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Short Communication

Simple Protocol to Extract DNA from Bone Fragments-A Preliminary Study

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Abstract DNA extraction from bone samples is greatly useful for forensic applications but it is more difficult to be conducted compared with extracting DNA from other tissues due to rigid structure of the bones and contamination from the environment, such as, bacterial colonization within bone tissues. This research aimed to determine a simple and cost-effective protocol for extracting DNA from bone fragments. Canine leg bones were used as samples and canine *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) gene-specific primers were used to confirm successful DNA extraction through PCR. We developed a protocol that was able to extract DNA from “fresh” as well as “old” bones that had been buried in soil or stored underwater in a pond for three months. Although the effectiveness decreased in the old bones, this protocol only requires common devices and chemicals readily available in general molecular laboratories and can be completed in less than 24 hours, offering an alternative lower-cost and less time intensive method for forensic research.
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Keywords : DNA extraction, bone, forensic sciences

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Introduction

DNA in bone samples can provide important information for forensic sciences. However, obtaining DNA from bone fragments is very difficult. The quality and quantity of the extracted DNA can be affected by many factors including low copy number due to the fact that the majority of bone is not cells (Loreille et al., 2007), incomplete or broken DNA fragments (Jones, 2007), and contamination by PCR inhibitors

(Chilvers et al., 2008). Isolating and purifying DNA from bones poses challenges for researchers.

Extracting DNA from bones normally requires a series of complicated steps and special tools, such as microconcentrators, recommended by many researchers to help condense DNA (Fondevila et al., 2008; Gonzalez-Andrade & Sanchez, 2005; Imaizumi et al., 2005; Latham & Ritke, 2002), and a bead-column to help remove other smaller molecules (Latham & Ritke, 2002). Some studies also used silica particles (Yang et al., 2004) and enzymes (Pusch & Scholz, 1997) during extraction. These chemicals increase the extraction cost compared to the simple manual DNA extraction method.

In our study, we modified some low-cost and effective DNA-extraction methods, using chemicals and tools readily available in most molecular laboratories. This paper reports the modified protocol that successfully extracted DNA from bone fragments.

Materials and methods

1. Bone samples

Canine leg bones from recently deceased dogs were collected from the Faculty of Veterinary Medicine, Chiang Mai University and from the Small Animal Hospital, Chiang Mai, Thailand. The bones were from 2 different breeds of domestic dogs, which are a poodle (labeled as P) and a golden retriever (labeled as G). Flesh and skin were removed, as much as possible, from the bone fragments. Fragments from the Faculty of Veterinary Medicine were stored at -20 °C (the “fresh” bone samples). Fragments from the Small Animal Hospital were separated into two groups: one was buried in the soil (labeled as 1) and the other was stored underwater in a pond nearby (labeled as 2). Both groups were kept under these conditions for three months (the “old” bone samples).

Before the DNA extraction process, fresh bones were washed with 70% ethanol and old bones were washed with 6% Sodium hypochlorite and then placed under UV light for 30 minutes. Subsequently, the epiphysis was separated from the diaphysis (labeled as E and D, respectively).

2. DNA extraction

This protocol was adapted from Imaizumi et al.(2005). The harder fresh bones were ground using a normal kitchen blender (SharpTM, EM-11), while the softer old bones were powdered using a pestle and mortar. The weight of bone powder was varied as 0.1 - 1.0 g for each sample.

Bone powder of each sample was put in 0.5M EDTA, pH 8.0 at 56°C overnight. The supernatant was then removed and the remaining powder was washed twice with distilled water and once with Tris-EDTA-NaCl (TEN) buffer, which consists of 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), and 100mM NaCl. Thereafter, 3ml of extraction buffer (TEN, 0.5% SDS, 0.5 mg/ml proteinase K) was added and the sample tubes were kept at 56°C for 4 hours. The supernatant was then placed in a new 1.5ml microcentrifuge tube and the equal volume of phenol : chloroform (1:1, v/v) was added before

centrifugation at 13,000 rpm for 5 minutes. The supernatant was placed in a new tube and 1,200 μl of chloroform was added before centrifugation at 13,000 rpm for 5 minutes. The supernatant was placed in a new tube and 60 μl of 3M Sodium Acetate and 600 μl of isopropanol were added before centrifugation at 13,000 rpm for 30 minutes. Then, the supernatant was removed and the remaining DNA was dried at 37 °C before added to 15 μl of distilled water.

3. Polymerase Chain Reaction

PCR was performed by using MJ Mini Personal Thermal Cycler (Bio-Rad, USA) with 40 cycles of following temperature conditions: denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, extension at 72 °C for 30 seconds. A pair of primers (Forward primer: 5’-AGTATGATTCTACCCACGGC-3’; Reverse primer: 5’-CGAAGTGGTCATGGATGACT-3’; Amplicon size 362 bp) that are specific to canine *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) gene (Siengdee et al., 2010) was used in PCR to confirm the success of DNA extraction. The 15 μl of PCR mixture contained 1 μl of template DNA, 0.083 mM of dNTP, 0.333 μM of each primers, 1X of reaction buffer (RBC, Taiwan), and 1 unit of Taq polymerase (RBC, Taiwan). The PCR products were detected by gel electrophoresis using 1.5% (w/v) agarose. If the PCR products could not be detected, re-amplification would be performed by using 1μl of those PCR products as templates. The condition of PCR components for re-amplification was the same as PCR mixture described above.

Results

The protocol was successful in extracting DNA from all fresh bone samples, including four diaphysis and four epiphysis replications, with 0.3 grams of bone powder as the minimal weight of tissue samples. The 362 base-pair PCR products were detected by electrophoresis (Figure 1).

For the old bone samples, the minimal weight of bone powder was 0.5 grams. However, when PCR was performed with DNA samples, only PCR product from one burial diaphysis sample (Gd1) was obviously seen. The PCR products from two underwater diaphysis samples (Gd2 and Pd2) and one underwater epiphysis sample (Ge2) were very dim and the rest were not visible (Figure 2). These PCR products were then re-amplified to increase the copy number. After re-amplification, more visible bands, seven out of eight samples, were detected (Figure 3).

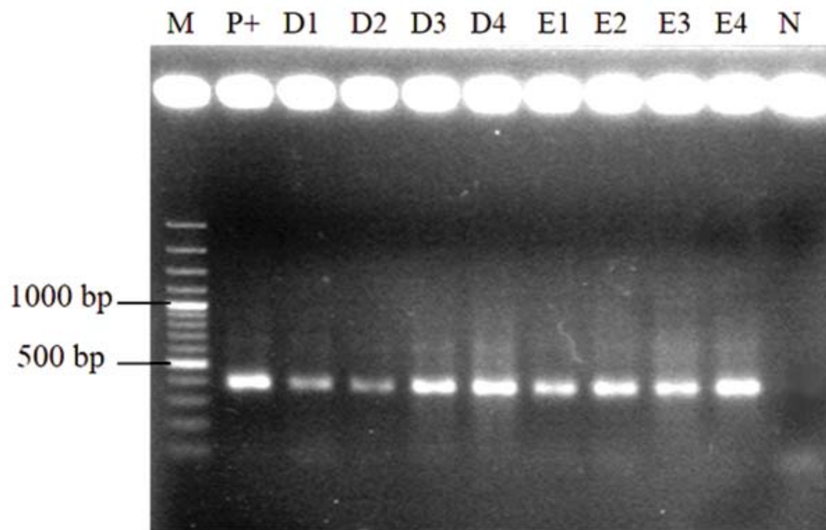

Figure 1 PCR product from fresh bones. M indicates marker, P+ indicates a positive control and N is a negative control. D1-D4 refers to diaphysis, while E1-E4 refers to epiphysis.

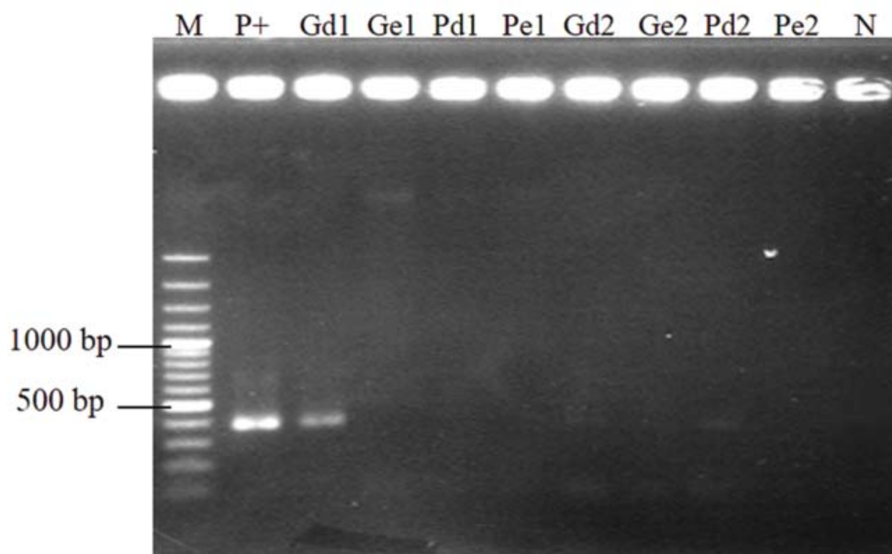

Figure 2 PCR product from old bones. M indicates marker, P+ indicates a positive control and N is a negative control. Gd1, Ge1, Pd1, and Pe1 were samples buried in soil while Gd2, Ge2, Pd2, Pe2 were samples kept underwater (G = golden retriever, P = poodle, d = diaphysis, e = epiphysis).

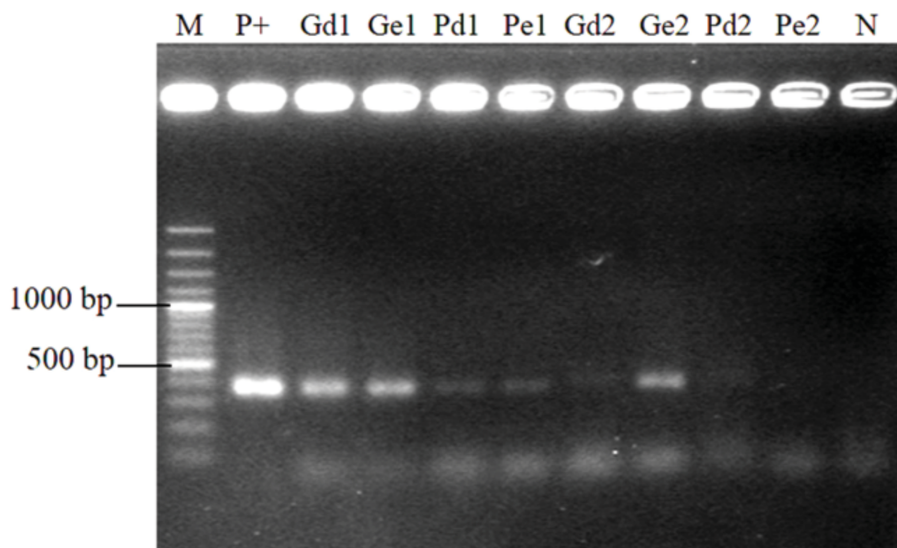

Figure 3 PCR re-amplification products of DNA extracted from old bones. There are five clearly visible bands of product from the sample Gd1, Ge1, Pd1, Pe1 and Ge1, respectively. There are also two faint bands from the sample Gd2 and Pd2. M indicates marker, P+ indicates a positive control and N is a negative control. Gd1, Ge1, Pd1, and Pe1 were samples buried in soil while Gd2, Ge2, Pd2, Pe2 were samples kept underwater (G = golden retriever, P = poodle, d = diaphysis, e = epiphysis).

Discussion

This study developed a protocol that effectively extracted DNA from all fresh bone samples, using only 0.3 grams of sample. However, the effectiveness decreased with the 0.5 gram samples of old bone powder, whether kept in soil or underwater for three months. The re-amplification result (Figure 3) showed that the protocol did not fail to extract DNA, but the copy numbers of most PCR products were too low to be seen. Increasing the amount of bone powder or increasing the number of PCR cycles may solve this problem.

The significant steps of this protocol are the addition of EDTA solution in the first step and subsequent washing with TNE (Tris-NaCl-EDTA) buffer. EDTA not only demineralizes bone structure but also inhibits DNase enzyme, so it helps protect DNA from degradation (Loreille et al., 2007). However, EDTA may inhibit PCR due to its ability to bind with the Mg2+ ions, a co- factor of *Taq* polymerase (Khosravinia et al., 2007). Therefore, an effective EDTA removal step is necessary, and TNE buffer was the key in this protocol.

In contrast to previously published protocols (Imaizumi et al., 2005; Hochmeister , 1995), this modified protocol did not use a multi-bead shocker to grind the bone into powder and did not need centricon-100 microconcentrator tubes to concentrate the DNA. No difference between the epiphysis and diaphysis samples was observed. There was not

enough data to determine the difference between the buried and underwater samples.

In conclusion, this modified protocol was a very simple protocol that did not need any special devices or chemicals. Moreover, it took less than 24 hours to complete. This modified protocol offers a lower cost and less time intensive method for extracting DNA from bone samples. However, further research is required to improve the effectiveness of the protocol applied to older bone samples with small amount.

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การสกัดดีเอ็นเอจากกระดูกอย่างง่าย-การศึกษาเบื้องต้น

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[2]ห้องปฏิบัติการวิจัยกระดูกและข้อในสัตว์ ภาควิชาชีวศาสตร์ทางสัตวแพทย์และสัตวแพทย์สาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ สกัดดีเอ็นเอจากตัวอย่างกระดูกเป็นประโยชน์อย่างมากสำหรับการใช้งานทางนิติวิทยาศาสตร์ แต่มันเป็นเรื่องยากมากที่จะดำเนินการเมื่อเทียบกับการสกัดดีเอ็นเอจากเนื้อเยื่ออื่นๆ เนื่องจากโครงสร้างแข็งของกระดูกและการปนเปื้อนจากสภาพแวดล้อมเช่นแบคทีเรียภายในเนื้อเยื่อกระดูก การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อตรวจสอบวิธีการที่ง่ายและประหยัดค่าใช้จ่ายที่มีประสิทธิภาพสำหรับการสกัดดีเอ็นเอจากกระดูก กระดูกขาสุนัขถูกนำมาใช้เป็นตัวอย่างและสุนัข ยีน dehydrogenase glyceraldehyde-3-phosphate (GAPDH) ถูกนำมาใช้เพื่อยืนยันดีเอ็นเอโดย PCR วิธีการนี้สามารถที่จะสกัดดีเอ็นเอจากกระดูกสดเช่นเดียวกับกระดูกเก่ากระดูกที่ได้รับการฝังอยู่ในดินหรือใต้น้ำที่เก็บไว้ในบ่อเป็นเวลาสามเดือน แต่พบว่าการสกัดมีประสิทธิภาพลดลงในกระดูกเก่า วิธีการนี้ใช้อุปกรณ์และสารเคมีที่มีในห้องปฏิบัติการโมเลกุลทั่วไปและใช้เวลาน้อยกว่า 24 ชั่วโมง จึงเป็นการนำเสนอทางเลือกที่ต้นทุนต่ำและใช้เวลาน้อย เหมาะสำหรับการวิจัยทางนิติวิทยาศาสตร์

คำสำคัญ: การสกัดดีเอ็นเอ, กระดูก, นิติวิทยาศาสตร์