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APPLICATION OF PROTEINASES FOR DNA ISOLATION OF

BONE SPECIMENS

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ABSTRACT

One of the greatest challenges when attempting to identify victims of mass fatality
incidents is the analysis of DNA from bone. The bone is more difficult to process and
extract for DNA. Additionally, the initial cleaning and sampling of the bone sample is a
labor-intensive and time-consuming step prior to isolating DNA. Thus, it is difficult to
adapt the current method for automation. In addition, quantities of specimens recovered
may be too small to properly isolate the DNA. To obtain an adequate quality and
quantity of DNA templates, strategies to improve the yield of DNA isolation are needed.
To address these issues, we conducted the following studies consisting two projects.

***Developing a simple sample processing method for DNA isolation from bone
specimens.*** We have adapted the trypsin bone maceration method for processing bone
samples prior to DNA isolation. Our results demonstrated that this method is effective
for the removal of soft tissues and the outer surface of bone fragment samples. The Short
Tandem Repeat (STR) analysis revealed that no adverse effect on DNA profile was
detected after trypsin treatment. The yield of DNA isolated from trypsin-treated bone
samples was sufficient for STR analysis. However, the DNA yield of trypsin-treated
bone samples was lower than that of untreated bone samples. Our data suggest that this
trypsin method is an alternative cleaning method to physical cleaning procedures, such as
sanding. This method potentially has a low risk of cross-contamination between samples
and diminishes safety concerns for laboratory analysts due to exposing bone powder.
This method could be adapted for automated DNA isolation for human identification of
bone samples, namely, from mass fatality incidents.

***Developing a high-yield DNA isolation method using proteinases for bone
specimens.*** It is known that the matrix protein network plays an important role in the
structure of the bone. The matrix could be digested by a number of proteinases. The
hypothesis of the study is that the digestion of the matrix protein network by application
of proteinases would lead to a degradation of the physical barrier surrounding the
osteocytes, thus facilitating DNA extraction. Our study revealed that the
clostridiopeptidase A is potent for bone degradation. The application of
clostridiopeptidase A can achieve speedy and better bone degradation. The STR analysis
revealed that no adverse effect on DNA profiles was detected after clostridiopeptidase A
treatment. Our results demonstrated that this method improved the DNA yield of bone
samples.

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EXECUTIVE SUMMARY

1. Developing a Simple Sample Processing Method for DNA Isolation from Bone Specimens

State of the Problem

Skeletal remains are challenging biological samples for DNA isolation since bone samples are difficult to process. The initial cleaning and sampling of the bone, a labor-intensive and time-consuming step, is required prior to isolating DNA from the bone samples. Due to the potential of having co-mingled remains, contamination by physical contact, environment-borne inhibitors, and bacterial contamination that interferes with forensic DNA analysis, the outer surface of the bone fragment must be cleaned by using a current method such as sanding. However, to avoid cross-contamination between samples, the bone dust generated by sanding the bone must be cleaned and removed. Additionally, special safety protection equipment and procedures are necessary to protect laboratory personnel from exposure to blood-borne pathogens.

Purpose of the Study

To address these issues, we have developed a simple processing method using trypsin solution (Li *et al*., in press). Trypsin, secreted in the digestive system, is a proteolytic enzyme which breaks down proteins, a process referred to as proteolysis. Trypsin was chosen due to its ability to degrade various types of proteins. Trypsin also has been utilized in enzymatic maceration methods for processing bone samples in anthropological laboratories. In our previous study, the trypsin maceration technique was adapted to the sample processing method prior to DNA isolation from bone samples. The soft tissue and outer surface of the bone fragment samples can be removed by trypsin treatment (Figure 1). Our data suggest that this method can be used in the initial sample preparation for cleaning the outer surface of human bone samples prior to DNA isolation. In this study, the application of the sample processing method for DNA isolation was further studied: 1) the effect of trypsin treatment on DNA yield, and 2) the effect of trypsin treatment on the quality of DNA isolated.

Method and Research Design

The Effects of Trypsin on Surface Cleaning of the Bone Sample. To determine the optimum incubation time for the standard assay, trypsin activity was compared after various lengths of incubation. Experiments were prepared by placing a piece of bone fragment (approximately 1 g) in trypsin solution. The trypsin treatment was carried out at various lengths of time. At set time points of incubation, the effect of trypsin treatment was visualized using light microscopy and Scanning Electron Microscopy.

The Effect of Trypsin Treatment on DNA Yield. To find out if this trypsin treatment method causes any adverse effect on DNA yield, the following studies were conducted: 1) The yield of DNA isolation with or without trypsin treatment was compared, and 2) the effect of various durations of the trypsin treatment on DNA yield was studied. The trypsin-processed bone fragments were pulverized, and 0.1 g of pulverized bone sample was decalcified and then was used for DNA isolation. DNA

quantitation was performed, and the DNA yield isolated was expressed as µg DNA/0.1 g bone.

   The Effect of Trypsin Treatment on the quality of DNA isolation. Experiments were conducted to find out if this processing method caused any adverse effect on DNA quality, such as DNA degradation. The DNA isolated from above was evaluated by Short Tandem Repeat (STR) analysis. Thus, the quality of the DNA isolated was evaluated.

Findings and Conclusions

   Discussion of findings. We have developed a simple sample processing method for bone specimens for DNA isolation. In this study, the surface cleaning of the bone samples (human radius, within 7 days postmortem) was achieved by the application of trypsin solution (30µg/µl). Light microscopy and Scanning Electron Microscopy (SEM) results indicated that 2-hr trypsin treatment was sufficient to remove surface materials of bone samples. The STR analysis revealed that no adverse effect on DNA profile was detected after trypsin treatment. The yield of DNA isolated from trypsin-treated bone samples (0.8 – 1.4 µg DNA/0.1 g bone) was sufficient for subsequent STR analysis. However, the DNA yield of trypsin-treated bone samples was lower than that of untreated bone samples (2.0 µg DNA/0.1 g bone).

   Implications for policy and practice. We have adapted the trypsin bone maceration method for processing bone samples prior to DNA isolation. Our results demonstrated that this method was effective for the removal of soft tissues and the outer surface of bone samples. Our data suggested that this method could be used in the initial sample preparation for cleaning the outer surface of human bone samples prior to DNA isolation. This trypsin method is an alternative cleaning procedure to physical cleaning procedures, such as sanding. Trypsin is reasonably inexpensive; thus it is practical to utilize a large volume of trypsin solution to treat various sizes of bone samples without cutting. This method potentially has a low risk of cross-contamination between samples and diminishes safety concerns for laboratory analysts due to exposing bone powder. This method could be adapted for automated DNA isolation for human identification of bone samples, namely, from mass fatality incidents.

   Implications for further research. Fresh human bone samples were used in this study as proposed. However, additional studies are needed to examine the yield and quality of the DNA isolated from aged bone samples. In addition, the yield and quality of the DNA isolated from bone samples from various environmental conditions such as heat, moisture and decomposition should also be studied.

2. Developing a High-yield DNA Isolation Method Using Proteinase for Bone Specimens

State of the Problem

   One of the greatest challenges when attempting to identify victims of mass fatality incidents is the analysis of DNA from bone (Holland *et al*., 2002; Lawler, 2001). The bone is more difficult to process for extracting DNA. In addition, quantities of samples recovered may be too small to properly isolate sufficient amounts of DNA. To obtain an

adequate quality and quantity of DNA templates, strategies to improve the yield of DNA isolation are needed.

The majority of the DNA in bone is located in the osteocytes (Hochmeister *et al.*, 1991). Frost (1961) and Martin *et al.* (1989) estimated that there are 20,000 to 26,000 osteocytes per cubic millimeter of calcified bone matrix. Hochmeister *et al.* (1991) estimated that microgram quantities of DNA could potentially be extracted from a gram of bone. Thus, bone tissue should contain sufficient DNA for analysis. However, the skeletal fragments recovered from mass fatality incidents often have experienced a series of decomposition changes. High humidity and temperatures are factors that affect the degradation rate of DNA (Perry *et al.*, 1988). Decomposition may be expected to significantly degrade both chromosomal and mitochondrial DNA. The identification of partial DNA profiles or a failure to obtain a DNA profile was reported from the World Trade Center case (Shaler, 2002; Prinz, 2002).

The osteocytes containing DNA are embedded in a calcified matrix. The calcified matrix is a barrier preventing isolation of the DNA in the osteocytes during the extraction process. Therefore, it is necessary to remove the matrix to improve the yield of DNA. The application of proteinase K by a number of groups is another approach to digest the matrix barrier (Perry *et al.,* 1988; Cattaneo *et al.,* 1995 and 1997; Hochmeister *et al.*, 1991). It is expected that the proteinase K could digest the bone matrix and thus may increase the yield of DNA harvested. However, limited knowledge of the effect of proteinase on the yield of DNA isolation from bone samples is available in the literature. Thus, the effect of the proteinase on the yield of DNA isolation is yet to be characterized.

Purpose of the Study

To address the issue as described above, we have conducted this study to characterize the effect of proteinase on the yield of DNA isolation from bone samples. It is known that the matrix protein network plays an important role in the structure of the bone. The matrix could be digested by a number of proteinases (Wang *et al.*, 2001). The hypothesis of this study is that the digestion of the matrix protein network by application of proteinases would lead to a degradation of the physical barrier surrounding the osteocytes, thus facilitating DNA extraction (Figure 2). We are interested in identifying the proteinases for digesting the matrix of bone tissue and optimizing proteinase treatment. In particular, collagenases are known for playing a role in the degradation of the bone matrix proteins (Mallya et al., 1992). Clostridiopeptidase A is one of the most potent collagenase; thus it was chosen for this study. In this study, 1) the characterization of the effect of clostridiopeptidase A on bone degradation was carried out and 2) the characterization of the effect of collagenase treatment on DNA yield and quality from bones was conducted.

This study has developed a modified DNA isolation method for bone tissue. The method increased the yield of isolated DNA. An increased yield of isolated DNA should provide more copies of DNA templates from bone samples. Therefore, it may increase the success rate of generating the genotype profiles of DNA analysis.

Method and Research Design

Characterization of the effect of clostridiopeptidase A on bone degradation. To determine the optimum incubation time for the standard assay, clostridiopeptidase A

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activity was compared at various incubation periods. The procedures for clostridiopeptidase A digestion was carried out based on the manufacturer's protocols. Pulverized bone samples (0.1 g) was decalcified and incubated with 0.3 ml clostridiopeptidase A solution (5 mg/ml) at 37°C with gentle agitation. Bone degradation by clostridiopeptidase A was measured according to the following procedure. The reaction was initiated by adding clostridiopeptidase A solution to the bone sample. After incubation, samples were centrifuged and the supernatant was removed. The remaining amount of undigested bone powder was measured. Bone degradation activity was measured as a reduction in the mass of bone powder after the clostridiopeptidase A digestion.

Characterization of the effect of collagenase treatment on DNA yield from bones. The application of collagenase selected on DNA isolation was studied. DNA quantitation was performed using the real-time PCR method. The yield of DNA isolated was calculated and was expressed as µg DNA/0.1 g bone. Since proteinase K is currently used in bone DNA isolation by a number of laboratories, the DNA yield using proteinase K was compared with the DNA yield using clostridiopeptidase A.

Characterization of the Effect of Clostridiopeptidase A treatment on the quality of DNA isolation. Experiments were conducted to find out if this clostridiopeptidase A treatment caused any adverse effect on DNA quality, such as DNA degradation. The DNA isolated from above was evaluated by STR analysis. Thus, the quality of the DNA isolated was evaluated.

Findings and Conclusions

Discussion of findings. Our study revealed that the clostridiopeptidase A is potent for bone degradation. The bone degradation activity was observed after 1-hr of clostridiopeptidase A treatment.   0.1 g of pulverized and decalcified bone sample was completely digested with 1-hr clostridiopeptidase A treatment followed by a 10-min proteinase K treatment. Thus, the entire digestion time is 1 hr and 10 min. The STR analysis revealed that no adverse effect on DNA profiles was detected after clostridiopeptidase A treatment. The yield of DNA isolated from clostridiopeptidase A-proteinase K treated bone samples was 3.5 µg DNA/0.1 g bone. The DNA yield was higher than that of clostridiopeptidase A untreated bone samples (2.0 µg DNA/0.1 g bone).

Implications for policy and practice. This method can achieve speedy and better bone degradation by the application of clostridiopeptidase A-proteinase K testament. First, this method reduces the digestion time. The entire digestion time is 1 hr and 10 min, which is less than that of conventional methods (6-8 hr). Second, our results demonstrate that this method improved the DNA yield of bone samples. An increased yield of isolated DNA provides more copies of DNA templates from bone samples. Therefore, it may increase the success rate of generating genotype profiles of DNA analysis. This method could be applied by forensic laboratories for DNA analysis of skeletal samples especially when small quantities of specimens are recovered.

Implications for further research. Fresh human bone samples were used in this study as proposed. Additional studies focusing on the yield and the quality of DNA isolated from aged bone samples are needed. The yield and the quality of DNA isolated

from bone samples from various environmental conditions such as heat, moisture and decomposition should also be studied.

I. INTRODUCTION

Human remains undergo a series of changes during decomposition. The rate of degradation of human remains varies greatly with environmental conditions (climate, bacterial growth, and insect and animal scavengers). After a period of time, soft tissues may be lost, while bone tissue may remain stable. Forensic scientists are called upon to attempt to identify human skeletal remains in a variety of situations, including mass fatality incidents, identification of the skeletal remains of military personnel recovered from war, fires, explosions, and in cases involving the identification of skeletal remains in criminal matters. A number of methods are used to identify human remains. The most common of these methods includes identification of facial characteristics, recognition of individualizing scars, marks, or other special features, matching dentition with premortem dental X-rays, or by fingerprint comparisons. In many situations, these methods cannot be used because of extensive putrefaction or destruction of the remains. The mass fatality terrorist attack on the World Trade Center provides a prime example of which common methods for identification may not be useful. Large quantities of compromised human skeletal fragments were recovered at the fatality site. In this type of case, DNA typing is a powerful tool for identifying human remains (Shaler, 2002).

One of the greatest challenges when attempting to identify victims of mass fatality incidents is the analysis of DNA from bone (Holland *et al*., 2002; Lawler, 2001). The bone is more difficult to process for extracting DNA. In addition, quantities of samples recovered may be too small to properly isolate sufficient amounts of DNA. To obtain an adequate quality and quantity of DNA templates, strategies to improve the yield of DNA isolation are needed. To address these issues, we conducted the following studies consisting two projects.

1. Developing a Simple Sample Processing Method for DNA Isolation from Bone Specimens

The initial cleaning and sampling of the bone, a labor-intensive and a time consuming step, is required prior to isolating DNA from bone samples. Due to the potential of having co-mingled remains, contamination by physical contact, environment-borne inhibitors, and bacterial contamination that interferes with forensic DNA analysis, the outer surface of the bone fragment must be cleaned by using a physical method such as sanding. However, to avoid cross-contamination between samples, the bone dust generated by sanding the bone must be cleaned and removed. Additionally, special safety protection equipment and procedures are necessary to protect laboratory analysts from exposure to blood-borne pathogens. Additionally, the sanding method is difficult to be adapted for automation.

To address these issues, we have developed a simple processing method using trypsin solution (Li *et al*., in press). Trypsin, secreted in the digestive system, is a proteolytic enzyme which breaks down proteins, a process referred to as proteolysis. Trypsin was chosen due to its ability to degrade various types of proteins (Buck *et al*., 1962; Walsh, 1970). Trypsin also has been utilized in enzymatic maceration methods for processing bone samples in anthropological laboratories (Hangay and Dingley, 1985; Hendry, 1999). In our previous study, the trypsin maceration technique was adapted to

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the sample processing method prior to DNA isolation from bone samples. The soft tissue and outer surface of the bone fragment samples can be removed by trypsin treatment. Our data suggest that this method can be used in the initial sample preparation for cleaning the outer surface of human bone samples prior to DNA isolation. In this study, the application of the sample processing method for DNA isolation was further studied: 1) the effect of trypsin treatment on DNA yield, and 2) the effect of trypsin treatment on the quality of DNA isolated.

2. *Developing a High-yield DNA Isolation Method Using Proteinase for Bone Specimens*

The majority of the DNA in bone is located in the osteocytes (Hochmeister *et al*., 1991). Frost (1961) and Martin *et al*. (1989) estimated that there are 20,000 to 26,000 osteocytes per cubic millimeter of calcified bone matrix. Hochmeister *et al*. (1991) estimated that microgram quantities of DNA could potentially be extracted from a gram of bone. Thus, bone tissue should contain sufficient DNA for analysis. However, the skeletal fragments recovered from mass fatality incidents often have experienced a series of decomposition changes. High humidity and temperatures are factors that affect the degradation rate of DNA (Perry *et al*., 1988). Decomposition may be expected to significantly degrade both chromosomal and mitochondrial DNA. The identification of partial DNA profiles or a failure to obtain a DNA profile was reported from the World Trade Center case (Shaler, 2002; Prinz, 2002).

The osteocytes containing DNA are embedded in a calcified matrix. The calcified matrix is a barrier for access to the DNA in the osteocytes during the extraction process. Therefore, it is necessary to remove the matrix to improve the yield of DNA. The application of proteinase K by a number of groups is one approach to digest the matrix barrier (Perry *et al.,* 1988; Cattaneo *et al.,* 1995 and 1997; Hochmeister *et al*., 1991). It is expected that the proteinase K could digest the bone matrix, and thus may increase the yield of DNA harvested. However, limited knowledge of the effect of proteinase on the yield of DNA isolation from bone samples is available in the literature. Thus, the effect of the proteinase on the yield of DNA isolation is yet to be characterized.

To address the issue as described above, we have conducted this study to characterize the effect of proteinase on the yield of DNA isolation from bone samples. It is known that the matrix protein network plays an important role in the structure of the bone. The matrix could be digested by a number of proteinases (Wang *et al.*, 2001). The hypothesis of this study is that the digestion of the matrix protein network by application of proteinases would lead to a degradation of the physical barrier surrounding the osteocytes, thus facilitating DNA extraction. We are interested in identifying the proteinases for digesting the matrix of bone tissue and optimizing proteinase treatment. In particular, collagenases are known for playing a role in the degradation of the bone matrix proteins (Mallya *et al*., 1992). Clostridiopeptidase A is one of the most potent collagenase; thus it was chosen for this study. In this study, 1) the characterization of the effect of clostridiopeptidase A on bone degradation was carried out and 2) the characterization of the effect of collagenase treatment on DNA yield and quality from bones was conducted.

This study has developed a modified DNA isolation method for bone tissue. The method increased the yield of isolated DNA. An increased yield of isolated DNA should

provide more copies of DNA templates from bone samples. Therefore, it may increase
the success rate of generating the genotype profiles of DNA analysis.

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II. MATERIALS AND METHODS

Sample Preparation

Sampling was carried out in a sterilized laminar flow cabinet. The bone fragments (approximately 1 g) of human radius (within 7 day postmortem) were used for this study. Soft tissue on bone surface was removed using a surgical scraper. The bone fragments were rinsed with 100% ethanol and left to air-dry. The pulverized bone powder was prepared by using a cryogenic impact grinder (SamplePrep 6770 Freezer Mill, SPEX, Metuchen, NJ). The procedure was programmed according to the manufacturer's protocols: 10 min pre-cooling followed by 3 cycles (2 min grinding at rate of 20 impacts/s, and 2 min cooling).

Enzymatic Digestion

Clostridiopeptidase A was obtained from Sigma-Aldrich, and trypsin was obtained from Fisher Scientific.

Trypsin Digestion. The procedures for trypsin digestion were those developed previously (Li *et al*., in press). The standard assay employed approximately 1 g of bone fragment. The reaction was initiated by adding 5 ml of trypsin solution (30µg/µl) to the bone sample and then was incubated at 55°C with gentle agitation. After incubation, the supernatant was removed. The bone sample was rinsed with water and air dried. Bone degradation by trypsin was then observed using light microscopy and scanning electron microscopy. For trypsin treatment longer than 2 hr, the trypsin solution was replaced at every 2 hr-interval.

Clostridiopeptidase A Digestion. The procedures for clostridiopeptidase A digestion was carried out based on the manufacturer's protocols. Pulverized bone samples (0.1 g) was decalcified and incubated with 0.3 ml clostridiopeptidase A solution (5 mg/ml clostridiopeptidase A; 50 mM TES, pH 7.4; 0.36 mM $CaCl_2$) at 37°C with gentle agitation. Bone degradation by clostridiopeptidase A was measured according to the following procedure. The standard assay employed 0.1 g of pulverized bone powder. The reaction was initiated by adding clostridiopeptidase A solution to the bone sample. After incubation, samples were centrifuged at 7,000 x g for 1 min. The supernatant was removed. The remaining amount (wet mass) of undigested bone powder was measured. The bone degradation activity was measured as a reduction in the mass of bone powder after the clostridiopeptidase A digestion.

Scanning Electron Microscopy

For Scanning Electron Microscopy (SEM) observation, samples were dehydrated based on the standard procedures. The specimen was observed and photographed using a variable pressure scanning electron microscope (Vega 5136 mm).

DNA Isolation

DNA isolation was performed based on Isenberg (2005). 0.1 g of pulverized bone sample was decalcified by incubating with 1.6 ml 0.5 M EDTA (pH8.0) overnight at

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room temperature. The sample was then digested with 300 µl proteinase K (200 µg, Fisher Scientific) solution (10 mM Tris, 100 mM NaCl, 39 mM dithiothreitol, 10mM EDTA, 2% SDS; pH 8) at 56°C for 6 hr with gentle agitation. DNA isolation of bone samples was performed using the QIAamp® DNA Micro kit (QIAGEN) according to the manufacturer's protocols. The final volume of eluted DNA was 60 µl.

For samples to be digested with clostridiopeptidase A, 0.1 g of pulverized bone sample was decalcified by incubating with 1.6 ml 0.5 M EDTA overnight at room temperature. The sample was then digested with clostridiopeptidase A at 37°C for 1 hr with gentle agitation. After incubation, samples were centrifuged at 7,000 x g for 1 min. The supernatant was removed, and placed in a fresh microcentrifuge tube. The remaining undigested bone powder was further digested by 300 µl proteinase K (200 µg) solution (10 mM Tris, 100 mM NaCl, 39 mM dithiothreitol, 10mM EDTA, 2% SDS; pH 8) at 56°C for 10 min. DNA isolation of bone samples was performed using the QIAamp® DNA Micro kit (QIAGEN) according to the manufacturer's protocols. The final volume of eluted DNA was 60 µl.

DNA Quantitation

DNA quantitation was performed using the Quantifiler Human DNA Quantitation kit (Applied Biosystems) according to the manufacturer's protocols. DNA yield was expressed as µg DNA/0.1 g bone. Internal positive controls were used to monitor PCR inhibitors and negative controls and reagent blanks were included to monitor contamination.

STR Analysis

STR analysis was performed using the Amp*fl*STR Cofiler™ amplification kit (Applied Biosystems). DNA amplification was performed according to the manufacturer's protocols. Amplified products were separated on ABI 310 Genetic Analyzer (Applied Biosystems) and analyzed using GeneMapper® software with the allelic ladders provided by the manufacturer.

III. RESULTS

1. Developing a Simple Sample Processing Method for DNA Isolation from Bone Specimens

The Effects of Trypsin on Surface Cleaning of the Bone Sample

To determine the optimum incubation time for the standard assay, trypsin activity was compared after various lengths of incubation. The procedures for trypsin treatment were those described in the Materials and Methods. Experiments were prepared by placing a piece of bone fragment (approximately 1 g) in trypsin solution, and it was then incubated under the conditions described in the Materials and Methods. The trypsin treatment was carried out at 2, 4, 6, and 12 hr increments, respectively. At set time points of incubation, the effect of trypsin treatment was visualized using light microscopy.

The morphological examination of bone is to facilitate the evaluation of the removal of surface layers of bone samples. Human cortical bone contains structures called Haversian canals that contain blood vessels and nerves (Figure 3). The Haversian canals are connected to one another by Volkmann's canals (Figure 3) which run perpendicular to Haversian systems. Figure 4 shows the surface of an untreated bone sample. Exposure of the Volkmann's canals, due to removing of surface layers from bone samples, was observed after 2 hr trypsin treatment (Figure 5A). After longer incubation (4 -12 hr), more surface materials were removed as indicated by the observation of exposure of both the Volkmann's and the Haversian canals (Figure 5B-D). The maximal activity for trypsin was achieved at 12 hr (Figure 5D).

To further examine the surface cleaning effect of trypsin treatment, SEM was employed. After 2 hr of trypsin incubation, surface degradation of the bone sample was observed (Figure 6) as indicated by the observation of appearance of the periosteal zone or outer portion of bone. This showed that 2 hr trypsin treatment was sufficient to remove surface materials of bone samples.

The Effect of Trypsin Treatment on DNA Yield

To find out if this bone processing method using trypsin treatment causes any adverse effect on DNA yield, the following studies were conducted: 1) The yield of DNA isolation with or without trypsin treatment was compared, and 2) the effect of various durations of the trypsin treatment on DNA yield was studied. The trypsin-processed bone fragments were pulverized and 0.1 g of pulverized bone sample was decalcified and then was used for DNA isolation. DNA quantitation was performed and the DNA yield isolated was expressed as µg DNA/0.1 g bone. Our results showed the DNA yield of untreated bone was 2.0 µg DNA/0.1 g bone. In contrast, the DNA yield of trypsin-treated bone samples was lower (ranged from 0.8 to 1.4 µg DNA/0.1 g bone) than that of untreated bone samples (Figure 7).

The Effect of Trypsin Treatment on the Quality of DNA Isolation

Experiments were conducted to find out if this processing method had any adverse effect on DNA quality, such as DNA degradation. The quality of DNA isolated from above was evaluated by STR analysis according to the procedures described in the

Materials and Methods. Figure 8 shows successful DNA amplification with the AmpflSTR Cofiler^{TM} amplification kit and the DNA profiles of untreated and trypsin-treated bone samples. No adverse effect on DNA profile was detected after trypsin treatment.

2. Developing a High-Yield DNA Isolation Method Using Clostridiopeptidase A for Bone Specimens

The Effect of Clostridiopeptidase A on Bone Degradation

To determine the optimum incubation time for the standard assay, clostridiopeptidase A activity was compared at various incubation periods. Sampling procedures and proteinase treatment procedures were those described in the Materials and Methods. 0.1 g of pulverized bone sample was decalcified and incubated with clostridiopeptidase A for 0, 1, 2, 3, 4, 5, and 6 hr. Bone degradation by clostridiopeptidase A at various incubation durations was illustrated in Figure 9. The bone degradation activity occurred after 1, 2, 3, 4 and 5 hr of incubation. However, the highest activity for clostridiopeptidase A was achieved after 6 hr incubation (Figure 9). The maximum degradation might not have been achieved, since longer digestion times were not examined.

The Effect of Clostridiopeptidase A Treatment on DNA Yield from Bones

The application of clostridiopeptidase A on DNA isolation was studied. 0.1 g of pulverized bone sample was decalcified and was then used for DNA isolation. The bone sample was digested with clostridiopeptidase A for 1 hr. The clostridiopeptidase A-treated sample was further treated with proteinase K for 10 min according to the procedure described in the Materials and Methods. DNA was extracted, and the yield of DNA isolated was 3.5 µg DNA/0.1 g bone (the value was the mean of 3 determinations and standard deviation was 0.1).

Since proteinase K is currently used in bone DNA isolation by a number of laboratories (Perry *et al.,* 1988; Cattaneo *et al.,* 1995 and 1997; Hochmeister *et al*., 1991), the DNA yield using proteinase K was compared with the DNA yield using clostridiopeptidase A-proteinase K. Our results showed that the DNA yield of clostridiopeptidase A-proteinase K method was higher than that of proteinase K alone (2.0 µg DNA/0.1 g bone; the value was the mean of 3 determinations and standard deviation was 0.2).

The Effect of Clostridiopeptidase A Treatment on the Quality of DNA Isolation

Experiments were conducted to find out if the clostridiopeptidase A treatment had any adverse effect on DNA quality, such as DNA degradation. The quality of DNA isolated from above was evaluated by using STR analysis. Figure 10 shows successful DNA amplification with the AmpflSTR Cofiler^{TM} amplification kit and the DNA profiles of proteinase K and clostridiopeptidase A-proteinase K treated bone samples. No adverse effect on DNA profile was detected after clostridiopeptidase A-proteinase K treatment (Figure 10).
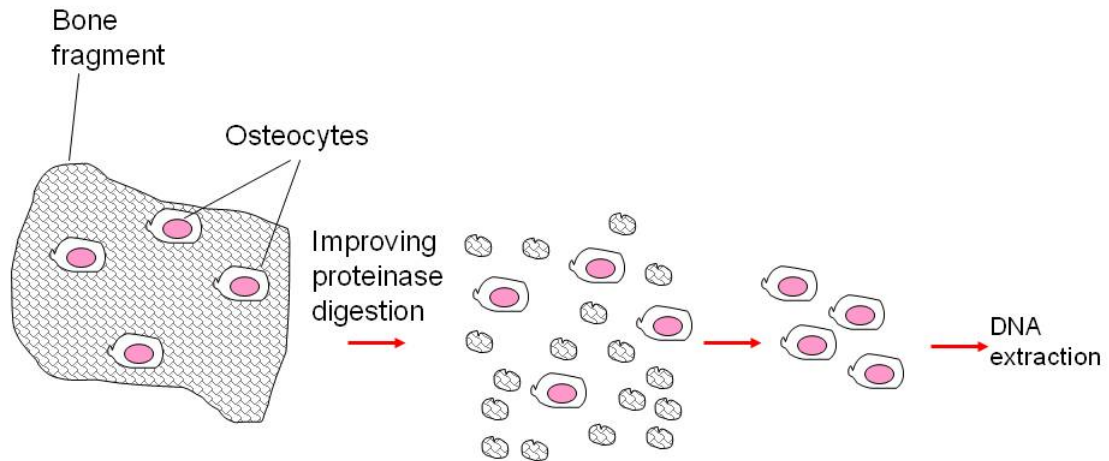
FIGURES

Figure 1. Schematic illustration of project 1
Developing a simple sample processing method for DNA isolation from bone specimens
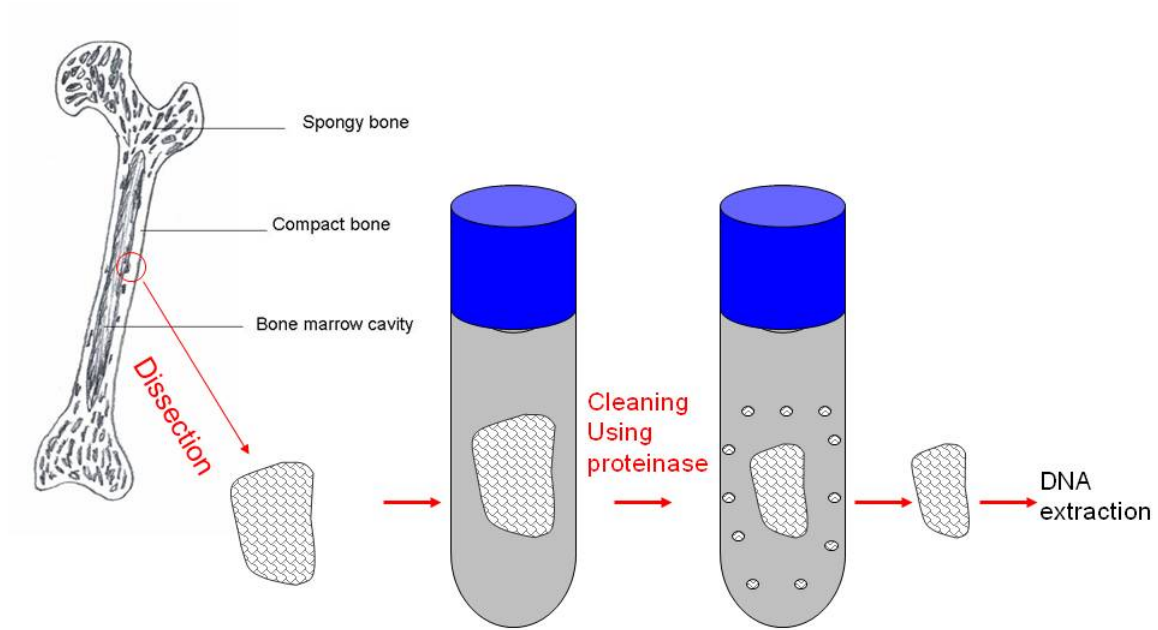
Figure 2. Schematic illustration of project 2
Improving the yield of a DNA isolation method using proteinases for bone specimens

Figure 3. Diagram of bone structure

The human bone is composed of two types of bone: cortical and cancellous bone. The cortical bone is hard and is located at the exterior of the bone. The cancellous bone, also referred to as the spongy bone, is comprised of an arrangement of bony spicules, which is located at the interior of bone. Human cortical bone contains structures called Haversian canals that contain blood vessels and nerves. The Haversian canals (black arrow) are connected to one another by Volkmann's canals (white arrow) which run perpendicular to Haversian systems.
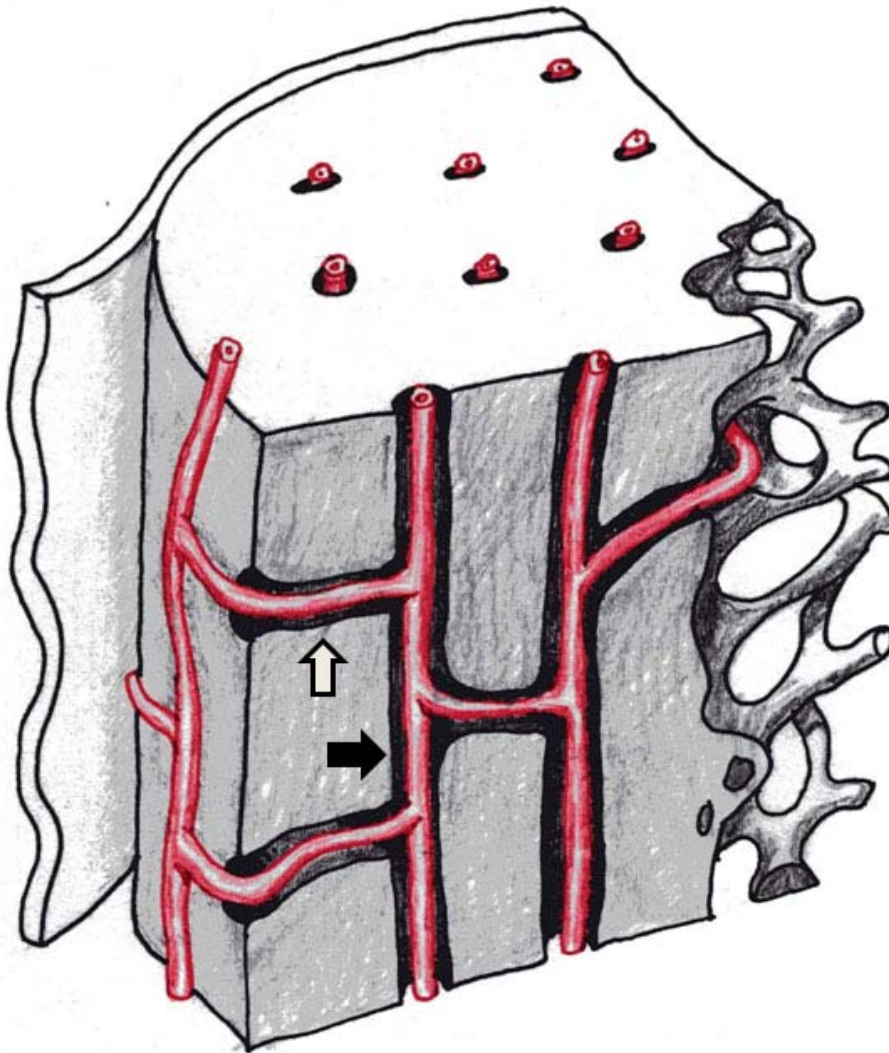
Figure 4. Light microscopic observation of bone surface
The bone fragments (approximately 1 g) of human radius (within 7 days postmortem)
were used for this study. A) bone surface, B) soft tissue residue, C) fibers, and D) dirt
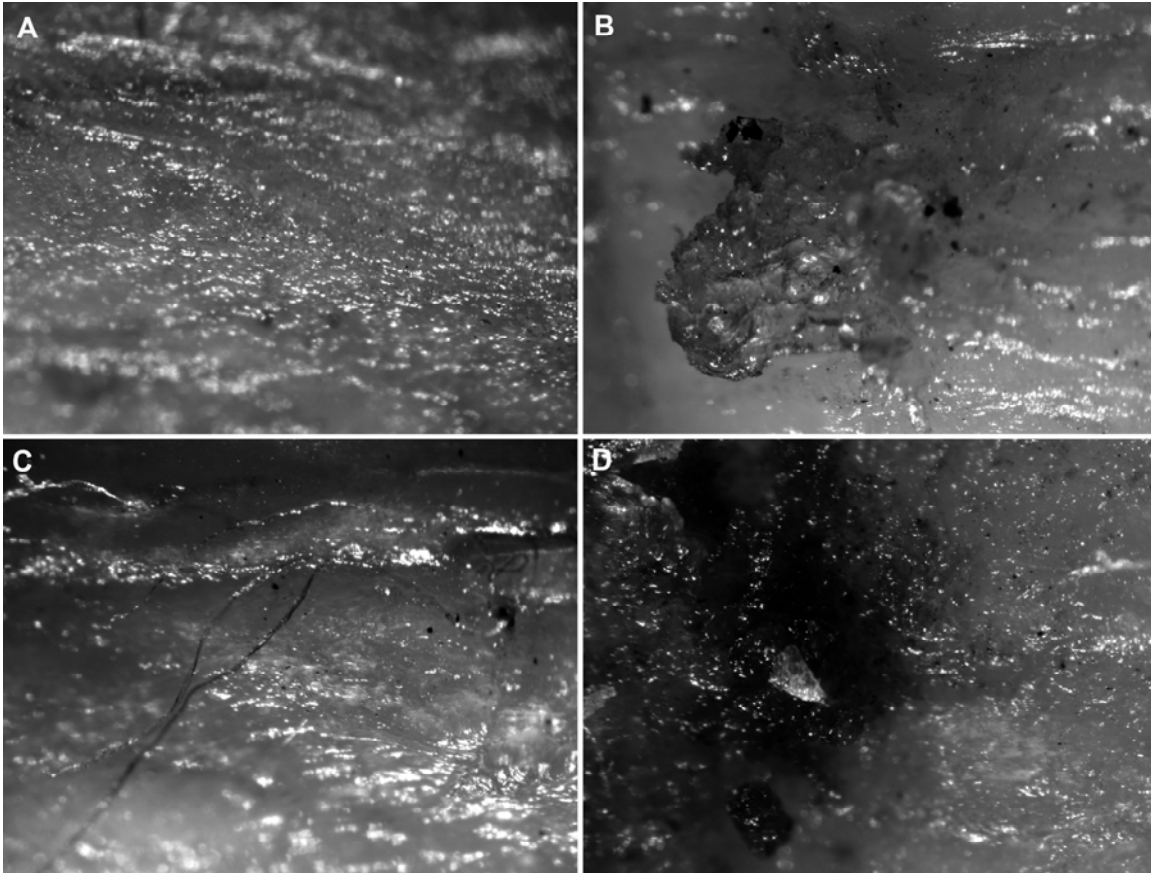
Figure 5. Light microscopic observation of trypsin treated bone surface
Experiments were prepared by placing a piece of bone fragment in 5 ml of trypsin solution (30µg/µl) and were then incubated at 55°C. The trypsin-treated bone fragment was examined and photographed. Trypsin activity was compared at A) 2 hr, B) 4 hr, C) 6 hr, and D) 12 hr incubation. White arrow = Volkmann's canal; Black arrow = Haversian canal.
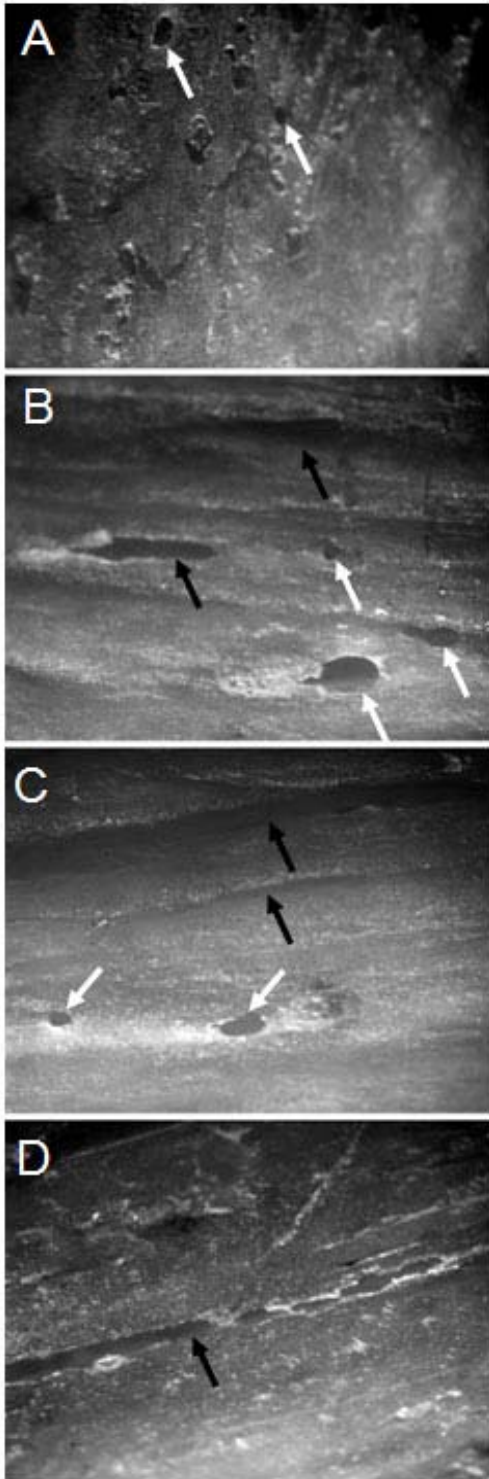
Figure 6. Scanning electron microscopic observation of the trypsin treated and control samples

Figure 6A and Figure 6B represent the samples magnified at X200.

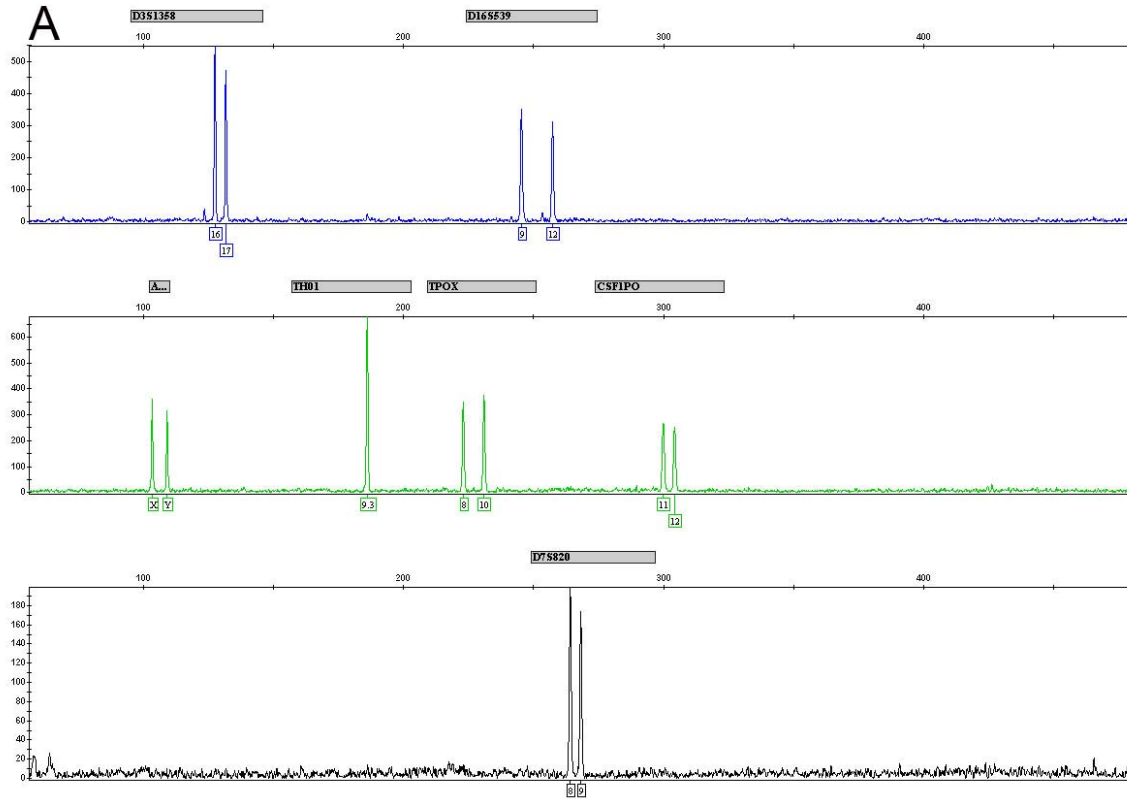A) untreated control sample, B) trypsin (30µg/µl; 2 hr at 55°C) treated bone fragment
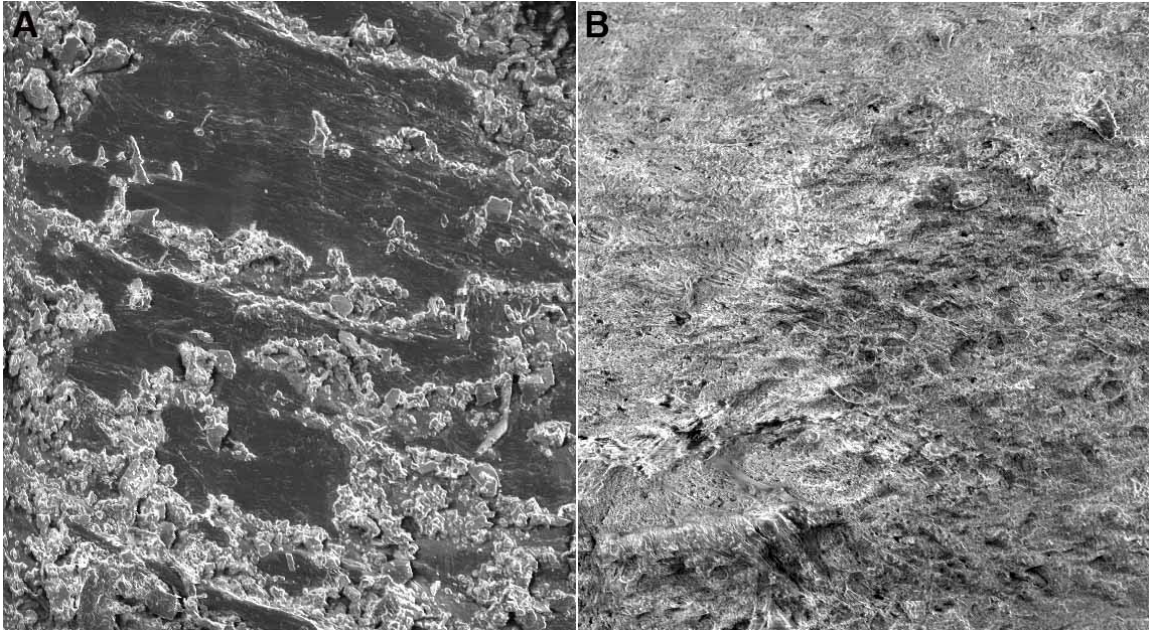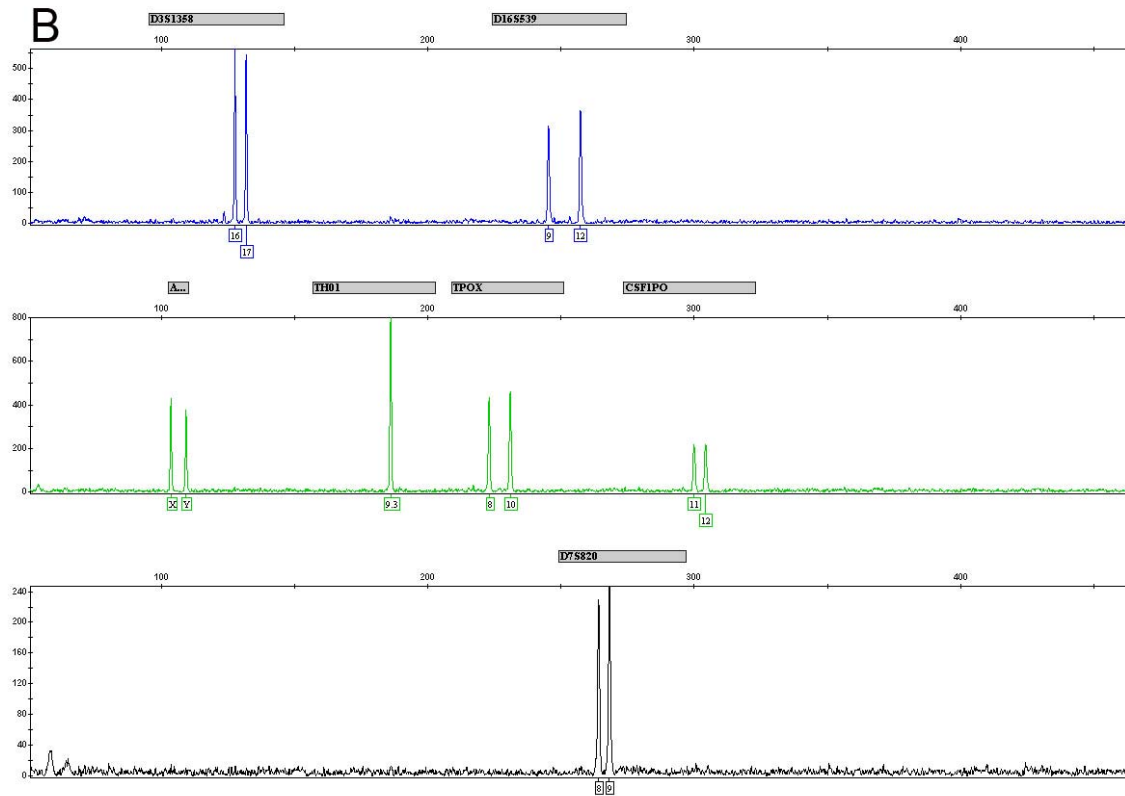
Figure 7. The effect of trypsin treatment on DNA yield
The trypsin treatment (30 µg/ µl) was carried out at 2, 4, 6 and 12 hr increments,
respectively. DNA yield was expressed as µg DNA/0.1 g bone. The values were the
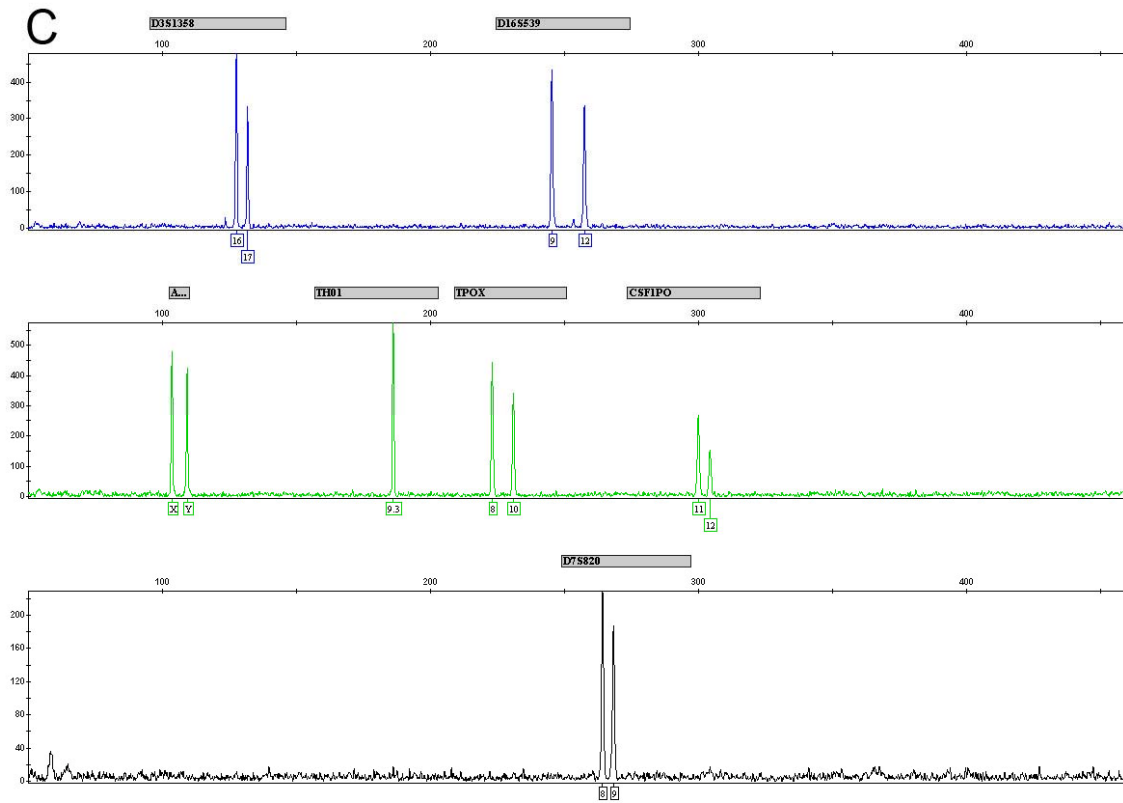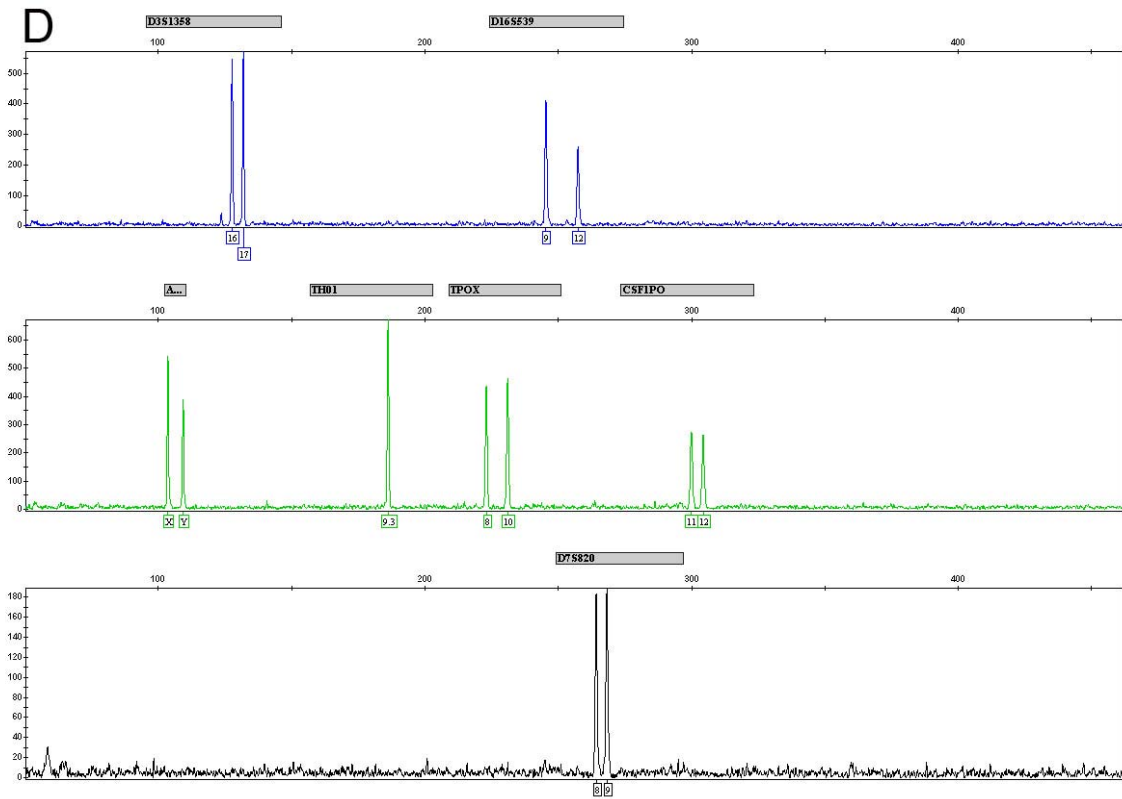means of three determinations.   Standard deviations are in parentheses.
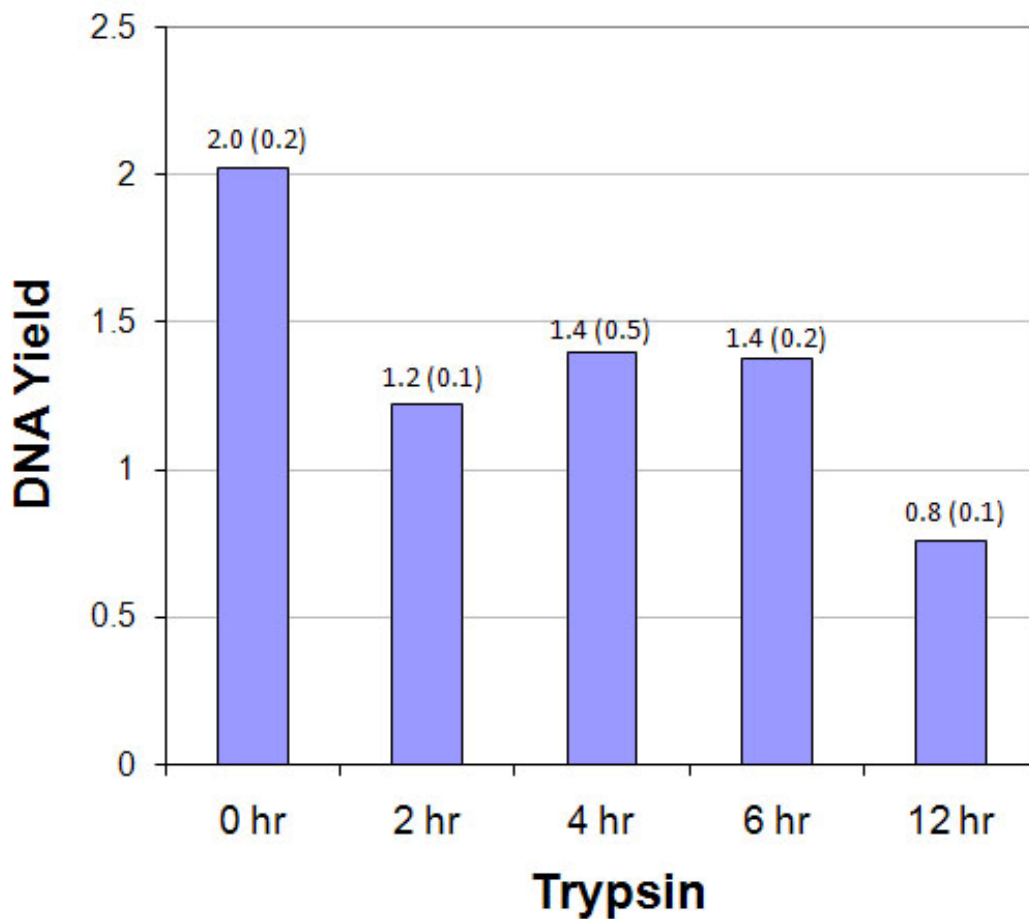
Figure 8. The effect of trypsin treatment on DNA profiles. STR profiles were obtained with AmpflSTR Cofiler[TM] amplification kit. Electropherograms of A) untreated, B) 2 hr, C) 4hr, D) 6 hr, and E) 12 hr trypsin treatments are shown.

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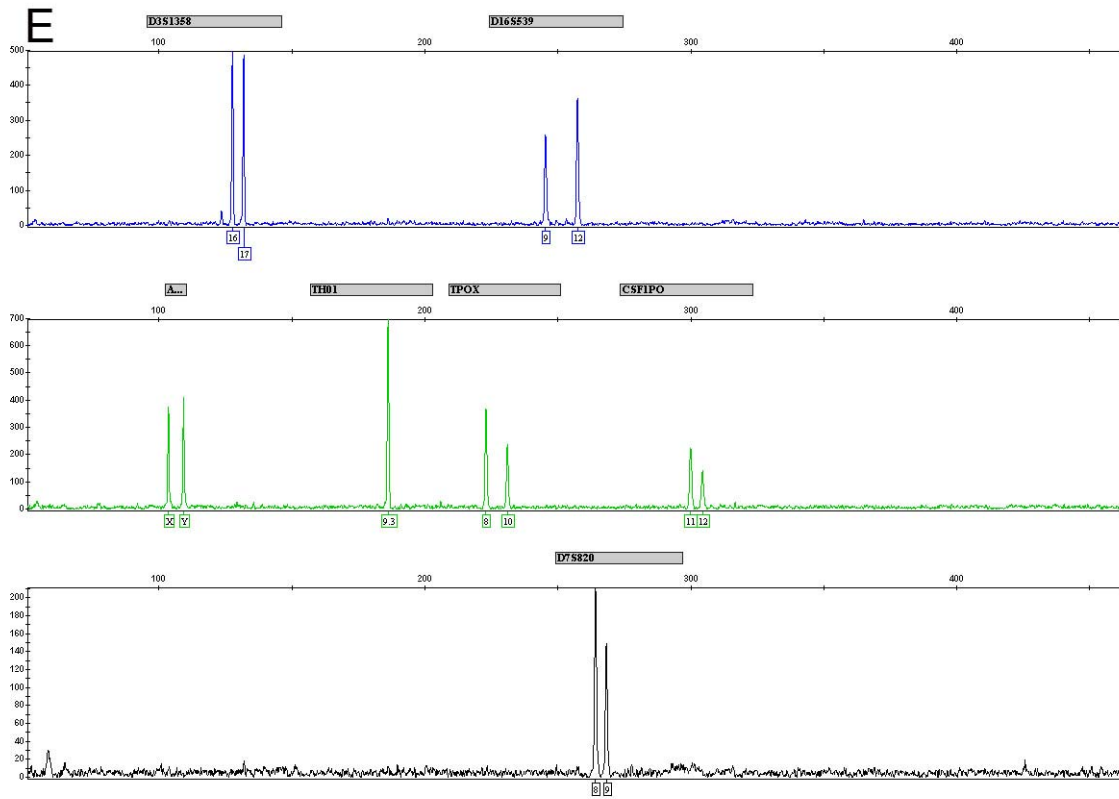
2006-DN-BX-K010

Figure 9. The effect of incubation time on clostridiopeptidase A activity
The proteolysis activity of clostridiopeptidase A was compared at 0, 1, 2, 3, 4, 5, and 6 hr of incubation. The amounts (mass) of undigested bone powder after various lengths of clostridiopeptidase A digestion were normalized to the mass of untreated sample which was set at 100%. The experiment was performed twice with similar results and one representative experiment is shown.
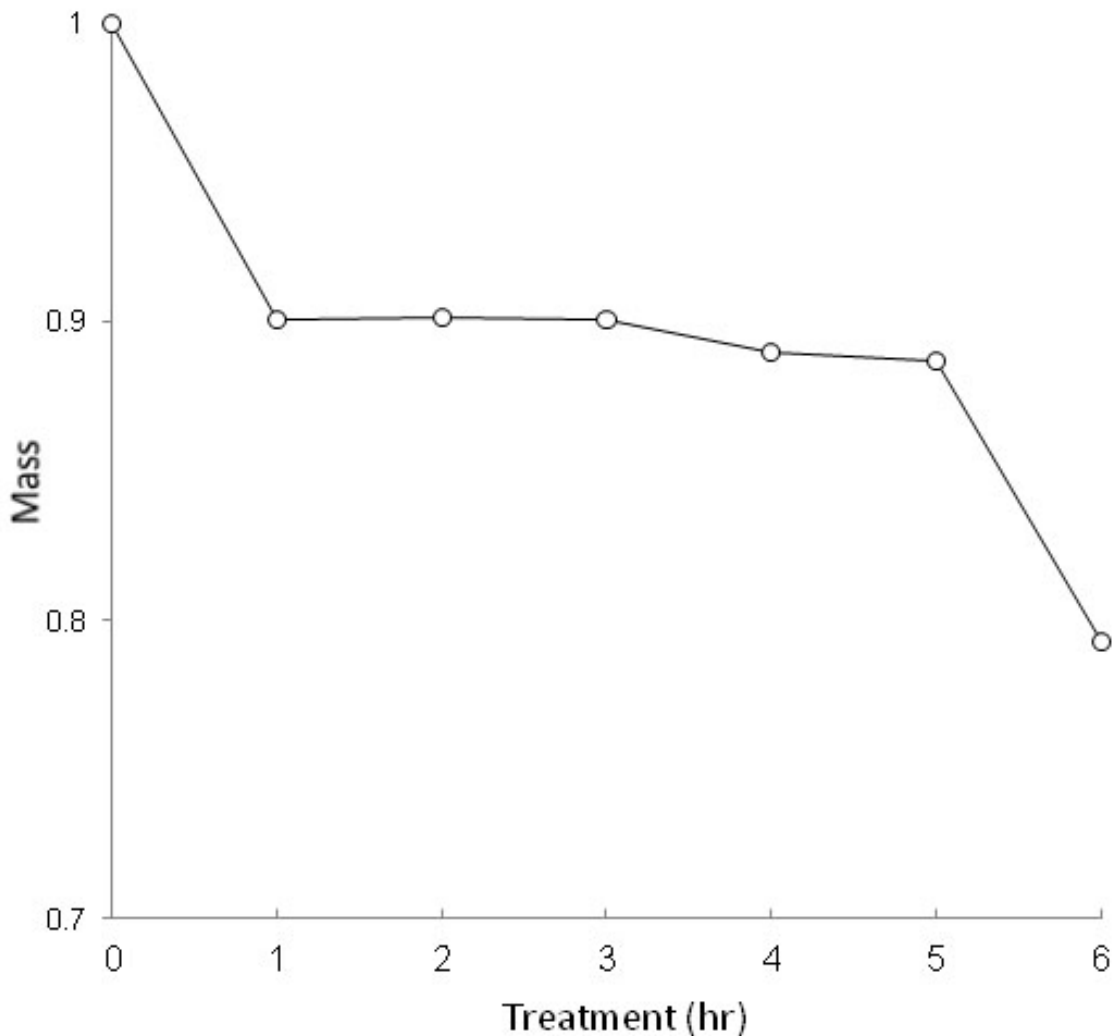
Figure 10. The effect of clostridiopeptidase A treatment on the DNA profiles
STR profiles were obtained with AmpflSTR Cofiler™ amplification kit. Electropherograms of A) proteinase K treated and B) clostridiopeptidase A-proteinase K treated are shown.
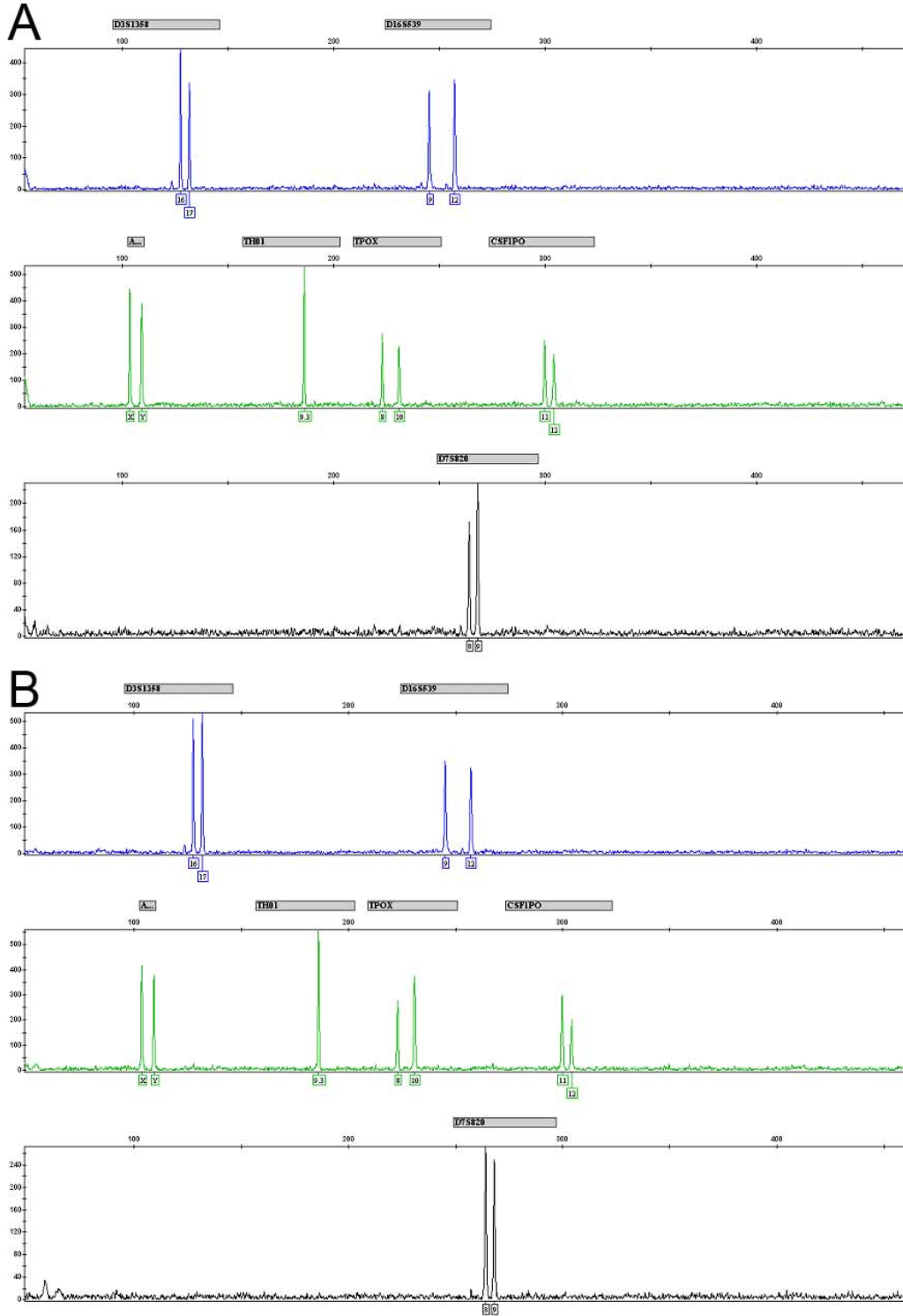
IV. CONCLUSIONS

1. Developing a simple sample processing method for DNA isolation from bone specimens

Discussion of Findings

We have developed a simple sample processing method for bone specimens for DNA isolation. In this study, the surface cleaning of the bone samples (human radius, within 7 days postmortem) was achieved by the application of trypsin solution (30µg/µl). Light microscopy and SEM results indicated that 2-hr trypsin treatment was sufficient to remove surface materials of bone samples. The STR analysis revealed that no adverse effect on DNA profile was detected after trypsin treatment. The yield of DNA isolated from trypsin-treated bone samples (0.8 – 1.4 µg DNA/0.1 g bone) was sufficient for subsequent STR analysis. However, the DNA yield of trypsin-treated bone samples was lower than that of untreated bone samples (2.0 µg DNA/0.1 g bone).

Implications for Policy and Practice

We have adapted the trypsin bone maceration method for processing bone samples prior to DNA isolation. Our results demonstrated that this method was effective for the removal of soft tissues and the outer surface of bone samples. Our data suggested that this method could be used in the initial sample preparation for cleaning the outer surface of human bone samples prior to DNA isolation. This trypsin method is an alternative cleaning method to physical cleaning procedures, such as sanding. Trypsin is reasonably inexpensive; thus it is practical to utilize a large volume of trypsin solution to treat various sizes of bone samples without cutting. This method potentially has a low risk of cross-contamination between samples and diminishes safety concerns for laboratory analysts due to exposing bone powder. This method could be adapted for automated DNA isolation for human identification of bone samples, namely, from mass fatality incidents.

Implications for Further Research

Fresh human bone samples were used in this study as proposed. However, additional studies are needed to examine the yield and quality of the DNA isolated from aged bone samples. In addition, the yield and quality of the DNA isolated from bone samples from various environmental conditions such as heat, moisture and decomposition should also be studied.

2. Developing a high-yield DNA isolation method using proteinases for bone specimens

Discussion of Findings

Our study revealed that the clostridiopeptidase A is potent for bone degradation. The bone degradation activity was observed after 1-hr of clostridiopeptidase A treatment. 0.1 g of pulverized and decalcified bone sample was completely digested with 1-hr clostridiopeptidase A treatment followed by a 10-min proteinase K treatment. Thus, the

entire digestion time is 1 hr and 10 min. The STR analysis revealed that no adverse effect on DNA profiles was detected after clostridiopeptidase A treatment. The yield of DNA isolated from clostridiopeptidase A-proteinase K treated bone samples was 3.5 µg DNA/0.1 g bone. The DNA yield was higher than that of clostridiopeptidase A untreated bone samples (2.0 µg DNA/0.1 g bone).

Implications for Policy and Practice

This method can achieve speedy and better bone degradation by the application of clostridiopeptidase A-proteinase K testament. First, this method reduces the digestion time. The entire digestion time is 1 hr and 10 min, which is less than that of conventional methods (6-8 hr). Second, our results demonstrate that this method improved the DNA yield of bone samples. An increased yield of isolated DNA provides more copies of DNA templates from bone samples. Therefore, it may increase the success rate of generating genotype profiles of DNA analysis. This method could be applied by forensic laboratories for DNA analysis of skeletal samples especially when small quantities of specimens are recovered.

Implications for Further Research

Fresh human bone samples were used in this study as proposed. Additional studies focusing on the yield and the quality of DNA isolated from aged bone samples are needed. The yield and the quality of DNA isolated from bone samples from various environmental conditions such as heat, moisture and decomposition should also be studied.

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VI.   DISSEMINATION OF RESEARCH FINDINGS

Journal Article

Li, R., Chapman, S., Thompson, M., and Schwartz, M. (in press) *Legal Medicine*

Abstract

Li, R. (2008) Application of proteinases for DNA isolation of bone specimens. . in *Annual NIJ Conference*, Washington, D.C.

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