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REVIEW ARTICLE

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Microbial forensics: the next forensic challenge

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Abstract Pathogens and toxins can be converted to bioweapons and used to commit bioterrorism and biocrime. Because of the potential and relative ease of an attack using a bioweapon, forensic science needs to be prepared to assist in the investigation to bring perpetrators to justice and to deter future attacks. A new subfield of forensics—microbial forensics—has been created, which is focused on characterization of evidence from a bioterrorism act, biocrime, hoax, or an inadvertent release. Forensic microbiological investigations are essentially the same as any other forensic investigation regarding processing. They involve crime scene (s) investigation, chain of custody practices, evidence collection, handling and preservation, evidence shipping, analysis of evidence, interpretation of results, and court presentation. In addition to collecting and analyzing traditional forensic evidence, the forensic investigation will attempt to determine the etiology and identity of the causal agent, often in a similar fashion as in an epidemiologic investigation. However, for attribution, higher-resolution characterization is needed. The tools for attribution include genetic- and non-genetic-based assays and informatics to attempt to determine the unique source of a sample or at least eliminate some sources. In addition, chemical and physical assays may help determine the process used to prepare, store, or disseminate the bioweapon. An effective microbial forensics program will require development and/or validation of all aspects of the forensic investigative process, from sample collection to interpretation of results. Quality assurance (QA) and QC practices, comparable to those used by the forensic DNA science community, are being implemented. Lastly, partnerships with other laboratories will be requisite, because many of the necessary capabilities for analysis will not reside in the traditional forensic laboratory.

Keywords Microbial forensics · Epidemiology · Bioterrorism · Biocrime · Virus · Bacteria · Fungi · Toxin · Quality assurance · Quality control · Validation

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Introduction

One of the most significant threats to society is a serious disease outbreak, be it naturally occurring or intentional. Such an event can wreak havoc by resulting in harm or death, causing disruption, creating fear, and affecting economic well being. Fortunately, advances in molecular and pathogen biology have and will continue to provide tools for combating a number of emerging and reemerging diseases. Unfortunately, these same technological boons to modern medicine and agricultural biology can also be utilized to generate new devastating biologic weapons that can be used to commit acts of terrorism and cause serious harm to humans, animals, and plants.

In the same manner that pathogens or toxins can be used to commit acts of terror, they can be used as weapons in the commission of a crime. Biocrimes are similar in many ways to traditional crimes where specific individuals are harmed; the difference is that the weapon is biological in nature instead of, for example, a gun or a knife. Bioweapons can be used to commit murder or bodily injury and can be used as instruments of revenge, extortion, or personal vengeance.

There are numerous documented examples of the use of pathogens in major conflicts or terrorism over the course of history (Table 1) [http://www.usamriid.army.mil/content/BioWarCourse/HISTORY/HISTORY.html; 1–6]. One classic and devastating example was the delivery by the Conquistadors and British of smallpox and measles on blankets and clothing provided to an immune-naive population of

Table 1 Examples of confirmed or highly probable cases using bioweapons in biocrime, bioterrorism, or state-sponsored action where pathogen was identified[a]

Perpetrator	Victim/target	Location/date	Pathogen/toxin	Introduction manner
Biocrimes				
Henri Girard	Multiple people	France/1909–1918	*Salmonella typhi*	Injection, food contamination
Patrick O'Brien de Lacy, Vladimir Patchenko	Vassilli Buturlin	Russia/1910	Diphtheria toxin	Injection
Karl Hopf	His child	Germany/1913	Cholera, typhus	Unknown
Benoyendra Chandra pandy	Amarendra pandy	India/1933	*Yersinia pestis*	Injection
Tei-Sabro Takahashi	Hisako Inukai, Yoshiko Yanagida, Yorinobu Nalayama	Japan/1936	*Salmonella typhi*	Food contamination
Kikuko Hirose	Ritsuo Kato	Japan/1939	*Salmonella typhi*, *Salmonella paratyphi*	Food contamination
Mitsuru Suzuki	Health colleagues	Japan/1960s	*Salmonella typhi*	Food contamination
Eric Kranz	Roommates	Canada/1970	Ascaris suum eggs	Food contamination
Arafin Nesset	Elderly patients	Norway/1977	Curacit	Injection
Graham Farlow	Geoffrey Pearce	Australia/1990	HIV-tainted blood	Injection
Brian Stewart	Son	Missouri/1992	HIV-tainted blood	Injection
Richard Smidtke	Janice Trahan	Louisiana/1994	HIV-tainted blood	Injection
Iwan E	Gina O	Holland/1994	HIV-tainted blood	Injection
Debora Green	Michael Farrar	Missouri/1995	Ricin	Food contamination
Diane Thompson	Hospital workers	Texas/1996	Shigella dysenteriae type 2	Food contamination
Uncertain	Rabbits	New Zealand/1997	Rabbit hemorrhagic fever virus	Injection, food contamination
State-sponsored				
Assyrians	Enemies	Sixth century BC	Rye ergot	Water supply
Romans	Enemies	Ancient Rome	Carrion	Water supply
Tartar	Kaffa	Crimea/1346	*Yersinia pestis*	Infected corpses
Spanish	Native Americans	Americas/15th century	Smallpox	Contaminated clothing
British	Native Americans	North America/18th century	Smallpox, measles	Contaminated clothing
German Secret Service	Horses, mules	Maryland/1915–1917	*Burkholderia mallei*	Injection via wound
Japanese	Chinese	China WWII	*Yersinia pestis*	Infected fleas
Russians	Civilians	Russia post WII	Fusarium	Contaminated food
Unknown Assassin	Georgi Markov	London 1978	Ricin	Injection
Unknown Assassin	Vladimir Kostov	Paris/1978	Ricin	Injection
Terrorism				
Rajneeshee cult	Town populace	Oregon/1984	*Salmonella typhimurium*	Food contamination
Aum Shinrikyo cult	Populace	Japan/1990s	*Bacillus anthracis*, botulinum toxin	Aerosol
Mau Mau	Livestock	Kenya/1952	African milk bush toxin	Injection via wound
Unknown	Populace	USA/2001	*Bacillus anthracis*	letters

There are many criminal and civil cases involving HIV or ricin, too many to list. Thus, the examples listed here are but a subset of the number of cases

[a]Cases selected from 1, 3, 5 and 6

Native Americans. It is believed that such tactics by the latter influenced the outcome of the French and Indian wars (http://www.usamriid.army.mil/content/BioWarCourse/HISTORY/HISTORY.html). Examples of biocrimes abound [1, 7–14] and one example occurred in Dallas, TX, in 1996 involving the use of *Shigella dysenteriae* type 2 and food contamination. Twelve people of the laboratory staff of the St. Paul Medical Center hospital developed severe, acute diarrheal illness. All outbreak patients reported eating pastries in a break room. *S. dysenteriae* type 2, recovered from the patients' stool specimens, from an uneaten muffin, and from the medical laboratory's stock strain, were indistinguishable by pulsed-field gel electrophoresis. A criminal investigation ensued focusing on a laboratory technician who was subsequently convicted on several felony assaults (intentional infection by a pathogenic organism) and falsifying laboratory documents [9].

Many of the pathogens used in past biocrimes are not as lethal as, for example, *Bacillus anthracis*, smallpox, or ebola, but they are readily accessible. The delivery of an

infectious agent in a biocrime can be carried out without sophisticated technology, such as by dispersal over a salad bar or by direct inoculation of the victim with a needle. These attacks do not require a large infrastructure or considerable expertise. As shown in Table 1, confirmed biocrimes greatly outnumber the bioterrorism cases. Thus, from a forensic perspective, biocrimes may be more likely to be encountered. However, an act of bioterrorism evokes greater public response and consequence. As it is impossible to anticipate the next organism that will be used for illicit purposes, the potential list of agents that might be used is enormous.

There is every reason to believe that pathogens are still considered viable weapons and may be the weapon of choice for some. Pathogens are readily accessible and relatively easy to generate. Perpetrators, who would use such weapons, need to be stopped, interdicted, brought to justice, or better yet, deterred or dissuaded from such actions. Forensic science will play an active and crucial role in addressing and resolving such horrendous acts [15–18]. In fact, the anthrax letters attack of 2001 raised the awareness of our susceptibility and the potential importance of forensic analysis for attribution purposes of biological and nonbiological samples used in a terrorism incident. Forensic science can also support the investigation of hoaxes, which also cause disruption, economic loss, fear, and diversion of first responders (such as firemen, police, and paramedics), from normal duties and are also considered acts of terrorism. The security, trust, and confidence of the public also may be at stake.

Forensic science provides a special investigative role, although not always definitive. However, in some scenarios, science can be quite revealing through the characterization of physical evidence found at a crime scene for probative and attribution purposes. In addition, a forensic science investigation relies on a combination of diverse areas of science, lending credence to conclusions arrived at (or obtained) by complementary and corroborative approaches. Both biothreat agent and traditional forensic evidence, which may be contaminated with a microbe or toxin, will be collected, analyzed, and interpreted.

A forensic investigation of a case, where the weapon was a pathogen or toxin, will attempt to determine the identity of the causal agent and source of the bioweapon in much the same manner as in an epidemiologic investigation. Forensics and epidemiology should not be thought of as separate disciplines in the context of a crime, but rather should be integrated. For decades, epidemiologists have used general forensic practices to identify causative agents and the etiology of diseases for public health concerns. Epidemiology studies the combination of clinical presentation of disease, identification of the pathogen, the distribution in a population, and anecdotal factors to deduce where an infection began and how it spread throughout a population [15, 19–21]. These studies provide us with a number of characteristics to consider epidemiologically to raise suspicion that an outbreak may have been caused intentionally (Table 2). In microbial forensics, the epidemiologic issues are identification and characterization of specific disease-causing pathogens or their toxins, their modes of transmission, and any manipulations that may have been done intentionally to amplify their effects against human, animal, or plant targets. Microbial forensics goes one step further than most epidemiologic investigations; evidence is characterized to assist in determining the source of the sample in order to ultimately determine the identity of the perpetrator of the attack and the methods, means, processes, and locations involved.

Table 2 Epidemiologic considerations that may signal a bioterrorist attack

1. Disease caused by an uncommon agent (such smallpox)
2. Unusual, atypical, genetically engineered, or antiquated strain of agent
3. High morbidity or mortality associated with a common disease or syndrome
4. Failure of patients to respond to usual therapy
5. Disease with an unusual seasonal or geographic distribution
6. Increase in normal incidence
7. Atypical disease transmission (such as *Shigella* in muffins)
8. Illness in people who are exposed to same ventilation system
9. More than one unusual or unexplained disease existing in a person
10. Illness that affects a large disparate population
11. Illness that is unusual for a population or age group
12. Unusual death or pattern of illness in animals preceding or accompanies death or illness in humans and vice versa
13. A number of ill persons seeking treatment or obtaining medicine at the same time
14. Same strain or genetic type from spatially or temporally disparate sources
15. Simultaneous cluster of disease in noncontiguous areas
16. Large number of unexplained diseases or deaths

To both prepare for the next attack, as well as deter attacks, a strong microbial forensic program is being developed [15–18, 22]. Microbial forensics is an evolving subdiscipline of forensic science, which leverages several scientific disciplines, directed at characterization toward attribution by analyzing evidence from a bioterrorism act, biocrime, hoax, or an inadvertent release [15, 22]. Until recently, the discipline of microbial forensics was slow to develop, being considered only as a minor program predominately in a variety of government agencies. While obviously important, microbial forensics, as its own discipline, was not considered a legitimate component of a biosecurity "tool kit" or a potential deterrent; it was thought to have limited capability to attribute the source and to assist in identifying the perpetrator. However, over the last decade, an acute awareness has developed of the imminent threat of pathogen and toxin weapons, technical capabilities have improved, and microbial forensics has become necessary. This has brought renewed interests, an influx of resources, and new rules of engagement among government agencies, laboratories, and individual scientists.

This article describes the field of microbial forensics and its needs. The intent is to provide the reader with insights as to the directions the field is headed, to describe the chal-

lenges to consider, and to point out that although there are still many unanswered issues, investigations can be carried out and provide meaningful results. Lastly, our intent is to motivate and/or seek collaboration, or at least guidance, from the greater scientific community on effective ways to develop this burgeoning field.

Threats and accompanying challenges

There are some advantages to using microbes or their toxins as weapons to commit crimes. These include ease of access, the ability to amplify pathogens (by culture), and relatively low cost to generate the pathogen. Although some security initiatives have been enacted to stem the dissemination of pathogens, it is difficult to eliminate their development as weapons and thwart all possible attacks, even with heightened security. Many pathogens can be found in nature and are endemic. To exacerbate the problem, microbes can be disseminated in a number of ways, not all requiring sophisticated expertise or technology. Some organisms are fairly stable in the environment and can persist well after dissemination (such as *B. anthracis*).

Civilian populations, or subgroups in the population (such as immune compromised people and the elderly), are susceptible to infection by pathogens, and high morbidity and mortality can result. Once initially disseminated, some microorganisms are transmittable (such as smallpox, plague, hemorrhagic fever); thus, only one or a few focal point attacks could result in high mortality and/or substantial health problems. Without prior warning, many physicians would not be prepared to properly diagnose the disease, and even if diagnosed, some diseases have no known treatment. The use of biological weapons creates such panic that public and medical services can quickly become overwhelmed, causing substantial disruption. Moreover, a successful covert attack may go unnoticed for some time after the event. Thus, a perpetrator can escape detection long before the disease takes effect, and the original crime scene may be difficult to locate. Because basic microbiology equipment is available in hospitals, universities, and industry, there is ready access to production capabilities. Production is relatively inexpensive (compared with developing other devastating weapons such as nuclear weapons) and can be effectively prepared in small quantities making it difficult to detect. In the US, much attention has been paid to the effects of bioweapons on humans. However, agricultural targets are of extreme concern because these assets are generally unprotected; many pathogens that could be used as a weapon pose no threat to the perpetrator or human populations and yet could still have massive, albeit different, effects on society.

Because of the potential of an attack, forensic science needs to be prepared to assist in the investigation. Developing methods for identification and attribution of potential microbial weapons is challenging. For forensic identification of humans, the focus was on only one species, and a set of 10–17 microsatellite loci enabled attribution for the majority of scenarios encountered in crimes [24, 25]. However, for microbial forensics, there is a multitude of species to characterize, being made even more complex by their biological and ecological dynamics. For the number of potential bioweapons against human targets, consider that naturally occurring infectious diseases account for 29 out of the 96 major causes of human mortality and morbidity and about 25% of global deaths (i.e., 14 million) per year. Of the 1,415 species known to be pathogenic to humans, 217 are viruses and prions, 538 are bacteria and rickettsia, 61% are zoonotic (i.e., transmitted between humans and animals), 33% are transmissible among humans, and only 3% have humans as their main reservoir [26]. Thus, there is an abundance of potential pathogens that can be exploited. However, not all are of high consequence.

The properties that make microorganisms useful biological weapons have been defined (Ecker et al., unpublished data; [27, 28]). The most important criteria include (1) accessibility; (2) culturability; (3) capability for large-scale production; (4) stability during preparation; (5) ability to retain potency during transport and storage; (6) ability for dissemination; (7) stability and retention of potency after dissemination; (8) incubation period; (9) infectivity; (10) lethality; (11) pathogenicity; (12) toxicity; (13) transmissibility; and (14) virulence.

The Centers for Disease Control and Prevention (CDC) (and others) (http://www.cdc.gov/agent/agentlist.asp, http://www.niaid.nih.gov/biodefense/bandc_priority.htm, http://www.cbwinfo.com/Biological/Bacteria.html) has identified and categorized biologic agents that potentially could be used as weapons based on the criteria of public health impact, ease of dissemination or transmission, requirements for public health preparedness, and social disruption (Table 3). CategoryA contains the microorganisms of highest concern. They are easily disseminated or transmitted from person to person, can cause high mortality, have major impact on public health, and cause substantial social disruption. Category B contains microorganisms that are moderately easy to disseminate and cause moderate morbidity, but usually low mortality. Category C contains the emerging pathogens that could be engineered for mass dissemination, are available, are relatively easy to produce, and have potential for high morbidity and mortality. While Categories A, B, and C constitute only a small subset of the 1,415 known human pathogens, the task is still daunting for the forensic scientist to characterize these microorganisms at the same level that has been achieved for humans in forensic science.

Humans are not the only potential targets. Agriculture and food are extremely tempting targets because of the economic, social, and political impact ([29–34], D Franz, personal communication, Kansas State University). In many countries, societies take the robustness and security of their food supply for granted. Every country relies on its agriculture to feed its people, and a number of countries have large sectors of the economy based on agriculture. For example, the US food and fiber industry generates nearly $1 trillion in revenue annually [35]. Both pre- and postharvest times are susceptible to attack.

Food-borne diseases are another potential manifestation of infectious agents and thus can be potentially used for bioterrorism and biocrimes. Each year in the US, there are

Table 3 CDC high-consequence pathogens and toxins

Category A	*Bacillus anthracis* (anthrax)
	Yersinia pestis (plague)
	Variola major (smallpox)
	Francisella tularensis (tularemia)
	Clostridium botulinum toxin
	Hemorrhagic fever filoviruses (Ebola and Marburg fever)
	Hemorrhagic arenaviruses (Lassa fever, Junin, Machupo)
Category B	*Brucella* spp.
	Burkholderia mallei (glanders)
	Burkholderia pseudomallei (melioidosis)
	Coxiella burnetti (Q fever)
	Cryptosporidium parvum (protozoan) (cryptosporidiosis)
	Escherichia coli O157:H7
	Salmonella spp.
	Shigella spp.
	Vibrio cholerae
	Chlamydia psittaci (psittacosis)
	Rickettsia prowazekii (typhus fever)
	Alphaviruses (Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis)
	Epsilon toxin (from *Clostridium perfringens*)
	Ricin (from *Ricinus communis*—castor bean)
	Staphylococcus enterotoxin B
Category C	Hantavirus
	Nipah virus
	Tick-borne hemorrhagic fever viruses
	Tick-borne encephalitis virus
	Yellow fever
	Multi-drug resistant tuberculosis (*Mycobacterium tuberculosis*)

~76 million cases of food-borne illness, including ~300 thousand hospitalizations and 5 thousand deaths per year due to naturally occurring food-borne illness (in the US alone) [36]. This natural food-borne pathogen background of disease can confound recognition of an intentional, covert attack.

Compared with weapons for human targets, less technical sophistication is needed for generating a delivery system at agriculture or food targets. In some cases, a perpetrator may only need a readily available infectious agent and not even need a dissemination device. As an example, all one needs to do to attempt to spread foot-and-mouth disease virus is to place manure from an infected cow on his/her shoes and walk through a herd of cattle. A nonendemic plant pathogen could be introduced by placing infested plant debris in contact with a targeted crop. A fairly simple dissemination method was used by cult followers of Baghwan Sri Rajneesh who in 1984 attempted to affect the outcome of a local election in Dalles, OR. They attempted to incapacitate the population and to prevent them from voting by successfully contaminating salad bars in 10 restaurants with *Salmonella typhimurium*; 751 people developed food poisoning [14]. No sophisticated equipment was needed to disseminate the microbe; it was simply sprinkled on food.

Based on economic impact, or because they are zoonotics, the pathogens listed in Table 4 are of the highest consequence for animals (http://www.aphis.usda.gov/ppq/permits/agr_bioterrorism/, http://www.aphis.usda.gov/vs/ncie/pdf/agent_toxin_list.pdf). For plant pathogens, the USDA priority threat pathogens are displayed in Table 5 (http://www.aphis.usda.gov/ppq/permits/agr_bioterrorism/). Obviously, the important threat pathogens for plant and animal agriculture will be different from country to country and will

Table 4 USDA high-consequence pathogens and toxins

Bacillus anthracis (anthrax)
Burkholderia mallei (glanders)
Burkholderia pseudomallei (melioidosis)
Cowdria ruminantium (heartwater or cowdriosis)
Coxiella burnetti (Q fever)
Brucella spp.
Coccidioides immitis (fungal) (true systemic (endemic) mycoses)
Francisella tularensis (tularemia)
Mycoplasma capricolum (contagious caprine pleuropneumonia)
Mycoplasma mycoides mycoides (contagious bovine pleuropneumonia)
Akabane virus
African horse sickness virus
African swine fever virus
Avian influenza virus
Blue tongue virus
Camel pox virus
Classical swine fever virus
Eastern equine encephalitis virus
Foot-and-mouth disease virus
Goat pox virus
Hendra virus
Japanese encephalitis virus
Lumpy skin disease virus
Malignant catarrhal fever virus
Menangle virus
Newcastle disease virus
Nipah virus
Peste des petits ruminants virus
Rift valley fever virus
Rinderpest virus
Sheep pox virus
Swine vesicular disease virus
Venezuelan equine encephalitis virus
Vesicular stomatitis virus
Bovine spongiform encephalopathy agent
Clostridium botulinum toxin
Epsilon toxin (from *Clostridium perfringens*)
Shigatoxin
Staphylococcal enterotoxin
T-2 toxin

Table 5 USDA high-consequence plant pathogens

Plum pox potyvirus
Liberobacter africanus and *L. asiaticus* (citrus greening)
Ralstonia solanacearum race 3, biovar 2 (brown rot)
Xylella fastidiosa (citrus-variegated chlorosis)
Xanthomonas oryzae (bacterial leaf streak of rice)
Sclerophthora rayssiae var. *zeae* (brown-striped downy mildew)
Peronoscleroppora philippinensis (Philippine downy mildew of corn)
Synchytrium endobioticum (potato wart)
Phakopsora pachyrhizi (soybean rust)

depend on the crops and animals raised, the endemic pathogens, and the economic impact.

These high-consequence pathogen lists help focus research efforts on target organisms, but inadvertently may misdirect resources from developing forensic signature assays on other likely and devastating pathogens that may be used as bioweapons. For example, the exotic plant pathogens listed in Table 5 are those not currently found within the borders of the US. However, there are many endemic plant pathogens that cause serious economic damage, are readily accessible, and could be used in a biocrime or bioterrorism act such as *Tilletia indica* (karnal bunt), *Xylella fastidiosa* (Pierce's disease of grapevines), *Ustilago maydis* (maize smut), *Xanthomonas axonopodis* var. *citri* (citrus canker), to name a few. For forensic purposes, the number of potential bioweapons is greater than those on threat lists.

Bioengineering should also be cause for concern and could be appealing to some bioterrorists. Many of the Category C pathogens could be modified by engineering. Using recombinant technology, microbes can be readily modified such that they can become more infectious or pathogenic and/or they may be made resistant to current treatments.

Of great concern is the ability today to synthesize or enhance pathogenicity of a virus. The poliovirus ENRfu53 has been synthesized [37]. Although this is one of the smallest viral genomes known, the capability exists and suggests that even eradicated viruses, such as smallpox, can be recreated someday in vitro, particularly since the genomes of many disease-causing viruses have been sequenced and are readily available.

Forensics

Bioterrorism and biocrime fall into two categories, i.e., cases where the crime is overt or where the crime has been perpetrated covertly. In an overt attack, the perpetrator announces that an attack is imminent (or has occurred) or the attack is obvious. An overt attack could be as straightforward as a package containing a biologic agent placed in a public place. Law enforcement, and thus forensic scientists, will become involved immediately, taking the lead in the investigation. Public health may be contacted by local officials before (or simultaneously) alerting law enforcement because of potential health risks. A covert attack will be identified by the unlikely occurrence of a disease in a single individual or several people (or animals or plants). Public health will take the lead and contact law enforcement if an attack is suspected. Thus, the partnership between public health and law enforcement is critical.

Forensic microbiological investigations are essentially the same as any other forensic investigation regarding processing. They involve crime scene(s) investigation, chain of custody practices, evidence collection, handling and preservation, evidence shipping, analysis of evidence, interpretation of results, and court presentation. Without recognition of the crime scene, which could extend over a wide area and involve more than one site, detection of the disease and its etiologic agent, and identification of physical evidence of an attack (or a credible admission), an intentional incident may not be suspected or discovered for some time, if ever. Fortunately, the public health sector has developed a sentinel system and implemented linked databases to identify outbreaks faster and better than ever before [Public Health Laboratory Information System (PHLIS), http://www.cdc.gov/ncidod/dbmd/phlisdata/default.htm; US Department of Agriculture's Animal and Plant Health Inspection Service (USDA APHIS) http://www.aphis.usda.gov/vs/index.html; WHO Department of Communicable Disease Surveillance and Response (CSR) http://www.who.int/csr/en/; 38–44]. Effective surveillance systems enable timely dissemination of information. A thorough understanding of the normal occurrence of diseases is imperative for informed investigation of suspicious outbreaks. Determining routes of exposure will also aid in the interpretation of suspicious outbreak. A pathogen could be disseminated by aerosol, by dermal exposure, by ingestion, or by vector (such as an insect). Because public health officials will likely be involved prior to law enforcement in many cases, they should be trained on critical aspects of crime scene investigation to include evidence collection and chain of custody. This situation begs for the interoperability or even integration of microbial forensics and epidemiology.

Current forensic science laboratories are not equipped to handle dangerous pathogenic samples and contaminated traditional physical evidence. They do not have Biosafety level 3 or 4 containment facilities to handle pathogens safely, and most forensic scientists are not trained in microbiology practices. Therefore, partnerships will need to be developed in which the forensic laboratory will likely serve as a case coordinator, and a laboratory with expertise and equipment will be engaged to carry out the necessary analyses. However, such microbiology laboratories will not have the requisite experience for analyzing traditional forensic evidence. Because any traditional forensic evidence associated with the microbiological evidence could be contaminated, a forensic interoperability program should be developed where forensic scientists, properly trained, can work in the specialized containment facility. In addition, a biocontainment facility may have to be modified to accommodate the types of evidence that may be received. It is not possible to place an automobile under a safety hood.

The Federal Bureau of Investigation (FBI), for example, will partner with the newly instituted Bioforensics Analysis Center (BFAC) as the primary federal microbial forensic

laboratory. The BFAC is part of the National Biodefense Analysis and Countermeasures Center (NBACC) of the Department of Homeland Security. It will reside on the Interagency Biodefense Campus at Fort Detrick, Frederick, MD [15, 22]. The laboratory will have a Knowledge Center (Hari et al., unpublished data) composed of databases of genomics, microbiology, forensic methods, associated materials, and related evidence analysis methods and will foster strong partnerships among existing government, academic, and private sector assets. These partnerships will include those with Plum Island Foreign Animal Disease Laboratory, the Department of Defense, the Department of Energy National Laboratories, the Department of Health and Human Services, the National Science Foundation, specialty technology laboratories, and other centers of excellence. An advisory arm for the BFAC (the Scientific Working Group on Microbial Genetics and Forensics—see below) will address current and emerging challenges and opportunities. Because of the demands of a dynamic microbial forensic program, it will take an international effort to be responsive, comprehensive, and anticipatory. Development of international partnerships is necessary and underway.

Methodology

There are a number of recent outbreaks of emerging and recurring diseases, and some acts of bioterrorism and biocrimes that have occurred in the past few years (http://www.usamriid.army.mil/content/BioWarCourse/HISTORY/HISTORY.html; [1–14]) that could serve well as the basis for case studies, to include investigations of cholera or food-borne disease outbreaks. Such studies should be carried out to better understand crimes and crime scene investigation. Once a crime (and scene) is identified, evidence collection and analysis ensue. There are existing protocols for sample collection and handling, analytical methods, epidemiology practices, and chain of custody procedures for attribution (http://www.bt.cdc.gov/lrn/factsheet.asp; [45–47]). Yet, to date, many of these have not been rigorously validated for microbial forensics applications. Further, better capacity and flexibility are needed to address next-generation attack(s) more effectively. End-to-end retrospective analyses of past cases would be extremely beneficial to determine how to better establish a robust microbial forensics program and how to improve sample processing. Such analyses may provide guidance for expeditiously differentiating between natural and intentional outbreaks, and how best to interface epidemiological and forensic investigations with law enforcement and national and international security investigations.

The proposed review of past cases should comprise everything that could have facilitated sample identification and selection, collection, handling and preservation, method selection, casework analysis, selection of new methods, interpretation and communication of results, validation, and quality assurance. Typically, particular parts of the analytical process tend to be controlled more so than others (such as a DNA-typing protocol), but there is still no comprehensive validated set of those methods and processes. For example, if identification, collection, handling, and preserving the samples are not performed efficaciously, subsequent analyses could be compromised. Biological specimens must be handled properly to preserve bacterial, viral, or toxin viability and integrity.

Each case could present a unique scenario, with regard to the types, quantities, and matrices of the evidence such that no single-sample and no single-recovery procedure will apply. Thus, a "tool kit" approach is warranted. Yet, some general practices need to be formalized and fully validated. Also, flexibility in protocols should be considered such as with sample types to be encountered, not all scenarios can be predetermined. Stringent standard operating collection and preservation protocols may be too restrictive and actually hinder effective sample collection. Bulk collection processes have different demands than collecting trace materials.

The collection of live microbes requires different strategies and practices than that for dead microbes. In fact, some collection and storage procedures may actually kill the microorganism, thus rendering the sample unculturable. Culturing, when possible, can be used to identify the microorganism, as well as propagate material, so that forensic genetic attribution analyses can be conducted. Toxins will require altogether different protocols.

All evidence collection procedures need to be developed with the intent, wherever possible, to preserve traditional forensic evidence, such as hair, fibers, fingerprints, human DNA, documents, and computer media. Decision trees should be built to assist the crime scene investigator and analyst in making the most judicious decision for selecting a course(s) of action for various circumstances. Lastly, investigators will have to don protective equipment that will limit the time one can devote to crime scene evidence collection; thus, collection methods may have to be expedient with the triage of samples carried out subsequently under biocontainment conditions. Some proven processes exist, yet they may not have been designed or validated for maximum or efficient evidence recovery. The areas of sample selection, collection, and preservation should be a main focus of microbial forensics. Biocontainment conditions and procedures must also apply to the analytic processes that target both the biothreat agent and contaminated traditional evidence.

Over the past 20 years, the forensic community has concentrated its efforts in forensic biology on molecular biology analysis. Developments in the field of molecular biology make possible forensic analyses once not thought feasible. DNA/RNA typing will figure prominently in the cadre of analytical tools for microbial identification and characterization purposes. Further, the extraction and recovery of minute quantities of nucleic acids from dilute samples and complex matrices are very significant issues. In addition, because the PCR is fundamental to most molecular biology assays, it will be necessary to remove inhibitors that copurify with nucleic acids. Inhibitors are likely to be in many samples due to environmental contamination. Many of the same methods currently used by DNA-typing forensic laboratories will be useful for extracting

small amounts of DNA and mollifying the effects of PCR inhibitors [48, 49]. Another significant issue is sample throughput per case, especially if culture repositories are to be analyzed.

It is not surprising that similar approaches to those used for forensic human DNA analysis and those of molecular epidemiologists tracking outbreak sources are being pursued to trace the source of microorganisms whose genetic material is present in forensic samples. These include microsatellite and minisatellite loci typing, SNP analyses, sequencing, and real-time PCR. The multilocus VNTR analysis (MLVA) technique for *B. anthracis* strain identification is analogous to the multiplex STR-typing procedures employed for human identification [50] (Fig. 1). The more stable canonical SNPs for defining *B. anthracis* strains use the same real-time PCR assays used for human SNP detection and quantification of human DNA [51, 52] (Fig. 2).

The goal in a forensic analysis is attribution, the desire being to individualize a sample so as to uniquely identify the source. One needs to be cautious when conveying source attribution of a pathogenic agent as having the same the meaning as that of a genetic "fingerprint" often applied to human DNA analyses. Unique genetic identification of a microorganism may never be possible because of the clonal nature of many microorganisms, the considerable genomic and ecologic dynamics of microbes, the less than optimal population and phylogenetic data, and, in some instances, the limited historical and epidemiological information. Nonetheless, by understanding the limits of the analyses and data, interpretations of genetic evidence can be made. Qualitative statements may suffice and may be all that is needed for some scenarios.

Consider the attempt by the Aum Shinrikyo cult (known for its sarin nerve gas attack in a Tokyo subway) in 1993 to spread anthrax in Tokyo from the roof top of an eight-story building owned by the Cult. The purported attack did not cause any illness from the anthrax agent. The spores were subsequently analyzed using a MLVA. The profile was compared to a database of *B. anthracis* strains and isolates, and the strain was determined to be the nonvirulent vaccine strain Sterne [53]. In this case, the knowledge of the strain was sufficient for the investigation to determine that the strain was relatively harmless. A more recent example using the same MLVA is the determination that the *B. anthracis* spores from the 2001 attack in the US were the Ames strain. Simply knowing that the strain in the letters is rare in nature but common in the laboratory focused, the investigation process toward laboratory sources.

Qualitative results from a genetic analysis can be useful in past case investigations resolving weapons treaty violations. In 1979, in Sverdlovsk (Russia), at least 66 people died of anthrax. The official Soviet government position was that the victims were infected by eating contaminated meat. Much later after the event, a MLVA demonstrated that four different strains of anthrax were present in preserved tissue specimens from some of the victims. Multiple strains in a sample were inconsistent with the government's claim of consumption of tainted meat. The epidemiology also did not support gastrointestinal anthrax, but supported the proposition that the disease was inhalational anthrax due to an accidental aerosol emission from a secret military weapons facility [54].

When genetic profiles from microbial evidentiary and reference samples are compared, a variety of questions can arise [55, 56]. These include: What might be deduced about the source of the evidentiary sample? Are the samples from the same source or lineage? Are the genetic differences too few to conclude the samples are from different sources (or different lineages)? Are these differences sufficient to consider that the samples are from different sources? Is it possible that the two samples have a recent common ancestor? What are alternative explanations and interpretations for the results obtained? The degree that these questions can be addressed depends on the context of the case and exploitable information from the sample. In some situations, the pathogen preparation could be maintained under controlled conditions, such as laboratory stocks, and may show little diversity compared with samples found in nature. Thus, one or two genetic differences between evidentiary and reference samples may be significant if the weapon originated from a laboratory-maintained culture. Alternatively, some genes in bacteria (e.g., *E. coli* and *Salmonella*) have elevated mutation rates (~100-fold) due to defects in mismatch repair, presence of mutator genes, etc. [57–60]. Thus, two recently linked laboratory-maintained culture isolates may differ at these rapidly evolving sites and yet may not be excluded as belonging to the same lineage. Such knowledge as general source (i.e., laboratory, nature), stability of genetic sites or elements, storage conditions, mutagenic treatments, etc. can further refine interpretations and conclusions.

Genomics is emerging as a powerful tool for improving forensic preparedness and response to the needs of an in-

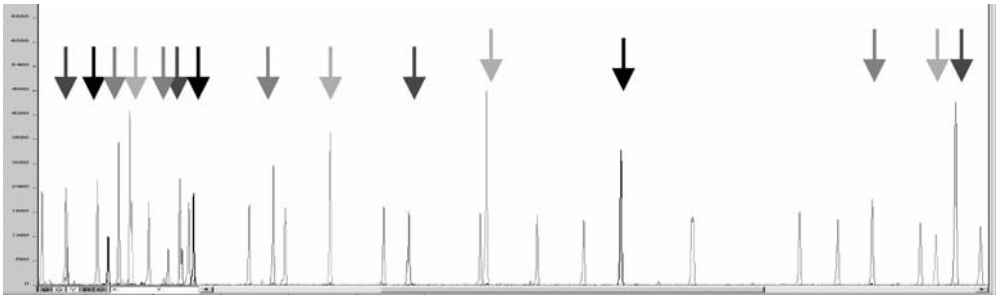

Fig. 1 A multi locus VNTR profile of the *Bacillus anthracis* Ames strain. There are 15 loci analyzed in this electropherogram (indicated by *arrows*). The other peaks are from an internal reference. Kindly provided by Paul Keim (Northern Arizona University)

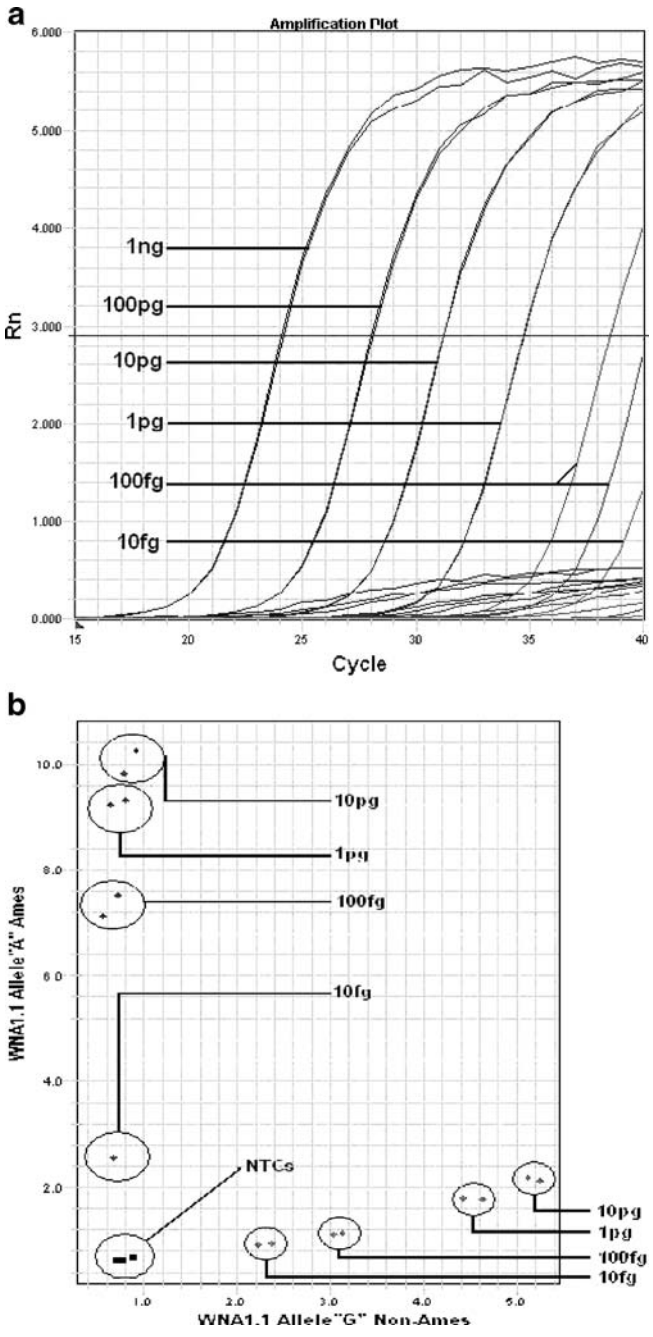

Fig. 2 **a** A real-time PCR plot from an assay utilizing TaqMan minor groove-binding probes for a SNP specific for *Bacillus anthracis* Ames strain. Two probes, labeled with different fluors, were designed for the SNP and combined into a single-assay run on an ABI 7900. The assay can detect the target over a wide range and has sensitivity near 10 fg (almost single-template molecule detection levels). Kindly provided by Paul Keim (Northern Arizona University). **b** TaqMan end-point Allele Determination Plot displaying results of interpretation of fluorescence detection in each sample following PCR amplification. The *plot* displays an analysis of the SNP specific for detection of the *B. anthracis* Ames strain. Samples along the *Y*-axis (*blue circles*) amplified because they contain the Ames-specific SNP state. Samples along the *X*-axis (*red circles*) amplified because they carry the SNP state found in other strains of *B. anthracis*. Samples near the origin (*black circles*) are negative controls. Kindly provided by Paul Keim (Northern Arizona University)

vestigation. There are complete genome sequences of multiple well-chosen representative strains of most threat-level biological agents. These are available to scientists designing diagnostic assays or for interpreting evidentiary results. An important finding from genomics is that many pathogens are remarkably similar to their nonpathogenic relatives, suggesting that intimate genomic knowledge of near neighbors will be needed to distinguish pathogenic from nonpathogenic strains [61–63] and for assessing a positive signal in an environmental sample. In addition, environmental samples may contain microbial communities of which some species may exchange genetic material [64–66] including markers used in diagnostic assays or forensic analyses.

Genomic technologies, such as DNA sequencing and bioinformatics tools, are constantly being improved. It is now possible to sequence, assemble, and analyze a bacterial genome to draft quality in less than a week [67]. Still, technologies that can reduce the time and cost of sequencing, particularly of large genomes, are needed. One of the challenges in a forensic investigation is to identify the genetic marker(s) that distinguishes one sample (or lineage) from another and provide the most useful information toward attribution. This is not a trivial exercise for a bacterial or fungal pathogen that has a relatively large genome. Very rapid, intelligently tailored sequencing would be a more viable and efficient approach to pursue for investigative purposes of this sort; but it does not yet exist.

Typically, repeat sequences are investigated because they evolve rapidly compared with, for example, SNPs. However, at times, the repetitive elements may not be sufficiently (or optimally) informative especially for extensive lineage tracing. Without DNA sequencing (be it be by traditional sequencing or by chip technology), finding rare mutational events is almost impossible. Also, a forensic genomic "toolbox" tailored for forensic analyses and comparisons is needed, which is well founded on microbial molecular evolution and tightly coupled with bioinformatics. User-friendly analytical tools that enable the posing and answering of pertinent questions and proper interpretation of results obtained should be developed and validated. Such an analytical toolbox would be useful for determining the set of informative sites (markers), or set of alternatives, with their respective power to answer specific questions. Examples of tools could be: (1) attempting to find the most genetically affine group of organisms that resemble the evidence sequence (by its single/multimarker profile) and placing a degree of confidence or uncertainty on the "matching" result. These will depend on the density with which the database has been populated, the type and number of markers used, or the length of the sequence examined; and (2) detecting signature profiles that relate diversity to function (such as virulence). While such software packages exist, they currently are applicable only under constrained evolutionary scenarios. Sequence data generally will require development of effective lineage-based approaches for reconstructing evolutionary history; gene conversion/recombination approaches will likely require using coalescence-based approaches [68]. Sequence alignment needs to

be considered, as well as phylogenetic algorithms, incorporating features such as presence of repeat motifs (of variable length), heterogeneity of recurrent as well as unique substitution rates, recombination, gene conversion, and population factors (e.g., demographic changes of population size and sympatry/allopatry of sister taxa) (Table 6).

DNA typing alone may not enable identification to the level of uniqueness or at least to a very limited number of putative sources. Therefore, chemical and physical analyses of microbial forensic evidence may increase the potential to achieve attribution [70]. Matching of sample properties can help establish the relatedness of disparate incidents, and mismatches might have exclusionary power or signify a more complex etiology of the events under investigation. A main issue, which is the same for traditional forensic analyses, is how to prioritize such measurements in the face of a limited sample material and how to render samples safe for handling in the analytical laboratory. Reference databases will be extremely helpful and increase the power and utility of chemical and physical analyses.

Forensic scientists often analyze materials for comparison purposes to eliminate them as potential sources of the evidence. However, chemical characterization of a microorganism or its matrix may assist an investigation by providing information regarding the processes used to grow the pathogenic agent. Thus, it may be possible to determine one or more plausible "recipes" by which the material was made or constructed. Such information may indicate the sophistication of the perpetrator, how recently the materials were made, provide signatures if certain unique or unusual materials were used, and suggest whether the process lends itself to producing large or small quantities of the agent. A systematic understanding of the many different possible recipes and processes for generating agents is needed. In addition to more standard methods, bioterrorists are likely to utilize information from a broad range of sources including open scientific literature, the internet, underground "cookbooks", and information that has been reported by the news media; thus, these recipes should be characterized as well (S. Velsko, Lawrence Livermore National Laboratories, personal communication).

In many imaginable scenarios, the only agent samples available for analysis will be those recovered from contaminated surfaces, ductwork, or filters from building air-conditioning systems, or material on the filter units used in urban air samplers. Such samples may be admixed to varying degrees with other contaminating materials. Thus, the analyst will be required to identify and isolate the agent particles from a complex mixture. There are a number of instruments that can measure chemical and physical properties of trace quantities of an agent, even single-agent particles (such as X-ray diffraction, scanning electron microscopy, and secondary ion mass spectroscopy). Generalized techniques need to be developed for mounting and handling samples to permit the use of different instruments to analyze the same particle.

A number of non-DNA-based approaches to consider for the microbial forensic panoply of assays have been described [71] and are listed in Table 7. Such analyses and the combination of results from nucleic acid and other methods, integrated with traditional analyses on associated contaminated evidence, will extend the ability of the microbial forensic scientist to attribute a sample.

Table 6 Components of a bioinformatic genetic toolbox for microbial forensics[a]

(1) Algorithm(s) for DNA marker alignment encompassing pattern heterogeneity of the various types of genetic markers and for detecting genes such as for pathogenicity and antibiotic resistance
(2) Phylogenetic algorithm(s) for clonal and sexually inherited markers, recombination, gene conversion, and horizontal gene transfer
(3) Capability to identify informative markers and their power to address specific forensic issues
(4) Better understanding of mutation rates and the effects of environment and host on these rates
(5) Discrimination and match criteria to quantitatively interpret results with confidence bounds
(6) Capability to relate diversity to function
(7) Capability for comparative and functional genomics
(8) Contain or access curated (genetic marker) databases on pathogens and near neighbors and their background occurrence with epidemiological history, when available
(9) Data management with the capability to access and process large amounts of diverse genetic data and to communicate data rapidly with stringent informational security (i.e., fully functioning information interoperability)

[a]From Hari (unpublished data); [29, 45, 55, 56, 69]

Table 7 Non DNA-based tools for the microbial forensics toolbox

(1) Characterization of physical attributes acquired during preparation
(2) Isotope analyses to approximate the age and source
(3) Physiologic methods (e.g., fatty acid composition, phage typing, serotyping)
(4) Analysis of growth media and media components adhering to the microorganisms
(5) Analysis of stabilizers and additives used in the preparation of a sample
(6) Identification of incidental biocontaminants, such as environmental pollen and fungi, for location and time of year of preparation
(7) Better understanding of bacterial endemism for identifying unique strains that may exist in only one location or few locations
(8) Monitoring changes in the immunological response of a host to a pathogen or toxin such as temporal IgG and IgM responses
(9) Improvements in immunoassays (and antibodies) for more effective rapid detection and field deployable assays

Quality assurance

Typically when a field is developing, scientists first concentrate on developing technology and only after that is well established are quality assurance (QA) practices considered. The forensic science community is no stranger to the importance, facets, and intricacies of a robust QA program. For microbial forensics, it was deemed important to establish some QA guidelines from the outset because of "lessons learned" from traditional forensics and their potential impact on documented biocrime and bioterrorism cases. Although epidemiologists have been using practices that are analogous to those used in forensics, there were no universal standards or general QA guidelines available.

To lay a proper QA foundation for the field of microbial forensics, the practice employed for human DNA forensic analyses was followed and a scientific working group known as the Scientific Working Group on Microbial Genetics and Forensics (SWGMGF) was created. This working group provides a vehicle for scientists from diverse disciplines, within the government and academia to come together in a collaborative, peer-consensus atmosphere to address issues and to develop guidelines related to the practice of microbial forensics. The SWGMGF has published QA guidelines for laboratories performing microbial forensic casework analyses [22, 72]. The general categories are typical of those in a forensic DNA QA program: References, Scope, Definitions, Organization and Management, Personnel, Facilities, Sample control, Validation, Analytical procedures, Equipment calibration and maintenance, Reports, Technical review, Proficiency testing, Corrective action, Audits, Safety, and Subcontractors. It is not necessary herein to describe all features of the QA guidelines because many are quite familiar to the forensic science community. Instead, only two issues are discussed to focus on the unique demands of a microbial forensics program: preliminary validation and subcontracting.

It is well accepted that new analytical methods, as well as those currently implemented, need to be validated. This requirement provides information on the performance and limitations of a protocol and provides confidence regarding the results obtained from an analysis. However, because there are many potential pathogens and toxins and myriad possible attack scenarios, it is unlikely that a microbial forensic laboratory will have validated standard operating protocols to cover all possibilities. Thus, some biological crimes and terrorism acts will require analysis by methodologies that may not have undergone the rigorous review process to the level of standard operating protocols. To effectively respond to demands and expectations for public safety and security, it will be imperative that investigators make use of these techniques. If the results of one of these less validated procedures are used for other than investigative leads, then a preliminary validation assessment should be carried out.

Preliminary validation is the acquisition of limited test data to enable an evaluation of a method to be used during a biocrime or bioterrorism investigation. One approach to acceptably achieve a preliminary validation is to convene a panel of experts, in some instances with proper security clearances, to assess the utility of the rapidly developed method, and to define the limits of interpretation and conclusions. Such an approach has been employed in the field of human DNA forensics for victim identifications in the disasters at the twin towers of the World Trade Center in New York caused by the terrorist acts of September 11, 2001.

As stated above, it is likely that traditional forensic laboratories will engage in partnerships to carry out microbial forensic analyses and thus will be subcontracting biothreat agent analyses. All laboratories, both principal and affiliates, should operate under the established QA guidelines, where applicable. These encompass the service rendered as well as the certification of compliance. Before analyzing evidence, the contract laboratory procedures must be reviewed and the integrity of the data received must be verified. For legal proceedings and QA, maintenance and documentation of chain-of-custody practices, information management, and security will be established. The subcontracting laboratory should notify the contacting laboratory in writing of any technical personnel changes in a timely (agreed upon) fashion.

The SWGMGF QA guidelines will be implemented in the national microbial forensics laboratory system, other partner laboratories, and subcontracted laboratories, where applicable. Each laboratory partner should demonstrate compliance and be audited.

Conclusion

In 2001, the US was subjected to an act of bioterrorism in which *B. anthracis* spores were delivered via the mail system. The mechanism of dispersal was simplistic and demonstrated our vulnerability to bioterrorism and biocrime. The attack spawned a massive response with the expectation of being better prepared for future incidents. Yet, some have asked, and still ask, why there is so much concern about bioterrorism. After all, there was only one attack in the US resulting in only a few deaths and illnesses. As described above, the anthrax letters are not the only cases to have occurred where a bioweapon has been employed. History is replete with examples from as far back as the Assyrians and Romans using dead carrion to poison enemy water supplies to developing nations using pathogens in the 20th century warfare to health-care workers assaulting acquaintances using accessible hospital strains (Table 1). In the 21st century, the concerns are greater. Terrorists are more committed, and they have expertise and readily available resources, knowledge, and technologies for developing bioweapons that can inflict massive damage or death on unsuspecting and unprotected societies. Because such events have occurred many times before, it is likely that bioterrorism, biocrime, and biowarfare will occur again; pathogens are readily accessible, and technology is making their use as a weapon more feasible. As part of the response for preparedness, it is imperative to establish robust microbial forensic capabilities.

An effective program will require development and/or validation of all aspects of the forensic investigative process, from sample collection to interpretation of results. Moreover, there will be a need to rely on other existing and emerging capabilities beyond the traditional forensic laboratory and its practitioners. To ensure that requisite quality is performed when analyzing evidence and the results will be admissible in court or accepted by senior government decision and policy makers, it is necessary that the engaged laboratories carry out QA and QC practices comparable to what have been embraced by the forensic DNA science community.

Forensic science provides a special investigative role particularly in the criminal justice system and, at times, can be significant in supporting or refuting hypotheses generated by parties involved in an investigation. Moving forward, forensic science applied to illicit biology should be extended to national and global security. The challenge today is to apply our knowledge and forensic capabilities to the characterization of bioweapons for attribution. There will always be more that could be known about each microorganism and process used to generate the material. Presently, any data collected and validated on these agents will greatly improve the practice of microbial forensics. Yet, much more needs to be done. With proper appreciation of the limitations of the tools available currently and intelligent design, appropriate validation, and application of a suite of tools and methods, microbial forensics can serve as an effective witness in the resolution of a biocrime or bioterrorism act. Forensic scientists worldwide should contribute to the field of microbial forensics and enhance its capabilities to aid in bringing perpetrators of these heinous attacks to justice.

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