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DNA Degradation and Postmortem Interval: Preliminary Observations and Methods

Rebecca Roberts Anderson
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To the Graduate Council:

I am submitting herewith a thesis written by Rebecca Roberts Anderson entitled "DNA Degradation and Postmortem Interval: Preliminary Observations and Methods." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts, with a major in Anthropology.

Lee Meadows Jantz, Major Professor

We have read this thesis and recommend its acceptance:

Karla J. Matteson, Lyle W. Konigsberg

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Anne Mayhew_____
Vice Chancellor and
Dean of Graduate Studies

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DNA DEGRADATION AND POSTMORTEM INTERVAL:
PRELIMINARY OBSERVATIONS AND METHODS

A Thesis
Presented for the
Master of Arts
Degree
The University of Tennessee, Knoxville

Rebecca Roberts Anderson
August 2005

DEDICATION

This thesis is dedicated to my mother, Fran Anderson, who introduced me to a
world of books and the art of forensics and has never stopped encouraging me to
go after my dreams. I love you.

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iii

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ABSTRACT

As deoxyribonucleic acid (DNA) research advances, anthropologists are finding more ways to use this technology to their advantage. Establishing postmortem interval (PMI) is a primary goal of forensic anthropology. It is known that DNA degrades, or breaks down, after an organism dies. Although several researchers have studied DNA degradation, few have focused on DNA's rate of decay in relation to time. In this project, degradation was examined in blood using both a controlled atmosphere and exposure to environmental and substrate effects.

This study was intended to gather information on PMI, using DNA degradation as a measure of time. Based on the current literature and knowledge of the properties of DNA, it was hypothesized that DNA would decay in a time-dependent manner in a controlled atmosphere. In addition, it was hypothesized that DNA exposed to environmental and substrate effects would decay more rapidly than in a controlled environment.

It was found that DNA of all four fragment lengths, ranging from 110 to 782 base pairs (bp), survived for at least eight days in a controlled atmosphere. Samples exposed to environmental and substrate effects exhibited what might have been degradation, but a way to quantitate the amount of DNA present in each sample is needed. One way to achieve this goal is to use fluorescently labeled PCR products and compare the intensities of the PCR product across samples and time.

The efforts put forth in this project have lead to the development of a system that is likely to be useful in the analysis of degraded DNA. It is clear that DNA analysis will continue to be a tool anthropologists should and will use in the continual effort to determine PMI.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Introduction

Scientists continue to search for ways to more accurately establish postmortem interval (PMI). Anthropologists observe stages of decomposition, entomologists collect and study insects, and botanists examine plant life at the crime scene. All these fields have yielded ways to narrow the time frame to seasons, months, or weeks, but few can pinpoint PMI to hours or sometimes even days. A noted exception is the work of Vass et al. (2002), which can discern PMI accurately up to three weeks postmortem based on chemical reactions if tissue is still identifiable.

The Anthropological Research Facility was created to study PMI. There is much work to be done in the area of deoxyribonucleic acid (DNA) degradation, especially concerning the relationship of DNA degradation and PMI. If degradation can be studied in detail under laboratory conditions and a methodology developed for consistent analysis, then the process can be used on actual decomposed tissue, bones, or other organic material from the body.

A major question is whether degradation occurs at a particular rate; if it does, can this rate be determined using laboratory methods? Theoretically, this information can be used to establish PMI for an individual who has been dead a number of days or hours. This preliminary study concentrates on the degradation rate of DNA in blood under both fixed laboratory conditions and less controlled conditions outdoors. Blood was dried onto plastic trays, and DNA was extracted after specified time periods. Future testing of this methodology can be used on

1

actual tissue, bone, or other material that has decomposed at a known rate. The ultimate goal of this study is to develop a degradation timeline for DNA in blood.

DNA and Forensic Anthropology

DNA analysis typically falls under the umbrella of forensic science, but anthropologists are beginning to recognize the benefits of DNA research in their own field. While physical anthropologists generally employ DNA analysis in a variety of ways, this section will focus on the benefits and uses of DNA in forensic anthropology. There are three main ways in which DNA has been studied in a forensic anthropological context: identification, ancient DNA studies, and degradation studies (which will be discussed further in Chapter 2).

Identification

The level of accuracy at which DNA can identify humans has revolutionized science and the legal system. Nuclear DNA, that which is found in the nuclei of all cells, is unique to each individual, with the exception of identical twins. In cases when nuclear DNA is unable to be amplified, mitochondrial DNA (mtDNA) can often be used for comparison and identification purposes. Mitochondrial DNA lasts longer than nuclear DNA and exists in a much higher copy number. For example, Pfeiffer et al. (1999) found that when analyzing DNA from teeth exposed to soil for only six weeks, mtDNA PCR amplification was much more successful than nuclear DNA amplification. In addition, mtDNA is maternally inherited, whereas nuclear DNA is inherited from both parents

2

(Holland et al., 1993; Lewis, 2001; Kaestle and Horsburgh, 2002; Rudin and Inman, 2002).

DNA is useful in a forensic anthropological setting where a large number of victims are close in age and/or all the same sex, as is often the case with mass graves. These graves may not yield nuclear DNA, but mtDNA can often be extracted and amplified after extended periods of time. It should be noted that a recent study revealed that nuclear DNA can be recovered from remains submerged in water for three years (Crainic et al., 2002). Despite its longevity, mtDNA is useful only as corroborative evidence if the sequences of the comparative population do not exhibit enough variation to match relatives to remains (Boles et al., 1995). When dealing with only one set of remains, it is easier to make a positive identification, assuming living maternal relatives exist to provide reference mtDNA samples (Holland et al., 1993).

Ancient DNA

Ancient DNA (aDNA) allows researchers to study a number of issues, including population movements over time, the relationships of multiple populations, and collective behavior of a society. In some cases, it is possible to determine the sex of remains as well (Stone et al., 1996; Faerman et al., 1998; Schultes et al., 1999; Cunha et al., 2000; Kaestle and Horsburgh, 2002).

The analysis of aDNA began in 1984, when it was extracted from an extinct zebra relative, the quagga; aDNA was first extracted from a human one year later (Higuchi et al., 1984; Pääbo, 1985; Lindahl, 1993). Like forensic DNA

3

research, aDNA research greatly benefited from the invention of polymerase chain reaction (PCR) methods. Suddenly, it became possible for the miniscule amounts of DNA that were recovered to be amplified multitudes of times (Mullis and Faloona, 1987; Saiki et al., 1988). Contrary to previous research, studies now indicate that DNA survives for approximately 130,000 years (Loreille et al., 2001; Kaestle and Horsburgh, 2002).

Nuclear and mitochondrial DNA offer different advantages to researchers. When nuclear DNA can be recovered from sex chromosomes, the sex of individuals can be identified; pedigrees and inherited disorders can also be studied (Faerman et al., 1998; Schultes et al., 1999; Cunha et al., 2000). Stone et al. (1996) developed a way to sex skeletons using a gene located on the sex chromosomes called amelogenin; this procedure correctly identified sex in a sample of 20 contemporary known individuals and 19 (of 20) ancient individuals (these were sexed osteologically). Mitochondrial DNA can differ by populations due to its ability to mutate quickly, and this quality allows scientists to study the movements of populations over time (Kaestle and Horsburgh, 2002).

Scientists' isolation of aDNA from bacteria and viruses sheds light on diseases suffered by past populations (Kaestle and Horsburgh, 2002). An example of ancient bacterial DNA analysis is the Mays et al. (2002) paper entitled "Investigation of the Link Between Visceral Surface Rib Lesions and Tuberculosis in Medieval Skeletal Series From England Using Ancient DNA." As the title suggests, the researchers attempted to find DNA from tuberculosis bacteria in the rib lesions of skeletons from medieval England. Although they

4

were unable to find any *Mycobacterium tuberculosis* DNA, the implications of this study are important. The lack of tuberculosis bacteria DNA suggests that rib lesions in skeletons of this time period should not necessarily be associated with the tuberculosis disease.

As always when dealing with human remains or cultural artifacts, ethical issues are a primary consideration for anthropologists; this same idea holds true for aDNA analysis. One major problem when dealing with aDNA is the necessary destruction of material. Tests should only be performed with good reason and when there is relative certainty of obtaining DNA. A portion of material should be preserved for upcoming consideration or reanalysis as well (Kaestle and Horsburgh, 2002).

Is a deceased individual able to give permission for examination of his or her remains? Individuals who died within the past several years can give this permission before death, but it is difficult to make choices regarding individuals who died longer ago. One view is that "this type of study can be done 'without seeking the consent of the dead person's descendants of his present-day culturally affiliated cultural community' in most cases" (Holm (2001) in Kaestle and Horsburgh, 2002:107).

Another ethical consideration of aDNA studies is their effects on populations today. Kaestle and Horsburgh (2002) mention the petitioning of Native Americans for more land and governmental recognition based on ancestral DNA. Repatriation is another avenue for aDNA testing wherein native people can request rights to skeletons and cultural artifacts based on genetic information.

When interacting with living groups, scientists need to be careful and culturally aware of each group with whom they communicate. Researchers should be culturally sensitive to the groups whose history they are interpreting.

Chapter 2: DNA, PCR, and Degradation

The DNA molecule is often described as a double helix. It has a sugar-phosphate backbone, with interior rungs consisting of complimentary nitrogenous bases. These bases (adenine, guanine, thymine, and cytosine) attach to one another via hydrogen bonds. In the DNA molecule, adenine always pairs with thymine, while guanine matches up with cytosine (Lindahl, 1993; Parsons and Weedn, 1996; Malacinski and Freifelder, 1998; Rudin and Inman, 2002).

Degradation of DNA begins directly after death, when the body's natural DNA repair mechanisms are halted (Parsons and Weedn, 1996). The breakdown process is influenced by several environmental factors, including heat, light, and humidity; this natural variation is greater than that of the degradation between individual samples (Perry et al., 1988; Arismendi et al., 2004; Bender et al., 2004; Schneider et al., 2004).

There are three main ways in which the DNA molecule degrades in a living person: hydrolysis, oxidation, and methylation. In the process of hydrolysis, purines (guanine and adenine) are freed from the molecule with the addition of a molecule of water, often as a result of an acidic environment. Excess enzymes that typically repair the DNA can cause damage during oxidation; these enzymes are produced by bacteria in response to exposure to oxygen. Extra methyl groups can be added to DNA through methylation, thus causing more code errors. Natural repair processes exist in the body to handle all these mechanisms. After death, "it can be predicted that deprived of the repair mechanisms provided in living cells, fully hydrated DNA is spontaneously

7

degraded to short fragments over a time period of several thousand years at moderate temperatures" (Lindahl, 1993:713).

PCR is a process whereby small amounts of DNA can be amplified exponentially and analyzed thereafter; Figure 1 demonstrates this process. Primers (small artificial pieces of single-stranded DNA) are designed to produce a specific section of DNA; nucleotides, buffer, water, and Taq polymerase enzyme complete the PCR mixture. The Taq polymerase is taken from underwater bacteria that can withstand very high temperatures, as the heating cycle of PCR does not denature it (Mullis and Faloona, 1987; Saiki et al., 1988; Rudin and Inman, 2002).

The sample is cycled through a process of heating and cooling. Heat denatures DNA and causes the double strand to break apart; the sample is then cooled to a calculated temperature specific to the primers being used. This allows the primers to anneal to their target sites and begin replication. Finally, the temperature is raised slightly so that extension can begin (Mullis and Faloona, 1987; Saiki et al., 1988; Parsons and Weedn, 1996; Rudin and Inman, 2002). The cycle is repeated a number of times (in this study, 25), and an amplicon is created.

Degradation Studies

Over the years, a number of degradation studies have been performed with varying success. The continual development of better molecular techniques allows scientists to improve upon past results. In 1988, Perry et al. examined

8

Plate 5   PCR — round 1.
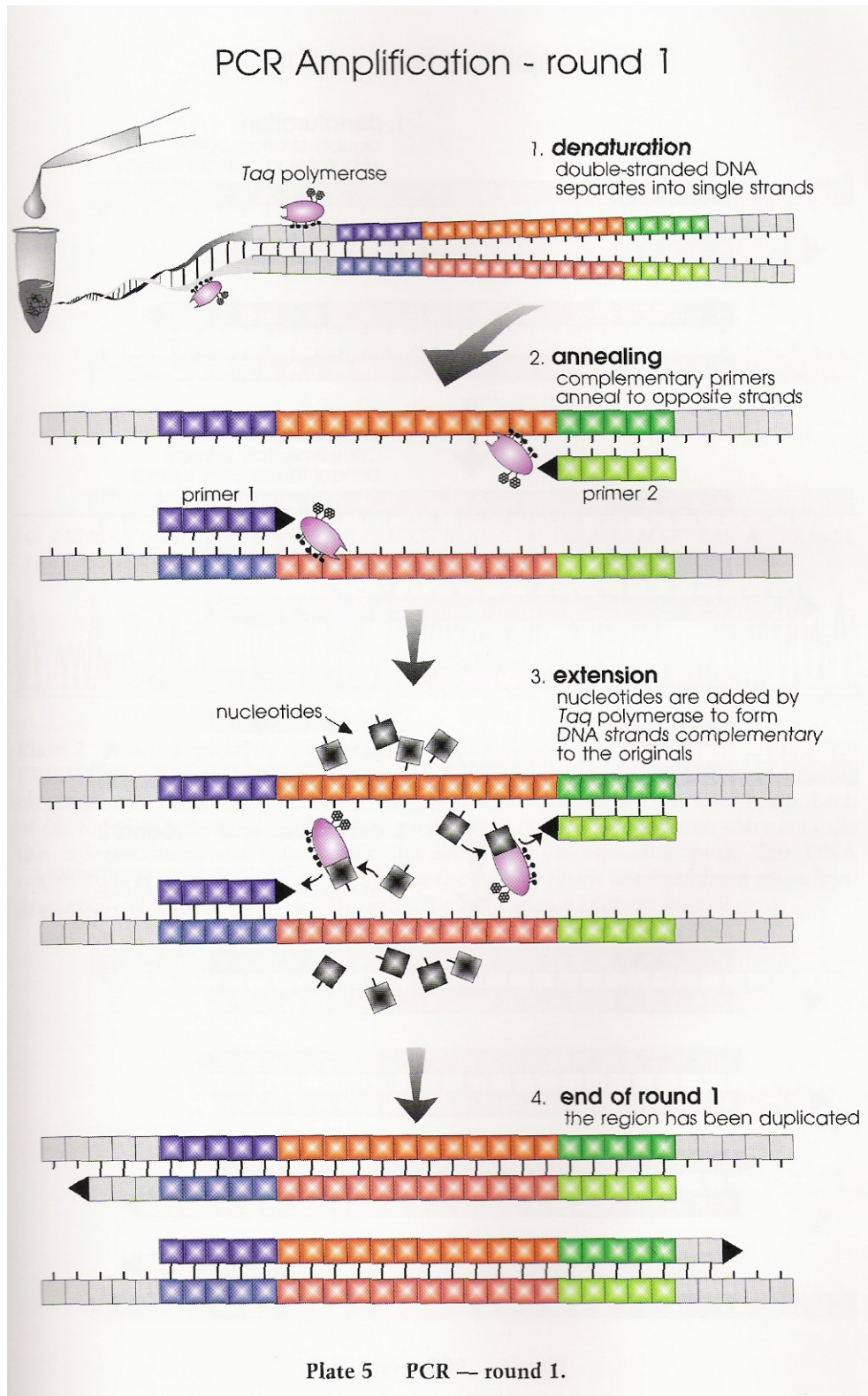
Figure 1. PCR Amplification- round 1 (Rudin and Inman, 2002: Plate 5)

9

DNA degradation in human rib bone with the goal of establishing PMI. Rib samples were sterilized, and DNA was extracted after the ribs were exposed to either high or low humidity in the laboratory at room temperature for a specified number of weeks. Autoradiograms were used to quantitate DNA after electrophoresis and Southern blot procedures. It appeared that the variation between samples of different individuals was less than variation caused by natural factors such as humidity.

The stability of DNA after death is variable in different body organs. According to Bär et al. (1988:68-69), the best stability after the longest period of time (3 weeks) occurs in the "brain cortex, lymph nodes and psoas muscle." Using minisatellites, it was found that DNA retained its structure in the spleen and kidney for five days before badly degrading. The blood results were inconsistent; clots yielded large amounts of DNA. It was found that the postmortem stability did seem associated with length of PMI, but there were exceptions wherein the DNA degraded very quickly after death. Evidently, "elevated environmental temperature at the site of death and/or infectious diseases were the main factors for the occurrence of disproportionate autolysis" (Bär et al., 1988:69).

The feasibility of DNA fingerprinting after various PMIs was studied in 1993. At this point in time, DNA fingerprinting required "high molecular weight DNA (HMWDNA)" (Ludes et al., 1993:686). This clearly limited the length of PMIs in testing, and the authors noted that DNA in blood and kidney tissue degraded after one week (so DNA fingerprinting was not practical). According to

10

the authors, fingerprinting was possible using DNA obtained from the cortex of the brain after 85 days.

Microsatellites, also known as short tandem repeats (STRs), are short stretches (two to five base pairs) of DNA that vary in number of repetitions from person to person (Rudin and Inman, 2002). In one study that utilized STRs from degraded samples, semen was found to be the best material from which to extract DNA (Cotton et al., 2000). Hoff-Olsen et al. (2001) questioned whether mutations in STRs occurred during decomposition. Among the samples taken from deceased individuals with no evidence of decomposition at death, the STRs appeared stable. The same results were achieved when testing samples from decomposed individuals, so it appears that STRs are appropriate for identification purposes even when tissue is heavily decomposed.

At the time of the Johnson and Ferris (2002) study, the best approximation of PMI only came within an eight-hour interval. They used single-cell gel electrophoresis, or the comet assay, to reveal a definitive DNA degradation pattern that is "organ and time-dependent" (Johnson and Ferris, 2002:46). This research demonstrated the breakdown of DNA into fragments using human blood cells. Pig tissues were used to demonstrate DNA behavior over a longer period of time. Nuclear DNA degradation intensified as PMI grew from 3-56 hours.

Degraded DNA has also been studied for the purpose of improving STR methodology. Genomic DNA was treated with sonication and DNase I to produce standardized fragments between 200 and 300 base pairs (bps) long (Bender et al., 2004). This artificial DNA was used in 17 European countries to

11

test various STR kits. The testing produced many questions, as the results were based on three factors: "correct allele detection, peak height and balance as well as the occurrence of artefacts" (Schneider et al., 2004:124). Problems and potential strategies for improved testing were detected. However, these studies did not address degradation in relation to time.

12

Chapter 3: Materials and Methods

The 11th exon of the breast cancer gene BRCA1 was used for this project because its sequence is long and well-known. In humans, this gene prevents tumors in breast cancer cells (NCBI, 2005).

Primer Design

DNA fragments of particular length can be produced using defined primer sets. For this project, primers were designed to create fragments of three lengths: 110 bp, 490 bp, and 782 bp. Table 1 lists the primer sequences and their corresponding fragment sizes. These PCR reactions were designed as a nested set, which means that all the fragments have a common beginning, and only the endings vary. Hemachromatosis primers HEMO 3 (H3) and HEMO 4 (H4) were used to create the 250 bp fragment. It should be noted that H3 and H4 are designed to adhere to a portion of the HLA-H gene, which is associated with the major histocompatability complex (Feder et al. 1996). This is a different gene and locus than the sequences highlighted by the other primer sets only because we were unable to create a 250 bp fragment on BRCA1. These lengths were selected in order that degradation might be observed over time. It was assumed that the 110 and 250 bp lengths would survive all lengths of time tested; however, it was thought that the 490 and 782 bp lengths would disappear over a relatively short time as the DNA degraded.

13

Table 1: Primer Sequences

Name	Length	Sequence
Forensic 3 & Forensic 4	110 bp	F3: 5' AACTTCCATGAATTCTAGTCC 3' F4: 5' CTGGAGGTTCAAAAATCTCA 3'
Forensic 3 & Forensic 8-3	490 bp	F8-3: 5' CTCTGCTGTGGAAGAATTGAG 3'
Forensic 3 & Forensic 5-2	782 bp	F5-2: 5' AGGGCCTCATTCTGGAAGGA 3'
HEMO 3 & HEMO 4	250 bp	H3: 5' ACATGGTTAAGGCCTGTTGC 3' H4: 5' GCCACATCTGGCTTGAAATT 3'

14

Sample Preparation and Extraction

The sample material in this study was blood taken from one of the investigator's arms. All preparation of new samples began with freshly drawn blood. On Day 1, blood was drawn, and 250 microliters of blood was pipetted onto a small plastic tray. A disposable plastic pipette was used to spread the blood on the plastic so it would dry more quickly. The plastic tray was then placed on a shelf in a 37 degree Celsius incubator for a specified amount of time. Nine identical trays were prepared at the same time in this manner.

After dry sample preparation, two samples were immediately prepared from the fresh blood for use as controls. There are multiple ways to extract DNA from organic substances (Hoff-Olsen et al., 1999). In this project, the Generation DNA Purification Capture Column Kit was used to prepare the DNA (Gentra Systems, Minneapolis, MN). Two hundred microliters of blood was placed on a purification matrix and purified per instructions from the kit. After blood was bound in the matrix, the sample was washed to remove proteins, heme, and RNA, which can inhibit PCR reactions. The sample was washed with an elution solution to further clean the DNA. All samples were labeled with the date and sample number before placement in a –80 degree Celsius freezer.

Three dried samples were removed from the 37 degree Celsius incubator after each time period; one day, five days, and eight days. The samples were rehydrated using 250 microliters of 0.9% sodium chloride (NaCl) solution. The 0.9% NaCl was pipetted onto each dried sample and then mixed for approximately one minute with a disposable plastic pipette. From each plate, 200

15

microliters were pipetted onto the matrix for purification as described above. Once prepared, these samples were labeled with the date and sample number and placed in a –80 degree Celsius freezer.

PCR and Gel Electrophoresis

Each PCR reaction consisted of one unit of Taq polymerase, 1 microliter of each 10 mM nucleotide (dATP, dCTP, dGTP, dTTP), 50 pmol of each primer, 1 microliter of DNA, 1X buffer (containing 50 mM KCl, 1.5 mM MgCl$_2$, and 10 mM Tris-HCl), and water in a final volume of 25 microliters. The method of preparing reactions developed in stages. Initially, one master mix, containing water, buffer, nucleotides, and Taq polymerase was prepared and split into individual aliquots, and the appropriate DNA and primers were then added to these aliquots. When this method appeared to lack consistent results, likely due to the small amounts of DNA and primers that had to be pipetted, the system was changed. In the second version, one master mix was made for each primer set containing water, buffer, nucleotides, Taq polymerase, and primers. These mixtures were split into individual aliquots (24 microliters each), and one microliter of DNA was added to each tube. While this method improved consistency, it was still not possible to get consistent results repeatedly.

Therefore, a third procedure was developed as follows: a large single master mix containing polymerase, nucleotides, buffer, and water was split into four sub master mixes, one for each primer set. Primers were added to each of these four sub master mixes as follows: F3/F4 (110 bp), H3/H4 (250 bp), F3/8-3

16

(490 bp), and F3/5-2 (782 bp). Finally, each of these four mixes was split into five aliquots (one for each of 3 DNA samples, one negative control water blank, and one positive control DNA sample). DNA was added as the final step to the mixture preparation. Using the finalized methodology, each PCR run tested only one DNA (i.e. Day 1 Sample 1, Day 5 Sample 1, and Day 8 Sample 1 were tested together with all four primer sets).

The negative control (water blank) contained all the aliquot ingredients, and water was substituted for DNA. This practice ensured that reagents were not contaminated. The positive control consisted of DNA prepared the same day blood was drawn (as described above). This positive control should always yield DNA product if the PCR is prepared and run properly.

As previously mentioned, PCR consists of a repeated cycle of various temperatures. For this project, the process began with a five-minute heating period at 94 degrees Celsius to denature genomic DNA. The cycle consisted of one minute at 94 degrees Celsius (denaturation) one minute at 50 degrees Celsius (annealing), and two minutes at 72 degrees Celsius (extension) (Lewis, 2001; Rudin and Inman, 2002). This cycle was repeated 25 times, and a seven-minute step at 72 degrees Celsius finished the PCR. The PCR products were held at 4 degrees Celsius in the machine until they could be stored in a refrigerator at 4 degrees Celsius.

After PCR, the samples were analyzed using polyacrylamide gel electrophoresis. DNA is a negatively charged molecule and travels toward the positive charge established by an electric current. Smaller fragments travel faster

17

and appear closer to the bottom of the gel (Rudin and Inman, 2002). A 100-bp marker was used to identify the size of the fragments (Promega, Madison, WI). The gels were stained with ethidium bromide and photographed on an ultraviolet light box. Positive results were shown by the existence of one band per lane, corresponding to the appropriate place on the marker.

Anthropological Research Facility Samples

It became clear that the laboratory samples, while yielding useful information, were not exhibiting degradation as quickly as expected. In order to observed degradation in a more natural state, it was decided that blood samples would be placed at the Anthropological Research Facility (ARF); exposure to sunlight, varying temperatures, and moisture have been shown to affect degradation (Perry et al., 1988; Arismendi et al., 2004; Bender et al., 2004, Schneider et al., 2004). Rather than only experimenting with degradation of blood on the plastic trays utilized in the lab, three other materials were also included in this portion of the study.

On Day 1, eight samples were placed at the ARF; eight plastic trays, identical to those used in the laboratory experiments, were housed in a plastic Tupperware box. The materials tested were gravel (gathered outdoors in the hospital parking lot), plastic (the trays themselves), metal (can openers), and cloth (4 cm by 4 cm centimeter portions of a white cotton towel); two samples of each type were prepared. The gravel and metal were washed, and two 4 cm by 4 cm squares of cloth were cut. The gravel, metal, and cloth were placed on six of the

18

individual plastic trays, while the remaining two served as plastic controls; there were two trays of each sample material. Then, 250 microliters of blood were pipetted onto each material, and disposable plastic pipettes were used to spread the blood on the plastic and metal. Blood absorbed into the cloth, so spreading had little effect and was not attempted on the gravel samples. Four holes were drilled into the Tupperware box for air circulation, and a plastic lid covered the samples. The box was clear plastic, so as to expose the samples to sunlight.

Half an hour after the samples were prepared, the box was placed on a tall tree stump at the ARF. After three days, four of the samples (one of each type) were brought to the lab and prepared according to the Gentra Systems protocol mentioned previously. The metal sample was rehydrated using 250 microliters of 0.9 % NaCl solution, and approximately 200 microliters of rehydrated sample were placed on the matrix. The plastic sample was also rehydrated with 250 microliters of 0.9 % NaCl. The sample containing gravel as part of the matrix required additional liquid in order to produce enough free liquid to remove an adequate amount of DNA for isolation. The sample dried on cloth required approximately 2000 microliters of 0.9% NaCl before enough liquid could be removed.

 The remaining samples were gathered from the ARF after eight days and prepared in the same way.

19

Chapter 4: Results

The controlled atmosphere samples were kept in a 37 degree Celsius incubator with no added humidity, no light, and room air. After one, five, and eight days, these samples were removed from the incubator, and DNA was extracted; three trays were removed each day to allow for replicate analysis. At the beginning of the project, one master mix containing water, buffer, Taq polymerase, and nucleotides was divided into individual aliquots, and DNA and primers were added to each aliquot. Inconsistent results proved to be a major obstacle, and weeks of notes revealed that the DNA samples were apparently degrading after two weeks in a -20 degree Celsius freezer. This problem was apparently due to the DNA extraction procedures, which were not intended to purify the DNA extensively; it was solved by moving the DNA samples to a -80 degree Celsius freezer for the remainder of the study.

Initially, it was thought that the larger DNA fragments were exhibiting degradation after eight days in the controlled atmosphere, judging by the disappearance of bands on the gels. It later became clear through repeated testing that the longer fragments were lasting at least eight days, but inconsistency was still a problem. Because of this difficulty, a second master mix system was devised. Four master mixes were made, one for each primer set, and split into individual aliquots; DNA was then added to each aliquot. This change in the master mix was done to reduce variation, but repeated trials still showed marked inconsistency, i.e., replicate samples from identical DNAs did not produce the

20

same results. Additionally, different DNA samples treated identically did not yield the same results in repeated tests (see Table 2).

The final change in the master mix system yielded consistent results. A master mix containing water, buffer, Taq polymerase, and nucleotides was split into four sub master mixes, one for each primer set. Primers were added, and the sub master mixes were split into five aliquots each (one for each DNA sample, a water blank, and a positive control). The only variation between these samples was in the pipetting of one microliter of DNA into each aliquot. Table 3 contains the same information as Table 2, but only includes the PCR results based on this system. Of all the PCR runs listed in Table 3, only two positive controls failed, and these occurred when the PCR machine malfunctioned.

DNA of all four lengths (110, 250, 490, and 782 bp) survived for eight days in the controlled atmosphere. Figures 2 through 4 show the fragments of each length of DNA after one, five, and eight days. The lane labeled "M" is a 100-bp marker (Promega, Madison, WI); each band marks base pair lengths from 100-1000 in increments of 100 bp. The brighter band in the middle of the ladder is equivalent to 500 bp. If DNA is present, it will appear as a band in the lane, corresponding to the correct position on the marker.

The ARF samples were exposed to ultraviolet light, humidity, and cool temperatures, as well as different substrates. The plastic samples served as controls in this portion of the research. As previously mentioned, an ARF sample

21

Table 2: PCR Results of Controlled Atmosphere Samples

| Sample Number-Length	Day 1		Day 5		Day 8	
	Attempts	Successes	Attempts	Successes	Attempts	Successes
1-110	6	4	6	6	6	3
1-250	5	5	5	4	5	5
1-490	4	2	4	4	4	1
1-782	6	4	6	3	6	5
2-110	5	5	5	4	5	2
2-250	5	5	5	2	5	5
2-490	5	4	5	1	5	1
2-782	6	6	6	3	6	2
3-110	5	5	5	2	5	5
3-250	5	5	5	2	5	5
3-490	5	4	5	2	5	5
3-782	6	4	6	4	6	3

Note: DNA 1-490 has one less trial and DNA 1-110 has one extra because during one trial, 110 bp primers were erroneously added to the 490 bp aliquot.

Table 3: PCR Results of Controlled Atmosphere Samples Using the Third Master Mix

Sample Number-Length	Day 1		Day 5		Day 8	
	Attempts	Successes	Attempts	Successes	Attempts	Successes
1-110	3	3	3	3	3	3
1-250	1	1	1	1	1	1
1-782	3	3	3	2	3	2
2-110	2	2	2	2	2	2
2-250	1	0	1	0	1	1
2-490	1	0	1	0	1	0
2-782	3	2	3	2	3	1
3-110	2	2	2	2	2	2
3-250	1	1	1	1	1	1
3-490	1	1	1	1	1	1
3-782	3	2	3	2	3	2

Notes: The PCR machine malfunctioned during one run of DNA 2, most likely altering results. One run involved fluorescently labeled primers, which yielded PCR product with all the 110 bp fragments, but only one of the 782 bp fragments.

22

1 M A B C D E F G H
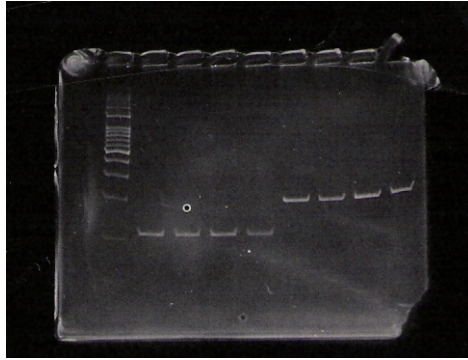
Figure 2. 110 and 250 bp lengths of DNA 3

1:	**110 bp water blank**
M:	**100 bp marker**
A:	**110 bp Day 0 positive control**
B:	**110 bp Day 1**
C:	**110 bp Day 5**
D:	**110 bp Day 8**
E:	**250 bp Day 0 positive control**
F:	**250 bp Day 1**
G:	**250 bp Day 5**
H:	**250 bp Day 8**

23

2 3 M J K L N
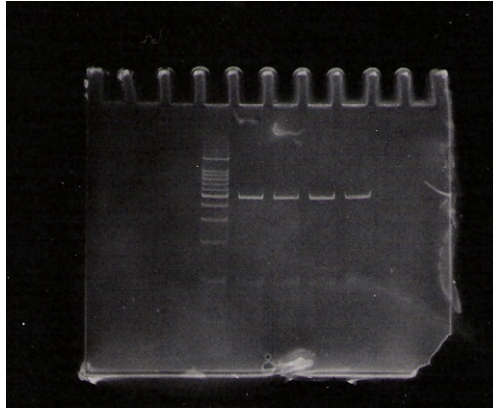
Figure 3. 490 bp lengths of DNA 3

2:   **250 bp water blank**
3:   **490 bp water blank**
M: **100 bp marker**
J: **490 bp Day 0 positive control**
K: **490 bp Day 1**
L: **490 bp Day 5**
N: **490 bp Day 8**

4 M O P Q R
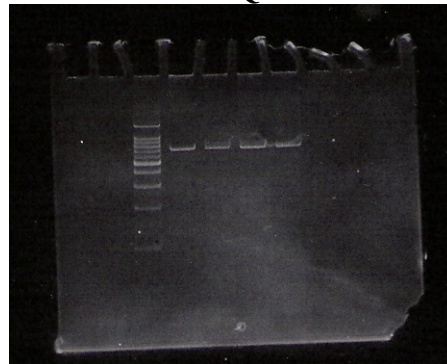
Figure 4. 782 bp lengths of DNA 3

4:   **782 bp water blank**
M: **100 bp marker**
O: **782 bp Day 0 positive control**
P: **782 bp Day 1**
Q: **782 bp Day 5**
**R:**   **782 bp Day 8**

of each type of substrate was analyzed after three and eight days, using the method developed with the controlled atmosphere samples (see Table 4). Figures 5 and 6 show that both 110 and 782 bp fragments survived on the metal and plastic substrates after eight days. The bands in the photographs indicate that degradation may be taking place over time; the 782 bp band is markedly lighter after eight days for both the metal and plastic samples. The gravel and cloth samples did not yield any PCR products (see Figures 7 and 8). This is most likely due to the extraction process; larger amounts of 0.9% NaCl were used to rehydrate these samples than were used with the plastic and metal samples, therefore any DNA that was isolated was diluted.

In the absence of complete loss of bands on the gels, methods to interpret the apparent decrease of the 782 bp fragments were devised. We proposed to examine the ratio of intensity between 782 and 110 bp bands. In this way, any small variation in the amount of DNA pipetted into the samples would be negated, as the ratios of the signals will be compared, not actual amounts. Production of DNA fragments using fluorescently labeled primers and capillary fragment separation was attempted. Additional work is required to make this method operable, but preliminary results suggest that it will work.

25

Table 4: PCR Results of Anthropological Research Facility Samples

Sample Type-Length	Day 3		Day 8	
	Attempts	**Successes**	**Attempts**	**Successes**
Plastic-110	2	2	2	2
Plastic-782	2	2	2	2
Metal-110	2	2	2	2
Metal-782	2	1	2	1
Cloth-110	2	0	2	0
Cloth-782	2	0	2	0
Gravel-110	2	2*	2	0
Gravel-782	2	0	2	0

Note: The * refers to a band on the gel that is extremely faint. Fragment analysis would determine if any DNA is actually present.

1 M A B C D E
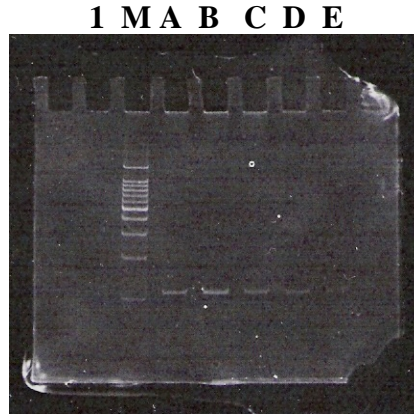
**Figure 5. 110 bp fragments from metal and plastic samples
at the Anthropological Research Facility**

1:	**110 bp water blank**
M:	**100 bp marker**
A:	**110 bp Day 0 positive control**
B:	**110 bp Day 3 Plastic**
C:	**110 bp Day 8 Plastic**
D:	**110 bp Day 3 Metal**
E:	**110 bp Day 8 Metal**

2 M F G H J K
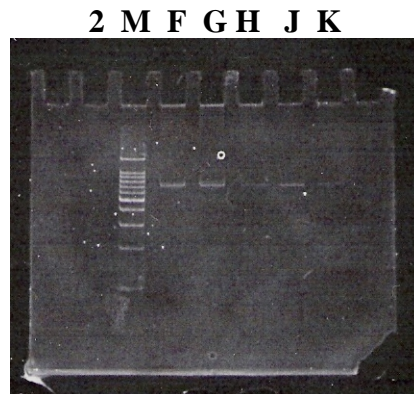
**Figure 6. 782 bp fragments from metal and plastic samples
at the Anthropological Research Facility**

2:	**782 bp water blank**
M:	**100 bp marker**
F:	**782 bp Day 0 positive control**
G:	**782 bp Day 3 Plastic**
H:	**782 bp Day 8 Plastic**
J:	**782 bp Day 3 Metal**
K:	**782 bp Day 8 Metal**

27

1 M A B C D E
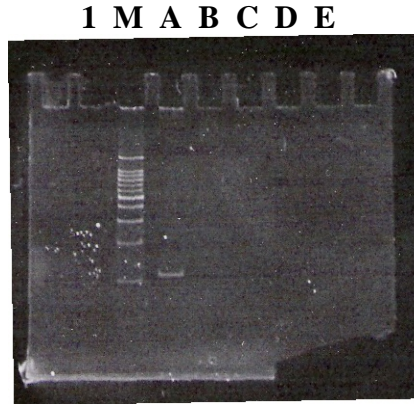
**Figure 7. 110 bp fragments from cloth and gravel samples
at the Anthropological Research Facility**

1:	**110 bp water blank**
M:	**100 bp marker**
A:	**110 bp Day 0 positive control**
B:	**110 bp Day 3 Cloth**
C:	**110 bp Day 8 Cloth**
D:	**110 bp Day 3 Gravel**
E:	**110 bp Day 8 Gravel**

2 M F G H J K
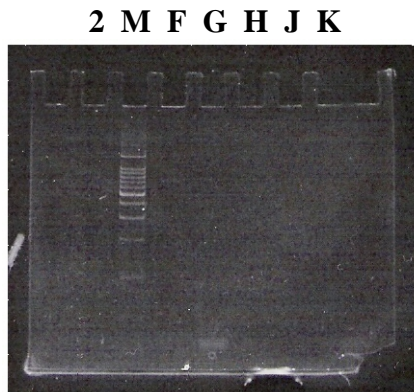
**Figure 8. 782 bp fragments from cloth and gravel samples
at the Anthropological Research Facility**

2:	**782 bp water blank**
M:	**100 bp marker**
F:	**782 bp Day 0 positive control**
G:	**782 bp Day 3 Cloth**
H:	**782 bp Day 8 Cloth**
J:	**782 bp Day 3 Gravel**
K:	**782 bp Day 8 Gravel**

28

Chapter 5: Discussion and Conclusions

This project was intended to gather information on PMI, using DNA degradation as a measure of time. Based on the current literature and knowledge of the properties of DNA, it was hypothesized that DNA would decay in a time-dependent manner in a controlled atmosphere. In addition, it was hypothesized that DNA exposed to environmental and substrate effects would decay more rapidly than in a controlled environment.

Many challenges were encountered during this study. DNA research is time-consuming and has potential for errors in a multitude of ways, but with careful planning and the use of controls, much of this error can be avoided. It was found that some primer sets yielded better results than others, and finding the correct temperatures at which to run the PCRs was a trial-and-error process. In addition, both the PCR process and master mix system required refinement to produce consistent results. These efforts have lead to the development of a system that is likely to be useful in the analysis of degraded DNA.

At the beginning of the study, the hypothesis assumed that the 782 bp fragments would degrade much more rapidly than was actually observed. In fact, the survival of the longer fragments is especially surprising because the environment in the 37 degree Celsius incubator (approximately 98 degrees Fahrenheit) would seemingly warrant faster degradation. However, the controlled atmosphere may have protected against effects of ultraviolet light and humidity, which are known to speed degradation. The apparent increased rate of degradation in the ARF samples would lend support to this idea.

29

In order to take this system to the next level, a way must be found to quantitate the amount of degradation that has occurred. In the time frame studied, disappearance of larger fragments was not observed. Therefore, we must examine relative amounts of the larger fragments over time. One way to achieve this goal is to use fluorescently labeled PCR products and compare the intensities of the PCR product across samples and time. If the 110 bp fragment is used as an internal control, i.e., it shouldn't decrease, a ratio between larger and smaller fragments would represent the relative amount of the larger fragment present in each sample. Quantitation of the fluorescently labeled samples requires capillary DNA sequencing equipment. An initial attempt at analyzing samples with fluorescently labeled primers was only partially successful, because an incorrect label was used. However, the results were encouraging enough to warrant further study.

In addition to the use of ratio analysis, efforts must be made to isolate DNA from samples in more realistic environments. Other extraction methods might be more appropriate for smaller amounts of DNA, such as might be found on substrates like the gravel and cloth ARF samples. These substrates can be examined more closely for inhibitors as well.

A logical next step to aid anthropologists would be to apply these methods to DNA extracted from bones that had been exposed to the elements for extended periods of time. A degradation study modeled after Vass et al.'s (2002) chemical research would yield much information; in that work, bodies were left at the ARF, and various organs and tissues were sampled periodically. While the present

30

study focused on the properties of DNA decay in blood, degradation should be studied in each tissue, because the rates will likely differ. Prior researchers have studied these differences using different techniques, and the specimens used were taken from autopsy cases (Bär et al., 1988; Hoff-Olsen et al., 1993). It is obvious that there is much work to be done in this field and equally certain that DNA analysis will continue to be a tool anthropologists should and will use to their advantage.

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