Evaluation of Mini-scale EDTA-decalcification for Degraded Bones DNA Analysis in Mocked Forensic Soil-Buried Condition

การประเมินประสิทธิภาพ Mini-scale EDTA-decalcification เพื่อวิเคราะห์สารพันธุกรรมจากกระดูกเสื่อมสภาพในสภาวะจำลองทางนิติวิทยาศาสตร์ด้วยการฝังดิน

Sineenat Sirijintana (สินีนาฏ ศิริจินตนา)* Dr.Achirapa Bandhaya (ดร.อจิรภาส์ พันธัย)**
Dr.Nathinee Panvisavas (ดร.ณัฏฐินี พันธ์วิศวาส)***

ABSTRACT

Forensic bone DNA analysis requires bone decalcification step in order to remove calcium prior to DNA extraction and recovery. This study aimed to evaluate the efficiency of mini-scale EDTA-decalcification modified with silica-based DNA extraction method for DNA recovery from degraded or treated bones in mocked forensic soil-buried condition for 4 and 8 weeks by using porcine bone as a model. Mitochondrial and nuclear DNA fragments were evaluated using 2 sets of pig *cyt* b and *β-actin* primer pairs. Results showed estimated total DNA recovery per 0.5 g of bone powder from 4- and 8-week samples were 60.33±26.88 ng and 41.23±7.65 ng, respectively. All bone samples in both treatment time periods were successfully amplified with pig *cyt* b 161-bp primer pairs while the 211-bp *β-actin* DNA fragments were amplified from most of the samples. This suggested that the mini-scale EDTA-decalcification modified with silica-based DNA extraction method could efficiently be used to obtain DNA from the degraded or treated bone samples in mocked forensic soil condition.

บทคัดย่อ

การตรวจวิเคราะห์สารพันธุกรรม (ดีเอ็นเอ) จากตัวอย่างกระดูกจำเป็นต้องมีขั้นตอนการดึงแคลเซียมออกจากตัวอย่างกระดูก (bone decalcification) ก่อนทำการสกัดและตรวจเก็บสารพันธุกรรม การศึกษานี้มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพของ mini-scale EDTA-decalcification modified with silica-based DNA extraction method เพื่อหาปริมาณดีเอ็นเอจากตัวอย่างกระดูกที่เสื่อมสภาพในสภาวะจำลองทางนิติวิทยาศาสตร์ด้วยการฝังดินเป็นเวลา 4 และ 8 สัปดาห์ ไมโตคอนเดรียลดีเอ็นเอและนิวเคลียร์ดีเอ็นเอของหมูถูกนำมาใช้ประเมินผลโดยใช้เป็น DNA marker จากยีน *cyt* b และ *β-actin* ค่าเฉลี่ยปริมาณดีเอ็นเอทั้งหมดที่ตรวจพบในตัวอย่างผงกระดูก 0.5 กรัมที่ฝังดิน 4 และ 8 สัปดาห์ มีค่าเท่ากับ 60.33±26.88 นาโนกรัม และ 41.23±7.65 นาโนกรัม ตามลำดับ นอกจากนี้ผลการทำ PCR สามารถตรวจพบชิ้นส่วนไมโตคอนเดรียลดีเอ็นเอจาก *cyt* b ขนาด 161 คู่เบสในทุกตัวอย่าง ขณะที่ยีน *β-actin* สามารถตรวจพบชิ้นส่วนนิวเคลียร์ดีเอ็นเอขนาด 211 คู่เบสได้มากที่สุด ดังนั้นวิธี mini-scale EDTA-decalcification ที่นำมาใช้ร่วมกับการสกัดสารพันธุกรรมด้วยหลักการ silica-based สามารถนำมาใช้วิเคราะห์หาดีเอ็นเอจากตัวอย่างกระดูกที่เสื่อมสภาพได้

Key Words : Bone decalcification method, DNA recovery, degraded bone
คำสำคัญ : วิธีดึงแคลเซียมออกจากตัวอย่างกระดูก การตรวจเก็บสารพันธุกรรม ตัวอย่างกระดูกที่เสื่อมสภาพ

* *Student, Master of Forensic Science Graduate Programme, Department of Forensic Science, Faculty of Science, Mahidol University*
** *Lecturer, Department of Forensic Science, Faculty of Science, Mahidol University*
*** *Assistant Professor, Department of Plant Science, Faculty of Science, Mahidol University*

Introduction

Bone is an essential biological evidence that is normally left in the crime scene because of its calcified structure which provides strength, protects bone cells and DNA damage from various adverse environment. Therefore, DNA recovery from bone is very useful for human DNA profiling, especially for identification of an unknown person without tissue or body fluid left. Previous studies suggested that compact bone from femur was the best sample for obtaining reproducible results in bone forensic DNA analysis (Barbaro et al., 2008) because it was the largest long bone which provides greatest amount and thick layer of compact bones to promote strength and high quality of the DNA extracts. Although the compact bone was a good sample, but its protective extracellular matrix was the main problem for DNA analysis. Multi-matrix layers make bones difficult to break open cells and calcium in hydroxyapatite (HAp) , the main minerals in the compact bone can interfere PCR. As a consequence, bone decalcification step is required to remove the divalent calcium ion prior to DNA extraction and recovery. Forensic bone DNA analysis routinely uses EDTA as decalcifying agent since it provides high quality of DNA and maintains the integrity of bone tissue. Recently, Khaiprapai et al. (2012) reported an optimization of bone EDTA-decalcification and DNA extraction method for small scale preparation of total nucleic acid called “mini-scale EDTA-decalcification and DNA extraction of bone” which was modified from Edson et al. (2004) by using pig bones as a model. The recommendation for the amount of bone as a starting material was 2 g. However, in the case of degraded bone samples which are found in various forensic environmental conditions such as buried in soil, DNA degradation is increased due to skeletal weathering process. Therefore, the efficiency of this method needs to be evaluated when applied in forensic samples which are usually degraded and contain small amount of DNA. In addition, although DNA obtained from organic extraction is effective, the solvents used are known as health hazards and handling in a safety fume hood is required. Therefore, non-organic extraction method is an alternative choice. The majority of these methods use the ability to reversibly bind DNA to silica particles which allow quick and efficient purification (Marshall et al. 2014).

Objectives of the study

The aim of this study was to evaluate the efficiency of mini-scale EDTA-decalcification modified with silica-based DNA extraction method to recover DNA from degraded or treated bones in mocked forensic soil-buried condition for 4 and 8 weeks by using porcine bones as a model.

Methodology

Preparation of pig bone samples in mocked soil-buried condition

Porcine femurs are treated in mocked soil buried condition that is one of commonly encountered in forensic case work. Fresh femurs were buried 1 m (3.28 feet) deep in field soil following the recommendation of Institute of Cemetery and Crematorium Management (2004). The diameter of each hole was approximately 50 cm. Type of soil used in this study was sandy-clay, which was normally found in all areas in Thailand. Durations of the samples buried in soil were 4 and 8 weeks. Then, the level of bone degradation was evaluated using Behrensmeyer' s skeletal weathering staging systems (Misner et al., 2009) which classified bone morphological weathering stages as 0 to 5 scales with the following details.

Stage 0: Bone surface shows no signs of cracking or flaking resulting from aging (weathering).

Stage 1: Bone shows some cracking, usually longitudinal in long bones.

Stage 2: Some cracking and flaking is apparent, especially on the outermost concentric thin layers of the bone.

Stage 3: Bone surface has rough patches of weathered compact bone; external concentric layers have been removed, but weathering does not penetrate deeper than 1.0–1.5 mm.

Stage 4: Bone surface is coarse and splinters may exist; weathering reaches into inner cavities.

Stage 5: Bone has large splinters and is easily broken; original bone shape may be undeterminable.

After pig femurs were buried in soil for 4 and 8 weeks, they were collected and sliced to obtain tissues attached to the bones. Then, the surfaces of bones were mechanically cleaned using sterile scalpel and chemically cleaned by immersing with 50% commercial bleach, containing 6% sodium hypochlorite for 15-30 min. The bones were cleaned with toothbrush to remove soft tissues. They were washed 4-5 times with sterile distilled water and rinsed with absolute ethanol. The cleaned pig femurs were dried in hot air oven at 56°C for 2-3 days. Dried bones were crushed to small pieces using a chisel and hammer, and then completely dried in hot air oven at 56°C overnight prior to grinding into fine powder under liquid nitrogen, using the Spex CertiPrep 6750 Freezer/Mill Cryogenic Grinder (SPEX® SamplePrep, USA). Pig bone powder was stored in paper bags at room temperature.

Bone decalcification and DNA extraction

Bone decalcification was carried out according to Khaiprapai et al. (2012). An aliquot of 0.5 g of pig bone powder was put into 1.5 ml sterile microcentrifuge tube. A volume of 750 µl of extraction buffer [10 mM Tris (pH 8), 100 mM NaCl, 50 mM EDTA (pH 8), 0.5% (w/v) SDS] was added into the tube. Then, 25 µl of 20 mg/ml proteinase K was added, mixed well by vortex mixer and incubated at 56°C overnight with gentle agitation. After that, centrifugation was performed at maximum speed for 5 min and the supernatant was discarded. The pellet was collected to for further DNA extraction by Wizard® SV Minicolumns (Promega Corporation, USA) according to manufacturer's protocol.

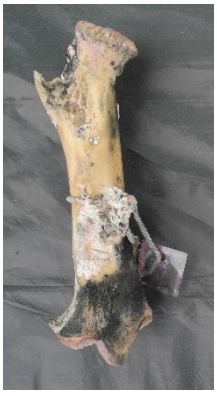
DNA concentration extracted from treated bones was quantified by fluorometric assay using Qubit® 3.0 fluorometric quantitation kit (Thermo Fisher Scientific Inc., USA) according to manufacturer's instruction.

DNA analysis by polymerase chain reaction

Analysis of DNA by PCR was performed by using two sets of pig primer pairs which were composed of 5 *Beta-actin (β-actin)* primer pairs and 2 *Cytochrome* b *(cyt* b*)* primer pairs according to Phengon et al. (2008) and Khaiprapai (2013) in order to investigate nuclear DNA and mitochondrial DNA fragments. The total volume of PCR reaction mixture was 25 µl. The reaction mixture consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 1 unit of *Taq* DNA polymerase, 2.5 pmol of each forward and reverse primer, and 2 ng of DNA template. Amplification of nuclear *β-actin* primer pairs was performed in Gene Amp 9700 (Applied Biosystem, USA) using the following thermocycling condition; initial denaturation at 95ºC for 5 min, then 35 cycles of denaturation at 95ºC for 30 sec, annealing at 63ºC for 30 sec, extension at 72ºC for 1 min and final extension at 72ºC for 7 min. Except for the PCR product size 289-bp, the annealing temperature was 65ºC. The sizes of the PCR products were 148-bp, 211-bp, 289-bp and 366-bp. For the mitochondrial *cyt* b primer pairs, the annealing temperature was at 59ºC and the sizes of the PCR products were 161-bp and 323-bp. The PCR products were separated and detected in 2% (w/v) ethidium bromide-stained agarose/Tris-borate-EDTA (TBE) gel.

Results

The evaluation of the level of bone degradation using Behrensmeyer's skeletal weathering staging systems following Misner et al. (2009) revealed that both femurs treated in mocked soil condition were categorized into "stage 0", the bone surfaces showed no signs of cracking or flaking resulting from aging (weathering), but showed color changed from beige to slightly yellowish.



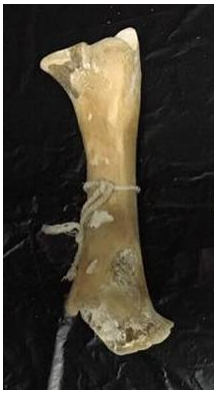


Figure 1 (A) Pig femur treated in soil for 4 weeks (B) Pig femur treated in soil for 8 weeks

1. Estimated total DNA recovery

Estimated total DNA obtained from 0.5 g of bone powder treated in mocked soil condition for 4 and 8 weeks with mini-scale EDTA-decalcification method compared to untreated bone sample are shown in Table 1. The experiment was conducted for 5 times. The average estimated total DNA yield obtained from 4- and 8-week samples were 60.33±26.88 and 41.23±7.65 ng, respectively. The untreated bone sample gave a total DNA yield of 4432±197.28 ng. Results showed that DNA recovered from the degraded bones treated for 4 weeks was slightly higher than 8 weeks. However, DNA obtained from untreated bone was higher than soil-treated bones approximately 100 times.

Table 1 Comparison between estimated total DNA obtained from 0.5 g of bone powder treated in mocked soil condition and untreated bone with mini-scale EDTA-decalcification

No. of replicate	Estimated total DNA (ng) per 0.5 g bone powder		
	4 weeks	8 weeks	Untreated bone
1	44.0	38.6	4600
2	56.9	32.7	4640
3	99.3	42.7	4200
4	41.1	50.9	4460
5	31.9	39.3	4260
Average ± SD	60.33±26.88	41.23±7.65	4432±197.28

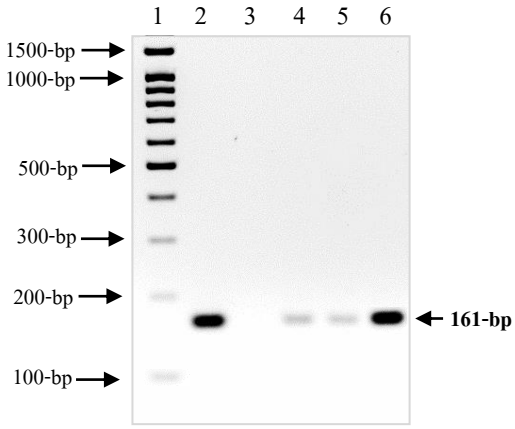
2. DNA fragmentation

PCR analysis of 4- and 8-week soil buried bone samples using the mitochondrial *cyt* b primer set showed reproducible amplification results for the 161-bp PCR product (Figure 2A), while no 323-bp amplification product was obtained. Analysis of the 4-week soil buried bone sample using the nuclear β-*actin* primer set showed that amplification of 148-bp, 289-bp, 289-bp*, and 366-bp PCR products were not reproducible. The amplification of 211-bp was reproducible (Figure 2B), as 4 out of 5 gave PCR products. For the 8-week soil buried bone sample, no PCR product was obtained from the amplification of 148-bp, 289-bp*, and amplification of the 211-bp, 289-bp, and 366-bp were not reproducible. The untreated bone sample gave reproducible amplification for all DNA markers used (Table 2).

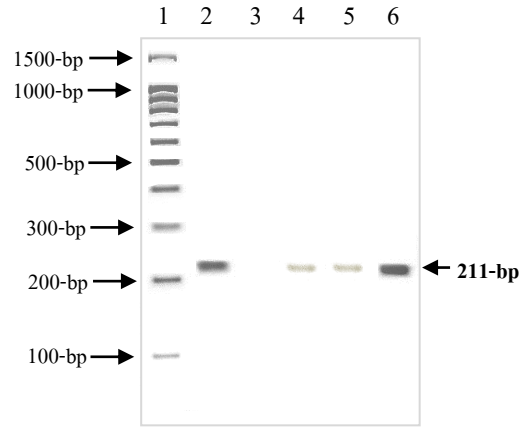
Table 2 Summary of analysis of DNA recovered from treated pig bones with mini-scale EDTA-decalcification

Treatment period	DNA markers						
	β-actin					*cyt* b	
	148-bp	211-bp	289-bp	289-bp*	366-bp	161-bp	323-bp
4 weeks	1/5	4/5	1/5	1/5	1/5	5/5	0/5
8 weeks	0/5	2/5	1/5	0/5	3/5	5/5	0/5
Untreated bone	5/5	5/5	5/5	5/5	5/5	5/5	5/5

Note: DNA fragments of length 289-bp were results of pig *β-actin* F1R2 and F2R3* primer pairs.



(A) The 161-bp mitochondrial pig *cyt* b PCR product



(B) The 211-bp nuclear pig *β-actin* PCR product

lane	Description	lane	Description
1	100-bp DNA ladder	4	Treated bone in soil for 4 weeks
2	Positive control	5	Treated bone in soil for 8 weeks
3	Negative control	6	Untreated bone

Figure 2 Photographs of 2.0% (w/v) ethidium bromide stained agarose/TBE gels depicting PCR amplification products of the 161-bp mitochondrial pig *cyt* b (A) and the 211-bp nuclear pig *β-actin* (B) DNA fragments

Discussion

The results of DNA extraction from 0.5 g of treated pig femur bones in mocked soil condition using mini-scale EDTA-decalcification according to the protocol of Khaiprapai et al. (2012) protocol applied with silica-based DNA extraction by Wizard® SV purification kit showed that the estimated total DNA recovery per 0.5 g of bone powder were 60.33±26.88 ng and 41.23±7.65 ng in 4 and 8 weeks, respectively (Table 1). DNA obtained from the bone samples were decreased as the treatment time periods increased due to skeletal weathering process that caused DNA degradation.

Then, after PCR was done using 2 sets of pig *cyt* b and β-*actin* primer pairs, results showed all bone samples were successfully amplified with the mitochondrial pig *cyt* b 161-bp DNA fragments in both treatment time periods while no sample was amplified with the mitochondrial pig *cyt* b 323-bp DNA marker. This was due to the sensitivity of these primer pairs that was quite low in the bone samples (required at least 20 ng DNA to present clearly visible DNA band). For the nuclear pig β-*actin* gene, 211-bp fragments were mostly amplified in mocked soil buried samples for 4 weeks followed by 148-bp, 298-bp, and 366-bp which presented DNA fragments in 1 of 5 replicate. Treatment for 8 weeks produced only PCR products of 211-bp, 298-bp, and 366-bp (Table 2). Comparison to the untreated (fresh) bone when using the same bone decalcification and extraction protocol, it showed higher total DNA recovery (4432±197.28 ng) that could be successfully amplified all DNA fragments using both pig *cyt* b and β-*actin* primer pairs (Tables 1 and 2).

This suggested that mini-scale EDTA-decalcification modified with silica-based DNA extraction could be used to obtain DNA from treated or degraded bone samples but could not reproducibly generate PCR products because of poor nature of samples. It may require higher amount of starting bone materials for bone decalcification. In addition, elution volume used in the Wizard® SV Genomic DNA Purification System should be further adjusted. Small elution volume should make the DNA extracts become more concentrated and suitable for degraded bone DNA analysis.

Conclusion

Mini-scale EDTA-decalcification modified with silica-based DNA extraction method could be used to recover DNA from degraded or treated bone samples in mocked forensic soil-buried condition for 4 and 8 weeks by using porcine bones as a model.

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References

Barbaro A, Cormaci P, Barbaro A. Validation of DNA typing from skeletal remains using the Invitrogen Charge Switch1 Forensic DNA Purification Kit. Forensic Sci Int. Genetic Supplement Series. 2008;1(1):398–400.

Edson SM, Ross JP, Coble MD, Parsons TJ, Barritt SM. Naming the Dead - Confronting the Realities of Rapid Identification of Degraded Skeletal Remains. Forensic Sci Rev. 2004;16(1):63-90.

Institute of Cemetery and Crematorium Management. Policy relating to Shallow Depth Graves [online] 2004 [cited 2016 May 22]. Available from: http://www.iccmuk.com/iccm/library/ ShallowGraves.pdf

Khaiprapai P, Waiyawith W, Smith S, Panvisavas N. Mini-scale EDTA-decalcification and DNA extraction from Bone. The Asian Forensic Science Network (AFSN) Conference 2012; 2012 Nov 25-28; Bangkok, Thailand. [in Thai].

Khaiprapai P. Development of acid decalcification technique for mini – scale DNA recovery from bone [Master Thesis in Forensic Science]. Bangkok: Faculty of Graduate Studies, Mahidol University; 2013. [in Thai].

Marshall PL, Stoljarova M, Schmedes SE, King JL, Budowle B. A high volume extraction and purification method for recovering DNA from human bone. Forensic Sci Int Genet. 2014;12:155-60.

Misner LM, Halvorson AC, Dreier JL, Ubelaker DH, Foran DR. The correlation between skeletal weathering and DNA quality and quantity. J Forensic Sci. 2009;54(4):822-8.

Phengon P, Wongwiggarn S, Panvisavas N. Analysis of DNA from degraded tissue. Forensic Sci Int: Genetics Supplement Series. 2008;1:439-441.

Promega Corporation. Wizard® SV Genomic DNA Purification System Technical Bulletin [online] 2012 [cited 2016 July 20]. Available from: https://worldwide.promega.com/resources/protocols/technical-bulletins/101/wizard-sv-genomic-dna-purification-system-protocol/

Thermofisher Scientific. Qubit® 3.0 Fluorometer user guide [online] 2014 [cited 2016 July 15]. Available from: https://tools.thermofisher.com/content/sfs/manuals/qubit_3_fluorometer_man.pdf

Thermofisher Scientific. Qubit® dsDNA HS Assay Kits [online] 2015 [cited 2016 July 15]. Available from: https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_HS_Assay_UG.pdf