

an unusual seasonal or geographic distribution, 6) a number of ill persons seeking treatment or obtaining medicine at the same time, and 7) unusual death or pattern of illness in animals or plants, among other indicators (4-8). Public health will initially be involved in the investigation from an epidemiologic perspective and law enforcement will be contacted only if an attack is suspected. Indeed, attending physicians and other health care related officials will likely serve as a nation's best biosensors in the detection of a covert attack. Thus, the partnership between public health and law enforcement is critical. Public health officials should be trained on important aspects of crime scene investigation to include evidence collection and chain of custody. Protocols need to be developed for these first responders, so the integrity of the evidence can be preserved as best as is possible. There is also a need to train law enforcement officials to understand the viewpoints of public health officials.

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. Moreover, there will be a need to rely on other existing and emerging capabilities beyond the traditional forensic laboratory and its practitioners. The challenge is to apply our best practices and capabilities to the characterization of bioweapons for attribution, even if they have not been through the extensive validation currently found in the forensic human DNA identification arena. For human forensic DNA typing, only one species was targeted, there are many practitioners, and a common core set of loci was sought to be able to exchange data and make national felon DNA databases effective investigative tools. In contrast, the specific method of analysis used in a microbial forensic investigation may be performed by a very few qualified individuals. Whereas, in principle, extensive validation is desirable for each method and marker system prior to use in a microbial forensics analysis, such requirements may not always be effective. In some instances, the "best" attribution markers will be discovered during the investigation of an ongoing case. Fortunately, the methodologies used to detect and apply the discovered genetic polymorphism(s) are well grounded in molecular biology and microbiology, so experience and a foundation for reliability will al-

ready be established. However, validation should not be discarded because of "on the fly" developments. The Scientific Working Group on Microbial Genetics and Forensics (SWGMEG) has proposed the concept of "preliminary validation," where acquisition of limited test data can be used to evaluate a method for assessing materials derived from a biocrime or bioterrorism event (9). The process would still rely on expert peer-review but in a more timely process. Even if not fully validated, scientific evidence can assist in developing an investigative lead. Ideally, evidence used for investigative leads would arise from a validated method and thus be admissible in legal proceedings. However, there is no requirement that only admissible evidence be used for investigative purposes (10). A parallel to this concept is the use of low copy number (LCN) analysis (11-13) in forensic human identification. Even though the assay may not always be reproducible and suffers from allele drop-in and allele drop-out, it has been used for investigative leads.

Basic Biology

The pathogens and toxins that can be converted to weapons can be derived from organisms from all kingdoms of life, with the majority arising from viruses, bacteria, and fungi. A brief description of microorganisms and toxins is provided to lay a basis for discussion. Also, because nucleic assays will figure prominently in the panoply of microbial forensic assays, genetics will be emphasized.

Viruses are parasites that infect all forms of life, from prokaryotes to eukaryotes, and are the simplest form of life. However, a virus cannot replicate without assistance of the metabolic machinery of its host. A basic virus is composed of a nucleic acid genome, typically carrying a minimum number of genes, which is contained within a protein coat (sometimes with an envelope). Some viral genomes are composed of DNA, and others are composed of RNA. Due to the lack of proofreading of the RNA polymerases and to the high error rate of reverse transcriptases (14,15), RNA viruses (such as human immunodeficiency virus [HIV], severe acute respiratory syndrome [SARS], influenza, foot and mouth disease [FMD]) exhibit high mutation rates, which result in genetically heterogeneous viral populations even within a host (14-20). Viral genomes are the smallest of all

known organisms, ranging in size from approximately 1 kilobase to approximately 800 kilobases (21). Therefore, in the forensic context, it is feasible to sequence the entire genome of a virus as a basic analytical characterization tool of the laboratory. In fact, databases on viral genome sequences are relatively well populated. There are at least 1,195 complete viral genomes represented in publicly available databases (21). These sequences will be invaluable for diagnostics development and interpreting genetic variation. However, it is anticipated that for routine forensic analysis of viral genetic evidence, sequencing of well-defined regions will be employed and sufficient, as is the case for biocrimes involving HIV. The number of species and overall degree of viral diversity remains unknown (22,23).

Bacteria are single celled free-living organisms that generally carry all the genes necessary for their life cycle. Like viruses, the number of species and the degree of diversity are unknown (24-26). A bacterium consists of genetic material, DNA chromosome(s), cytoplasm and a cell membrane. Bacterial genomes are larger and more complex than viral genomes and typically are a few million bases in length. Genes can also be carried on plasmids (extrachromosomal DNA) that replicate independently of the chromosome and can be transferred between bacteria. Genes for virulence and resistance factors may be found on chromosomes or plasmids. The variation among bacteria is immense and a number of genetic elements contribute to this variation (also found in higher level organisms). Insertions and deletions occur regularly and are responsible for most of the variation among bacterial species (21). A major class of elements is "pathogenicity islands." Pathogenicity islands, which are large blocks of self-mobile DNA, can be transferred from one bacterium and inserted into another. These islands carry genes that enable an organism to act as a pathogen and sequences that allow the islands to excise from one genome and integrate into a new host genome (21,27-30). There are also smaller gene packets, referred to as pathogenicity islets, which can carry major virulence determinants (21). Mobile elements, recombination, and gene duplication are major contributors to genetic diversity. Single nucleotide polymorphisms (SNPs) also occur throughout the genome in non-coding regions as well as in regions that can inactivate or modify

genes, another manifestation of the diversity in bacteria. It was once thought that the bacterial genome did not contain repetitive elements. Today, it is well known that many bacterial species carry microsatellites and minisatellites, similar to those exploited for forensic human DNA typing. Genomic variation is also enhanced by gene conversion, where pieces of repetitive and other sequences of DNA are shuffled around the genome to create unique gene sequences (21). Horizontal gene transfer is another method for increasing variation by transferring genetic elements between species. Lastly, variation can be obtained through viral DNA that integrates into the genome of a bacterium prophage (31,32). Sequencing of a bacterial genome is possible for forensic analyses, as was done in analyzing the *Bacillus anthracis* strain Ames from the anthrax letters (22). However, it is still costly and not practical to sequence an entire bacterial genome by the application-oriented laboratory, which may have to analyze several to hundreds of genomes in any one case, or when generating a database. Diagnostics for the foreseeable future will focus on specific informative sites. However, microarrays, or chips, offer the possibility for resequencing of bacterial genomes rapidly and at much lower costs than whole genome sequencing; thus, scanning genomes for forensically important genetic variants may become routinely feasible.

Fungi are eukaryotic organisms. Estimates suggest that 5 million species may exist (21,33). Whereas viruses and bacteria are the typical focus for disease, some fungi are harmful pathogens to plants (animals and humans as well). At least 8,000 fungal species are known to cause plant diseases, but less than 100 of these are known pathogens of humans (33). Many fungi are harmful because they produce toxins, such as mycotoxins. One cannot overstate the impact of fungi on the world economy; they cause over 80% of plant diseases and cost the world over US \$200 billion in lost products annually (21). Fungal genomes range in size from 10 to 240 megabases (Mb), with the average genome being approximately 40 Mb (33). The large genome size makes it difficult to sequence routinely. Thus, genetic analyses of fungi are unlikely to be solely based on sequencing technology; but when applied, it will likely be targeted against specific variable sites in fungal genomes.

Biologic toxins are molecules produced by an organism(s) that are poisonous to other species (34). Some biologic toxins, such as botulinum toxin, ricin (readily accessible from castor bean, *Ricinus communis*), staphylococcal enterotoxin B, and *Clostridium perfringens* epsilon toxin, are so potent that they are classified as threats on bioterrorism agents lists (35). Since toxins are not living organisms, they cannot be cultured to facilitate identification. Some preparations of the toxin may contain the genome of the organism from which the toxin was derived. Immunoassays have been used traditionally for identification of toxins, although mass spectrometry assays are beginning to be used.

HIV Biocrime Case Involving Genetic Analyses and Epidemiology

There will always be more that could be known about each microorganism and the process used to generate the material or a more informative assay to characterize the microorganism could be developed. This is no basis for not using available technologies, but instead should be an impetus to enhance capabilities. One approach to provide direction on closing knowledge gaps that exist in microbial forensics is to perform an end-to-end retrospective analysis of past cases to determine how to better establish a robust microbial forensics program and how to improve sample processing. Such analyses may provide guidance for developing a better interface between the epidemiological investigations and law enforcement activities, and show areas where microbial forensic scientists might focus their efforts on maximizing the amount of information that can be obtained and reliability issues. Below is an example of a past case demonstrating the interoperability of different disciplines and the application of a combination of existing techniques for a biocrime to the interpretation of microbial forensic evidence. The questions raised and answers obtained during the investigation, analysis, and legal deliberations at times can reveal reliability issues and suggest review of specific practices. Such an evaluation will help microbial forensic practitioners to be prepared by better understanding their processes and focusing resources on gaps and issues that may arise.

As in any investigation involving biologic evidence, there is a science component and the more traditional investigative or fact gathering component. Genetic analysis can assist in elucidating biological and forensic relationships to allow inferences to be derived. To illustrate, an investigation involving the human immunodeficiency virus type 1 (HIV) is described. HIV is an example of a natural disease-causing pathogen that, although not considered as a potential weapon for bioterrorism, has been used in the commission of biocrimes (36-40). Investigations in HIV cases routinely involve both gathering factual information (ie, epidemiological investigation) and genetic and/or serologic data. Because HIV is a rapidly evolving RNA virus (18,41-44), it is illustrative of some of the complexities in a genetic investigation that go beyond simple direct matching of DNA profiles. It is highly likely that two HIV samples even with a recent common origin will differ at a number of nucleotides within the genome. Also, since there are multiple HIV variants within a host, sequences from the donor may not exactly match those found in a direct transmission recipient. Initially, questions to be considered may be "are these two samples directly related" or more appropriately "could the two samples be related?" Such questions as to what constitutes a match (or dissimilarity) must be considered. Tools, such as phylogenetic analysis, at the disposal of the microbial forensic analyst and epidemiologist, will be useful in supporting or refuting relationships of isolates that have an alleged recent common ancestry compared with those isolates that do not. Bioinformatic tools already exist to assist in such genetic analyses (37,39-41,45).

It is unlikely that two recent origin samples will ever have the exact same RNA sequence. Based on evolutionary theory, similarity among genes, individuals, populations, and/or species is attributed to common descent or a relationship with a common ancestor (although convergent evolution may be considered at times, possibly when only one trait is evaluated). Therefore, even though direct matching, as employed in human DNA forensic analyses, is not meaningful for more rapidly evolving genes and organisms, there are methods available to assess the inherent variation in a sample (population) derived from a victim, alleged donor, and control samples.

Epidemiology and phylogenetics were used in the forensic case of the gastroenterologist Dr Richard J. Schmidt (RS), who was accused of second degree attempted murder of his paramour Janice Trahan (JT) by injection with HIV (37). In 1994, RS was accused of preparing a mixture of blood from two of his patients, one infected with HIV (DM) and one infected with hepatitis C (LL). JT had recently ended a 10-year extramarital relationship with RS, but they still continued to communicate. On August 4, 1994, RS purportedly gave JT a vitamin B12 injection. According to JT, that shot was more painful than previous ones administered by RS and from then on her health deteriorated. On January 3, 1995, JT learned that she was HIV-positive, and sometime thereafter, it was determined that she also suffered from hepatitis C. After the diagnosis, JT complained to the authorities, and RS was indicted for attempted second degree murder. A forensic/epidemiologic investigation ensued.

As a part of the epidemiologic/forensic process, the lifestyle of JT, the victim, was investigated. She reported a sexual history of contact with seven men, including the doctor RS. All provided blood samples and tested HIV negative. JT was a nurse, but had no documented needle sticks. She did report the splashing of saliva onto her skin by a HIV infected patient in the mid 1980's, but subsequently tested negative for HIV. In fact, JT was a routine blood donor and tested HIV negative annually up through early 1994. Thus, the investigation focused on the doctor and the alleged vitamin B12 injection. On August 2, 1994, RS's nurse drew blood from a hepatitis C positive patient LL and did not follow typical office procedure. A second incidence of improper blood drawing occurred on August 4, 1994, in which RS obtained blood from an HIV positive patient DM. According to LL, RS offered to draw a blood sample for his private research project involving hepatitis C and there would be no charge. The doctor's nurse obtained the sample from LL at the doctor's office. That was the only time a sample of LL's blood was drawn at the doctor's office. Typically, LL's blood was drawn at a local hospital to assure that the expense would be covered by her insurance.

The last entry in the doctor's patient log-book was a blood draw from the HIV positive patient on August 1994. The investigators' hypothe-

sis was that the blood from the HIV positive patient DM was the source of the HIV found in the victim JT. Blood was collected from the HIV positive patient, the victim, and control samples (ie, HIV positive individuals in the vicinity where the patient and victim reside – about 30 local control samples and two database samples). Sequence data were generated from two genes (gp 120 and RT) from HIV isolates from all the samples and subsequent phylogenetic analyses demonstrated a clustering of sequence types from the patient and victim. This supported the hypothesis that the HIV variants from DM and JT were closely related (37). Dr Schmidt stood trial and was found guilty of second degree attempted murder and is currently serving a 50 year sentence.

The admissibility of the phylogenetic analysis was challenged on the basis that there was no legal precedent for establishing similarities between the viral infections in different individuals. An admissibility hearing determination is usually an all or nothing proposition, ie, the evidence is usually deemed admissible or not. If the scientific evidence being challenged is critical and is excluded at this point, it may be necessary to dismiss the case. On the other hand, if the evidence is ruled admissible in spite of a pre-trial challenge by experts, those experts may still present their views during the ensuing trial.

At the conclusion of this pre-trial hearing, during which the competing experts testified, the trial court deemed the evidence admissible. This decision was reviewed by the appellate court before the trial took place (46). The Court of Appeal of Louisiana held that: 1) evidence that RS caused blood to be drawn from hepatitis patient LL without keeping proper records was admissible as other acts of evidence to show that the doctor had the opportunity to acquire tainted HIV blood with which he allegedly infected the victim; 2) the DNA test methodology used by the experts to conclude that the variants were closely related satisfied Daubert criteria (47) for admissibility of expert testimony; and 3) whether protocols applicable to each step of DNA testing process were properly applied was a question for the trier of fact rather than for the trial court, as part of its gate keeping function, where there were accepted protocols for each methodology applied in the testing process. An appeal was rejected by the Louisiana State Supreme Court in 2000 (Personal communication, B.

Korber Lawrence Livermore National Laboratory; S Sinha, Reliagene, New Orleans, LA).

This case demonstrates a number of issues that can arise in a microbial forensic case. Genetic analyses and traditional epidemiological investigation combined to develop support for the proposition that the gastroenterologist was the perpetrator and the weapon was HIV from a patient with no direct access to the nurse. It also points to the demands that may arise: the need for proper software and phylogenetic analyses, knowledge of the genetics of the microorganism, analysis of a large number of samples, and understanding and managing uncertainty.

Some limitations of the available or underlying knowledge that can arise in such an investigation (as may be in any criminal investigation) will impact on the level of certainty or uncertainty. For example, as in the case described above, the number of sexual partners reported by the victim relies on her veracity. She may or may not disclose all relationships. In this case, all reported partners provided a blood sample. But, suppose that some or all of the alleged partners would not provide a sample. Additional uncertainty may be unknown factors that can impact the analysis, such as the within-host sample size, the stage of disease, drug therapies, and drug effectiveness. Further, as in this case, a representative control population was based on limited sampling and those willing to participate. Metzker et al (37) exercised caution and did not exceed the bounds of permissible data analysis when interpreting the results. The genetic data could not demonstrate the route of transmission. A patient-to-victim route was equally likely as a victim-to-patient route. The epidemiology and probable contacts helped support the proposed transmission mode. Had the sample from the victim and DM been collected soon after infection, a phylogenetic analysis may have been able to shed light on the direction of transmission. A paraphyletic relationship of the victim's viral sequences with respect to the viral sequences of the patient sample might have elucidated whether a patient-to-victim transmission direction was possible. This relationship signature may be lost over time due to lineage extinction and substitutions accumulating in the viral genome. Additionally, the phylogenetic data could not determine if additional individuals were in-

involved between the transmission line of the patient and victim.

There were methodological challenges raised by the defense regarding the molecular epidemiological analysis. These included: separation of reagents and separate use of prepared reagents, documentation of storage of sample aliquots and the specimen, sufficiently detailed statements of the protocols used so that they could be reproduced or understood by any individual with ordinary skills within the field, and consideration of contamination, either by ruling it out or detecting a potential source of contamination. The defense also argued that the local control samples should have been derived from individuals who had the same sexual preferences as JT and who were infected at approximately the same time. Thus, the sampling regimen and database were argued to be inadequate. Although methodology issues were raised, the components of the methodology were deemed to be based on valid methods, and thus the procedure used for this case was admissible. We do not address in this paper whether the methodology had flaws or could be improved (obviously any method no matter how reliable can be improved); we simply point out that the challenges to methodology are similar to those raised regarding the admissibility of human forensic DNA analysis.

A second challenge was on the interpretation of the genetic evidence. The defense experts challenged that the RNA genome mutates so rapidly that any testing would be unreliable – the samples compared were drawn eight years apart. The supporting experts stated that phylogenetic analysis is a theoretically sound, widely used methodology and comparisons of samples up to 15 years apart can provide meaningful results. Another challenge was that more analyses should have been carried out and more data should have been collected. Also posed was that while the molecular epidemiology did use phylogenetic analysis, it also should include obtaining additional information for use in the final analysis. An epidemiological investigation includes interviewing the individuals who submitted samples in the study to determine any sexual partners, the lifestyle of the subject and partners, potential risks for contracting HIV in the workplace, and other risk factors, such as a blood transfusion. Obviously, much epidemiological information was collected. The court held

that epidemiological investigation is a factual investigation and not a scientific one. Thus, the findings would only go to the weight of the conclusions (37).

A typical issue often raised in science admissibility challenges revolves around the question that the science is attempting to answer. Through its scientific evidence, the government proposed to demonstrate that two HIV samples (from JT and DM) were "closely related" and not that they were identical. In other words, the genetic data were just another piece of the circumstantial evidence puzzle. In objecting to the evidence, the defense attempted to re-characterize the goal as one of identity and because of the high mutation rate of the HIV RNA genome, the defense claimed that the current method was incapable (and thus flawed) to state that the samples were identical. The foregoing illustrates, that in any use of scientific evidence in a legal proceeding, it is important to state the question to be answered clearly and maintain focus on that issue.

Conclusion

We can expect similar challenges to microbial forensic evidence. It is a defense attorney's responsibility to challenge such evidence and to attempt to create doubt, even if the attorney is personally convinced of the client's guilt. In the courtroom, one may exploit the standard practice of science "to question" as lack of consensus, even if most, if not all, agree that the approach is reliable. The issues raised in *LA v. Schmidt* and other such cases (36,38-40,48) are not new. But they do focus on issues particularly relevant to microbial forensics. There will be a number of well-validated methods applied to analyzing microbial evidence. These should pass muster. However, because of the myriad possible pathogens that may be used as weapons, the methods used most likely will be validated "on the fly." The components of the methodology will likely be well validated and used for some analysis and target microorganism or toxin. The difference will be the newer target.

The SWGMGF can assist by developing requirements for the many labs providing microbial forensic services as was done with the SWGDAM for human forensic identity testing (3,4). Such processes will play a significant role factor in ensuring that newer forms of microbial forensic methods will meet scientific requirements

and thus also will pass legal admissibility challenges. Peer review, general acceptance, accreditation, and/or the formal approval of the SWGMGF are not necessarily prerequisites to a favorable legal admissibility determination, but they do provide a solid basis for the scientific community, as well as a court's determination that there is enough confidence in the proffered evidence to allow a jury to hear it.

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