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Preventing the Sixth Plague: Microbial Forensics in the War against Terrorism

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As thesis advisor for Amanda Mickley

I have read this paper and find it satisfactory.

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Abstract

Bioterrorism utilizes viruses, bacteria, fungi and toxins to cause mass sickness or death in people, animals, or agriculture. The use of bioterrorism may be ideal for terrorists, because these agents are difficult to detect, and people may not become sick for a few hours or even days, allowing for the ability to cover their trail. Microbial Forensics is an up and coming field which utilizes the basic concepts of forensic biology in the application of pathogens. Some of the current technologies include rapid response hand-held bioassays, and genomic sequencing of the pathogens. Advanced technologies in the field are currently being further developed to investigate bio-crimes and bioterrorism threats such as the post 9/11 anthrax attacks. The field is vital to the prevention of bioterrorism and the conviction of bioterrorists. Since it is a relatively new field, standards need to be set to make it a valid practice, so it should be improved and further invested in for the sake of national security.

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Introduction

Bioterrorism utilizes viruses, bacteria, fungi and toxins to cause mass sickness or death in people, animals, or agriculture[2]. It is used with the intent of debilitating a nation by killing or incapacitating ones enemy. Most commonly the agents used in bioterrorism are already found in nature, but they are often modified to cause more severe illness, or to prevent them from being cured by medication[2]. Biological agents can be spread through a variety of different mediums, including the air, food, and water[2]. The use of bioterrorism may be ideal for terrorists, because these agents are difficult to detect, and people may not become sick for a few hours or even days, allowing for the ability to cover their trail[2].

In the 14th century bioterrorism was used by the Tartar army to besiege the city of Kaffa[3]. The bubonic plague broke out among the troops of the Tartar army in 1346[3]. Once soldiers started to die, in an attempt to spread the disease onto the residents of the city, the survivors would catapult the dead bodies of soldiers over the walls into the city[3]. The fleas which spread the disease on the bodies could therefore spread to the residents of the city causing an epidemic[3]. The people of Kaffa then evacuated the city to get away from the plague, leaving it open for the Tartar army to take over[3]. Bioterrorism was also seen in the times of ancient Egypt. The Bible tells of God's demand for the Israelite slaves to be freed by the Pharaoh[3]. When the Pharaoh did not comply, God sent upon Egypt ten deadly plagues[3]. The sixth plague was Shkhim, and was caused when Moses sprinkled soot into the air[3]. Shkhim is a skin disease which is most commonly described as one which causes black boils all over the body (see Figure 1) [3]. Many believe that Anthrax was the cause of these boils because the spores can be spread in a similar fashion [3]. Whoever the true writers of the Bible were, this story shows their knowledge of or experiences with biological warfare. Stories such as this show the destruction that biological

weapons can cause.

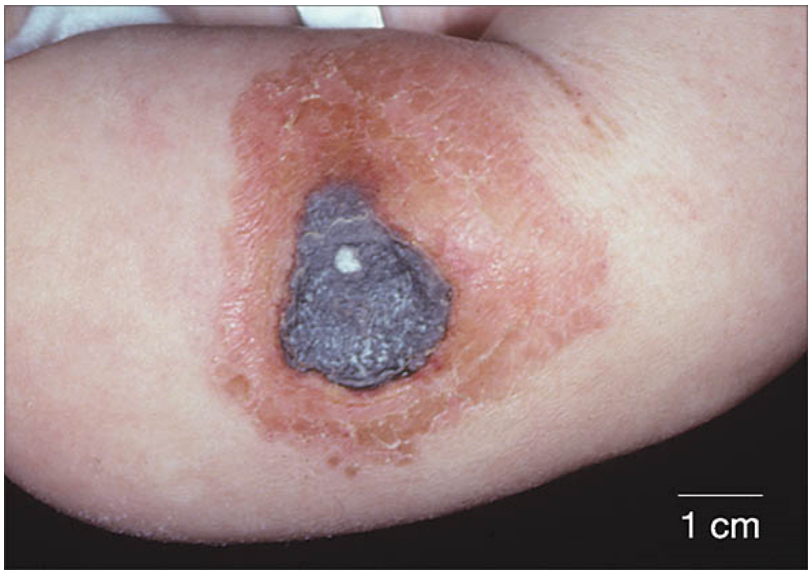

Figure 1: Lesion of Cutaneous Anthrax[14].

Bioterrorism is not solely part of the past. In September of 2001 anthrax spores were sent in about 5 letters through the mail within the United States Postal Service to several different locations in Florida, New York City, and Washington D.C.[4]. All of the letters came from Trenton, New Jersey and from these mailings, twenty-two cases of anthrax infection were found, five of which resulted in death of the victim[4]. It was found that the anthrax in the letters were of the Ames strain, with each letter containing approximately 2 grams of powder containing the anthrax spores[4]. This powder is reported to contain between 100 billion and 1 trillion anthrax spores per gram[4]. If more letters were sent out at this time, to places all over the United States, the casualties could have been devastating.

Another recent case which used microbial forensics in the investigation was the 1990 human immunodeficiency virus (HIV) exposure to five patients from a Florida dentist[6]. The first

patient to be identified with acquired immunodeficiency syndrome (AIDS) had no risk factors to be infected by the virus except for exposure through the dentist[6]. She and two of the other patients whom were believed to be infected by the dentist were tested with DNA sequencing[6]. All three of these patients had strains of the virus which were closely related to that of the dentist[6]. It is unlikely that they contracted the virus from another source, because their strain does not match the strain which is of people of that geographic area[6]. The second patient was an older woman, whom also did not have any risk factors for HIV[6]. She had no history of drug use, blood transfusion, or other illnesses compatible with a retroviral syndrome[6]. Her husband, with whom she was married to for more than 25 years, did not test positive for the HIV antibody[6]. The third patient had multiple sexual partners, but all tested negative for HIV, while the fourth and fifth patients had numerous risk factors but no proven infection before a visit to the dentist[6]. Through DNA sequencing and statistics, it was found that there was a "low probability ($p=0.006$, Wilcoxon rank-sum statistic) that the HIV DNA sequences from patients 1, 2, and 3 would be closer by chance alone to the sequence from the dentist than to the sequences from the eight controls indicates that the viruses from patients 1, 2, and 3 are significantly more similar to the dentist's virus than to the viruses of the controls" [6]. Also, an extremely unique pattern called a signature sequence of amino acids encoded by V3 nucleotides was found in patients 1, 2, and 3, and was also in the dentist's strain of HIV[6]. The evidence found in the case strongly suggests that the first three patients were infected by the Florida dentist with HIV[6]. A summary of the evidence is as follows, 1) no prior exposure to HIV, 2) all three of the patients had invasive procedure performed by the HIV infected dentist, 3) the DNA sequences are extremely close, and distinct from other persons with the sickness[6]. Use of microbial forensics helped to expose unsafe practices at this dental office, and allowed for the Center of Disease Control to look closer

at safe practices in places such as dentist and other medical offices[6].

Microbial forensics utilizes the identification and individualization techniques used in forensic biology to solve human criminal cases, to the application of microbial substances. It "combines principles of public health, epidemiology and law enforcement to identify patterns in disease outbreak, determine which pathogen may be involved, and trace the organism to its source"[9]. Microbial Forensics is an up and coming field which utilizes advanced technologies to investigate bio-crimes and is currently further developing these technologies to be better suited for bioterrorism threats in the future.

The Origins of Microbial Forensics: Forensic Biology

The main goal in forensic biology is to take a sample left at the scene of the crime, identify what it is (blood, semen, saliva, ect.), and determine who left this substance at the scene. By determining who left this sample behind, it may lead to answering more questions about the crime or bring the investigators to who committed the crime. This is done by DNA (deoxyribonucleic acid) testing, which can be used to determine if someone has a genetic disease, human origin, paternity or maternity, or in the forensic point of view, the source of the DNA[5]. By generating a DNA profile from the evidence at the scene, and comparing it to suspects, one can find which suspect's profile matches that of the profile from the scene[5]. If there is a match, this means that this suspect was at the scene.

Genetic information is passed down from parents to progeny through DNA replication. When sexual intercourse occurs, the gametes (sex cells) combine causing the progeny to receive an allele, a sequence variant at a specific site or locus, from both the mother and father[1]. These

two alleles can be determined for each locus on a chromosome, for all human beings[1]. Certain errors and genetic variation in genomes cause a unique code for each individual[1]. Genetic information and errors in the genetic code are both conserved from generation to generation[1]. In forensic biology, genetic variations determine whether or not individuals are closely related[1]. PCR made determining the sites of differentiation in the human genome possible[1]. It is the main component which is used in DNA sequencing[1]. There are 4 steps in DNA sequencing which include the extraction of DNA, DNA quantitation, DNA amplification, and detection of DNA which allow for the determination of genetic sequences in a human being[5].

The extraction of DNA is the step which utilizes numerous techniques to physically remove the DNA from the nucleus of the cell, and to purify or clean it[5]. The main goals are to break open the cellular membranes of the cells, separate the DNA from the lipids, organelles and proteins, and to minimize the degradation of the DNA[5]. The most popular technique to extract DNA from a sample is the use of Bio-Rad Chelex 100[5]. First, 1mL of distilled water is added to the biological sample, which is mixed and incubated at room temperature[5]. This step lyses (breaks open) the cellular membranes[5]. The samples are spun at 15,000g in a centrifuge for 2 to 3 minutes[5]. This pellets the nuclei and substrate at the bottom of the tube[5]. The supernatant is discarded to get rid of any potential PCR inhibitors such as porphyrins[5]. The Bio-Rad Chelex 100 is added to the sample; the iminodiacetate ions chelate polyvalent metal ions such as magnesium[5]. Since polyvalent metal ions increase nuclease activity, Chelex actually reduces it[5]. The sample is then incubated 15 to 30 minutes at 56˚C which is the optimal temperature for Chelex activity[5]. It is then mixed, and incubated at 100˚C for 8 minutes to rupture the nuclear membrane, denature the proteins, and release the nuclear single stranded DNA into the supernatant (surrounding liquid)[5]. The DNA is then ready to move onto quantitation.

The goal of DNA quantitation is to determine how much DNA is actually in the sample[5]. In order to generate a successful DNA profile, one usually needs about 1 nanogram of DNA[5]. If there is too much or too little DNA in the sample, the results will not be ideal. One of the most widely used techniques of DNA quantitation is the use of the Quantifiler Human DNA Quantification Kit on a Real-Time PCR System[5]. This system monitors the amount of DNA which accumulates as it is being amplified[5]. The DNA is tagged with a fluorescent dye which gives off a wavelength of light when it is activated[5]. The fluorescent intensity then overcomes the threshold of visibility, and can be detected by the machine[5]. Since the cycle number is proportional to the amount of DNA present, a standard curve can be used to determine the correct amount of DNA in the sample[5]. Once the amount of DNA is know, the needed loci can then be amplified.

DNA amplification uses the polymerase chain reaction to make many copies of the loci to be analyzed[5]. In the United States, 13 different loci is the standard for matching two DNA profiles[5]. These two profiles must have the same 13 loci match to be reliable. PCR makes billions of copies of these 13 loci, so that it is easy to make a profile of the regions actually needed[5]. The reaction mix used in PCR and the DNA are heated and cooled in cycles causing denaturation, annealing, and extension of the DNA[5]. The steps of PCR can be seen in Figure 2.

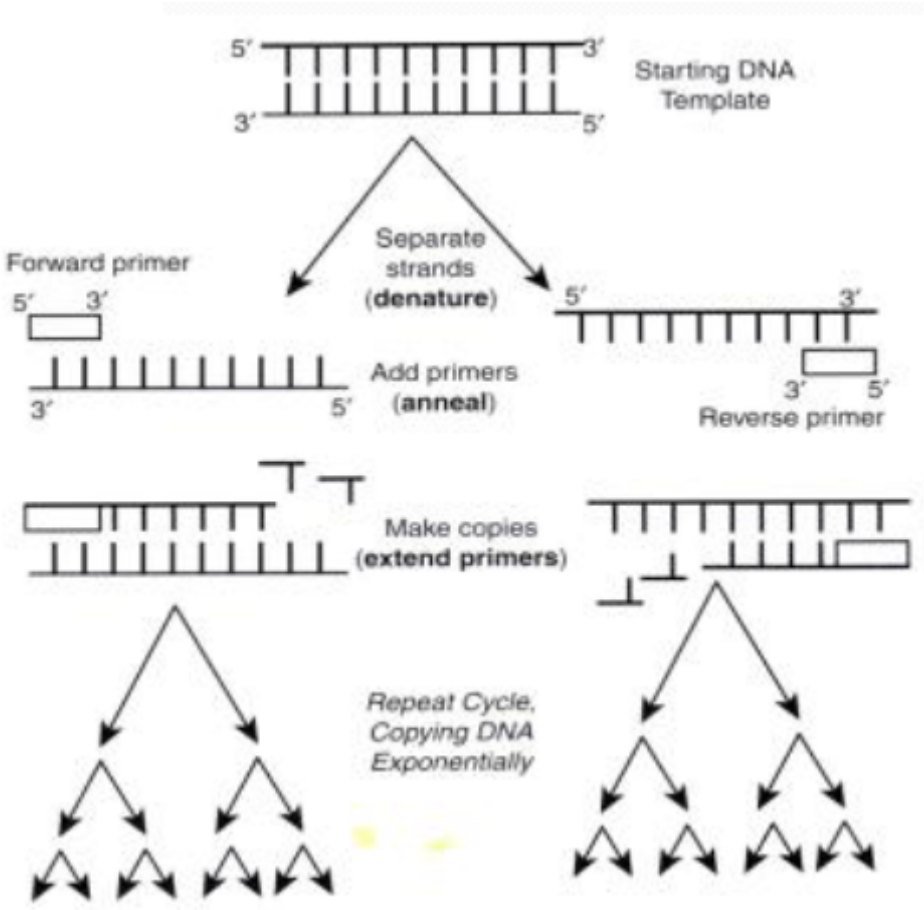

Figure 2: Steps of the Polymerase Chain Reaction[5].

The final step of DNA sequencing is DNA detection and analysis. This technique is most widely done by Capillary Electrophoresis[5]. The tools needed for a Capillary Electrophoresis schematic include a sample vial, source and destination vials, a capillary, electrodes, a high voltage power supply, a detector, and a data output and handling device[5]. Inside the capillary there is an aqueous buffer[5]. An electric current is run from the source vial to the destination vial, causing the sample to migrate and separate based on the ionic charge of the segments of DNA[5]. The information from the detector is sent to a computer, which analyzes the data and shows separated chemical compounds as different peaks[5]. Figure 3 shows a simple schematic of

capillary electrophoresis.

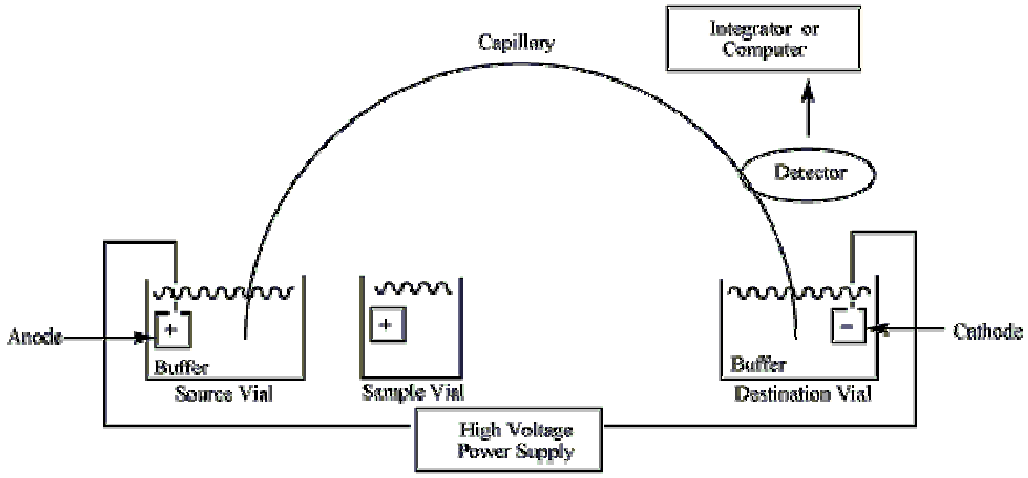

Figure 3: Capillary Electrophoresis[5].

CODIS is the combined DNA index system[7]. This system was developed as a place to compare convicted offenders DNA profiles with those found at the scene of the crime[7]. This helps to give investigative leads to the detectives working on the case[7]. The system is a national computer index, which can be used through the internet to compare profiles all over the country[7]. It can also be used to link different crime scenes together[7]. If a DNA profile was found at one scene, and also at another scene, then that means that the same person most likely committed the crime[7]. Figure 4 shows the number of matches found through the CODIS system in the United States.

Offender/Forensic Profiles & Total Offender Hits

Offender/Forensic Profiles & Total Offender Hits

	2000	2001	2002	2003	2004	2005
Offender Profiles	460,365	750,929	1,247,163	1,493,536	2,038,514	2,826,505
Forensic Profiles	22,484	27,897	46,177	70,931	93,956	126,315
Investigations Aided	1,573	3,635	6,670	11,220	20,788	30,455
Forensic Hits	507	1,031	1,832	3,004	5,147	7,071
National	26	167	638	1,151	1,864	2,855
State	705	2,204	4,394	7,118	11,991	18,664
Total Offender Hits	731	2,371	5,032	8,269	13,855	21,519

Offender/Forensic Profiles & Total Offender Hits (cont'd.)

	2006	2007	2008
Offender Profiles	3,977,433	5,372,773	6,539,919
Forensic Profiles	160,582	203,401	248,943
Investigations Aided	43,156	62,059	80,948
Forensic Hits	9,529	11,750	14,122
National	4,276	6,508	8,479
State	28,163	43,305	58,304
Total Offender Hits	32,436	49,813	66,783

Figure 4: CODIS DNA profile matches[7]

The Application of Forensic Biology to Pathogens: Microbial Forensics

The same techniques used to identify and individualize human DNA can be used in microbial forensics. Bacteria and other pathogens are much more difficult to obtain a genotype for, because their DNA makeup is much more diverse than that of humans[1]. Unlike a human's genome which is 99.9% the same for all humans, each bacteria has a very different genome from

other types of bacteria, or even different strains of the same bacteria[1]. This is because their oldest common ancestors stretch back to even more than three billion years ago, while the eukaryotic domains oldest known relatives were from about 500 million years ago, giving them much more time to diversify, especially to the harsh conditions that existed on earth during that time[1]. The diversity of bacteria can be shown through studying a phylogenetic tree. On a phylogenetic tree, one can see the relation of different species to one another (Figure 5). The animal kingdom occupies a single branch on the Eukaryotic domain which encompasses all of the animals known to existence; each branch has a similar genomic makeup[1]. In contrast, the Bacterial domain contains nine different branches of differing bacterial kingdoms, containing thousands of species in each[1].

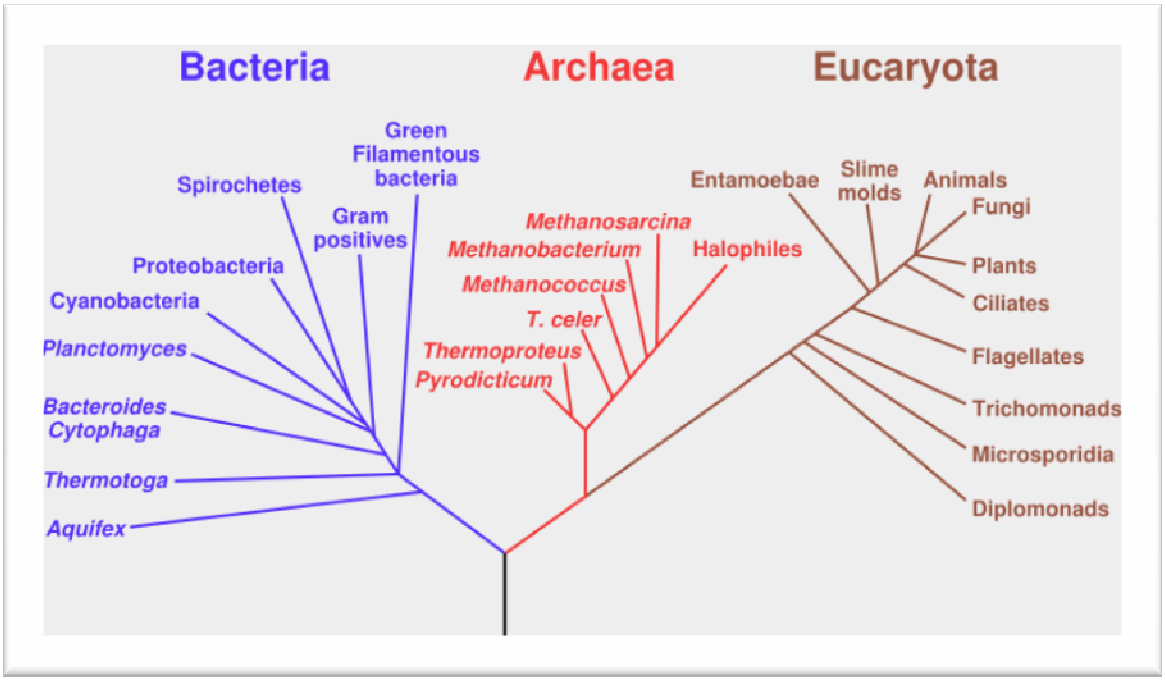

Figure 5: Universal Phylogenetic Tree[15].

Though the genomes of pathogens are much more diverse than humans, there are a few techniques which allow for the DNA sequencing of these organisms. One of the first techniques used in DNA analysis was the use of restriction fragment length polymorphism (RFLP)[5]. This method uses restriction enzymes to cut DNA into fragments at specific sites, and then these fragments are separated by size on an agarose gel electrophoresis[5]. A Southern blot procedure is then used, to transfer the fragments to a membrane, which can be hybridized to a DNA probe that allows for the complimentary strands to be seen[5]. RFLP occurs when this sequence or "barcode" is different for different individuals or organisms[5]. The difference in the barcode between family members can be seen in Figure 6. Though this technique is no longer used for human DNA analysis, it is quite effective for pathogens. Since pathogens have a relatively small genome, the entire genome of the pathogen can be analyzed and compared this way[1]. Some disadvantages of this method are that species that are not really diverse between different isolates may have the same restriction sites, therefore would give the same barcode, and a live sample is needed to conduct this type of analysis, which is not always available[1].

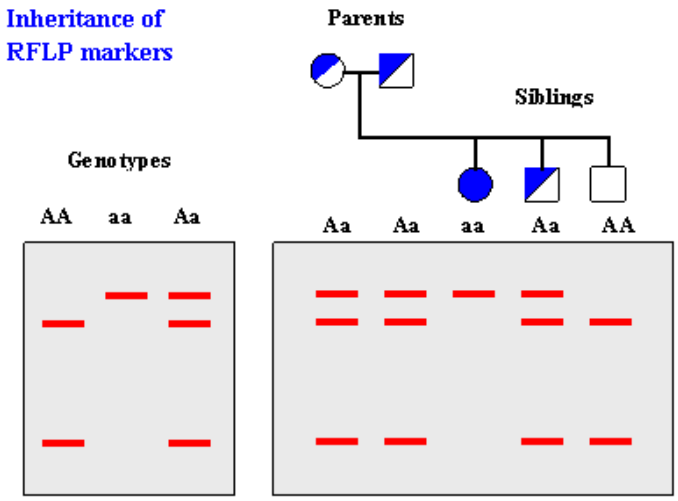

Figure 6: Inheritance of RFLP markers[11]

The next method used to analyze the genomes of pathogens was amplified fragment length polymorphism (AFLP)[12]. AFLP is similar to RFLP, but differs in that after the DNA is broken into fragments from restriction enzymes, select parts of the DNA are amplified with PCR primers, and then they are run through electrophoresis[12]. This method has a higher reproducibility, resolution, and sensitivity than RFLP when analyzing an entire genome[12]. It can also amplify between 50 and 100 fragments at once[12].

Multiple-locus sequence typing (MLST) uses the nucleotide sequences of housekeeping genes to differentiate between different isolates of pathogens[1]. Comparing the housekeeping genes of two isolates of a pathogen can be an advantage because they maintain the basic functions of the cell, therefore they are in all cells (including all bacteria cells)[1]. In comparison to rRNA genes, they also have a higher rate of evolution; therefore there is a higher probability that these sites would be different for two different isolates of a pathogen[1]. "The original idea behind MLST was to identify 7–10 genomically distributed conserved regions that could be sequenced from a collection of isolates from a single pathogen and then analyzed to determine the genetic relationships among isolates"[1]. This type of sequencing has taken a step towards validating microbial forensics, because it has a database associated with it, one of the closest there is to the CODIS system for human DNA[1]. The site is www.mlst.net, and is kept up to date by laboratories with specialties in the different types of pathogens[1]. A disadvantage to this type of analysis is that it cannot provide specific details to newly evolved pathogens[1].

Variable number of tandem repeats (VNTR) are quickly evolving sites in both humans and pathogens[1]. VNTR is a location on the genome of an individual where nucleotide sequences are repeated[5]. VNTR or more specifically short tandem repeats (STRs) are used in identifying

DNA sequences in forensic biology[5]. The standard of the CODIS system calls for 13 STR loci to match to individualize a DNA sample to a suspect[5]. Since this is the technique used in human identification, at this time it should also be the standard in microbial forensics in order to get the best results. This type of analysis gives the best resolution in determining the individualizing characteristics of pathogens in microbial forensics[1].

A technique which would give the greatest amount of certainty as to whether or not the DNA at the scene is the same as the suspect would be a whole genomic sequence[1]. If the whole genomic sequence was available for comparison, there would be no question as to whether or not the two samples are a match. The current cost of DNA sequencing of the whole genome of a pathogen is about $500[1]. Though this technique is expensive now, in the future more cost effective technologies may be available. In the view of bioterrorism, it is reasonable to sequence the whole genome of a pathogen, because determining who committed the crime may be vital to saving lives in the future[1].

Other Technology

Though it is a relatively new field, there are various techniques used to detect, quantify, and individualize pathogens both at the scene of the crime and in the lab. The first step in any biocrime investigation is to detect/identify the pathogens used at the scene. Universal Detection Technology is a company that focuses their efforts on "post and pre incident planning, bio-terror detection for large events, and drafting customized security plans for customers interested in taking a proactive approach against bio-terrorism"[10]. Some of their products and services include airborne pathogen detection, surveillance technologies, radiation detection, bioterrorism detection kits, training movies and DVDs[10]. The BSM 2000 is a bacterial pore detection system

which is specific to anthrax[10]. The system samples the air at continuous intervals, and heats up the samples to release dipicolinic acid (a chemical which is specific to anthrax) [10]. When dipicolinic acid is released, it mixes with a chemical sensor inside the system, causing a bright green luminescence that corresponds with concentration when viewed with ultraviolet light[10]. If anthrax spores are detected an alarm sounds and the building security and emergency services are contacted via land line and wireless network connected to the device[10]. The science behind the BSM 2000 system can be seen in Figure 7 while the actual system can be seen in Figure 8.

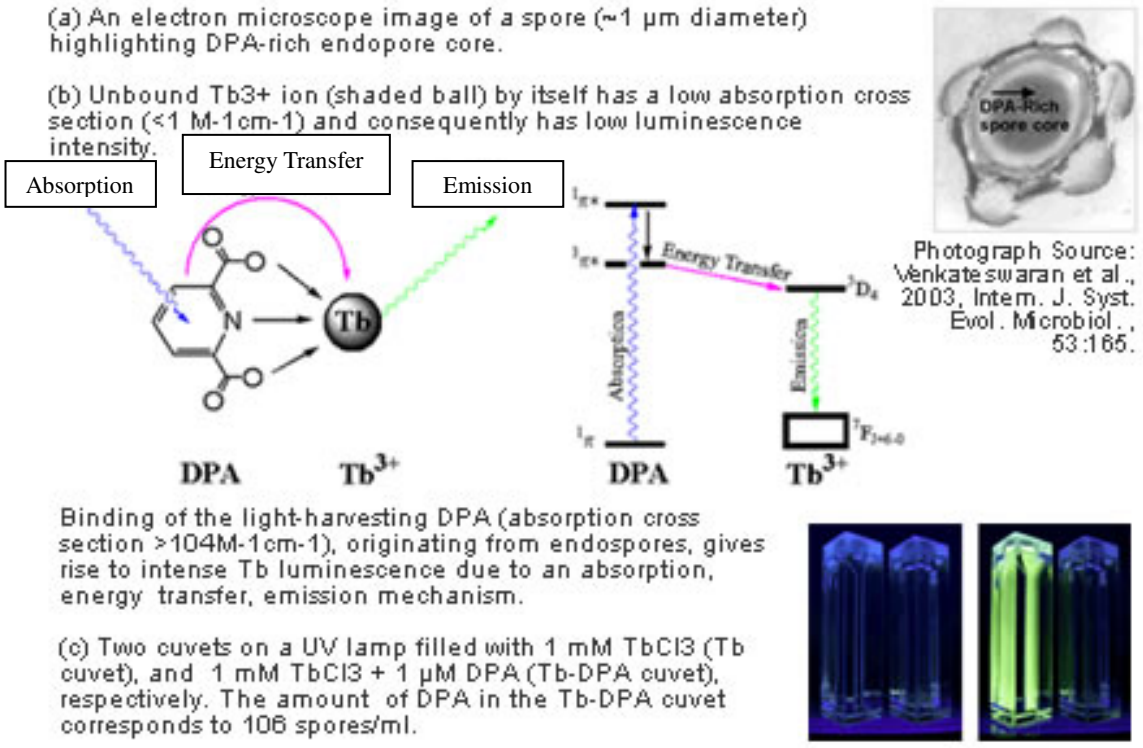

Figure 7: BSM 2000 Mechanism[10]

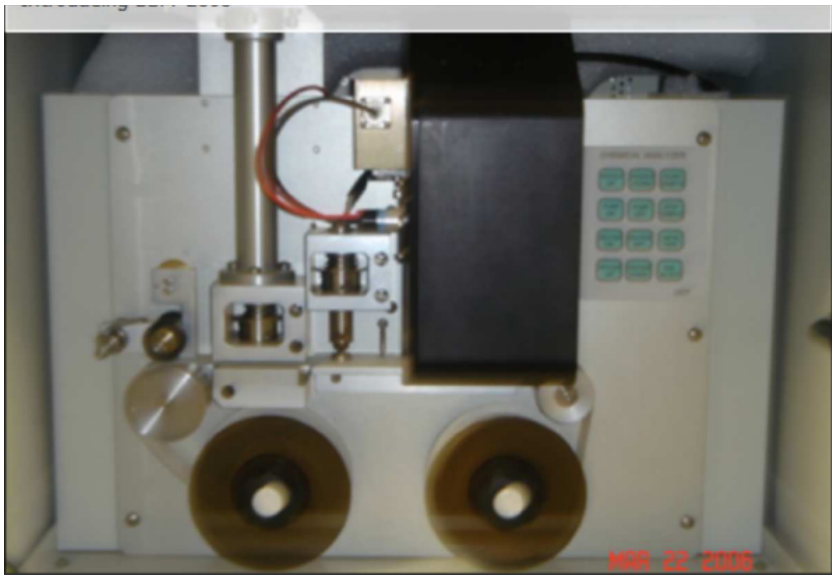

Figure 8: BSM 2000[10]

Universal Detection Technology also has a line of bio-weapon detection kits. The TS-10-5-agent biodetection kit is the industry's only hand-held assay for biological weapons[10]. It is designed to detect/identify five different pathogens using only one sample in a small portable device[10]. The biological warfare agents the device can test for are anthrax, ricin, botulinum toxin, Y. pestis (plague), and Staphylococcal Enterotoxin B (SEB), all done in less than three minutes[10]. With this biodetection kit, there are a variety of features that make this type of test convenient, such as: "no cross-reactivity with near neighbor strains, no cross-reactivity to household powders, no set up time, no expensive reader needed, no decontamination requirements, no false positives, no false negatives, and no hook effect"[10]. Features such as this are important in tests used in microbial forensic analysis, because misleading results may put the first responders and investigators on the scene in danger. For example, if the test gives off a false negative for anthrax, then the people working on the scene may not be prepared to handle

anthrax contaminated items, therefore they themselves may become infected. The TS-10-5-agent biodetection kit can be seen in Figure 9.

Figure 9: TS-10-5-Agent Biodetection Kit[10]

Post 9/11 Anthrax Attacks

In September and October of 2001, five letters were mailed containing *Bacillus anthracis* (anthrax) to United States Senators Patrick Leahy, Thomas Daschle, and various media organizations (for copies of the letters see Figure 10)[13].

Letters to "Tom Brokaw NBC TV" and "Editor New York Post"

09-11-01

THIS IS NEXT
TAKE PENACILIN NOW

DEATH TO AMERICA
DEATH TO ISRAEL

ALLAH IS GREAT

Letters to "Senator Leahy" and "Senator Daschle"

09-11-01

YOU CAN NOT STOP US.
WE HAVE THIS ANTHRAX.
YOU DIE NOW.
ARE YOU AFRAID?

DEATH TO AMERICA.
DEATH TO ISRAEL.
ALLAH IS GREAT.

Figure 10: Letters Used in Anthrax Attacks[13]

From these letters, 22 Americans contracted anthrax, killing 5 of the victims[13]. Over ten thousand other people were tested and deemed at risk for contraction anthrax[13]. In 1981 the same strain used in the attack (Ames) was found in Texas, where it was isolated and sent to the United States Army Medical Research Institute for Infectious Diseases (USAMRIID), and was never seen again in a natural outbreak[13].

Preliminary forensic testing of the powder found in the envelopes used a variety of different methods and techniques to try and individualize the powder[13]. The five main

techniques used include; Microscopy, to identify spore size, shape, and quality, Inductively Coupled Plasma – Optical Emission Spectroscopy, to determine the elemental composition of the anthrax, Gas Chromatography Mass Spectroscopy, to determine the presence of agar used to grow the spores, Accelerator Mass Spectroscopy, to identify the age of the anthrax with the use of carbon dating, and Stable Isotope Ratio Analysis, to try and find information regarding geographic location[13]. It was found that

> they exhibited an electrostatic charge, showed no signs of genetic engineering, and were non-hemolytic, gamma-phage susceptible, antibiotic and vaccine sensitive, and devoid of aerosolizing enhancers (*e.g.*, fumed silica, bentonite, or other inert material). These characteristics were and are inconsistent with weapons-grade *anthracis* produced by offensive, state-sponsored biological weapons programs. In addition, the spores in the Washington, D.C. letters were of exceptional purity. Spores of this quality are often used in biodefense research, including vaccine development

meaning whoever produced the anthrax used in the attacks had exceeding experience with anthrax[13]. From this preliminary information, investigators made three main conclusions about the perpetrator/s; 1. The Ames strain must have come from a laboratory in the United States, because the only natural occurrence recorded was in 1981 in Texas, where the pathogen was isolated. There are 15 known laboratories in the United States with the Ames strain, 2. The perpetrator/s is/are familiar with microbiology. The only form of the Ames strain in the laboratories is in a slurry form, so whoever worked with the anthrax must have known that the sample must be dried out with a lyophilizer or speed-vac system to dry it out to be used in the powder form, and 3. Whoever worked with the anthrax must have come in contact with it, so they most likely have a vaccination to prevent contracting anthrax[13].

By November 2001, the genetic analysis of the anthrax spores began[13]. This is one of the key events to occur in the field of microbial forensics, because at this time, it was not even known if it was possible to use DNA sequencing techniques on pathogens[13]. Subpoenas were given to the investigators to get samples of the anthrax from each of the 15 labs, and 3 foreign labs that were known to have the Ames strain[13]. The Institute of Genomic Research was hired, to perform genetic sequencing and analysis of the Ames strain found in the letters, to determine mutagenic regions for comparison against evidence from the labs[13]. Four mutation sites were found, and assays were developed to test for the presence of these mutation sites in the evidence collected during investigation[13]. These assays were known as Morph A1, Morph A3, Morph D, and Morph E[13]. Over the next few years the assays were used on about 1,070 samples and it was determined that eight different samples from the letters all came from the common source known as RMR-1029, which was only found at the USAMRIID[13].

It was determined that the perpetrator was Dr. Bruce Edwards Ivins[13]. Dr. Ivins "was a senior microbiologist in the Bacteriology Division of USAMRIID", and was the leading expert in the nation of *Bacillus anthracis*[13]. There are many findings which lead to the conclusion that he was the perpetrator. He was in charge of the anthrax stores being held at the USAMRIID, so whoever was behind the attacks had to go through him to get the samples[13]. His laboratory notebook states "RMR-1029: :99% refractile spores; < 1% vegetative cells; < 1% non-refractile spores; : 1% debris", which are all components consistent with the spores used in the attacks[13]. In his log books, there is a total of 100ml of anthrax slurry missing and unaccounted for in transfers[13]. His senior technician accounts that she was constantly making spores, which she thought was being put into storage to use in testing, but in fact it was never logged that these spores were ever placed into storage[13]. It was also found that Dr. Ivins was in the lab off hours

almost every day by himself for 3 hour time periods the week before the attacks, which is the amount of time consistent with preparing anthrax spores (none of this time is accounted for in his lab books)[13]. Finally, a long stretch of suspicious behavior, and his eventual suicide, all point to Dr. Ivins[13].

Recommendations for Improvement

Like any scientific field, forensic science is constantly being reexamined for improvement. As technology and the understanding of science improve, forensic science is being modified to provide valid, more reliable evidence. Not only does new technology allow for more reliable evidence, but universal standards in the field can help to provide better handling of said evidence. When there are no discrepancies as to how evidence should be handled, it will have a stronger impact on the results of trials. Being a relatively new specialization in forensic science, microbial forensics lacks these universal standards. The America Academy of Microbiology has set forth a list of recommendations to improve the subfield of microbial forensics[8]. This list includes recommendations in evidence gathering, identification of biocrime organisms, tracing of the source of these organisms, investigation behind the crime, and in education, training and communication[8].

Biocrime scenes have shown to be frantic in the past, with investigators trying to balance between protecting the crime scene, protecting people in the crime scene, and collecting usable evidence[8]. The solution to this problem put out by the American Academy of Microbiology, is to establish better chains of communication throughout the investigation[8]. In a crime scene with dangerous pathogens at hand, different workers tend to have different agendas to complete. The investigators are trying to preserve evidence for the case, and may not know the harms of the

environment around them, while public health workers are doing their best to protect the people at the scene, and make sure the pathogen is not spreading further[8]. With safety number one on the public health workers list, they may neglect the fact that evidence is needed to build a case, which may lead to contamination of the evidence. This can be avoided by establishing "permanent communication and cross-discipline education programs for public health and law enforcement communities", establishing groups of first responders who specialize in biocrime incidents, establishing a team of biologist and scientists who know the organisms most likely to be used in a biocrime situation and can be called upon in the event of a biocrime, and by developing "standard operating procedures for sample collection, documentation, access, storage, and transmittal so that samples are not compromised" [8]. If the investigators are trained ahead of time to handle such a situation, fewer errors which compromise evidence and safety will occur. Biologists can help to set forth protocols for different types of pathogens to protect both evidence and evidence collectors. Standard operating procedures which are used in all subfields of forensic science, such as establishing a chain of custody, should also be used to protect the validity of the evidence at hand.

The identification of the pathogen used in the biocrime is critical in protecting the workers at the scene, treating those who have been victims of the crime, and to the prosecution of those who committed the crime. In order to determine what organism or organisms are present at the scene of the crime, a variety of tools and methods, resources, and analysis are needed to be done both in the lab, and at the scene[8]. Tools used at the scene of the crime and in the lab should be standardized, to assure for reliable evidence[8]. Funding from the government needs to be made available to create more reliable identification and individualization techniques that are specific to certain pathogens[8]. Further research in the field and publications of the work

in reputable journals, will validate it, and allow for the development of more advanced technologies to provide reliable evidence in court[8]. Full genomic sequences of likely biothreats, with at least three different strains available for each of those threats, should be done and placed in the database for quick comparison and identification[8]. Resources for comparison should include national computerized networks and databases that track diseases and symptoms, databases with genomic sequences on file to promote research and allow for comparison of pathogens (much like CODIS for human DNA), and databases for biocrime strains in geographical regions to determine if the threat was truly a biocrime or if it was just from the environment[8]. Finally, as with all sub-disciplines of forensic science, statistical and probability analysis needs to be done for the pathogen and strain identification[8]. For example, when there is a rape case and DNA sequencing is performed on the suspects DNA and compared to that of the semen found on the victim, a statistical analysis is done to strengthen the argument of the probability that the semen belongs to the suspect. In the analysis, the probability is determined in the form of the likelihood that this DNA sequence is the same in someone else in the world. There will not be the same level of certainty in pathogens that there are in sexually reproducing organisms such as humans, but "identification capacity should be defined for each set of microbial markers for various biocrime agents" [8]. Analysis such as this will provide information to the legal system that is consistent with other fields of forensic science, and will further validate the field of microbial forensics[8].

As with any forensic investigation, there should be standards as to how to approach the investigation of biocrimes. Microbial forensics will need to accommodate both the forensic and scientific communities in their standards of investigation since it is mostly a laboratory science, so it should abide by the rules of laboratory procedures[8]. The American Academy of

Microbiology has set forth recommendations in quality assurance and quality control, quantitative evaluation of false-positives and false-negatives of certain procedures, peer-review, and a multi-tiered laboratory system with specializations[8]. By establishing quality assurance and quality control procedures, it will establish confidence in microbial forensics[8]. It is recommended that the FBI report "Quality Assurance Standards for Forensic DNA Testing Laboratories", be applied to microbial forensics and changed to better fit microorganisms by a team of experts in the field[8]. False-negatives and false-positives are prevalent in many forensics tests, and should be determined for microbial tests also. False-negative results may be dangerous in the field of microbial forensics, because first responders may let their guard down if they believe that there is no pathogen present when there actually is[8]. It is recommended that proficiency and validation testing be adopted to eliminate, or determine the cause of false-negative and positives, and the rate at which they occur[8]. In many forensic disciplines there is a system of peer review set up, to make sure that the work being submitted to court is valid and fits the standards of the institution. In the Office of the Chief Medical Examiner for example, workers must submit their work to their managing supervisor before it can be submitted to the court. This double check method reduces "erroneous or misleading results or conclusions" [8]. For microbial forensics however, it is recommended that a panel of forensic and microbiology experts peer review the results of tests before they are submitted to court[8]. If the information is reviewed by not only forensic experts but also microbiology experts, it will "help to promote confidence in the results, both by the public and by judicial bodies" [8]. The final step in the investigation of crimes is the lab work on the evidence. It is recommended that the forensic science field instate a multi-tiered laboratory system, where all testing does not need to be done in the local labs, but can be sent to more specialized laboratories in the system[8]. There would be

four levels (A through D) in which evidence could be tested[8]. Level A would be the lowest tier, including all hospitals and commercial reference laboratories able to use presumptive testing to determine the presence of biological pathogens[8]. Further testing would move up to Level B, which would be made up of about 200 laboratories able to perform confirmatory testing on the pathogens[8]. Level C would be made up of about twenty to thirty laboratories "that perform antimicrobial susceptibility testing and typing of isolates" [8]. Finally, Level D would be only about two laboratories that would be able to perform all the tasks of the other laboratories, and can also complete full genomic sequencing of the pathogens[8].

The last set of recommendations set forth by the American Academy of Microbiology include the education, training, and communication issues in the field of microbial forensics[8]. The field of forensic science is constantly being evaluated for up to date practices. With technology constantly changing and developing, workers in the field must undergo regular training so they can effectively complete their jobs in the most efficient way. Microbial forensics laboratories should also have professional standards by which the staff is trained by. It is recommended to have a set of both professional and public education standards to keep those working in the field and affected by biocrimes prepared, accredited and efficient[8]. Since microbial forensics is a very specialized field, there should be a full time staff with the same specialized knowledge[8]. It should consist of microbiologists trained in forensic analysis[8]. Training in such a field should also be available as a specialization at the university level, giving people interested in the field specialized training so they are ready to work right out of college[8]. Updated training should be made available at various forensics conferences, such as the American Academy of Forensic Sciences, providing the importance of microbial forensics to "forensic scientists, microbiologists, and law enforcement officials[8]. Not only does the forensic

staff need proper training in the field, but training should be provided to the first responders[8]. First responders at the scene need to know how to handle a potential biocrime scene, by being able to determine the possibility of the presence of biothreats, how to protect themselves if there is a threat present, how to investigate a biocrime scene, or determine whether or not the scene is too dangerous to begin evidence collection at the time of arrival[8]. This training should be done by medical professionals, microbiologists, and forensic scientists to combine the disciplines efficiently[8]. Education of the public can also have positive effects on the field. If the public is educated in the purpose of microbial forensics, it is more likely that the field will have their support. "An understanding about potential bioterrorism agents, preventive measures, manifestations of biocrime pathogens, and how they are detected and contained" is an important step in gaining public support and to avoid panic if a biocrime occurs[8]. It is recommended that information about the field be conveyed to the public through local news media, radio, collaborations with the American Society of Virology and the Centers for Disease Control and Prevention to provide information, and by creating a website for information exchange[8]. Finally, better communication should be established between the fields by setting up journals of research focusing on the combination of the disciplines[8]. It would be ideal to have full journals on the topic of microbial forensics to discuss biothreat response and technology, but it may be sufficient enough to create sections of microbial forensic research in current forensic and microbiology journals[8].

Conclusions

In current times, one of the most feared events in the American public eye is that of terrorism. The events of 9/11 not only destroyed thousands of lives, but it scarred the memories

of millions of Americans. Life in America will never be the same, with heightened security and fear around every corner. Bioterrorism and biological warfare have been seen throughout history, but in a time of new technology, the threat is much greater. With advances in microbiology and other sciences, pathogens can be altered to cause more destruction. A small sample of a pathogen can cause immense destruction and death, without even being noticed by the people around it. This was seen in the post 9/11 anthrax attacks, where anthrax spores were sent through the mail, and thousands of people were at risk for developing the anthrax infection, with 5 people actually being killed by it. Through the use of microbial forensics, it was determined that the perpetrator was Dr. Bruce Edwards Ivins, who was one of the only people with the same strain of anthrax that was used. Microbial forensics uses the concepts of forensic biology, DNA sequencing, and other sciences to determine the source of biological threats. It is a relatively new field that should be further developed to help protect the American public. To validate this field, and to make it more effective, the American Academy of Microbiology has made suggestions in evidence gathering, identification, tracing of the source, investigation, education, training, and communication, all focusing on the standardization of procedures, development of technology, and preparation in the case of a bioterrorist attack. Being prepared for such a situation is critical in preventing mass casualties and destruction. Current technologies such as DNA sequencing and identification tests are extremely important foundations in the field, but are not the most effective. The most effective way to protect the American citizens from bioterrorism is to develop new technology, standardize procedures in the field, and to invest time and money into specialized microbial forensics units. By not having such advances in the field, it is a question of national security as to whether or not the American people are actually safe, and can get the best treatment if a bioterrorist attack is to happen.

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