciences, Blacksburg, VA, United States

Sarah E. Schmedes Association of Public Health Laboratories, Silver Spring, MD, United States

Steven E. Schutzer Department of Medicine, Rutgers New Jersey Medical School, Newark, NJ, United States

Orin C. Shanks U.S. Environmental Protection Agency, Office of Research and Development, Cincinnati, OH, United States

Frank P. Simione American Type Culture Collection (ATCC), Manassas, VA, United States

Tom Slezak Lawrence Livermore National Laboratory, Livermore, CA, United States

Jenifer A.L. Smith District of Columbia Department of Forensic Sciences, Washington, DC, United States

Carla S. Thomas University of California, Plant Pathology Department, Davis, CA, United States

Stephan P. Velsko Lawrence Livermore National Laboratory, Livermore, CA, United States

Richard R. Vines American Type Culture Collection (ATCC), Manassas, VA, United States

Amy Vogler The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

David M. Wagner The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Karen L. Wahl Pacific Northwest National Laboratory, Richland, WA, United States

Charles H. Williamson The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Mark Wilson MRW Analytics, LLC, Fredericksburg, VA, United States

August Woerner Center for Human Identification, University of North Texas Health Science Center, Fort Worth, TX, United States; Department of Microbiology, Immunology and Genetics, University of North Texas Health Science Center, Fort Worth, TX, United States

David S. Wunschel Pacific Northwest National Laboratory, Richland, WA, United States

Foreword

I am honored that the authors have asked me to contribute the foreword to the third edition of *Microbial Forensics*. My connection to the book and to the field in general was as a facilitator rather than as a researcher, and it was in the distant past, 20 years ago!

Microbial Forensics received input and direction in a series of meetings held at the Banbury Center, a conference center at Cold Spring Harbor Laboratory. Indeed, some meetings held at the Banbury, which predate the emergence of Microbial Forensics as a formalized discipline, laid some of the groundwork for this must-needed forensic field. Banbury has an international reputation for intensive discussion meetings with fewer than 30 participants of topics in the life sciences that would benefit from critical review, topics that are frequently controversial and often involve issues of policy as well as science.

In 1999, Suzanne Vernon and Bill Reeves of the Centers for Disease Control (CDC) approached me about a meeting to be funded by CDC to discuss how it would be possible to detect and develop tests for unknown pathogens. CDC was interested because it had been proposed that chronic fatigue syndrome (CFS) was an infectious disease, but the pathogen detection techniques used by CDC—serology, culture, PCR—had failed to identify an infectious agent associated with CFS. By 1999, new techniques were coming online, and CDC wanted to review how these might be used to find novel or previously uncharacterized pathogens associated with CFS.

Banbury already had held a series of meetings on what was still a "newly emerged" disease, Lyme disease. The first meeting was held in 1991, only nine years after Willy Burgdorfer had identified the previously unknown pathogen, the spirochete, *Borrelia burgdorferi*. I put Suzanne in touch with the organizers of those meetings, Steve Schutzer and Ben Luft, and the three organized the April 2000 meeting "Strategies for Detection and Identification of Unknown Pathogens."

The primary focus of this meeting was health, and the word "forensic" did not appear. However, one of the participants was Roger Breeze, then with the US Department of Agriculture. While the title of the meeting he organized in October 2000 had the rather innocuous title "The Challenge of Infectious Diseases in the 21st Century," his view of unrecognized infectious disease was quite definitely forensic; the invitation referred to the need for strategies to deal with new and emerging threats "…of which biological terrorism is the latest."

Finally, in November 2002, Schutzer and Breeze with Bruce Budowle (then with the FBI Laboratory in Quantico) organized the first "Microbial Forensics" meeting, followed by additional four meetings. The final meeting in 2010 was on "Microbial Forensics in the Era of Genomics," which reviewed techniques for DNA sequencing and bioinformatics that were unfathomable mere eight years earlier.

Despite these and other dramatic advances in technology, it is not surprising that the same

broad issues remain, some of which are identified in the authors' introduction, for example, the differing needs of the worlds of public health and forensics; the latter required meeting the legal standards for evidence that might be used in the court.

The authors refer to the issue of whether microbial forensics will be centralized, for example, at the National Bioforensics Analysis Center, or become distributed. If the latter, adequate resources—graining and infrastructure—will be needed, but this may be hard to justify for what one hopes will be rare events.

In some ways, I am reminded of the early days of forensic DNA fingerprinting. The techniques and associated population genetic analyses had been developed in academic laboratories, and there were many obstacles to be overcome before DNA fingerprinting was accepted. It required training in techniques and theory, as well as an acceptance by the public, law enforcement, and defense attorneys, before it became a powerful tool for the justice system. I am proud to recall that a Banbury Center meeting in 1988 (attended by Bruce Budowle) contributed to cementing this technology as the gold standard of the forensic sciences.

Finally, I fully endorse the authors' closing remarks. Scientific evidence carries great authority over and above its evidentiary value. This responsibility requires that those employing scientific techniques in the service of forensic science do so to the highest standards possible. It took several years for DNA fingerprinting to reach that position, and it will take the same commitment for microbial forensics to attain the same level of credibility and acceptance. But without a doubt, it will be well worthwhile as this burgeoning discipline continues to fulfill its promise.

Jan A Witkowski PhD
Professor
Cold Spring Harbor Laboratory

Introduction

Stephen A. Morse[1], *Bruce Budowle*[2], *Steven E. Schutzer*[3]

[1]IHRC, Inc., Atlanta, GA, United States; [2]Center for Human Identification, University of North Texas Health Sciences Center, Fort Worth, TX, United States; [3]Department of Medicine, Rutgers New Jersey Medical School, Newark, NJ, United States

Deliberate dissemination of a biological agent via several different routes, including air, water, food, infected vectors, and manmade avenues, presents a challenge to global health and security. The ability to identify the perpetrator(s) in an expeditious and effective manner is of paramount importance as both a deterrent and for gauging an appropriate response. Eliminating those individuals who are falsely associated with evidence or a crime is of equal importance.

The past decade has seen major advances in the scientific discipline of microbial forensics, whose core objective is attribution: the investigative process aimed at identifying the perpetrators of a biowarfare, bioterrorism, or biocrime event and bringing them to justice. This highly interdisciplinary field, which has largely emerged through US initiatives (Tucker and Koblentz, 2009; U.S., 2010), encompasses traditional law enforcement and forensic investigative methods as well as the detailed characterization of samples containing biological agents. Moreover, with the advent of high throughput technologies, such as massively parallel sequencing, microbial forensics as a discipline has expanded beyond its primary focus on microbes and their products involved in bioterrorism and biocrimes to other applications such as exploiting the human microbiome for human identification, human geolocation, and determining post mortem interval.

Microbial forensic investigations and molecular epidemiologic investigations share many aspects but are performed for different reasons. An epidemiologic investigation aims to identify the source of an outbreak and to understand routes of transmission in order to prevent further spread and reduce the risk of future outbreaks through effective preventive measures. A microbial forensic investigation is undertaken to identify the perpetrator of a crime and to gather high quality evidence that can be used for indictment and conviction of perpetrators or to help decision makers prepare an appropriate response to an attack. A microbial forensic investigation has been described as consisting of three interrelated stages: identification of the biological agent(s) responsible for an event; characterization of the event as intentional, unintentional (e.g., due to negligence or carelessness), or naturally occurring; and, if the event is deemed illegitimate (i.e., intentional or unintentional), attribution of use to a specific perpetrator(s) (Koblentz and Tucker, 2010). Additionally, analyses may be used to determine the process(es) used to produce a bioweapon, which can provide additional forensic evidence and insight into the sophistication of the production process. Regardless if an event was intentional or accidental, an immediate goal is to prevent additional attacks and protect the public.

Many of the questions asked during the first two stages of a microbial forensic investigation are identical to those of an epidemiologic investigation, and the same methods and technologies are generally used to answer them (Sjodin et al., 2013). However, the attribution step is unique to microbial forensics. In addition to traditional forensic analyses of recovered evidence from the crime scene (analysis of human DNA, fingerprints, fibers, etc.), detailed analyses are conducted of the responsible biologic agent (bacterium, virus, fungus, or toxin). It is possible that the results of an epidemiologic investigation may suggest a deliberate or unintentional release of a biological agent and thus lead to the initiation of a microbial forensic investigation. The intentional release of a biological agent can be done overtly, in which case the perpetrators will announce their responsibility, or covertly, in which case they will not call attention to the release of a biological agent (Budowle et al., 2005). It is likely that most unintentional releases will be covert-like in nature (Zimmer and Burke, 2009).

Since the publication of the first edition of this book in 2005, the field of microbial forensics has evolved substantially in its ability to attribute the source of microorganisms and toxins that have been used in cases of bioterrorism and in biocrimes. The 2001 anthrax mail attack (often referred to as "Amerithrax") fortunately remains the worst and best-known example of successful bioterrorism on US soil (Chapters 2 and 3). At the time, the US was ill prepared to investigate the unique types of microbiological evidence for attribution that were generated by that investigation, although traditional types of forensic evidence could readily be collected, analyzed, and interpreted within the framework of a statistically sound scientific foundation. The first edition of this book had only limited reference to the anthrax mail attack because much of the scientific examination was still in progress and methodologies were still under development. Early capabilities were initially limited to detection and identification and did not include detailed characterization and comparative analyses. Thus, the first edition was developed as a foundational text to stimulate scientists, legal experts, and decision makers responsible for analyzing and interpreting evidence from an act of biological warfare, bioterrorism, biocrime, or inadvertent release of a microorganism or toxin for attribution purposes and to describe the discipline. In addition, the initial edition of this book discussed some of the future opportunities and challenges for the microbial forensic field.

The second edition of this book, published in 2011, contained several chapters that brought the Amerithrax investigation to a conclusion. The anthrax mail investigation, one of the most intense and expansive investigations to date by the Federal Bureau of Investigation and US Postal Service (http://www.fbi.gov/antrhrax/amerithraxlinks.htm), is now considered closed. However, the third edition of this book revisits this case and provides a first-hand account of the investigation as well as lessons learned on what should be done in microbial forensics to support an investigation and equally important what should be avoided.

Many of the elements discussed in the first and second editions of this book are echoed in the 2009 US National Research and Development Strategy for Microbial Forensics (U.S., 2010), which identifies threat awareness, prevention and protection, surveillance and detection, and response and recovery as the essential pillars of US national biodefense policy. "Attribution"—the investigative process by which the US government links the identity of a perpetrator or perpetrators of illicit activity and the pathway leading to criminal activity—is part of the surveillance and detection pillar. Making a determination of attribution for a planned or actual perpetrated biological attack would be the culmination of a complex investigative process drawing on many different sources of information, including traditional forensic analysis of material evidence collected

during an investigation of a planned attack or material evidence resulting from an attack and relevant subject matter experts. One of the key sources of attribution information in a biological attack is the result of a microbial forensic investigation.

Future investigations of biological warfare, bioterrorism events, or biocrimes are likely to be as multifaceted as the Amerithrax investigation, if not more so, and will demand more integration and better communication among government agencies. Fortunately, the technologies today provide much better capabilities to respond with a strong microbial forensic program. At the same time, microbial forensics is still a developing field facing numerous scientific challenges. For these and other reasons, governments are making investments, often for public health purposes, to provide a robust capability to detect, identify, and characterize biological agents. Advances in this area have been sufficiently exciting and significant to make this third edition of *Microbial Forensics* both timely and necessary. There is growing interest and application of microbial forensic principles in food safety, water quality, and bioinformatics, and thus this edition contains chapters to cover a broader audience interest than that of national security. We recognize that traditional detective investigation, coupled with scientific analysis of evidence and frequently additional ongoing and novel experimentation, can generate investigative leads. Thus, chapters on microbial forensic approaches to human identification and the determination of postmortem interval have been included.

The first edition of this book was developed for individuals entering the field of microbial forensics who were looking for a single source for initial guidance and information. The second edition supported that need with a combination of basic texts and chapters on more sophisticated technologies, such as nonbiological analytical tools and next-generation sequencing methods. The rapid advance in technology and its application have led to the third edition. This edition recognizes the combination of diverse disciplines that must be exploited to analyze evidence, including biology, microbiology, medicine, chemistry, physics, statistics, population genetics, computer science, and computational biology. New techniques must be developed and employed to extract the most information from forensic evidence obtained from terrorist and criminal events, especially when more traditional forms of evidence are either not available or very limited in content. Yet microbial forensics—a maturing discipline—depends very heavily on some traditional and frequently overlooked scientific values: willingness to share often priceless samples with others across international boundaries, rigorous curation of microbial repository samples over decades, and organization and execution of international collaborative studies with recognition of all involved.

Over the past century, science has played an increasingly greater role in criminal investigation (Budowle et al., 2008, 2009). Microbial forensic science will continue this tradition. But scientific analysis is only part of the process. Forensic science results must be integrated with other information, and attention must be paid to steps that will assure the quality of results as well as support their admissibility in a court of law (see Chapter 23—27). We stress quality control and quality assurance as the means to ensure integrity of the evidence. Practices such as adherence to chain of custody procedures, documentation of activities, and the use of validated reagents, calibrated equipment, negative and known positive control samples, validated procedures, standard operating procedures, and so on are the essence of reliability and confidence. These criteria in turn ensure admissibility in court.

The scientific foundations of microbial forensics will be strengthened, built upon, and likely remodeled by our present and future colleagues. Their accomplishments over the past eight years

have led to this new edition. We look forward to their continued input, interaction, and insight.

References

Budowle, B., Bottrell, M.C., Bunch, S.G., Fram, R., Harrison, D., Meagher, S., et al., 2009. A perspective on errors, bias, and interpretation in the forensic sciences and direction for continuing advancement. J. Forensic Sci. 54, 798–809.

Budowle, B., Schutzer, S.E., Ascher, M.S., Atlas, R.M., Burans, J.P., Chakraborty, R., et al., 2005. Toward a system of microbial forensics: from sample collection to interpretation of evidence. Appl. Environ. Microbiol. 71, 2209–2213.

Budowle, B., Schutzer, S.E., Morse, S.A., Martinez, K.F., Chakraborty, R., Marrone, B.L., et al., 2008. Criteria for validation of methods in microbial forensics. Appl. Environ. Microbiol. 74, 5599–5607.

Koblentz, G.D., Tucker, J.B., 2010. Tracing an attack: the promise and pitfalls of microbial forensics. Survival 52, 159–186.

Sjodin, A., Broman, T., Melefors, O., Andersson, G., Rasmusson, B., Knutsson, R., et al., 2013. The need for high-quality whole-genome sequence databases in microbial foremsics. Biosec. Bioterror. 11 (Suppl. 1), S78–S86.

Tucker, J.B., Koblentz, G.D., 2009. The four faces of microbial forensics. Biosecur. Bioterror. 7, 389–397.

U.S., 2010. National Science and Technology Council, Executive Office of the President, National Research and Development Strategy for Microbial Forensics. http:www.whitehouse.gov/files/documents/ostp/NSTC%20Reports/National%20MicroForensics%20R&DStrategy%202009%20UNLIMITED%20DISTRIBUTION.pdf.

Zimmer, S.M., Burke, D.S., 2009. Historical perspective – emergence of influenza A (H1N1) viruses. New Engl. J. Med. 361, 279–285.

CHAPTER

1

The Kameido anthrax incident: a microbial forensic case study

Arnold F. Kaufmann[1], *Paul Keim*[2]

[1]United States Public Health Service, Stone Mountain, GA, United States; [2]The Pathogen and Microbiome Institute, Northern Arizona University, Flagstaff, AZ, United States

Introduction

The Aum Shinrikyo, an apocalyptic religious sect based in Japan, first came to worldwide attention in 1995 as the result of their deadly sarin gas attack on the Tokyo subway system (Hudson, 1999; Smithson, 2000; Tu, 2002). Subsequent investigations revealed that the Aum Shinrikyo had launched earlier attacks with both chemical and biological agents. The biological attacks utilizing *Bacillus anthracis* spores and botulinum neurotoxin were notably unsuccessful, with failure to produce any casualties in at least seven alleged attempts over several years beginning in 1990. In this chapter, an attack in 1993 that was launched from the Aum Shinrikyo headquarters building then located in Kameido, a Tokyo suburb, will be discussed, with emphasis on laboratory and epidemiological studies (Takahashi et al., 2004; Keim et al., 2001).

The Aum Shinrikyo: a brief history

To put the Kameido incident into context, a brief history of the Aum Shinrikyo and its founder, primarily adapted from the reviews by Hudson, Smithson, and Tu, is useful (Hudson, 1999; Smithson, 2000; Tu, 2002). The Aum Shinrikyo was founded by Shoko Asahara, whose birth name was Chizuo Matsumoto. Born into poverty in 1955 and suffering severe visual impairment due to infantile glaucoma, Matsumoto was sent at a young age to a government-subsidized boarding school for the blind. He purportedly felt abandoned by his family, which may have later led to an Aum Shinrikyo rule that followers were to cut off relationships with their parents to attain the supreme truth.

Having limited vision in one eye, Matsumoto developed influence over the other blind students, who paid him for various services. During his student years, he developed a reputation as a bully and con artist. After high school graduation in 1975, Matsumoto established a successful acupuncture clinic, but he had to move to Tokyo in 1977 due to his involvement in a fight that resulted in injury to several persons. About this time, his stated ambitions included becoming the leader of a robot kingdom or the Prime Minister of Japan.

https://doi.org/10.1016/B978-0-12-815379-6.00001-5

In Tokyo, he found work as an acupuncturist and enrolled in a preparatory school for the Japanese college entrance examination, with a goal of qualifying for matriculation in Tokyo University. Failing the entrance examination, Matsumoto married and established an acupuncture clinic and a natural foods shop. As a sideline, he concocted an alcohol extract of tangerine skins that he marketed as a miracle drug for weight loss and a variety of other conditions. His success in sales of this product attracted the attention of the police and subsequent arrest and imprisonment for violating the Japanese Cosmetics and Medical Instruments Act. This experience may have contributed to his animosity toward established authority.

After his move to Tokyo, Matsumoto became interested in religion and, in 1981, joined Agon Shu, a new religion based on Buddhism and yoga. In 1984, Matsumoto quit the Agon Shu and established Aum Shinsen, a yoga club which rapidly grew from 15 to more than 1000 members. He also changed his name to Shoko Asahara or Bright Light in Japanese. Following a trip to India in 1986–87, Asahara changed the name of the yoga club to Aum Shinrikyo. Aum is a Hindu syllable representing the spoken essence of the universe, and Shinrikyo is derived from the Japanese words for "supreme truth." In 1989, Aum Shinrikyo was officially recognized as a religious sect in Japan, giving the sect tax advantages as well as the ability to claim the members' work in the sect's various enterprises as voluntary. The sect's growth continued with spread to other countries, including the United States, Germany, and Russia.

Sect members lived a Spartan life and were expected to cut off all associations from their past life, to take a chastity vow, and to turn over all their assets to the Aum Shinrikyo. They were subjected to a heavy diet of their master's "wisdom," often simultaneously undergoing food and sleep deprivation. Members were expected to voluntarily labor in the sect's various commercial enterprises, such as sales of herbal teas and natural medications, operation of noodle shops, health clubs and baby-sitting services, and computer-related services. Those who balked were driven ever harder, drugged and confined. In some extreme cases, defectors were murdered.

To carry out its activities, the Aum Shinrikyo developed a complex organizational structure consisting of 22 ministries plus the Offices of Religious Members. The latter was charged with recruitment of persons having needed skills, such as members of the Japanese Self Defense Forces and scientists. In effect, the Aum Shinrikyo assumed the form of a shadow government which could supplant the existent Japanese government if Asahara's ambitions were fulfilled.

By 1990, with membership in the tens of thousands spread over six countries and an estimated 300 million to a billion dollars available, the Aum Shinrikyo was well positioned to further Asahara's ambitions and delusions. The Aum Shinrikyo initially attempted a conventional approach to political power by fielding a slate of 25 candidates for the national elections that year. None of the candidates, including Asahara, were even modestly successful. Asahara believed that the Japanese government had cheated him rather than that the electorate was put off by the doomsday overtones of the candidate's speeches. His belief was reinforced by the fact that the number of votes received by all the candidates was far fewer than the number of Aum Shinrikyo members in Japan.

Based on a pastiche of apocalyptic scenarios drawn from various religions, Asahara preached that Japan was destined to suffer a number of overwhelming catastrophes, including a poison gas attack by the United States. Asahara and his followers would survive the looming Armageddon and evolve into a super race dominating the world. He became more vocal in expressing this belief after the humiliating electoral defeat in 1990. Not content to allow the catastrophes to occur in their own time, Asahara initiated development of chemical and biological weapons to speed up the process.

Only the chemical weapons program had some success. After overcoming initial production problems, the Aum launched an attack with sarin gas in Matsumoto City in June 1994. The attack targeted judges presiding over a land use dispute between the Aum Shinrikyo and a local real estate agent. Suspecting that the judges would make a decision unfavorable to the cult, Asahara ordered their assassination. This gave an opportunity to test the effectiveness of their sarin on humans. The sarin release utilized a spray device and resulted in 311 known casualties, with 58 hospitalized, including 7 deaths. The judges were unharmed.

Investigation of the Matsumoto City attack proceeded slowly, without definitive evidence linking the crime to a specific individual or group. The Aum Shinrikyo was suspected, and a sarin degradation product was detected in soil near a building in an Aum Shinrikyo compound, the Seventh Satayan, in Kamakiuishiki. The police did not seek a warrant to search the facility because of a conservative interpretation of pertinent laws. In an unrelated kidnapping case, however, the police found fingerprint evidence that an Aum Shinrikyo member was involved. This gave a justification for obtaining a warrant to investigate the facility. In March 1995, Asahara learned of the plans for a police raid from Aum members within the Japanese Self Defense Forces. In a ploy to distract the police and buy time, Asahara decided to release sarin in the Tokyo subway system. Two days later, the attack was carried out, resulting in several thousand casualties, including 12 fatalities. During the same period (1994–95) they also produced 100–200 g of VX, which was used to attack three persons (one died). Hence, the Aum's chemical weapons program was more extensive than sarin.

The Aum Shinrikyo cult had been thought to be an odd group and even a nuisance on occasion. Investigations after the Tokyo sarin attack revealed a more sinister aspect of the sect and its leader. In addition to the chemical weapons development program, the Aum Shinrikyo was found to have been actively pursuing biological weapons, albeit without success due to incompetence. In particular, the ineffective release of *B. anthracis* spores in Kameido was discovered, leading to the investigations discussed in this chapter.

The Kameido anthrax incident

On June 29–30, 1993, complaints about foul odors were registered with local environmental health authorities in Kameido in the Tokyo metropolitan area (Takahashi et al., 2004). The odors originated from the eight-story headquarters building of the Aum Shinrikyo. Some of the exposed persons reported appetite loss, nausea, and vomiting. Birds and pets were also reportedly ill, but the nature of these illnesses was not defined. The environmental health officials requested permission to inspect the headquarters building, but Aum Shinrikyo members at the scene refused. The officials checked the building's exterior, collected air samples, and began surveillance of activities at the building. Other than the nuisance posed by the odor, definitive human health risks could not be identified.

On the morning of July 1, neighbors began to complain about loud noises and an intermittent mist originating from one of two structures on the roof which were thought to be cooling towers (Fig. 1.1). As the day progressed, 118 complaints about foul odors were received from nearby residents, primarily to the south of the building. Winds (2–4 m/s) that day blew from a northeasterly direction (Takahashi et al., 2004). Light rain (7 mm total) fell in the early morning, with cloudy conditions thereafter. The temperature ranged from 16.9 to 19.9°C through the day.

A "gelatin-like, oily, gray-to-black" fluid from the mist coming off the "cooling towers" collected on the side of the building (Takahashi et al., 2004). Samples of the fluid were collected by the environmental health officials and stored in a refrigerator (4°C) for later testing.

FIGURE 1.1 Mist being dispersed from spray device on the roof of the Aum Shinrikyo headquarters building, Kameido, Japan, July 1, 2006 (Takahashi et al., 2004).

The next day, July 2, Shoko Asahara agreed to stop using the rooftop devices and to clean and vacate the building (Takahashi et al., 2004). An environmental inspection found no equipment, including the rooftop devices, remaining in the building, and black stains on the walls were the only notable observation.

The problem, apparently being resolved, was largely forgotten until 1996. Police investigations of the sarin attack on the Tokyo subway system revealed that the Aum Shinrikyo was also involved in bioterrorism. Following the conservative Japanese policy of not revealing criminal evidence until the time of trial in court, the true nature of the Kameido incident was first disclosed to the public in May 1996 when Asahara was arraigned (Smithson, 2000; Takahashi et al., 2004). Aum Shinrikyo members had confessed that the odors resulted from efforts to aerosolize a liquid suspension of *B. anthracis* spores. The motive was to trigger an inhalational anthrax epidemic and a subsequent world war. The war would culminate in the Aum Shinrikyo members becoming a super race which would rule the world in accord with Asahara's preaching (Takahashi et al., 2004).

Many questions about the incident remained unanswered. For example, did the attack actually occur? If so, were *B. anthracis* spores utilized? Could the specific *B. anthracis* strain and its origin be identified? Was the strain virulent? Why did the attack apparently fail? Had illnesses occurred but gone undetected? Investigations were initiated to better characterize the alleged attack and its consequences.

Microbial forensic investigation

Fluid, that had been collected from the Aum Shinrikyo headquarters building in July 1993 and subsequently stored at 4°C, was examined in January 2000 for bacterial content (Keim et al., 2001). Polymerase chain reaction (PCR) screening of the fluid was positive for *B. anthracis*. Microscopic examination of the fluid after staining with malachite green and safranin revealed spores, nonspecific debris, and bacterial cells other than large bacilli. The fluid was cultured by spreading on sheep blood agar plates and incubating at 37°C under ambient CO_2 concentration (Fig. 1.2). Based on the number of

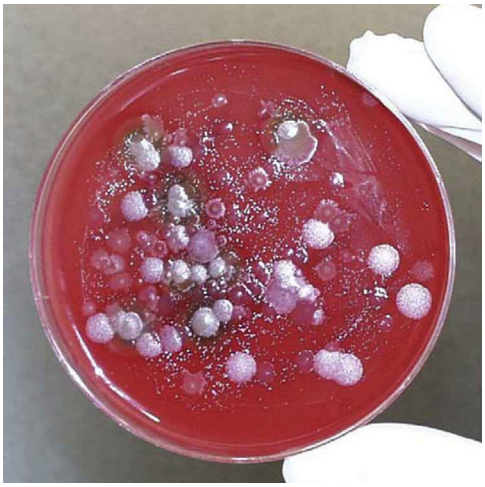


FIGURE 1.2 Blood agar plate of the sampling from the Kameido site (Takahashi et al., 2004).

bacterial colonies observed on the plates after incubation, the fluid contained approximately 4×10^4 bacterial colony-forming units (CFUs) per mL. Most colonies grew only weakly and were morphologically inconsistent with normal *B. anthracis* characteristics when grown under these conditions. The poorly growing bacteria were not further characterized.

About 10% of the colonies on the plates were typical of *B. anthracis*, being large and having a nonhemolytic, "gray ground glass" appearance (Fig. 1.2). The number of observed colonies consistent with *B. anthracis* was consistent with a concentration of 4×10^3 CFU per mL of the fluid. Forty-eight of these colonies were purified by single-colony streaking and subjected to the 8-locus multiple-locus variable-number tandem repeat analysis (MLVA) (Keim et al., 2000). All 48 colonies were *B. anthracis* and had an identical MLVA genotype, though the VNTR marker on the pX02 plasmid failed to amplify. The genotype of all 48 strains was vrrA, 313 bp; $vrrB_1$, 229 bp; $vrrB_2$, 162 bp; $vrrC_1$; 583 bp; $vrrC_2$; 532 bp; CG3, 158 bp; pX01-aat, 129 bp; pX02-at, no amplification. The lack of PCR amplification at the pX02 markers is consistent with strains that are missing the pX02 plasmid entirely. Amplification of these loci can occur in closely related *Bacillus cereus* strains, but the actual amplicon sizes had been previously observed only in *B. anthracis*. The MLVA genotype observed was consistent with results obtained with the Sterne anthrax vaccine strain (Fig. 1.3). The Sterne strain is a member of the A3.b diversity cluster, and in a study of 419 isolates only four naturally occurring *B. anthracis* strains in the electronic database of worldwide isolates had the same seven-marker genotype, though these strains were pX02 positive (Keim et al., 2000).

As the ability to perform whole genome sequencing has improved, one isolate collected

FIGURE 1.3 Multiple-locus, variable-number tandem repeat analysis of a Kameido isolate and the Sterne strain of *Bacillus anthracis* (Takahashi et al., 2004).

at the release site was DNA sequenced using short read Illumina technology (Keim, unpublished data). The resulting sequence was identical to that of the Sterne genome available at NCBI. This suggests that the cult directly used a commercial stock without manipulation or extensive laboratory growth.

The Sterne $34F_2$ anthrax vaccine strain is commercially available in Japan for veterinary use. It had been previously reported that the Aum Shinrikyo had obtained a veterinary vaccine strain of *B. anthracis* which may have been used by them for bioterrorist attacks (Hudson, 1999). Our results are consistent with this previously unsubstantiated report. The Sterne strain has low virulence due to lack of the pXO2 plasmid, which is the location of genes coding for the poly-D-glutamate capsule, a major virulence factor of *B. anthracis*.

Epidemiologic investigation

Culture-confirmed anthrax is a nationally notifiable disease in Japan, with physicians being required to report all cases (Takahashi et al., 2004). Only four human anthrax cases were reported during the 1990s, with a single case being reported in Tokyo. The Tokyo case was diagnosed in a man who was in his eighties and resided in Sumida ward which is adjacent to Kameido ward. The case occurred in August 1994 and had no obvious association with the 1993 Kameido incident.

Could additional anthrax cases from the 1993 attack have gone unrecognized or unreported? A retrospective case-detection survey was conducted in 1999 to address this question (Takahashi et al., 2004). Using the official registry of "foul odor" complaints, the residences of the 118 complainants were mapped to identify the area of presumed highest risk. The 0.33 km^2 high-risk area determined by this approach contained approximately 3400 households and 7000 residents. A telephone survey was conducted of physicians at 39 medical facilities (15 internal medicine, 7 dermatology, and 15 other specialties) serving the area. None of these physicians had treated cases of anthrax, unexplained serious respiratory illnesses, or hemorrhagic meningitis, a common complication of systemic anthrax in residents of the high-risk area (Holty et al., 2006).

Discussion

Several lessons can be learned from the Kameido incident. The investigation suffered from a failure to detect the incident at the time. The Aum Shinrikyo did not attract much official attention until the sarin attack in Matsumoto City in 1994. The Japanese culture is very tolerant of varying religious beliefs, an attitude reflecting Japanese constitutional guarantees of religious freedom. The Aum Shinrikyo was but one of more than 180,000 minor religions active in Japan (Hudson, 1999). The police policy of conservative interpretation of pertinent laws was another factor. In addition, the policy of not revealing details about a criminal investigation until the time court procedures are initiated slowed communications between pertinent agencies, delaying investigation even after the Aum Shinrikyo's attempt to utilize biologic weapons first became known during the investigation of the 1995 sarin attack on the Tokyo subway (Tu, 2002). Awareness of potential threats, a low threshold of suspicion, and active sharing of information between governmental agencies having pertinent expertise and/or authority are some key components in early detection of terrorism incidents.

Early characterization of the Aum Shinrikyo biological weapons program was primarily based on statements by the perpetrators and expert opinions (Hudson, 1999; Smithson, 2000; Tu, 2002). Physical evidence and independent corroboration of the claims were notably absent. Fortunately, health officials had collected a specimen of fluid from the building at the time of the

Kameido incident although it was not been analyzed at the time. The fluid was kept as potential evidence in the event disease could be associated with the incident. A policy of keeping evidence for a period of years allowed its examination in 2000, more than 6 years after the incident. From a forensic perspective, however, the lack of formal chain-of-custody documentation might be an issue in some jurisdictions.

The MLVA analysis of the *B. anthracis* strain isolated from the fluid from the building revealed a genotype consistent with that of the Sterne $34F_2$ anthrax vaccine strain. A direct comparison, however, was not made with the Sterne $34F_2$ strain used to produce anthrax vaccine in Japan. In addition, the MLVA genotypic match was not unique to Sterne as the published database contained additional samples with identical (excluding pXO2) genotypes. No samples from Japan had ever been characterized using MLVA and, as such, the natural background *B. anthracis* was unknown. As noted above, whole genome sequencing has been recently performed and the exact identity between the Kameido isolate and the commercial Sterne strain genome strongly supports the position that this is what the cult used. In addition, no effort was made to isolate *B. anthracis* from the building's environment or adjoining areas of Kameido to provide evidence that the strain isolated from the fluid had been dispersed and the extent of the dispersal. The ability of *B. anthracis* to survive many years in contaminated environment makes such an effort potentially productive (Turnbull, 2008).

Why did no disease result from the attack? One possibility is a small inhaled dose of spores for exposed persons. Although presumably strain dependent, the minimum inhaled dosage capable of causing human disease is unclear. However, unknowns such as aerosol dispersal device efficiency in generating a fine particle aerosol, concentration of spores in the fluid being dispersed, and aerosol cloud movement preclude making defensible estimates of inhaled dosage for persons exposed during the attack.

Another possible factor in lack of detected disease is the low virulence of the Sterne strain for humans. Despite frequent accidental inoculations of the vaccine during immunization of domestic animals, no documented cases of associated disease have been reported. The Sterne strain lacks a capsule, and other strains of *B. anthracis* lacking a capsule rarely produce human illness. Only three reports of illness associated with nonencapsulated strains have been made, with one case being in an immunocompromised person and the cause–effect relationship of the isolate to the illness observed in the other two cases being uncertain.

Serologic studies were not done at the time of the event but could have been potentially useful. For example, antibody and cell-mediated immune responses in persons who were exposed in the 2001 bioterrorism attack at the United States capitol were used to demonstrate infection without resultant disease (Doolan et al., 2007). If a similar study had been done in Kameido at the time of the attack, the question of whether infection had occurred in absence of disease could have been addressed. The long lapse of time between the Kameido event and the epidemiologic study would compromise the validity of such studies at this time.

The isolation of *B. anthracis* and the results of the MLVA testing might have proven useful in prosecuting some Aum Shinrikyo members, but this will have to remain conjectural. Criminal charges related to the biologic weapons development and attacks were not made, and, hence, the microbiological evidence was never tested in court. However, 13 Aum Shinrikyo members, including Asahara, have been convicted for perpetrating the sarin attack on the Tokyo subway and condemned to death. Three of the Aum Shinrrikyo members including Asahara were executed by hanging in 2018 after appeals had been exhausted for all of the accused.

Despite the convictions of many leaders, the Aum Shinrikyo continues to exist, having been renamed Aleph, the first letter of the Phoenician

alphabet. A splinter group, Hikari no Wa or Ring of Light, broke off from Aleph following discord within the Aleph leadership. Surveillance of the two groups for potential terrorist activities has been maintained, and they have been formally designated terrorist organizations by several countries, including the European Union, Russia, Canada, Kazakhstan, and the United States.

Challenges

The Kameido incident underscored a number of essential concepts in microbial forensics. Implementing these concepts must be a high priority for law enforcement jurisdictions. Early communication and information sharing between appropriate governmental agencies, particularly law enforcement and public health, are essential to an optimal outcome of microbial forensic studies. Public health agencies have a major role in the investigation of suspected and documented bioterrorism incidents, but the similarities and differences in public health and law enforcement investigations must be understood and coordinated so that both can be more effective (Butler et al., 2002). Investigations must, of necessity, be multidisciplinary and draw on the best available expertise whether or not located in a governmental agency. Specimen testing must follow established chain-of-custody procedures, and all involved groups must be trained in these procedures. Strain subtyping is a powerful investigative tool for tracing the origins of microbial agents, but the procedures must be validated and have yet to be accepted by the courts. Epidemiologic studies are essential to putting laboratory findings into the context of a bioterrorist event.

References

Butler, J.C., Cohen, M.L., Friedman, C.R., Scripp, R.M., Watz, C.G., 2002. Collaboration between public health and law enforcement: new paradigms and partnerships for bioterrorism planning and response. Emerg. Infect. Dis. 8 (10), 1152–1156.

Doolan, D.L., Freilich, D.A., Brice, G.T., Burgess, T.H., Berzins, M.P., Bull, R.L., Graber, N.L., Dabbs, J.L., Shatney, L.L., Blazes, D.L., Bebris, L.M., Malone, M.F., Eisold, J.F., Mateczun, A.J., Martin, G.J., 2007. The US capitol bioterrorism anthrax exposures: clinical epidemiological and immunological characteristics. J. Infect. Dis. 195 (2), 174–184.

Holty, J.E.C., Bravata, D.M., Liu, H., Olshen, R.A., McDonald, K.M., Owens, D.K., 2006. Systemic review: a century of inhalational anthrax cases from 1900 to 2005. Ann. Intern. Med. 144 (4), 270–280.

Hudson, R.A., 1999. The Sociology and Psychology of Terrorism: Who Becomes a Terrorist and Why? Library of Congress, Federal Research Division Report Library of Congress, Washington, DC.

Keim, P., Price, L.B., Klevytska, Smith, K.L., Schupp, J.M., Okinaka, R., Jackson, P., Hugh-Jones, M.E., 2000. Multiple-locus VNTR analysis (MLVA) reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. 182 (10), 2928–2936.

Keim, P., Smith, K.L., Keys, C., Takahashi, H., Kurata, T., Kaufmann, A., 2001. Molecular investigation of the Aum Shinrikyo anthrax release in Kameido, Japan. J. Clin. Microbiol. 39 (12), 4566–4567.

Smithson, A.E., 2000. Rethinking the lessons of Tokyo. In: Smithson, A.E., Levy, L.E. (Eds.), Ataxia: The Chemical and Biological Terrorism Threat and the US Response. Stimson Center Report No. 35. Simpson Center, Washington DC, pp. 71–111.

Takahashi, H., Keim, P., Kaufmann, A.F., Keys, C., Smith, K.L., Taniguchi, K., Inouye, S., Kurata, T., 2004. *Bacillus anthracis* incident, Kameido, Tokyo, 1993. Emerg. Infect. Dis. 10 (1), 117–120.

Tu, A.T., 2002. Chemical Terrorism: Horrors in Tokyo Subway and Matsumoto City. Fort Collins CO: Alaken Inc.

Turnbull, P. (Ed.), 2008. Anthrax in Humans and Animals, fourth ed. World Health Organization, Geneva.

CHAPTER

2

The FBI's Amerithrax Task Force and the advent of microbial forensics

R. Scott Decker, Terry L. Kerns

Bio-Logic Security, LLC, Las Vegas, NV, United States

Concern for the future

The 1990s saw a dramatic rise in awareness of the use of biological weapons and terrorism. Secretary of Defense William Cohen startled the nation one Sunday morning by equating the amount of anthrax[1] powder with a bag of sugar and its capability to kill one-half of Washington, D.C.'s population; United Nation's inspectors uncovered the extent of Iraq's biological weapons program; Richard Preston's best-selling book, *The Hot Zone*, followed by *The Cobra Event* brought microbes gone awry to the public's eye (Decker, 2018).

Those in government circles took note and realized the nation was unprepared for a biological attack they feared was looming on the horizon. In the Federal Bureau of Investigation (FBI) Laboratory Division, Deputy Assistant Director Randall S. Murch stood up a new unit, the Hazardous Materials Response Unit (HMRU). Its mission was to respond to crime scenes involving use of a weapon of mass destruction, collect and preserve the evidence, and coordinate its forensic exploitation. At the Centers for Disease Control and Prevention (CDC), US Public Health Service medical officer Scott Lillibridge with the help of microbiologist Stephen A. Morse initiated the Bioterrorism Preparedness and Response Program (BPRP). Richard F. Meyer from Plum Island and Richard B. Kellogg soon joined Morse, and they began identifying state-of-the-art technology for characterizing microbes.

Laboratory Response Network

In addition to building the CDC's BPRP and the FBI's HMRU, Morse and Murch developed the idea for a national Laboratory Response Network (LRN)—a concept simple, elegant, and novel—highly effective and efficient. The CDC's headquarters in Atlanta hosted the January 1999 kick-off meeting. Attendees included representatives from the Departments

[1] Anthrax is defined as the disease caused by the bacterium, *Bacillus anthracis*. However, in conversational usage, anthrax and *Bacillus anthracis* are often used interchangeably.

https://doi.org/10.1016/B978-0-12-815379-6.00002-7

of the Army and Navy and the American Public Health Association. Top public health officials from around the country and the FBI's Laboratory Division joined them. That meeting began efforts for the standardization of methods and use of equipment for the detection and identification of microbes used in bioterrorist and criminal events.

The Department of Health and Human Services through the CDC rebuilt the nation's public health infrastructure by providing funds to state and large county public health labs for the purchase of state-of-the-art equipment for microbiological identification. At the same time, scientists from national institutions such as the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), the Naval Medical Research Laboratory, and CDC's Rich Meyer would develop, standardize, and validate protocols. Standardized reagents and training would also be provided. It was an ambitious undertaking, but one soon desperately needed. In the months following 9/11, the LRN would provide invaluable resources and rescue the nation from near-panic over white powder letters and hoaxes.

While the public health labs were well attuned to processing public health samples, they now learned to process evidentiary samples. In the Phoenix FBI Division, for example, agents added chain of custody documentation to the existing public health accession paperwork and processes were developed for cross-referencing evidentiary samples with public health cataloging systems. On arrival at an LRN member lab, the FBI's evidence would be stored in a dedicated and secure area. Following testing for biological threat agents, laboratory personnel would transfer the evidence to a second, secure area to avoid cross-contamination with new incoming samples. Entry to secure areas was limited and records of ingress/egress maintained. Following intake of suspected samples, the LRN labs would communicate daily with the submitting FBI field office; test results were relayed immediately to victims and their designated healthcare professionals.

The equipment provided to the LRN was available for day-to-day public health work, and in the instance of a suspected biological attack or threat, available to analyze evidence collected through the coordination of FBI Joint Terrorism Task Forces. Evidence that tested positive for a pathogen or toxin (e.g., ricin) would be transferred to a national-level facility—CDC or a Washington, DC area Department of Defense lab—for confirmation and forensic analysis. Specialized testing, such as genome analysis, would take place at partner laboratories such as Paul Keim's laboratory at Northern Arizona University.

The primary advantage of using local facilities was timely turn around. In 1998, any threatening material, such as an envelope filled with a powder, was transported to a national-level lab for testing, taking days before an answer, usually negative, could be given to victims, risking unnecessary administration of antibiotics or in the case of a positive test, costly delay. Should the country be deluged with anthrax hoax letters as in 1998, the volume of testing would threaten to overwhelm national assets.

Beginning in 1998, the FBI added microbial forensics to its list of available investigative approaches. It built and strengthened relationships with the country's microbiology experts and was able to provide rudimentary testing to its field offices around the United States. Contracts and Memoranda of Agreement were put in place as vehicles for funding and reimbursement. Microbial forensics would not be a stand-alone tool to prove innocence or guilt, but one of many available to generate investigative leads (Fig. 2.1).

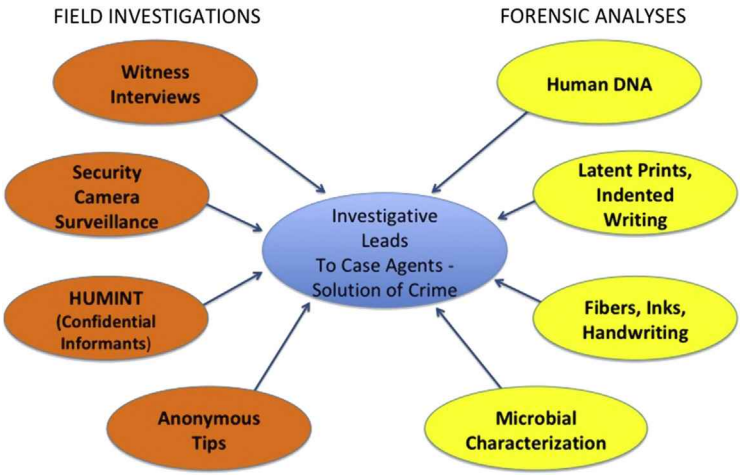


FIGURE 2.1 Flow chart for investigation of use of a biological weapon of mass destruction as presented to first responders and public health personnel by the authors while assigned to the FBI's Hazardous Materials Response Unit (c. 1998–2001).

Post-9/11: the second wave of attack

The events of September 11, 2001, are one of those days in history that everyone will remember where they were. The near simultaneous airliner attacks on the World Trade Center towers (Fig. 2.2) and the Pentagon, along with the diverted attack to a coalfield in Pennsylvania, remain scorched in our national collectiveness along with Japan's 1941 attack on Pearl Harbor and the 1963 assassination of President John F. Kennedy.

Members of the FBI's HMRU responded to all three of the 9/11 crime scenes. In New York City, epidemiologists from the CDC began monitoring area hospitals for mysterious illnesses.

FIGURE 2.2 The Ground Zero Pile. A Caterpillar excavator clears steel and concrete from the wreckage of Manhattan's World Trade Center complex following an attack by hijacked commercial airliners on September 11, 2001.

Concern ran through government of a biological bomb aboard one of the airliners. Officials worried about a biological attack coming on the heels of 9/11. Around Washington, D.C., the Department of Energy deployed its Biological Aerosol Sentry and Information System (BASIS) technology to sample the air for biological aerosols, *Bacillus anthracis* at the top of their list. USAMRIID tested the small filters collected hourly from BASIS monitoring stations deployed throughout the National Capital region. While the locations of the stations were confidential, USAMRIID staff were aware of the testing, and it generated speculation about what was coming next.

Three weeks after 9/11, early in the morning, Robert Stevens of south Florida admitted himself to the JFK Medical Center located between West Palm Beach and Boca Raton. He had been vomiting and incoherent during the night. By the time he arrived at JFK, delirium had taken over. Initially, doctors suspected bacterial meningitis and administered multiple antibiotics. Six hours later he suffered a seizure and was placed on a ventilator (Cole, 2003). Farther south, Stevens' coworker, Ernesto Blanco, a mail room worker at American Media, Incorporated (AMI) checked himself into Miami's Cedars Medical Center as he became increasingly disorientated. The medical staff soon administered intravenous antibiotics and hooked him up to a mechanical respirator. A definitive diagnosis for Blanco would elude them for days.

An examination of Stevens' cerebrospinal fluid revealed gram-positive bacilli and a Florida Public Health laboratory, using the new LRN protocols, identified the bacteria as *B. anthracis,* 44 h after Stevens was admitted to JFK. The bacteria were sent to Atlanta where the CDC's BPRP confirmed the diagnosis of anthrax. BPRP staff inoculated samples of Stevens' *B. anthracis* onto an agar slant and flew it to Northern Arizona University. There, working through the night, Paul Keim and his staff identified the *B. anthracis* as the Ames strain, a research strain used to assess vaccine efficacy. At the same time, the CDC's Alex Hoffmaster, using the Multiple Locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA) developed by the Keim lab, confirmed the Ames identification (Keim et al., 1997, 2000). Finding that a laboratory strain infected Stevens and not an unknown environmental strain pointed toward an intentional act—potentially the first act of deadly bioterrorism in the United States. On October 5, 2 days after entering JFK Medical Center, Stevens succumbed. He was the first case of lethal inhalational anthrax in the United States since 1976.

Blanco's diagnosis was not as straightforward. Repeated attempts to identify viable bacteria in Blanco failed. The CDC case definition required viable *B. anthracis* for a diagnosis of anthrax. However, in his Rapid Response and Advanced Technology lab at CDC, Rich Meyer had validated a new assay, a highly sensitive and quantitative immunoassay he termed Time-Resolved Fluorescence (TRF). Applying TRF to Blanco's pleural fluid, Meyer detected high levels of inert *B. anthracis* antigens, convincing Meyer that Blanco had inhalational anthrax. But CDC executive leadership needed more convincing. After several days of tension-filled meetings and terse discussions, the CDC front office agreed that Ernesto Blanco was the second inhalational anthrax victim since 1976 (Blanco's case would lead to the CDC revising their criteria for anthrax diagnosis). While the extraordinary high levels of intravenous antibiotics may have confounded an early diagnosis, they undoubtedly saved Blanco's life. He would go on to a full recovery (UCLA, Fielding School of Public Health).

On Friday, October 12, 2001, 9 days after Stevens' initial diagnosis, suspicions of a bioterrorist attack were confirmed. After a circuitous search, a letter addressed to NBC news anchor Tom Brokaw in Manhattan was recovered containing dry *B. anthracis* spores. The envelope was postmarked "Trenton, NJ." Three

days later, a second spore-filled letter was recovered in the Capitol Hill offices of US Senator Tom Daschle—the handwriting and eagle prestamped envelope identical to the Brokaw letter. However, the Daschle letter, also postmarked "Trenton, NJ," bore a New Jersey return address—which proved to be fake. One week after recovering the Brokaw letter, a *B. anthracis*–filled letter was recovered addressed to the editor of the *New York Post* in Manhattan. In early November, a letter filled with spores was recovered in quarantined mail. This one was addressed to US Senator Patrick Leahy. Four letters were recovered in all, the *Post* and Leahy letters were unopened. Recovery of the Leahy letter allowed for a range of destructive forensic analyses.

The two letters sent to New York City contained granular dark brown dried spores mixed with debris; the two Capitol Hill letters sent to Daschle and Leahy contained light gray powder, very fine and little to no debris (Fig. 2.3). The dried powder in these letters consisted of tiny clumps of spores and single spores. When the Daschle letter was opened in his office, the powder behaved as an aerosol and spread through office buildings on Capitol Hill. Cleanup took months. In New Jersey, New York, Maryland, Washington, D.C., Florida, and Connecticut, victims presented with cutaneous and inhalational anthrax. By the end of November 2001, there were 22 total cases of anthrax, 11 inhalational and 11 cutaneous. The inhalational form claimed five lives, one in Florida, one in Connecticut, two in Maryland, and one in New York City. Seventeen of the victims recovered from their infection; many remain disabled or disfigured.

FIGURE 2.3 Powder of dried *Bacillus anthracis* spores recovered from envelope and letter addressed to US Senator Patrick.

Quality of spore material—behavioral assessment

The Senate letters were of the greatest interest in terms of purity and concentration. Estimates are that 0.8–1.0 g of powder filled the letter mailed to Senator Leahy, with a density of 2×10^{12} spores per gram. The *NY Post* letter material was of considerably lesser purity with estimates of 2×10^{11} spores per gram. For the two Capitol Hill letters, the two New York letters and assuming one letter at the AMI building, the FBI Laboratory estimated the attacker mailed approximately 3.7×10^{12} spores. Conclusions about the amount of time required to produce the material varied widely, from 2 to 3 days to months, assuming liquid culture in shaking flasks or a fermenter (Gast et al., 2011).

Studies of the material in the Daschle and Leahy letters revealed a bimodal particle distribution. The powder consisted of single spores with a 1.5 um diameter that represented 1% of the total spore mass. The remainder consisted of clumps approximately 20 um in diameter. These measurements were conducted with an Aerosizer (TSI, Inc.) following aerosolization of the powder. Scanning electron microscopy (SEM) confirmed a bimodal particle distribution:

single particles with a morphology consistent with *B. anthracis* spores and clusters of spores. However, the powder was subjected to autoclaving before SEM analysis and that may have affected size distribution (Gast).

The level of purity provided a forensic clue to the sophistication of the material's producer(s). The transition of the crude New York letter material within 3 weeks to the fine powder in the Capitol Hill letters indicated a quick learner and astute microbiologist. This information was factored into the behavioral assessment being developed by FBI profilers in Quantico.

Amerithrax Task Force

Anthrax victims were located in six FBI divisions, but FBI headquarters recognized the attacks were likely the work of one person or a single group and consolidated the growing investigation. The Washington Field Office (WFO) in Washington, D.C. was designated Office of Origin (OO). In FBI parlance, the OO would bear responsibility for solving the case.

The WFO quickly formed a task force; headquarters gave the investigation a codename, AMERITHRAX (AMX). It included FBI agents with science backgrounds and United States Postal Inspection Service inspectors. Together with scientists from the FBI Laboratory's Forensic Science Research Unit, the science agents began brainstorming sessions. Originally, it was hoped that forensic methods already in place at the FBI would provide investigative leads, but those hopes quickly dissipated. The envelopes had been taped shut, and there were no stamps with water-soluble adhesive, leaving little hope for recovering human DNA. Nor were latent impressions found that could be fed into the Automated Fingerprint Identification System. Examiners quickly determined that the ink used to write the envelopes' addresses was common and of no value for attribution. Neither letters nor envelopes contained indented writing. The mailbox used to post the letters was eventually located in Princeton, NJ, but a search of the area turned up no surveillance cameras. Traffic citations issued in the area were of no value and nearby shop owners and residents provided no clues.

The brainstorming sessions identified novel science applications. Considered were stable isotope analysis for attribution to a geographic area; the presence of hemoglobin as an indication blood agar had been used to culture the microorganism; origin of the silicon identified in the spore coat; dating the spores with carbon-14; analysis of plasmid DNA for mutations specific to the mailed *B. anthracis*. Of the techniques considered, three provided leads or corroborated investigative conclusions.

Colony morphology and DNA sequencing

As often happens in science, great discoveries begin as accidents, followed by astute observations. Such was the biggest lead in the anthrax investigation. While characterizing the spores from the Daschle and *NY Post* letters (most of the powder in the Brokaw letter was lost before its transportation to the CDC), USAMRIID microbiologist Terry Abshire set up culture plates and diluted the spores to allow single colony propagation. On one occasion, she let the plates remain in the incubator at 37°C, rediscovering them several days later. She noticed a minority of the colonies had morphologies different than the predominant wild-type *B. anthracis*. Based on their experience, Abshire and her supervisor, John Ezzell, suspected the variants might be due to mutations in the sporulation pathway.

Patricia Worsham, also a USAMRIID microbiologist, was the institution's expert on bacillus sporulation. She began working with the Amerithrax Task Force to characterize the morphology variants—soon known as "morphs." She found them in all three letters, Daschle, Leahy, and

NY Post. The variants were stable in culture, an indication of being the result of DNA mutation. She isolated several and designated them A1, A2, A3, B, C, D, and E, estimating they accounted for 10% of the total colonies.

Luck, fueled by persistence and deliberation, comes into play during law enforcement investigations as well as those of science. During a consent search of refrigerators in conjunction with interviews of USAMRIID personnel, the original agar slant of the Ames isolate was discovered. Its tube was dated February 1981 and viable spores remained. Finding the 1981 Ames gave the FBI an ideal candidate to provide a baseline, unaltered DNA sequence of the genome from the original sample (Ravel et al., 2009). A pioneer in whole genome sequencing, The Institute for Genomic Research (TIGR) of Rockville, MD, was soon under agreement to perform the work.

Before the isolation and initial characterization of the morphology variants and whole genome sequencing of the 1981 Ames, the FBI had begun an ambitious project. Beginning with select agent transfer regulation records from the CDC, coupled with literature and patent reviews, and field interviews, the FBI tracked down locations of laboratories possessing the Ames strain of *B. anthracis*. The effort would take agents to 16 laboratories in the United States, laboratories in Canada, the United Kingdom, and Sweden; the involvement of at least one foreign embassy and two trips across the Atlantic by private aircraft. Collecting Ames samples began in the fall of 2001 and was completed during fall 2006 (Fig. 2.4).

Following completion of the 1981 Ames' genome DNA sequence, TIGR began sequencing DNA isolated from the morphology variants. Worsham isolated and propagated A1-3, B, C, D, and E colonies; the bacteria were transferred to Paul Keim for DNA isolation (TIGR was not approved to work with live select agents); the DNA was sent to TIGR for genome sequencing; and lastly, samples containing putative mutations returned to Keim for confirmation. All scientists received coded samples and did not know their identity or origin.

FIGURE 2.4 One of 1082 samples of *Bacillus anthracis* Ames strain collected worldwide. Each sample was submitted to the FBI repository in duplicate. One of the duplicates samples was sent to Paul Keim at the Northern Arizona University for strain confirmation and emergency backup storage in case of loss of power, fire, water line break, etc., at United States Army Medical Research Institute of Infectious Diseases. *Photo courtesy of Tom Reynolds, NEXT Molecular Analytics, LLC.*

During 2003, the efforts of the FBI, Worsham, Keim, and TIGR began to pay off. The B variant's morphology was attributed to a single point mutation—this would prove unrealistic for developing a screening assay with technology that was available. However, an A variant was found to contain a 2024 base pair (bp) insertion in one of the eleven 16s RNA genes. This insertion was amenable to developing a screening assay. Mutations were also identified in additional A, and C, D, and E variants (Rasko et al., 2011). For a complete list, see the 2011 report from the National Research Council of the National Academy of Sciences (Gast).

The FBI contracted the comparison of the evidentiary morphology variants and the Ames exemplars in their repository to Commonwealth Biotechnologies, Incorporated (CBI). With the identification of the morphology mutations, CBI began developing Taqman real-time polymerase chain reaction (PCR) assays. The FBI imposed strict specifications. Each sample in its Ames repository would be screened in

FIGURE 2.5 The −70°C freezer used to transport the repository of collected Ames exemplars to Commonwealth Biotechnologies, Inc., accompanied by members of the FBI's Hazardous Materials Response Unit. *Photo courtesy of Tom Reynolds, NEXT Molecular Analytics, LLC.*

triplicate on different days by different technicians. In addition, both strands of the resulting PCR DNA amplicon would be sequenced. Following validation of CBI's screening assays using blinded samples provided by Pat Worsham, CBI began screening for the A1 (2024 bp) and A3 (823 bp) mutations during the spring of 2004 (Fig. 2.5). Collection for the Ames repository continued while CBI screened for mutations. Screening for D and E morphologies was added. By the conclusion of these analyses, 8 matches were identified out of 1082 samples. All matches derived from a single source, a flask of reference Ames spores, RMR-1029, in the custody of Bruce Ivins, a civilian microbiologist employed by USAMRIID.

Carbon-14 dating

During the initial brainstorming sessions, scientists of the FBI's Forensic Science Research Unit identified recent technology that enabled carbon-14 dating of minute samples. The use of carbon-14 radioactive decay to estimate the age of organic material is attributed to University of Chicago scientist, Willard Libby, who was awarded a Nobel Prize in 1960 for his work. Unfortunately, carbon-12/carbon-14 ratios fluctuated over time using Libby's original method making the range of error in carbon dating significant.

In late 1963, all nuclear weapon testing in the atmosphere or ocean came to an end when President Kennedy signed the Nuclear Test Ban Treaty. During the prior decade, nuclear testing had created a "bomb spike," measurable as a dramatic rise of radioactive carbon in the atmosphere. The banning of all open-air and underwater testing halted this steep increase in carbon-14 levels, and the radioactive isotope began a sharp and predictable decline. Coupled with this phenomenon, the application of accelerator mass spectrometry, known as AMS, replaced the original method of measuring radioactive carbon. AMS requires 1000 to 2000 times less material to measure carbon-14 levels than past methods. Material can now be dated to an accuracy of one to 2 years (Taylor and Bar-Yosef, 2014).

Milligram amounts of the Leahy letter powder were subjected to carbon-14 dating. Two independent laboratories tested the spores: Lawrence Livermore National Laboratory's Center for Accelerator Mass Spectrometry and the National Ocean Sciences AMS Facility at the Woods Hole Oceanographic Institute. Results indicated that the Leahy spores went through at least one growth step after 1998. Thus, the spores were likely prepared between 1998 and September 2001. This timeframe ruled out the mailed spores coming from stockpiles of the disbanded United States' Biological Weapons (BW) Program of the 1950s–60s. The identification of the mailed spores as Ames strain confirmed that the mailed *B. anthracis* did not come from old stockpiles; Ames was isolated in 1981 and the US BW Program weaponized the Vollum strain.

Flexographic print defects

All four recovered envelopes were sold by the United States Postal Service. They bore postage stamps printed with blue and gray ink, designated Federal Eagle stamps. The formal term for the inked stamps is "pre-franked." The stamp is an image of an eagle standing on a bar with out-stretched wings. Under the talons is "USA" and under that is "34," denoting a denomination of 34 cents. As each envelope cost $0.34 to purchase, the same as individual adhesive stamps, the Postal Service treated the envelopes as currency and kept exacting manufacture and shipment records. The reverse side of the envelope also bore printing: "THIS ENVELOPE IS RECYCLABLE AND MADE WITH 100% RECYCLED PAPER, 30% POST-CONSUMER CONTENT." In addition, the envelopes bore phosphorescent markings, visible with alternate light sources.

A private corporation under contract to the Postal Service manufactured the envelopes using flexographic printing. The process uses printing plates made of a flexible polymer that hardens when exposed to ultraviolet (UV) light. A film negative of the printing is placed on the surface of the polymer and exposed to UV light; exposure hardens the exposed and the protected areas of polymer remain gummy and are removed by washing. The finished plates are then placed on large printing rollers and inks applied to the exposed areas. During repetitive inking/printing, the polymer surface accumulates wear; minor abrasions appear and disappear. One plate printed approximately one million Eagle envelopes before being replaced.

Postal records indicated that 45 million Eagle envelopes had been produced from December 6, 2000, through March 3, 2002. Manufacturing production runs took place on 57 days during the 15 months. On January 9, 2001, the Postal Service ordered a change in ink formulation; the four recovered envelopes bore the new ink formulation, indicating manufacture after January 9, 2001. Thirteen production runs took place before January 2001 and seven production runs took place after the mailing of the two Capitol Hill letters. Thus, the mailed envelopes were manufactured during one of 37 runs, totaling 31 million envelopes.

The Task Force turned to the expertise of the United States Secret Service Forensic Services Division. The Secret Service has long been recognized as the country's experts in detecting slight imperfections and subtle differences of inked impressions. Over the years, they have developed methods to examine microprinting, evaluate water-markings, and analyze microscopic lenses of colored plastic making up the flaxen and cotton paper used in today's currency.

Examination of the evidentiary envelopes under a dissecting microscope revealed a number of defects in the inked printing. Examiners found a slight blue line along the bottom edge of the stamped Eagle's right wing. Imperfections also appeared on the reverse side of the envelopes; a faint line appeared between the "P" and "S" of "USPS." Matching defects appeared on the

Daschle and *Post* envelopes, likewise identical defects appeared on the Brokaw and Leahy envelopes, but different than those on Daschle and *Post* (Decker; The United States Department of Justice, 2010).

While the Secret Service examined the evidentiary envelopes, Amerithrax agents and inspectors visited post offices, collecting remaining, unsold Eagle envelopes (the Postal Service terminated sale of the envelopes when the first attack envelope was identified). Collecting the envelopes in their shipping boxes, which bore manufacturing lot numbers, the Task Force matched the envelope exemplars with the shipping records maintained by the Postal Service. Each box of collected envelopes could be identified by its time of manufacture with the day of shipping and arrival at its final postal destination. A total of 200,245 exemplars were collected.

Defect analysis in flexographic printing had not been used before in an investigation, nor had it been documented in the scientific literature, and the Task Force asked the manufacturer to conduct a controlled production run. Over the course of 14 1/2 h, 525,000 envelopes were printed and packaged. As the Secret Service scientists examined the envelopes, they saw that defects appeared, changed, and finally disappeared as production continued. They concluded that a defect appeared and remained during production of 2,000 envelopes before disappearing. The occurrence of defects and the number of envelopes exhibiting a defect with similar characteristics was low (LaPorte et al., 2010). They also noted that a specific defect appeared on every other envelope in a box until it disappeared. Alternatively, the intervening envelopes bore completely different defects, which also appeared and then disappeared. The chief examiner hypothesized that this alternate pattern was due to the use of two printing plates in a production run—plates on opposite sides of the cylindrical printing roller—that accumulated different defects.

During examination of the collected exemplars, examiners identified a box of envelopes from Elkton, Maryland, bearing defects very similar to those on the evidentiary envelopes. A review of the shipping and distribution records found boxes had been sent to Elkton, Severna Park, Cumberland, and Galena, Maryland, and Fairfax and Machipongo, Virginia, on the same day and bore nearly identical defects. Furthermore, the records showed a box of 1,000 envelopes was shipped to the post office in Frederick, Maryland, that day. Unfortunately, the Frederick shipment had been destroyed before agents could retrieve it. Nonetheless, based on the results of the controlled run and the similarity of defects, the Secret Service chief examiner concluded that the evidentiary envelopes most likely originated at the Frederick, Maryland post office; the same post office where the investigation's prime subject leased a mailbox (Decker; Department of Justice).

Bacterial contamination

One item of microbial evidence was identified which had the potential for proof of attribution rather than limited to lead potential. The Brokaw and *NY Post* letters contained non−*B. anthracis* bacteria. First noticed in cultures on agar plates, it was originally thought to be a strain of *Bacillus licheniformis*; DNA sequencing subsequently identified it as *Bacillus subtilis*. While the arduous journey of the Brokaw letter would likely have led investigators to conclude that the contamination was introduced after the envelope had been opened, the *Post* letter was recovered unopened. Sequence analysis showed the *B. subtilis* isolates from the Brokaw and *Post* letter were indistinguishable, although the *B. subtilis* DNA from the Brokaw letter was not fully sequenced. Estimates put the level of contamination at 1%−5% of the total bacteria.

DNA screening tests, based on TaqMan technology, were designed for the *B. subtilis*. One marker within the *sbo*A locus was generic to all *B. subtilis* strains tested. Three markers in additional loci were specific to the *B. subtilis* found in the New York letters. The presence of the *B. subtilis* contamination in the NY letters was the subject of search warrant affidavits and searches were conducted, but a match was never found. Likewise, screening of the repository of Ames *B. anthracis* exemplars found that 322 of the 1082 samples contained *B. subtilis*, but not the strain that contaminated the New York letters (Gast).

Operational concerns—collection and preservation of microbial evidence

The FBI's HMRU and field division Hazardous Materials Response Teams (HMRTs) are trained to process chemical, biological, radiological, and nuclear (CBRN) crime scenes. The majority of scenes processed by HMRU and the HMRTs contain evidence visible to the human eye, such as powders, equipment for fabrication, and literature. Visual evidence provides guidance on how and where to collect. There are also a variety of detectors that will locate nonvisible chemical, radiological, and nuclear evidence. A bioterrorism attack that leaves behind visible evidence can be processed according to standard and tested protocols. However, in the case of the anthrax attacks, the AMX Task Force encountered crime scenes containing evidence not visible by the naked eye, which necessitated additional collection procedures be developed. Examples of these include the search for the Leahy letter, the search for the mailbox(s) used for the mailings, and locating the source of *B. anthracis* in the AMI and Brentwood buildings.

From 1998 through mid-2001, FBI HRMTs collected biological evidence using sterile, individually packaged cotton swabs and screw-cap polypropylene tubes. Attempts were made by HMRU and the field HRMTs to collect samples directly on culture medium, such as agar in Petri dishes. Incorporating this method into their collection and processing protocols proved difficult. Downrange hot-zone[2] operators, working in encapsulating personal protective equipment (PPE) and filtration masks under strict time constraints, tore the culture medium when attempting to transfer the material from the cotton swab. Their protocols required consistency and reproducibility between operators; this directive proved elusive. Had they been successful, the improvement would have saved time once samples arrived at microbiology lab(s).

Following 9/11, anthrax-laden letters appeared in the mail and compounded the state of fear and nervousness pervading our country. The anthrax letters in turn led to copy-cat hoax mailings during October, November, and December 2001 and threatened to overwhelm LRN resources across the United States. Requests by the FBI for environmental testing came on top of public health's primary mission: testing clinical samples from persons potentially exposed to *B. anthracis*. More efficient methods of collecting biological evidence were needed.

At the FBI Laboratory, microbiologist Doug Beecher experimented with sampling techniques utilizing Petri dishes containing sheep blood agar. In contrast to a traditional concave agar surface below the plastic rim, agar in Beecher's culture plates rose into a convex surface above the rim. A hot-zone operator could remove the dish's lid, press the agar to the area in question, and replace the lid for transport. The innovation

[2] A Hot-Zone is an area that is considered dangerous. In the case of bioterrorism, it is the maximum area that may be contaminated by a select or biological threat agent.

greatly decreased time downrange, eliminated tedious manipulation of swabs and screw-caps, and decreased processing time at LRN labs.

Processing a biological terrorism crime scene when reacting to an attack or executing a preventive search warrant requires an operation plan. During the Amerithrax investigation, operational plans included personnel paired with individual expertise, a sampling scheme, type of PPE, decontamination protocols, and medical contingencies. Potential risks were factored in, including slip/trip/fall hazards and weather conditions. Time downrange is inverse to temperature. Considered was operator time in the hot-zone and the decontamination line while still wearing PPE. The sampling plan was often based on visible physical evidence and interviews of witnesses that may have been downrange before the realization a bioterrorism crime scene existed. This occurred on numerous occasions during Amerithrax: arrival at Senator Daschle's office, and processing the myriad buildings on Capitol Hill. Often, as with the Daschle and Brokaw letters, Evidence Response Team and HAZMAT personnel were not the first to arrive, but took control of rapidly evolving situations—nerves and emotions near breaking points.

No matter what operation plan is used for CBRN-contaminated crime scenes, guidelines and principles are followed in contrast to stringent standard operating procedures. In the case of the Amerithrax investigation, the guiding principles of traditional crime scene processing were combined with expertise from alternate disciplines to devise a sampling plan. If microbial evidence was to be collected from a large area such as a floor or a desktop, what was the best sample collection tool and method to collect that evidence? The ultimate goal was exploitation of the items collected, whether by traditional forensic examinations or by microbial analysis (Budowle et al., 2006). Often, initial sampling for microbial evidence will assist in focusing an investigation. Such was the case processing the AMI building. Investigators conducted a limited and directed sampling first. It established the presence of *B. anthracis* spores and suggested mail as the mode of delivery. A thorough, exhaustive sampling followed.

The processing of crime scenes and execution of search warrants during the anthrax investigation were complicated processes that incorporated the expertise of many disciplines and agencies. Safety of the public and the responders was paramount. Shortcuts could not be taken; processing Amerithrax crime scenes were not trivial undertakings. FBI and Environmental Protection Agency HAZMAT operators recovered the unopened Senator Leahy letter under extremely dangerous circumstances. A multitude of considerations went into planning, slowing processes but ensuring well-being of personnel and preserving microbial evidence and chains of custody. All the time incorporating newly discovered information and adapting to changes of investigative direction.

References

UCLA, Fielding School of Public Health, AMIemployeegoback, 07 August 2005; accessed 29 July 2019. http://www.ph.ucla.edu/epi/bioter/AMIemployeegoback.html.

Budowle, B., Schutzer, S.E., Burans, J.P., Beecher, D.J., Cebula, T.A., Chakraborty, R., et al., 2006. Quality sample collection, handling, and preservation for an effective microbial forensics program. Appl. Environ. Microbiol. 72, 6431–6438.

Cole, L.A., 2003. The Anthrax Letters: A Medical Detective Story. Joseph Henry Press, Washington, DC, p. 4.

Decker, R.S., 2018. Recounting the Anthrax Attacks: Terror, the Amerithrax Task Force, and the Evolution of Forensics in the FBI. Rowman & Littlefield, Lanham, MD, p. 4.

Decker., Recounting the Anthrax Attacks. 160.

Decker., Recounting the Anthrax Attacks. 179, 201.

Gast., The National Academies. 79.

Gast., The National Academies. 116–119.

Gast., The National Academies. 104–105.

Keim, P., Kalif, A., Schupp, J.M., Hill, K., Travis, S.E., Richmond, K., et al., 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. 179, 818–824.

Keim, P., Price, L.B., Klevytska, A.M., Smith, K.L., Schupp, J.M., Okinaka, R., et al., 2000. Multiplelocus

variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. 182, 2928–2936.

LaPorte, G.M., Stephens, J.C., Beuchel, A.K., 2010. The examination of commercial printing defects to assess common origin, batch variation, and error rate. J. Forensic Sci. 55, 136–140.

Rasko, D.A., Worsham, P.L., Abshire, T.G., Stanley, S.T., Bannan, J.D., Wilson, M.R., et al., 2011. *Bacillus anthracis* comparative genome analysis in support of the Amerithrax investigation. Proc. Natl. Acad. Sci. U.S.A. 108, 5027–5032.

Ravel, J., Jiang, L., Stanley, S.T., Wilson, M.R., Decker, R.S., Read, T.D., et al., 2009. The complete genome sequence of *Bacillus anthracis* Ames "Ancestor". J. Bacteriol. 191, 445–446.

Taylor, R.E., Bar-Yosef, O., 2014. Radiocarbon Dating. Left Coast Press, Walnut Creek, CA.

The National Academies. In: Gast, A.P., et al. (Eds.), 2011. Review of the Scientific Approach Used during the FBI's Investigation of the 2001 Anthrax Attacks. National Academy Press, Washington, DC, pp. 75–80.

The United States Department of Justice, February 19, 2010. Amerithrax Investigative Summary, p. 53.

The United States Department of Justice., Amerithrax Investigative Summary. 51–56.

CHAPTER

3

Microbial forensic investigation of the anthrax letter attacks: how the investigation would differ using today's technologies

Paul J. Jackson

Adjunct Professor, Nonproliferation and Terrorism Studies, James Martin Center for Nonproliferation Studies, Middlebury Institute of International Studies at Monterey, Monterey, California, United States

The 2001 bioterrorism attack using *Bacillus anthracis* spores perpetrated very shortly after the September 11 terrorist attacks, infected 22 people, caused 11 cases of inhalational anthrax, and resulted in 5 deaths. From a purely medical standpoint, the attack did not infect most of the people who were exposed to the anthrax spores. However, the attack clearly demonstrated that the dissemination of *B. anthracis* spores sufficient to infect a number of individuals and to significantly contaminate public and private infrastructure could be accomplished without the use of sophisticated equipment, instead simply using the US Postal System to deliver and disseminate the spores. The letters also demonstrated the outscale effect of a small-scale attack; the social and political response to the letters was extreme, costly, and long-lived, largely because of a lack of knowledge about the impact of spore release and persistence of viable spores in a public venue.

Methods used to analyze the attack isolate

Until the mid-1990s, no forensic methods were available that could differentiate among different *B. anthracis* strains. In 1996, Andersen et al. identified a variable number tandem repeat (VNTR) in *B. anthracis* that differed among different strains and thus allowed the sorting of all *B. anthracis* isolates into several categories based on the number of tandem repeats present in a particular strain (Andersen et al., 1996). In 1997, Keim et al. demonstrated that amplified fragment length polymorphism analysis could be used to identify genetic differences among different isolates of what was found to be a

Microbial Forensics, Third Edition
https://doi.org/10.1016/B978-0-12-815379-6.00003-9

genetically very monomorphic species (Keim et al., 1997). In 1997, Jackson et al. showed there were additional VNTRs in *B. anthracis* (Jackson et al., 1997), and in 2000, Keim et al. demonstrated that a large collection of *B. anthracis* isolates could be subdivided into 81 different genotypes based on signatures created using 8 different VNTR loci (Keim et al., 2000). This method became to be known as multiple locus variable number tandem repeat analysis or MLVA.

In the late 1990s and during the first decade of the new millennium, DNA sequencing was expensive and labor-intensive, and the cost and time required to sequence even the two large *B. anthracis* plasmids was considerable (Okinaka et al., 1999). Sequencing and assembling the entire 5.2 million nucleotides of a single *B. anthracis* genome required a level of effort and cost that was beyond the means of most laboratories and investigators. Such research was normally conducted by a very few specialty laboratories and results were published in prestigious journals. However, directed sequencing of specific genetic loci (multilocus sequence typing) was done routinely, and in *B. anthracis*, strain-specific sequence differences were demonstrated in the *pagA* gene that encodes a key protein in anthrax pathology and vaccine development (Okinaka et al., 1999; Price et al., 1999). More extensive single-nucleotide polymorphism (SNP) analysis of *B. anthracis* was conducted as DNA sequencing technology improved and costs declined, but this work did not proceed until well after the investigation into the 2001 anthrax letters had begun (Van Ert et al., 2007a,b).

The Federal Bureau of Investigation (FBI) was responsible for investigating the anthrax letter attack. However, at that time, they had only just begun reviewing different forensic methods that could be applied to *B. anthracis* and none of the methods had yet been fully validated. Indeed, the development of many of the microbial forensic methods eventually applied to the letters and their spore contents proceeded more rapidly because of the investigation. The development of new forensic analysis methods necessarily requires extensive testing and validation to demonstrate the accuracy and limitations of the methods. Consequently, microbial forensic analysis of the anthrax letter samples proceeded, sometimes at a slow pace, through most of the first decade of the 21st century. The FBI relied on the scientific expertise and experience of the country's academic resources, national laboratories, and private industry to assist with the development, validation, and application of forensic methods to the Amerithrax samples. The scientific team included personnel from the FBI, the Department of Defense, the Centers for Disease Control and Prevention (CDC), the National Institutes of Health, the Department of Homeland Security, the National Science Foundation, the national laboratories, academia, and industry. The large number of people involved often led to extensive discussions and, sometimes, disagreements about the value and validity of the forensic methods applied. It must be emphasized that microbial forensics, in itself, is not sufficient to provide the information needed to identify the perpetrator of such a crime; rather, forensic results are used to provide leads and clues that, in turn, lead to further analysis that may eventually identify the perpetrator.

The attack strain was quickly identified by Northern Arizona University and the CDC as the Ames strain. MLVA analysis generated a fragment pattern for the attack strain that was identical to the pattern generated from an archived Ames strain sample (Hoffmaster et al., 2002; Cole, 2009). The Ames strain of *B. anthracis* (originally isolated from an infected animal) has been used extensively in laboratory studies and for vaccine development and testing because of its high virulence. However, at the time of the anthrax letters, there were relatively few *B. anthracis* isolates available for comparison. Thus, the fact that the MLVA profile of the attack isolate matched that of the Ames strain

did not rule out the possibility that it also might match one or more other *B. anthracis* isolates not yet characterized via MLVA. In fact, the Amerithrax MLVA profile also matched that of a *B. anthracis* isolate collected in 1997 from a Texas goat (Van Ert et al., 2007a,b).

In 2001, the only method known to provide more resolution than MLVA profiling was genome sequencing. The cost to sequence a single *B. anthracis* genome in 2001 was approximately $150,000 (US) and it took several months to complete and assemble the sequence. However, simply sequencing the genome of a single strain provided little information of value because there were no other full genome sequences available for comparison. Therefore, the FBI in consultation with anthrax experts decided that the genomes of 20 different *B. anthracis* genomes, selected for their genetic diversity, would be sequenced by The Institute for Genomic Research (TIGR) at an estimated cost of $3 million (US) (Enserink, 2002). TIGR had previously sequenced an Ames isolate cured of its two large plasmids and had found differences between this isolate and the Ames isolate collected from the Florida patient, the first Amerithrax victim (Read et al., 2002, 2003). However, because the process of curing *B. anthracis* isolates of their two plasmids often involves the use of high temperature and chemical mutagens that can introduce mutations into the plasmid-cured isolates, this original comparison was of little value. While 20 isolates were a relatively small number, the sequencing effort was limited by cost and time. The Keim laboratory selected the strains to be sequenced based on the known diversity within the species as determined primarily by MLVA analysis.

The early whole-genome sequencing effort did not provide useful investigative leads, but it did confirm earlier investigative results that showed the isolate had not been genetically modified. The information derived from these sequences was also used to develop SNP assays that provided a four-SNP signature specific to the Ames strain, which could then be used for real-time polymerase chain reaction (PCR) analysis. Real-time PCR assays that identified these SNPs were much faster and far less expensive than whole genome sequencing or MLVA analysis. As more microbial genomes were sequenced and added to databases, microarray analysis and other hybridization methods became more valuable in the interrogation of medical, veterinary, and environmental *B. anthracis* samples (Jaing et al., 2015; Thissen et al., 2014; Be et al., 2014).

Bacillus anthracis strain archives

The size of the reference population is critical to any system that intends to specifically identify an isolate based on its specific DNA signature. A reference sequence database of 20 selected *B. anthracis* isolates is far too small to be able to draw conclusions about the species in general or a specific isolate in particular, especially if the starting collection is biased in any way. An apparent match to an isolate already present in a collection only demonstrates that the isolate in question has a genetic signature identical or very similar to something that has already been collected and studied. It is not known how frequent that same signature may occur in the environment. Moreover, as specific isolates are transferred among different laboratories and are grown under differing conditions, formerly identical isolates will begin to diverge as a result of mutations and selections during culturing and handling.

A good example of this is the presence of morphological variants in the spores found in the 2001 anthrax letters. Staff at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) with extensive experience in culturing and handling *B. anthracis* noticed variants in colony morphology when growing plates of the Amerithrax spores (Rasko et al., 2011). Such colony morphology differences likely

would not have been noticed or would have been dismissed as variations in agar plates or other experimental biases by scientists with less experience handling this pathogen. These variants exhibited altered sporulation phenotypes. All of the anthrax letter variants identified by colony morphology were poorly sporogenic when compared to the Ames Ancestor (the earliest known archived culture of the Ames strain and the original stock from which all other Ames cultures were derived). The genomes of these variants were sequenced and the sequences compared to the genome sequence of the Ames Ancestor, revealing SNPs, insertions, deletions, and large duplications often close to genes involved in sporulation and its regulation. A thorough analysis of the differences found in the anthrax letter spores and a comparison of those results to the FBI's 1077 repository samples, collected during the investigation, revealed that only eight repository samples contained the same variant signatures. All eight samples were derived from a USAMRIID spore stock known as RMR1029. Genome analysis results were consistent with subculturing of the RMR1029 stock to produce the attack spores. The match of the variant profile of the attack spores to the RMR1029 stock narrowed the investigation to a small number of possible suspect samples, but it did not identify any single individual who might have perpetrated the attack.

Analysis using today's advanced forensic methods

Given the advances in molecular biology and microbial forensics that have occurred since 2001, how would the letter samples be analyzed today? (We note that immunologic and other detection and characterization methods have also made substantial progress over this same period.) The microbial forensic analysis of the *B. anthracis* spores in the Amerithrax letters used methods developed shortly before and during the investigation. Indeed, the investigation accelerated the development of new, groundbreaking methods, and many of these assays and methods have now been fully validated and are readily available. DNA sequencing and microarray analysis, in particular, have led to major advances in microbial genetics since the early 2000s (Goodwin et al., 2016; Levy and Myers, 2016).

Such an analysis today could be done much more rapidly, more economically, and in much greater depth than was possible during the initial investigation. The cost of DNA sequencing and related methods has been reduced significantly, and the ease with which a microbial genome can be completely sequenced using very small amounts of DNA has placed the sequencing of even large genomes and large populations of genomes within the reach of almost any laboratory. (Many research laboratories no longer maintain a DNA sequencing capability of their own, choosing instead to contract with companies that specialize in this technology.) With the availability of large numbers of microbial DNA sequences, the information derived from these sequences has been used to develop different DNA-based approaches to sample analysis. The reagents required for these molecular assays can now be readily and rapidly obtained simply by specifying and ordering online the oligonucleotides and other necessary reagents from a number of reputable companies.

If a similar bioterrorist attack occurred today, direct DNA sequencing would replace many of the analysis methods used on the 2001 anthrax letters. However, DNA sequencing would not be the only technique used. It would still be useful to conduct SNP analysis of any attack isolates and compare these to archived profiles to help focus the DNA sequencing effort on relevant isolates, especially if archived isolates are not immediately available for sequencing (as might be the case for isolates from foreign collections). Once DNA sequences are available, genome annotation methods developed over the last

decade would be used to thoroughly characterize the attack strain's genome (Fricke and Rasko, 2014; Tanizawa et al., 2018). Instead of just comparing sequences among different strains, such characterization makes it possible to look for specific changes in the genome that might affect the organism's phenotype, including possible resistance to different antibiotics and other factors related to survival of the spores upon release or medical treatment scenarios for exposed individuals. Indeed, the mere presence of genome differences associated with genes known to play a role in pathogenesis, survival of spores, or other factors that might influence the impact of an attack would be an indication that the attack strain was intentionally manipulated and released.

If such changes were identified, deep DNA sequencing would then be applied. Deep DNA sequencing refers to the sequencing of a genome or region of a genome hundreds or even thousands of times. This method allows the detection of rare cells within a population and might alleviate the need to collect and culture rare mutants existing in a microbial population that depend on colony morphology differences for identification (Lasken and McLean, 2014; Salk et al., 2018). Such methods can also be used to identify other microbial species that might be rare contaminants within the original release material, which would provide a unique, sample-specific molecular signature.

Limits to sample analysis and other issues

Although significant advances have been made over the past decade in DNA sequencing and annotation as well as in the development of deep sequencing methods and rapid assays for genome characterization, some significant hurdles still exist. In particular, the number of archived *B. anthracis* isolates available for comparison is very limited, and often there is only minimal information about individual isolates in the archives. Many of the archived isolates were collected from different geographic origins, but all that is known about them is the date and location of collection and the material from which they were collected. In most cases, virtually nothing is known about the specific pathogenic properties of these isolates. Most of these isolates have not been extensively studied in vitro or in vivo, and for many, it is not even known whether or not they are pathogenic. Without specific information about the pathogenic and other properties of these archived strains, they have no value in studies to better understand these properties and their genetic control in *B. anthracis* based on its DNA signature. This lack of information about so many of the archived isolates also limits the use of comparative methods in a forensic investigation. The match of an attack isolate to an isolate in a strain collection only demonstrates that those two isolates are identical within the limits of the analysis method that was used. It does not rule out the possibility that the attack isolate is also identical to one or more isolates that have not yet been collected or that exist in an archive but have not been genetically characterized. The failure of an attack isolate to match archived isolates is actually more valuable in a forensic analysis because the lack of a match rules out those previously characterized isolates as attack isolate candidates.

Isolates from many foreign microbial archives are not always readily available to researchers, and the "select agent" regulations (C.F.R Part 33; C.F.R Part 73) that were enacted after the 2001 anthrax letters attack make it unlikely that such isolates could be rapidly obtained in the event of a similar attack today. Moreover, these regulations have significantly curtailed academic research efforts to collect and characterize new isolates or further study the pathology and other characteristics of the available isolates. In the absence of such studies, it is unlikely that archives of this pathogen will grow significantly or that new or improved methods to effectively

treat *B. anthracis* infections will be developed anytime in the near future.

It was fortuitous for the Amerithrax investigation that several of the anthrax letters were collected intact, allowing analysis of the population of spores within each letter. Such an analysis eventually led to the conclusion that the spores were propagated from the RMR1029 stock. If intact letters had not been available for analysis and spores could only have been collected from environmental sources or victims, it would have been impossible to establish the frequency and distribution of the rare mutations that eventually provided the molecular signature that tied the letter spores to the RMR1029 flask. Collection and forensic analysis of spores already released into the environment and not from a defined source would make source attribution much more difficult or impossible.

Advances in DNA sequencing and related diagnostic technologies have been accompanied by significant advances in methods that allow easy and direct modification of a microbe's genome. In 2001, when the anthrax letters were mailed, genetic manipulation of such microorganisms was possible but only within the realm of specialty laboratories working on those pathogens. Today, genetic manipulation is relatively easy with inexpensive kits and reagents that can be purchased online to introduce specific genetic modifications into *B. anthracis* and other pathogens. Some would argue that such manipulation would reduce the possibility that the modified microbe would still be highly pathogenic, as it is well known that extensive handling of pathogenic microbes in a laboratory may reduce their pathogenic properties. However, as demonstrated by the 2001 anthrax attack (which infected only a handful of individuals), terrorist use of an intentionally modified threat agent with only moderate pathogenicity would be massively disruptive both socially and politically.

What sort of changes might a perpetrator attempt to introduce into *B. anthracis*? What comes to mind are changes to increase the virulence of the pathogen and its survival during handling and dissemination and following release as well as changes that would make the prevention or treatment of infection more difficult. Successful introduction of such changes would require an in-depth understanding of the physiology, biochemistry, and genetic modifications underlying the changes, including identifying the specific gene(s) involved and their regulation. Although the introduction of antibiotic resistance in some cases involves changing only one or several nucleotides, changes that affect other phenotypic characteristics are typically much more complicated and in many cases not yet understood. It is also conceivable that a perpetrator would seek to make changes to disguise the source of the attack or perhaps confuse or defeat the systems in place to detect an environmental release of spores. The introduction of such changes would require a high level of expertise in the long-term growth and handling of *B. anthracis* as well as an in-depth understanding of the capabilities of deployed detection and analysis.

Conclusion

Significant advances in DNA detection, characterization, sequencing, and annotation have been made since the 2001 anthrax letters, which would enable a much more rapid and in-depth analysis should such an attack occur today. Despite these advances, there are still limitations to our microbial forensic analysis capabilities. Archives of *B. anthracis* isolates remain limited. While expanding the size and geographic representation of these archives would be of great benefit, such efforts are extremely difficult as a result of changes to the "select agent" regulations implemented since the Amerithrax investigation. Expansion of the archives must be accompanied by a more detailed analysis of the archived samples to better understand differences in their pathogenic properties and the genetic changes

responsible for these. Advances in microbial forensics have been accompanied by advances in the methods available to genetically modify microbes. While much of the physiology, biochemistry, and molecular biology of this pathogen must still be understood to introduce genetic changes that impact phenotypic traits of this pathogen, this information will become more readily available as investigations of the specific pathogen continue. The potential target of an investigation today might be significantly different than the unmodified Ames strain mailed in the 2001 letters. Forensic tools are therefore needed to rapidly identify the signatures associated with such changes and the impact they might have on the viability, virulence, and other properties of a released isolate. Rapid, in-depth DNA sequencing will continue to be used to provide a molecular signature of any released isolate. Methods to rapidly determine the phenotypic characteristics of an isolate based on its DNA signature are still not mature and require a better understanding of the pathogen and its interaction with its hosts and the environment.

References

Andersen, G.L., Simchock, J.M., Wilson, K.H., 1996. Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. J. Bacteriol. 178, 377–384.

Be, N., Allen, J., Brown, T., Gardner, S., McLoughlin, K., Forsberg, J., et al., 2014. Microbial profiling of combat wound infection through detection microarray and next-generation sequencing. J. Clin. Microbiol. 52, 2583–2594.

C.F.R. Part 33, 9 C.F.R. Part 121 and 42 C.F.R. Part 73 HHS and USDA Select Agents and Toxins.

C.F.R. Part 73 Possession, Use and Transfer of Select Agents and Toxins.

Cole, L.A., 2009. The Anthrax Letters: A Bioterrorism Expert Investigates the Attack that Shocked America. Skyhorse Publishing.

Enserink, M., 2002. TIGR Attacks Anthrax Genome. http://www.sciencemag.org/news/2002/02/tigr-attacks-anthrax-genome.

Fricke, W.F., Rasko, D.A., 2014. Bacterial genome sequencing in the clinic: bioinformatics challenges and solutions. Nat. Rev. Genet. 15, 49–55.

Goodwin, S., McPherson, J.D., McCombie, W.R., 2016. Coming of age: ten years of next-generation sequencing technologies. Nat. Rev. Genet. 17, 333–351.

Hoffmaster, A.R., Fitzgerald, C.C., Ribot, E., Mayer, L.W., Popovic, T., 2002. Molecular subtyping of *Bacillus anthracis* and the 2001 bioterrorism-associated anthrax outbreak, United States. Emerg. Inf. Dis. 8, 1111–1116.

Jackson, P.J., Walthers, E.A., Kalif, A.S., Richmond, K.L., Adair, D.M., Hill, K.K., et al., 1997. Characterization of the variable number tandem repeats in vrrA from different *Bacillus anthracis* isolates. Appl. Environ. Micro. 63, 1400–1405.

Jaing, C.J., Thissen, J.B., Gardner, S.N., McLoughlin, K.S., Hullinger, P.J., Monday, N.A., et al., 2015. Application of a pathogen microarray for the analysis of viruses and bacteria in clinical diagnostic samples from pigs. J. Vet. Diagn. Invest. 27, 313–325.

Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S.E., Richmond, K., et al., 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. 179, 818–824.

Keim, P., Price, L.B., Klevytska, A.M., Smith, L.L., Schupp, J.M., Okinaka, R., et al., 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. 182, 2928–2936.

Lasken, R.S., McLean, J.S., 2014. Recent advances in genome DNA sequencing of microbial species from single cells. Nat. Rev. Genet. 15, 577–584.

Levy, S.E., Myers, R.M., 2016. Advancements in next-generation sequencing. Ann. Rev. Genomics Hum. Genet. 17, 95–115.

Okinaka, R.T., Cloud, K., Hampton, O., Hoffmaster, A.R., Hill, K.K., Keim, P., et al., 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. J. Bacteriol. 181, 6509–6515.

Price, L.B., Hugh-Jones, M., Jackson, P.J., Keim, P., 1999. Genetic diversity in the protective antigen gene of *Bacillus anthracis*. J. Bacteriol. 181, 2358–2362.

Rasko, D.A., Worsham, P.L., Abshire, T.G., Stanley, S.T., Bannan, J.D., Wilson, M.R., et al., 2011. *Bacillus anthracis* comparative genome analysis in support of the Amerithrax investigation. Proc. Natl. Acad. Sci. U.S.A. 108, 5027–5032.

Read, T.D., Salzberg, S.L., Pop, M., Shumway, M., Umayam, L., Jiang, L.X., et al., 2002. Comparative

genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. Science 296, 2028–2033.

Read, T.D., Peterson, S.N., Tourasse, N., Baillie, L.W., Paulsen, I.T., Nelson, K.E., et al., 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature 423, 81–86.

Salk, J.J., Schmitt, J.W., Loeb, L.A., 2018. Enhancing the accuracy of next-generation sequencing for detecting rare and subclonal mutations. Nat. Rev. Genet. 19, 269–285.

Tanizawa, Y., Fujisawa, T., Nakamura, Y., 2018. DFAST: a flexible prokaryotic genome annotation pipeline for faster genome publications. Bioinformatics 34, 1037–1039.

Thissen, J.B., McLoughlin, K., Gardner, S., Gu, P., Mabery, S., Slezak, T., et al., 2014. Analysis of sensitivity and rapid hybridization of multiplexed microbial detection microarray. J. Virol. Methods 201, 73–78.

Van Ert, M.N., Easterday, W.R., Simonson, T.S., U'Ren, J.M., Pearson, T., Kenefic, L.J., et al., 2007a. Strain-specific single-nucleotide polymorphism assays for the *Bacillus anthracis* Ames strain. J. Clin. Microbiol. 45, 47–53.

Van Ert, M.N., Easterday, W.R., Huynh, L.Y., Okinaka, R.T., Hugh-Jones, M.E., Ravel, J., et al., 2007b. Global genetic population structure of *Bacillus anthracis*. PLoS One 2, e461.

CHAPTER

4

Foodborne outbreaks

E.W. Brown, M.W. Allard

Division of Microbiology, Office of Regulatory Science, CFSAN, US FDA, College Park, MD, United States

Whole genome sequencing for foodborne outbreaks

Whole genome sequencing (WGS) has been applied to the source tracking of foodborne pathogens where often there was insufficient resolution of existing subtyping tools (Allard et al., 2012, 2013; Hoffmann et al., 2014; Kupferschmidt, 2011; Lienau et al., 2011). The primary application and use of WGS is for pathogen characterization and to cluster genomes from a recent common ancestor, between clinical, food, and environmental isolates. Close phylogenetic matches define WGS linkage which may support additional investigation and/or direct additional inquiry into the causes of the observed genetic signal (Bell et al., 2015; Dallman et al., 2016a,b; Gonzalez-Escalona et al., 2016; Waldram et al., 2018). The ability to reconstruct the evolutionary history of very closely related isolates enables the identification of a recent common ancestor and often the determination of the root cause of the contamination event (Chen et al., 2017a,b; Haendiges et al., 2016; Hoffmann et al., 2016). These WGS phylogenetic methods are reproducible and accurate, often providing the first physical evidence showing a connection between isolates in an epidemiological investigation. Regulatory decisions are made based on a three-legged stool consisting of (1) the genetic signal supporting shared ancestry of isolates; (2) evidence gathered from inspections and investigations along the farm-to-fork continuum of the implicated production line documenting contamination on site and possibly recovering additional foodborne bacterial evidence; and (3) epidemiological evidence based on exposure questionnaires to various foods that sickened people share. Genetic evidence and linkages are not by themselves regulatory proof that the source of the outbreak has been found but are one piece of the combined evidence. A strong genetic signal can assist so that investigations are acted on rapidly. In an ideal pathogen surveillance network based on WGS data, diversity in the genomic database should represent the real-world, global, microbial diversity (Allard et al., 2016; Gardy and Loman, 2018). Contamination events identified in the United States (US) may involve foods that were traded half-way around the world (Kwong et al., 2016a,b). As more countries share the genomic data from the contaminants that they discover, we are seeing more outbreaks linked through global trade (Lambert et al., 2017). The GenomeTrakr database, which was developed by the US Food and Drug

Microbial Forensics, Third Edition
https://doi.org/10.1016/B978-0-12-815379-6.00004-0

Administration (FDA), is publicly available in real time to support global public health and food safety (Allard et al., 2016). The database is housed within the National Center for Biotechnology Information (NCBI) at their Pathogen Detection website to leverage the NCBI tools (https://www.ncbi.nlm.nih.gov/pathogens/). The Pathogen Detection portal is updated daily to provide WGS linkages and phylogenetic trees. Additionally, the PulseNet Network also uploads WGS data to the Sequence Read Archive at NCBI as does the US Department of Agriculture's Food Safety and Inspection Services. Together these data comprise an integrated one-health—oriented database where clinical, domestic animal, food, and environmental WGS data are combined to discover novel linkages among foodborne pathogens. As WGS data provide detailed information and an increased degree of certainty in identifying the sources of foodborne contamination, these phylogenetic tools are examined daily to identify new clusters of interest to the regulatory offices. The GenomeTrakr database is leveraging WGS data in an open access database to improve public heath through more rapid identification of the root sources of foodborne illness, which in turn allows a faster public health response that will reduce morbidity and mortality. Providing large amounts of genomic information also fosters innovations in new rapid tests kits, methods, and equipment, by providing more detailed information on the genetic variation and diversity of foodborne pathogen present in populations. WGS has been deployed across FDA field laboratories and is now being applied to many isolates of *Salmonella enteritidis, Escherichia coli, Campylobacter jejuni*, and *L. monocytogenes* as they are isolated from food and environmental sources. WGS is now a regular tool used by federal and state laboratories to support foodborne outbreak detection and investigations. In the daily monitoring of the NCBI Pathogen Detection website, federal and state investigators are looking for matches to the isolates that they have uploaded. Important signals that are monitored are whether any clinical isolates match food or environmental ones. Matches to recent clinical isolates are a possible signal that a problem is current, while older clinical cases indicate that the pathogen has been virulent in the past and may represent an emerging risk. Clusters of interest to the federal laboratories are prioritized and watched to see if they are increasing in size, and matches are followed up with other state, federal, and international collaborators that provided the WGS of the clustered isolates. Approximately 200 clusters are identified each year and are further investigated. FDA investigators also reanalyze clusters identified on the NCBI Pathogen Detection website using validated software (Davis et al., 2015). A public version of this validated software pipeline is also available on the Galaxy server for state, industry, and international collaborators.

FDA watches the NCBI Pathogen Detection database to discover new contamination events and to see if older known events have been cleaned up from the food supply. The initial assumption is that contamination occurs in the food supply chain along the farm-to-fork continuum, though other sources of contamination may arise. One new WGS application is to include sequences in the database of all known positive controls used in the laboratory, to determine if contamination is arising in the testing laboratory as a false positive. Another potential use of WGS might include the discovery of an intentional contamination event. For example, in 1984, the *Salmonella* outbreak in the Dalles, OR, was initially attributed to a natural outbreak until an informant came forward. Much later, it was realized that it was an intentional contamination event to influence an election (https://en.wikipedia.org/wiki/1984_Rajneeshee_bioterror_attack). The foodborne pathogen strain used in this bioterror attack was a laboratory strain obtained from the ATCC. So, if the Genome Trakr had been available in 1984, the genome sequence of the strain would have indicated that it was a laboratory strain and likely

the result of intentional contamination. FDA and many of the GenomeTrakr members are part of the Food Emergency Response Network which is able to respond to emergencies involving biological, chemical, or radiological contamination of food with the goals of prevention, preparedness, and response.

'Omics and global scientific and regulatory trends

With the globalization of the human food supply over the past two decades, the importance of food safety has become a collective concern, and efforts aimed at the prevention of and response to foodborne illness have become an ever-more important focus by the world food community. As food imports and exports continue to rise globally so too does the reach of the agents of foodborne illness previously associated with certain commodities. For example, *Salmonella*, which persisted as a bacterial contaminant of poultry and eggs for many decades in the US, has recently reemerged in a number of unexpected food sources including dry spices, numerous produce commodities in the fresh-cut market, and processed products such as peanut butter and cereal. As many of these pathogens now follow the trade routes of the food reservoirs in which they reside, 'omic tools, capable of providing extensive genotypic and phenotypic detail about a foodborne pathogenic strain, are poised to mitigate our response to these challenges. In particular, in the areas of detecting, identifying, and responding to foodborne outbreaks, genomic technologies such as next-generation sequencing (NGS) may provide the basis for the rapid and highly specific global monitoring and surveillance of the food supply for dangerous pathogens. Examples continue to amass in the scientific literature reporting on the use of NGS to augment infectious disease outbreak investigations (i.e., foodborne, nosocomial, and community-acquired) by (i) delimiting the scope of a contamination event; (ii) providing detailed information on the potential reservoirs or sources of pathogen contamination; and (iii) illuminating the vehicle for spread and/or dissemination of the causative agent through a population. Additionally, such a system allows for enhanced and more detailed pathogen risk assessments and preventive control strategies for various sectors of the food supply (Franz et al., 2016). Indeed, economic, scientific, and public health drivers do exist for the continued development and deployment of such 'omic technologies. In the case of pathogen detection, identification, and traceability, a framework for the operation and regulation of such a system has recently emerged. Of course, precisely how the data from such systems will be shared among global stakeholders including developing countries remains an important question to be answered. Here, we frame the ethical, regulatory, and scientific challenges surrounding the global emergence of a genomic system capable of establishing the next paradigm for foodborne pathogen detection, identification, and subtyping.

Drivers for scientific development

Arguably, the greatest drivers behind the development and deployment of 'omic systems on a global scale come from the need for enhanced foodborne pathogen detection tools in both public and private health. From a public health and food safety perspective, NGS provides a new opportunity to develop worldwide food pathogen sequence databases that are portable, updated in real time, and completely transferrable among governmental public health stakeholders focused on surveillance and detection of pathogens in the food supply. However, the drive for such a system does not emanate only from public health settings. The point-of-care testing community also drives the demand for a genomic pathogen identification system.

A single genomic system containing an extensive pathogen genome sequence database would provide unprecedented accuracy for global testing and surveillance of the food supply. Moreover, such a system will also serve those who are diagnosing and treating foodborne illnesses and understanding the root cause for infection control, a single platform from which to extract extensive detail about a particular microbial food contaminant or bacterial/viral agent of foodborne illness. Diagnostic information gleaned from a next-generation pathogen database could include information on drug susceptibility (i.e., multidrug resistance), determinants of virulence and pathogenicity, other genetic factors associated with persistence or tolerance to heat, desiccation or oxidative assaults (e.g., preservatives), and important biomarkers for clustering for traceability and molecular epidemiological tracking. Perhaps most importantly, global deployment of such a database by its very nature provides a standardized approach to frontline stakeholders such as the food testing and food quality assurance communities. This, in turn, will make harmonization for testing much more achievable between countries as much of the information learned about a contaminant will be derived from a common shared global database, and with a common data-input platform, e.g., whole genomic sequences (Cheung and Kwan, 2012; Eng, 2004; Gwinn et al., 2017; Howard et al., 2013; Niesters et al., 2013).

One of the single most important drivers of 'omic technologies in food safety and public health is the eventual loss of culture in clinical microbiology and therefore pathogen isolates, many of which are associated with foodborne illness. Recently, the CDC projected, with impending urgency, that clinical isolates of foodborne pathogens will continue to dwindle due to the availability of rapid, culture-free diagnostics which continue to be licensed for use by the point-of-care testing community for the diagnosis of infection by pathogenic microbes, including many foodborne species such as *S. enteritidis*, shiga-toxin producing *E. coli*, and *L. monocytogenes*. The eventual loss of clinical cultures associated with foodborne illness will have a marked deleterious effect on the food safety and surveillance community's ability to detect and react to disease outbreak clusters on a global scale. PulseNet, the current global subtyping network administered by the CDC and used in most of the developed and developing world, requires a pure culture for PFGE analysis. The resulting fingerprint is uploaded into an expansive database to query for matches from the food supply and among individuals sickened by particular food sources. Loss of culture capacity is driving the development of future 'omic methods, which will enable the capture of important and detailed strain information including those strain attributes listed in the preceding paragraph such as antimicrobial resistance, virulence, and discriminatory markers for subtype investigation. While many 'omic approaches involve the extraction and purification of DNA from pure cultures, it is envisaged that metagenomic approaches, akin to those being used currently in the human microbiome effort, will ultimately be deployed to obtain essential genotypic details about a pathogen directly from contaminated food sources or from associated clinical or environmental samples as well. The data could then be fed directly into the cross-compatible databases described above (i.e., MetaGenomeTrakr) (Ottesen et al., 2016).

Although partially encompassed under public health or food safety, additional drivers from both academia and the regulatory sector can be envisioned for the advancement and deployment of a globally distributed next-generation 'omic system. From an academic perspective, a database comprised of comprehensive, sharable, and standardized data (e.g., genome sequence data) provides a single data mining vehicle along with a cornucopia of biomarkers and other diagnostic targets for international scientists to engage in more