Role of Law Enforcement Response and Microbial Forensics in Investigation of Bioterrorism

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The risk and threat of bioterrorism and biocrime have become a large concern and challenge for governments and society to enhance biosecurity. Law enforcement plays an important role in assessing and investigating activities involved in an event of bioterrorism or biocrime. Key to a successful biosecurity program is increased awareness and early detection of threats facilitated by an integrated network of responsibilities and capabilities from government, academic, private, and public assets. To support an investigation, microbial forensic sciences are employed to analyze and characterize forensic evidence with the goal of attribution or crime scene reconstruction. Two different molecular biology-based assays – real time polymerase chain reaction (PCR) and repetitive element PCR – are described and demonstrate how molecular biology tools may be utilized to aid in the investigative process. Technologies relied on by microbial forensic scientists need to be properly validated so that the methods used are understood and so that interpretation of results is carried out within the limitations of the assays. The three types of validation are preliminary, developmental, and internal. The first is necessary for rapid response when a threat is imminent or an attack has recently occurred. The latter two apply to implementation of routinely used procedures.

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The use of bioweapons throughout history has been well-documented (1,2 and references within); however, it was the delivery of *Bacillus anthracis* through the US postal system in 2001 that raised to a new level public awareness of the need for biosecurity (3,4). The risk and threat of bioterrorism and biocrime are greater concerns today because of the relative ease of acquiring and preparing a pathogenic organism and the recognition that methods of dissemination need not be sophisticated or complex. These concerns for biosecurity have been exacerbated due to the recent natural outbreaks of Severe Acute Respiratory Syndrome (SARS), avian influenza, and monkeypox. Yet, the letter attacks of 2001 and subsequent exaggerated reactions by the public revealed the need for law enforcement organizations to be able to respond to such an attack more effectively to identify the perpetrator of the crime, to exclude those not associated with the crime, to interdict, to deter, and to prevent future crime, and to maintain public safety and security.

This paper describes the role of law enforcement in investigative and assessment activities involved in a bioterrorism or biocrime event. Then an overview is presented of how the field of microbial forensics can assist investigations by analyzing and characterizing forensic evidence, with the goal of attribution or crime scene reconstruction, and includes some of the challenges to meet that goal. For the analytical part, two very different molecular biology-based assays are described: real time PCR (RT-PCR) (5-11) and repetitive element PCR (rep-PCR) (12-19); these procedures are used to exemplify how molecular biology tools may aid in an investigative process. The former approach offers high throughput, sensitivity, specificity, and resolution. The latter method is less likely to be used routinely but is more generic in application, offering a tool useful in characterizing evidence when genomic signatures are not known, and is available as a commercial kit. Finally, the need for validation is stressed, to ensure that the methods used are understood and that interpretation of results is carried out within the limitations of the assays and existing supporting data.

Role of law enforcement

The use or potential use of biological agents or their by-products as weapons to inflict harm or to terrorize creates biological security risks (Figure 1). Law enforcement must prepare to address these risks by taking established approaches and adapting them. This is based on an appreciation of novel issues that arise with the growth and preparation of microorganisms, the preparation of toxins, the various approaches for weaponization and dispersal of biothreat agents, and the use of synthetic biology. Because of the dual use of biotechnology, ease of access to technology and information, and low cost of development, it may never be possible to detect all threats and to prevent anti-societal actions. However, to best meet the needs of biosecurity, governments and the public should work together and take responsibility to prevent and minimize threats arising from the malicious use of microorganisms. Increased awareness and early detection of these threats are keys to facilitating investigation and attribution as well as deterring some potential criminals. Foremost, an integrated network of responsibilities and capabilities should be de-

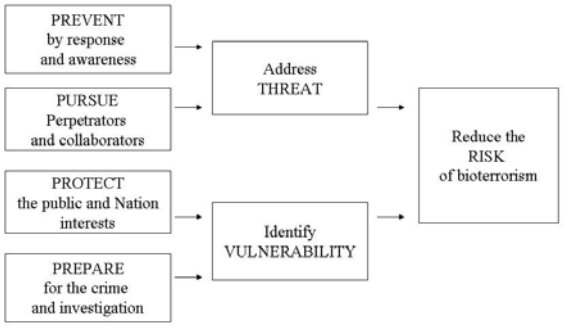

Figure 1. Flowchart for law enforcement for identifying and reducing risk from bioterrorism and biocrime.

veloped from government, academic, private, and public assets. Primary to this process is that the legitimate use of biotechnology is promoted and that beneficial developments in these areas are not deterred by excessive oversight by law enforcement.

In the United States, the Federal Bureau of Investigation (FBI), Department of Justice, is the lead agency responsible for investigating acts of domestic bioterrorism (20). Briefly, any actual or threatened use of a disease-causing microorganism or biological material (such as a toxin) directed at humans, animals, plants, or materiel is regarded as a crime. The possession of a biological agent, toxin, or delivery system that cannot be justified by a prophylactic, protective, *bona fide* research effort, or other peaceful purpose, can result in arrest, prosecution, fines, or imprisonment (21). Thus, today in the United States, the burden of proof has shifted to the defendant to prove that possession of the material was justified. The Select Agent Rule (22) defines those particularly harmful microorganisms (and in some cases nucleic acids) and toxins that are monitored and restricted (Table 1). These are considered to be of the greatest threat; but genetic engineering and synthetic technology may in the future broaden the threat list. Moreover, it does not matter whether the perpetrator actually possesses the bioagent; the intent to obtain and use the bioagent is sufficient for arrest and prosecution (21). Thus, interdiction and prosecution can occur even for those attempting to develop a weapon or for those who perpetrate hoaxes.

If an alleged incident is brought to the attention of the FBI, an immediate response begins with a rapid and comprehensive assessment of the potential threat. A Threat Credibility Assessment is carried out to determine whether the threat is technically feasible and operationally practical. This assessment is carried out by consultation with other area experts from within the government (such as the Department of Health and Human Services – HHS, Centers for Disease Control and Prevention – CDC, the US Department of Agriculture – USDA), academia and industry. The results of the assessment are incorporated into critical decisions involving the deployment of FBI response assets, along with the request, coordination, and deployment of other US government assets, and the notification of state and local authorities.

Table 1. Center for Disease Control and Prevention high-consequence pathogens and toxins (21-23)*

Category A – microorganisms that are easily disseminated or transmitted from person to person, can cause high mortality, have major impact on public health, and cause substantial social disruption. The toxin in this category is very lethal:
Bacillus anthracis
Yersinia pestis
Variola major
Francisella tularensis
Clostridium botulinum toxin
Filoviruses (Ebola and Marburg)
Arenaviruses (eg, Lassa virus, Junin virus, and Machupo virus)
Category B – microorganisms that are moderately easy to disseminate and cause moderate morbidity, but usually low mortality. Toxins in this category can cause mortality.
Brucella spp
Burkholderia mallei
Burkholderia pseudomallei
Coxiella burnetti
Cryptosporidium parvum
Escherichia coli O157:H7
Salmonella spp
Shigella spp
Vibrio cholerae
Chlamydia psittaci
Rickettsia prowazekii
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 – emerging pathogens that could be engineered for mass dissemination, are available, are relatively easy to produce, and have potential for high morbidity and mortality.
Hantaviruses
Nipah virus
Tick-borne hemorrhagic fever viruses
Tick-borne encephalitis virus
Yellow fever virus
Mycobacterium tuberculosis

*These pathogens are those of concern for humans. There is a list of pathogens from the US Department of Agriculture for plants and animals (ref 1 and citations within).

Microbial forensics

If an actual event were to occur (or a potential threat was interceded), authorities would use national and international scientific technical capabilities to analyze evidence to assist in attribution of the bioagent to a source and/or reconstruction of the crime. To best exploit the value of fo-

rensic evidence, a robust microbial forensics field has been developed which is dedicated to the characterization, analysis, and interpretation of evidence for attribution purposes from a bioterrorism act, biocrime, hoax, or inadvertent agent release (3). Attribution in this context is the information obtained regarding the identification or source of a material to the degree that it can be ascertained (3,25). The ultimate goal of attribution is the identification of those involved in the perpetration of the event, which is necessary for criminal prosecution, to prevent additional criminal acts, or for useful background for national policy decisions and actions.

The field of microbial forensics is multidisciplinary, relying heavily (but not exclusively) on the foundations of traditional forensic sciences, epidemiology, microbiology, and molecular biology. Forensics laboratories have well-established procedures for all aspects of investigating crime scenes through the scientific analysis of evidence. Similar methods are being developed for microbial forensics cases. The goal is to develop an infrastructure so that microbial forensic evidence will be collected, stored, analyzed, and interpreted in a manner that is scientifically robust and thus legally defensible.

Intentional bioattacks can be classified as either overt or covert (1,26). The difference between them is that an overt attack is often recognized immediately; while a covert attack may not become known for some time, if at all. Covert biological attacks are by their nature more difficult to discover than are overt attacks. Complicating factors include the background of commonly occurring food-borne illnesses and endemic cases of emerging and re-emerging infectious diseases. Separating naturally occurring outbreaks from those that are the result of an intentional attack requires an in-depth understanding of common domestic pathogens and their epidemiologies. Therefore a significant challenge exists in developing awareness, surveillance measures, and methods of detection (1,27-29). The type of forensic information sought in such cases may evolve over the course of the investigation, but evidentiary collection is initiated at the first sign of an attack or potential attack. Indeed, the anthrax letters attack began as a covert attack and became an overt attack with the discovery of the anthrax tainted letters. Regardless, whether an attack is overt or covert, public (which includes agriculture) health officials will likely be the first ones involved. Law enforcement and microbial forensic scientist involvement would likely follow the discovery of a criminal act.

Once collected and preserved, the biological evidence is sent to a laboratory for analysis. Within the laboratory, a core group of analytical tools is available to aid in characterization of the evidentiary material. Because microbial forensics focuses on tracking and linking microorganisms to individuals and locations, different strategies may be implemented, depending on the nature of the attack and the type(s) of evidence collected. In an overt attack, for example, the package, the weapon, and associated materials, in addition to traditional forensic evidence (eg, hairs, fibers, fingerprints), may be analyzed. In a covert attack, the evidence may be more limited to medical histories, diagnoses, and isolates taken from victims. In the case of the 2001 attacks, a combination of these methods has been and is being utilized in the investigation. As such, an investigative analytical plan may involve many and diverse strategies (Figure 2).

While many methodologies are available, molecular biological tools figure prominently in the repertoire of the microbial forensic scientist. Comparative genetic sequence analyses of Select Agent pathogens and their near neighbors are an important part of the attribution process. Methods that enable recognition of nucleic acid signatures will be relied on for screening potential candidate sources to facilitate an investigation. There are a variety of genetic markers (Table 2) and methods that allow highly specific and accurate characterization of microbial diversity (30).

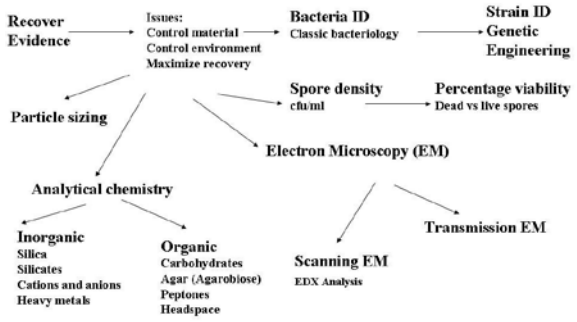

Figure 2. A hypothetical sample microbial forensic analysis flowchart for characterizing evidence from an overt attack. The flowchart will vary depending on the type, quantity and quality of the microbial forensic evidence. An alternate flowchart may be considered for traditional forensic evidence.

Table 2. Genetic markers that can be selected for analysis for attribution (29)

Genetic marker:
Single nucleotide polymorphisms
Repetitive sequences
Insertions and deletions
Mobile elements, including bacteriophage, insertion elements, transposons, integrons, and plasmids
Pathogenicity islands
Virulence and resistance genes
House keeping genes
Structural genes
Whole genomes

For forensic purposes, assaying rapidly evolving markers enables better affiliation to recent common sources, while more stable markers provide better lineage-based evolutionary interpretations, such as strain and sub-strain definition. Since bacteria, viruses, and some fungi reproduce asexually, their genomes are considered to be clonal and portions of their genomes may be very stable and uninformative for distinguishing samples. Therefore, it may not be possible to individualize the source of a sample by genetic analysis alone (as often is accomplished in human DNA identity testing). Since many microbial genomes have relatively short generation times, in an overnight culture, a single microbe could have reproduced its genome over a million times, increasing the chance of mutation that may be seen within the culture. Thus, some variation, and hence a forensic signature, may occur during asexual reproduction. However, there also may exist sexual reproduction, horizontal gene transfer, conjugation, transduction, lysogeny, gene conversion, mobile elements, recombination, reassortment, gene duplication, rearrangements, and mutational hotspots that can be exploited for the purposes of seeking forensic attribution (30,31).

The forensic comparison of a genetic profile from a reference sample with that of an evidentiary sample can have three possible general outcomes: "match" or "inclusion," "exclusion," or "inconclusive." With microbial genetic information, it is less likely to have a prescribed interpretation policy for what constitutes a match and what does not. Some questions may be difficult to answer unequivocally based on extant data (Table 3). Uncertainty is greater than what is experienced for human DNA identity testing because of unknown diversity, limited databases, unknown manipulations, and limited genetic testing. However, the power of microbial forensic tools is increasing rapidly with ever advancing technology. Still, the questions, such as those listed in Table 3, must be addressed to utilize the full extent of microbial forensic analyses.

Even with the current degree of uncertainty, capabilities exist that enable analysis and inter-

Table 3. Selected questions that genetic analyses might address (25,27,29,30)

What might be deduced about the nature and source of the evidentiary sample?
Is the pathogen detected endemic or introduced?
Do the genetic markers provide a significant amount of probative information?
Does the choice of markers allow the effective comparison of samples from known and questioned sources?
If such a comparison can be made, how definitively and confidently can a conclusion be reached?
Is it possible that the two samples have a recent common ancestor, or how long ago was there a common ancestor?
Can any sample be excluded as contaminants or recent sources of the isolate?
Are there alternative explanations for the results obtained?
Where does the divergence occur within an individual gene and what are the mechanisms of variation?
What mechanisms are responsible for the genetic changes that are detected (to include horizontal gene transfer, gene conversion, recombination, gene duplication, random mutation, mutation hot spots, mobile elements, etc.)?
Have host selective pressures contributed to the genetic composition of a sample?
What are the effects of laboratory stresses and manipulation on genetic variation?
Is there evidence of genetic engineering?
Can natural mutational events be discerned from creative or subtle genetic engineering?

*The degree to which these questions can be addressed depends on the context of the case and the available knowledge of the genetics, phylogeny, and ecology of the target microorganism.

pretation of results that can be meaningful for investigation purposes. At times, interpretations of genetic data can be made qualitatively (such as strain identification, ref. 1). Typically, qualitative statements are made based on characteristics that are shared or not shared between evidentiary and reference samples. Genetic analysis methods used by epidemiologists to track infectious disease outbreaks employ interpretation guidelines to evaluate the results and to determine case relationships in epidemics (32). Forensic analyses can often use the same or similar methods but may require additional criteria, such as identification of individualizing characteristics for higher resolution source attribution.

Technology

Methodologies are available to enable attribution to the level possible based on existing genetic data. Samples may be viable microorganisms and, if culturable, there will be available copious material for forensic analyses. For non-viable materials, the challenge is greater. The evidence can be limited in quantity and quality, sometimes being partially or totally degraded, contaminated with inhibitors and/or other background microorganisms from the environment. PCR analysis will figure heavily in the molecular analyses of such genetic evidence because of the exquisite sensitivity and specificity that those particular assays afford. Producing PCR amplicons of lengths of 60-80 base pairs make it more possible to type highly degraded samples. The markers most suited for analysis with small length amplicons are Single Nucleotide Polymorphisms (SNPs). A variety of methods is available for SNP detection (30); the one described briefly below is RT-PCR (5-11).

Real-time PCR

The PCR approach used for many assays is designed for end-point analysis. Thus, the PCR products are examined at the "yield plateau" of the reaction. Endpoint assays, although effective, tend to have limited dynamic range, are at best only semiquantitative, and tend to be laborious in that they require several manipulations. The RT-PCR assay involves continuous monitoring of the generation of PCR product, particularly during the linear reaction phase. This provides both qualitative identification and quantitative estimation of target amounts in a single tube by calibrating reaction kinetics to known standards (6,7,11). The TaqMan® PCR assay (8,10), a 5′ nuclease based approach, makes RT-PCR facile and reliable. This assay relies on the fluorescent detection of unquenched reporter dye moieties from displaced digested probes (by Taq polymerase) that were hybridized to a target sequence of interest. The fluorescence signal should be proportional to the hydrolyzed probe within the region defined by the primers in any one cycle of the PCR, and this is directly correlated with the amount of PCR product.

For SNP detection, probes can be designed to identify specific SNP states such that any non-complementarity between the probe and the template will disrupt the duplex conformation and probe hybridization, so that digestion is less favored (5). The use of minor groove binder (MGB) probes has greatly enhanced the performance of RT-PCR SNP assays because the T_m (melting temperature) of a probe is increased, as compared with equivalent non-modified probes (9). The stability allows for MGB probes to be shorter and yet still achieve an acceptable probe T_m. The benefits of shorter probes during RT-PCR are: 1) specificity is enhanced because a single mismatch will more likely destabilize the probe/template duplex and 2) fluorescent signal-to-noise ratios are improved because the efficiency by which the fluorescence of unhybridized probe is quenched is directly related to the distance between the quencher moiety and reporter dye. Shorter probes tend to allow for a closer proximity of the reporter dye to the quencher moiety, thus reducing background fluorescence

without affecting the signal yield that results after cleavage. Other modifications, such as use of Peptide Nucleic Acid molecular beacons, have been developed to enhance RT-PCR parameters for genotyping (33).

The primary benefits of the RT-PCR assay are:

- Extremely low limits of detection (approaching single copy detection);
- Inherent specificity that enables design of highly discriminating assays that are capable of distinguishing closely related molecular species;
- A broad dynamic range of quantification (up to 7 orders of magnitude);
- A single step reaction contained within a closed tube, which reduces manipulations and laboratory contamination; and
- Rapid automated detection in a 96-well or 384-well format.

For most diagnostic applications, a SNP is a stable marker likely to have occurred only once in the phylogenetic history of the species (particularly at the population level). This stability can be invaluable for defining strain groups, such as major phylogenetic divisions, as well as specifically defining a particular terminal branch strain (eg, *B. anthracis* Ames strain). Moreover, when available, multiple SNPs along a branch provide the same phylogenetic information but are diagnostically redundant. When multiple SNPs that define a branch are available, then an assay can be optimized by choosing the SNP that contributes to the most robust (based on sensitivity and reproducibility) assay. Alternatively, multiple SNPs may be needed to increase confidence in interpretation when knowledge on population data and diversity are somewhat limited.

In addition to their use as phylogenetic research tools, RT-PCR SNP assays also have been developed for microbial forensic application (4,34-37). The most notable have been SNP assays designed for identification of *B. anthracis* strains/isolates (34,38-40). Thousands of SNPs have been discovered in *B. anthracis* through whole genome sequencing (40). Indeed, Keim et al (34,41) have identified a number of SNPs that can define major genetic groups in *B. anthracis*. These diagnostic "canonical" SNPs (canSNPs) identify particular phylogenetic points in the evolutionary history of *B. anthracis* (1). Both Amplified Fragment Length Polymorphisms (AFLP) (42) and Variable Number of Tandem Repeats (VNTR) (43) markers provided a phylogenetic framework across several major clades (38,43) for assessing the informative value of potential canSNPs (against a diverse set of 26 strains from a collection of more than 1300 *B. anthracis* isolates). One representative SNP marker from each of the major evolutionary branches was then selected as the defining SNP for that clade (Figure 3). Finally, each canSNP candidate was tested against the strain collection to support or refute the validity of its canonical designation (34).

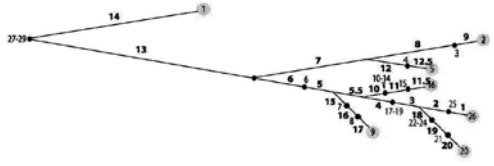

Figure 3. Phylogeny based upon SNPs with branch designations. Branch designations are indicated in bold text and strains are comparable to previous phylogenetic hypotheses (33). Highlighted numbers refer to strains in reference 33.

Six SNPs specific for the *B. anthracis* "Ames" genetic cluster (ie, the strain identified in the anthrax letters attacks of 2001) were identified. Four chromosomal SNPs (designated Br1-7, Br1-26, Br1-28, Br1-31) and one plasmid SNP (designated PS-52 on pXO2) are diagnostic for the Ames strain (34) (Tables 4 and 5). Another

Table 4. *B. anthracis* Ames specific Single Nucleotide Polymorphisms and genome position

Ames Specific SNP	SNP Δ	Genome position	GENBANK accession No.
PS-1* (SNPO1)	A-G	7452	NC_003980
PS-52 ($SNPO_2$)	A-C	72924	NC_003981
Br1-7	C-A	433277	NC_003997
Br1-26	T-C	4624132	NC_003997
Br1-28	T-G	4929186	NC_003997
Br1-31	G-A	2749543	NC_003997

*PS-1 does cross react with isolates closely related to Ames.

Table 5. Ames specific Single Nucleotide Polymorphisms compared to close genetic relatives (33)

	SNP profiles for selected isolates of *Bacillus anthracis*						
SNP	A0462 Ames	A1115 Texas	A1117 Texas	A0394 Texas Goat	A0728 China	A0584 China	A0488 Vollum
PS-52	A	C	C	C	C	C	C
Br1-7	C	A	A	A	A	A	A
Br1-26	T	C	C	C	C	C	C
Br1-28	T	G	G	G	G	G	G
Br-31	A	G	G	G	G	G	G
PS-1*	G	G	G	G	G	A	A

*PS-1 does cross react with isolates closely related to Ames.

SNP on the pXO1 plasmid (PS-1) shows slightly less specificity as it shares SNP identity with four other closely related strains (Table 5); but this SNP shows greater sensitivity of detection.

A dual probe allelic discrimination assay using RT-PCR (and MGB probes) has been developed for typing these canonical SNPs. The assays rely upon differential cleavage of allele-specific probes to score the SNP state of unknown *B. anthracis* DNA templates (Figure 4). The real-time and endpoint analysis is performed on an AB 7900HT instrument (Applied Biosystems, Foster City, CA, USA). Quantification of DNA by PicoGreen® (Invitrogen, Carlsbad, CA, USA) analysis has demonstrated that as little as 10 fg (approximately 1.6 genome equivalents) of starting DNA is sufficient to obtain robust and reliable real-time amplification results (based on the Poisson distribution). The Ames canSNP assays appear robust and have been successfully performed under a range of validation criteria (our unpublished results).

Repetitive element PCR for bacteria identification

Repetitive sequences have been found in many bacterial genomes (12-15). Some stable and conserved repetitive elements occur in lengths of 33 and 40 bp and comprise about 1% of the bacterial genome. This translates into 500 to 1000 copies per genome (16,17). Another repetitive element, known as enterobacterial repetitive intergenic consensus or ERIC, is 124 to 127 bp in length and occurs at 30 to 150 copies per genome (18). These repetitive elements are stable

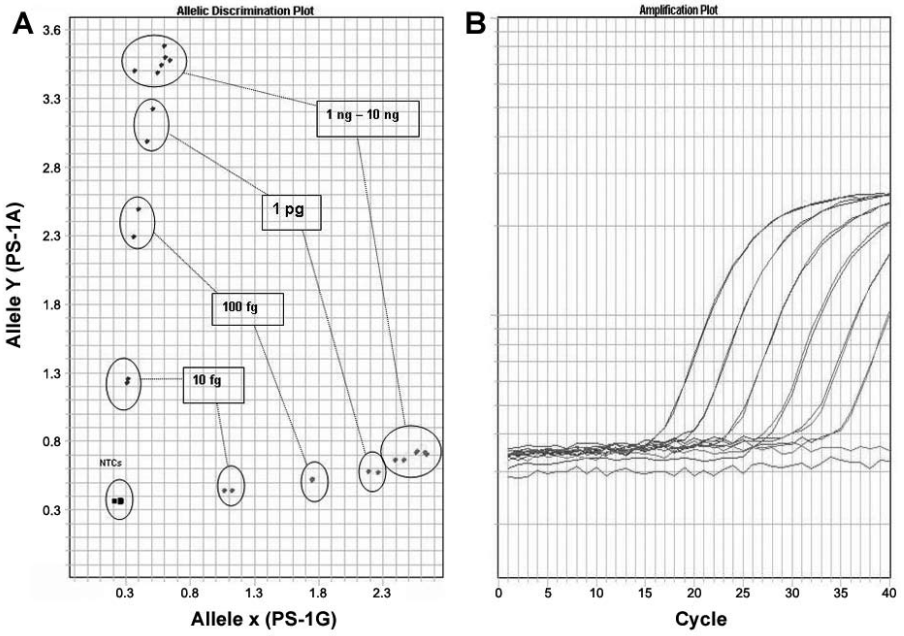

Figure 4. (**A**) The real-time PCR analysis of Ames strain template ranging from 1.0 ng to 10 fg. (**B**) The endpoint analysis of Ames strain (y-axis) and non-Ames strain DNA templates (x-axis) ranging from 10 pg-10 fg.

but differ in their copy number and chromosomal locations within and among bacterial species. This variation can be used for species, subspecies, and at times strain differentiation by a procedure known as rep-PCR (12-14).

The rep-PCR procedure exploits the variable distribution of these repetitive elements throughout the genome of the microorganism by using oligonucleotide primers complementary to the repetitive element(s) in a PCR amplification of the microbial genomic DNA target. Because of the variation in number and location of repetitive elements within a bacterial genome, many different length amplicons will be generated. The various length rep-PCR amplicons are fractionated by electrophoresis. DNA patterns from different isolates or strains can then be compared to enable some degree of resolution within and among bacterial species. Rep-PCR is not as random as is AFLP, since its primers target known conserved regions (19).

Commercially-available kits (Bacterial Barcodes, Athens, GA, USA) are available to facilitate analyses and offer increased complexity of the repetitive element profiles by using primer sequences for several distinct repetitive elements. Therefore, more fragments may be generated, resulting in better differentiation of strains.

In the United States, there were 33589 documented cases of *Salmonella* infection in 2003 (44). Thus, *Salmonella* is an epidemiologically important microorganism. This organism also has been used previously as a bioweapon to intentionally contaminate public food sources (2,45). Consequently, *Salmonella* is well-characterized (46-48) and is a good model for testing the efficacy of genetic tools for attribution. The rep-PCR analysis is not expected to be used routinely to examine *Salmonella,* but *Salmonella* typing can exemplify the degree of resolution that may be attained (49).

Figure 5 displays profiles from several strains of *Salmonella* that can be differentiated along with a tree derived from the data using Diversilab version 3.1 (Diversilab, Athens, GA) analytical software. Thus, by using this rapid assay some strain resolution is demonstrated. For example, the discrimination potential between *S. newport* and *S. infantis* is very good. However, four distinct strains of *S. typhimurium* have very similar rep-PCR profiles, making it difficult to differentiate these strains, and one strain of *S. infantis* is more similar to the *S. typhimurium* strains than to another example of *S. infantis*. Therefore, while the rep-PCR technique can generally distinguish differences between strains, the presence of the same profile may not provide strong evidence of common origin.

There may be times when an urgent need for a quick characterization of a pathogenic organism is needed; in such situations the rep-PCR may be one viable option. The advantages of the rep-PCR are that it does have some discriminatory power, can be implemented rapidly so results can be obtained quickly, does not necessarily require *a priori* knowledge of genetic signatures, and the cost of implementation and use is relatively low. The primary disadvantages are the lower discriminatory power of the analysis compared with more specific assays, and the fact that the rep-PCR process is more refractory to template quality and quantity. In contrast, the RT-PCR focuses on specific sites of high resolution for lineage or for forensic discrimination and is more likely to provide results from highly degraded and limited quantity samples. However, RT-PCR requires substantially more development time to identify the markers of interest and validate the methodology.

Validation

The reliability and proper interpretation of results rely substantially on understanding the performance and limitations of an assay. Validity and rigor are essential for the tools of microbial forensics, for determination of quality and for establishing confidence in results attained in anal-

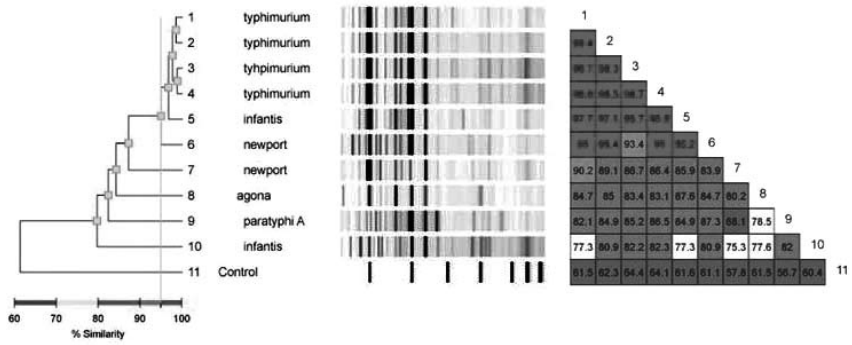

Figure 5. Rep-PCR profiles, relatedness tree, and similarity index for ten *Salmonella enteritidis* serotypes. Note lack of diversity in 4 typhimurium strains and greater similarity between an infantis strain and typhimurium strains than with another infantis strain. Also, note discriminatory power for two Newport strains and two infantis strains. All isolates were collected from unrelated events. The DNAs were kindly provided by Eugene LeClerc (FDA).

yses. Quality Assurance guidelines have been developed (3,25) and are similar to those adopted for human DNA forensic analyses (50). The section (Section 8 of the guidelines) related to validation is listed below:

8.1 The laboratory should use validated methods and procedures for analyses.

8.1.1 Developmental validation should be appropriately documented and should address specificity, sensitivity, reproducibility, bias, precision, false-positives, false-negatives, and determine appropriate controls. Any reference database used should be documented.

8.1.2 Preliminary validation is the acquisition of limited test data to enable an evaluation of a method used to provide investigative support to investigate a biocrime or bioterrorism event. If the results are to be used for other than investigative support, then a panel of peer experts, external to the laboratory, should be convened to assess the utility of the method and to define the limits of interpretation and conclusions drawn.

8.1.3 Internal validation should be performed and documented by the laboratory.

8.1.3.1 The procedure should be tested using known samples. The laboratory should monitor and document the reproducibility and precision and define reportable ranges of the procedure using control(s).

8.1.3.2 Before the introduction of a new procedure into sample analysis, the analyst or examination team should successfully complete a qualifying test for that procedure.

8.1.3.3 Material modifications made to analytical procedures should be documented and subjected to validation testing commensurate with the modification and have documented approval.

Validation is an essential process by which a procedure is evaluated to determine its efficacy and reliability for analysis. The fundamental categories of validation are developmental validation, internal validation and preliminary validation. Developmental validation is the acquisition of test data and the determination of conditions and limitations of a newly developed methodology for use on samples. Internal validation is an accumulation of test data within the laboratory to demonstrate that established methods and procedures perform within determined limits in the laboratory. These two types of validation are crucial for addressing the reliability and robustness of any method routinely implemented in the laboratory.

Preliminary validation, however, is not described in the human DNA forensic arena, but is essential when addressing biodefense and biosecurity. One cannot predict which microorgan-

ism, virus, or toxin will be used in the next attack (planned, attempted, or successful) or the next hoax. Yet, authorities will need to respond expeditiously to protect the public and the country's assets. If there are no established validated standard operating protocols to identify or characterize the bioweapon, it is incumbent upon the microbial forensic scientists and others to identify any tools that are available to assist in the investigation even if they have been used previously for research purposes only. It would be irresponsible to wait for months or years (as is done for human identity testing) for validation of an analytical procedure when an attack is under way and biosecurity is threatened. However, quality of the methods used and understanding the limitations of a methodology should not be overlooked. Therefore, the concept of preliminary validation was developed. Preliminary validation is the acquisition of limited test data to enable an evaluation of a method used to assess materials derived from a biocrime or bioterrorism event. The evaluation is based on peer review of extant data, typically by a panel of experts that determines the extent of use and limitations of the technology or methodology and makes recommendations of evaluations or studies that may be needed prior to processing evidentiary material or studies that may be carried out subsequent to analyses and results are obtained. The goal is to be able to respond expeditiously, effectively, and efficiently while maintaining scientifically valid and rigorous approaches.

Validation of a procedure includes addressing many parameters. Some of these are listed in Table 6. While not all parameters may be applicable for any one assay, they should be considered to ascertain whether they apply. By using appropriate validation criteria, the reliable conditions can be determined for the methodology or for the interpretation of the analytical results. In addition, conditions can be defined under which the results or the standard interpretation are not valid.

Table 6. Minimum validation criteria (24,50,51)

Sensitivity – is the minimum amount or concentration of analyte required to generate a reliable result
Specificity – is the ability to measure the intended target analyte or signature
Reproducibility – is the closeness of agreement between the results of successive measurements of the same analyte under similar but not necessarily identical conditions
Precision – is the degree that measurements are similar
Accuracy – is the degree that the measured material or analyte is similar to its true value*
Resolution – is smallest difference between measurements that can be meaningfully distinguished
Reliability – is the closeness of agreement between the results of successive measurements of the same analyte under the same conditions of measurement
Robustness – ability of analytical performance under challenged conditions or when analyzing challenged samples
Specified samples – are those necessary to test the performance of the assay (eg, reference panels and mock or non-probative materials) corresponding with the intended application of the assay
Purity – is the quality required for extracted analyte to be analyzed
Input values – is the range of quantity of analyte that can be analyzed reliably
Quantitation – is the amount or concentration of material for input
Dynamic range – is the range of values or limits where precision is held
Limit of detection – is the lowest concentration of analyte that can be consistently detected
Controls – are defined test materials for the measured analyte (includes blind samples, negative and positive controls)
Window of performance for operational steps of assay – parameters such that slight analytical condition variation will not substantially affect performance or reliability
Critical equipment calibration – are those requiring calibration prior to their initial use and on a regular basis thereafter
Critical reagents – are determined by empirical studies or routine practice to require testing on known samples prior to use with evidentiary materials in order to prevent unnecessary consumption of forensic samples
Databases – a collection of data to be used to support interpretation of results

*The term "analyte" is used here in a broad sense and can range from intact viable microorganism to an ion.

Conclusion

Many nations are faced with the possibility of a biological attack, and discovering, attributing, and prosecuting these cases pose serious challenges for law enforcement. The best approach for preparedness to detect and/or respond to an event is an effective integrated network with government, academia, industry, and the public all contributing to this common goal. The field of microbial forensics has been implemented to enable better confidence in scientific analyses of forensic evidence and to gain greater confidence in the interpretations of the results obtained. Progress in microbial forensics is currently focused on a number of areas to address (52-54), including:

- Sample collection and preservation strategies,

• Novel methods and analyses for typing non-viable and/or trace materials,

• Development of validated methodologies that define the limits of an analysis,

• Identification of genetic markers and match criteria that can establish baselines for comparing reference and evidence samples, and

• Interpretation criteria,

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