A SYSTEMATIC STUDY OF DNA YIELD AND INTEGRITY IN BOVINE LONG BONES

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Bachelor of Science in Cell and Molecular Biology

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2006

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2013

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I. Introduction

Since the discovery of bones as a great source of deoxyribonucleic acid (DNA), scientists have applied the genomic information found in skeletal remains in a variety of scenarios. Genetic material from bones have answered questions about migratory patterns of ancient people[1] and the identity of victims of mass disaster and war.[2–5] When performed correctly, the application of DNA extraction from bone allows forensic scientists to produce interpretable genetic profiles. However, in order to reliably produce STR profiles, forensic analysts face challenges ranging from prioritizing available samples to choosing the most effective DNA analysis protocol to extract genetic information from a sample. Although these challenges exist in every forensic case, they are especially significant in a case where bone is the primary evidence.

As one might imagine, either ancient or modern bones are often less than pristine. Since the 1980s, amplification and analysis of genetic material has improved greatly providing scientists with several investigative options involving DNA.[4] Depending on the nature of the results desired and the suspected quality of the bone sample, one can opt to extract either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA), or both. Severely degraded bone generally requires the analysis of mtDNA for identification because it contains higher copy numbers per human cell than nDNA and nucleotide sequencing of even small, degraded, mtDNA sequences is possible. Mitochondrial DNA analysis is also the choice when only limited amounts of genetic material are available for use. A

limitation of mtDNA analysis is that it is maternally inherited and subsequently less discriminatory than nDNA when genetic profiling is utilized for identification purposes.[6] In cases involving the identification of missing persons, genetic profile discrimination is of the utmost importance. The challenge of identification is typically not a factor in ancient bone cases; however, conclusive identification is the end goal for much of modern forensic science practices.

One decision a forensic analyst must make is which method of DNA isolation to use. Of course, the quality of the DNA recovered aids in the decision process and allows scientists to choose how best to proceed with DNA analysis. Protocols for the classical phenol-chloroform extraction, crystal aggregate extraction, total demineralization, and numerous commercial kits are all valid options.[7–10] Each method is accompanied by a set of pros and cons, usually pertaining to feasibility and ease, cost effectiveness, and end-product quality. Additionally, each of the above methods performs best under certain circumstances generally determined by the age of the bone, intended use of DNA, and likely presence of PCR inhibitors. Choosing a method that isolates as much intact-purified DNA as possible is always the goal.

Selecting a reliable method for DNA quantification is also important because the quantity and quality of template DNA is critical to all downstream applications. A reliable DNA profile often cannot be generated when too much or too little starting template is present in a reaction. Too much template likely produces DNA profiles with such intense fluorescence that the genetic analyzer cannot yield a reliable profile.[11] Furthermore, incomplete nucleotide addition (i.e. “minus A”) can occur if too much DNA template is added to the typing reaction, complicating the electropherogram by generating

a split allelic peak. When too little starting template is used, allelic dropout can occur, resulting in a partial or altogether failed profile. Using Quantitative real-time PCR (RT-qPCR) to determine quantity, degradation, and inhibition is an acceptable option. RT-qPCR holds benefits such as its one-step closed–tube design, short assay time, and high sensitivity. However, the consequences of RT-qPCR include the addition of extraordinary equipment to forensic laboratories, the requirement for expensive reagents, and the failure to produce additional information such as gender or mixture identification. Quantitative Template Amplification Technology (Q-TAT) is a two-step method capable of determining all the features that RT-qPCR can plus gender and/or mixture identification. Moreover, Q-TAT does not generally require additional laboratory equipment. Q-TAT is more cost effective than qPCR, but lacks the sensitivity. In conclusion, either qPCR or Q-TAT represent acceptable methods for quantitative and qualitative analysis of DNA extracts.

Determining the ideal sampling location is the final decision faced and probably one of the more important to the overall process. With all forensic evidence, the analyst is challenged with where to obtain a sample; sometimes, it is not obvious. In order to achieve good results, an area with a high cell number and relatively good preservation is best.

All of the questions surrounding genetic analysis using bone as the starting material call for research in the area of improving the quantity and quality of DNA extracts. Although Irwin[3] claims that improved amplification techniques will overcome the hindrances surrounding nDNA genetic analysis, identifying the location of an optimal sampling spot within a long bone will also assist in the reliable production of STR

profiles from this type of forensic sample. The extracted DNA is only as good as the starting material, so localizing the optimal sampling area only seems logical. This study aims to localize the region of bone that yields the most abundant and intact DNA by subjecting DNA isolated from *Bos indicus* (the cow) femora to quantitative and qualitative analyses. The information collected will help streamline human identification efforts whenever possible.

II. Review of Literature

Mass disasters are a global problem, exist as either natural or man-made, and unfortunately can cause loss of extensive loss of life. Often, skeletal fragments are the only remains recovered from mass disaster sites. Further complicating victim identification is the comingling of remains. Without sufficient DNA profiling methods, victim identification would often be virtually impossible.

Victims from numerous airline crashes, mass graves, natural disasters, and terrorist attacks have been identified using DNA typing. In September 1998, Swissair Flight 111 crashed near Nova Scotia. The airline logs indicated that 229 passengers were aboard the flight, all of which died, and were subsequently identified using visual recognition, latent fingerprints, and primarily DNA typing.[12] Regions of the world, including Yugoslavia, the Balkans, Mexico, and Colombia, have suffered from genocide or mass homicide leaving victims buried in clandestine mass graves. For example, an estimated 40,000 unidentified bodies are located in unmarked mass graves in the former Yugoslavia.[12] A twelve-year study aimed at identifying 1155 remains recovered from the former Yugoslavia used traditional forensic methods to identify 577 victims between 1993 to 1999.[2] From 2000-2004 DNA typing was utilized to identify 109 additional victims.[2] The authors also noted that the extraction of nDNA from some samples in terms of quantity or quality was not sufficient and thus mtDNA analysis had to be used yielding less discriminatory results. In 2009, Victoria, Australia was swept by an intense brushfire

that destroyed thousands of homes and businesses. One hundred seventy-three people were killed.[13] Due to the nature of the disaster, forensic analysts received charred skeletal remains along with some blood and tissue samples. Of the 263 samples belonging to 163 individuals, all were successfully profiled and thus all victims were positively identified. Forensic analysis was employed in the above-mentioned scenarios because victim identification helps to solve crimes and, more importantly, bring justice and closure to families of the dead.

As unfortunate as the 2001 World Trade Center Disaster was, the situation afforded forensic scientists a wonderful opportunity to further develop human identification using partial skeletal remains. Due to the sheer number of victims and the nature of the attack, scientists were forced to substantiate identification based on small fragments of bone rather than intact bones or an entire skeleton.[14] Bone proved itself as an excellent source of DNA for testing and is especially useful when alternative material, such as blood, muscle, or soft tissue is unavailable. By mid-2010, advancements in DNA technology contributed to the successful typing of 12,769 remains (59%) from a total of 21,802 recovered which enabled 1626 victim identifications.[12]

The anatomical structure of bone creates a protective layer surrounding the genetic material preserving it from the detriment incurred by exposure to environmental extremes.[10] The role of this protective matrix helps to preserve the integrity of nDNA and mtDNA which is for the identification of human remains recovered in the wake of mass disasters, clandestine graves, or arson.

Critical to all downstream applications of DNA extraction is the quantity and quality of template. Ideally, both characteristics should be determined prior to genetic analysis in order to produce a reliable profile. Too much starting template will likely produce results with such intense fluorescence in STR alleles that a reliable profile cannot be produced.[11] Furthermore, incomplete nucleotide addition (i.e. "minus A") can occur if too much DNA template is added to the typing reaction, which complicates the electropherogram by generating a split allelic peak. When too little starting template is used, allelic drop out can occur resulting in a partial or failed profile.

The quality of DNA is determined by the amount of degradation and presence of polymerase chain reaction (PCR) inhibitors. Degradation is the random fragmentation of DNA over time and can be stimulated by environmental factors such as pH, ultraviolet light, moisture, and heat.[5] Depending upon the state of degradation of a DNA sample, it may be impossible to produce an STR profile, or, produce only a partial profile because only small pieces of intact template are eligible for the amplification process. Quality can also relate to levels of PCR inhibitors that may be co-extracted with DNA from an evidentiary item. If present, inhibitors will cause reduced PCR efficiency or may prevent amplification altogether. Crucial to PCR amplifications and subsequent genetic analysis is the removal of inhibitors from template material. Thus, for successful STR analysis to occur, DNA must be available at an optimal concentration, lack degradation, and be free of PCR inhibitors. Methods for confirming the quantity and quality of DNA will be discussed in further detail below.

2.1 Key terms and search strategies

Prior to the current study, a search of the literature revealed a considerable investigation of DNA extraction of specific skeletal elements. A search of PubMed, PubMed Central, Science Direct, and various other search engines was carried out using the search terms "DNA extraction bone," "DNA human remains," "DNA human identification," "*Bos indicus* anatomy," "*Bos indicus* atlas," and "bone histology." A search of the *Journal of Forensic Sciences* and *Journal of Forensic Sciences International* also yielded many relevant articles.

2.2 Limitations of current research

The literature offers numerous approaches to human identification using skeletal remains by comparing DNA isolation methods, assessing DNA degradation, and detecting and minimizing the effects of PCR inhibitors[5,15,16]. Currently, there is no optimal STR typing method involving bone as a source of DNA.

2.2.1 DNA isolation from bone

Bone is a complex material composed of dense bone (also known as compact), and spongy or cancellous (also known as trabecular) layers.[17] Each of these layers consists of mineral, collagen, water, noncollagenous proteins, lipids, vascular elements, and cells. Methods for DNA extraction from bone generally require the following steps:

I. Initial decalcification breaks down the inorganic solid matrix of bone by removing the metal ions that contribute to the boney matrix.

II. This makes the demineralized cell matrix that remains sensitive to protease and detergent. Cell lysis dissociates cell membranes and liberates genetic material.

III. Extraction isolates DNA from RNA, proteins and other cellular components.

IV. Final clean-up and concentration of an extract purifies the recovered DNA and prepares the extract for quantitative, qualitative, and genetic analysis.

Jakubowska et al[7] and Rucinski et al[8] compared several methods of DNA extraction from human bones exhibiting varying degrees of degradation. Both studies compared DNA extraction methods commonly used in forensic laboratories— classical phenol-chloroform, crystal aggregate extraction, and total demineralization. Results indicated that each method performed better than the other methods under certain circumstances. In general, the total demineralization method yielded the highest DNA concentration.[7] Extraction from bone crystal aggregates produced more concentrated and highly purified DNA in extremely degraded bones; while classical phenol-chloroform performed poorly across all variables and should be used when bones are fresh. In contrast, a recent study by Rabe[18] suggests that classical phenol-chloroform outperforms a double magnetic bead extraction using the Promega DNA-IQ system (Promega Corp., Madison, WI); a 5-fold increase in DNA yield specifically.[18] Also observed by Rabe[18] was a decrease in pRL intensity suggesting an increase of PCR inhibitors in samples extracted using phenol-chloroform. Although DNA purity is important for genetic analysis, a sufficient yield is more imperative.

2.2.2 Yield Gel

DNA concentration and the level of degradation can be determined in a number of ways. The simplest method is the use of agarose gel electrophoresis, referred to as a yield gel. Sample DNA is mixed with a glycerol-dye loading buffer that adds weight to the sample causing it to sink when loaded into a well of an agarose gel. An electric current is applied to the gel and, due to the specificity of nucleic acid charges, the sample migrates through the agarose directionally. Smaller molecular weight fragments of DNA migrate faster than larger fragments, thereby separating the entire sample based on molecular weight. Upon completion of the run, staining the gel with an interchelating dye allows for visualization under ultra violet light. Quantification of DNA yield occurs by comparing the signal intensity of the unknown sample to that of a sample with known concentration, typically Lambda DNA. The amount of degradation is determined based on the tightness of the sample band resolved. Intact DNA is observed as single, discreet band, whereas fragmented or degraded DNA is observed as multiple bands or a continuous smear down the gel track respectively.[19]

2.3 Quantitative Real-time PCR (RT-qPCR)

RT-q PCR can be used to measure concentration, degradation, and inhibition in DNA samples.[8,10,20] As with all PCR amplification reactions, regions of exponential, linear, and plateau exist. During the exponential phase, the number of amplicons double with each cycle thereby producing a linear relationship between cycle number and the log scale of the DNA concentration.[21] PCR products are detected as they accumulate by a special interaction between target specific primers and a fluorogenic probe designed to

anneal between the forward and reverse primer. As the steps of PCR are carried out, the DNA polymerase activity cleaves a quencher dye from the probe thus allowing it to fluoresce. This fluorescent signal is detected by the thermal cycler camera and the time is recorded in a unit of "cycles.". The point at which exponentially increasing fluorescence signal exceeds an arbitrary threshold is referred to as the cycle threshold value (C_T). The initial DNA concentration correlates proportionally to the C_T making it possible for a plot of DNA concentration vs. C_T to create a standard curve.[22] Samples with unknown DNA concentration can be analyzed using the standard curve and the initial concentration is produced. Furthermore, the use of multiple fluorogenic probes each with a different reporter dye and target region allows for the analysis of several PCR products in a single reaction

During degradation, large segments of DNA are randomly broken into smaller pieces. Determining degradation within a sample is achieved by quantifying the amplification of large PCR products. By coupling the same 5`-forward primer with different 3`-reverse primers, targeted at sequences downstream (thus amplifying increasingly larger regions, in basepairs), degradation can detected by a change in the proportion of low and high molecular weight amplicons produced from a given sample.[10] A ratio of DNA yield between two amplicons can also act as a measure of degradation provided the oligos used to amplify each locus perform at similar efficiencies. Depending on the level of degradation, large regions will fail to amplify since the sequence is likely discontinuous due to degradation. In contrast, smaller regions amplify more readily since these short sequences more likely do not contain a random break. Internal PCR controls (IPC), such as Lambda DNA or *Renilla rentiformis* luciferase gene (pRL), can be added

to each reaction to measure the presence and/or level of PCR inhibitors in each unknown sample.[8,10] The IPC has a relatively constant C_T that increases significantly if PCR inhibitors are present in the sample of interest.

There are benefits to using qPCR such as its one-step closed–tube design, short assay time, and sensitivity. However, the consequences include the addition of extraordinary equipment to forensic laboratories, the failure to produce additional information such as gender or mixture identification, and the requirement for expensive reagents.

2.4 Quantitative Template Amplification Technology

Quantitative Template Amplification Technology (Q-TAT) is a two-step method for DNA quantification that allows for several variables to be determined in one reaction.[18,21,23,24] Initial standard endpoint PCR co-amplifies unknown and serially diluted standard DNA using several fluorescently labeled primers, specifically targeting the sex-determining loci (AMEL) and *Renilla rentiformis* luciferase gene (pRL) sequences.[18,21,23,24] The AMEL primers are designed to amplify regions of varying product length, offering the capacity to measure degradation. Amplification of the pRL recombinant plasmid is sensitive to PCR inhibitors such as Ethylenediaminetetraacetic acid (EDTA), hemin, blue denim dye, and humic acid.[21] Because pRL amplification is inhibited by PCR inhibitors so effectively, the amplicon produced from the pRL plasmid serves as a reliable indicator of PCR inhibitors. The second step of Q-TAT is capillary electrophoresis, which utilizes equipment standard for all forensic science laboratories. Fluorescently labeled PCR products are size separated and a CCD camera captures their

respective fluorescence. Genemapper ID software supplied for use with the genetic analyzer allows for the conversion of peak area and fluorescence of loci within a sample to relative fluorescent units (RFU).[21,23,24] RFU vs. known DNA concentration is used to create a standard curve in pg/μL which is then used to quantify sample DNA. Consideration of several variables (quantitation, degradation, inhibition, and gender determination) occurs in tandem using Q-TAT methodology. Q-TAT has a sensitive detection range of 20-1000pg of DNA; however, the coefficient of variation in DNA concentration estimates is 35%.[23]

2.5 Discussion

A search of scientific literature supports the need for a systematic comparison of DNA quantity and quality recovered from bones, including a sampling study of anatomical regions (epiphysis, metaphysis, diaphysis), and layers within the diaphysis region (Figure 1). Although comparative studies, limited to a few types of bone, have been performed (rib vs. tooth, weight-bearing bone vs. non-weight bearing bone, long bones, small bones), a study investigating areas within each bone has not been carried out.[5,15,16,25,26] A study using an experimental design of this type would help to make a confident conclusion about which area of bone yields the most concentrated and well-preserved DNA.

DNA typing provides the greatest chance of positively identifying human remains. In a situation where bone is the only source of genetic material available for analysis, a satisfactory and efficient protocol for the extraction and recovery of genomic DNA is required. This study aims to localize the region of bone that yields the most

abundant and intact DNA. Harvested from several *Bos indicus* (the cow) femora, DNA extracted from various anatomical regions and layers of bone at each region was subjected to quantitative and qualitative analysis.

III. Methods

DNA testing can be used to identify intact or partial remains recovered from a disaster site, provided there is sufficient DNA present to obtain a DNA profile and a reference sample profile is available for comparison.[12] The current study was designed to determine the ideal sampling location for DNA isolation from bone. Due to the availability and easy manipulation of weathering conditions, *Bos indicus* bones were selected as the material source. Femora were recovered from their respective locations and labeled sufficiently. Bones recovered from above ground were in general clean and free from dirt and debris. However, buried bones were covered with wet dirt and required a mild cleaning prior to experimentation. Gently scrubbing the bone after soaking in a 10% solution of Era detergent (Proctor & Gamble, Cincinnati, OH) and water was performed to remove soft tissue and soil found on the bone. Three categories of *Bos indicus* femora obtained from steers less than one year old were identified for use in this study:

1. Bleached: Exposed year-round for an unknown length of time displaying sun-bleached characteristics, (n= 5)
2. Fresh: Exposed to summer/autumn seasons for approximately six months, (n=2)

3. Buried: Buried in a shallow grave for longer than three years, (n=2)

Each of the groups above represents a bone sample type frequently encountered by analysts in active forensic science laboratories. The first and second groups containing exposed bones represented those often left uncovered, scavenged by animals, or weathered under typical Oklahoma climate conditions. Buried bones represented possible victims buried in clandestine graves for varying lengths of time. Additionally, these bones represent victims of mass disasters buried under heaps of debris.

3.1 Extraction of DNA from bone

Methods for cleansing, demineralizing and phenol-chloroform extraction, described below are modified from the procedures currently being used by the Human Identity Testing Laboratory on the OSU-CHS campus.[27]

3.1.1 Excision and Demineralization of Specimen

All work with intact bone samples occurred strictly in a fume hood and safety precautions, such as the use of personal protective equipment, were taken. The working surface of the fume hood was cleaned with a 10% bleach solution between samples and allowed to air dry. Clean bench paper was placed to cover the entire working surface. Lastly, arrangement of an elaborate system within the fume hood included:

1. Dremel tool (Dremel, Racine, WI) in a fixed vertical position using an articulating drill-press workstation (Part# 222-01, Dremel, Racine, WI)

2. Raised platform which held a sterilized weigh-boat for bone chip collection

3. Paristaltic pump for irrigation while excising bone chip

The Dremel rotary tool was affixed with a Diamond wheel (Part# 545, Dremel, Racine, WI) and was used to excise a 1.5”x1.5” bone chip of relative flatness from the cranial region of each bone at five locations (Figure 1A):

1. spongy proximal (SP)

2. spongy distal (SD)

3. epiphyseal plate proximal (EPP)

4. epiphyseal plate distal (EPD)

5. compact medial (CM)

Additionally, two subsections of compact medial bone were collected (Figure 1B):

6. Osteoclast cells (OC)

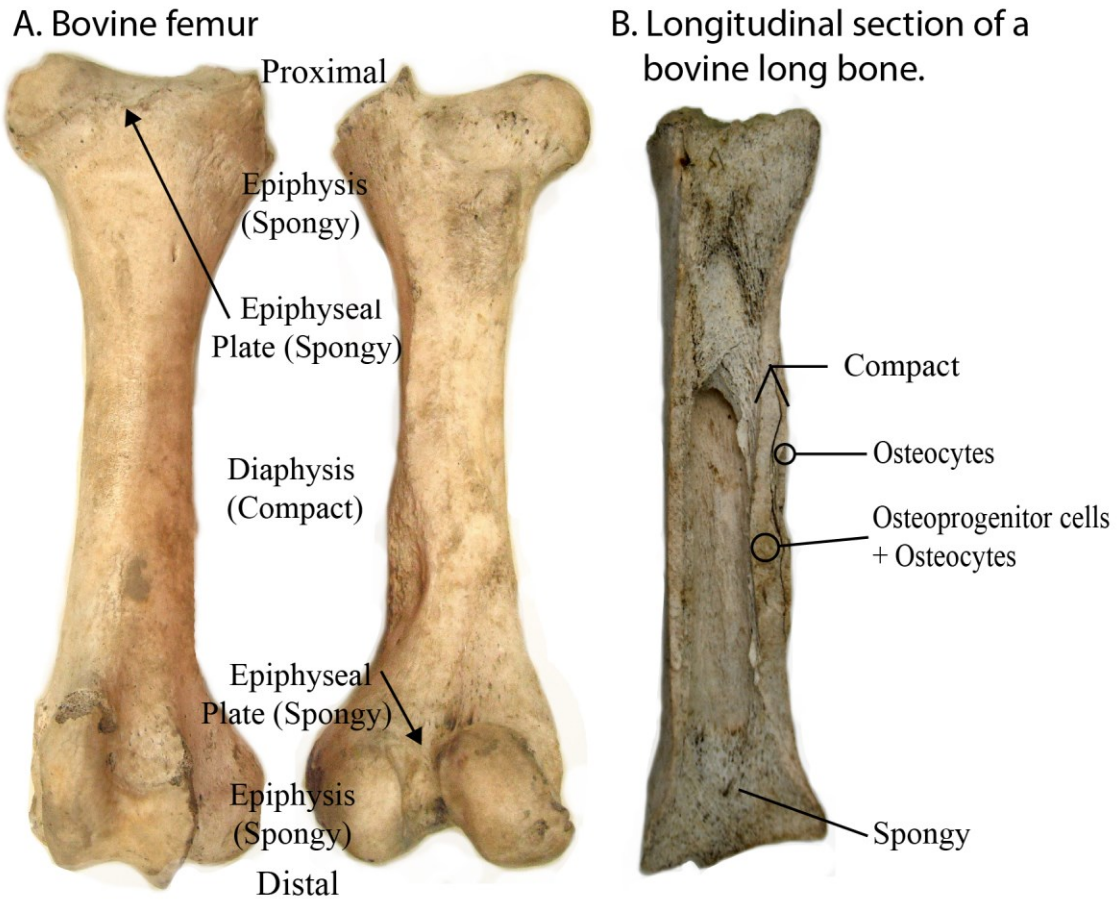
7. Osteoclast containing Osteoprogenitor cells (OCOP)

Irrigation while excising the chip effectively weighed down the bone dust and minimized bone dust contamination within the workspace. Sterilization of pump tubing using a 10% bleach solution, followed by copious rinsing with deionized water and irrigation using deionized water, reduced the risk of introducing foreign contaminants into the bone powder. Furthermore, a pipette tip attached to the pump hosing provided

controlled directionality of irrigant and easy replacement between samples minimizing contamination.

Using extreme caution, each bone chip was firmly held by a pair of vise-grip pliers and cut into numerous smaller chips capable of fitting in a sterile 5 ml polypropylene tube. Chips were stored at -80°C for 10-20 minutes. Next, chips were placed in a steel mortar and pestle previously washed with detergent, wiped with absolute ethanol, and allowed to air dry. A hammer was used to exert force on the pestle subsequently pulverizing the bone chips into a fine powder. Each set of chips was smashed until the same powder consistency was reached, i.e. compact bone required more smashing than spongy bone. Bone powder was retrieved from the mortar by inverting the mortar on fresh weigh paper and gently tapping to loosen the powder. The bone powder was transferred to a fresh eppendorf tube and stored at room temperature until demineralization.

Figure 1. Anatomical regions of bone chip excision.[17]



(A) Left: A weathered *Bos indicus* left femur (caudal view). Right: A *Bos indicus* left femur (cranial view) showing the five areas of bone chip excision. Note the absence of the Greater Trochanter, presumably broken off during the weathering process. (B) A translongitudinal section of a *Bos indicus* tibia showing spongy and compact bone. Inset: The location of compact bone subsampling representing Osteocytes and Osteocytes containing Osteoprogenitor cells.

A recent study by Rabe[18] defined parameters of efficient DNA extraction from small amounts of bone powder. The following methods were modified from Rabe's results and validated procedures employed by the OSU-CHS Human Identity Laboratory.

Demineralization of 150-250 mg of bone powder was carried out in 15 ml conical tube containing 10 mL of 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.[18,27] The exact starting amount of each sample was recorded and used to normalize results. Samples were continuously rotated for 24 hours at room temperature using a platform orbital shaker. Following the first demineralization incubation, the bone powder was pelleted by centrifugation at 2,000 × g for 5 minutes. After decanting the supernatant, a second 10 mL aliquot of EDTA was added to the powder pellet and vortexed. Incubation on the platform shaker continued for an additional 24 hours. Centrifugation and removal of the supernatant was repeated a second time. Addition of 10 mL of TE-4 buffer, followed by centrifugation, removed any remaining EDTA inhibitors.

3.1.2 Phenol-Chloroform Extraction

The demineralized bone powder pellet was resuspended in 500 µL of extraction buffer containing 482.5 µL TNE (10 mM Tris-Cl, pH 8.0; 0.2 M NaCl; and 0.1 mM EDTA), 12.5 µL 20% SDS, and 5.0 µL Proteinase K (20 mg/mL stock) and incubated at 65^{o}C for 90 minutes with occasional vortexing.[27] An additional 2.0 µL of Proteinase K was added to the tube, vortexed, and returned to incubation for an additional 30 minutes. Two µL of RNase A (100 mg/mL) was then added to the sample and vortexed, followed by incubation at 37^{o}C for 20 minutes. The sample becomes clear and viscous once digestion and lysis is complete. Following lysis, the solution was transferred to a 2 mL eppendorf tube. DNA extraction continued with the addition of an equal volume of Tris-equilibrated-phenol:chloroform:isoamyl alcohol solution (9:0.96:0.04 vol/vol/vol). The sample was vortexed and centrifuged at 15,000 × g for 3 minutes. The upper-aqueous phase was transferred to a fresh 2 mL tube and an equal volume of chloroform:isoamyl

alcohol (24:1 vol/vol) was added. The sample was then vortexed and centrifuged at 15,000 × g for 1 minute. Again, the aqueous phase was transferred to a 1.5 mL eppendorf tube for DNA capture and purification using the Zymo Clean and Concentrator kit (Zymo Research, Orange, CA). Target DNA was purified following the manufacturer's instructions and eluted from the Zymo spin column with 2 × 15 µL aliquots of hot (65^{o}C) TE-4 buffer. A total volume of approximately 28 µL was stored at 4^{o}C until use.

3.2 Quantitative Template Amplification Technology (Q-TAT)

3.2.1 Q-TAT PCR Set Up and Amplification

As previously discussed, Q-TAT is a two-step method for DNA quantification that allows for several variables to be determined in one reaction.[18,21,23,24] Standard endpoint PCR co-amplifies unknown and serially diluted known DNA using fluorescently labeled primers, specifically designed to amplify the sex-determining loci (AMEL) and *Renilla rentiformis* luciferase gene (pRL) sequences.[18,21,23,24] Quantification of DNA present in an unknown sample is possible by comparison of the relative fluorescence units (RFU) in the peak areas of specific loci (*Bos* small AMEL, *Bos* large AMEL, and pRL) to a standard curve created from known concentrations of intact bovine DNA. [18,21,23,24] Each Q-TAT PCR reaction of 12.5 µL total volume prepared using the following reagents in the amounts specified in Table 1.

Table 1. Q-TAT PCR reagents per 12.5µL reaction volume.

Reagent	Volume per reaction, µL
dH_20	2.875
Q-TAT Primers[a]	0.625
GoTaq DNA Polymerase	7.50
pRL plasmid (0.5pg/µL)	0.50
DNA template	1.0

[a]10 µM each of *Bos indicus* small AMEL, large AMEL and 0.1 µM pRL primers.

Reagents and respective volumes in µL used in single Q-TAT amplification reactions. A master mix was prepared proportional to each volume so that the final volume was sufficient to amplify all samples in a single run.

Primers specifically designed to amplify the *Bos indicus* amelogenin loci were used. The target sequences and product sizes for *Bos indicus* and pRL primers are provided in Table 2.

Table 2. Gene Primers.

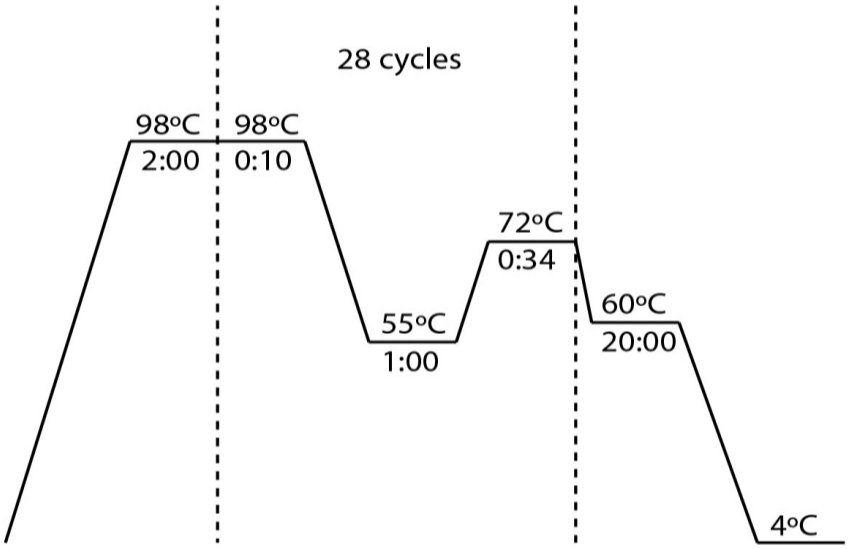
Primer	Forward Sequence	Reverse Sequence	Product size, (bp)
Bos Small AMEL	5`-TGGCCTAAAGAGCAGTTGA	5`- GCAGAGCACAGAATCTTGG	100
Bos Large AMEL	5'- AAATGGATTCCCAGATGCT	5`- ATGTTTGCCTAAGGCTGGT	210
pRL	5'- AAGGTGGTAAACCTGACGTTG	5`- TTCATCAGGTGCATCTTCTTG	203

To allow for accurate pipetting and a homogenous mixture, all Q-TAT PCR reagents except template were mixed in sufficient quantity to amplify all samples within a single run. The reaction mixture was then aliqouted in 11.5 µL volumes into the required number of reaction wells in a 96-well plate, followed by the addition of 1 µL

reference or unknown template DNA to the appropriate tubes. A negative control consisting of 1 µL dH_2O in place of DNA was mixed identically to the other samples. Including a negative control in the PCR set up demonstrates lack of contamination during the processing of samples. The PCR plate was then mixed by vortexing and briefly centrifuged.

An ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA) was used to complete Q-TAT PCR according to the parameters described in Figure 2. The PCR cycle finished with an indefinite hold at 4°C.

Figure 2. Q-TAT PCR parameters.



Q-TAT PCR parameters consisted of an initial denaturation set for 2 minutes at 98°C; followed by 28 cycles of amplification for 10 seconds at 98°C, 1 minute at 55°C, 34 seconds at 72°C; a final extension step for 15 minutes at 60°C; and an indefinite hold at 4°C.

Amplified samples were prepared for electrophoresis by adding a 1.0 µL aliquot of PCR reaction to a mixture of 14.9 µL of HiDi formamide and 0.1 µL GS500 LIZ sizing ladder (both from Applied Biosystems, Foster City, CA) and pipetted into 96-well plate. A rubber septum covered the plate and the samples were gently vortexed, followed by slow centrifugation. Next, the plate was loaded into an ABI 3130XL 16-capillary genetic analyzer (Applied Biosystems, Foster City, CA) and allowed to electrophorese through polymer (POP7 from Applied Biosystems, Foster City, CA) following a 25-second injection period.

3.2.2 Construction of a Standard Curve for DNA Quantitation Using Q-TAT

A 20 ng/mL stock solution of *Bos indicus* DNA was diluted with UV cross-linked dH_2O to a concentration of 1000 pg/µL. Serially diluting the reference DNA in dH_2O to the following concentrations generated a 3-fold dilution series: 1000, 333, 111, 37, and 12.3 pg/µL.[27] Six PCR reactions were set up as discussed to account for each of the five concentrations of reference DNA and one negative control. The standard serial dilutions also served as positive controls, demonstrating that the reagents performed as expected. Amplification and capillary electrophoresis of samples were completed using the parameters listed in Figure 2.

GeneMapper ID software (Applied Biosystems, Foster City, CA) was used to analyze raw data generated by capillary electrophoresis. As previously described, fluorescence emitted by the amplicons is expressed as RFU and reflects the amount of PCR product amplified. Furthermore, the amount of PCR product amplified is directly

proportional to the amount of starting template.[18] RFU data was obtained for each of the peak areas of *Bos* Small AMEL and *Bos* Large AMEL in the standard dilution. Results of 10 runs were recorded in a computational spreadsheet allowing for calculation of mean RFU and standard error of the mean RFU. The resulting data was then used to create a standard curve by plotting a linear regression trendline. Extrapolation of unknown sample RFU data using the respective standard curve produced a value of initial DNA concentration. Due to differences between the *Bos* Small AMEL and *Bos* Large AMEL trendline equations, the DNA yield for each locus was calculated using the respective standard curve. Subsequently, the two values representing DNA yield were averaged to provide a mean yield for each sample.

A method for measuring degradation involved analyzing the relationship between *Bos* Small AMEL and *Bos* Large AMEL peak areas. Severely degraded samples exhibit fragmentation of high molecular weight DNA, and therefore large PCR products fail to amplify during PCR with similar efficiency to low molecular weight products. A mean ratio of *Bos* Small AMEL peak area: *Bos* Large AMEL peak area in control samples provided an acceptable threshold ratio of 2.45 (STD=1.28, n=45). Unknown samples with a degradation ratio significantly greater than the acceptable value were classified as "degraded."

As previously discussed, Q-TAT is also used to determine the presence of inhibitors in an unknown sample. In human, the inhibition indicator, pRL, exhibits a slight response to increased concentrations of template DNA, which is evident when plotting pRL peak area as a function of DNA concentration.[18] However, pRL is unaffected by the amount of template DNA in *Bos indicus* (Figure 3). The pRL loci mean

peak area and standard error of the mean for each concentration of template DNA from 10 Q-TAT runs was calculated and graphed. The pRL value of an unknown sample was compared to the pRL value of a known sample with like concentration and percent inhibition was calculated. The mean percent inhibition for all unknown samples was also calculated and provided a threshold for comparison within the assay.

3.3 Quantitative Real-time PCR (RT-qPCR)

3.3.1 RT-qPCR Setup and Amplification

RT-q PCR is a sensitive assay used to measure concentration, degradation, and inhibitors as they accumulate.[8,10,20] The RT-qPCR assay used in this project utilized primers identical to those used for the Q-TAT portion of this study (Table 2) and specially synthesized TaqMan-MGB (minor groove bender) fluorogenic probes containing both quencher and reporter dyes (Table 3). TaqMan probes anneal to target sequences located on the PCR product. The quencher dye inhibits the fluorescent properties of the reporter dye as long as the two dyes are in close proximity. As PCR progresses, the quencher dye is cleaved from the probe thereby allowing the reporter dye to fluoresce. The fluorescence signal is negligible in initial cycles of amplification, however, as more product is generated the signal become significant enough for detection to occur. The time at which fluorescent signal is first detected is recorded as C_T and is directly proportional to the log amount of starting template. The use of varying TaqMan probes each with a different reporter dye allowed for amplification detection of multiple products in a single PCR reaction and is commonly referred to as multiplexing.

Table 3. RT-qPCR TaqMan-MBG fluorogenic probes.

Probe	Sequence	Reporter/Quencher Dye
Bos Small AMEL	5`-AGAAGCCAGCAAAGCTTGAA	VIC/MGBNFQ
Bos Large AMEL	5'- TTGAAAGGCCTCCAGAGAAA	6FAM/MGBNFQ
pRL	5'- ATCGGACCCAGGATTCTTTT	NED/MGBNFQ

Titration of each primer and probe set was performed to reach optimal performance when amplified both individually and in multiplex. Once the optimal concentration (primer efficiency between 90-110%) was determined a multiplex reaction containing all primer-probe sets, pRL plasmid, and DNA template was performed in a 25µL volume according to the parameters provided in Table 4. The volumes of each reagent were scaled proportionally to prepare a working solution with sufficient volume to amplify all the samples in a set.

RT-qPCR was performed on the ABI 7500 real time thermal cycler utilizing 96-well plates and optical sealing tape (Applied Biosystems, Foster City, CA) according the manufacturer's recommended universal thermocycling parameters: an initial hot start at 95°C for 10 minutes, followed by 40 cycles of annealing and extension at 95°C for 15 seconds and 60°C for 1 minute. Collection of the fluorescent signal by the instrument occurred during the extension period.

Table 4. RT-qPCR reagents per 25 µL reaction volume.

Reagent	Final Concentration per reaction, n*M*	Volume per reaction, µL
dH_20	—	9.49
TaqMan Fast Advanced Master Mix (2x)	—	12.50
Bos Large AMEL F/R primer[a]	360.0	0.36
Bos Large AMEL probe[b]	80.0	0.20
Bos Small AMEL F/R primer[a]	100.0	0.10
Bos Small AMEL probe[b]	50.0	0.125
pRL F/R primer[a]	100.0	0.10
pRL probe[b]	50.0	0.125
pRL plasmid (0.1pg/µL)	—	1.0
DNA template	—	1.0

[a]50 µM each of *Bos indicus* small AMEL, large AMEL and pRL primers.
[b]10 µM each TaqMan fluorogenic probe.

3.3.2 Construction of a Standard Curve for DNA Quantitation Using RT-qPCR

C_T values for each sample were obtained automatically by the SDS software and used to create a standard curve plotting C_T vs. DNA concentration for nine replicate known samples which were amplified simultaneously with unknown samples (Figure 4). Each sample was amplified in duplicate and the DNA concentration was quantified from extrapolation of the mean C_T from the standard curve. A 20 ng/mL stock solution of *Bos indicus* DNA was diluted with UV cross-linked dH_2O to a concentration of 1000 pg/µL. Serially diluting the reference DNA in dH_2O to the following concentrations generated a 3-fold dilution series: 1000, 333, 111, 37 pg/µL. For each run, five PCR reactions were set up as discussed to account for each of the four concentrations of reference DNA and

one negative control. The standard serial dilutions served as positive controls, demonstrating that the reagents performed as expected.

Degradation was measured by comparison of DNA yield in *Bos* Small AMEL and *Bos* Large AMEL products. Severely degraded samples exhibit fragmentation of large molecular weight loci, and therefore fail to amplify during PCR at similar efficiency to small molecular weight loci. Unknown samples with a degradation ratio significantly greater than the mean value for all unknown samples were classified as "degraded."

Inclusion of pRL in each amplification reaction served as an indicator of inhibition. Amplification reactions used for generation of the quantification standard curve also contained pRL and were subsequently used to determine a mean C_T for "uninhibited" samples. The mean pRL C_T value of an unknown sample was thus compared using a Student's T-test to the "uninhibited" pRL value (25.01, STD=0.30, N=54). A high degree of inhibition was classified by a significant p-value (<0.05) and lack of inhibition was classified as a non-significant p-value.

3.4 Statistical Analysis

As previously described DNA yield was determined by extrapolation of data from a linear regression analysis of the appropriate assay standard curve. Afterwards, the average of the yields produced by the smAMEL and lgAMEL markers was calculated and normalized to the starting amount of bone powder (weighed in milligrams). The Student's t-test was utilized for comparison between the 1) means of each group and 2) means of individual bones within a group.[28] Parameters for all two sample t-tests included α=0.05, two-tailed distribution, equal variance. Analysis of variance (ANOVA)

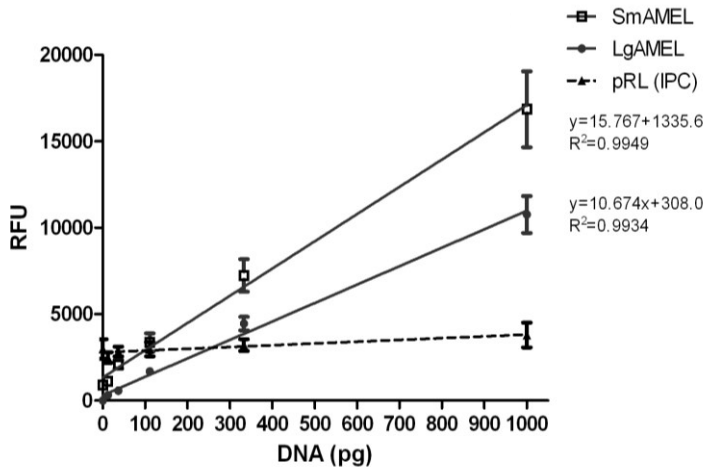
is the best statistical test for comparing three or more means because it limits type-I error associated with t-test.[29] However, ANOVA was not applied to the data obtained in this study. Lastly, Pearson correlation analysis was performed on all the data in a given group using GraphPad Prism 5.0 software (La Jolla, CA) . For this analysis a two-tailed distribution analysis was assumed and respective correlation coefficients and p-values were calculated by the software.[30]

IV. Results

4.1 Quantitative Template Amplification Technology (Q-TAT)

Ten replicate Q-TAT amplification reactions were set up as described in the Methods section to quantify known serial dilutions of *Bos indicus* DNA. The mean RFU at each dilution for small Amelogenin, large Amelogenin, and pRL loci were calculated individually and used to plot standard curves. GraphPad Prism 5.0 (La Jolla, CA) software generated the plots and performed a linear regression analysis that provided an equation used to quantify unknown template DNA at the respective loci (Figure 3). Points at the small Amelogenin locus generated a trendline with equation y = 15.767x+1335.6 and coefficient of determination (R^2) = 0.9949; large Amelogenin locus generated a trendline with equation y = 10.674x+308.0 and coefficient of determination (R^2) = 0.9934; pRL generated a trendline with equation y = 1.0263x+2793.3 and coefficient of determination (R^2) = 0.845.

Figure 3. Q-TAT Amplification Standard Curve using 10 replicate reactions of known *Bos indicus* DNA.



4.1.1 DNA Yield

DNA from 63 bone powder samples was isolated and quantified using Q-TAT as described in the Methods sections. Twenty-seven of the samples produced results (43%) with more than 85% of those samples originating in compact bone (Table 5). The mean yield for all samples quantified in the study was determined to be 32.0 pg per milligram of input bone powde (±26.7). Reasons for failed amplifications in over 50% of the samples may include degradation and/or the presence of PCR inhibitors, which were investigated in later experiments.

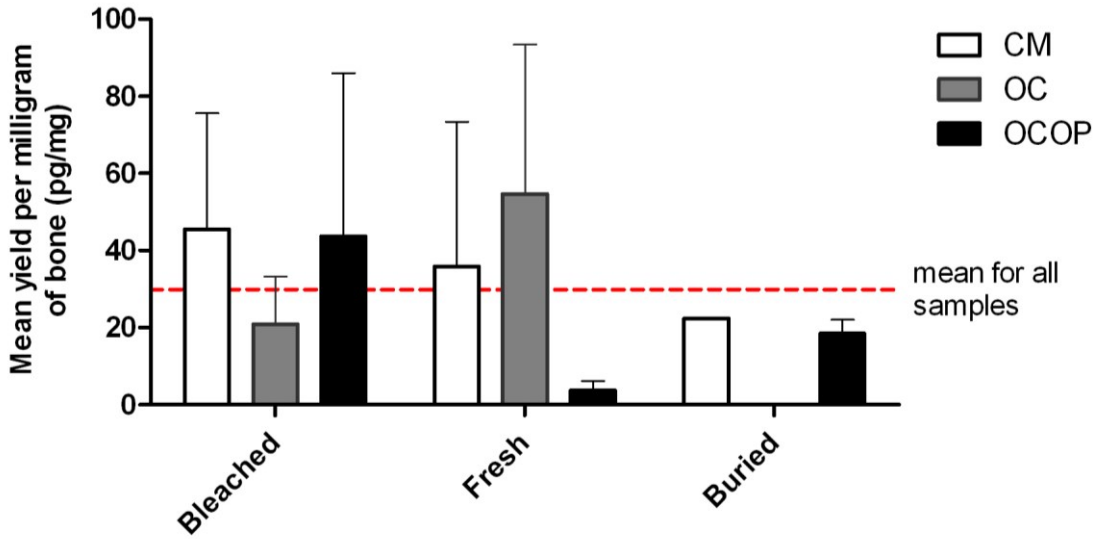
Table 5. Mean DNA yield (±STD) for samples amplified in duplicate-Q-TAT.

		Bleached						Fresh			Buried			Combined
Sample Type		Bone 1	2	7	8	9	MEAN, ±STD	3	4	MEAN, ±STD	5	6	MEAN, ±STD	MEAN, ±STD
SP	Mean Yield (pg/mg)		14.3					33.9						
SD	Mean Yield (pg/mg)							52.1	20.1	**36.14 ±22.6**				
EPP	Mean Yield (pg/mg)													
EPD	Mean Yield (pg/mg)													
CM	Mean Yield (pg/mg)	15.7	19.2	43.7	88.2	60.4	**45.50 ±30.1**	9.4	62.3	**35.9 ±37.4**	22.3			**40.2 ±28.1**
OC	Mean Yield (pg/mg)	22.4	39.9	18.9	17.4	5.6	**20.8 ±12.4**	54.7		**54.7**				**26.5 ±19.0**
OCOP	Mean Yield (pg/mg)	86.5	20.3	10.3	92.9	8.1	**43.6 ±42.3**	5.4	1.9	**3.7 ±2.4**	15.9	20.9	**18.4 ±3.5**	**29.1 ±34.9**
Mean	**Yield (pg/mg)**	**41.5**	**23.4**	**24.3**	**66.2**	**24.7**		**31.1**	**28.1**		**19.1**	**20.9**		

The blank results in the table were from PCR reactions in which one or more locus failed to amplify suggesting complete inhibition of the PCR process.

Since, compact bone samples represented the overwhelming majority of useable samples this group became the focus of subsequent analyses. DNA yield in bleached bones was significantly higher than from the buried bone group (p=0.017) (Figure 4). DNA yield in OCOP samples is comparable to compact medial (CM) samples in bleached and buried groups, yet only a small amount is recovered from fresh bones. In fresh bones, OC samples produce the greatest yield when compared to CM and OCOP samples, however CM samples produced the lowest yield in bleached and buried bones.

Figure 4. Mean yield of DNA per mass sampled-Q-TAT.



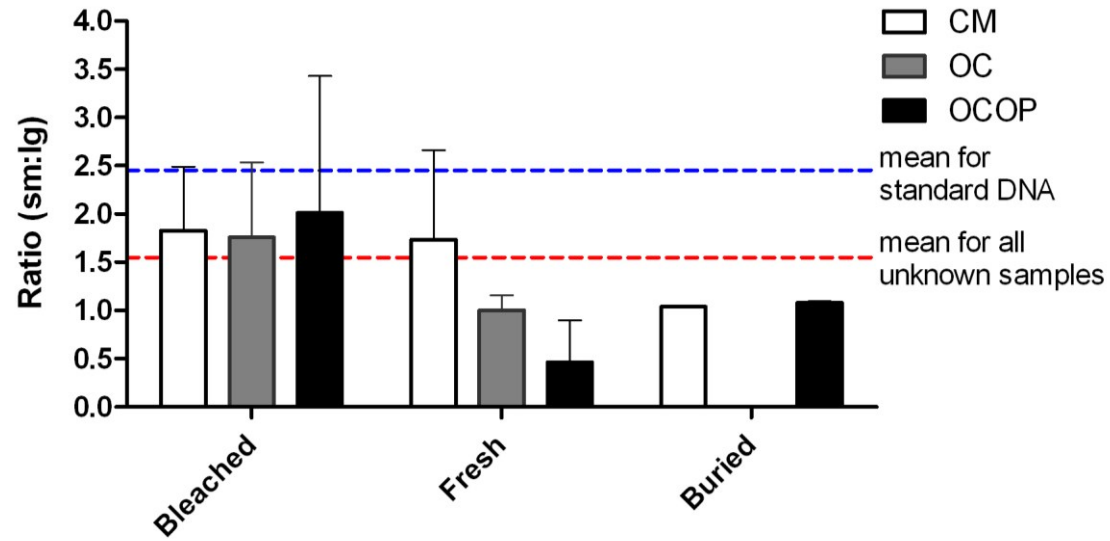
4.1.2 DNA Degradation

The ratio of mean DNA yield of the *Bos* small AMEL and *Bos* large AMEL products provides a method of estimating DNA degradation, this is only possible when both loci produce measureable product. Degradation was determined for compact bone

samples only (Figure 5). The mean degradation ratio for all Compact bone samples within the study was determined to be 1.55 (±0.86), compare with a ratio of 2.45 (±1.28) for all dilutions of the reference bovine DNA sample.

Fresh and buried bone groups demonstrated the most well preserved genomic material in the study as evidence by a ratio close to 1.0. Samples within the bleached group produced similar degradation values and as a whole were significantly more degraded compared to buried bones (p=0.005). Degradation ratios for individual tissue types within a group varied slightly but failed to suggest a single ideal source for that group.

Figure 5. Mean DNA degradation in Compact bone samples-Q-TAT.



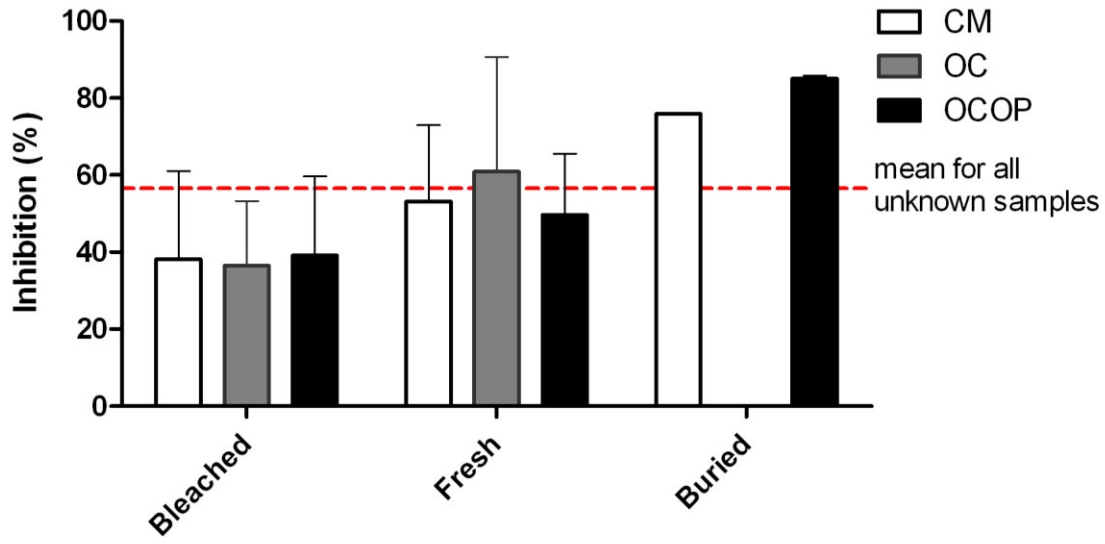
4.1.3 PCR Inhibitor Presence

Peak height for the pRL target in each unknown sample can serve as an indicator of the presence of PCR inhibitors. As previously described, the pRL plasmid is extremely sensitive to inhibitors, such as EDTA, heme and soil contaminants. A noticeable decrease or total dropout in pRL amplification is indicative of inhibitor contaminants within the sample. All samples in the study were co-amplified with the pRL plasmid as described in the Methods section, allowing for direct comparison of an "expected" pRL peak height value versus the "actual" pRL peak height value. Using the pRL standard curve and trendline equation (Figure 3) an expected pRL value based on DNA yield in a sample was computed. Next, percent inhibition of pRL activity was calculated using the actual and expected values:

$$\text{Percent inhibition} = \frac{Expected - Actual}{Expected} \times 100\%$$

Amplification of the pRL locus was exhibited in 35 bone samples and produced a mean percent inhibition of 56.5% (±24.5). As a whole, the bleached group was significantly less inhibited than the mean of all groups ($p=0.01$). As a whole, the buried group was significantly more inhibited than the mean of all groups ($p=0.03$). Percent inhibition in the fresh and buried groups was not significantly different from the mean of all groups. However, when percent inhibition between groups was compared the buried group exhibited significantly more inhibitor presence than the bleached and fresh groups ($P<0.05$). A significant difference between the bleached and fresh groups was not apparent. Furthermore, a single tissue type was not identified as significantly more or less inhibited than others.

Figure 6. Mean percent inhibition of pRL in Compact bone samples-Q-TAT.

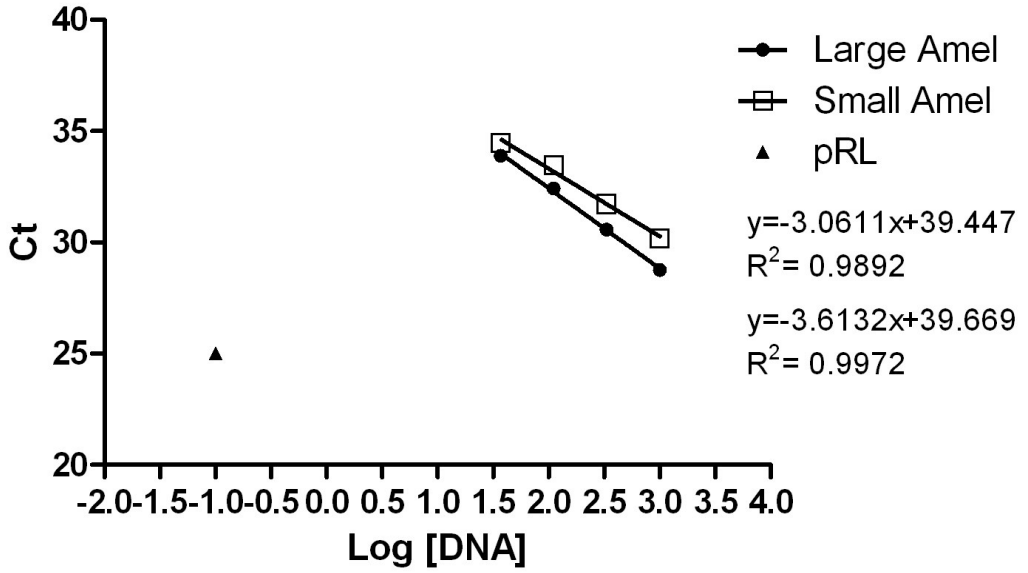


4.2 Quantitative Real-time PCR (RT-qPCR)

RT-qPCR was performed on the same DNA extracts using identical gene primers as was used in the Q-TAT portion of this study. RT-qPCR also required the use of TaqMan fluorogenic probes (Table 3). The sensitivity of the RT-qPCR assay requires all primer and probe concentrations in a multiplex design to be optimized; therefore, extensive titration of primers and probes was carried out until optimal concentrations were determined (Table 4). As described in the Methods section, nine replicate reactions using C_T values produced by standard *Bos indicus* DNA generated a standard curve for quantification of unknown samples (Figure 7). Linear regression analysis for each respective curve produced an equation for the curve and correlation of determination (R^2)

value. When input into the equation $E = 10^{-1/slope}$, the slope of the linear regression line provides a value of amplification efficiency[31]. In multiplex the primer efficiencies for *Bos* small AMEL and *Bos* large AMEL are 112% and 87%, respectively.

Figure 7. RT-qPCR Amplification Standard Curve using known *Bos indicus* DNA



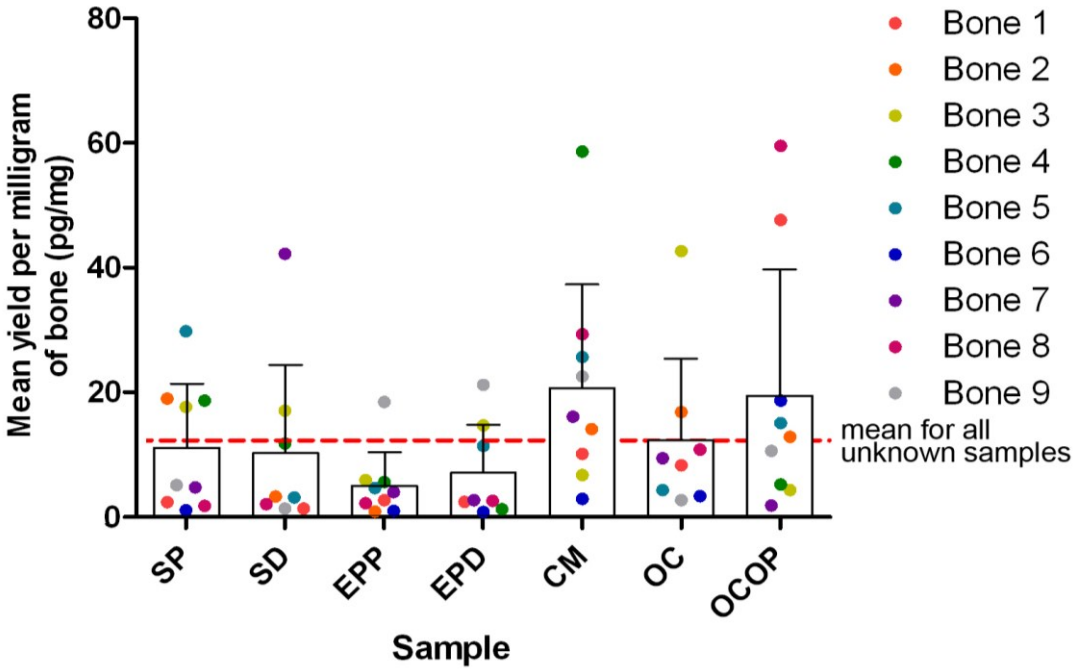
RT-qPCR Standard Curve, C_T of known concentrations of *Bos indicus* DNA, 37-1000pg. Linear regression of data generated from *Bos indicus* blood amplified using RT-qPCR methodology. *Bos* Small AMEL R^2=0.9892; *Bos* Large AMEL R^2=0.9972. Mean pRL = 25.01 cycles (STD ±0.3).

The RT-qPCR assay design demonstrated sensitivity capable of detecting C_T values for 4.1─1000 pg/uL standard *Bos indicus* template DNA in 100% of the samples tested. However, concentrations exhibiting a standard error of the mean greater than 0.3 cycles were omitted from the standard curve. The standard curve in Figure 7 consists of data generated by the following starting amounts of template DNA (pg/uL): 1000, 333, 111, 37.

4.2.1 DNA Yield

DNA was quantified from 63 unknown samples using RT-qPCR as described in the Methods section. All samples amplified at least one of the loci tested; however, three samples failed to amplify the *Bos* Large AMEL loci, making quantification based on the mean of the small and large AMEL loci impossible. The 60 bones quantified provided a DNA yield range of 1.0 ─ 60 pg/mg of bone powder and mean value of 12.5 pg/mg (STD 13.92 pg/mg) (Figure 8). Although groups CM, OCOP, and OC produced the greatest mean yields 20.7, 19.5, 12.3 pg/uL, respectively, none of the tissue types varied significantly from the mean of all unknown samples ($P>0.05$). Furthermore, only the group exhibiting the lowest mean yield, EPP, and the highest mean yield, CM, were significantly different ($p=0.01$). Even though mean yields were not significant, a trend grouping tissue types and consistency in mean DNA yield values is observed in Figure 8. For example, compact bone groups (CM, OC, OCOP) produced the highest yield; the next greatest DNA yield was found in spongy bone (SP and SD); and spongy bone extracted from the epiphyseal plate (EPP and EPD) consistently yielded the least amount of DNA.

Figure 8. Mean yield of DNA per mass sampled-RT-qPCR.



Mean yield of DNA for each tissue type sampled: spongy proximal (SP), spongy distal (SD), epiphyseal plate proximal (EPP), epiphyseal plate distal (EPD), compact medial (CM), osteoclasts (OC), osteoclasts + osteoprogenitor cells (OCOP). The bleached bone group is comprised of bones #1-2,7-9; the fresh bone group is comprised of bones #3-4; the buried bone group includes bones #5-6.

Individual bones producing greater than twice the mean DNA yield for all unknown samples were present in all three test groups (Table 6). The least amount of DNA was recovered from bones 6EPP, 2EPP, 6EPD (<1.0 pg/mg). The low yield in these bones may be attributed to PCR inhibitors or degradation since these samples belong to the bleached and buried groups.

Table 6. Bones with greatest mean yield of DNA per mass sampled-RT-qPCR.

Rank	Bone	Group	DNA yield (pg/mg)
1.	8OCOP	Bleached	59.56
2.	4CM	Fresh	58.65
3.	1OCOP	Bleached	47.68
4.	3OC	Fresh	42.68
5.	7SD	Bleached	42.21
6.	5SP	Buried	29.78
7.	8CM	Bleached	29.31
8.	5CM	Buried	25.67

4.2.2 DNA Degradation

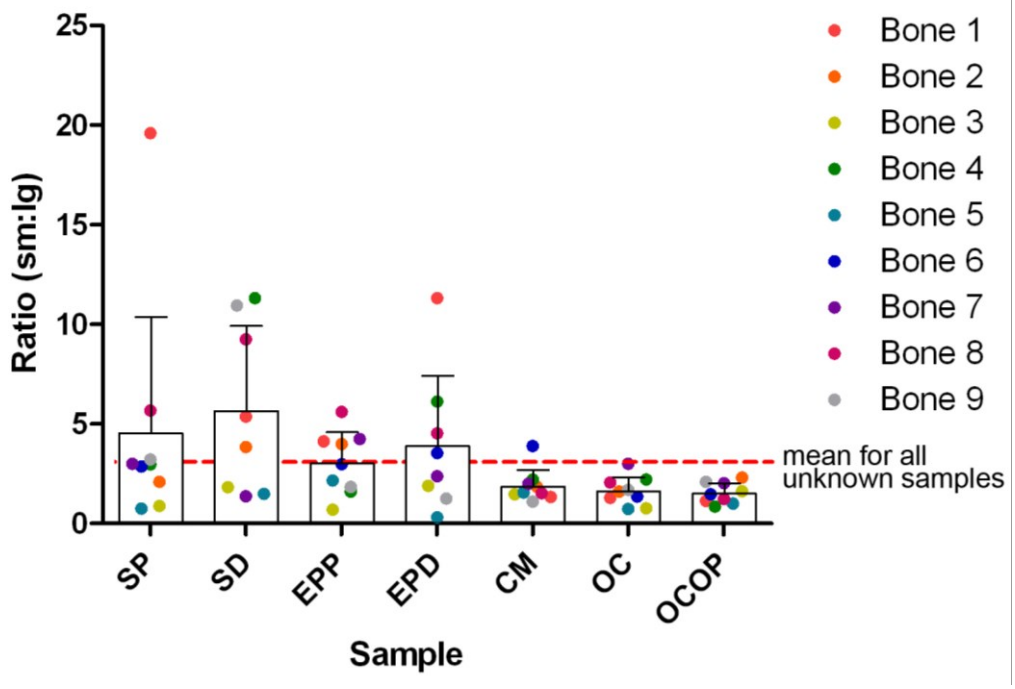
Sunlight, humidity, extreme heat, and soil contaminants are capable of breaking full-length segments of genomic material into random fragments, a process referred to as DNA degradation. Degradation can occur in varying degrees and may or may not inhibit PCR amplification. Determining the degree of degradation, if any, within a sample was done by calculating the ratio of DNA yield in a large molecular weight amplicon to that of a small molecular weight amplicon; samples failing to amplify either locus were omitted from this portion of the study. Specifically, a degradation ratio was calculated by dividing the yield value determined in the *Bos* Small AMEL by the value determined in the *Bos* Large AMEL:

$$\text{Degradation Ratio} = \frac{\textit{Bos Small AMEL}\ (\text{pg/uL})}{\textit{Bos Large AMEL}\ (\text{pg/uL})}$$

A degradation ratio close to 1.0 indicates approximately equal amplicon abundance regardless of length, while a value greater than 1.0 indicates an imbalance of small amplicon versus large amplicon suggesting genomic degradation.

Sixty-three bone samples were quantified using RT-qPCR as described in the Methods section. Because two samples failed to amplify the *Bos* Large AMEL locus, only 61 samples were eligible for degradation analysis. The range of degradation ratios for the 61 samples was 0.30─19.60, with a mean of 3.12 (±3.31) (Figure 9).

Figure 9. Mean DNA degradation ratios of each tissue type-RT-qPCR.



Mean yield of DNA for each tissue type sampled: spongy proximal (SP), spongy distal (SD), epiphyseal plate proximal (EPP), epiphyseal plate distal (EPD), compact medial (CM), osteoclasts (OC), osteoclasts + osteoprogenitor cells (OCOP). The bleached bone group is comprised of bones #1-2,7-9; the fresh bone group is comprised of bones #3-4; the buried bone group includes bones #5-6.

The mean degradation ratio of each bone group was not significantly different from the mean of all unknowns in the study. However, the mean of the bleached group

was significantly more degraded than the buried group (p=0.01). The mean degradation values for the fresh and buried groups were not significantly different (Table 7).

Table 7. Group mean degradation ratios-RT-qPCR.

Group	Mean ratio (±STD)	Range	N
Bleached[a]	3.82 (±3.83)	1.09─19.60	34
Fresh	2.60 (±2.86)	0.69─11.32	14
Buried[a]	1.85 (±1.14)	0.30─3.89	13
Note: Groups with like superscript notations are significantly different (p=0.01)			

Individual bones displaying the least degradation (ratio<1.0) belonged to the fresh and buried groups only (Table 8). Furthermore, the least degraded samples represented every tissue type suggesting that one type is neither protected nor susceptible to genomic degradation. With the exception of one, all bones exhibiting degradation ratios greater than 10.0 belonged to the bleached group (9SD, 4SD, 1EPD, 1SP). Unlike well-preserved samples, severely degraded samples were primarily spongy bone: 18 of the 20 most degraded samples are spongy.

Table 8. Least degraded samples-RT-qPCR.

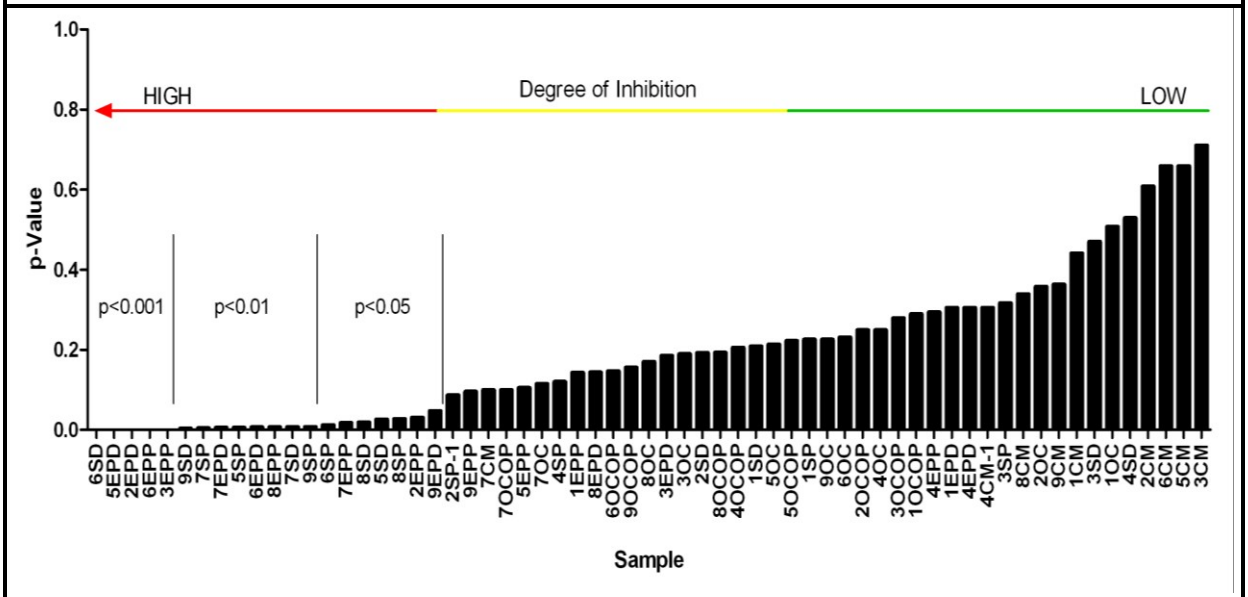
Rank	Bone	Group	Degradation Ratio
1.	5EPD	Buried	0.30
2.	3EPP	Fresh	0.69
3.	5OC	Buried	0.73
4.	5SP	Buried	0.75
5.	3OC	Fresh	0.77
6.	4OCOP	Fresh	0.85
7.	3SP	Fresh	0.88

4.2.3 PCR Inhibitor Presence

Every sample, including standard DNA used for the quantification curve, was co-amplified with the pRL plasmid. The C_T values of the pRL amplicon in each unknown sample was compared using a student's T-test to the mean C_T value of 54 reactions that utilized standard *Bos indicus* template DNA ($\bar{x}$=25.01, STD±0.30). All 63 bone samples in the experiment amplified the pRL product and therefore 100% of samples were analyzed for PCR inhibition, including those that suggested inhibitor presence in earlier portions of the project, i.e. failed AMEL amplification. The student's T-test reported a significant difference ($P<0.05$) in 20 of the samples, suggesting a high degree of PCR inhibitor presence (Figure 10).

All of the 20 samples displaying significant PCR inhibition are spongy bone samples: 50% spongy, 50% epiphyseal plate spongy, 0% compact bone (Figure 10). Moreover, the four least inhibited samples are compact medial bone specimens, 2CM, 6CM, 5CM, 3CM ($P>0.60$).

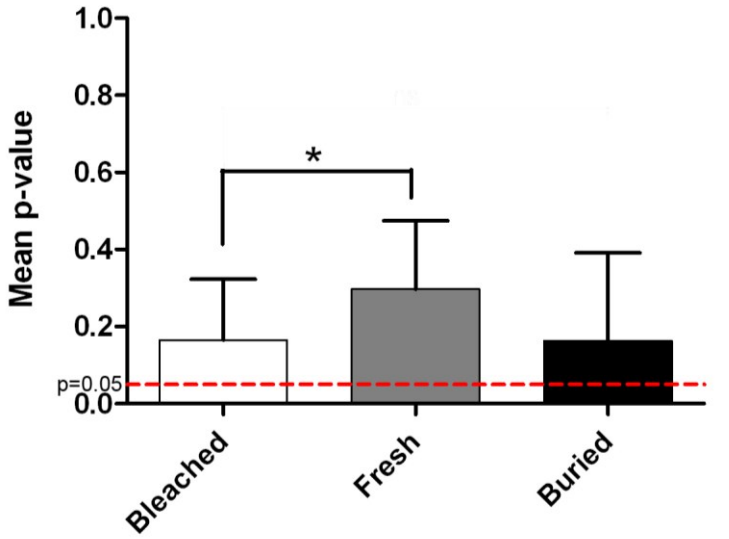
Figure 10. Significant inhibition of pRL activity-RT-qPCR



The resulting p-value for each sample pRL C_T versus standard pRL mean C_T (25.01±0.30). Samples with P<0.05 possess significant PCR inhibition. Samples with P>0.05 exhibit less inhibition, indicating the presence of fewer PCR inhibitors in the sample.

The significant inhibition of PCR by DNA extracted from spongy bones within a group had no effect on the respective group's mean inhibition (mean P>0.05) (Figure 11). Furthermore, no difference is seen between bleached and buried bone groups, or fresh and buried bone groups. Yet the fresh bone group is significantly less inhibited than the bleached group (p=0.02).

Figure 11. Significant PCR inhibition-RT-qPCR



The mean p-value for each group is plotted and indicates that none of the groups as a whole are significantly inhibited (P>0.05). However, there is significantly more PCR inhibitor presence seen in the bleached group than the fresh group (p=0.02). Mean p-value: Bleached (0.17±0.16), Fresh (0.30±0.18), Buried (0.16±0.23).

IV. Discussion

Skeletal remains recovered from crime scenes are often less than pristine and usually present a number of obstacles for forensic DNA analysts. Casework in the modern forensic laboratory often strives to identify such remains by use of STR or mtDNA typing technologies that require a certain minimal quantity and quality for template DNA . Several studies have aimed to improve methods quantitating DNA, assessing degradation, and diluting PCR inhibitor presence, as well as identifying specific skeletal elements best suited for forensic profiling. However, a study reporting on the strict use of long bones has not yet been published. The goal of this study was to localize a single source of genomic material that provided the most abundant, intact and pure DNA. The strategy for locating such a source included sampling spongy and compact tissues from five areas of *Bos indicus* femora.

5.1 DNA Yield

Two methods of quantification were utilized in this study 1) Q-TAT and 2) RT-qPCR. The two techniques vary largely in that Q-TAT is an end-point PCR design[21,23] and RT-qPCR measures the accumulation of template throughout the exponential phase of the amplification process.[32] RT-qPCR proved to be a more robust assay for quantifying DNA yield than Q-TAT (97% versus 44% of samples amplified respectively) Reasons for the variance in sensitivity may include instrumentation detection abilities and reagent

efficiency and susceptibility to PCR inhibition. Reaction buffers, polymerase enzymes, and primer efficiencies may have some effect on sample amplification. Careful consideration and planning was taken to minimize variability whenever possible, therefore leading to the use of identical oligo sequences between assays. Furthermore, both assays underwent thorough optimization of primer and/or probe multiplexing. Although the optimal conditions between assays vary, both demonstrated balanced primer binding efficiencies. Described later, Q-TAT appeared to be more affected by PCR inhibitors, therefore suggesting a possible reason as to why the majority of samples failed to amplify all together.

Because of the heightened sensitivity seen with RT-qPCR more bone samples were quantified and subsequently eligible for degradation and inhibition studies. Table 9 displays cumulative results obtained from both assays and all three categories of interest. Compact bone appears to be the best source for consistently obtaining enough DNA extract for forensic applications, regardless of a few instances where yield was less in compact bone than spongy bone. Furthermore, spongy bone sampled from the epiphyseal plate repeatedly presented the lowest DNA yield.

Table 9. Mean results produced by Q-TAT and RT-qPCR assays

		Q-TAT			RT-qPCR		
		Mean Yield (pg/mg)	**Mean Degradation Ratio**	**Mean Inhibition (%)**	**Mean Yield (pg/mg)**	**Mean Degradation Ratio**	**Mean Significant Inhibition (p-value)**
Bleached	Spongy (head)	14.36	1.75	83.61	8.34	6.43	0.078
	Spongy (Epiphyseal plate)	n/a	n/a	n/a	6.37	4.36	0.079
	Compact	36.68	1.87	37.91	18.20	1.75	0.281
Fresh	Spongy (head)	35.42	13.66	62.16	16.31	4.25	0.359
	Spongy (Epiphyseal plate)	n/a	n/a	n/a	6.88	2.58	0.196
	Compact	26.79	1.06	54.54	23.53	1.52	0.323
Buried	Spongy (head)	n/a	n/a	n/a	11.34	1.70	*0.011
	Spongy (Epiphyseal plate)	n/a	n/a	n/a	4.48	2.25	*0.028
	Compact	19.77	1.07	79.72	11.67	1.67	0.355

Significant mean PCR inhibition is indicated by (*), p-value<0.05.

5.2 DNA Degradation

The ratio of mean DNA yield produced by the small and large molecular weight loci provided a measure of genomic degradation. Again, samples that failed to amplify could not be accessed for degradation.

In Q-TAT, spongy bone isolated from the epiphyseal plate failed to amplify 100% of the time. Also in Q-TAT, spongy bone isolated from the epiphyseal head amplified in the bleached and fresh groups and provided degradations ratios of 1.75 and 13.66 respectively (Table 9). Compact bone analyzed by Q-TAT provided degradation ratios near 1.0 in the fresh and buried groups and 1.9 in the bleached groups. Results obtained by RT-qPCR are more complete demonstrating a conclusion that in general compact bone

is a better choice than spongy when considering degradation.[1] In spongy samples that yielded results using both assays, a distinct variation in degradation ratios is seen. In contrast, degradation ratios within compact bone samples remain consistent between the assays, ranging between 1.0 and 1.9.

According to a recent study by Smith[33] "samples containing degradation ratio values between 0.5 and 1.67 produced optimally amplified [human identification] data in which all the loci contained peaks of near equal signal intensity throughout the locus-to-locus size range."[33] Human samples displaying ratios greater than 1.70 exhibit a noticeable decrease in signal intensity of the larger molecular weight STR loci consistent with the genomic template being partially degraded. Using the threshold set forth by Smith, the majority of spongy bone samples are too degraded for forensic application, as evidenced primarily by RT-qPCR results (Table 9). All samples analyzed using Q-TAT produced a mean ratio of 1.55, and 3.12 using RT-qPCR. The increased ratio in RT-qPCR may be explained by the inclusion of spongy samples in the statistic, whereas spongy samples were absent from the Q-TAT mean ratio calculation.

[1] RT-qPCR results indicated that the least degraded individual bone samples consisted of spongy and compact bone. However, the broad conclusion that compact bone DNA is more intact than spongy bone DNA is made based on the mean results obtained from QTAT and RT-qPCR assays combined (Figure 9).

5.3 PCR Inhibitor Presence

The pRL plasmid was added at a constant concentration to each master mix, thereby amplifying the same number of copies in each reaction. When PCR inhibitors are absent, the RFU peak height or C_T is nearly constant depending on the quantitation method. When PCR inhibitors are present in a sample, pRL signal is diminished. Even in samples where pRL signal was partially reduced, PCR inhibition was found to be significant (Figure 10).

This study demonstrated that Q-TAT is more sensitive to PCR inhibition than RT-qPCR; total pRL dropout occurred in 43% and 0% of samples respectively. Out of 63 total samples assayed with Q-TAT, 42 amplified the pRL locus, 7 of which failed to co-amplify one or more AMEL loci. As was the case in DNA yield and degradation portions of this study, 78% of spongy bone samples failed to amplify the pRL plasmid in the Q-TAT assay. Where data is available, compact bone presented less pRL inhibition than spongy bones (Table 9). This conclusion is further supported by RT-qPCR results. Twenty samples showed significant pRL inhibition, 100% of those samples isolated from spongy bone sources (Figure 10).

A reason why spongy bone is more prone to PCR inhibition is not known. However, the microarchitecture of spongy tissue may harbor contaminates which are co-isolated during DNA extraction and purification. The phenol-chloroform isolation method used in this study may not be effective at entirely removing certain contaminates such as humic acid and urea.[34]

Rabe[18] recently reported a correlation between diminished pRL signal in PCR reactions containing high levels of input DNA.[18] The author suggested that competition for reagents in the reaction attributed to the decrease in pRL as more amelogenin product accumulates. As seen in Figure 3 and Figure 7, pRL signal remained relatively constant regardless of input DNA concentration, confirming that any change in internal pRL signal is indicative of inhibition.

5.4 Correlation Analysis

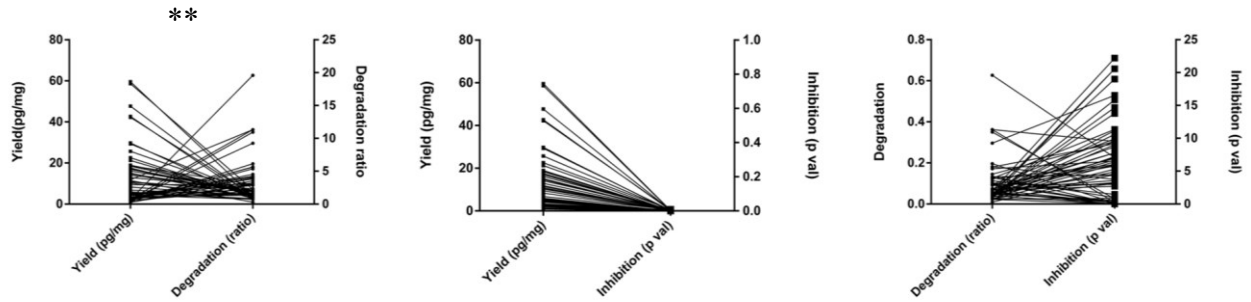
As evidenced by the RT-qPCR data, a trend between yield and degradation was suspected. Correlation analyses using GraphPad Prism (La Jolla, CA) statistical software was performed comparing combinations of the characteristics measured (i.e.Yield vs. Degradation) in the study using all eligible samples in the following groups:

1. All unknown samples, n=60 (Figure 12)
2. Bleached group, n=34 (Figure 13)
3. Fresh group, n=13 (Figure 14)
4. Buried group, n=13 (Figure 15)

A correlation with statistical significance was strictly observed when yield and degradation were compared in the analysis of all samples in the study (Figure 12) and those in the bleached group (Figure 13). The correlation coefficients, r, for the comparisons resulting in statistical significance display an inverse correlation, suggesting that as the yield of the sample increases the degradation ratio decreases. This interpretation seems logical since the design of the study measures yield based on the

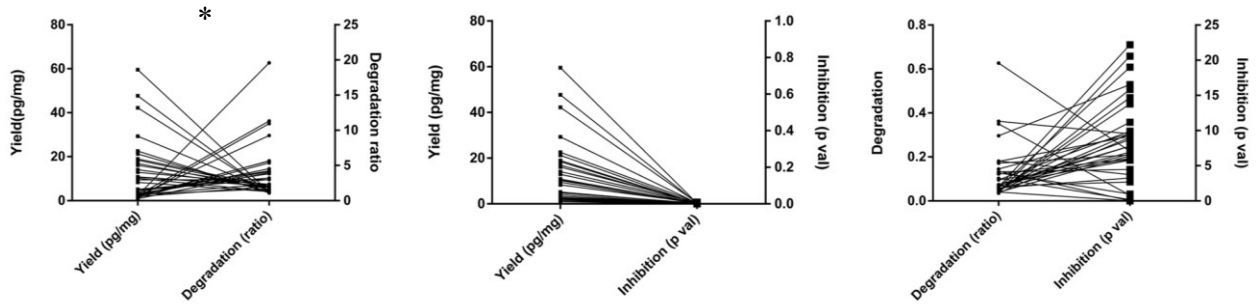
mean of high and low molecular weight loci. A sample with a low degradation value will likely yield more mean DNA since both loci are theoretically amplified with equal efficiency. The correlation analyses performed using data for the fresh and buried groups did not indicate a significant correlation between the components measured, most likely due to small sample numbers (n=13).

Figure 12. Pearson correlation analysis for all samples.



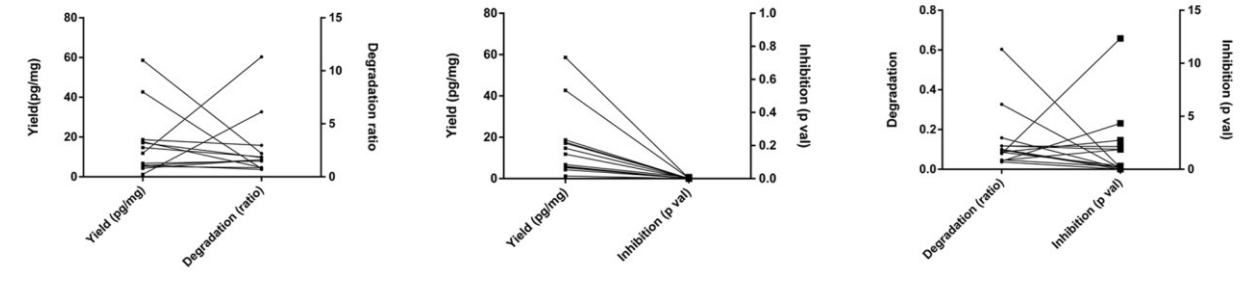
Pearson correlation analysis for all samples yielding results in the RT-qPCR portion of the study (n=60). Yield vs. Degradation, r=-0.335, p=0.008. Yield vs. Inhibition, r=0.115, p>0.05. Degradation vs. Inhibition, r=-0.048, p>0.05.

Figure 13. Pearson correlation analysis for bleached samples.



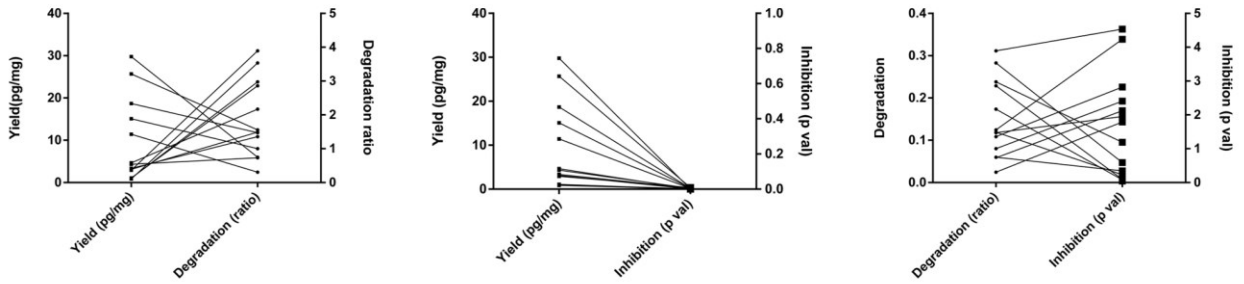
Pearson correlation analysis for bleached samples yielding results in the RT-qPCR portion of the study (n=34). Yield vs. Degradation, r=-0.4303, p=0.011. Yield vs. Inhibition, p>0.05. Degradation vs. Inhibition, p>0.05.

Figure 14. Pearson correlation analysis for fresh samples.



Pearson correlation analysis for fresh samples yielding results in the RT-qPCR portion of the study (n=13). Yield vs. Degradation, p>0.05. Yield vs. Inhibition, p>0.05. Degradation vs. Inhibition, p>0.05.

Figure 15. Pearson correlation analysis for buried samples.



Pearson correlation analysis for buried samples yielding results in the RT-qPCR portion of the study (n=13). Yield vs. Degradation, $p>0.05$. Yield vs. Inhibition, $p>0.05$. Degradation vs. Inhibition, $p>0.05$.

5.5 Areas of possible future investigation

Bovine long bones provided a good source of sample material for this study. Because characterized DNA is not commercially available, standard DNA was isolated from freshly drawn bovine blood. The *Bos indicus* blood used throughout the study performed as expected and in a manner comparable to human controls used previous studies.[18,33] Adding to the benefit of bovine as model is the readily available supply of skeletal elements. Group sample size varied between 2 and 5 bones per group with 7 excisions per bone. Due to time constraints, a larger sample size in each group was not utilized thereby allowing such an increase to be a great starting place for further studies.

Femora from yearling male cattle (steers) were the only bones collected. Additional research should be performed on alternate bovine long bones to check for consistency of the results.

Bones used in this study were collected within a 10-mile radius of one another and were therefore subjected to identical weather patterns. With the exception of bones scattered above or below ground level, temperature, humidity, precipitation, winds, and soil content was essentially the same for all groups. An interesting avenue to pursue might be additional environmental factors introduced when bones are recovered across a larger working distance, i.e. regions across the state. Additionally, collecting bones from the same general vicinity but with contrasting settings, perhaps shaded vs. direct sunlight or well drained vs. watershed, may provide new and different results.

During sampling, compact bone was crudely separated into layers of 1) osteoclasts and 2) osteoclasts containing osteoprogenitor cells using the naked eye and

Dremel sanding disc. An improved sampling technique that utilizes the use of a microscope and fine-tipped Dremel tool would likely prove beneficial and reduce/eliminate contamination between sample groups. Also, a close look at the microarchitecture of the bone by including additional cell types such as osteoblasts and osteocytes may produce interesting results relevant to the forensic science community.

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VITA

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Title of Study: A SYSTEMATIC STUDY OF DNA YIELD AND INTEGRITY IN BOVINE LONG BONES.

Major Field: Forensic Sciences

Abstract:

Scope and Method of Study: The purpose of this study was to identify a single source of bone that produced the most abundant, intact, and well-preserved deoxyribonucleic acid (DNA). The specific aim was approached through an experimental design using *Bos indicus* (the cow) femora presenting various degrees/modes of decomposition as sample material. Chips of bone were excised from seven locations on each femur including spongy and compact tissue types. DNA was isolated using procedures standard to functional forensic crime laboratories and subsequently quantified using Q-TAT and RT-qPCR assays. Furthermore, genomic degradation and the presence of PCR inhibitors for each sample was measured.

Findings and Discussion: Samples were successfully quantified using both Q-TAT and RT-qPCR technology and subsequently assessed for levels of degradation and PCR inhibition. Results from the Q-TAT assay suggested that spongy bone samples contained the presence of PCR inhibitors as evidenced by failed amplifications in 88% of reactions. Samples with failed amplification became ineligible for further analyses, making compact bone samples essentially the only tissues to produce data. The RT-qPCR assay amplified 97% of samples in the study allowing for a more comprehensive analysis of resulting data. Although not significant, compact bone samples yielded the most DNA and indicated low levels of genomic degradation and PCR inhibitors. Resulting data for compact bone samples was consistent across test groups, however variation in spongy bone was observed between bone groups. This finding suggests, that in general, compact bone is an ideal source for forensic analysis, however additional considerations can be made if spongy bone must be used.

Advisor's Approval: _____

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