ISOLATION AND DEVELOPMENT OF A SURROGATE BIOAGENT FOR ANALYSIS OF DISEASE TRANSMISSION BY INANIMATE OBJECTS

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ABBREVIATION DESIGNATIONS

API	Analytical Profile Index
BHI	Brain Heart Infusion
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
FDA	Federal Drug Administration
GF	Boone T. Pickens Football Field
LD	Lethal Dose
LR	Locker Room
MYP	Mannitol-Egg Yolk-Polymyxin
NA	Nutrient Agar
NG	No Growth
OSU-CHS	Oklahoma State University-Center for Health Sciences
PA	Protective Antigen
PCR	Polymerase Chain Reaction
PF	Practice Field
SBA	Sheep Blood Agar
SF	Soccer Field
TNTC	Too Numerous To Count
VNTR	Variable-number Tandem Repeat

CHAPTER I

INTRODUCTION

In recent years terrorism has become a household word. The first bombing of the World Trade Center in 1993 by foreign terrorists shocked the world and proved the United States was indeed vulnerable. In 1995, the bombing of the Murrah Federal Building in Oklahoma City brought the realization that terrorism existed on a domestic level. In 2001, the World Trade Center and the Pentagon were attacked by foreign terrorists flying highjacked planes and brought terrorism to a personal level in the minds of all Americans. The subsequent disbursement of a laboratory strain of anthrax spores via the United States Mail caused panic and social disruption. While terrorism has crossed all geographical, ethnic, and social barriers, it is the water, food supply, people, and the infrastructure of the United States that has proven to be vulnerable. At the present time terrorism may be either foreign or domestic and may take the form of nuclear, chemical, or biological assaults.

Neither public health issues nor biological warfare involving anthrax are new. Historically, "the plague of boils", one of the seven plagues of Egypt, is suspected to have been anthrax (*Bacillus anthracis* and Anthrax). In the 1600's, Black Bain (anthrax) is believed to have caused the death of 60,000 cattle in Europe (Timeline, 2001). A human case of anthrax was reported in Kentucky in the early 1800's. This case of cutaneous anthrax was subsequently found to be contracted by contact with infected

livestock (Todar, 2001). The disease became known as "Woolsorter's disease" because it affects people involved in the sheep industry. In 1876, Robert Koch isolated and identified *Bacillus anthracis* as the bacterial agent responsible for anthrax (Oxford University Press, Inc., 1998). In 1881, Pasteur developed the first immunization for livestock against anthrax.

In World War II, after having had success with chemical warfare (i.e. mustard gas) programs during World War I, Nazi Germany established programs using anthrax as a biological weapon (Milestones in Anthrax History, n.d.). German agents infected the horses, mules, and cattle used by the allied forces with anthrax. In 1937, Japan began a biological warfare program in Manchuria which included testing human subjects with anthrax (Dixon et al.,1999). During the Manchurian campaign, Japan performed extensive testing of anthrax as a bioweapon. It is estimated that by 1940, over 5 tons of anthrax had been manufactured for placement in bombshell casings (Albarelli, 2001). Extensive testing on casing types led to the development of a porcelain anthrax bomb that shattered on impact.

By 1942, the United Kingdom's biological warfare program included experimentation off the coast of Scotland on Gruinard's Island, which has only recently been decontaminated (Bhalla & Warheit, 2004). The United States Army began considering anthrax as a biological warfare agent in 1942 (Albarelli, 2001). This project was a joint effort with the Canadian military and produced 150 pounds of anthrax spore per month. The United States military converted a conventional munitions plant into a bioweapon

production facility in Vigo, Indiana. This plant would produce enough anthrax for 500,000 bombs in less than a month. The facility produced one million anthrax bombs, half of which were stockpiled in the United States. Believing that the Japanese were using hot air balloons as a transport for porcelain encased anthrax, Fort Detrick, Maryland placed bacteriologists on high alert in 1944 when large balloon sightings inscribed with Japanese writing were reported. The U.S. biological warfare program continued after World War II at Fort Detrick. In the late 1950's the military began clinical trials on humans for an anthrax vaccine. In 1969, President Nixon ended the biological weapons program, however, biological warfare defense programs continue today (Johnson, n.d.). In 1970, the FDA approved the anthrax vaccine (Gardner, 2004).

In 1972, international concerns of biological weapons resulted in outlawing the development and stockpiling of these weapons. In the late 1970's, a human anthrax epidemic broke out in Zimbabwe infecting 10,738 victims (Nass, 1992). The Soviet Union's biological weapons program continued in deference to the international bans. A military facility in Sverdlvosk accidentally released aerosolized anthrax spores in 1979 (Abramova et al., 1993). This well documented incident resulted in 64 deaths at a nearby community. The incident provided the opportunity for case studies of anthrax exposure for short term and long-term effects. Concerns of anthrax use as a biological weapon led to the vaccination of U.S. troops in 1991 as preparation for the Gulf War (Todar, 2001).

Anthrax has been used as a terrorist weapon since the early 1990's. In 1993, Aum Shinrikyo, a terrorist group, released anthrax from a mid-rise Tokyo office building

laboratory; no one was injured by this incident (Guillemin, 1999). By 1995, Iraq had produced 8,500 liters of concentrated anthrax (Todar, 2001). In 1997, the treatment of choice for anthrax was penicillin G (Penn & Koltz, 1977). The possibility of a penicillin resistant strain of anthrax being used as a bioweapon has led to a preferred treatment of dual antibiotics consisting of ciprofloxacin and doxycycline. In 1998 the Secretary of Defense, realizing the anthrax threat, approved anthrax vaccination for all military service members.

In 2001, subsequent to the World Trade Center bombing, anthrax spores were used by terrorists. These spores were distributed though the U.S. postal service. This attack resulted in 22 cases of anthrax with 4 deaths (Sanderson et al., 2004). The 2001 anthrax outbreak has been traced to *B. anthracis* strain 135. These isolates were confirmed and subtyped utilizing procedures described by Keim et al. (2000). During the 2001 outbreak, Florida postal workers at risk of exposure were offered prophylactic antibiotics (Quintiliani, R. Jr. & Quintiliani R., 2003).

The anthrax exposure via the U.S. mail has sparked renewed interest in anthrax research. The primary difficulty for treating anthrax is diagnosis. The United States Army categorizes agents for biological warfare into three groups (Kahn et al., 2000). Category A is designated for high priority agents. These agents are easily disseminated causing a high mortality rate. The effect of public panic and social disruption is critical and would require special and immediate action for public health preparedness. Category A agents includes smallpox, anthrax, plague, botulism, and Ebola. Category B agents are the

second highest priority agents and are moderately easy to disseminate. These agents cause moderate morbidity with low mortality. Category B agents include Q-fever, brucellosis, ricin toxin and staphylococcus enterotoxin B. Food and water pathogens such as *Salmonella*, *Shigella*, and *E. coli* are also considered Category B agents. Category C is designated for emerging pathogens with engineering potential in future use. These agents are characterized by availability, ease of production, and potential major health impact. Category C agents include hantavirus, yellow fever, tick-borne hemorrhagic-fever, and multi-drug resistant tuberculosis. Category C agents require further research including detection, treatment, and prevention.

Anthrax is suitable as a biological weapon. *B. anthracis* is the etiological agent of anthrax. Classified as a Category A agent, this spore forming gram positive rod is capable of causing a large number of deaths rapidly which would lead to social disruption (Lane et al., 2001). In order to be effective, bioweapons need to be inexpensive and easily produced (McGovern & Christopher, n.d.). Bioweapons have the added characteristic of being selective. These weapons can be developed to target humans, animals, or plants.

The primary disadvantage of using bioagents as weapons is the hazard to the user and the dependence on weather conditions. Bioagents with a short incubation period are more effective in a tactical setting. The bioagent would prevent a battle unit from carrying out its mission and subsequently overwhelm the medical and evacuation process (Goodman, 2004). Bioagents with a longer incubation period have more appeal to terrorist as they would cause social disruption. The World Health Organization estimates that 50kg of

anthrax spores released upwind in an urban area could result in a 20% fatality rate with an additional 25% requiring hospitalization (Reshetin & Regens, 2003).

The incident in Sverdlvosk provided an opportunity for studies on the effectiveness of anthrax as a bioweapon. Extensive studies of the location of victims relative to the source site have been preformed (Meselson et al., 1994). The total weight of spores accidentally released is estimated to be between 5mg to 1000mg. This small quantity caused 64 fatalities and 32 non-fatal causalities. This example clearly demonstrates the effectiveness of anthrax as a bioweapon.

B. anthracis can produce a fatal bacterial infection when the microorganism enters the body by cutaneous, ingestion, or inhalation means/mode. Generally, anthrax is a disease of domesticated herbivorous animals such as cattle, sheep, horses, mules, and goats (Baillei, 2001). Most human infections are a result of contact with contaminated animals or animal products. Woolsorter's Disease is an example of inhalation infection by spore-contaminated dust when animal hairs or hides are handled (Todar, 2001). Human-to-human cases of anthrax have not been documented. Cutaneous anthrax infection presents as a nondescript, painless, pruritic papule 3 to 5 days after exposure. These lesions quickly form a necrotic vesicle leaving a characteristic black eschar or scab surrounded by edema (Dixon et al., 1999). Gastrointestinal anthrax occurs by ingestion of either vegetative microorganisms or spores through a pre-existing GI lesion. The affected tissues show massive edema and mucosal necrosis. The peritoneal fluid may reveal numerous large gram-positive bacilli (Nalin et al., 1977). Inhalation anthrax is usually

associated with a pre-existing pulmonary disease or other medical compromises such as leukemia or diabetes.

Both the vegetative form and spore form of the microorganism can produce disease. The vegetative form of the microorganism is susceptible to a wide range of antibiotics because it does not localize in the lung similar to other "true pneumonias". However, there is difficulty in getting antibiotics to the microorganism (Penn & Koltz, 1977). The endospores of *B. anthracis* are the infectious particles of anthrax. Since anthrax is a spore former, killing the vegetative phase does not inactivate the spores. Spores are inhaled and deposited in the terminal alveoli where they are engulfed by macrophages and carried to the lymph nodes, germinating en route. The organism produces necrosis in the lymph nodes which allows for vascular dissemination and multi-organ failure, often resulting in death (Abramova et al., 1993).

Endospores are the dormant life stage produced by the genus *Bacillus*. The endospore is not the reproductive structure but rather a survival mechanism. These endospores are resistant to extreme conditions such as high temperatures, most disinfectants, and low energy radiation (The Prokaryotic Cell, 2004). *B. anthracis* spores are ellipsoidal, located centrally or terminally, and do not swell the sporangium (Turnball, n.d.). There are five distinct waves of gene expression as the *B. anthracis* cells proceed from exponential growth through sporulation. Many of the gene components responsible for the assembly and maturation of the spore are expressed from the exponential growth phase through sporulation (Lie et al., 2004). It is notable that this gene expression occurs even before

sporulation. Gene expression during sporulation is mainly related to the physical construction of the spore rather than the synthesis of the potential spore content.

The first anthrax vaccination for animal use was developed in 1881 by Louis Pasteur for animal use. Human vaccines were not developed until the middle of the 20th century (Baillei, 2001). Classic vaccines use non-living subunits based on protective antigens. Vaccination is the most cost effective form of protection. Medical professionals do not recommend the anthrax vaccine for general use by the public. Additional studies are needed to determine safety and efficacy of the vaccine in children, the elderly and immunocompromised individuals. The current anthrax vaccine, while effective, requires six shots over an 18 month period with an additional annual booster (Bioterrorism Week, 2004). This vaccine has shown to cause some side effects.

Research on alternative vaccines is in clinical trial stages. The immune responses to anthrax in non-human primates involve a Protective Antigen (PA). Anti-PA antibodies are currently being considered in anthrax studies for developing new vaccines (Anthrax, 2002). A vaccine based on a human monoclonal antibody to the *B. anthracis* protective antigen (ABthrax) is being developed by Human Genome Sciences, Inc. (Bioterrorism Week, 2004). The phase I clinical trial results have been presented with only occasional mild adverse side effects. Pharmacokinetic analysis shows a half-life of approximately three weeks. This vaccine does not disrupt the host derived anti-PA antibody production. Antibodies against Anthrax can provide a significant survival benefit without interfering with an individual's protective immune response. Early phase II results indicate

continued complete protection against anthrax for up to one year. The U.S. government has plans to stockpile anthrax vaccines. These long-range plans also include experimental vaccines that show promise. The National Institute of Health backs this plan even though many of the new vaccines are in the early stages of study. The constant threat of terrorism has forced this unconventional plan. The time frame required to produce and stockpile sufficient inoculum for twenty-five million people does not allow the government to wait for the clinical trial results (Bioterrorism Week, 2004).

Two plasmids are linked to the virulence of *B. anthracis*, pXO1 and pX02. The pX01 plasmid encodes for three toxin factors: the protective antigen, the lethal factor, and the edema factor (Bhatnagar & Batra, 2001). These three toxin factors are necessary to produce two exotoxins. The pX02 plasmid encodes a poly D-glutamic acid capsule. This capsule enables the bacterium to withstand phagocytosis (Jensen et al., 2003). When the pX02 plasmid is not expressed, the cell is incapable of establishing an infection. The Sterne anthrax vaccine is based on the bacterium becoming attenuated in this matter (Okinaka et al., 1999).

There is a strong genetic similarity between *B. anthracis* and other *Bacillus* species. Analysis of 16S rRNA sequences indicates a greater than 99% sequence similarity (Pepper & Gentry, 2002). Variable-number tandem repeat (VNTR) studies comparing *B. cereus* and *B. anthracis* show an exact match. This genetic analysis has led some researchers to believe that *B. anthracis* and *B. cereus* may be one species (Helgason et al., 2000). The conserved core genes between *B. anthracis* and *B. cereus* include factors

for invasion, establishment, and propagation of bacteria within the host (Ivanova, 2003). Both species have homologous mechanisms for protection against the host defense system. Pathogenicity-related genes are present in *B. anthracis* and *B. cereus.*

The evolution of *B. anthracis* and *B. cereus* from a common ancestor is linked to the acquisition of key plasmid-encoded toxin, capsule, and regulatory loci. This lethal toxin locus is located on pX01. The atxA regulatory gene controls toxin gene expression in *B. anthracis.* The chromosomal regulator plcR in *B. cereus* is incompatible with the atxA regulatory gene, thus inhibiting the lethal toxin expression (Read et al.). Additional genetics studies of *B. anthracis* and *B. cereus* show a commonality in the components of the exposporium. Eight genes encode structural components for the exosporium with more similarity between *B. anthracis* and *B. cereus* than other *Bacillus* species (Todd et al., 2003). In a study of the genomic sequences of ten non-anthrax-causing *Bacillus* species; there was a high likelihood of false-positives for DNA-based identification assays on chromosomal loci (Radnedge et al., 2003).

The increased incidents of bioterrorism caused the United States to develop a separate governmental department known as the Department of Homeland Security. This department regulates and oversees potential biological events. Currently *B. anthracis* is one of those agents being closely monitored by this department. All known cultures of *B. anthracis* are documented for reference. The commonality within the species allows for the study of *B. anthracis* through the use of the surrogate *B. cereus.* This study utilized *B. cereus* as a model for addressing issues of a bioterrorism agent.

B. cereus belongs to the group *Bacillus cereus sensu lato*, a gram-positive, rod-shaped, aerobic, spore-forming bacteria and is a soil-dwelling opportunistic pathogen (Rasko et al., 2004). *B. cereus* is associated with non-fatal cases of food poisoning as a result of two exotoxins, an emetic and a diarrheal (Opstal et al., 2004). The microorganism has also been linked to endophthalmitis (after eye trauma), periodontal disease, and fatal cases of pneumonia.

The life cycle for *Bacillus* species encompasses five stages: lag phase, exponential phase (cells in short chains with high motility, pH drops), stationary phase (cells form aggregates, pH increases, lactic acid metabolizes), early sporulation phase (gray spores formed), spore formation state (bright spores rapidly increase, aggregates of cells disassociates, cells lyse, spores released) (De Vries et al., 2004). The spores are resistant to heat, cold, radiation, desiccation, and disinfectants. The bacteria are ubiquitous with a wide distribution in soil, dust, and air (Zhang R. & Zhang C., 2003). It is commonly found in food products such as dairy items, rice, spices, vegetables, meats, and infant products (Faille et al., 2002).

The spores of *Bacillus* species persist for long periods of time. Forty years after the Gruinard's Island biological warfare test, viable *B. anthracis* spores were isolated (Manchee et al., 1982). The recovered spores represent only a small fraction of those deposited on the island. In a Norwegian study of soil isolates, genetic diversity is seen among strains of *B. cereus.* These samples were collected in a small geographic area yet displayed a high degree of genetic variation which is attributed to the highly polymorphic

characteristic of the species. The *B. anthracis* strain showed little variation reflecting its monomorhpic characteristic (Ticknor et al., 2001).

B. cereus possesses characteristics that make it easy to isolate and identify. The organism appears in chains. It is motile and has a distinct hemolytic pattern when grown on reduced blood agar. It is a producer of lecithinase and is penicillin-resistant. The gram-positive spores readily stain with Malachite green. These spores are ellipsoidal, central to subterminal, and do not swell the sporangium (Oxford University, 1998). The bacteria has a short generation time (20-30 minutes) and with heat shock, converts to spore form. On blood agar, *B. cereus* produces a zone on non-hemolysis surrounded by a zone of complete hemolysis (Beecher & Wong, 2000). (Figure1).

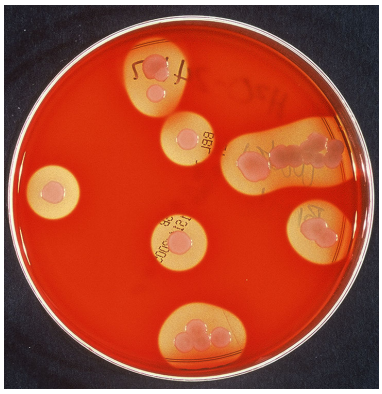


Figure 1**.** *B. cereus* colonies on SBA demonstrating various forms of hemolysis.

The use of Mannitol-egg yolk-polymyxin (MYP) allows for further identification of *B. cereus*. The egg yolk in the media provides lecithin for the *B. cereus* to hydrolyze

forming a precipitate on the media. *B. cereus* colonies are surrounded by precipitates which indicate lecithinase production. (Figure 2).

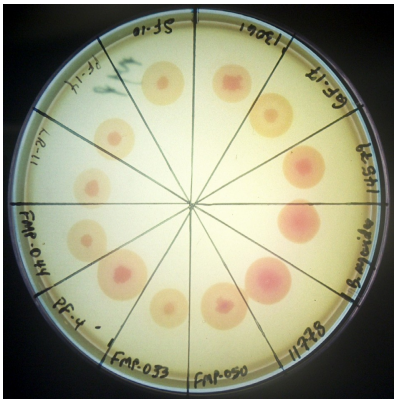


Figure 2. *B. cereus* colonies on MYP agar demonstrating various degrees and types of lecithinase production.

The polymyxin will inhibit the growth of most other bacteria (Jensen et al., 2003). The distinguishing characteristic of *B. anthracis* and *B. cereus* are compared in Table 1.

Table 1. Characteristics of *B. anthracis* and *B. cereus* on Blood Agar.

Characteristics	*B. anthracis*	*B. cereus*
Motility	Negative	Positive
Hemolysis	Negative	Positive
Lysis by Gamma Phage	Positive	Negative
Penicillin Susceptibility	Positive	Negative
Crystalline Parasporal Inclusion	Negative	Negative
Growth on 7% NaCl	Positive	Positive
Tyrosine Decomposition	Negative	Positive

**B. anthracis* colonies will appear grey-white/white in color and are generally smaller.
**B. cereus* colonies will have a green tinge appearance and are generally larger.

Finally, Interstate Highway-35 (I-35), the North-South highway that divides the state of Oklahoma, is considered to be one of the ecological dividing lines of the United States (Guided Spatial Analysis, n.d.). East of I-35, the environment is that of the Mississippi River Valley and West of I-35 is considered lower sonora. This rather abrupt change of the environment has an impact on the variety of microorganisms found in the soil. For example, *Histoplasma capsulatum* is known to thrive east of I-35, but will not grow west of I-35. This microbial diversity is thought to be related to the soil acidity and moisture content.

Research Questions

1. Is *B. cereus* easily found in soil?
2. What effect does the ecological dividing line have on ubiquity?
2. Can *B. cereus* be easily grown and made to sporulate?
3. Can the spore be transmitted by foodstuff (coffee), intimate contact (toothbrushes), and casual contact (doorknobs)?

The following research protocols were employed to answer these questions.

CHAPTER II

METHODOLOGY

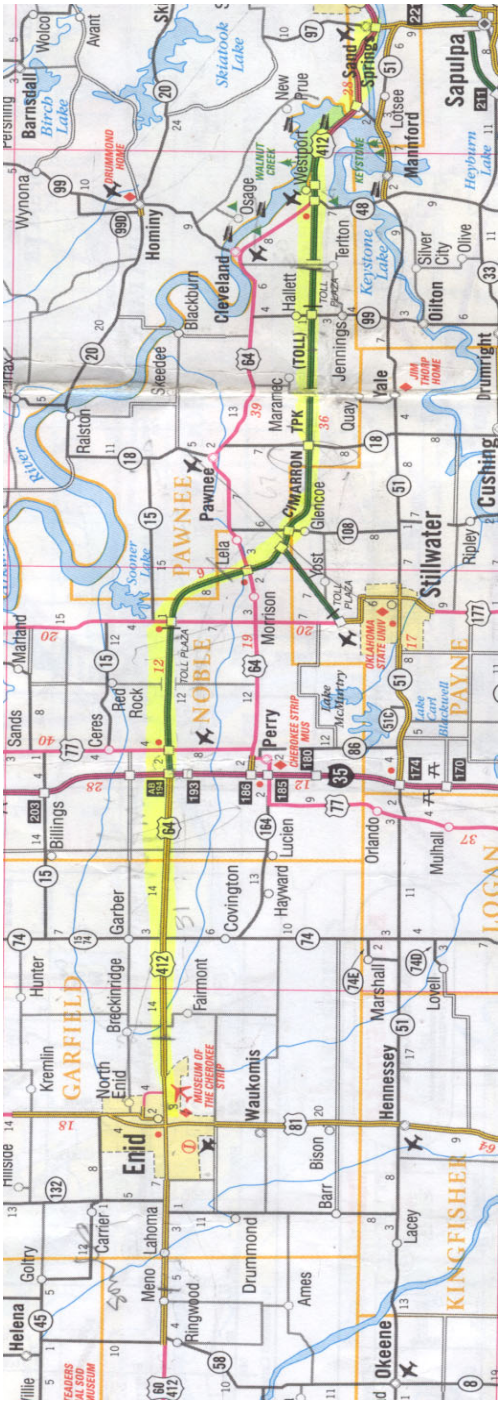
The materials used in these protocols and their sources are listed in the appendix A.

A. ENVIRONMENTAL DISTRIBUTION

1. SPECIMEN COLLECTION

In order to answer the question regarding the presence of *B. cereus* in the environment while addressing the question of the ecological dividing line (I-35), microbial specimens were obtained at 5-mile intervals from west of Enid, Oklahoma to just west of Tulsa, Oklahoma. (~50 miles on either sides of I-35) (Figure 3). Three soil specimens were taken from each site at ~25 foot increments. Samples were collected from 32 locations resulting in 96 total specimens. Mile markers were used as a reference for the sample sites. Aseptic techniques were utilized by dipping a standard bulb planter into a container of alcohol and flaming, the bulb planter was used to take the top one inch of the soil. The soil samples were placed in sterile Petri dishes, marked for identification, and sealed with tape. The specimens were transported to the OSU-CHS Infectious Diseases laboratory and allowed to dry at room temperature.

Figure 3. Map of Soil Collection



2. PASTEURIZATION

Prior to use, soil samples were pasteurized to determine the optimum temperature that inhibits other microorganisms without inhibiting *Bacillus cereus* growth. Two samples of *B. cereus* with known profiles from previous studies, a sample of *Staphylococcus aureus* ATCC 25923, and a sample of *Escherichia coli* ATCC 25922 were used. The specimens were placed in a flask of Brain Heart Infusion (BHI) broth and incubated overnight. The cultures were then subjected to a water bath for 10 minute increments at temperatures ranging from 30°C to 100ºC. After treatment at each temperature level, a sample of the solution was plated to Sheep Blood Agar (SBA).

3. SOIL CULTURES

One gram of each soil specimen was mixed with 3 ml of BHI broth and subjected to pasteurization at 70°C for 10 minutes. Microbial specimens that survived pasteurization were classified as spore formers. "Spore formers" were plated to SBA and MYP plates. Gross colonial morphology was used to select colonies for pure cultures. Distinguishing characteristics of motility, hemolysis, and penicillin susceptibility were identifying factors for *B. cereus*. (Manual of Clinical Biology, Sixth Edition). Specimens that were plated to SBA were also subjected to 10,000-units /ml penicillin test discs. Specimens inoculated on MYP were distinguished by catalase production, mannitol reaction, and lipase production. Specimens demonstrating the standardized characteristics were chosen for gram stain. Gram stain reactions showing a positive stain and spores that were ellipsoidal, spherical, central, paracentral and did not swell the sporangium were

considered diagnostic of *Bacillus cereus*. These specimens were frozen in BHI broth with glycerol for future use.

4. HEAT RESISTANCE OF *B. CEREUS* SPORES

In order to produce an adequate supply of spores for transmission studies, the *B. cereus* strains are heat treated to eliminate extraneous microbes. Test of 17 strains of *Bacillus cereus* were used. In four soil samples (5A, 18A, 27A, and 30A), *B. cereus* spores survived a heat shock treatment of 80ºC for 10 minutes. These four spore strains, ten *B. cereus* spore specimens with known heat shock profiles (SF-3, SF-19, PF-5, SF-20, LR-15, GF-6, PF-16A, FMP-44, LR-16, and SF-10), and three commercial stocks of *B. cereus* (ATCC 13061, ATCC 14579, and ATCC 11778) were used to produce killing curves to select the most heat resistant spore. Spore specimens were subject to heat shock from 30°C to 90ºC for 10 minutes. Serial dilutions were prepared, plated to BHI agar, incubated overnight at 37ºC and a colony count performed.

5. SPORE PREPARATION

Based on the serial dilutions and the results of the heat shock study, two *B. cereus* spore specimens were chosen: Arnold 5A, a strong toxin producing strain and Whimpy SF-19, a weak toxin producing strain. The specimens were placed in a sporulation media and incubated over the weekend at 37°C. The spore solutions were centrifuged at 12000 RPM for 10 minutes at 4ºC in a Beckman Avanti J-25. The Arnold and Whimpy spore pellets were then freeze dried in a lyophilizer. The spore pellets were weighed and frozen until used. Eight serial 10-fold dilutions were performed with 1/100 of each

dilution plated to BHI agar plates and incubated overnight at 37°C; colony forming units (CFU) were counted. Calculations of CFU's determined the amount of spores to be used. Aliquots of Arnold and Whimpy were each resuspended in sterile water and placed into a 70ml spray bottle for contamination transmission purposes.

B. CONTAMINATION TRANSMISSION STUDIES

Three contamination transmission studies were conducted. One study used coffee, to mimic ingestion transmission. Another study used toothbrushes to evaluate intimate ingestion transmission. The final studies used sterile doorknobs to simulate contact or casual transmission. All studies involved the spore form of the two previously discussed strains of *B. cereus.*

1. INGESTION TRANSMISSION: COFFEE

Coffee was used to demonstrate transmission through foodstuff. Because of the time lapse between contamination and end results, it would be considered an untraceable source. Approximately 12mg of Arnold (5A) spores and 12mg of Whimpy (SF-19) spores were mixed with 25 grams of coffee grounds separately. Coffee was brewed using these grounds in an automatic brewing coffee pot using sterile water. After brewing, 5 cups of coffee were prepared as follows: coffee only, coffee with Sweet-n-Low, coffee with sugar, coffee with Coffee-Mate French Vanilla/International Delight French Vanilla creamer (liquids) for Arnold, and Cain's Coffee Creamer (powder) and Lando'Lakes half and half (liquid) for Whimpy. The cups of coffee were allowed to sit

for 20 minutes. Serial dilutions were performed and inoculation on BHI Agar plates. The BHI Agar plates were incubated overnight at 37°C and the data recorded.

2. INTIMATE TRANSMISSION: TOOTHBRUSH

Sterile toothbrushes were used to demonstrate ingestion transmission through personal intimate objects. Spores were resuspended in sterile water and distributed via a spray bottle. Three sets of ethylene oxide sterilized toothbrushes were utilized in this experiment. The first set was sprayed with sterile water. The second set was sprayed with the spore solution. The third set was sprayed with spore solution, incubated, and then dipped in a liquid suspension of toothpaste and sterile water to simulate brushing. At 0 hours, 24 hours, and 48 hours, each toothbrush was brushed in an X pattern onto a SBA plate. The 24 hour and 48 hour toothbrushes were placed in a vented hood and were undisturbed during the allotted time period. The SBA plates were incubated overnight at 37ºC and the data recorded.

3. CONTACT OR CASUAL TRANSMISSION: DOORKNOB

Doorknobs were used to demonstrate transmission through casual contacts. The doorknobs were sterilized using ethylene oxide. Two sets of sterile doorknobs of varying materials (glass, brushed metal and brass-plated) were mounted double sided to a wooden platform to simulate a door frame. The wooden platform with its porous nature was covered in vinyl contact paper to facilitate cleaning. Controls were constructed by spraying SBA plates with the sterile water and the spore solution. The spore solution was sprayed onto each doorknob at a precise distance (6”) to maintain uniformity. The

doorknobs were allowed to dry for 15 minutes and touched to SBA plates for a baseline determination. The doorknobs were thoroughly cleaned with an alcohol solution and resprayed with the spore suspension and allowed to dry. Each doorknob was touched to a SBA plate after a 24 hour period. The process was repeated again after a 48 hour time period. The SBA plates were incubated overnight at 37ºC and the data recorded. During each experiment, the doorknobs were kept in a vented hood and allowed to dry undisturbed for the allotted time.

CHAPTER III

RESULTS

A. ENVIRONMENTAL DISTRIBUTION

1. SPECIMEN COLLECTION

The soil sample collection was performed on October 28, 2004. The weather condition was overcast with periodic light precipitation. The preceding days' weather was moderate rainfall, typical of Oklahoma fall weather. These conditions resulted in soil samples, which were moist to varying degrees. The data is summarized in Table 2.

Sample sites varied in soil texture and consistency. No single site presented unusual or notable characteristics (i.e. large population of insects or visible parasites). Sample sites varied in terrain types from bordering croplands and wooded areas to bordering salt flats. The path of sample collection included a sampling from a medium-sized town. (Enid)

Table 2. Soil Sample Collection.

Soil Sample Collection
28-Oct-04
Fairview, OK to Prue Exit, west to east
Weather conditions - overcast and rain
Dina Miller, Dr. Glass, Dr. Conrad, Jay Bullard

Site	Mileage	Mile Marker	Time	Photo #
1	71688		12:22	222,221,220
2	71693		12:26	219
3	71697		12:45	218
4	71702		12:56	217
5	71707		1:09	216
6	71712		1:18	215
7	71717		1:28	214,213
8	71722		1:39	212
9	71727	1 mile west of feed lot	1:50	211
10	71732	West side of Enid	2:03	210
11	71737	East side of Enid	2:24	209
12	71742			209
13	71747			208
14	71752			207
15	72757	10 miles west of I-35		206
16	71762		3:25	205
17	71767	West of I-35		204
18	71767	East of I-35		203
19	71772	5		203
20	71777	10		202
21	71782	15	4:20	201
22	71787	20	4:35	200
23	71792	25, rest area		198.199
24	71797	30		197
25	71802	35	5:00	195
26	71807	40		194
27	71812	45	5:29	193
28	71817	50		192
29	71822	55		190,191
30	71827	entrance to East 64		187,188,189
31	71833			186
32	71837	Prue Exit		185

Three samples taken at each site~10 feet increments at 45 degree angles. Samples labeled A, B, and C. Total of 96 samples.

The weather conditions required the soil samples to be dried upon returning to the OSU-CHS laboratory. The petri dishes of soil were vented to facilitate the drying process. Visual inspection of the soil samples determined that the soil types range from red clay to dark enriched soil. Some samples contained small amounts of decaying vegetative matter. Most sites presented consistent composition. The soil sample from site 19 presented the largest color range from light red clay to dark brown soil. Sample site 26 presented a gray pigment in the soil. Sample C from site 30 appeared black in color, which may be attributed to products from the roadway. Soil sites and soil sample specimens are demonstrated in Appendix C.

The midpoint of the path of the soil sample collection crossed the ecological dividing line (I-35). This ecological dividing line separates the temperate eastern United States from the arid western United States. This broad generalization separates areas relative to agricultural factors. The ubiquity of *B. cereus* was found to cross this divide.

2. PASTEURIZATION

An initial culturing of sample 8C was performed by sprinkling a small amount of soil sample directly to a BHI agar plate. This culture had diverse growth of multiple types of bacteria that were too numerous to count (TNTC). Prior to testing for *B. cereus*, pasteurization of the soil specimens was performed to eliminate extraneous microorganisms. Known strains of *B. cereus* were tested in conjunction with *S. aureus* and *E. coli*, common non-spore forming bacteria. The results of temperature sensitivity for the three microorganisms are summarized in Table 3.

Table 3. Pasteurization Test of Microorganisms

Pasteurization Test of Microorganisms			
	B. cereus	*S. aureus*	*E. coli*
Room Temp/ 0 minutes	TNTC	TNTC	TNTC
Room Temp/10 minutes	TNTC	TNTC	TNTC
30ºC/10 minutes	TNTC	TNTC	TNTC
40ºC/10minutes	TNTC	TNTC	TNTC
50ºC 10 minutes	TNTC	TNTC	TNTC
60ºC/10 minutes	TNTC	TNTC	TNTC
70ºC/10 minutes	~18 colonies	NG	NG
80ºC/10 minutes	3 colonies	NG	NG
90ºC/10 minutes	NG	NG	NG
100ºC/10 minutes	NG	NG	NG

*TNTC – Too Numerous To Count, NG – No Growth

Cultures grown from samples heated at 50°C and below showed numerous colonies. These colonies were normal in appearance, size, and color. At 60°C, the *B. cereus* colonies were TNTC and showed normal morphology. While the growth of *S. aureus* and *E. coli* colonies was reduced and the colony morphology appeared irregular and degraded. At 70°C no growth was observed on the *S. aureus* and *E. coli* cultures and reduced growth was observed with *B. cereus*. Minimal growth was seen with *B. cereus* at 80°C with no growth in the *S. aureus* and *E. coli* cultures. At 70°C and 80°C the *B. cereus* colonies appeared healthy with normal morphology. Growth at 80°C is indicative of spore formation. From this data, 70°C was selected for the ideal pasteurization temperature. When pasteurization at temperatures was greater than 80°C, no growth was seen. The optimum pasteurization with the preservation of *B. cereus* was 70°C for 10 minutes.

3. SOIL CULTURES

All soil samples were pasteurized (70°C for 10 minutes) to inhibit growth of non-spore forming bacteria. The soil samples were tested for the presence of *B. cereus* spore. Some soil samples may have contained other spore forming microbes. However, only cultures that presented typical with *B. cereus* characteristics were subsequently evaluated. The results are summarized in Table 4.

Gross colony morphology was used as the initial criteria to select colonies for further identification. Subsequent identification was based on motility, hemolysis, and penicillin susceptibility. Colony and sub-colony morphology for sites A, B, and C isolates are summarized in the appendix B. On this basis, 65 of the 96 soil samples tested positive for *B. cereus*; 27 of the soil samples tested negative for the presence of *B. cereus*. Four soil samples showed no growth of *B. cereus*, however these four samples did show growth of spore forming bacteria. These colonies demonstrated indeterminate levels within the identification criteria. The ambiguity of these samples could not be defined as a negative and are therefore classified as no data. The results are summarized in Table 4. The distinction between sites A, B, and C were arbitrary. Site C was located closest to the pavement of the roadway. Sites A and B were equidistant from the roadway. (Approximately 25 feet from the pavement edge, approximately 25 feet apart) Each sample site is independent and not mutually exclusive. The sampling sites were approximately 5 miles apart. The negative sites located 25 feet from a positive soil site

would seem improbable. It is pertinent to note the presence of *B. cereus* was independent of the ecological dividing line. The mean probability of obtaining *B. cereus* spores from a site is 0.705 (70.5%). The results are summarized in Table 5.

Table 4. Detection of *B. cereus* in Soil Sample Collections.

Detection of *B. cereus* in Soil Sample Collections							
West of Highway I-35				East of Highway I-35			
Sample Site	Sample A	Sample B	Sample C	Sample Site	Sample A	Sample B	Sample C
1	+	+	+	18	+	+	+
2	+	-	+	19	-	+	-
3	No data	No data	+	20	+	+	+
4	-	-	-	21	+	-	+
5	+	+	-	22	-	+	+
6	+	-	-	23	+	+	+
7	No data	+	+	24	-	+	+
8	-	-	-	25	-	+	+
9	+	-	+	26	-	+	+
10	+	+	+	27	-	+	-
11	+	-	-	28	No data	+	+
12	+	+	-	29	+	+	-
13	+	-	+	30	+	+	+
14	+	+	+	31	+	+	+
15	+	+	+	32	+	+	+
16	-	+	+				
17	-	+	+				
Total (+)	11	9	11		8	14	12

*(+) – Positive for B. cereus, (-) – Negative for B. cereus, No data – no growth

Table 5. Probability of *B. cereus* in Soil Samples.

Site	Positive Sites	Probability
A	10 of 29	0.655
B	23 of 31	0.742
C	23 of 32	0.719
Overall	65 of 92	0.707

4. HEAT RESISTANCE STUDY

Heat resistance tests were performed to produce a killing curve of 17 strains of *B. cereus.* Killing Curves for 5A (Arnold) and SF-19 (Whimpy) are demonstrated in Appendix E. Ten *B. cereus* strains were obtained from previous studies, three commercial strains, and four *B. cereus* strains obtained from the soil sample studies were tested. The growth patterns of the *B. cereus* strain were consistent with the previous pasteurization study with inhibition of growth occurring at 70°C. Two strains of *B. cereus* were selected for the transmission studies. The 5A strain showed healthy growth at 70°C with classic colony morphology. (Arnold) The SF-19 *B. cereus* strain showed reduced growth at 70°C, but did exhibited characteristics meeting the *B. cereus* criteria with diminished hemolysis or lecithinase production. (Whimpy) The data of 1:10 dilutions, 1:100 dilutions, and 1:1000 dilutions are summarized in Tables 6A, B, and C.

Table 6A. The Heat Resistance Study of *B. cereus* Spores – 1:10 Dilution.

Heat Resistance of *Bacillus* Spores - 1:10 Dilution							
Sample	30°C	40ºC	50°C	60°C	70°C	80°C	90ºC
B. cereus ATCC 14579	TNTC	TNTC	TNTC	TNTC	TNTC	10	0
B. cereus ATCC 13061	TNTC	TNTC	TNTC	TNTC	0	1	0
B. cereus ATCC 11778	TNTC	TNTC	TNTC	2	TNTC	0	0
30A	TNTC	TNTC	TNTC	10	TNTC	3	1
5A	TNTC	TNTC	TNTC	4	TNTC	2	0
18A	TNTC	TNTC	TNTC	2	0	0	0
27A	TNTC	TNTC	TNTC	1	2	2	0
LR-16	TNTC	TNTC	TNTC	20	16	3	1
SF-10	TNTC	TNTC	TNTC	3	0	0	0
LR-15	TNTC	TNTC	TNTC	TNTC	4	4	0
SF-3	TNTC	TNTC	TNTC	TNTC	TNTC	5	0
GF-6	TNTC	TNTC	TNTC	16	11	12	1
FMP-44	TNTC	TNTC	TNTC	TNTC	13	5	0
SF-20	TNTC	TNTC	TNTC	TNTC	TNTC	24	0
PF-5	TNTC	TNTC	TNTC	25	13	9	0
PF-16A	TNTC	TNTC	TNTC	TNTC	15	10	1
SF-19	TNTC	TNTC	TNTC	TNTC	13	2	0

*Numbers represent CFU.

Table 6B. The Study of Heat Resistance of *B. cereus* Spores – 1:100 Dilution.

Heat Resistance of *Bacillus* Spores - 1:100 Dilution							
Sample	30°C	40°C	50°C	60°C	70°C	80°C	90°C
B. cereus ATCC 14579	TNTC	TNTC	TNTC	5	TNTC	4	0
B. cereus ATCC 11778	TNTC	TNTC	TNTC	1	TNTC	0	0
B. cereus ATCC 13061	TNTC	TNTC	TNTC	4	3	0	0
30A	TNTC	TNTC	TNTC	6	TNTC	2	0
27A	TNTC	TNTC	TNTC	0	0	0	0
5A	TNTC	TNTC	TNTC	2	TNTC	0	0
18A	TNTC	TNTC	TNTC	0	0	0	0
LR-16	TNTC	TNTC	TNTC	4	3	0	0
SF-10	TNTC	TNTC	TNTC	1	0	0	0
LR-15	TNTC	TNTC	TNTC	5	0	0	0
SF-3	TNTC	TNTC	TNTC	TNTC	TNTC	0	0
GF-6	TNTC	TNTC	TNTC	1	1	0	0
FMP-44	TNTC	TNTC	TNTC	3	2	1	0
SF-20	TNTC	TNTC	TNTC	16	2	2	0
PF-5	TNTC	TNTC	TNTC	5	1	0	0
PF-16A	TNTC	TNTC	TNTC	TNTC	6	0	0
SF-19	TNTC	TNTC	TNTC	12	0	0	0

*Numbers represent CFU.

Table 6C. The Study of Heat Resistance of *B. cereus* Spores – 1:1000 Dilution.

Heat Resistance of *Bacillus* Spores - 1:1000 Dilution.							
Sample	30°C	40°C	50°C	60°C	70°C	80°C	90°C
B. cereus ATCC 14579	TNTC	TNTC	TNTC	3	6	1	0
B. cereus ATCC 11778	TNTC	TNTC	TNTC	3	13	0	0
B. cereus ATCC 13061	TNTC	TNTC	TNTC	0	1	0	0
30A	TNTC	TNTC	TNTC	0	11	0	0
27A	TNTC	TNTC	TNTC	0	0	0	0
5A	TNTC	TNTC	TNTC	0	9	0	0
18A	TNTC	TNTC	TNTC	0	0	0	0
LR-16	TNTC	TNTC	TNTC	0	0	0	0
SF-10	TNTC	TNTC	TNTC	0	0	0	0
LR-15	TNTC	TNTC	TNTC	1	0	0	0
SF-3	TNTC	TNTC	TNTC	TNTC	5	0	0
GF-6	TNTC	TNTC	TNTC	0	4	0	0
FMP-44	TNTC	TNTC	TNTC	0	0	0	0
SF-20	TNTC	TNTC	TNTC	4	1	0	0
PF-5	TNTC	TNTC	TNTC	1	0	0	0
PF-16A	TNTC	TNTC	TNTC	13	1	0	0
SF-19	TNTC	TNTC	TNTC	4	0	0	0

*Numbers represent CFU.

5. SPORE PREPARATION

The Arnold strain provided 1 colony when diluted 10^{-10}, yielding 10^{10} CFU's per gram. The Whimpy strain provided 1 colony when diluted 10^{-8}, yielding 10^{8} CFU's per gram. The stock culture for each strain was grown in 3 liters of sporulation media for 72 hours. The culture was centrifuged, dried and weighed. The final weight of the Arnold strain was 1.615 grams. A total of 1.615×10^{10} spores were produced in 3 liters of media after 72 hours, a short time period. The final weight of the Whimpy strain was 872mg giving a total of 8.72×10^{10} spores produced from the culture.

The spore suspension used to simulate transmission contained 10^{6} CFU/spray. A filter paper was sprayed with sterile water to obtain the weight of a single aerosol spray from the spray bottle. The mean weight of the aerosol spray was 148mg. A specimen of 473mg of Arnold spores is needed to make 70ml of spore suspension. A sample of 50mg of the Arnold and Whimpy spores were used to make the spores suspension for the transmission studies. For Arnold, the spore suspension contained 1.06×10^{7} CFU/spray. For Whimpy, the spore suspension contained 1.06×10^{6} CFU/spray. These spore suspensions were used in the transmission studies involving toothbrushes and doorknobs. Calculations are demonstrated in Appendix D.

B. TRANSMISSION STUDIES

1. INGESTION TRANSMISSION: COFFEE

Coffee was selected as a model to simulate ingestion transmission of a pathogen via food product. 12mg of spores were added directly to the coffee grounds prior to brewing. This study utilized the two *B. cereus* strains; Arnold and Whimpy. *B. cereus* spores were successfully transmitted via brewed coffee. The temperature of the brewed coffee was 78ºC which is below the threshold limit from the heat resistant studies. The results are summarized in Table 7.

Table 7. Detection of *B. cereus* in Coffee.

ARNOLD		
Serial Dilution	10µl	1µl
Additive	CFU	
Black	9	2
French Vanilla (International)	TNTC	2
French Vanilla (Coffee Mate)	TNTC	2
Sweet-n-Low	17	2
Sugar	20	2

WHIMPY		
Serial Dilution	10µl	1µl
Additive	CFU	
Black	7	2
Creamer (Half & Half, liquid)	5	2
Creamer (Cain's, powder)	3	2
Sweet-n-Low	7	2
Sugar	6	2

Four types of creamer were tested: Coffeemate French Vanilla (liquid), International Delight French Vanilla (liquid), Lando'Lakes half and half (liquid), Cain's coffee creamer (powder). Initial cultures of the cream were performed to determine extraneous microbes present. These cultures yielded no growth. A culture of the uninfected coffee grounds yielded a colony growth which was subsequently tested using an API test system. This test indicated the presence of a *Flavobacterium* species. A recent study of *B. cereus* in coffee samples showed that *B. cereus* was not detected. PCR tests were performed and amplicons were only found in inoculated coffee samples. (Manzano et al., 2003).

The Arnold strain was selected because of its full complement of toxins. A sample of 12mg was added to the coffee grounds. The coffee was brewed with 1000ml of sterile water. The coffee was aliquotted into five cups. Each cup had 2.4 X 10^7 CFU. 10µl of the coffee was plated to BHI agar plates for culture. Theoretically, a 10µl sampling should contain 120 CFU. The 10µl sampling of black coffee formed 9 colonies. The 10 fold loss of colony forming units can be attributed to the brewing process. The coffee with the sugar and artificial sugar added demonstrated a higher growth. This would indicate that the sweeteners stimulate germination prior to ingestion. The coffee with the creamer added demonstrated a growth rate TNTC. This indicates that the creamers provide a nutrient rich environment that stimulates germination for *B. cereus*.

The Whimpy strain was selected as an example of a less toxic *B. cereus* strain. A sample of 12mg was added to the coffee grounds. The coffee was brewed with 1000ml of sterile water. The coffee was aliquotted into five cups. Each cup had 2 X 10^7CFU/200ml coffee x 10µl X 1 ml/1000µl = 100 CFU. Theoretically, a 10µl sampling should contain 100 CFU. The 10µl sampling of black coffee formed 7 colonies. The effects of coffee additives with the Whimpy strain were consistent with Arnold. This indicates that the ingestion transmission is less effective for spore transmission when additives are present. The inoculation of spores did not change the appearance of the coffee. The additives to the coffee increased the germination rates of the spore but had the appearance of normal coffee. All of the cups of coffee appeared untampered.

2. INGESTION TRASMISSION: TOOTHBRUSH

Toothbrushes were selected as a model of transmission of a pathogen via personal intimate objects. The toothbrushes were sprayed with a spore suspension to simulate transmission with a spore-based bioweapon. Viable *B. cereus* spores were recovered from all of the inoculated toothbrushes. All of the cultures showed prominent growth from viable spores. The data is summarized in Table 8.

Table 8. Detection of *B. cereus* from Toothbrushes.

Detection of *B. cereus* from Toothbrushes			
Toothbrush	0 Hour	24 Hour	48 Hour
Sterile Water	-	+	+
Spore Suspension	+	+	+
Spore Suspension/Toothpaste	+	+	+

*All positive plates – too numerous to count
Sterile water at 24 hour < 20 colonies
*(+) Positive, (-) Negative
Spore Suspension prepared with 500mg of spores in 70ml sterile water.

The negative control in the study was a toothbrush inoculated with sterile water. The negative control toothbrush was positive when incubated for 24 and 48 hours. The toothbrush was stored with the spore inoculated toothbrushes separated by a distance of four inches. The amount of growth was relatively poor – less than 20 colonies. The presence of viable spores in the negative control indicates a small level of transmission from the inoculated toothbrushes. The toothbrushes did not change in appearance when inoculated with spores. The texture of the bristles camouflaged the presence of the inoculum. The toothbrushes appeared untampered.

3. CONTACT/CASUAL TRANSMISSION: DOORKNOBS

Doorknobs were selected as the model for transmission of a pathogen via casual objects. The doorknobs were sprayed with a spore suspension to simulate contamination with a spore-based bioweapon. *B. cereus* spores were recovered from all inoculated doorknobs. The growth rate at all time intervals indicates that viable spores were present. The prominent growth at the 48-hour interval suggests that spore recovery is not diminished over time. The visual inspection of the doorknobs at the 24-48 hour time intervals

revealed a watermark residue. The watermark residue was more prominent on the brushed metal doorknob. The uneven tones of the brass-plated doorknob decreased the visualization of the watermark residue. The glass doorknob disguised the watermark residue with its refractive nature. The doorknobs appeared untampered. The data is summarized in Table 9.

Table 9. Detection of *B. cereus* from Doorknobs.

Detection of *B. cereus* from Door Knobs			
Door Knobs	0 Hour	24 Hour	48 Hour
Glass	+	+	+
Brushed Metal	+	+	+
Brass-plated	+	+	+

*All colonies were too numerous to count
(+) Positive

CHAPTER IV

DISCUSSION

The current climate of terrorist awareness has led to renewed interest in biological warfare defense. *B. anthracis* has already been used as a bioweapon. The phylogenetic nature within the *Bacillus* group allows for *B. cereus* to be used as a surrogate for *B. anthracis* research.

A total of 96 soil samples were collected along a highway that crossed a significant ecological dividing line. *B. cereus* strains were isolated and cultured uniformly throughout the collection trail. Seventy percent (70%) of the soil samples were positive for *B. cereus*. The lack of *B. cereus* in the negative sites can be attributed to the high level of diversity of natural flora. An individual site may contain a plethora of normal flora that could retard the growth of *B. cereus* (von Stetten et al., 1999). This high probability implies that if a terrorist collected three dirt samples from a random site, 2 of the samples would be positive for *B. cereus* spores. This emphasizes the ubiquity of *B. cereus*. Pasteurization studies reveal a temperature of 70°C as ideal for treating the soil samples. This high temperature eliminates extraneous microbes without inhibiting the germinating potential of *B. cereus* spores.

The events of 2002 renewed interest in biological warfare defense and led to the development of Homeland Security. With anthrax being a known threat, the government now scrutinizes known cultures and strains of *B. anthracis*. The access to anthrax strains

are well documented. An obvious crack in this surveillance system is the potential of a terrorist to obtain isolates from nature. While colonies exhibiting anthrax characteristics were not rigorously definitive, it is easy to perceive that a terrorist could obtain anthrax spores from the soil to be grown for use as a bioweapon from an untraceable origin.

The heat resistant studies yielded results consistent with the heat resistant nature of spores. High temperatures (>80°C) are needed to inactivate spore germination. The growth patterns of the *B. cereus* strains were uniform regardless of the strain source. A simple spore culture was grown for 72 hours yielding greater that 10^8 CFU's. The 1979 Sverdlovsk incident which resulted in a 2 % fatality rate involved the accidental release of spores equivalent to the quantity of CFU's grown from three liters of 72 hour cultures (Meselson et al. 1994). Sverdlovsk has a population greater than 1.2 million but the release of anthrax spores was on the southeast point of a non-residential area. The wind pattern from the north caused the dispersal of spores to spread in a sparsely populated rural area. Had the wind shifted to the north, the fatality rate would have undoubtedly been larger. Information from the Sverdlovsk outbreak of 1979 is limited as the KGB confiscated hospital and public health records of the epidemic. Detailed information has been extrapolated from death records, records of compensated families, grave markers, interviews with survivors and victim family members, and interviews with health care professionals.

The U.S. Defense Department estimates that the LD_{50} of anthrax for humans is between 8,000 and 10,000 spores (U.S. Department of Defense, 1986). The 2% fatality rate of

Sverdlovsk was caused by a dose of 9 spores. This dose would be inhaled by an individual breathing $0.03m^3$/minute. Using a Gaussian plume model, the researchers determined that $4 \text{ X } 10^9$ spores were released at the source site (Meselson et al., 1994).

The estimated amount of anthrax spores accidentally released in Sverdlovsk is equivalent to the amount of *B. cereus* spores used in the 70ml aerosol spray bottle. A terrorist intent on creating a bioweapon could easily stockpile enough *Bacillus* spores to achieve a LD_{50} for a major metropolitan area. The spores could be obtained from the soil outside of the current surveillance focus of the Department of Homeland Security.

Coffee grounds were used as a model for ingestion transmission. *B. cereus* spores were readily recovered from the coffee after brewing. The presence of coffee additives yielded a higher growth of *B. cereus*. This would indicate that the nutrient-rich additives and the heat shock are stimulating germination prior to ingestion. If germination occurs prior to ingestion, the amount of viable spores is diminished reducing the effectiveness of transmission for terrorists.

Visual inspection of coffee grounds did not suggest tampering from an outside source. A terrorist could inoculate the grounds during the manufacturing stage. The presence of the spores would not be visually detected while packaged tampering could be noticeable. Minimally processed foods are not typically treated to inactivate bacterial spores. A study of *B. cereus* spores in milk indicated that treatment with high pressure successfully inactived bacterial spores (Opstal et al., 2004). Nisin is added to crumpets, liquid eggs,

and other food products to inhibit the growth of gram-positive pathogens such as *B. cereus*; however, this additive does not inactivate spores (Beuchat et al., 1997). The 10µl sampling of coffee yielded viable CFU's. If an individual swallowed 10ml of the infected black coffee, approximately 9,000 viable CFU's would be consumed with the Arnold strain and 70,000 viable CFU's would be consumed with the Whimpy strain. The target victim would not need to consume the entire cup of coffee in order to achieve the LD_{50} rate.

An additional ingestion transmission study utilized toothbrushes as a source of infection. Successful transmission of viable spores was found. The presence of toothpaste stimulates spore germination prior to ingestion that might reduce transmission effectiveness. Cross-contamination from a contaminated toothbrush to an uninoculated toothbrush was performed. In both transmission studies, the inoculums were not visually evident due to the uneven texture of the toothbrushes that tended to mask the presence of the spore inoculums. Visual inspection of the toothbrushes did not suggest tampering from an outside source. The scenario for this study shows the potential for terrorist threat, however; the infection of a bioagent on a toothbrush requires access to the toothbrush. A terrorist would need access to the toothbrush prior to packaging, perhaps by obtaining a job at a toothbrush manufacturing plant. An outbreak could cross geographic barriers during distribution of the contaminated toothbrushes and the subsequent investigation to trace the infectious source would be more difficult. Investigation would first need to isolate the source of infection as the toothbrush to be

traced back to the supplier. This scenario gives a new perspective to the concept of "untraceable origin."

Toothpaste was ineffective in killing *Bacillus* spores in the transmission studies. Rather, the toothbrushes with the toothpaste (simulating brushing) had more prominent growth than toothbrushes without toothpaste. The bacteria appeared to flourish in the nutrient-rich toothpaste, indicating the ingredients in toothpaste enhance *B. cereus* growth. Toothpaste does promote spore germination, making the transmission of spores less effective. Studies of toothpaste indicate that triclosan retards the growth of some microbial contamination (McGhee, n.d.). While this additive may inhibit some microbial growth, it would not inactivate the methodically inert spores. This would indicate that more of the spores would reach the target. Having a microbial inhibitor may reduce the incidence of viral or bacterial outbreaks, but this would not be effective for spore-based bioweapons.

A contact transmission study was performed utilizing doorknobs as a source of infection transmission. The *Bacillus* spores were successfully retrieved from three different doorknob surface types. This model of contamination is ideal for the bioterrorist. The inoculum was not visibly apparent and the source of infection would be difficult to trace. Visual inspection of the doorknobs at the 24-48 hour time interval revealed a watermark residue. The watermark residue was more prominent on the brushed metal doorknob. The uneven tones of the brass doorknob decreased the visualization of the watermark residue. The glass doorknob disguised the watermark residue with its refractive nature.

The 2001 anthrax outbreak was traced to transmission via the United States postal service. Witnesses from the outbreak reported a white powder contaminant. In an atmosphere of heightened awareness to bioterrorist activities, individuals reported the presence of unusual powder residues. The Illinois Department of Public Health received over a thousand environmental specimens from the Chicago area during the two-month period of October and November 2001 (Dworkin & Golash, 2003). The high level of public anxiety led to the submission of samples that included powdered doughnuts, a telephone, a frozen dinner, and mail items. It is relevant to note that the "suspicious" public looked for a powdery substance.

The public at large does not examine a doorknob when entering a public facility. This study clearly demonstrates the ease of transmission of a bioterrorism agent via doorknobs. If a terrorist chose a doorknob in a public facility, the source of the contaminant would be difficult to trace. The victims profile would indicate the source of infection to be the restaurant, a department store, or even a church. Once the location was identified by investigators, time would be lost in attempting to rule out the staff and all internal items as sources. The terrorist could infect multiple sites within a target facility, making the identification source of infection more difficult. This infection would tend to cross economic barriers and in the case of multiple sites, geographic barriers. The victims would be of varying ages and racial backgrounds. This transmission would encompass cutaneous infection as well as ingestion as most individuals do not wash their hands before eating a meal in public.

CHAPTER V

CONCLUSION

The focus of national security relies heavily on known sources for bioagents. This study demonstrates the ease of obtaining a bioagent from an undocumented and untraceable source such as dirt. The bioagent could be dispersed covertly, yielding a potentially widespread outbreak of disease. Specifically, the findings of the present studies revealed that:

1. *B. cereus* could be found in 70% of the soil samples tested.
2. The ecological dividing line that separates the arid west from the temperate east, does not affect *B. cereus* growth.
3. *B. cereus* can be easily grown and sporulated to produce a sufficient quantity of spores for bioterrorism use.
4. *B. cereus* can be transmitted by ingestion of foodstuff such as coffee and/or creamers; by intimate inanimate objects such as toothbrushes; and by casual contact such as doorknobs.

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APPENDIX

APPENDIX A

MATERIALS

Standard bulb planter

Sheep blood agar plates (SBA): SBA is Trypticase® Soy Agar with 5% sheep blood (TSA II) (Troy Biologicals Inc., Troy, New York).

Mannitol-egg-yolk-polymyixin agar plates (MYP): The MYP agar plates were prepared in the laboratory using the formulation and procedure as given in the Difco Manuel.
Formula per Liter:
Bacto Beef Extract…….1 g
Bacto Peptone……...10 g
Bacto D-Mannitol…….10 g
Sodium Chloride……...10 g
Bacto Phenol Red……0.025 g
Bacto Agar……15 g
Antimicrobic Vial P: 30,000 units polymixin B

Brain Heart Infusion Broth (BHI) (Acumedia Baltimore MD)

Brain Heart Infusion Agar (BHI) (Acumedia Baltimore MD)

Freezing Media – Brain Heart Infusion Broth (BHI) (Acumedia Baltimore MD) with 10% glycerol (Sigma St. Louis, MO).

Sporulation Medium: Nutrient Agar (NA) (Difco) was supplemented with 0.05 g/liter of manganese sulfate (NAMS agar). (Beuchat et al.)

10,000-units/mL penicillin test discs. BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Disc (Benton Dickson and Co. Sparks MD)

Beckman Avanti J-25 Centrifuge

Lifealizer: Labconco Freeze Dry/System/Freezone 4.5

Coffeemaker: Proctor Silex

Coffee: Maxwell House regular roast grounds Creamer: French Vanilla Coffeemate, French Vanilla International Delight, Half and Half Land Lakes, Cain's coffee creamer – powder.

Sugar: S&H sugar, Sweet-n-Low

Toothbrushes: Oral –B, Sulcus

Door Knobs: 2 glass, 2 brushed metal, 2 brass-plated standard house door knobs. (Lowe's)

70 ml spray bottles

Toothpaste - Aim©2004 Church & Dwight Co., Inc., active ingredient – Sodium Monofluorophosphate (0.8%).

Gram Stain – Standard

APPENDIX B – COLONY MORPHOLOGY

Colony Morphology for All Site A Isolates						
Site	Blood Agar	Lecithinase	Lipase	Spore Form	Gram +	Penicillin
A1,1	Dis	+	+	Central/Ellipsoidal	+	R
A1,2	Dis	+	+	Central / Ellipsoidal	+	R
2A	Dis	+	+	Central/ Ellipsoidal	+	R
3A	No data	No data	No data	No data	No data	No data
4A,1	Nh	+	+	Gram negative	-	R
4A,2	Dis	+	+	Spores Absent	+	S
4A,3	Nh	+	+	Spores Absent	+	R
5A	W	+	+	Central /Spherodial	+	R
6A,1	Nh	+	+	Terminal / Ellipsoidal	+	S
6A,2	Dis	+	+	Spores Absent	+	R
6A,3	Dis	+	+	Central /Spheroidal	+	S
7A	No data	No data	No data	No data	No data	No data
8A,1	Dis	+	+	Paracentral/ Spheroidal	+	S
8A,2	Dis	+	+	Spores Absent	+	S
9A	Dis	+	+	Paracentral/ Spheroidal	+	R
10A,1	Dis	+	-	Paracentral / Spheroidal	+	S
10A,2	Dis	+	+	Paracentral /Spheroidal	+	R
10,3	Dis	+	+	Central / Ellipsoidal	+	S
11A,1	Dis	+	-	Central /Spheroidal	+	No tablet
11A,2	Dis	+	+	Paracentral/ Spheroidal	+	No tablet
11A,3	Dis	+	+	Paracentral/ Spheroidal	+	No tablet
12A,1	Nh	+	+	Paracentral/ Spheroidal	+	R
12A,2	W	+	+	Paracentral/ Spheroidal	+	S
12A,3	Dis	+	+	Paracentral/ Spheroidal	+	S
13A,1	W	+	+	No data	+	R
13A,2	W	+	+	Spores Absent	+	R
13A,3	W	+	+	Gram negative	+	No data
14A,1	W	+	+	Spores Absent	+	R
14A,2	W	+	+	Gram negative	-	R
14A,3	W	+	+	Gram negative	-	R
15A,1	W	+	-	Central / Ellipsoidal	+	R
15A,2	Nh	+	-	No data	+	R
16A,1	Nh	+	+	Paracentral/ Spheroidal	+	R
16A,2	Nh	+	+	Paracentral/ Spheroidal	+	R
16A,3	Dis	+	-	Central /Spheroidal	+	S
17A,1	Nh	+	+	Spores Absent	+	R
17A,2	Nh	+	+	Gram negative	-	R
17A,3	Nh	+	+	Gram negative	-	R
18A,1	W	+	+	Central /Spheroidal	+	R
18A,2	W	+	+	Gram negative	-	R
19A,1	Nh	+	+	Spores Absent	+	S
19A,2	Nh	+	+	Central /Spheroidal	+	S
19A,3	Dis	+	+	Central /Spheroidal	+	S
20A,1	Dis	+	+	Central /Spheroidal	+	R

20A,2	W	+	-	Gram negative	-	S
20A,3	W	+	+	Central /Spheroidal	+	R
20A,4	Nh	+	+	Central /Spheroidal	+	R
21A,1	Dis	+	+	Paracentral/ Spheroidal	+	S
21A,2	Dis	+	+	Paracentral/ Spheroidal	+	S
21A,3	W	+	+	Gram negative	+	R
22A,1	Dis	+	+	Paracentral / Spheroidal	+	S
22A,2	Nh	+	-	Central and Spheroidal	+	R
22A,3	Nh	+	-	Terminal /Spheroidal	+	R
23A,1	No growth	+	+	Paracentral/ Spheroidal	+	No growth
23A,2	Nh	+	+	Paracentral/ Spheroidal	+	S
24A,1	Dis	+	+	Paracentral/ Spheroidal	+	S
24A,2	Nh	+	+	Central /Spheroidal	+	R
24A,3	Nh	+	+	Terminal /Spheroidal	+	S
25A,1	Dis	+	+	Terminal /Spheroidal	+	S
25A,2	Nh	+	+	Paracentral/ Spheroidal	+	S
25A,3	Nh	+	+	Central /Spheroidal	+	S
26A,1	Nh	+	+	Terminal/Spheroidal	+	R
26A,2	Nh	+	+	Paracentral / Ellipsoidal	+	S
27A,1	Nh	+	+	Central / Ellipsoidal	+	R
27A,2	Dis	+	+	Central / Ellipsoidal	+	S
27A,3	Nh	+	+	Central / Ellipsoidal	+	R
27A,4	Nh	+	+	Paracentral/ Spheroidal	+	R
28A,1	Nh	+	+	Paracentral / Ellipsoidal	+	S
28A,2	Nh	+	+	Central /Spheroidal	+	S
28A,3	Nh	+	+	Central /Spheroidal	+	R
28A,4	No growth	+	+	No data	No data	No growth
29A,1	Dis	+	+	Central /Spheroidal	+	R
29A,2	Dis	+	+	Central /Spheroidal	+	S
30A,1	Dis	+	+	Central / Ellipsoidal	+	R
30A,2	Nh	+	+	Paracentral/ Spheroidal	+	R
30A,3	Dis	+	+	Central /Spheroidal	+	S
31A,1	W	+	+	Central /Spheroidal	+	S
31A,2	Nh	+	+	Paracentral / Ellipsoidal	+	S
31A,3	Dis	+	+	Paracentral / Ellipsoidal	+	R
32A,1	Dis	+	-	Paracentral / Ellipsoidal	+	R
32A,2	Dis	+	+	Central /Spheroidal	+	R
32A,3	Dis	+	+	Central /Spheroidal	+	R
*Dis(Discontinuous) *W(Weak hemolytic) *Nh(Non-hemolytic) *R(Resistant) *S(Non-resistant)						

Colony Morphology for All Site B Isolates						
Site	Blood Agar	Lecithinase	Lipase	Spore Form	Gram +	Penicillin
1B,1	Dis	+	+	Central / Ellipsoidal	+	R
1B,2	Dis	+	+	Central / Ellipsoidal	+	R
1B,3	Nh	+	+	Central / Ellipsoidal	+	S
2B,1	Dis	+	+	Central / Ellipsoidal	+	S
2B,2	Dis	+	+	Spores Absent	+	S
3B	No data	No data	No data	No data	No data	No data
4B,1	Nh	+	+	Spores Absent	+	S
4B,2	Dis	+	+	Paracentral/ Spheroidal	+	S
5B,1	W	+	+	Paracentral/ Spheroidal	+	S
5B,2	Dis	+	+	No data	No data	
6B,1	Dis	+	+	Spores Absent	+	S
6B,2	Dis	+	+	Central / Spheroidal	+	S
6B,3	Dis	+	+	Terminal / Ellipsoidal	+	S
7B,1	Dis	+	-	No data	No data	R
7B,2	Dis	+	+	No data	No data	R
8B,1	Dis	+	+	Paracentral/ Spheroidal	+	S
8B,2	Dis	+	+	Paracentral/ Spheroidal	+	S
9B,1	Dis	+	+	Paracentral/ Spheroidal	+	S
9B,2	Dis	+	+	Terminal / Ellipsoidal	+	S
10B,1	Dis	+	+	Spores Absent	+	R
10B,2	W	+	+	Paracentral/ Spheroidal	+	R
11B,1	Dis	+	+	Paracentral/ Spheroidal	+	S
11B,2	Dis	+	+	Spores Absent	+	S
12B,1	Dis	+	+	Paracentral / Ellipsoidal	+	R
12B,2	W	+	+	Paracentral / Ellipsoidal	+	R
12B,3	W	+	+	Paracentral / Ellipsoidal	+	R
13B,1	W	+	+	Paracentral/ Spheroidal	+	S
13B,2	W	+	+	Paracentral/ Spheroidal	+	S
13B,3	Dis	+	+	Paracentral/ Spheroidal	+	S
14B,1	W	+	+	Too small to visualize	+	S
14B,2	W	+	+	Spores Absent	+	S
14B,3	Dis	+	+	Paracentral/ Spheroidal	+	R
15B,1	W	+	+	Paracentral/ Spheroidal	+	S
15B,2	Dis	+	+	Paracentral/ Spheroidal	+	R
15B,3	W	+	+	Paracentral/ Spheroidal	+	S
16B,1	W	+	+	Paracentral / Ellipsoidal	+	S
16B,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
16B,3	W	+	-	Paracentral / Ellipsoidal	+	S
17B,1	W	+	+	Spores Absent	+	S
17B,2	Dis	+	+	Terminal /Spheroidal	+	R
17B,3	W	+	+	Spores Absent	+	S
18B,1	Dis	+	+	Paracentral / Ellipsoidal	+	R
18B,2	W	+	+	Paracentral / Ellipsoidal	+	R
18B,3	Dis	+	+	Paracentral / Ellipsoidal	+	R
19B,1	Dis	+	+	Paracentral / Ellipsoidal	+	R
19B,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
20B,1	W	+	+	Paracentral / Ellipsoidal	+	S

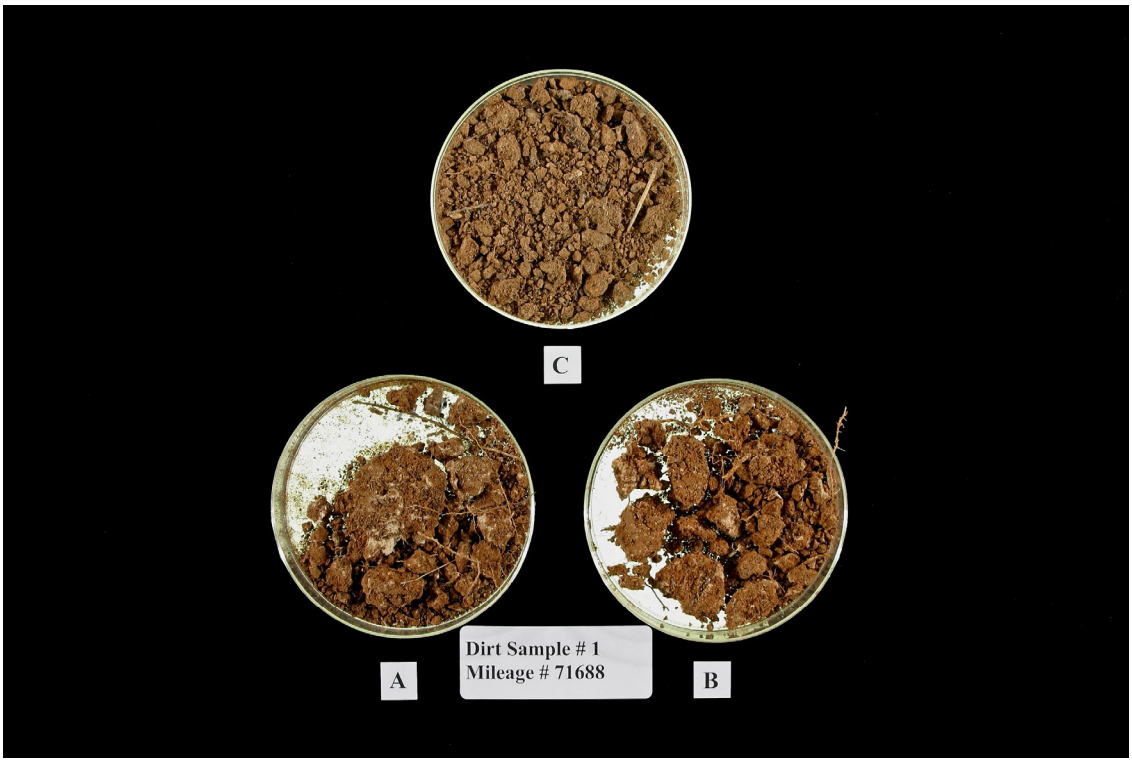
20B,2	W	+	+	Central and Ellipsoidal	+	S
20B,3	Dis	+	+	Paracentral / Ellipsoidal	+	R
21B,1	W	+	+	Spores Absent	+	S
21B,2	W	+	+	Spores Absent	+	S
22B,1	Dis	+	+	Paracentral / Ellipsoidal	+	R
22B,2	W	+	+	Paracentral/ Spheroidal	+	S
22B,3	Dis	+	+	Spores Absent	+	S
23B,1	Dis	+	+	Central / Spheroidal	+	R
23B,2	W	+	+	Spores Absent	+	S
23B,3	W	+	+	Terminal /Spheroidal	+	S
24B,1	Dis	+	+	Spores Absent	+	R
24B,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
25B,1	Dis	+	+	Paracentral / Ellipsoidal	+	R
25B,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
26B	Dis	+	+	Paracentral / Ellipsoidal	+	R
27B,1	Dis	+	+	Paracentral / Ellipsoidal	+	R
27B,2	W	+	+	Paracentral/ Spheroidal	+	S
28B,1	Dis	+	+	Paracentral / Ellipsoidal	+	R
28B,2	W	+	+	Paracentral / Ellipsoidal	+	R
29B,1	W	+	+	Spores Absent	+	S
29B,2	Dis	+	+	Paracentral/ Spheroidal	+	R
30B,1	W	+	+	Terminal /Spheroidal	+	R
30B,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
31B,1	W	+	+	Paracentral / Ellipsoidal	+	S
31B,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
32B,1	W	+	+	Paracentral/ Spheroidal	+	S
32B,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
*Dis(Discontinuous) *W(Weak hemolytic) *Nh(Non-hemolytic) *R(Resistant) *S(Non-resistant)						

Colony Morphology for All Site C Isolates						
Site	Blood Agar	Lecithinase	Lipase	Spore Form	Gram +	Penicillin
1C,1	Dis	+	+	Central / Ellipsoidal	+	R
1C,2	No growth	+	-	No data	No data	No growth
2C,1	Dis	+	+	Paracentral / Ellipsoidal	+	S
2C,2	Dis	+	+	Spores absent	+	R
3C,1	Dis	+	+	Central / Ellipsoidal	+	No data
3C,2	Dis	+	+	Terminal /Spheroidal	+	No data
3C,3	Dis	+	+	Paracentral / Ellipsoidal	+	No data
4C,1	Dis	+	+	Spores absent	+	S
4C,2	Dis	+	+	Paracentral / Ellipsoidal	+	S
4C,3	Dis	+	+	Spores absent	+	S
5C.1	Dis	+	-	No data	No data	S
5C,2	Dis	+	-	No data	No data	S
6C,1	W	+	+	Spores absent	+	S
6C,2	Dis	+	+	Central /Shperoidal	+	S
6C,3	Dis	+	+	Paracentral / Ellipsoidal	+	S
7C,1	Dis	+	+	Central /Shperoidal	+	S
7C,2	Dis	+	+	Terminal/ Ellipsoidal	+	R
7C,3	Dis	+	+	Terminal/ Ellipsoidal	+	R
8C,1	Dis	+	+	Paracentral /Spheroidal	+	S
8C,2	Dis	+	+	Paracentral /Spheroidal	+	S
9C	Dis	+	+	Spores absent	+	R
10C,1	Dis	+	+	Central / Ellipsoidal	+	R
10C,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
11C,1	Dis	+	+	Spores absent	+	S
11C,2	Dis	+	+	Spores absent	+	S
12C,1	Dis	+	-	No data	No data	S
12C,2	W	+	-	Gram negative	-	S
13C,1	Dis	+	+	Terminal/ Ellipsoidal	+	S
13C,2	Dis	+	+	Paracentral /Spheroidal	+	R
13C,3	Dis	+	+	Gram negative	-	R
14C,1	Dis	+	+	Terminal /Spheroidal	+	R
14C,2	Dis	+	+	Terminal/ Ellipsoidal	+	R
14C,3	Dis	+	-	Terminal/ Ellipsoidal	+	R
15C	Dis	+	+	Central /Shperoidal	+	R
16C,1	Dis	+	+	Terminal/ Ellipsoidal	+	R
16C,2	Dis	+	+	Terminal /Spheroidal	+	R
17C,1	Dis	+	-	Terminal/ Ellipsoidal	+	R
17C,2	Dis	+	+	Terminal/ Ellipsoidal	+	S
17C,3	Nh	+	+	Central /Shperoidal	+	S
18C	Dis	+	+	Paracentral /Spheroidal	+	R
19C	Nh	+	-	Terminal/ Ellipsoidal	+	S
20C,1	Dis	+	+	Central /Shperoidal	+	R
20C,2	Dis	+	+	Central /Shperoidal	+	R
21C,1	Nh	+	-	Terminal /Spheroidal	+	R
21C,2	W	+	+	Terminal/ Ellipsoidal	+	S
22C,1	Dis	+	+	Paracentral /Spheroidal	+	S

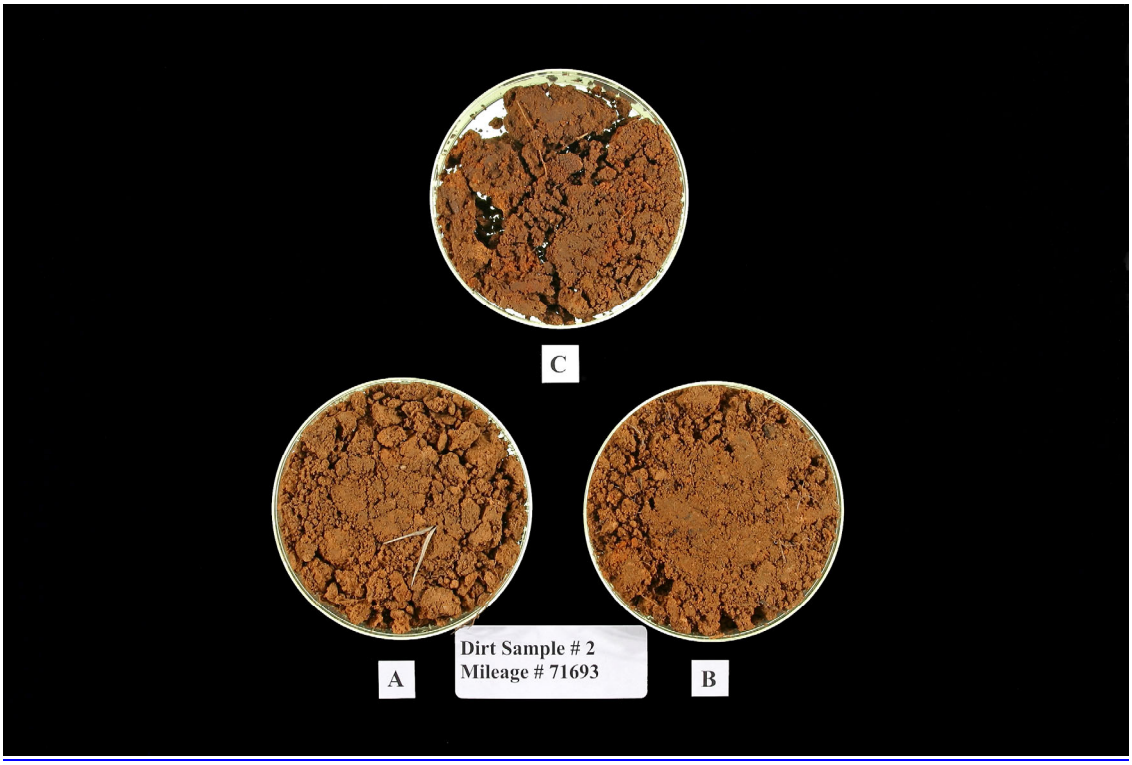
22C,2	Dis	+	+	Paracentral /Spheroidal	+	R
22C,3	Dis	+	+	Terminal /Spheroidal	+	R
22C,4	Dis	+	+	Terminal/ Ellipsoidal	+	R
23C,1	Dis	+	+	No data	No data	S
23C,2	Dis	+	+	Central /Shperoidal	+	S
23C,3	Dis	+	+	Paracentral / Ellipsoidal	+	R
23C,4	Dis	+	+	Paracentral / Ellipsoidal	+	R
24C,1	Dis	+	+	Terminal /Spheroidal	+	S
24C,2	Dis	+	+	Terminal/ Ellipsoidal	+	R
24C,3	Dis	+	+	Terminal/ Ellipsoidal	+	R
25C,1	Dis	+	+	Terminal /Spheroidal	+	R
25C,2	Dis	+	+	Paracentral / Ellipsoidal	+	R
25C,3	Dis	+	+	Terminal/ Ellipsoidal	+	R
26C,1	W	+	+	Central /Shperoidal	+	R
26C,2	Dis	+	+	Central /Shperoidal	+	R
26C,3	Dis	+	+	Paracentral / Ellipsoidal	+	R
27C,1	Dis	+	+	No data	No data	S
27C,2	Dis	+	+	Paracentral /Spheroidal	+	S
28C,1	Dis	+	+	Paracentral /Spheroidal	+	S
28C,2	Dis	+	+	Paracentral /Spheroidal	+	R
28C,3	Dis	+	+	Paracentral /Spheroidal	+	R
29C,1	Dis	+	+	Paracentral / Ellipsoidal	+	S
29C,2	Dis	+	+	Spores absent	+	S
30C,1	Dis	+	+	Central /Shperoidal	+	R
30C,2	Dis	+	+	Paracentral /Spheroidal	+	R
31C,1	Dis	+	+	Central /Shperoidal	+	R
31C,2	W	+	+	Paracentral /Spheroidal	+	R
32C,1	Dis	+	+	Terminal /Spheroidal	+	S
32C,2	Dis	+	+	Central /Shperoidal	+	R
32C,3	Dis	+	+	Central / Ellipsoidal	+	R
*Dis(Discontinuous) *W(Weak hemolytic) *Nh(Non-hemolytic) *R(Resistant) *S(Non-resistant)						

APPENDIX C – SOIL SITES AND SOL SAMPLES

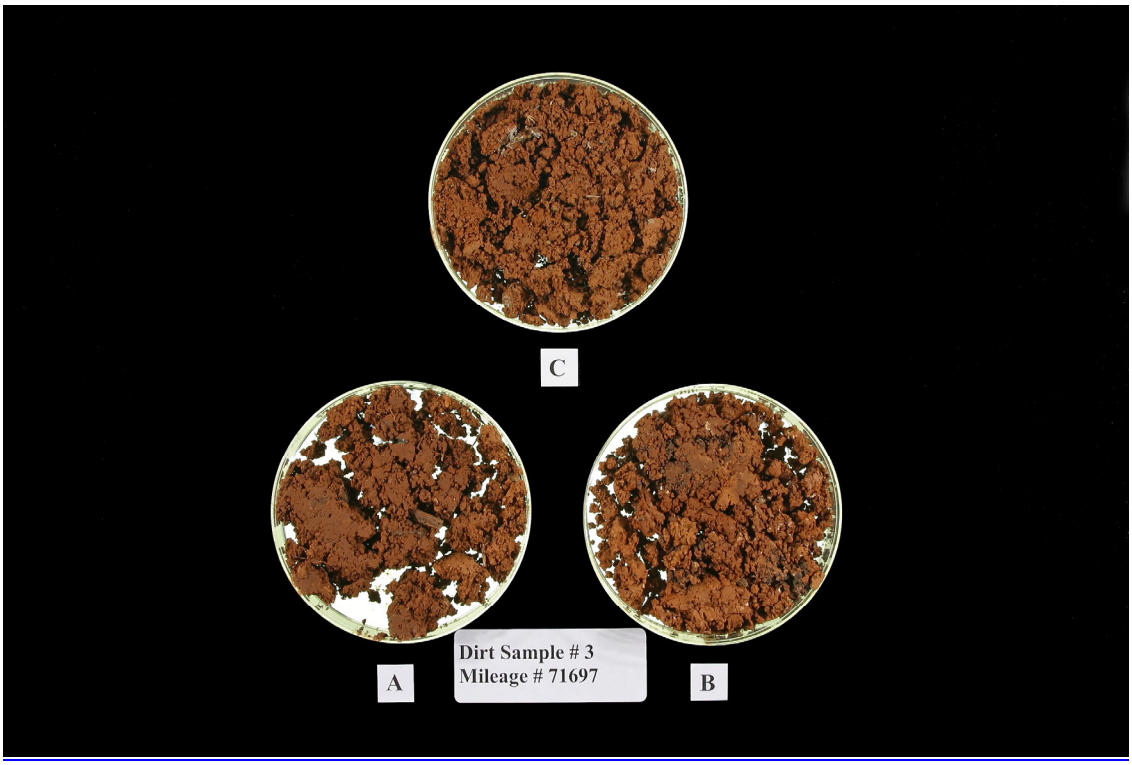
Site 1 and Soil Samples.



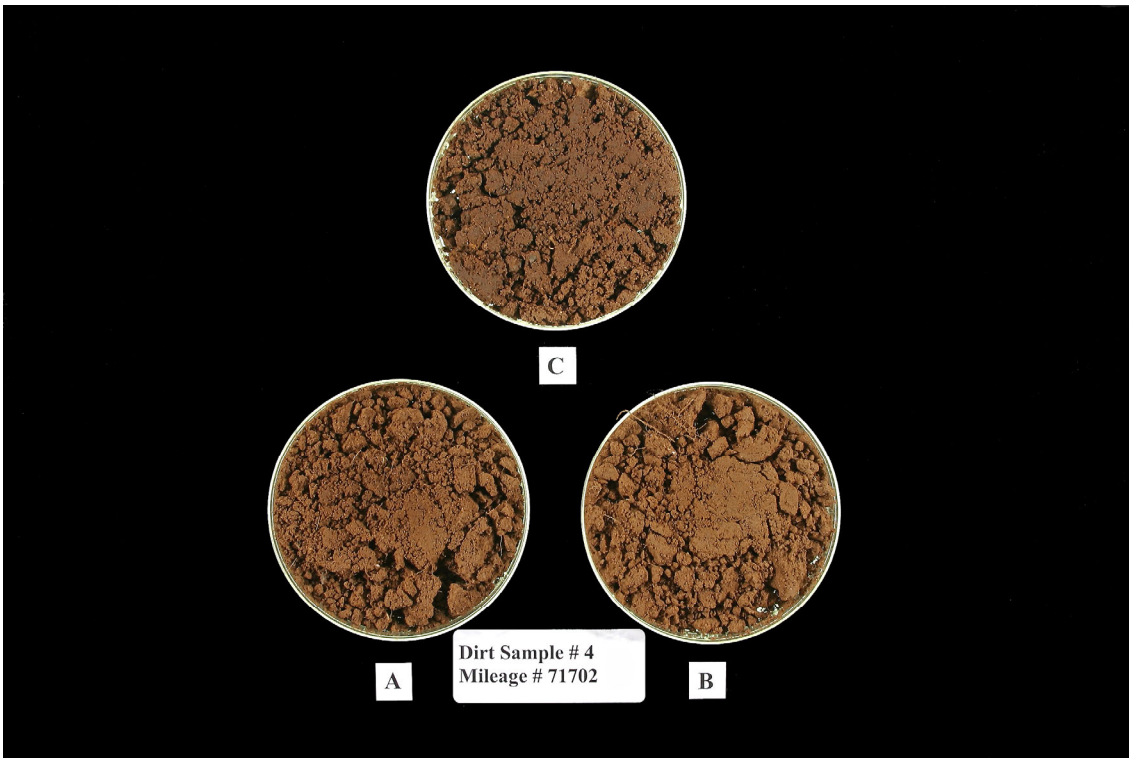
Site 2 and Soil Samples



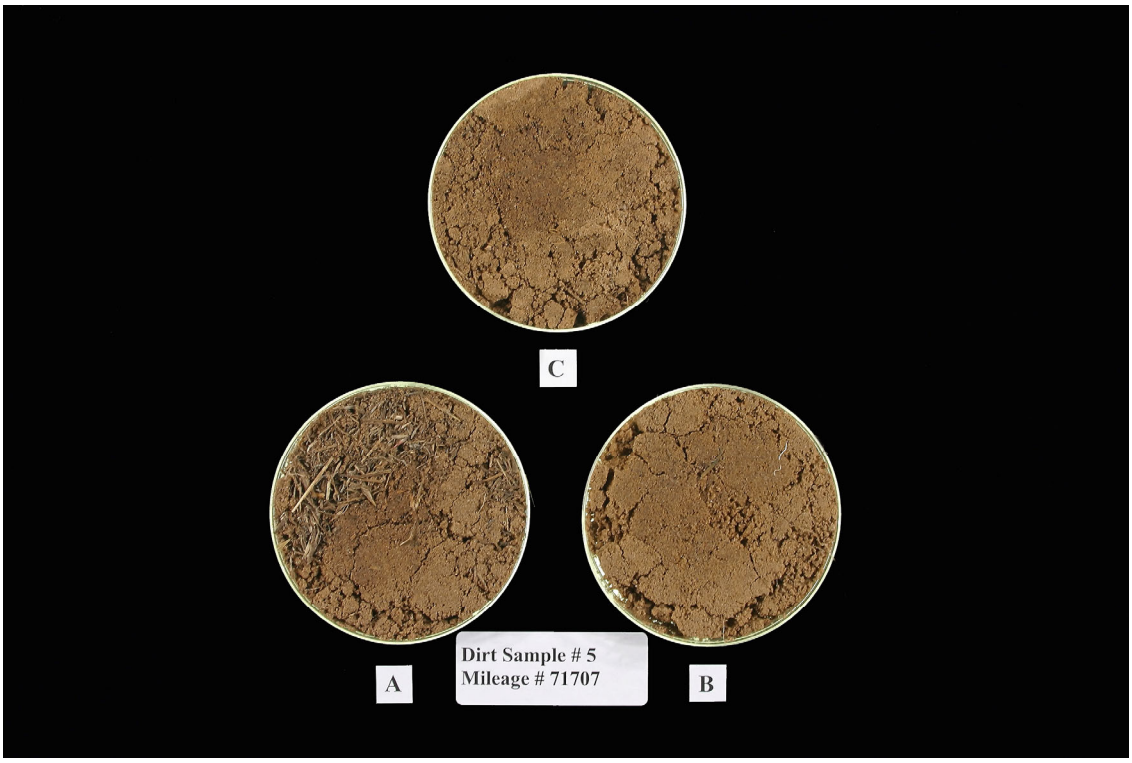
Site 3 and Soil Samples.



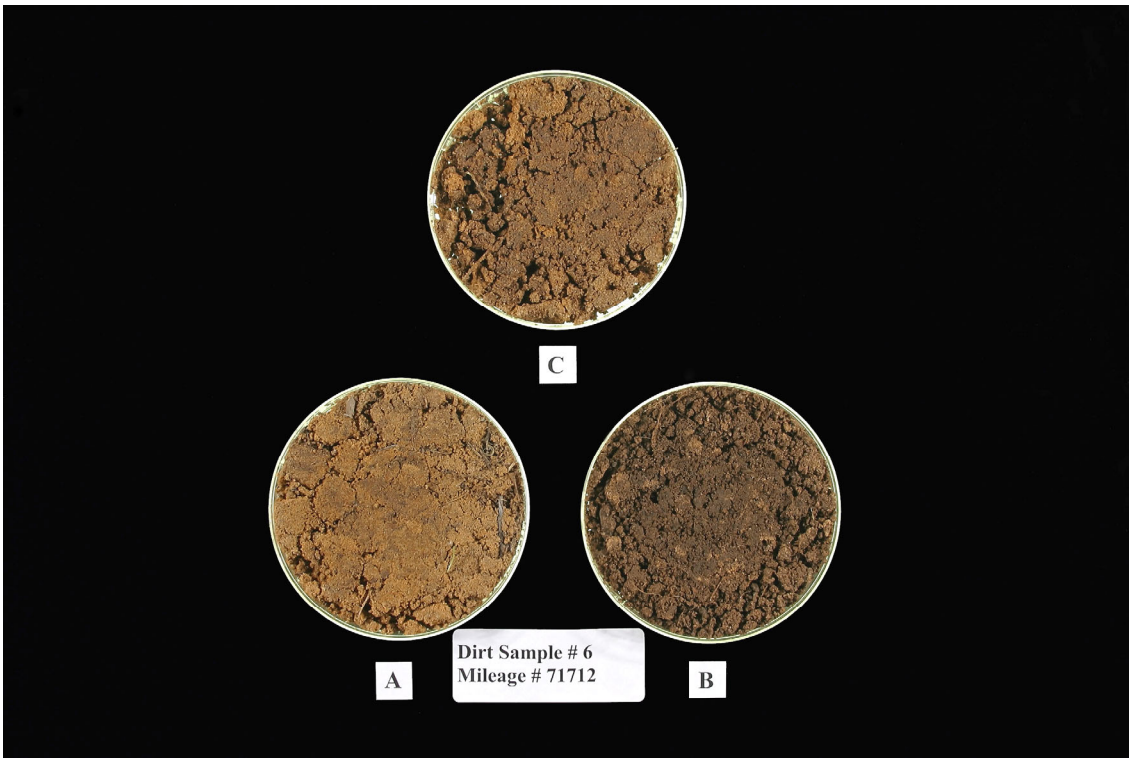
Site 4 and Soil Samples.



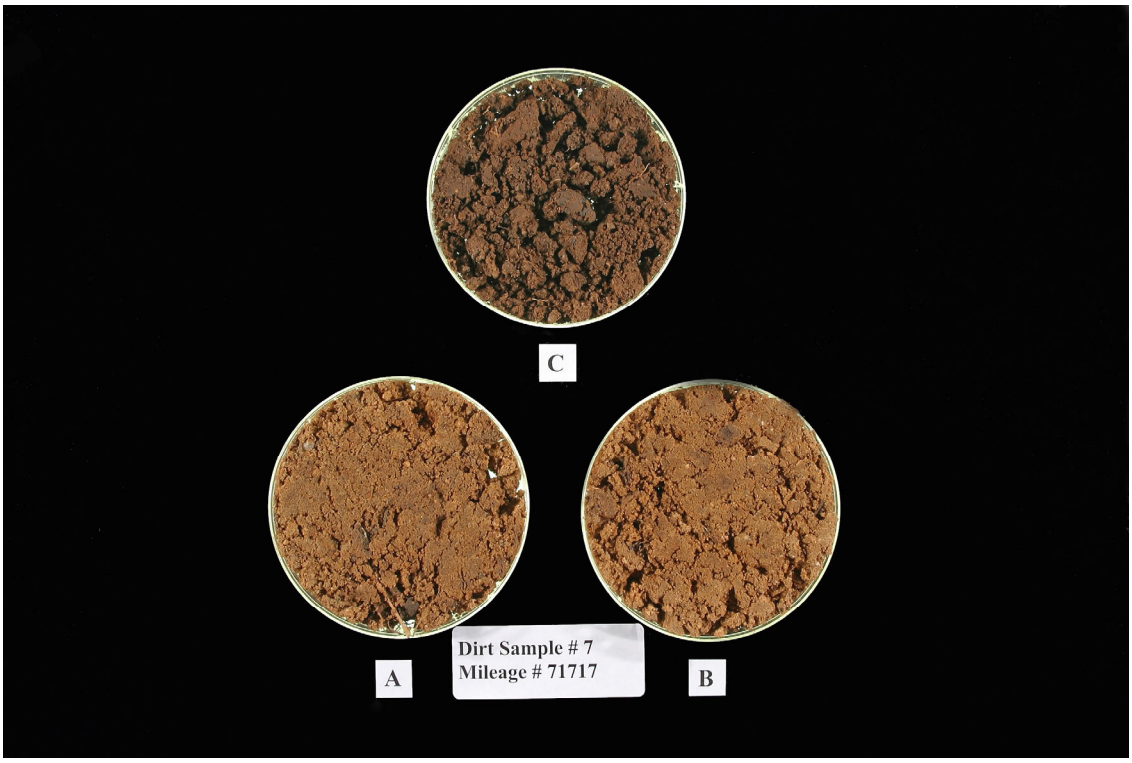
Site 5 and Soil Samples.



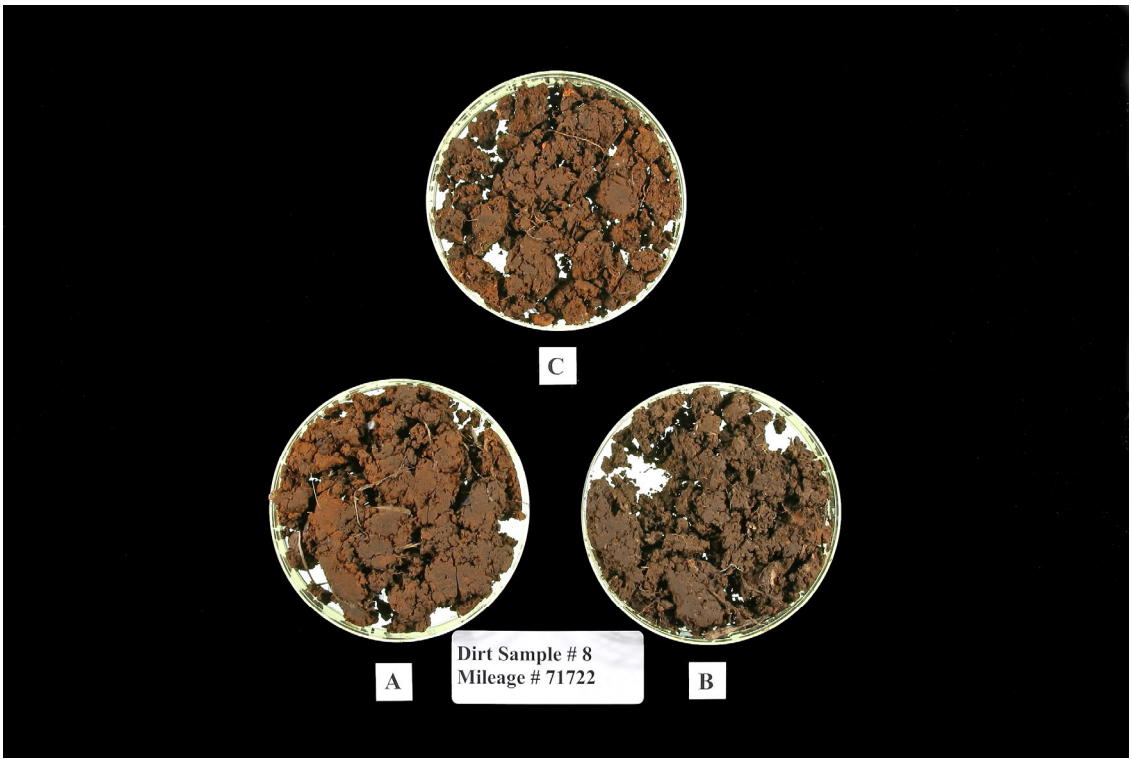
Site 6 and Soil Samples.



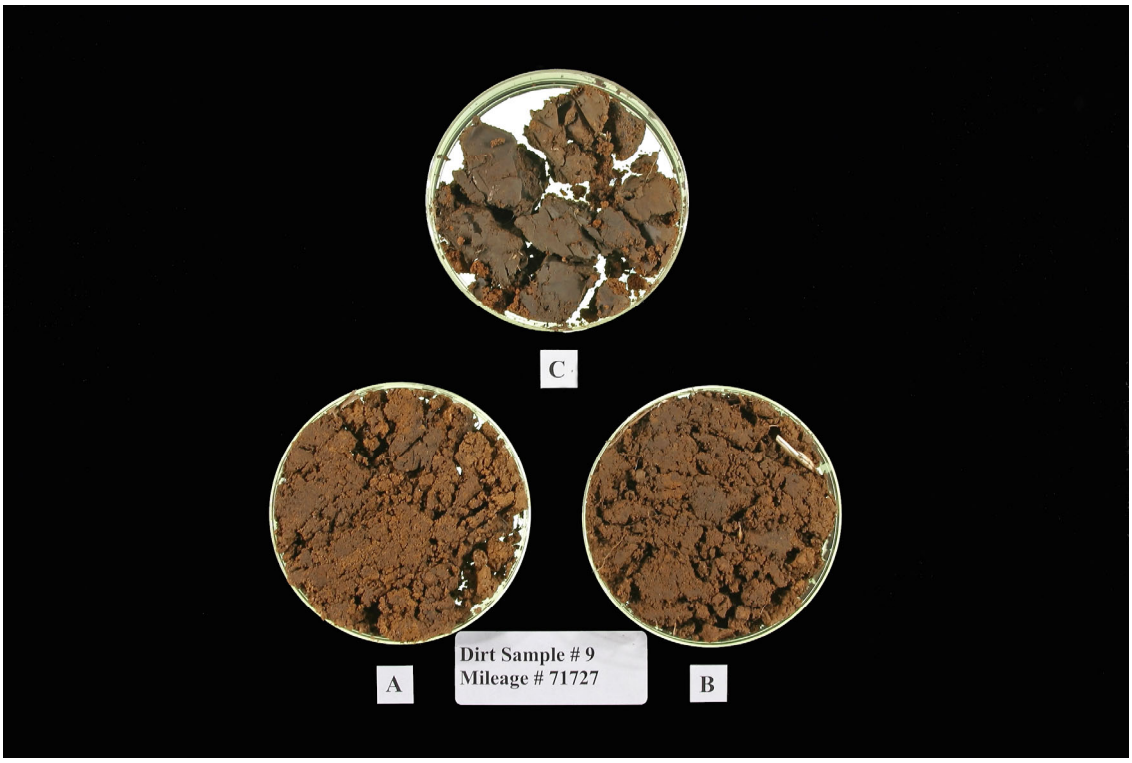
Site 7 and Soil Samples.



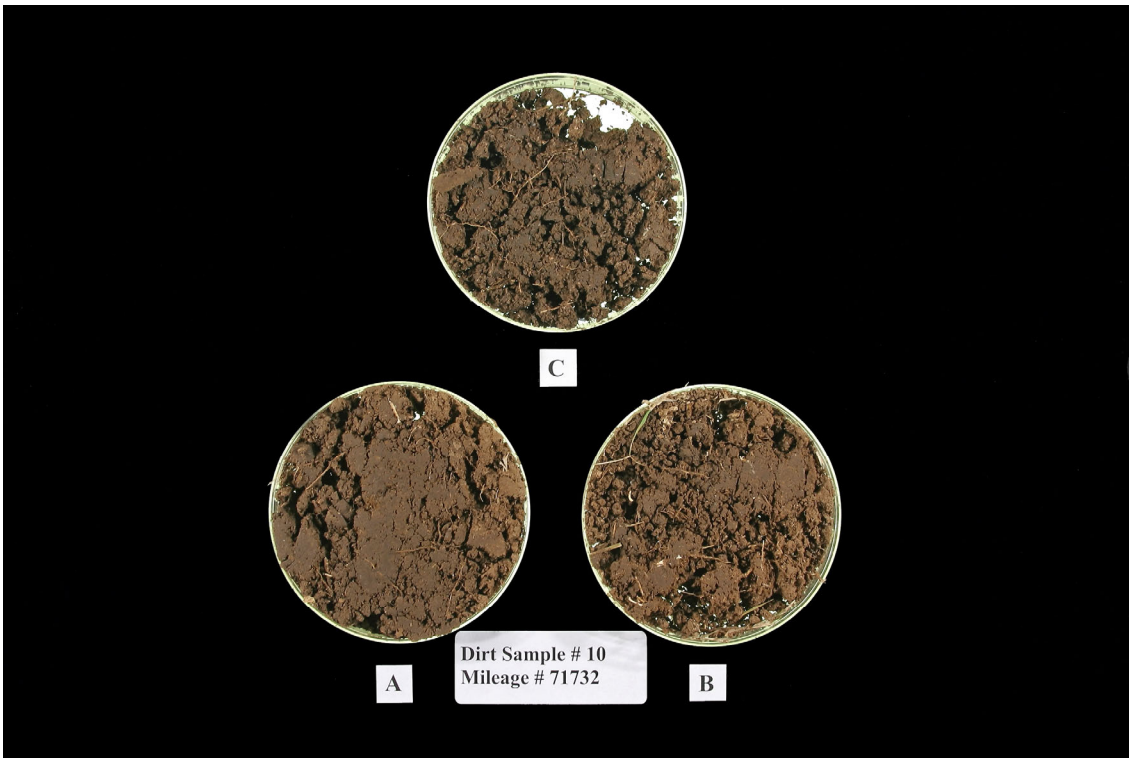
Site 8 and Soil Samples.



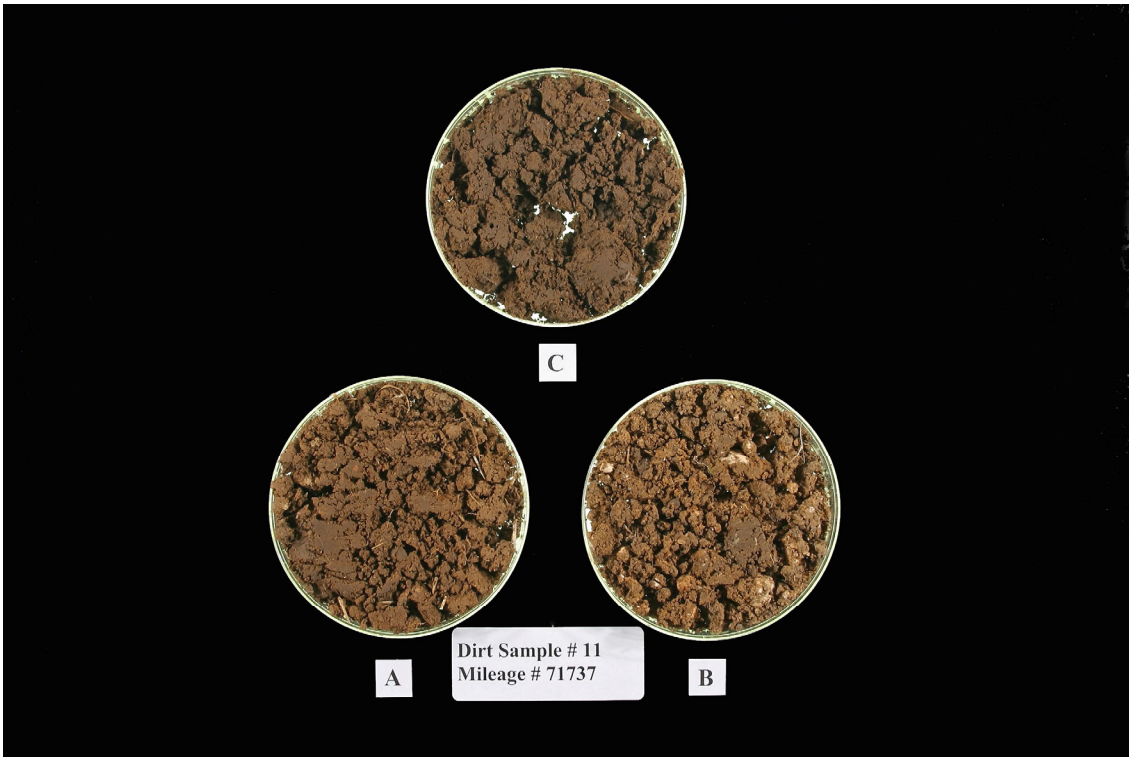
Site 9 and Soil Samples.



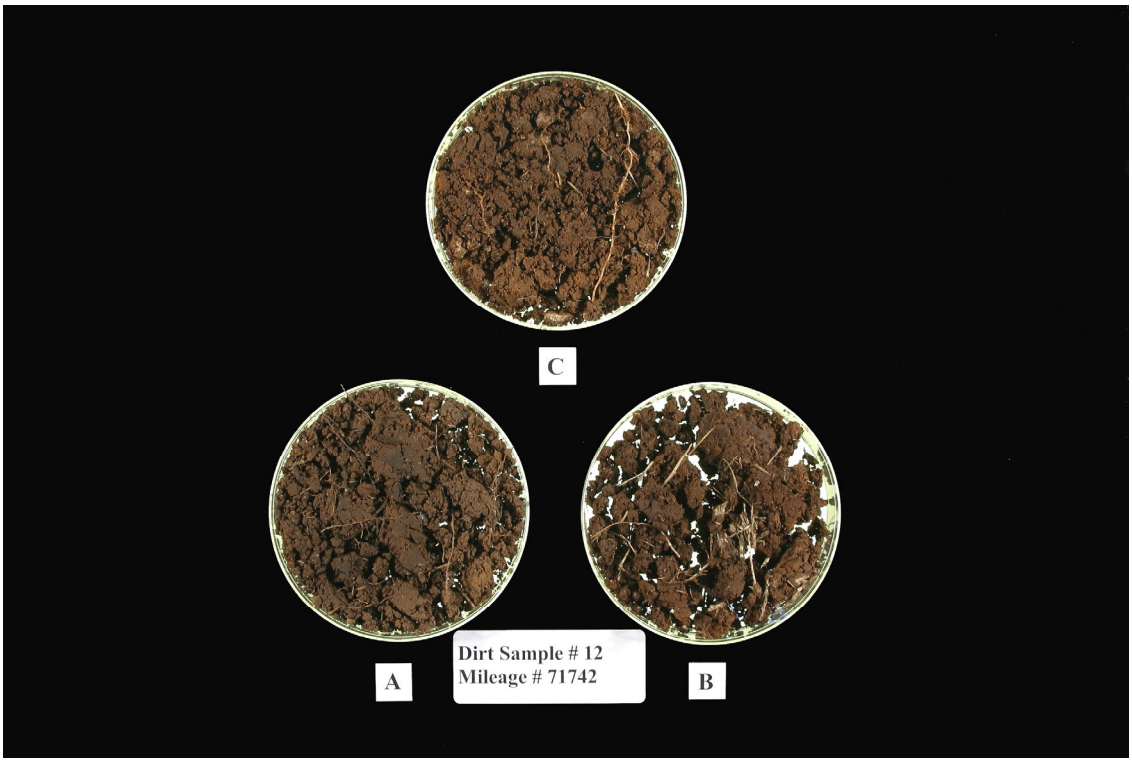
Site 10 and Soil Samples.



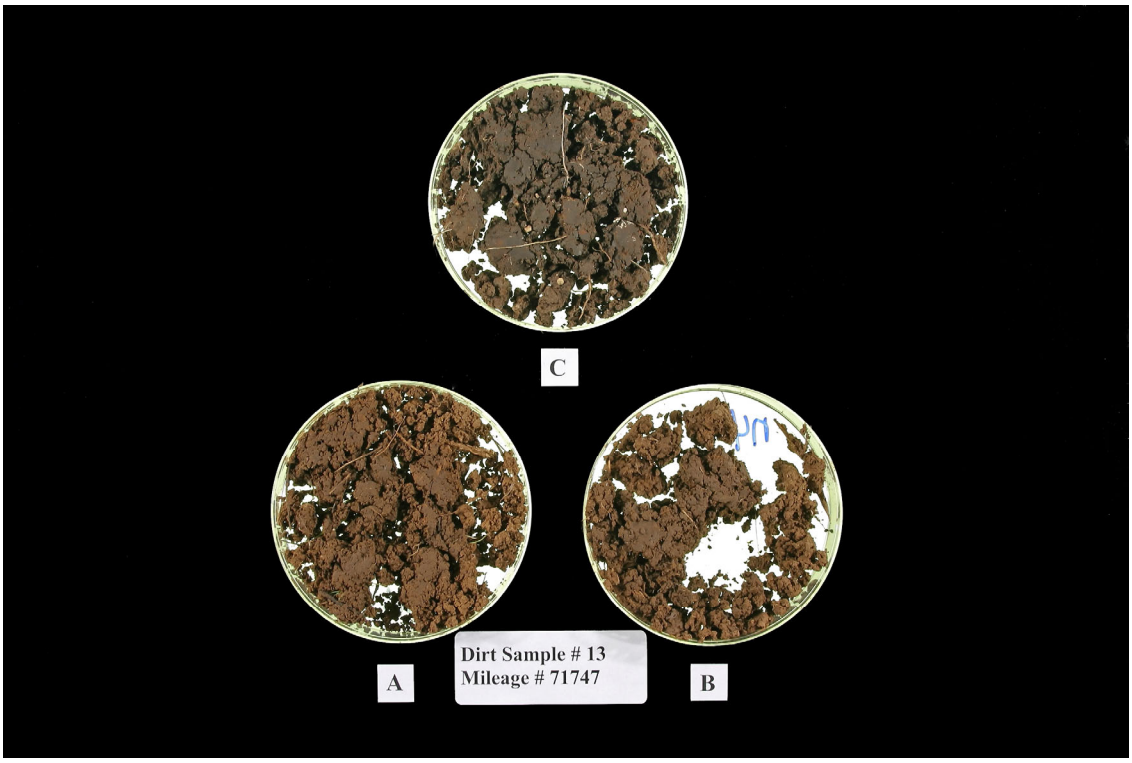
Site 11 and Soil Samples.



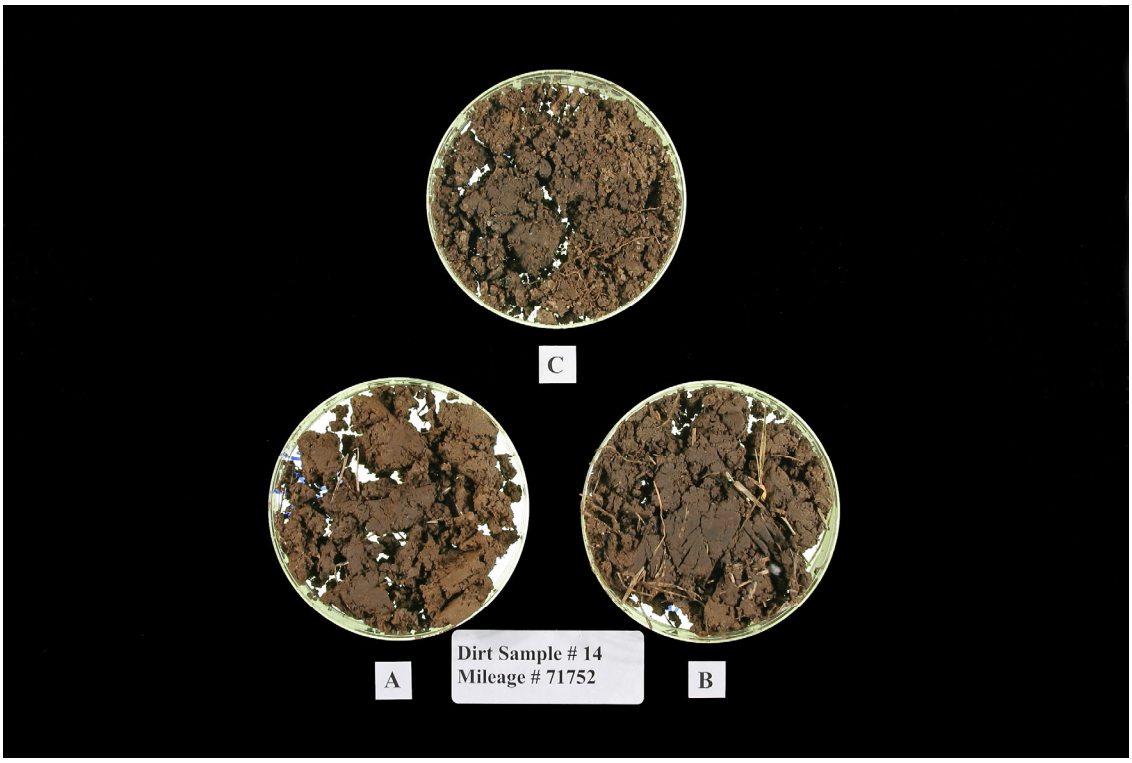
Site 12 and Soil Samples.



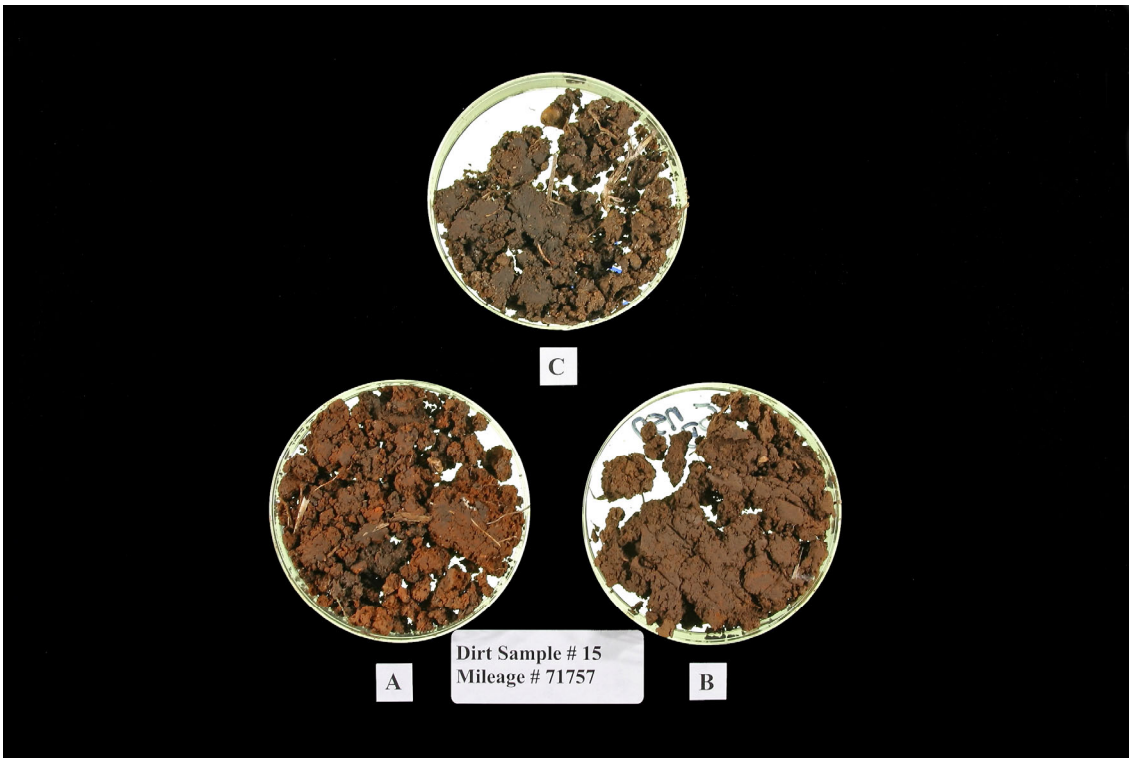
Site 13 and Soil Samples.



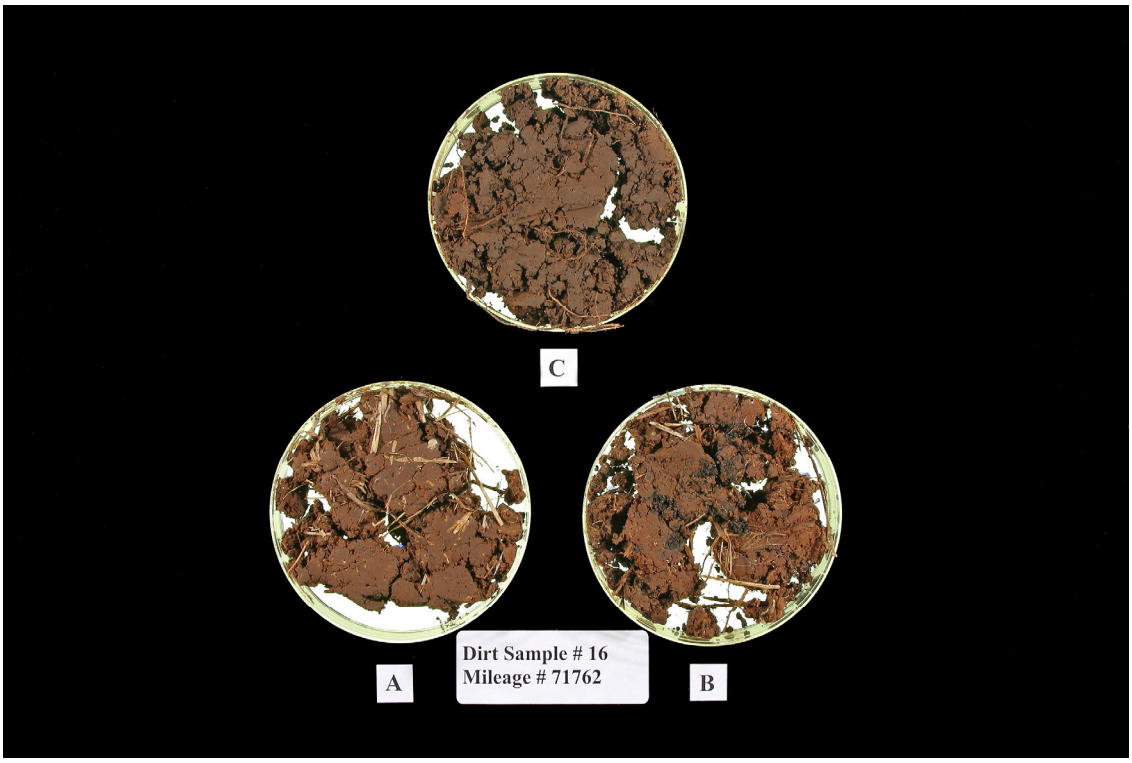
Site 14 and Soil Samples.



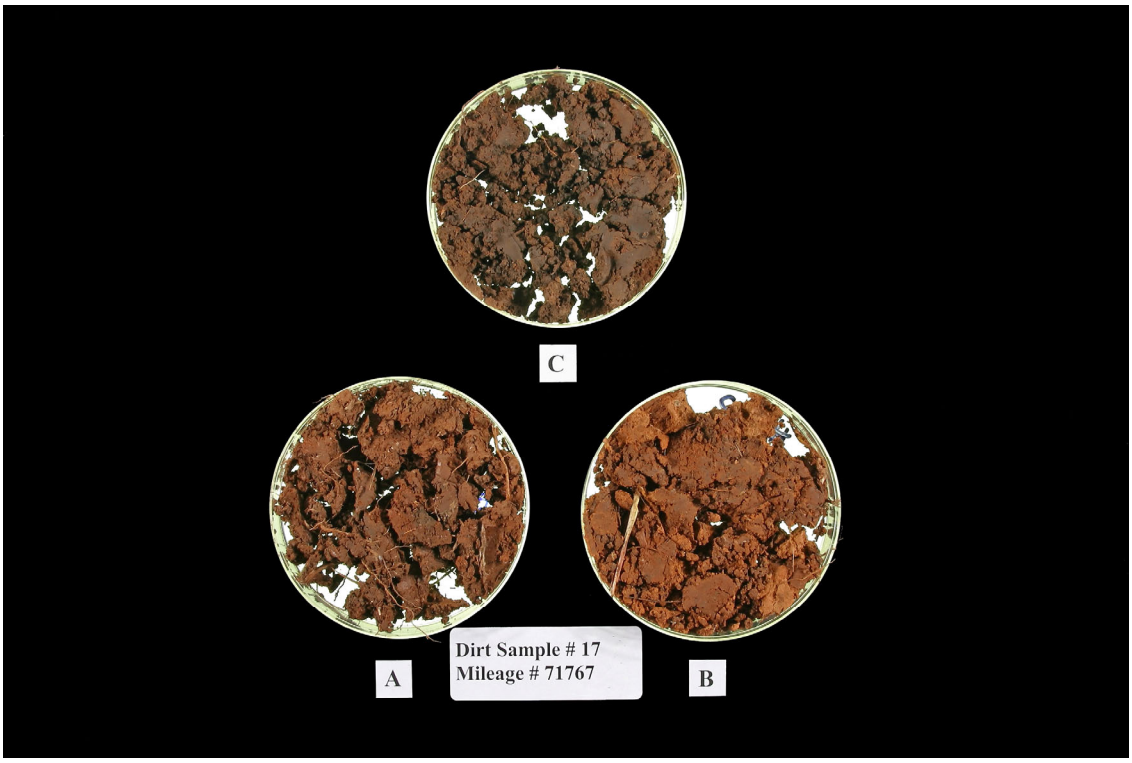
Site 15 and Soil Samples.



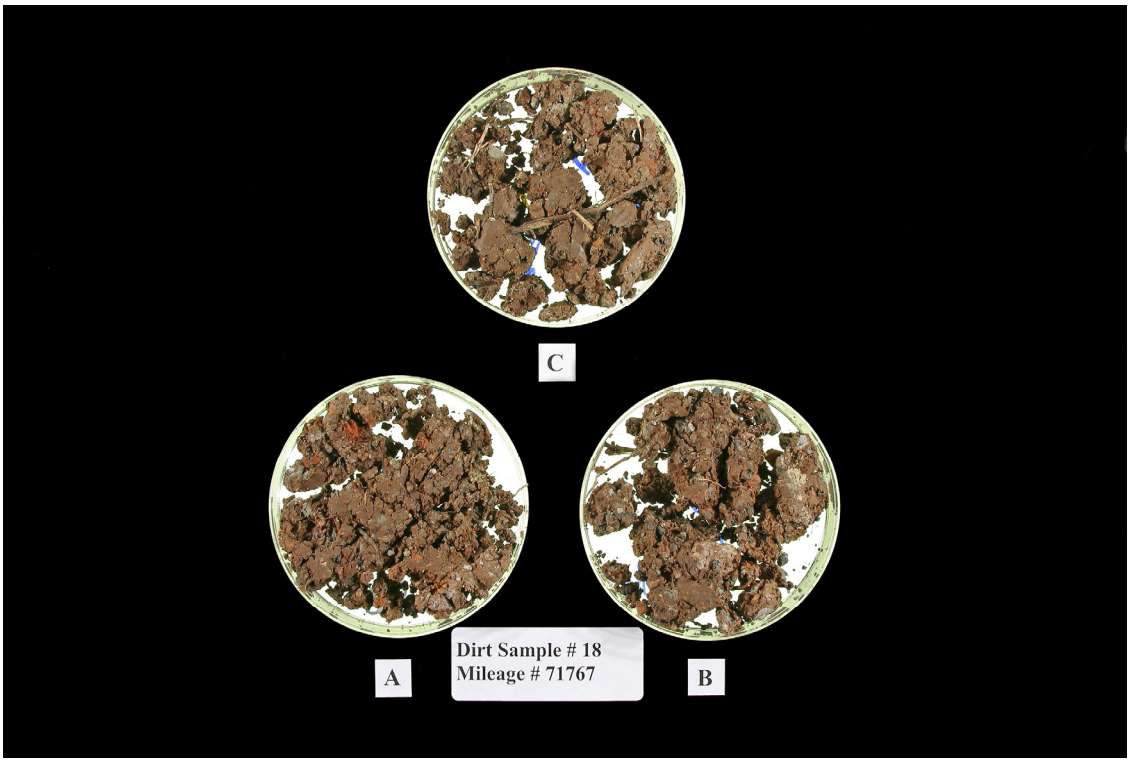
Site 16 and Soil Samples.



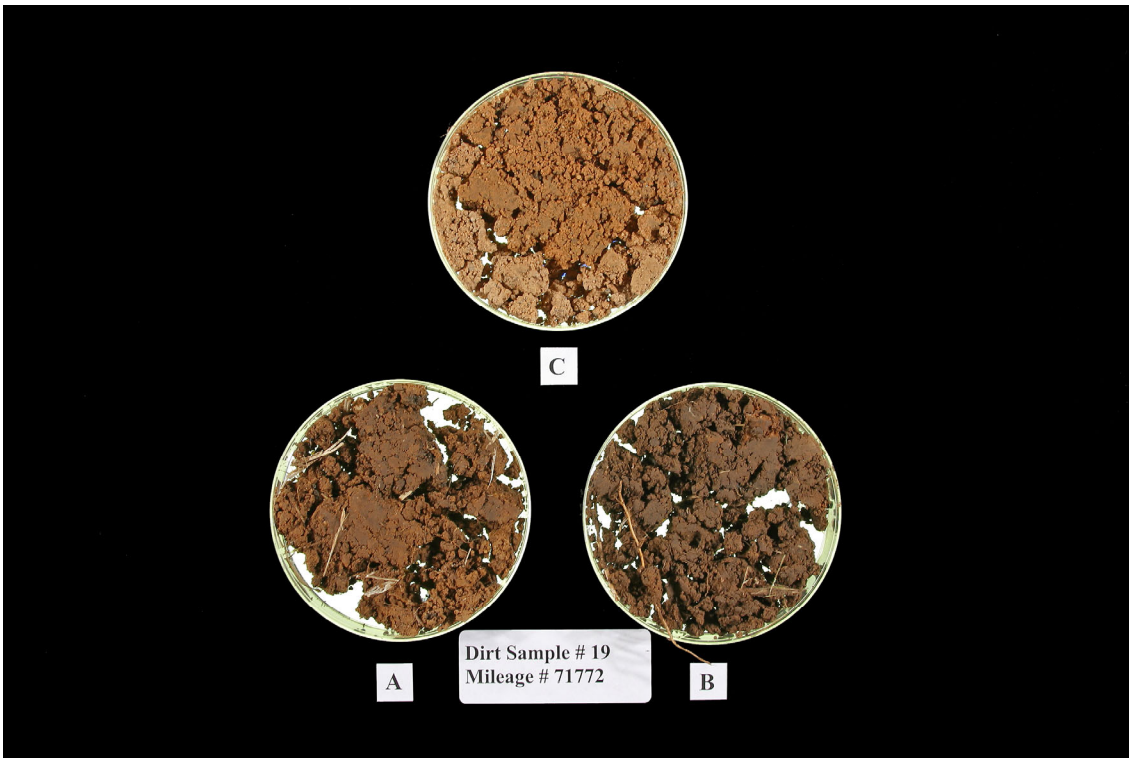
Site 17 and Soil Samples.



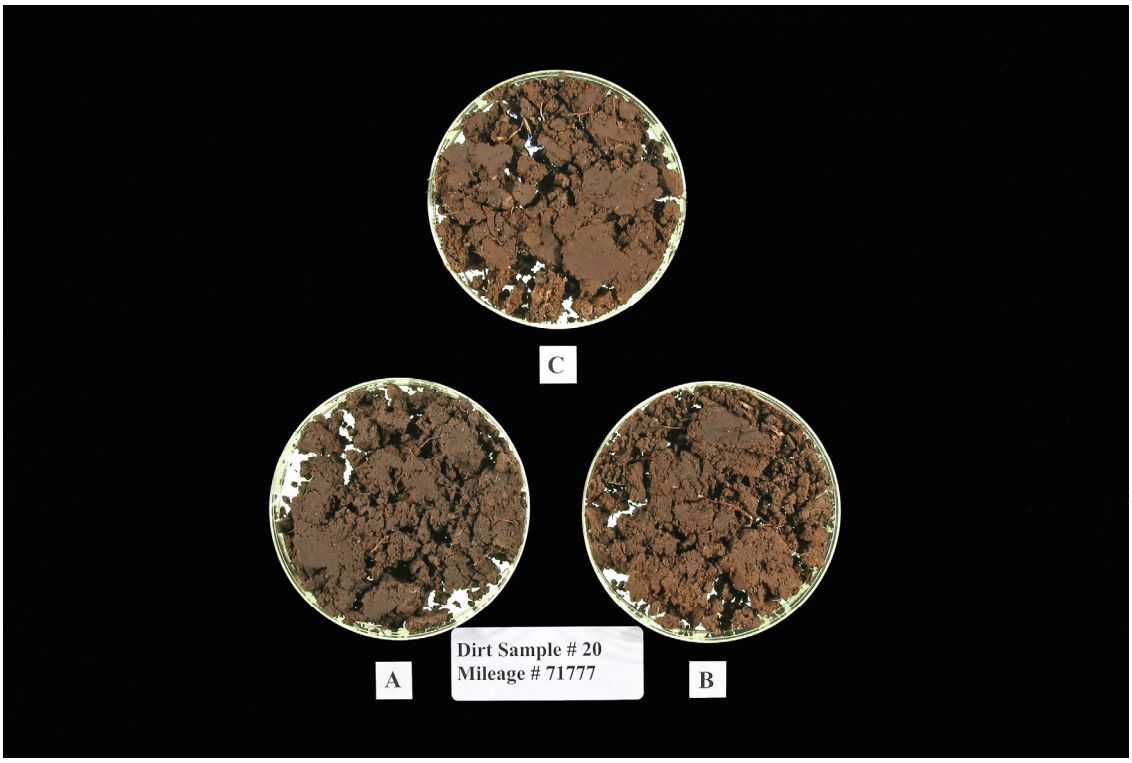
Site 18 and Soil Samples.



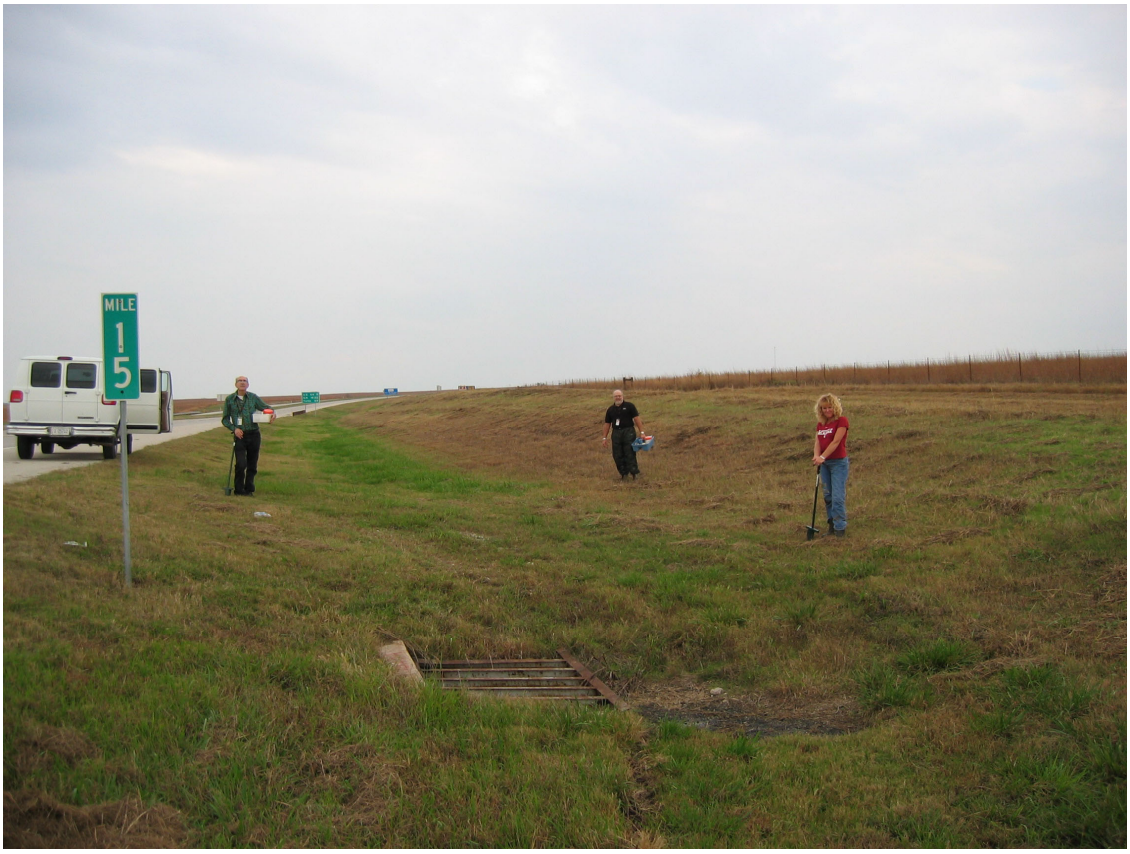
Site 19 and Soil Samples.

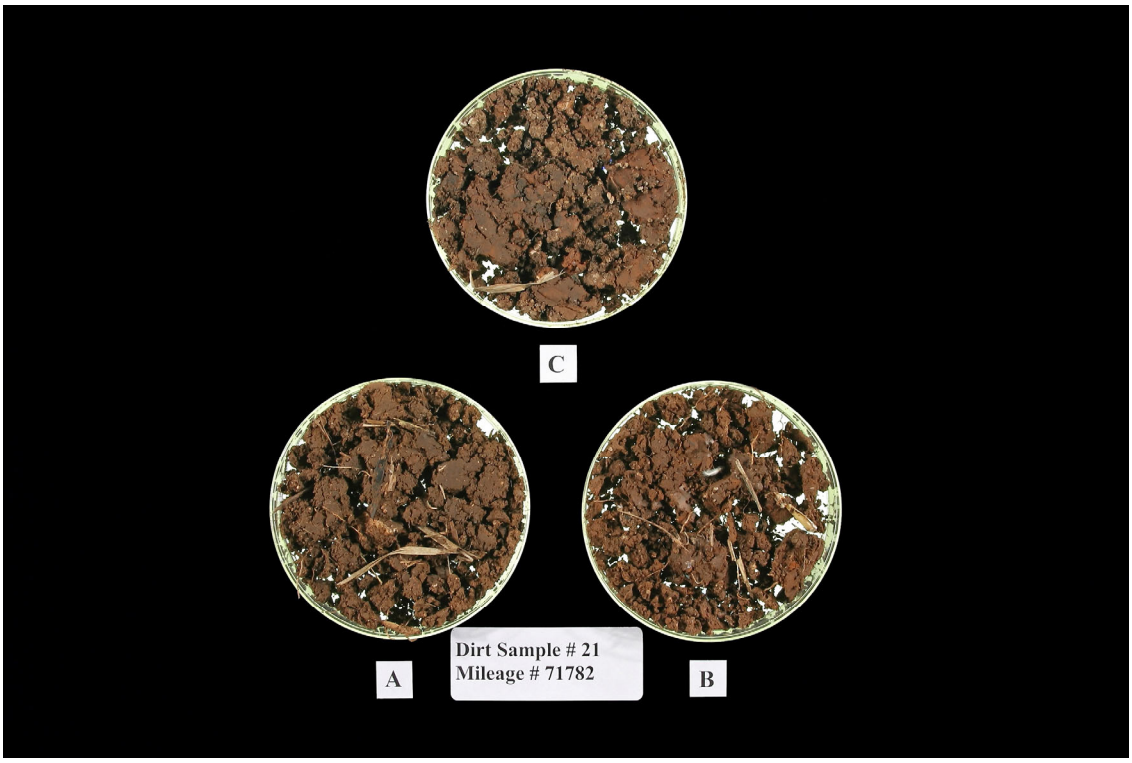


Site 20 and Soil Samples.

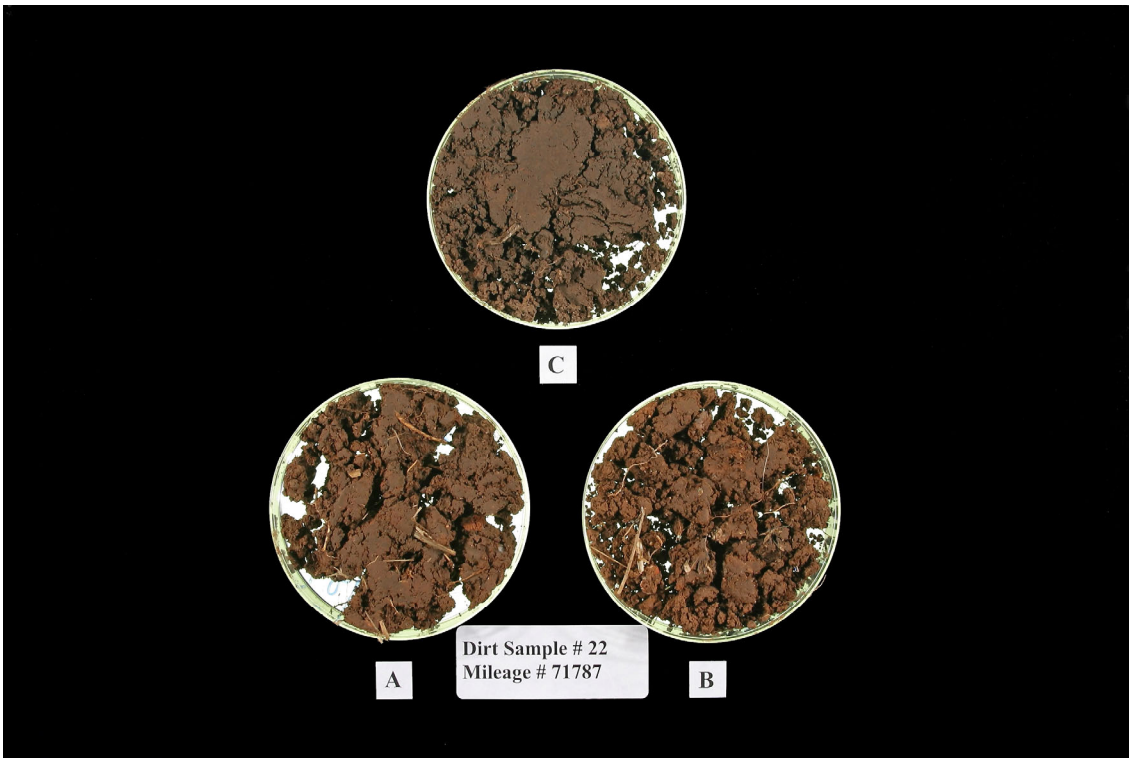


Site 21 and Soil Samples.

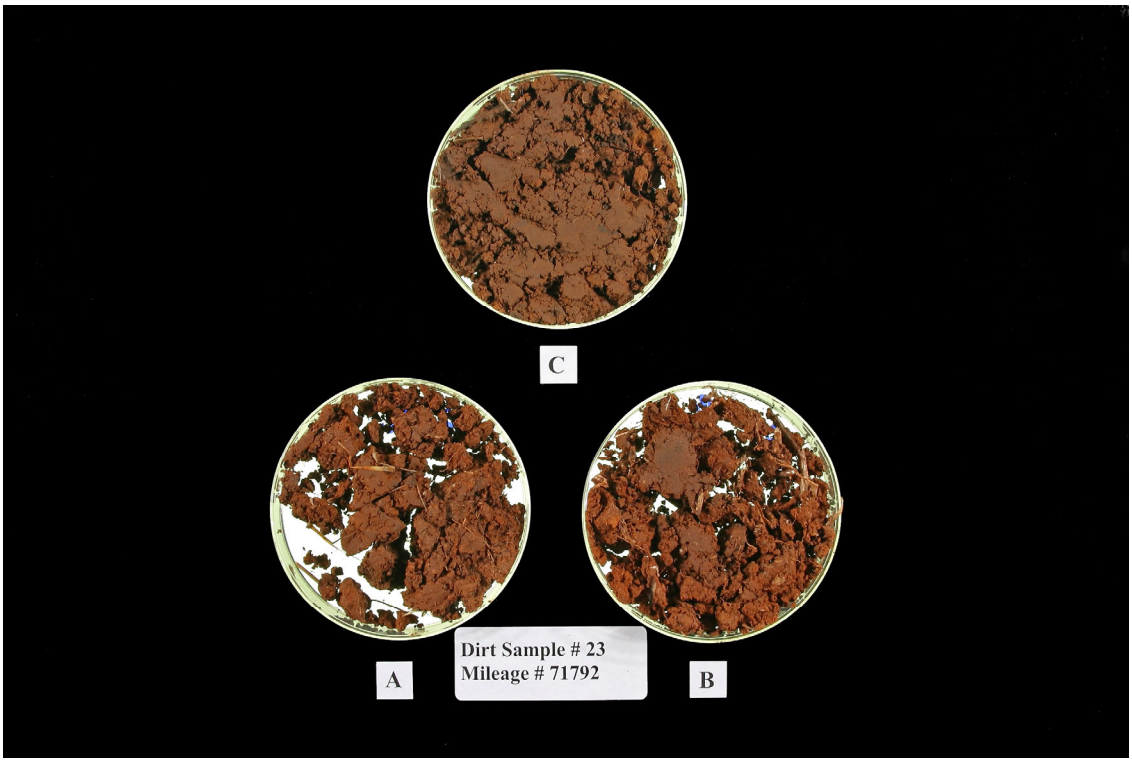




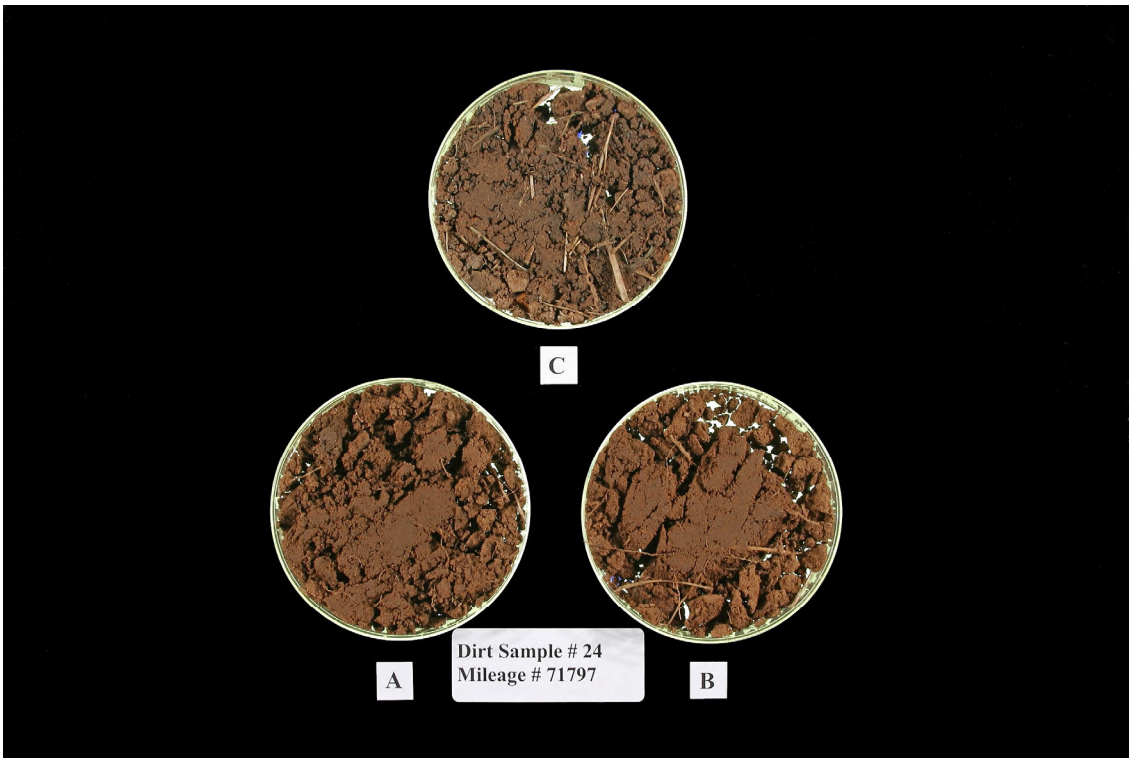
Site 22 and Soil Samples.



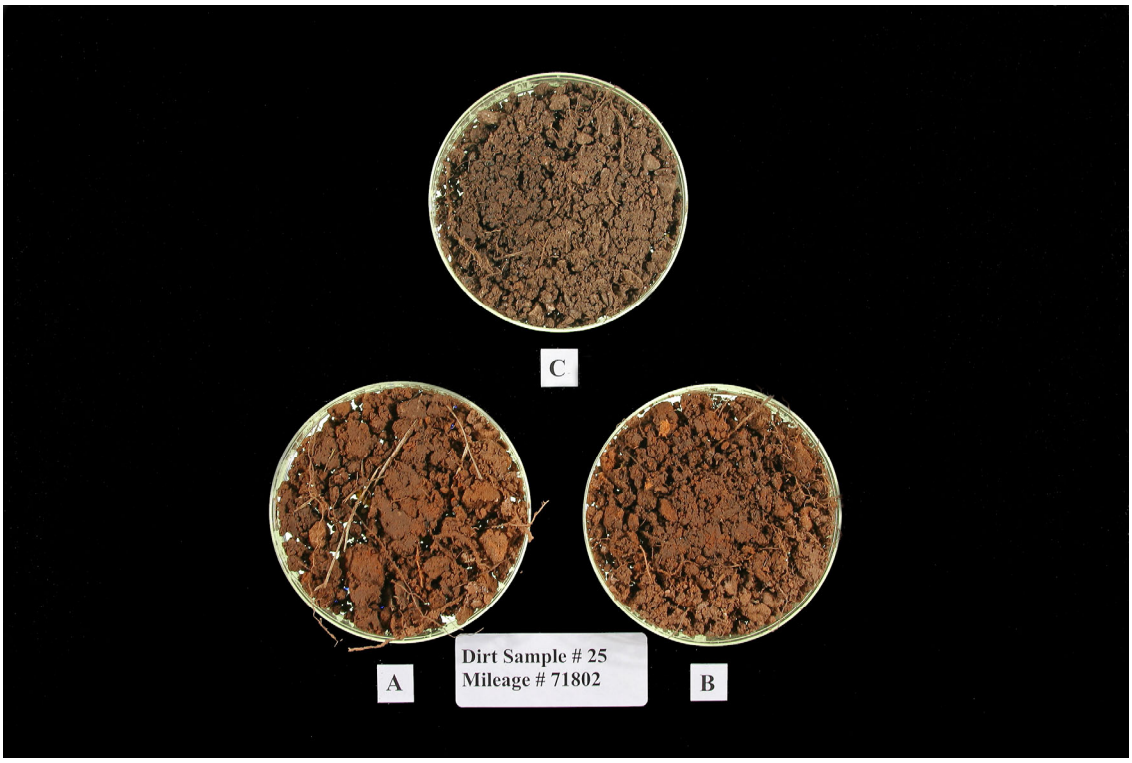
Site 23 and Soil Samples.



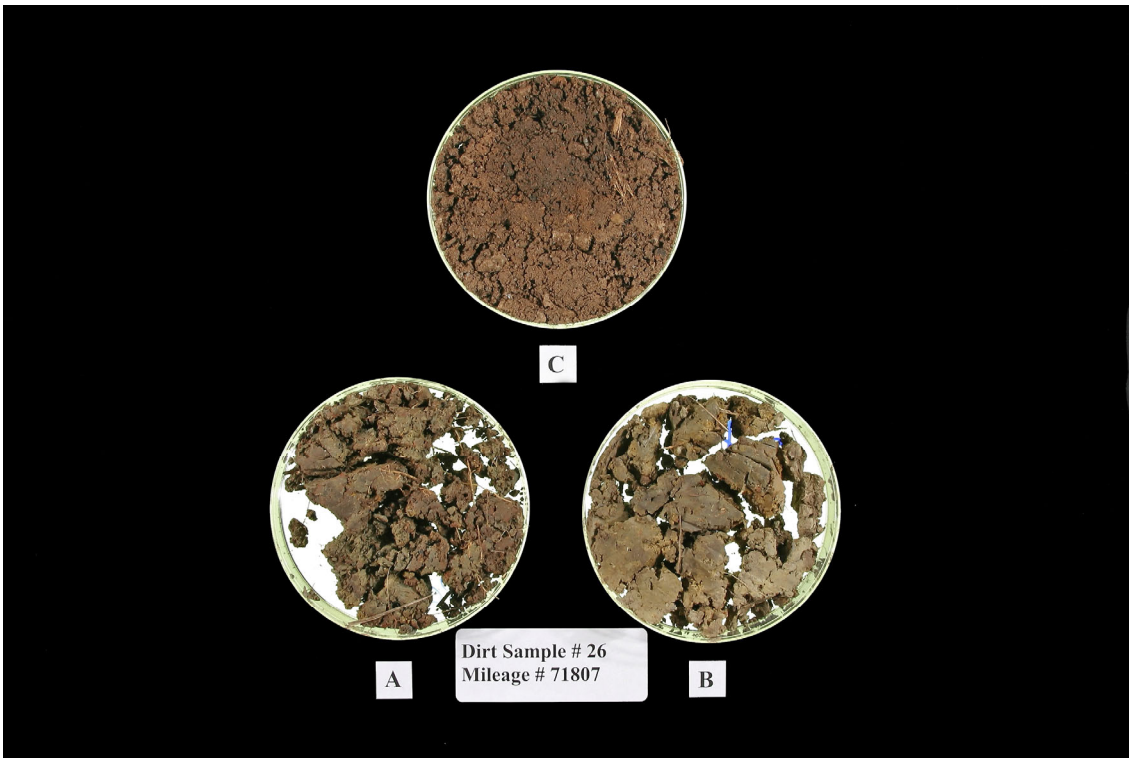
Site 24 and Soil Samples.



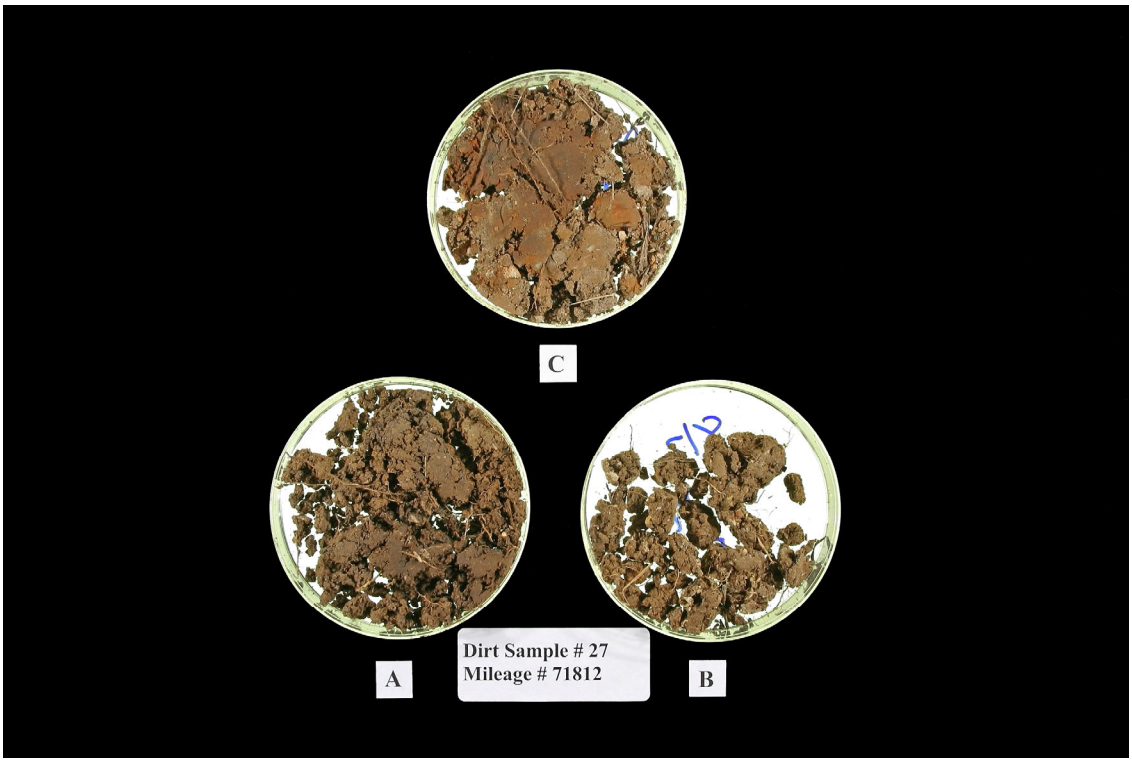
Site 25 and Soil Samples.



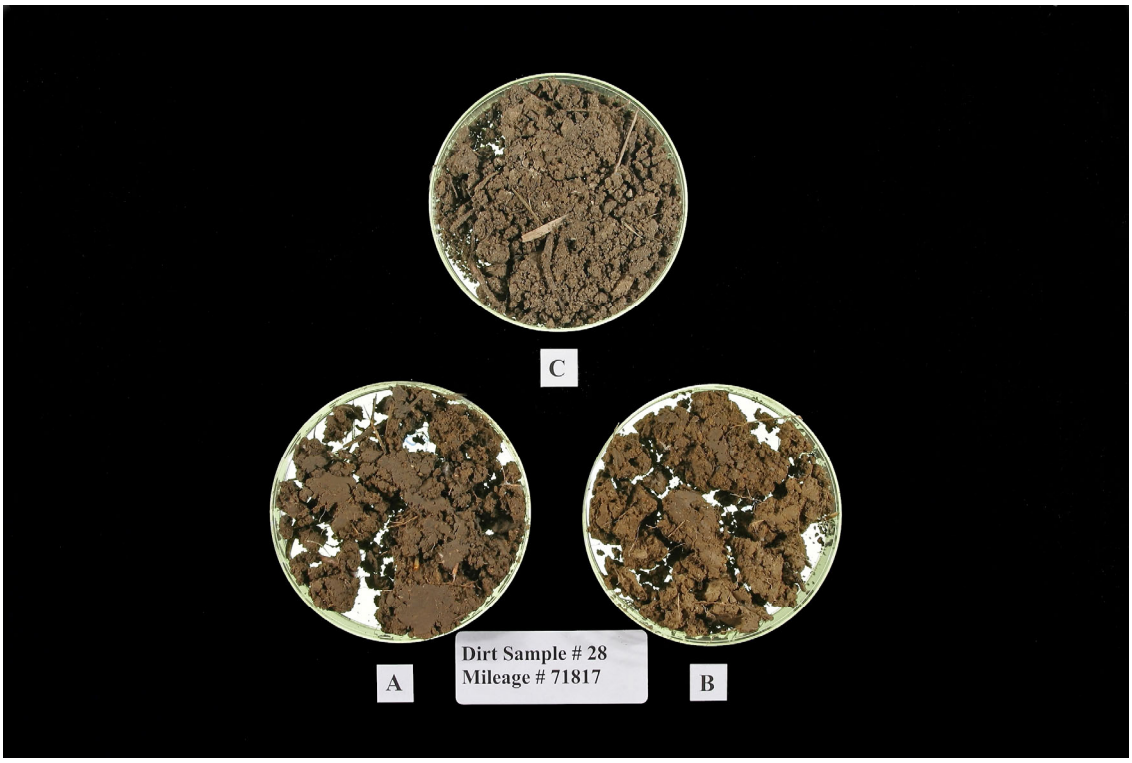
Site 26 and Soil Samples.



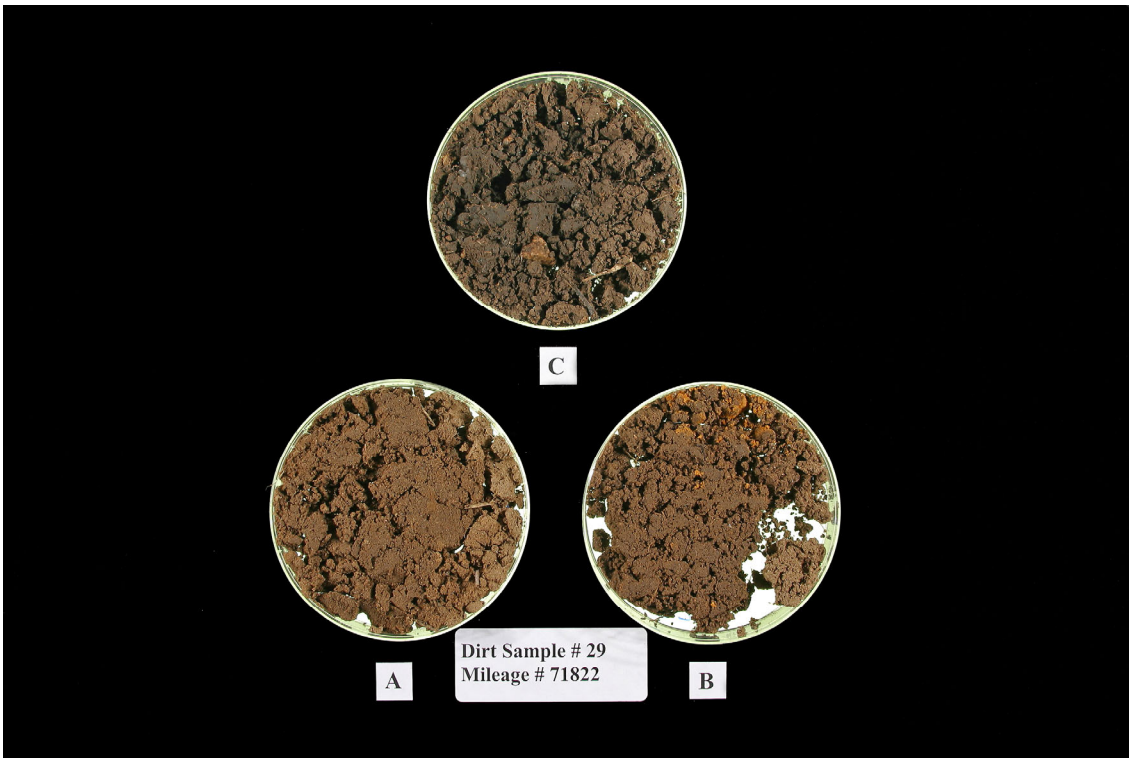
Site 27 and Soil Samples.



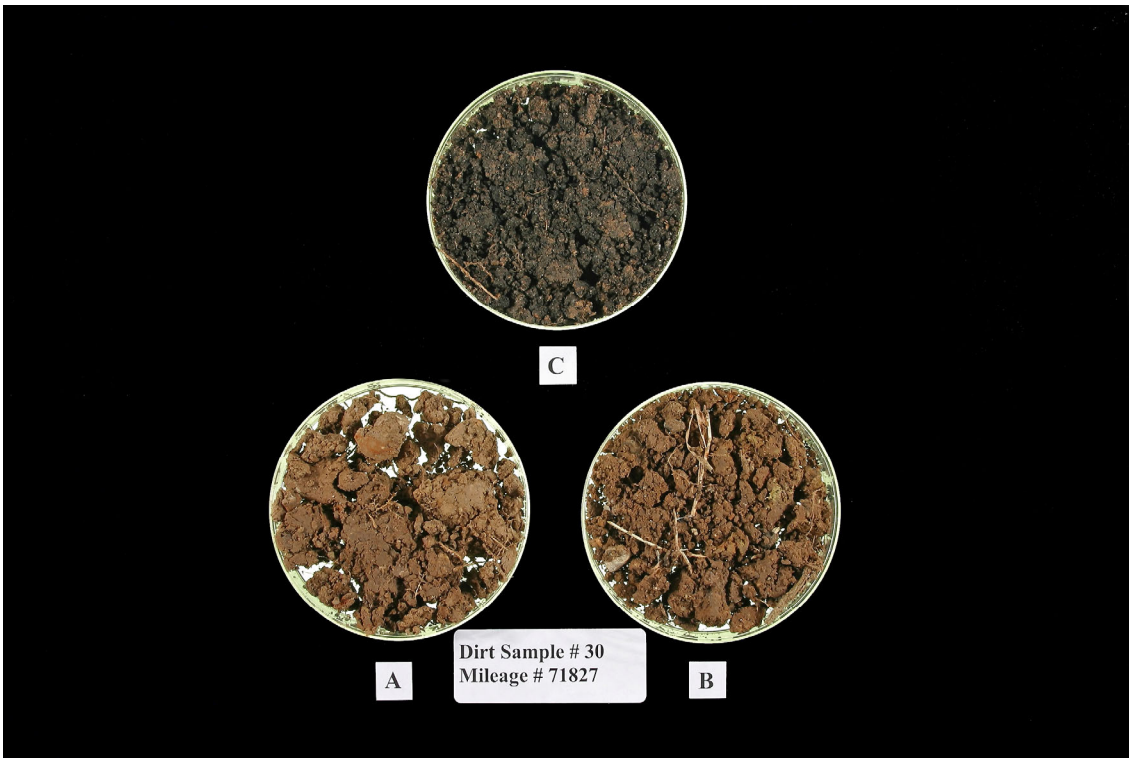
Site 28 and Soil Samples.



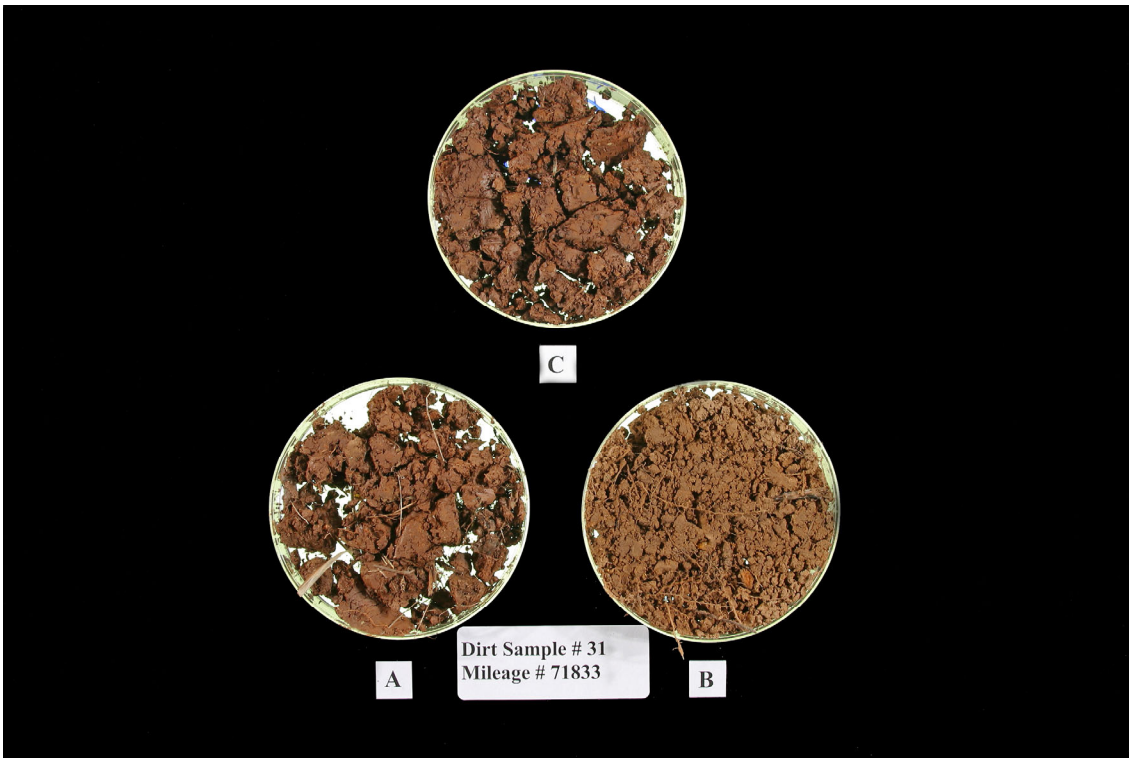
Site 29 and Soil Samples.



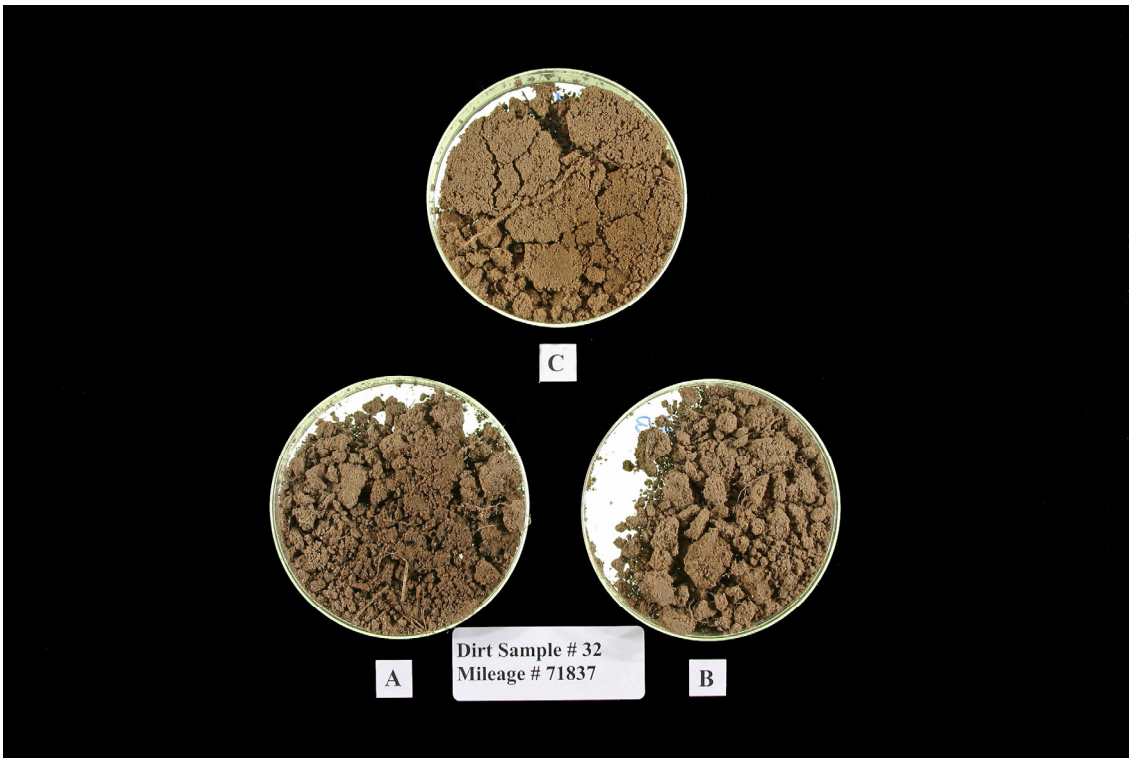
Site 30 and Soil Samples.



Site 31 and Soil Samples.



Site 32 and Soil Samples.



APPENDIX D – CALCULATIONS

Spore Preparation for Arnold
1 colony resulted at a dilution of 10^{-10}

1g of spore into 10ml = $\frac{10^{-3}\text{grams}}{1\text{ml}}$

Arnold spore powder weighed 1.615 grams

$\frac{\text{10-10 grams/ml}}{\text{1 colony}} = \frac{\text{1 gram/ml}}{\text{x colonies}}$

$x = \frac{1}{10^{-10}} = \frac{10^{-10}\text{ CFU}}{\text{1 gram}}$

Spray Preparation
Theory

Spray weight = 0.148 grams density of H_20 = $\frac{\text{1 gram}}{\text{ml}}$

70ml spray bottle $\frac{\text{70ml}}{0.148} = 472.973\ \frac{\text{sprays}}{\text{bottle}}$

Infectious rate = $\frac{10^6\text{CFU}}{\text{Spray}}$ $\frac{10^6\text{CFU}}{0.148\text{ ml}} = \frac{\text{CFU}}{\text{70ml}} = \sim 0.05$ grams

Experiment
0.05 grams added to 70ml of sterile water in spray bottle

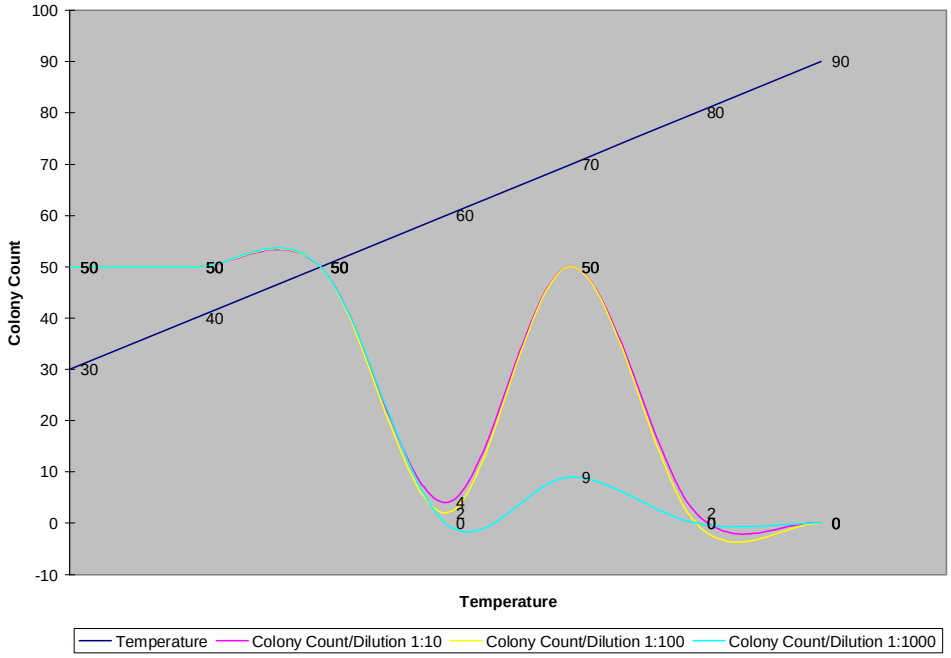
$0.05\text{grams} \times \frac{10^{10}\text{CFU}}{\text{1 gram}} \times \frac{1}{\text{70ml}} \times 0.148\text{ml} = \frac{1.06 \times 10^7\text{CFU}}{\text{1 spray}}$

Coffee Experiment
0.012 grams to make 1000ml of coffee
aliquot to 5 cups, ~ 200ml/cup

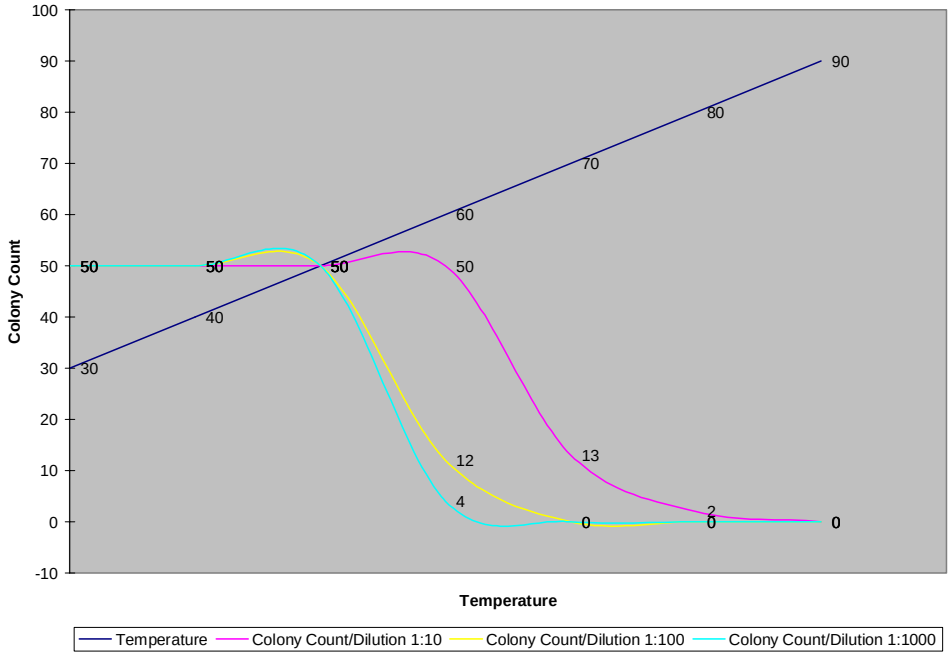
$\frac{0.012}{5} = \frac{0.0024\text{ grams}}{\text{cup}}$

$\frac{0.0024\text{ grams}}{\text{1 cup}} \times \frac{10^{10}\text{CFU}}{\text{gram}} = \frac{2.4 \times 10^7\text{CFU}}{\text{1 cup}} = \frac{2.4 \times 10^7}{200\text{ml}} = \frac{x\text{CFU}}{10\mu\text{l}} = 120\text{CFU}$

Killing Curve for Specimen 5A



Killing Curve for Specimen SF-19



VITA

Dina L. Miller

Candidate for the Degree of

Master of Science

Thesis: ISOLATION AND DEVELOPMENT OF A SURROGATE BIOAGENT FOR ANALYSIS OF DISEASE TRANSMISSION BY INANIMATE OBJECTS

Major Field: Forensic Science

Biographical:

Education: Graduated from Coweta High School, Coweta, Oklahoma in May 1981; received Associates of Radiological Science in 1986 from Bacone Junior College, Muskogee, Oklahoma. Received Bachelor of Science degree in Biology from Northeastern State University, Tahlequah, Oklahoma in May 2002. Will complete the requirements for the Master of Science degree with a major in forensic pathology at Oklahoma State University – Center for Health Science, Tulsa, Oklahoma in July, 2005.

Experience: Have worked as a radiological technologist for nineteen years. Employed at Saint Francis Hospital, Tulsa, Oklahoma for twelve years and currently employed at Jane Phillips Hospital, Bartlesville, Oklahoma for the past seven years. Performed internship in the spring of 2005 with the Oklahoma State Medical Examiner in Tulsa, Oklahoma. Participated in Sitlington Infectious Disease Symposium, April 2005. Biodefense, Bioterrorism, and Emerging Infectious Disease. Guest speaker for the Scientific Session of the Annual Meeting of the Association of GeneticTechnologist. June 2005 on the topic of Bioterrorism.

Professional Memberships: American Society of Radiologic Technologists.

ADVISER'S APPROVAL: Dr. Richard T. Glass

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