# FORENSIC USE OF RAPD ANALYSIS IN THE INVESTIGATION OF BIOTERRORISM

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FORENSIC USE OF RAPD ANALYSIS IN THE

INVESTIGATION OF BIOTERRORISM

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## NOMENCLATURE

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| RAPD | Random Amplification of Polymorphic DNA |
| VNTR | Variable number of tandem repeats |
| RFLP | Restriction fragment length polymorphism |
| CDC | Centers for Disease Control and Prevention |
| g | gravity constant |
| *B. anthracis* | *Bacillus anthracis* |
| *B. cereus* | *Bacillus cereus* |
| LIF | Laser-induced fluorescence |
| C | Celsius |
| uL | Microliter |
| ng | nanograms |
| UV | Ultra Violet |
| dH2O | distilled water |
| uM | Micromoles |
| RFU | Relative Fluorescence units |
| CDC | Charged-coupled device |
| PCR | Polymerase Chain Reaction |
| SBA | Sheep's Blood Agar |
| MYP | Mannitol-yolk-polymyxin |

| | |
|---|---|
| CE | Capillary Electrophoresis |
| PA | Protective antigen |

## 1. Introduction

The anthrax attacks that occurred in 2001 brought into sharp focus many issues concerning the vulnerability of the United States to the threat of bioterrorism. Bioterrorism, instead of being an event that occurred in distant places, became a reality on American soil. These attacks were a true test of how the United States could respond and react to bioterrorism and demonstrated that America needed to establish more effective programs of preparedness and quick response. An effective program should include everything from defense against a bioattack, to early detection and identification, isolation of affected victims, and availability of adequate supplies of vaccines and drugs for treatment.

One of the aspects of being prepared is the ability to quickly detect, identify, and determine the origin of a bioterrorism agent. In 2002, the government passed The Public Health Security and Bioterrorism Preparedness and Response Act of 2002. This legislation expanded, enhanced, and improved the capabilities of the Centers for Disease Control and Prevention (CDC) to respond to an attack. It also made available billions of dollars for enhanced training of responders, for production of vaccines, growth of facilities, and research (Public Health Security, 2002). State governments also tried to do their part in the fight on terrorism. Oklahoma, for example, hosted a seminar composed of key staff and representatives from federal and local agencies. The seminar, sponsored by the Oklahoma City National Memorial Institute for the Prevention of Terrorism,

MIPT, was held to discuss how to prepare, educate, and respond to a local bioterrorism attack (Validating Oklahoma Bioterrorism Planning, 2002). There was a strong interest in developing rapid, presumptive identification capability for bioterrorist agents at a local level due to the loss of critical time when a state lab sends specimens of suspected infectious agents to the CDC for analysis (Validating Oklahoma Bioterrorism Planning, 2002).

Bioterrorism was first documented in the French and Indian War when the British gave Native Americans blankets that had previously been used by smallpox victims. There have been reported instances of the intentional use of infectious agents in every major war since (Bioterrorism [CD Rom]). In addition, although there has been no recent intentional use of biological weapons, Germany, Japan, the United Kingdom, Iraq, the former Soviet Union, and the United States are all suspected of having experimented with anthrax (Timeline, 2001). In fact, Iraq admitted in 1995, to having 8,500L of concentrated anthrax and 19,000L of concentrated Botulism toxin on hand, which is enough to kill the entire human population almost three times over (Arnon et. al., 2001, Timeline, 2001). Based on past history, recent events, and the future threat of agents such as anthrax being used as biological weapons, research is needed to enhance the detection and identification of bacterial strains and viruses that pose a biowarfare threat.

| Category | A | B | C |
|---|---|---|---|
| Definition | High priority agents that pose a risk to national security | Moderate priority agents | Lowest priority agents and emerging pathogens |
| Examples | Anthrax, Botulism, Smallpox, Plague | Brucellosis, Salmonella, Q Fever, Typhus Fever | Nipah Virus, Hantavirus |

Table 1. CDC's categories of Bioterrorism Agents/Diseases. The CDC list the agents according to priority which is determined by mortality rates, ease of production, and possible social disruption and public panic (Bioterrorism Agents/Diseases, 2004).

Table 1 was created by the CDC and provides a list of possible bioterrorism weapons. One of the most notable bioweapons is anthrax. Anthrax has existed throughout recorded history. It is believed that the fifth plague described in Genesis was anthrax and it is also described in the early literature of the Greeks and Romans (Morse, 2002). One of the best documented natural outbreaks occurred in 1979/1980 in Zimbabwe, infecting animals and over 6,000 humans, killing almost 100 humans (Timeline, 2001). These outbreaks are relatively rare in the United States with only 236 cases of anthrax infecting human patients reported between 1955 and 1999 (Anthrax:NIAID Fact Sheet, 2002). Anthrax outbreaks occur because *Bacillus anthracis (B. anthracis),* the causative organism, is a normal inhabitant of soil and water. When the bacterium is in its spore stage, it has the capability of infecting organisms and elaborating its toxic properties. Grazing animals eat contaminated vegetation or soil and become infected. If a human comes into contact with the animals flesh, bones, hides, hair, or excrement, they too can become infected (Ryan, 1994).

Interestingly, *Bacillus anthracis* was the first bacterium to be identified as the causative agent of a disease. In 1876, Robert Koch injected livestock with *B. anthracis* and demonstrated that they then became ill. (Ryan, 1994). It wasn't until 1954, however, that

the toxic properties associated with *B. anthracis* infection were characterized (Todar, 2004). Included in these toxic factors are a protective antigen, an edema factor, and a lethal factor. The protective antigen is responsible for binding to cells of the infected life form and producing a channel through which the edema and lethal factors can enter. The edema factor, which has been identified as adenylate cyclase, causes fluid to accumulate in the lungs (Leppla, 1982). The lethal factor is a zinc-metallo protease , which disrupts normal hemoestatic function (Klimpel et. al., 1994, Hanna, 1999). In addition to toxic factors that cause harm to an infected cell, *B. anthracis* is protected by a cell wall capsule, consisting of a high content of poly-D-glutamic acid, which helps protect vegetative cells from elimination from the body of the infected host. (Ryan, 1994).

There are three different types of human infections caused by *B. anthracis*. In rare occurrences, a person can eat infected, undercooked meat or drink contaminated water and obtain one form of anthrax known as gastrointestinal anthrax. The symptoms include nausea, fever, abdominal pain, and vomiting of blood (Morse, 2002). The most common and the most treatable form of anthrax is the cutaneous form which occurs when spores enter the skin through a cut or opening (Figure 1). Cutaneous anthrax begins as an itchy bump that becomes fluid-filled and ruptures into a painless ulcer (Morse, 2002).

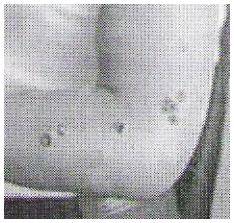

Figure 1. Cutaneous anthrax infection. Example of a human four day old anthrax infection (http://www.bt.cdc.gov/agent/anthrax/anthrax-images/cutaneous.asp).

The most deadly form of anthrax, and the infection that would be the goal of a bioterrorist attack, is inhalation anthrax. This form results when spores are inhaled into the lungs (Inglesby, 2002). Inhalation anthrax is deadly because the toxic factors described above destroy white blood cells, the body's first line of defense against the pathogen. The toxins also disable signaling of other elements of the immune system. The overall breakdown of the immune system allows the bacteria to remain undetected in the lungs and to survive and reproduce. When the first respiratory symptoms of inhalation anthrax appear, it is usually too late for treatment that will ensure the survival of the patient. This is why death seems to occur so quickly and unexpectedly after diagnosis. Death is apparently due to oxygen depletion, secondary shock, and respiratory and cardiac failure (Todar, 2004).

*B. anthracis* is a large, aerobic, gram-positive, rod-shaped, non-motile, spore-forming bacterium (Koneman et. al., 1998). The genome of *B. anthracis* is over 5.2 million base pairs long and exists as a single chromosome. *B. anthracis* also harbors extra-chromosomal, circular plasmids that generally carry the genes for all the toxic properties (Read et. al., 2003). At the genomic level *B. anthracis* is believed to be one of the most homogenous species known (Keim et. al., 2000). The small variation that is found among different strains could explain differences in antibiotic sensitivity (Read et. al., 2003). These subtle differences in DNA sequences are also of great importance to public health investigators hoping to pinpoint the origin of an outbreak.

The CDC classifies anthrax as a Category A agent. This means that it is considered the highest threat to national security due to its ease of transmission, potential for causing public panic, and high rate of death and illness, especially in the case of inhalation anthrax (Anthrax:NIAID Fact Sheet, 2002). Anthrax is a good biological weapon for terrorism for many reasons. First, it is relatively easy and inexpensive to produce in large quantities since the knowledge concerning how to culture the microbe is readily available. Once produced, anthrax spores have a long shelf life. Spores are persistent in the environment and could conceivably contaminate the environment for months or even years (University of Alabama at Birmingham, 2004). Other features of anthrax that make it attractive as a bio-weapon include the limited capabilities for detection and high rate of infection. One gram of anthrax spores is equivalent to 100 million infectious doses, making it 100,000 times more deadly than the deadliest chemical warfare agent (United States Department of Defense, 1998). The US Army views anthrax as a potential threat due to the fact that the spores are resistant to destruction, it can easily be spread through the air, and there is documented development and existing stockpiles of the bacterium in foreign countries (Anthrax, 2002).

*Bacillus cereus* (*B. cereus*) is a close relative of *B. anthracis* and is genetically very similar. Since this lab is not certified as a Biological Safety Level-2 lab and could not perform research on the dangerous organism, *B. cereus* was used as a model organism for *B. anthracis*. Bacteria belonging to the *Bacillus* genus are all spore-forming and, besides Clostridium, represent the only bacteria that have sporulation capability. *Bacillus* species are also ubiquitous, found mostly in soil, but also found in water, dust, and even as

natural flora in the human intestine (Koneman et. al., 1988). Similarities in phenotypes between *B. cereus* and *B. anthracis* include a rod shape, inhabitating the soil, and being gram positive. However, *B. cereus* is motile while *B. anthracis* is not (*Bacillus cereus* and other *Bacillus* spp., 2002).

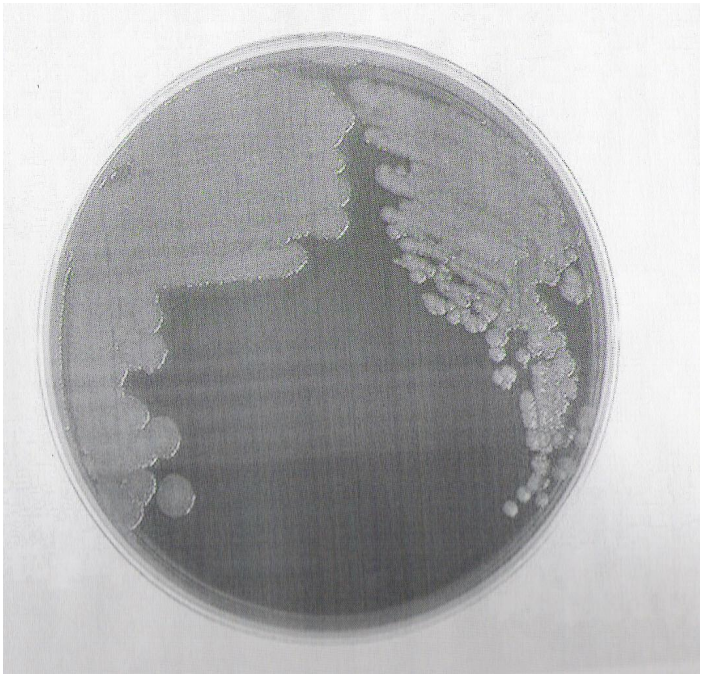

Figure 2. Hemolysis represented on blood agar. *B. cereus* colonies shown on left and *B. anthracis* colonies shown on right, providing a visual differentiation between the two strains of *Bacillus* (Todar, 2004).

*B. cereus* can be toxic causing a non-fatal, food-borne intoxication. *B. cereus* exhibits two different types of poisoning, an emetic form (causing vomiting) and a diarrheal form (Moravek et. al., 2004). The toxin that causes diarrhea is thought to be a non-hemolytic entertoxin, or NHE, isolated from food poisoning outbreaks. The nheAB genes encode NHE and are homologous to some genes found in *B. anthracis* (Mendelson, et. al, 2004). Another study suggested the existence of an additional toxin, called hemolysis BL-enterotoxin, or HBL, that complements NHE in causing food poisoning from the

ingestion of *B. cereus* (Moravek et. al., 2004). *B. cereus,* which is an opportunistic pathogen, can also cause diseases of the eye following trauma. *B. cereus* contaminated dust or soil that enters the eye following an injury can be very destructive and can lead to loss of vision (Howard, 1987).

Many studies have been conducted testing the relatedness exhibited among *Bacillus* species. One such study found that *Bacillus* species are genetically similar and *B. cereus* even contains a plasmid that is similar to one of the two that *B. anthracis* possesses (Rasko et. al., 2004). Read et. al. (2003) compared genomic hybridization tests of *B. cereus* and *B. thuringiensis* against *B. anthracis* and confirmed many genomic similarities between the species. In fact, when nineteen members of the *B. cereus* group were compared to *B. anthracis* they were found to share 66-92% of their chromosomal genes. In a study by Radnedge et. al. (2003), only 93 nucleotide sequences were identified in the genome of *B. anthracis* that were not found in the genome of other *Bacillus* species.

Traditional approaches for the comparison of *Bacillus* species have included colony morphology, biochemical pathway testing, and the use of Gram staining. These tests are time consuming, labor intensive, and interpretation of results can be subjective. At the DNA level, one group of researchers found that the Polymerase Chain Reaction, or PCR, could be used to distinguish among species, and even found different DNA fingerprints within each strain, suggesting subtypes exist and can be distinguished using PCR (Webb

et. al., 2003). Molecular assays such as PCR can exhibit a higher degree of specificity, are rapid to perform, and less labor intensive than classic methods for identification.

DNA typing, also known as DNA fingerprinting, was first described in 1985, by Sir Alec Jefferys. Jefferys found regions in human DNA associated with the myoglobin genes that contain sequences that are repeated consecutively over and over again (Jeffreys et. al., 1985). Moreover, Jeffreys found that the number of repeats differs among individuals. The regions of genomic DNA that are repeated became known as variable number of tandem repeats, or VNTRs(Nakamura et. al., 1987) . Jeffreys used the restriction fragment length polymorphism procedure (RFLP) to create a fingerprint of a DNA sample. This procedure uses restriction enzymes that target the areas surrounding the repeated sequences (Botstein et. al., 1980). Producing a complete DNA fingerprint usually takes about 4-6 weeks, is labor intensive, and, to process the sample, at least 100ng of intact DNA is needed (Butler, 2001). In the mid-1980s, Saiki et. al. (1985) used a process that was described by Kary Mullis and discovered it was possible to amplify VNTR loci using PCR. For reasons of speed, sample type and amount, and ease of use, PCR amplification of VNTR loci replaced RFLP mapping as the principle DNA typing methodology (Butler, 2001).

PCR is an enzymatic process used to copy short, known sequences of target DNA. The process is similarly modeled on natural DNA replication within a cell. PCR uses alternating cycles of heat denaturation and cooling during which thermostable DNA polymerase replicates a nucleotide sequence of interest. This sequence is specified by

oligonucleotide primers that are added to the reaction and are complementary to the 5' and 3' ends of the target DNA (Hartl and Jones, 2005). By performing 20-35 cycles of heating and cooling, millions of copies of a given DNA sequence can be produced (Figure 3). The advantage of PCR over RFLP is that it is relatively simple and reasonably fast, allowing for a quick turn-around of results (Budowle et. al., 2002). More importantly, an extremely small amount of DNA template (50-100pg) can be amplified even if it is partially degraded because the procedure is sensitive. The principal disadvantage of PCR also relates to sensitivity, meaning contamination becomes a greater threat to the validity of results (Budowle et. al., 2002).

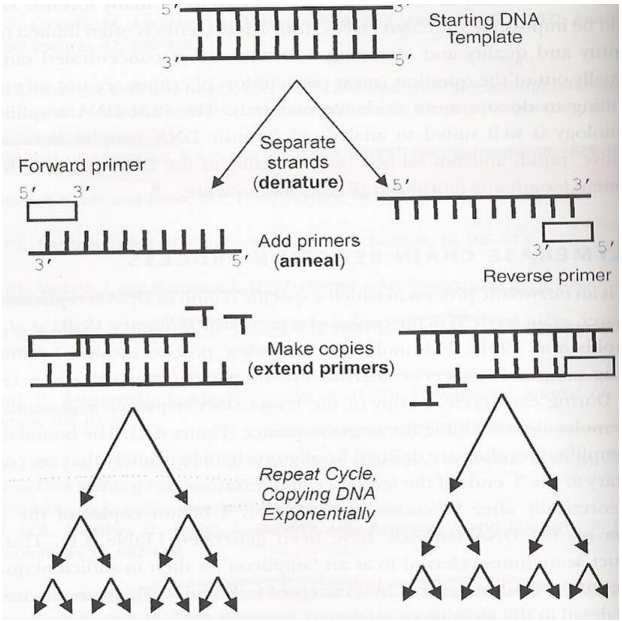


Figure 3. Representation of a typical PCR amplification with known primers (Butler, 2001).

In designing a typical PCR reaction, the sequence of the target area of DNA must be known. Knowing the nucleotide sequence of the target DNA allows primers to be synthesized that are complementary to the boundaries of the target sequence. The two

primers added to a PCR reaction bind to the 3'end of each strand of DNA and serve as the initiation points for replication catalyzed by DNA polymerase (Hart and Jones, 2005).

Random Amplification of Polymorphic DNA, or RAPD, is a variation of traditional PCR and is designed to be used with DNA templates whose sequences are not known or well characterized. Williams et. al. (1990), used single olignucleotide primers with arbitrary nucleotide sequences to amplify polymorphic DNA segments that are inherited in a Mendelian fashion and was able to construct genetic maps among members of plant and animal species. Because the primers used exhibited a high degree of sequence variability, polymorphic regions of the genome can be amplified in the absence of specific knowledge of nucleotide sequence (Williams et. al., 1990). In order for a RAPD product to be produced, the primers must hybridize to a target sequence in the genome and be spaced on opposite DNA strands in close enough proximity to form primer pairs that can efficiency amplify a PCR product. In figure 4, primers labeled as 2 and 5 are able to produce a product, product A, and primers 3 and 6 also have opposite orientations and are in close enough proximity to produce product B. Primers 1 and 4 are also in opposite directions, but are not close enough to direct the efficient amplification of a PCR product.

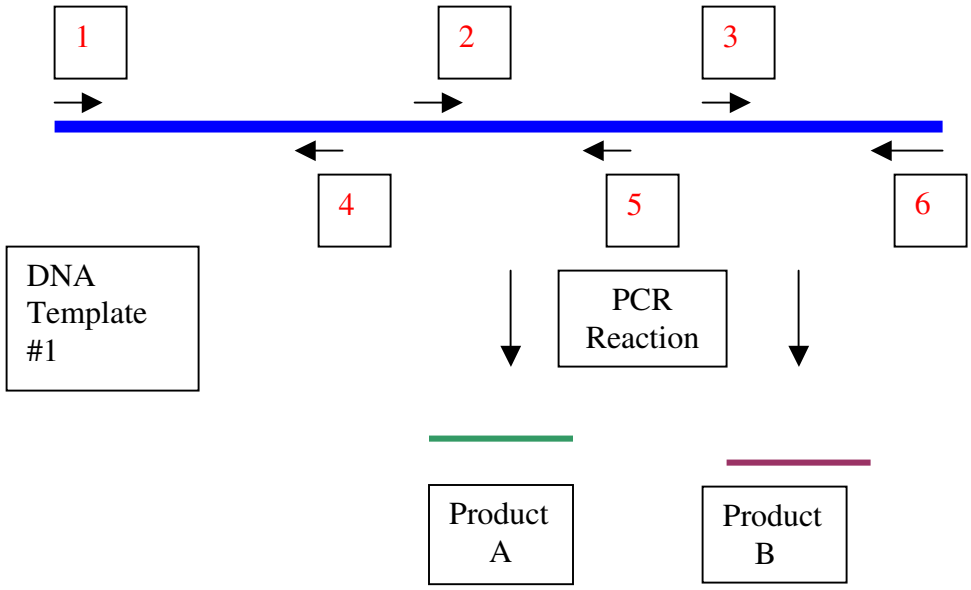


Figure 4. RAPD analysis producing two products. This example shows a simple example of RAPD primers binding to a DNA template in an orientation where two different size products were produced.

Variation in the products produced with all PCR procedures occurs due to nucleotide sequences differences occurring in the DNA template. When using RAPD analysis this variation can make primers bind in different places, different orientations, or they might not bind to the templates at all. In figure 5, the DNA template is different from the figure shown above, because the primer labeled number 2 does not bind to DNA Template #2 and product A is not produced.

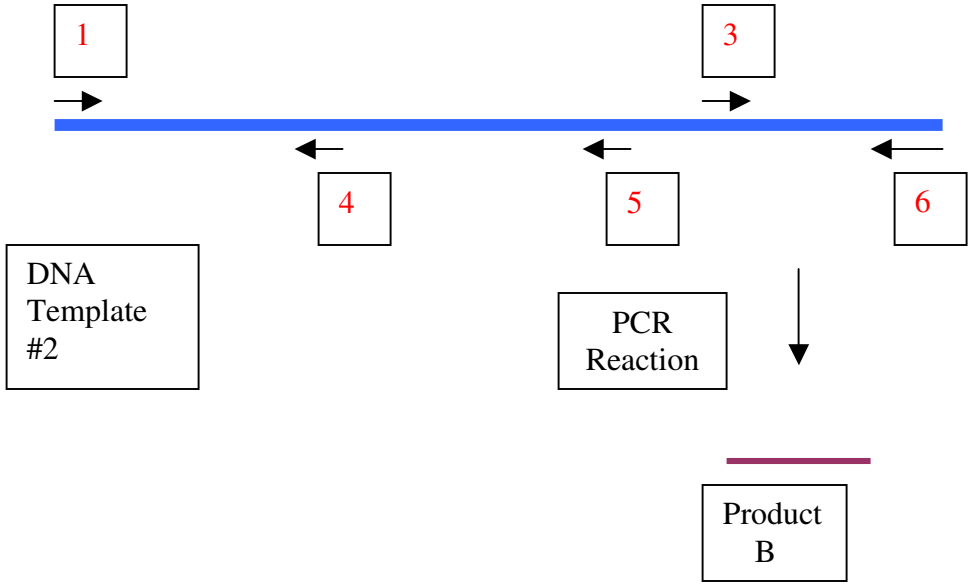


Figure 5. RAPD analysis producing one product. This picture shows a simple example of how a different DNA template has different binding patters and therefore, different products produced.

If amplification products from the templates shown in figures 5 and 6 were analyzed using gel electrophoresis, which separates DNA molecules based on size, results shown in figure 6 would be obtained.

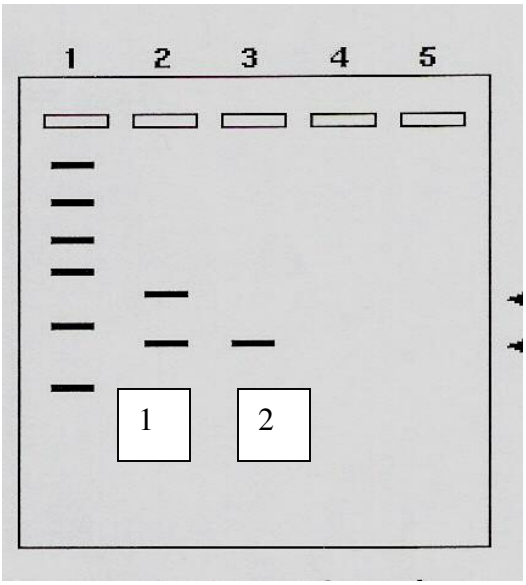


Figure 6. Results of the simple RAPD example. Agarose gel results of the two DNA templates. Lane one contains a size ladder, while lane 2 portrays the products produced in Figure 5 and lane 3 portraits the products produced from Figure 6.

Since these early studies other researchers have applied the RAPD procedure in different ways. One group performed parentage testing on burying beetles (*Nicrophorus tomentosus*: Silphidae) and was able to establish a relationship between parents and offspring. An exception was that they found occasional non-parental products that were detected at low frequencies in offspring (Scott et. al., 1992). Similar results were found by another group studying a flax rust fungus. Detailed analysis revealed that the spurious amplification products represented heteroduplex molecules that formed between two allelic sequences of different sizes, explaining the non-parental products visualized in the Scott et. al.(1992) study (Ayliffe et. al., 1994).

Harrison et. al. (1992), used RAPD in conjunction with DNA from *Rhizobium leguminosarum* to differentiate strains in order to examine genetic structure and relationships within the population. Other groups studied many other organisms with

many different random primers and all have found RAPD to be efficient, flexible, rapid, and useful as a tool for identifying and typing organisms (Soto et. al., 1999). All have demonstrated RAPD to reveal polymorphism in genomic DNA and therefore it is capable of producing a DNA fingerprint (Melcher, 2001). RAPD can be a powerful tool for gene mapping, population genetics, pedigree analysis, phylogenetic studies, surveying DNA for damage or mutation, and strain identification (Atienzar et. al., 2001). In most studies, 10bp oligonucleotide primers with at least a 50% GC content seem to represent optimal primers for RAPD analysis (Williams et. al., 1990).

An example of the forensic use of RAPD occurred when an investigator needed to quickly determine if maggots found inside of a body bag were the same as the pupae found on the floor under the corpse (Benecke, 1998). There was not enough time to let the pupa grow, so DNA testing was needed. Results of RAPD analysis showed the arthropods being compared were genetically identical, and the testing was considered successful (Benecke, 1998). Another example of the use of RAPD in forensic science occurred in May of 1992. A female was found dead under a Palo Verde tree in Arizona and a pager was found nearby. The pager was linked to Mark Bogen and he became the number one suspect, but the investigator needed more evidence that would place him at the scene of the crime. When they investigated the suspect's truck they found seed pods from a Palo Verde tree. One of the only ways to prove that the pods were from the same tree that the victim was found under was through DNA analysis, but no past genomic information was known about the Palo Verde tree. That is when the forensic scientist decided to use the RAPD procedure. Based upon testimony from Tim Helentjaris, and Dr.

Paul Keim, associate professor at the University of Arizona and professor at Northern Arizona University respectively, the evidence was allowed in court for the first time. Dr. Keim did testify about the downfall of RAPD stating that the procedure is not completely reliable or reproducible. Due to this testimony statistical evidence was not allowed in court but that did not seem to hinder the evidentiary value of the RAPD profiles for the jury. Dr. Helentjaris was allowed to testify that the DNA RAPD pattern of the seed pod was indistinguishable from the reference pattern obtained from the tree the victim was found under. The suspect was convicted of the murder based on upon the DNA and physical evidence and he was sentenced to life without the possibility of parole (Rudin and Inman, 2002).

The advantages of RAPD are that only a small amount of template DNA is needed, a specific primer is not needed, the sequence of the genome of the organism does not need to be known, and the procedure is relatively easy, quick, and inexpensive. Some disadvantages include the possibility of contamination and the fact that it is extremely sensitive to the reaction conditions which can compromise reproducibility (Treuren, 2000).

Agarose gel electrophoresis is the technology that has generally been used to separate the RAPD-DNA fragments produced. Thus, tracing the source of a DNA sample relies upon matching the spatial arrangement of polymorphic DNA fragments produced by RFLP analysis or PCR (figure 7). The agarose gel used for DNA fragment separation is a semi-solid matrix that has pores acting as a molecular sieve. The DNA is loaded into the gel

and migrates based on size to the anode when a direct current applied. Along with RAPD products, a ladder representing different size standards are often run in the gel so the unknown DNA can be characterized by size. Visualization of DNA in the gel often occurs following staining the gel with ethidium bromide and illumination with UV light (Butler, 2001).

Capillary gel electrophoresis (CE) has replaced the older slab gel electrophoresis technology in most forensic labs in the United States. Capillary electrophoresis is most often performed as part of automated DNA analysis systems like the ABI Prism 310 Genetic Analyzer that was introduced in 1995 by Perkin Elmer-Applied Biosystems (Foster City, CA). CE normally incorporates laser-induced fluorescence (LIF) to detect DNA fragments. Visualization of DNA profiles with LIF is more sensitive, easily automated, and is less toxic than the ethidium bromide used to visualize DNA in slab gels. In the capillary based methods, each amplified DNA product is labeled with a fluorescent dye (attached to one or more primer) and separation of fluorescent DNA molecules occurs in the capillary containing a sieving polymer (310 Genetic Analyzer Performance Optimized Polymer 4, PE Applied Biosystems, Foster City, CA). Fluorescent products are excited by the laser and a CCD, or charge-coupled device, camera will capture those emissions as the sample migrates through the capillary and passes a small window through which the laser enters. The genetic analyzer is interfaced with a Macintosh computer that stores electrophoretic and fluorescence data (Relative Fluorescent Units, or RFUs) to produce an electropherogram. The raw data can then be analyzed with a specialty software (Genescan or Genotyper) to create an easily analyzed

DNA profile for each sample that can be compared with the profiles produced from other samples (Butler, 2001).

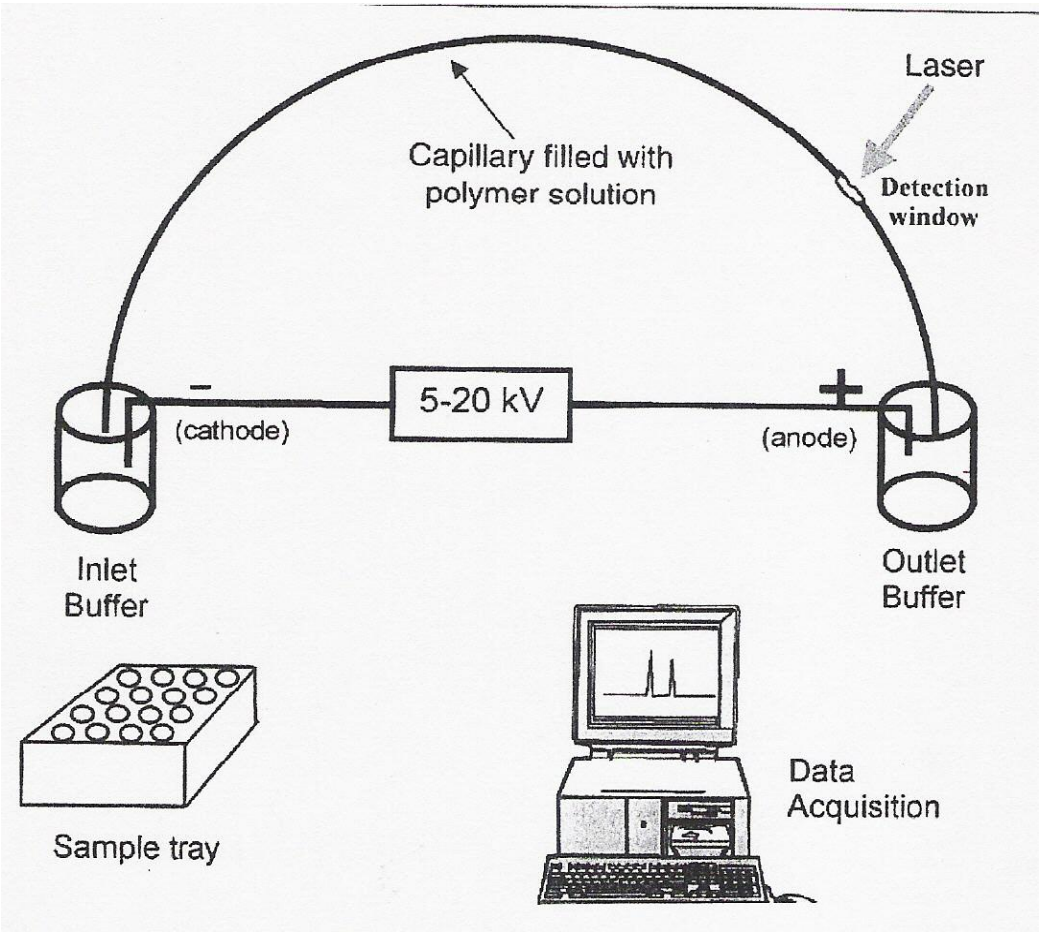


Figure 7. Representation of a Capillary Electrophortic Device (Butler, 2001).

CE has many advantages over slab gel electrophoresis including the fact that many of the steps are automated and thus can be run unattended. A smaller amount of sample is used and higher voltage (due to the small surface area of the capillary) is used allowing for the separation of DNA products in minutes rather than the hours required for slab gel electrophoresis. Another advantage is that the additional steps needed to analyze the gel

are not performed when using capillary electrophoresis and cross-contamination that can occur in gels is eliminated in the capillary. The final advantage is that capillary electrophoresis results are interpreted more precisely by a computer program, unlike the human generated results that accompany analysis of a slab gel. This allows for better reproducibility and the ability to estimate quantities of PCR products based on the amount of fluorescence produced (RFUs). One of the disadvantages is that since the samples are analyzed one at a time the throughput is not as high as a slab gel that can separate as many as 100 samples at once (Butler, 2001).

Presently, identification of bacteria through the use of morphological and physiological tests are the most commonly used to determine if a specimen is *B. anthracis*. The CDC produced a protocol for Level A Laboratories for the presumptive identification of *B. anthracis*. The protocol gives a flow chart of what testing a lab needs to do with a suspected *B. anthracis* sample. It states what specimens are to be collected from a human patient based on the type of anthrax suspected. For example, if inhalation anthrax is suspected, the lab is to collect blood or sputum from the lower reparatory tract. A Gram stain test is performed to look for large, Gram positive rods (Figure 8). A smear of the specimen is also performed and short chains of encapsulated bacteria are suggestive of *B. anthracis* (Morse, 2002).

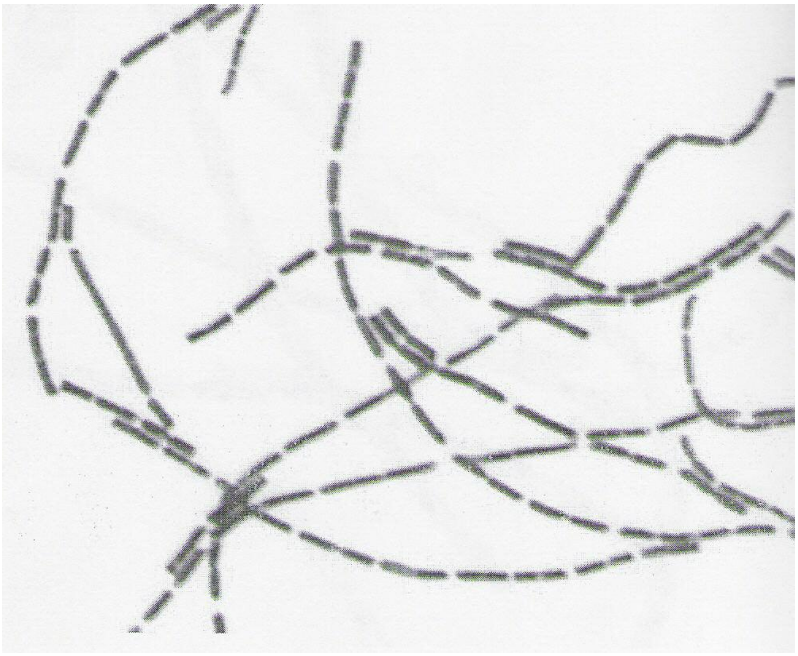

Figure 8. Gram Stain of *B. anthracis* (Morse, 2002).

Labs are also instructed to grow the bacterium on sheep's blood agar to look for hemolytic activity and also for the presences of oval intracellular spores within long chains of bacilli. Culture tests are to be performed including inoculating and streaking different plates at 35$^{o}$C with reading of the plates occurring daily for 3 days. Motility is tested on a wet mount slide to look for nonmotility because *B. anthracis* is nonmotile, this can be determined within 1-2 days. Table 2 provides a list of all approved tests for the detection of *B anthracis*.

| | Laboratory Level approved to perform test | | | |
|---|---|---|---|---|
| **Test Procedure** | **A** | **B** | **C** | **D** |
| Gram Stain | X | X | X | X |
| Capsule (Indian Ink) | X | X | X | X |
| **Routine culture:** | | | | |
| Colonial morphology | X | X | X | X |
| Hemolysis | X | X | X | X |
| Motility | X | X | X | X |
| Sporulation | X | X | X | X |
| **Confirmatory test:** | | | | |
| Lysis by gamma-phage | | X | X | X |
| Direct fluorescence assay (DFA) | | X | X | X |
| Antimicrobial susceptibility testing | | | X | X |
| Advanced technologies (PCR) | | | X | X |
| Molecular characterization | | | | X |

Table 2. CDC's approved test for Detection of *Bacillus* anthracis in the Laboratory Response Network (LRN) (Approved tests, 2001).

The CDC recommends that at least two of these tests be performed with results showing that the bacterium is not *B. anthracis* before it is ruled out (Morse, 2002). If it has all the features of *B. anthracis* it is to be reported as stated in the following protocol;

**Reporting/action**

a. Consult with state public health laboratory director (or designate) if *B. anthracis* is suspected.

b. General instruction and information

(1) Preserve original specimens pursuant to a potential criminal investigation and possible transfer to an appropriate LRN laboratory.

(2) Environmental/nonclinical samples and samples from announced events are not processed by Level A Laboratory; submitter should contact the state public health laboratory directly.

(3) The state public health laboratory/state public health department will coordinate notification of local FBI agents as appropriate.

(4) Assist local law enforcement efforts in conjunction with guidance received from the state public health laboratory.
(5) The state public health laboratory/state public health department may request transfer of suspicious specimens prior to presumptive testing.
(g) FBI and state public health laboratory/state public health department will coordinate the transfer of isolates/specimens to a higher-level LRN laboratory as appropriate; refer to Shipping Procedure.

c. Immediately notify state public health laboratory director (or designate) and state public health department epidemiologist/health officer if *B. anthracis* cannot be ruled out and a bioterrorist event is suspected.
d. Immediately notify physician/infection control according to internal policies if *B. anthracis* cannot be ruled.
e. If *B. anthracis* is ruled out, proceed with efforts to identify using established procedures (Morse, 2002).

When the CDC receives samples of suspected *B. anthracis* they perform their own morphological testing and ELISA (Enzyme Linked Immunosorbent Assay) testing. The morphological tests generally take approximately twelve to twenty-four hours (CDC's Laboratory Response to Suspicious Substances, 2004; J. Schwendinger, personal communication, June 6, 2005). ELISA is a conformation test that detects the immunoglobulin G (IgG) response to *B. anthracis* protective antigen (PA). This test is sensitive, has been shown to be very reliable, and can be performed in one hour. ELISA is approximately 80% specific, so to improve specificity, the CDC added an anti-PA IgG competitive inhibition ELISA as a second confirmatory step (Clinical issue, 2002). The anti-PA IgG ELISA is a direct extension of the standard ELSIA that utilizes different antibody concentrations. These two ELISA tests allow for rapid, sensitive, and specific detection of *B. anthracis* in human patients (Quinn et. al., 2002). Another test that the CDC performs to detect anthrax is a general PCR test that uses primers created specifically for *B. anthracis*. An example of primers used are ones that complementarily pair to loci in the 16S rRNA gene. This test, among a battery of such tests, takes 3-5

hours each and allows the CDC to detect specific strains of disease agents efficiently (CDC's Laboratory Response to Suspicious Substances, 2004; J. Schwendinger, personal communication, June 6, 2005).

During the anthrax mailing attacks of 2001, a procedure coined the "Keim Technique" played an important role in subtyping and identifying strains of *Bacillus anthracis* (Hoffmaster et. al., 2002). Keim testing uses multiple-locus variable-number tandem repeat analysis (MLVA) to examine the relationships among isolates of *B. anthracis*. The Keim assay uses RFLP analysis of eight VNTR loci located in the genome of anthrax. Among 426 different isolates of *B. anthracis,* only 89 distinct genotypes were identified, underscoring the genetic similarity of the species (Keim et. al., 2000). This procedure is used by the CDC because it proved to be useful in confirming that all isolates involved in the attacks of 2001 were the same strain (Hoffmaster et. al., 2002). Over 100 different strains of *B. anthracis* with distinguishing DNA fingerprints have now been found. At present however, these test are not considered confirmatory (Anthrax Q & A: Laboratory Testing, 2004). The tests take the CDC approximately six to eight hours to subtype bacteria whose genome is known (J. Schwendinger, personal communication, June 6, 2005).

Along with this procedure, Read et. al. (2002) used whole-genome analysis of the strains isolated from the victims of the attacks, and found 60 new DNA markers that included SNPs, or single nucleotide polymorphisms as well as the tandem repeats discussed above. These markers allow the bacterium to be divided into distinct families. DNA analysis

procedures are believed to help in the future investigation of infectious disease outbreaks when the genomic sequences of the agent are known.

The goals of this study include:

1) Determining if the Random Amplification of Polymorphic DNA method, or RAPD method, can be used in conjunction with capillary electrophoresis platform.

2) Determining if differences can be found using RAPD analysis between *Bacillus* and non-*Bacillus* bacteria.

3) Determine if differences can also be found using RAPD between *Bacillus cereus* and other *Bacillus* species.

4). The development of a working, reproducible procedure for the strain differentiation among isolates of *Bacillus cereus*.

5) Determining if RAPD can be used to match unknown forensic samples to each other.

The ultimate aim of the study was to develop a procedure that could be used to efficiently link forensic samples to each other. This is the same goal as would be in any other discipline of forensic science. The goal of fingerprint analysis is to match a suspect to a fingerprint left at a scene, the goal of document examination is to link a suspect's known writing sample to that on a questioned document, and when a fiber or hair is found at a scene of a crime the goal is to link that hair or fiber back to a suspect. During the anthrax mailings of 2001, it was important for investigators to know if all of the letters had been sent from one source, or if more than one source was involved in the attacks, and what was the origin of the source. This was eventually accomplished but was time consuming,

due to the fact that the complete genome of the Ames strain of *B. anthracis* had not yet been determined, and was not completely sequenced until May of 2003 (Read et. al., 2003). The aim of this project was to use the RAPD procedure in a mock terrorist attack to link isolates of *B. cereus* to each other and to an origin efficiently and effectively.

## 2. Material and Methods

### 2.1 Sample Selection

Soil samples were collected along Interstate 35 in Oklahoma, which is considered the horticultural dividing line of the United States. The top one inch of soil was collected every ten miles between Tulsa and Enid, Oklahoma. Soil samples were also obtained from sporting fields, including the football practice field of Oklahoma State University in Stillwater, Oklahoma.

*Bacillus* microbes were cultured from the soil samples by pasteurization at 70$^{o}$C in order to eliminate other unwanted bacteria present. The bacteria were plated on sheep's blood agar (SBA) and mannitol-yolk-polymyxin (MYP) plates. The plates were incubated at 38$^{o}$C for 24 hours and examined morphologically to identify candidate bacterial colonies representing *B. cereus*. B-hemolytic patterns on SBA, microbial interaction on MYP, production of lecithinase, and API test strips were all used to determine which colonies were *B. cereus*. Colonies positively identified as *B. cereus* were harvested with a loop and re-plated to obtain a pure culture. Gram staining was preformed to confirm the presences and purity of *B. cereus* isolates(Miller, 2005) .

## 2.2 Growth for DNA isolation

A loop of a particular *B. cereus* strain was inoculated into a labeled tube containing 2mL of prepared Mueller-Hinton Broth (Becton, Dickinson and Company, Sparks, MD) . The tubes were placed in an incubator-shaker overnight at 37°C with continual shacking to produce a stationary phase culture. Following incubation, each sample was centrifuged at 7000 xg for five minutes at room temperature to obtain the cellular pellet. The media was carefully removed as not to disturb the cell pellet.

## 2.3 DNA Extraction

The extraction of DNA from Gram positive bacteria is complicated by the fact that the cell wall (which contains D-amino acids) is resistant to proteolysis and must be disrupted mechanically to facilitate release of the DNA. In this method the combination of chemical and mechanical disruption liberates DNA from the cell. 35uL of 20% Sodium dodecyl sulfate, 25uL of 20mg/mL Protease K( in 10mM Tris-Cl, 20mM $CaCl_2$, 50% Glycerol), 440uL of TNE(10mM Tris-Cl pH8.3, .2M NaCl, 1mM EDTA), and .05g of glass beads (Sigma Chemical Company, St. Louis, Missouri), were added to each bacterial cell pellet. Cell pellets were resuspended and incubated at 65°C with vigorous, intermittent vortexing throughout the hour long incubation.

Extracts were then subjected to organic extraction with an equal volume of phenol:chloroform/isoamyl alcohol (9:0.96:0.4 v/v). Phases were separated by centrifugation at 8,000 xg for one minute at room temperature. The top, aqueous layer,

which holds the genetic material, was removed and added to a new, labeled tube. The sample was then extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) followed by centrifugation to separate the phases. The top aqueous layer was again removed and added into a new clean, labeled tube.

A commercial kit called DNA Clean and Concentrator $^{TM}$ -5 (Zymo Research, Orange, California) was use to obtain pure and concentrated DNA. The kit uses a binding buffer with a high salt content to promote binding of the DNA to silica particles in a small column. This binding buffer was added at 2:1 parts of buffer to each DNA sample. That mixture was then added to the silica column and the samples were centrifuged at room temperature at 10,000 xg for 10 seconds. As a sample is forced through the column, the DNA binds to the silica but allows all others components in the sample to pass through and collect in the bottom of a microfuge tube. The flow through produced was considered waste and was discarded. 200ul of the Wash Buffer, provided with the kit, was added to each filter followed by another round of centrifugation at room temperature for 10 seconds. The wash step was repeated one more time with the centrifugation time being raised to 30 seconds, and the wash fluid was also discarded.

Heat and $TE^{-4}$ (10mM Tris-CL pH 8, .1mM EDTA) was used to elute the DNA from the silica column. The column apparatus was placed into a clean, labeled tube, and 15uL of $TE^{-4}$ stored at 65$^{o}$C was added. The tube/column was then heated for one minute at 65$^{o}$C and centrifuged at 10,000 xg for 10 seconds at room temperature. This process was

repeated again and both 15ul elutes were combined into a single collection tube. DNA was stored at 4$^{o}$C until further use.

**2.4 DNA Quantitation**

The amount of DNA obtained from each bacterial sample varies and must therefore be quantified. This was accomplished using a .8% agarose yield gel. The gel was made by adding 0.6 grams of agarose (Sigma-Aldrich, Inc. St. Louis, Missouri) into 75mL of 1 X TAE buffer (900-1000ul dH2O, 48.4g Tris, 11.4mL Glacial Acetic Acid, 20mL .0.5 M EDTA). The agarose-buffer mixture was put in a microwave and heated, swirling frequently, until the agarose melted. The molten agarose was cooled to about 50$^{o}$C, poured into a mold containing a sample well comb, and allowed to set at room temperature until it has hardened (usually 15-30 minutes).

In order to determine the quantity of DNA in the bacterial extracts, known quantities of a reference DNA were electrophoresed alongside given volumes of the unknowns in the yield gel. Five quantitation standards containing different amounts of Lambda bacteriophage DNA (United States Biochemical, Cleveland, Ohio ) were prepared in 1 X TAE containing electrophoresis loading dye (bromphenol blue, xylene cyanol, 5X TAE w/ 25% Ficol), and distilled water as described in Table 3.

| Total Concentration Obtained | Amount of Lambda DNA | Amount of Loading Dye | Amount of Distilled Water |
|---|---|---|---|
| 500ug/uL | 2uL | 23uL | 0 |
| 250ug/uL | 1uL | 24uL | 0 |
| 125ug/uL | 0.5uL | 24uL | 0.5uL |
| 62.5ug/uL | 0.25uL | 24uL | 0.75uL |
| 31.25ug/uL | 0.125uL | 24uL | 0.875uL |

Table 3. Description of how size standards were produced for use in the yield gel to aid in the estimation of the amount of DNA present in the sample.

The agarose gel was placed into the gel box and enough 1X TAE Buffer to cover the gel (approximately 800mL) was added. Samples were loaded individually into the wells of the gel and electrophoresis was initiated at 50 volts until the samples entered the gel and then the voltage was increased to 75 volts. Electrophoresis was continued until the samples migrated half way down the length of the gel (approximately two inches), which required approximately one hour.

DNA was visualized in the gel after ethidium bromide staining (0.2% in dH2O, J.T. Baker, Phillipsburg, NJ) and illuminated with a UV light. The gel and running buffer were placed into a baking dish along with 100ul of ethidium bromide. The ethidium bromide interacts within the DNA helix and fluoresces when illuminated with UV light. A digital picture was taken of the illuminated gel through an orange filter and used to compare the fluorescence of the standards to theunknowns to estimate the amount of DNA present in each extract (Figure 9).

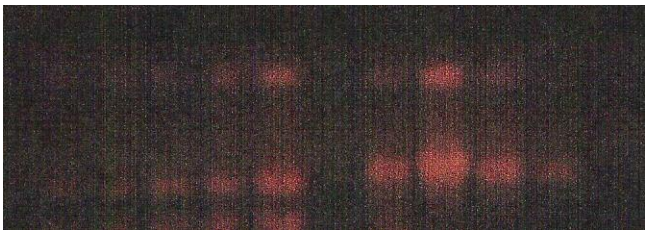

Figure 9. Photograph of a yield gel used in this study. The first 5 wells are the standards. The next well is empty. The next four rows are different isolates of DNA. An estimate of amount of DNA is made based on the intensity and width of the band. For example isolate number one is slightly larger than the standard for 500ug/uL, so it is estimated that its concentration is 550ug/uL.

## 2.5 Amplification

PCR reactions were prepared from stocks of concentrated components diluted with distilled water to achieve the desired concentration. A primer concentrate was made that contained equal molar amounts of each of the five primers used for the RAPD procedure. The mixture contained 1uL of each primer at 100uM concentration mixed with 20uL of $TE^{-4}$, which produced a final primer concentration in the stock of 10uM each. Some of the random oblionucleotide primers used were chemically linked to fluorescein, and therefore amplicons produced with these primers were fluorescently labeled. The five RAPD primers consisted of the following sequences:

1. 5'- fluoresceine –GGGTAACGCC-3'
2. 5'-fluorescein – GTGATCGCAG – 3'
3. 5'-fluorescein – AGCCAGCGAA -3'
4. 5'-CACACACACACACACARG-3' (R= can bind to Adenine or Guanine)
5. 5'-CACACACACACACACARY-3' (Y= can bind to Cytosine or Thymine)

Bacterial DNA recovered from each bacterial strain was diluted with distilled water in order to obtain a concentration of approximately 20ng/uL. Each PCR reaction was

programmed with 20ng of DNA sample (in 1 uL) along with 11.5ul of master mix consisting of primers, nucleotide triphosphates, Taq DNA polymerase, and buffer components, producing a total volume of 12.5ul (Figure 10). The master mix was composed of 8.5uL of dH2O, 1.25uL Gold Star 10X Buffer (Promega, Madison, Wisconsin), 1.25uL of the prepared primer mix, and .25uL of Taq Gold (Applied Biosystems, Foster City, California) for each sample. A blank was also amplified, prepared with 1ul of distilled water rather than DNA.

| Reactants | per Rx | Per # of Reactions 9 |
|---|---|---|
| dH20 | 8.75 | 78.75 |
| 10X Buff. | 1.25 | 11.25 |
| Primer 1,2,3,E1,E2 | 1.25 | 11.25 |
| DNA | 1 | |
| Taq | 0.25 | 2.25 |
| Amount Add | 11.5 | |

Figure 10. PCR set-up spreadsheet. Example of how the PCR reactions are calculated.

All samples were amplified with a PTC-200 thermalcycler (MJ Research, Las Vegas, NV). The cycling program consisted of 1 cycle at 95$^{o}$C for 11minutes,followed by 44 cycles of 94$^{o}$C for 1 minute, 40$^{o}$C for 1 minute, and 72$^{o}$C for 2 minutes. A final cycle of 72$^{o}$C for 45 minutes was added to complete product elongation and minimize the variability of the added 'Adenine' nucleotide that occurs slowly following the completion of each cycle.

### 2.6 Electrophoresis

Following amplification, amplified products were analyzed utilizing capillary electrophoresis in a Prism ABI310 Genetic Analyzer (Applied Biosystems, Inc. Foster

City, California). An internal size standard added to each sample allowed for automated sizing of amplicons in each electrophoretic run and to normalize the patterns across multiple runs. Each sample consisting of 5ul of amplified product had .5ul of the internal size standard, Genescan ROX 500 (Applied Biosystems, Foster City, California), added along with 24.5uL of Hi-Di Formamide (Applied Biosytems). The samples were heated at 95$^{o}$C for 3 minutes to denature the double stranded DNA and then immediately put in a frozen ice block to quickly chill the samples. The samples were placed into the ABI 310 sample tray and electrophoresed in succession. Each sample was electrophoresed for 32 minutes in order to allow for the detection of high molecular weight amplicons produced from bacterial samples. Raw electrophoresis data was analyzed using Genescan software to compute fragment sizes and quantitiate fluorescence. RFU, or relative fluoresence units were measured by the CCD camera as labeled PCR products pass the detection window in the capillary. Size estimates and fluorescence intensity were shown together in a GeneScan printout known as an electropherogram.

**2.7 Data Analysis**

Electropherograms allow an investigator to determine the size (in base pairs, or bp) of each product amplified from a template and an estimate of amplicon quantity reflected in the total RFUs in each PCR product. Electropherograms can be visually compared with one another to determine similarities and differences in the sizes and amount of products produced from different DNA templates. For this study, PCR products were also grouped into sized bins of 10bp over the entire size range spanned by an electropherogram (100bp

to 500bp). If there was at least one product in a 10bp size bin, then that bin was scored for a particular bacterial isolate. Thus matching the DNA profile produced from different strains of bacteria depended in large measure on the spatial relationship of PCR product sizes in narrowly defined size categories.

## 3. Results

### 3.1 Strategy of the RAPD procedure

The goal of this study was to evaluate the feasibility of using RAPD analysis methods to develop genetic profiles for different isolates of bacteria. It was important to develop a RAPD procedure that would produce DNA profiles with sufficient polymorphic complexity that a differentiation among strains of bacteria could be determined, but yet not be so complex as to make comparisons impossible. Initial experiments were performed to examine different steps in the RAPD procedure used in conjunction with the 310 Genetic Analyzer. The nature of RAPD analysis is such that mixtures of arbitrary primers direct the amplification of genomic DNA targets under conditions of low stringency. Thus, it was imperative to evaluate different primer combinations and to fastidiously control temperature during cycling to generate reproducible DNA profiles. Each aspect of the RAPD procedure was evaluated in turn to develop conditions that would produce DNA profiles that were reproducible.

### 3.1.1 Primer Investigation

Identification of suitable primers involved testing individuals or mixtures of each of five different primers available with DNA extracted from two morphologically distinct isolates of *B. cereus*. The nucleotide sequences of three of the primers evaluated were chosen because they had previously been shown to be useful in the RAPD method with DNA obtained from soil bacteria (Babalola et. al., 2002). The other two primers evaluated exhibited redundancy at the 3'end to enhance their ability to direct amplification of a wider collection of genomic DNA targets and were included in the study simply because they were available in the laboratory.

Each primer alone directed amplification of only a few DNA products, if they produced any at all. Thus, the use of a single primer would not direct the amplification of a sufficiciently complex DNA profile to distinguish strains of *B. cereus* (Figure 11).

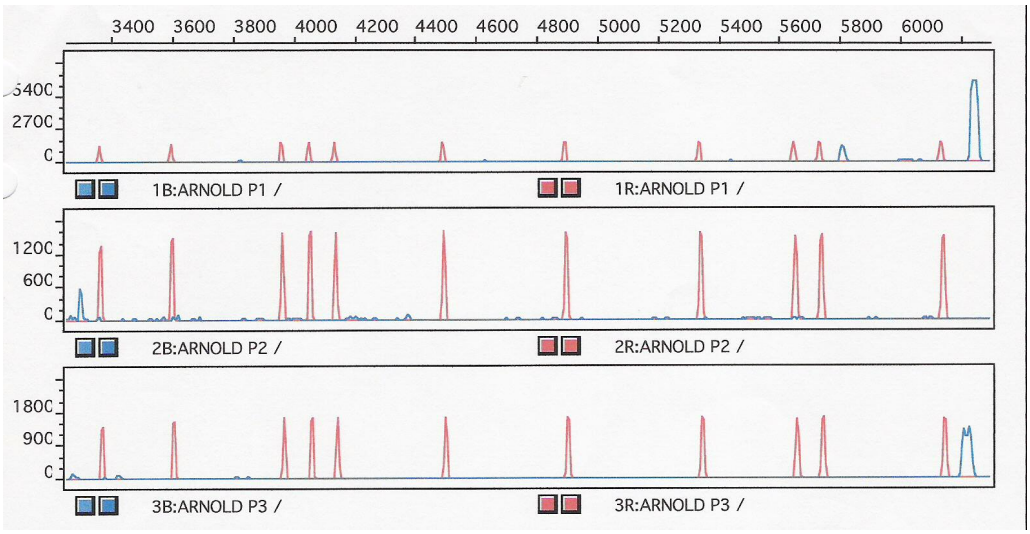


Figure 11. Single primer experimentation results. Electropherogram of the strain Arnold amplified with a single primer, shown with the internal standard (in red). P1 is the primer sequence GGGTAACGCC, P2 is GTGATCGCAG, and P3 is AGCCAGCGAA. As shown, these profiles were not complex enough to create a profile suitable to distinguish strains from each other.

In addition to single primers, mixtures of subsets of primers also did not produce DNA profiles of suitable complexity (not shown). However, a mixture of all five primers (figure 12) did produce DNA profiles that were sufficiently complex to allow two strains of *B. cereus* (Arnold and Whimpy) to be easily distinguished (Refer to section 3.2 on page 39 for more information about these strains).

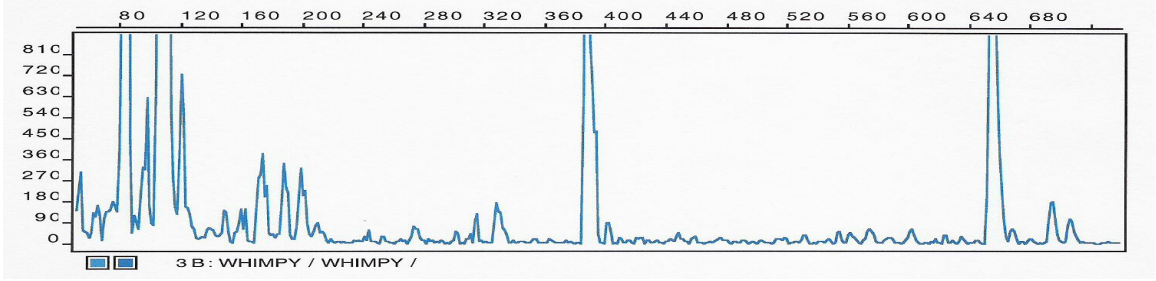


Figure 12. Electropherogram produced with all five primers on a reference strain of bacteria. These primers provide a profile sufficiently complex enough to allow for strain comparisons.

### 3.1.2 Annealing Temperature Investigation

Due to the fact that RAPD primers do not target specific, predetermined loci in genomic DNA, the annealing temperature used in the PCR cycling is important. PCR primers bind tightly to a DNA target that is perfectly complementary. In the RAPD method however, primers are usually not perfectly complementary, but may be sufficiently related to targets in a template to direct amplification of products at a low annealing temperature. Therefore, it was important to determine which annealing temperature gave the most reproducible DNA profile consisting of an adequate number of polymorphic products. This was empirically determined by varying the annealing temperature in the cycling program and seeing which appeared to produce the most reproducible and sufficiently complex profiles. Throughout testing, the optimal temperature was $40^{o}$C, which was used for the rest of the experiments (not shown).

### 3.1.3 Primer Dimer Test

A blank was included in each analysis to serve as a negative control to show that samples were not contaminated. Several small PCR products were typicallyvisible in the electropherogram of the blank. A test was performed to determine if these products were produced by contaminating DNA, or resulted from a phenomenon called primer dimer. This is caused when primers bind to each other, are amplified, and produce a product.

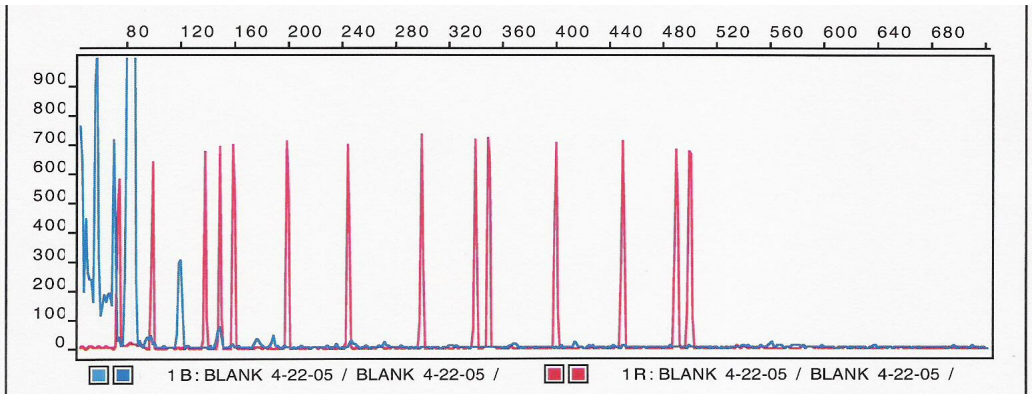


Figure 13. Evidence of the phenomenon, primer dimer, occurring. Small molecular weight products (in blue), most occurring under 80bp (sizes listed across the top of the electopherogram), were produced in ever blank sample. Suggesting that primer dimer has occurred.

| Product sizes | # of products |
|---|---|
| 50-59 | 6 |
| 60-69 | 5 |
| 70-79 | 2 |
| 80-89 | 7 |
| 120 | 1 |

Table 4. Size and average amount of products produced due to primer dimer.

Figure 13 is an example of the primer mix directing the amplification of products of low molecular weight in the absence of added DNA template. These low molecular weight products, listed in Table 4, appear to be primer dimers for several reasons:

1.) The products are typically small, exhibiting molecular weights of less than 100bp.

2.) They are produced in the absence of any added DNA template. Even sterile distilled water will result in their production.

3.) Amplification containing single primers or subsets of primers lack the products produced when all five primers are used together.

4.) Raising the annealing temperature during PCR cycling reduces the complexity of the low molecular weight products produced.

Due to the fact that primer dimer products generally (with the exception of the 120bp product) only occurred under 90bp, only products larger than 100bp were used for analysis.

### 3.1.4 Reproducibility Investigation

Since RAPD is an extremely sensitive procedure, variability in any step in DNA extraction or PCR amplification can affect the qualitative or quantitative nature of products produced. Therefore it is important that there is reproducibility in the DNA profiles produced from DNA extracted from a given strain of bacteria. Testing was done to determine if within the same run of the ABI Prism 310 Genetic Analyzer, a single bacterial isolate would produce the same DNA profile when repeatedly amplified. Then the same test was performed when the isolates were analyzed on different days. The results show that there is some variability both within and between runs on the genetic analyzer, but the variability did not preclude profile comparisons. As shown in Figures 14 and 15, most of the variability is quantitative in nature.

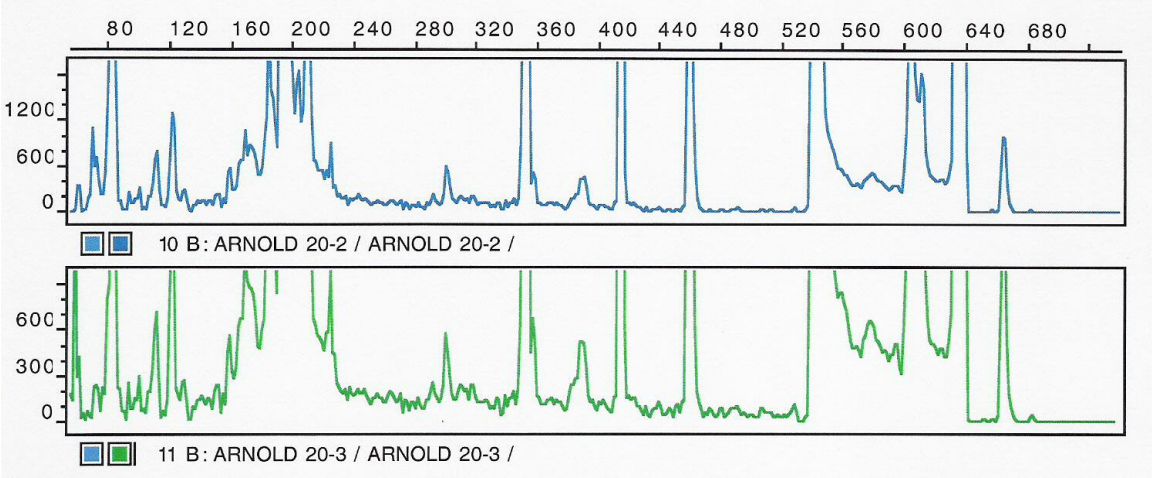


Figure 14. Evidence of reproducibility within the same run. The profiles that are shown were produced from genomic DNA extracted from the Arnold isolate. The peaks are all qualitatively the same, the only difference being the amount of product present as represented by the relative fluorescence, or RFUS, in each PCR product. These two profiles show that they are from the same isolate of bacteria.

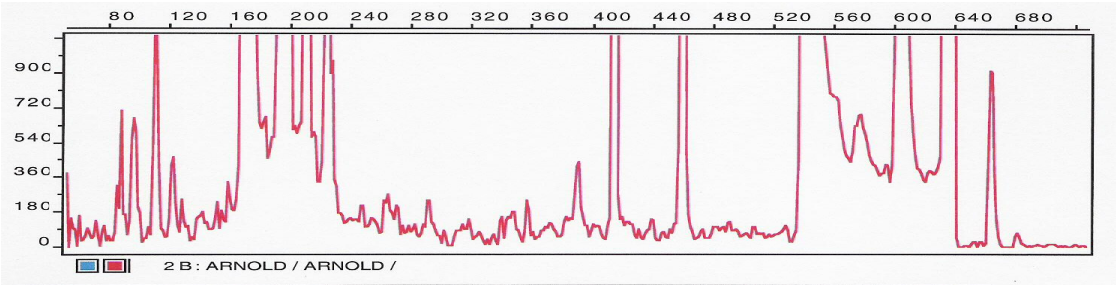


Figure 15. Evidence of reproducibility between different runs. The profile produced is very similar to the profiles produce in figure 14, and can be shown to be the same strain as the isolate in figure 14.

### 3.2 Isolate Differentiation

Two morphologically different isolates of *B. cereus* were selected to be the controls for the project and represent extremes in DNA profiles produced with *B. cereus* genomic DNA. One strain, named Arnold, exhibited phenotypic properties suggesting it would be the most toxic if one were infected with it. The Arnold strain also exhibits the most complex RAPD profile. The other strain, known as Whimpy, exhibits a phenotype suggestive of very few toxins and also exhibited a very simple RAPD profile. Their

profiles were easily distinguishable from one and another and thus represent extremes of DNA RAPD profiles for *B. cereus* (Figure 16).

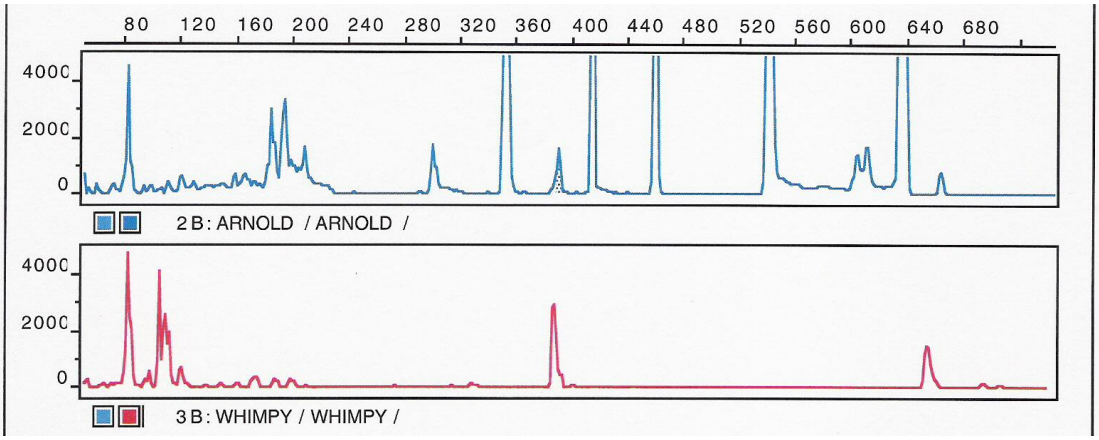


Figure 16. *B. cereus* strain vs. *B. cereus* strain. Electropherogram of Arnold in blue and Whimpy in red showing the differences in the profiles. Some of the products were shared, such as the 380bp product, as would be expected in related isolates.

The two references DNA were also compared based on the average products that were produced. The binning of these products underscores the significant difference in RAPD profiles between the two strains (Table 5).

| Product Size(s) (base pairs) | Arnold | Whimpy |
|---|---|---|
| 100-119 | X | X |
| 150-159 | X | |
| 160-179 | X | X |
| 180-189 | X | |
| 190-199 | X | X |
| 200-219 | X | |
| 220-239 | X | |
| 300-309 | X | |
| 320-329 | | X |
| 350-359 | X | |
| 380-399 | X | X |
| 410-419 | X | |
| 450-460 | X | |
| Over 500 | Avg. 6 products | Avg. 5 products |

Table 5. Binning of products produced in RAPD analysis. Products are placed in size bins of 10 to 20 base pairs. There are generally multiple products of slightly differing size occupying each bin. All products listed appeared in over 77% of runs. These two isolates became the reference phenotypes that profiles produced from unknown isolates were compared against during the rest of the experiments.

For any molecular assay to be useful for the identification of a bioterrorist agent, it must be able to distinguish closely related strains of organisms. In the case of anthrax identification, a molecular assay must be able to distiquish between *B. anthracis* and non-*Bacillus* species as well as different species of *Bacillus* and ideally even different strains or isolates of the same species of bacteria. The RAPD profiles produced with DNA from reference strains of *B. cereus* were compared against other *Bacillus* isolates shown morphologically to be non-*B. cereus*.

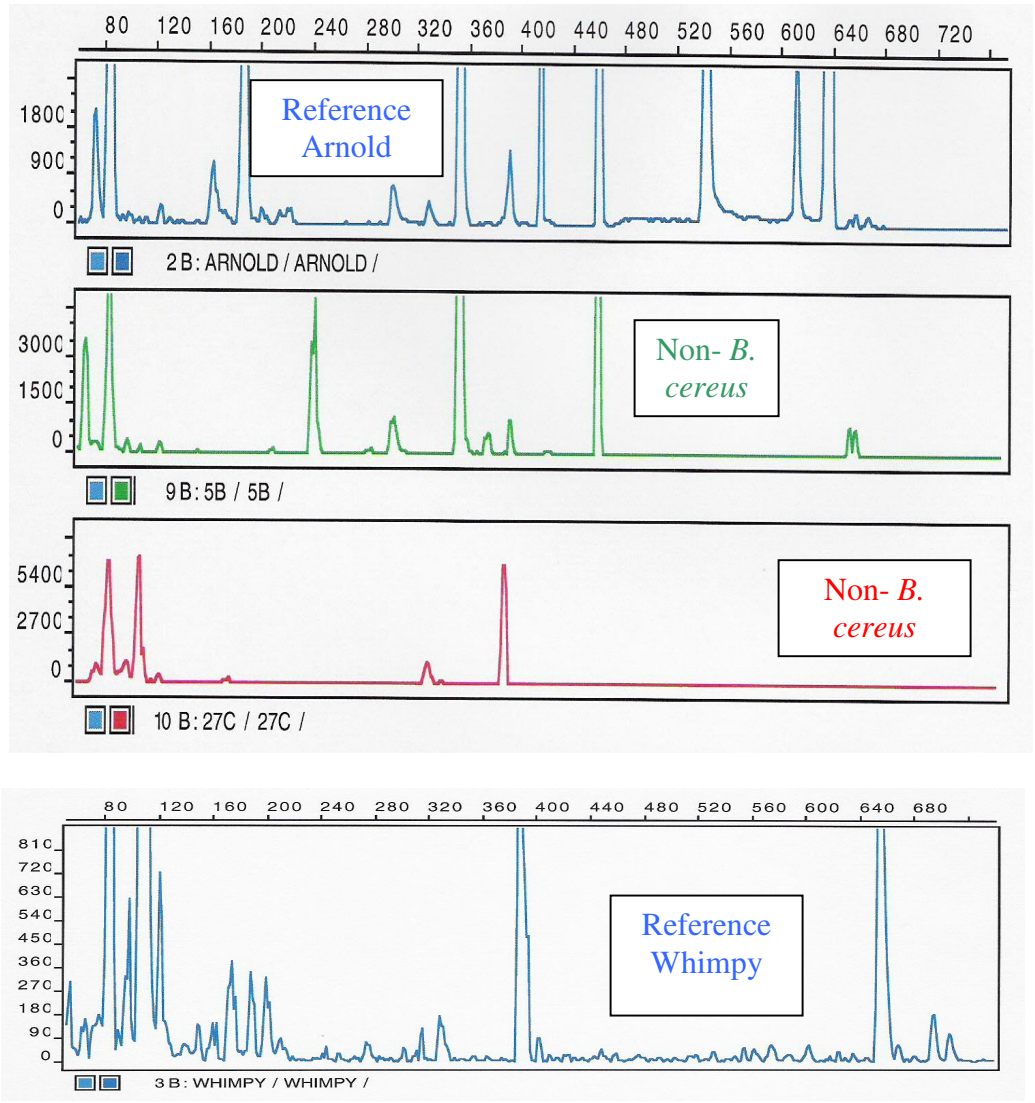


Figure 17. Non-*B. cereus* isolate electropherogram. Arnold and Whimpy (in blue) are the two reference samples that are examples of *B. cereus*.. The green, 5B, and red, 27C, are isolates that were known to be *Bacillus* species, but were not *B. cereus* as determined by morphological and physiological criteria.

Figure 17 provides evidence showing the RAPD profiles of non-*B.cereus* species are distinguishable from that of *B. cereus*, but can look similar as does sample 27C and reference sample, Whimpy. Isolate 5B exhibits clear similarity to the Arnold strain and yet harbors clear differences in the genome that are revealed through the RAPD procedure. If the Arnold strain and 5B strains were recovered from separate disease

outbreaks thought due to bioterrorism, one could possibly conclude the infectious agents responsible for the outbreaks originated from related, yet distinct sources.

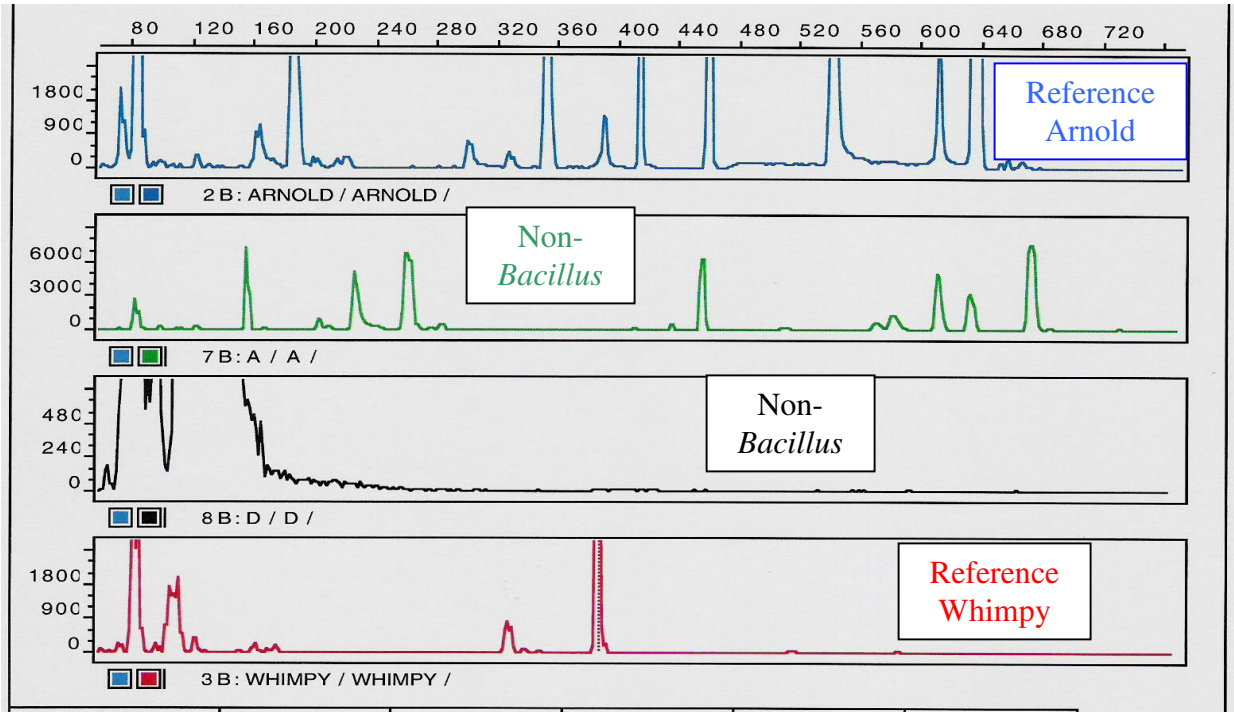


Figure 18. Non-*Bacillus* isolates electropherogram. Profiles of *Bacillus cereus* Arnold and Whimpy are shown again as reference to compare non-*Bacillus* isolates. The green profile, A, and the black profile, D, are isolates of bacteria that are not *Bacillus*.

In figure 18, RAPD profiles produced from non-*Bacillus* species are compared to the reference *B. cereus* strains, Arnold and Whimpy. The isolate labeled D shows a very different profile from either of the reference isolates. The other non-*Bacillus* isolate is distinct, but not as clearly as the isolate labeled D. It does have products that are sizes different than any *B. cereus* isolates, like the products in the 200-300bp range. Some products were shared by isolate A and Arnold, like the 460bp product. This might suggest there is some relatedness at the DNA level between the isolates, or it could just be a coincidence that the DNA from the different isolates directs the amplification of distinct products of similar size. Recall the RAPD procedure is useful because one need not

know any DNA sequence of a target and thus products of identical size may consist of unrelated nucleotide sequence.

### 3.3 Mock Forensic Investigation

A mock forensic investigation was performed to test the RAPD procedure. In an actual attack a terrorist could use any number of different methods to deliver an infectious agent including mailing spores to selected targets like the attacks of 2001. Other possible transmission routes exist and in a recent study from this graduate program (Miller, 2005), *B. cereus* spores were inoculated into coffee grinds that were subsequently brewed, were inoculated onto a toothbrush, and were sprayed onto doorknobs. In every case, viable bacteria could be cultured from the spores, demonstrating the potential for success in infecting a human target with anthrax. These scenarios of mock bioterrorist attacks also afforded an opportunity to challenge the RAPD assay to identify the strains of *B. cereus* used to inoculate the different items. Seven isolates of *B. cereus* from which the strains used in the mock attack were derived were tested using RAPD and the resulting DNA profiles were used as a reference database (Figure 19).

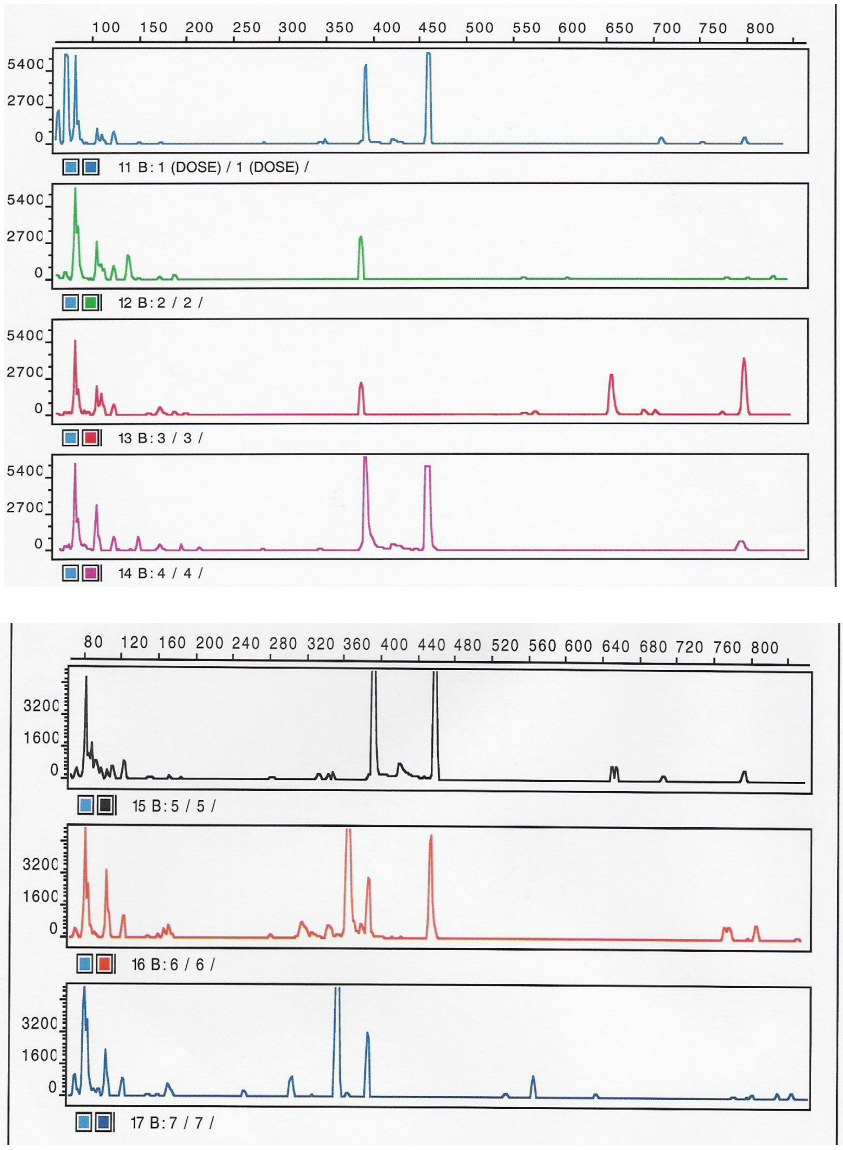


Figure 19. Electropherograms of the seven reference used in the mock forensic attack.

Two of the seven isolates were chosen to be used for the coffee scenarios and to spray the doorknobs. The spores recovered from the brewed coffee and from the doorknob were cultured in order to produce sufficient cells to be analyzed using the RAPD procedure. RAPD profiles produced from isolatesfrom the coffee and doorknob were compared against the database of RAPD profiles from the seven reference strains through simple

visual comparison and matching of the binning of main PCR products found in each of the isolates (Figure 20).

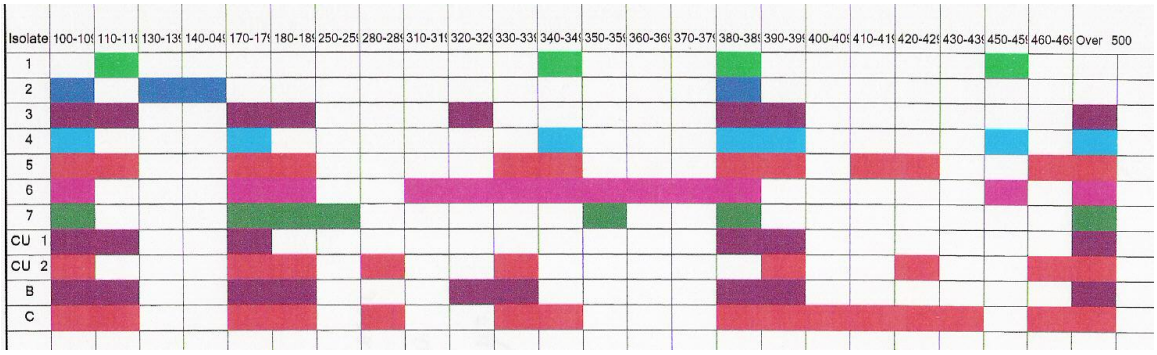


Figure 20. Products produced during RAPD analysis used in the bioterrorist experiment. Colored horizontal bars indicate the presence of PCR products grouped into size categories expressed as 10bp or 20bp ranges and listed across the top of the figure. The isolates recovered from brewed coffee are labeled Coffee 1 and 2 respectively (CU1 and CU2). The isolates cultured from spores sprayed on to the doorknobs are labeled B and C. Reference and unknown strains believed to be identical based upon their RAPD profiles are indicated with horizontal bars of the same color.

The two different isolates were used in both the coffee and doorknob test and in each case the references samples could be matched to each 'attack,' linking them to each other and a common source. The RAPD profiles recovered from bacteria used to inoculate coffee and to spray doorknobs show clear relatedness to reference strains #3 and #5, respectively (Figure 21). After consulting with the investigators who produced the attack experiment, it was learned that the correct identification of the unknowns had been made by the RAPD procedure.

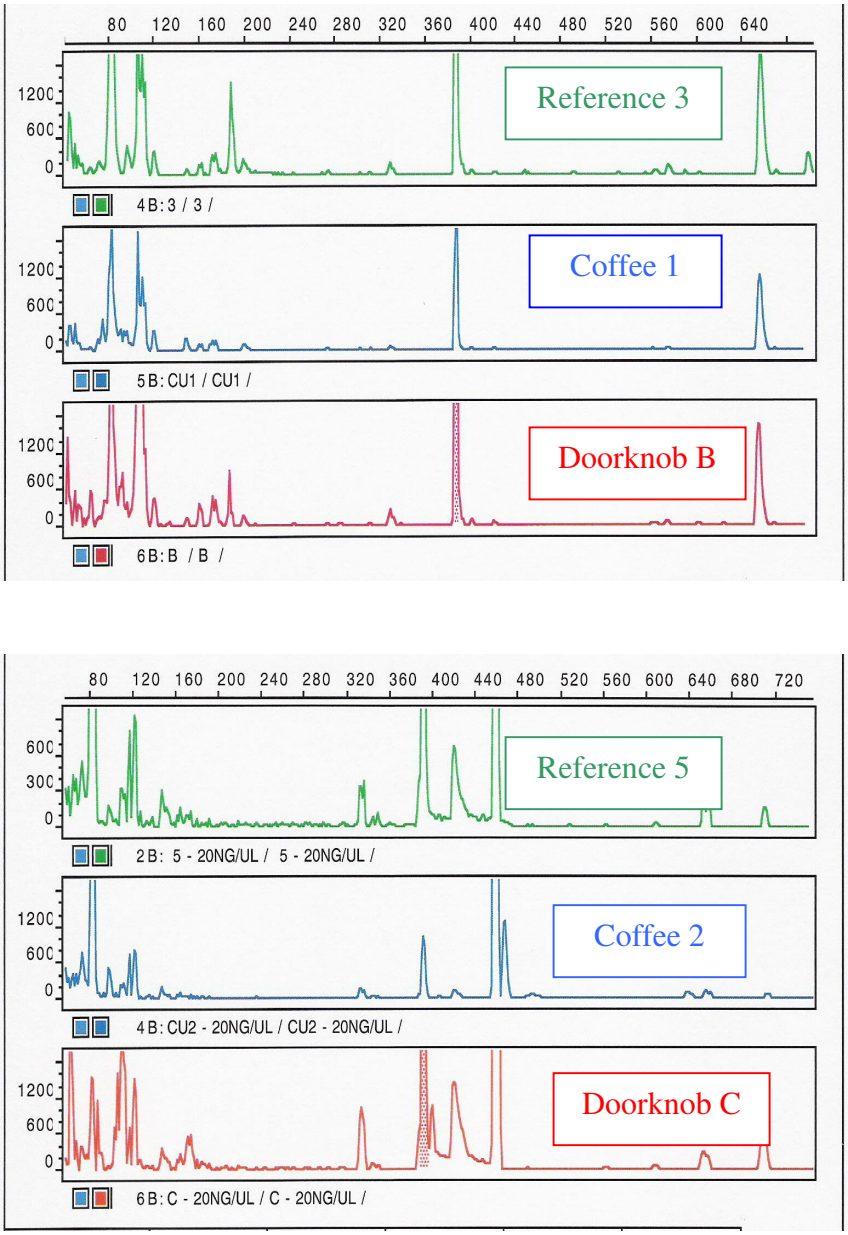


Figure 21. Electropherogram of linked isolates. Bacteria obtained after brewing the coffee and bacteria obtained from the doorknob were able to be linked to each other. They both were able to be linked to a references isolate, determining the origin of the bacteria. The green profiles are one of the seven isolates that were given as a reference profile. The blue profile is the isolate that was obtained from the mock coffee pot attack. The red profiles were the isolates obtained from the doorknobs.

Figure 21 shows that the reference (#3) matched the strain from the coffee pot (CU1) and one from the door handles (B). Likewise, Reference strain #5 matches the other isolates from the coffee pot attack (CU2) and door handle attack isolate (C). RAPD profiles from a common source are superimposed on one another to demonstrate similarities and

quantitative differences (Figure 22). Quantitatively the strains differ, which could be due to unequal concentrations of template DNA, but qualitatively the products produced are ultimately the same and these isolates can be said to be from the same strain of bacteria.

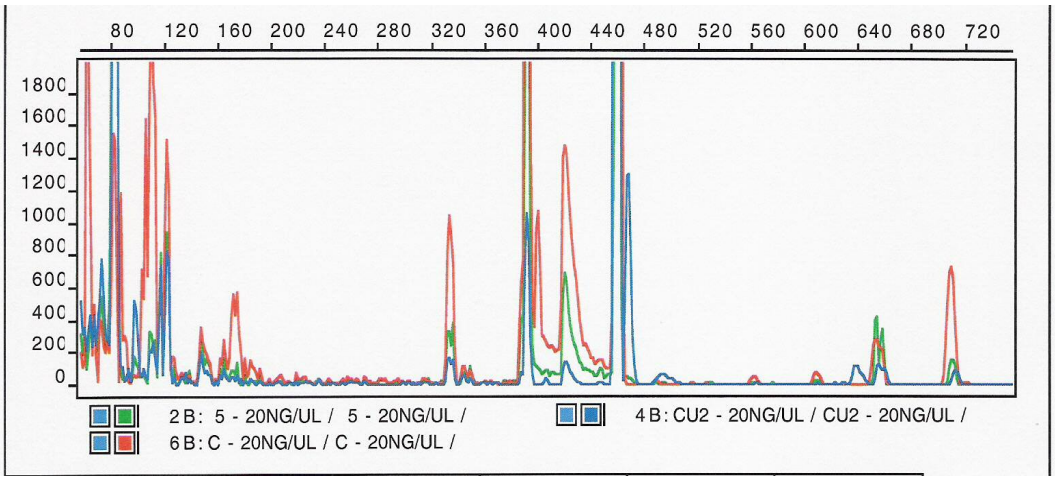


Figure 22. Superimposed electrpherogram of linked isolates. The two isolates used in the experiments and the references sample that was determined to be the origin of the attack, all were found to be identified. The reference isolate is in green and its profiles matches closely the profile of the isolate from the coffee, shown in blue and that produced from the door knob isolate (in red).

## 4. Discussion

### 4.1 Review of Results

Investigators need a reliable way to link terrorist attacks to each other or back to an original source. It is important that in a bioterrorism attack, separate outbreaks of infection can be quickly analyzed to link the outbreaks to a single infectious agent. This would help investigators determine if one source or multiple sources of agents were responsible. Genetic analysis of a bacterial strain can also help determine the origin of the bacteria. This occurred during the 2001 attacks, when it was determined, through genetic

analysis, that the anthrax strain used was identical to Ames strain. The Ames strain originated in the United States and was named for a strain that was isolated from cows in Ames, Iowa in the 1930's (MacKenzie, 2001). Genetic analysis also showed a high degree of homology between strains used in research and the strains used in the attack, suggesting the strains were not separated by many or any generations (MacKenzie, 2002). Al this information assisted investigators in their investigation of this crime.

The RAPD method provides a relatively quick, inexpensive, and reliable way to compare the DNA profiles of strains of bacteria. After extraction of DNA, this procedure can be accomplished in 5-8 hours. The faster such information becomes available, the faster an investigation can move forward to hopefully prevent further attacks. Moreover, with more research, if the CDC and state laboratories were to develop databases of RAPD profiles for strains of bacteria possibly used as a bioweapons, public health investigators could compare the profiles in the database against an isolate recovered from a suspected attack and perhaps identify the bacteria. When anthrax was sent through the mail in 2001, researchers had to test each sample against all strains known to be used in different countries. If there would have been databases the samples could have been tested quickly and the researchers would have known quickly that the strain used in each attack was the same, and from the American strain known as Ames.

The RAPD procedure developed in these experiments, could allow for strain differentiation among *Bacillus* species. It was also effective in revealing distinctions at the DNA level between *B. cereus* strains and non-*Bacillus* species. Most importantly, it

allowed for strain matching among different isolates of *B. cereus,* which, overall, exhibits a high degree of DNA sequence homology. During the testing it became apparent that many of the different strains shared some of the same products, as would be expected from related organisms. However, there was enough variability among the strains to differentiate them and be confident in matching strains to each other, to a reference, or to an original source.

Some of the isolates of bacteria were very similar in the products that they produced with the RAPD procedure. For example, in Figure 24, isolates labeled as 4 and 5 are shown in electropherograms. They show many of the same products, suggesting close relatedness, but there are high molecular weight products that isolate #5 possesses that isolate #4 does not. This information allowed the two strains to be distinguished, which was informative for the mock forensic examination. However as stated above, isolates 4 and 5 are clearly related, both are *B. cereus*, sharing a large portion of their RAPD profile (Figure 23).

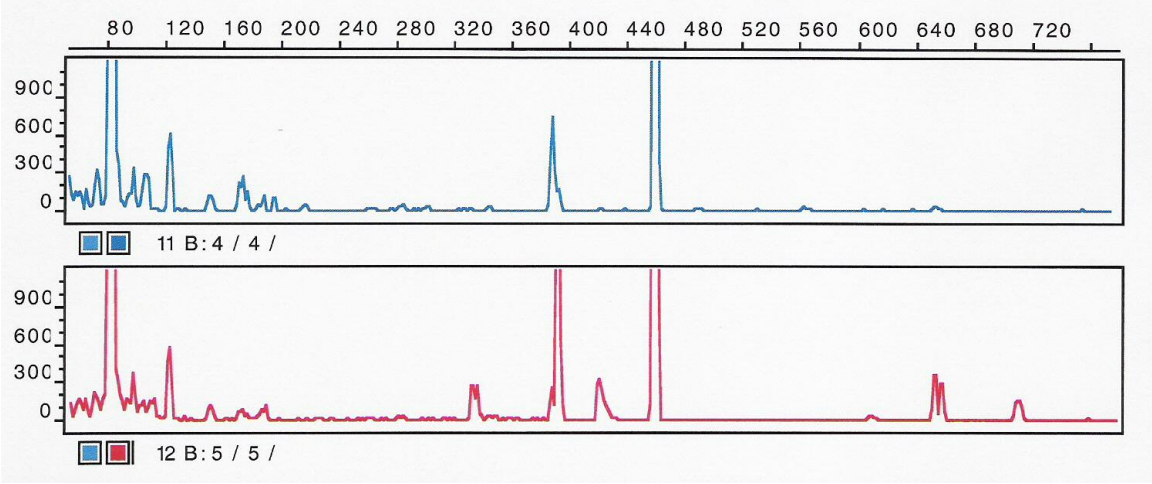


Figure 23. Electropherogram of similar profiles from two different isolates.

A mock forensic test was performed by another student in the Forensic Science Department, which in turn allowed for a practical application of the RAPD method. The exercise demonstrated RAPD analysis to be useful, but results produced with DNA from the same bacterial isolates sometimes produced RAPD profiles that varied somewhat. Since the procedure is sensitive to temperature and the concentration of template and primer, small variations in these PCR reaction conditions could have significant impact on the RAPD profiles generated. Variation in RAPD profiles included products that were missing in some runs that had been produced normally in other runs (Figure 24).

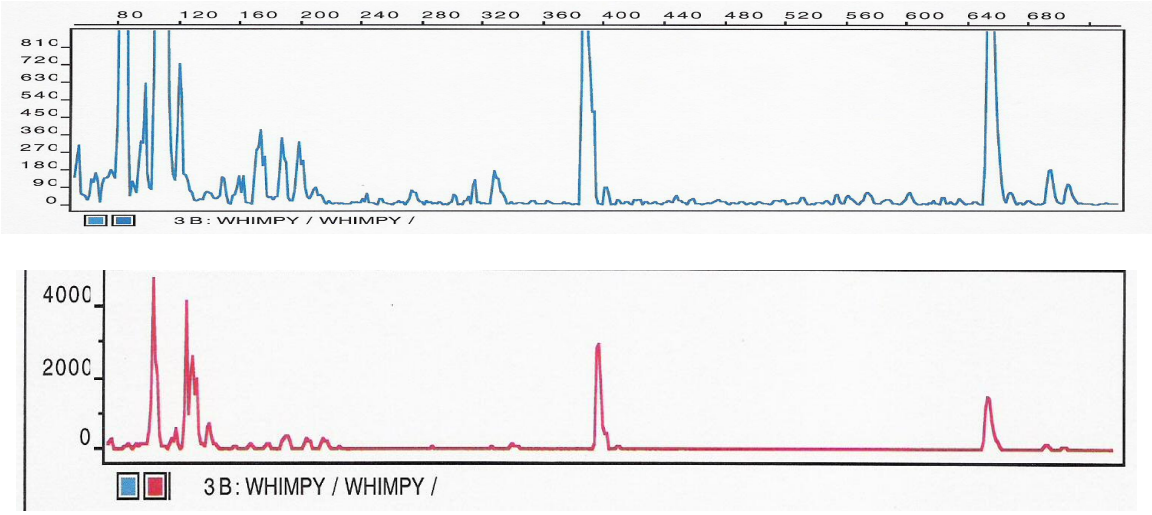


Figure 24. Electropherogram showing variability between different runs of the same isolate of *B. cereus*. Whimpy's profile was slightly different between these two runs. In the first electropherogram (shown in blue) products were produced in between 180 and 200bp and above 680bp that were not equally present in the second electropherogram (shown in red).

One possible explanation for this phenomenon is aplicon dropout. Aplicon dropout occurs when there is too little DNA template, primer binding is disrupted, or the product fails to amplify (Butler, 2001). Thus, in order to be sure a RAPD profile truly is representative, repeat runs could be performed on the DNA of an isolate, perhaps varying the amount of genomic DNA template added to the PCR reaction.

Among the variables responsible for inconsistency in RAPD profiles seen in our study, we feel variation in input template DNA amounts is the most likely cause. The quantity of DNA recovered from each bacterial culture was estimated using a yield gel that was a crude method to estimate the quantity present in each sample. If DNA quantitation were performed using a more accurate methodology, perhaps reproducibility would be enhanced.

The reproducibility of the procedure is one of the most important aspects in developing of effective RAPD procedure. Annealing temperature is one critical feature of a PCR cycling scheme and thus was tested to determine which temperature would give the most reproducible results. Different concentrations of template DNA were also tested to determine the smallest amount of DNA that would direct reproducible amplification. 20ng of input DNA template and an annealing temperature of 40$^{o}$C were found to represent PCR cycling conditions that resulted in acceptable reproducibility for the RAPD methodology. Future testing with different primers might reveal a more reliable and reproducible combination of temperature and DNA concentration.

A mock forensic experiment demonstrated that spores added to a hot liquid (i.e. coffee) or sprayed on inanimate objects, can be used to produce RAPD profiles that are suitable for comparison to profiles developed from the parent *B. cereus* strains originally isolated from the soil (Miller, 2005). Therefore, the chromosomal DNA and corresponding RAPD profiles do not change significantly when the recovered spores are germinated in liquid

culture and subjected to RAPD analysis. Thus, in a suspected bioterrorism attack involving *Bacillus*, spores recovered from multiple sites could be analyzed using the RAPD procedure to determine if they have a common source. This procedure would be a useful addition to the technologies currently in place in forensic labs.

After the United States became a victim of bioterrorism, many researchers determined that ways were needed to quickly investigate and identify bioterrorism agents. Vaillancourt et. al. (2001) developed a Multiplex PCR assay using SmartCycler® technology for three DNA targets in the plasmids and chromosome of *B. anthracis*. SmartCycler® Technology is a real-time thermal cycler that has up to 96 independently programmable reaction sites allowing multiple experiments with different protocols to be simultaneously run. The systems software monitors each reaction site during the run as the fluorescent signals develop and produces a growth curve as amplification occurs (SmartCycler® Technology). The claims of the researchers are that this procedure is effective for identification of the strains of *B. anthracis*, it is sensitive, and only takes about one hour. Bergeron et. al. (2003) also used the SmartCycler® to pinpoint single nucleotide polymorphisms (SNPs) found on plasmids pX01 and pX02 to quickly identify *B. anthracis*. One of the advantages of this analysis over the traditional culture methods is it is rapid, sensitive, and is subjected to computer analysis as compared to human analysis. This specific PCR assay is applicable for various clinical specimens, suitable for spore detection, and is specific to only *B. anthracis* (Bergeron et. al., 2003).

Another group found that they could identify *Bacillus* species and could differentiate between strains using automated repetitive sequencing based PCR, or rep-PCR, to find DNA fingerprints (Webb et. al., 2003). This method utilizes the Caliper 1000 Analyzer (Caliper Technologies, Corp, CA) and DiversLab software to analyze non-coding, interspersed repeat sequences. The analyzer and software allow for a report to be automatically generated that includes gel-like images of the fingerprint to be analyzed to determine which species of *Bacillus*, if any, is present (Webb et. al., 2003).

One of the advantages of the RAPD procedure developed over the procedures previously mentioned is that this method utilizes technology and instruments that are used everyday in most forensic labs. Thus, existing instrumentation and techniques allow the procedure to be used in any lab that utilizes capillary electrophoresis with no need for new, expensive equipment or training.

### 4.1 Future Testing

Results of our study raise some questions suitable for future study:

- Does the RAPD procedure work with DNA from other bacterial species or other potential biological weapons (i.e. Smallpox virus or *Clostridium botulinum* bacterium)?
- Does this procedure work with samples obtained from infected humans or animals?

- Can a process be developed that will allow a RAPD profile to be used as a screening test to determine what bioterrorist agent was used in an attack?
- Does the RAPD profile degrade based on factors that are known to cause DNA degradation (age of sample, heat, chemicals)?

There are issues that arose during the course of this study that would need to be considered before this procedure can be routinely used in a laboratory. One issue is the RFU threshold set for designating PCR products. The threshold is the minimum amount of RFUs required for a PCR product to be incorporated into the GeneScan software analysis. When deterring how to analyze the results, different threshold values were evaluated to determine the RFU threshold that gave the most reproducible profiles. 150 RFUs was chosen as the threshold that produced an acceptable level of reproducibility. More experimentation, especially with additional primers sequences might further enhance reliability and the usefulness of the RAPD procedure.

Future enhancements to this study include expanding the list of forensically important agents tested. For example, RAPD analysis could possibly be used to reveal polymorphisms in the DNA of naturally occurring drugs like marijuana. If a RAPD profile database can be constructed, it could be possible to link marijuana confiscated from one source to others, or linking it back to an original plant. Such information would be valuable in tracing the distribution network of drugs to their source. RAPD analysis could also be used by public health officials to investigate outbreaks of food borne illness from contaminated food sold to restaurants or grocery stores. Such outbreaks are

typically the result of bacterial contamination and represent a scenario very similar to applications of RAPD with bioterrorist attacks.

The ABI 310 Genetic Analyzer has been proven to be extremely useful in forensic testing and most forensic labs use the 310 routinely for DNA analysis. This RAPD procedure described here is compatible with the 310 DNA analysis platform and thus forensic labs will be able to incorporate the RAPD procedure without the need for additional spaces, money, and training for new equipment.

The RAPD procedure developed in our study provides a way to match bacterial isolates recovered from different sources to one another, which will be important in any future bioterrorist attacks. A future bioterrorist attack is inevitable whether it be small scale, as in the 2001 mailing, or a large aerosol attack of a major population center. It is believed that an attack will occur at some future time because bioterrorist agents have been used as weapons for hundreds of years. The fact that many countries are suspected of having active bioterrorism programs and stockpiles of infectious agents underscores the potential for an attack and tools that incorporate modern molecular methods, like RAPD, will hopefully better prepare this country and others to respond and effectively investigate such attacks and, ultimately help ensure our long term security.

## 5. Conclusion

In summery the works of this study provided evidence that under carefully controlled conditions the RAPD procedure coupled with capillary electrophoresis can be useful in linking isolates of *B. cereus* to each other and to an origin. Thus, it could be possible to use this procedure to link outbreaks of *B. anthracis* to each other. In developing this procedure a species of bacteria whose genome was known was used as an example. The nature of this procedure allows for investigations to occur without any past knowledge about the genome of the organism. Thus, it could be useful if there is ever any future attack with an organism (i.e. Hantavirus) whose genetic information is not known, to link them to each other and back to an origin without having any knowledge about the genetic sequence.

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