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Research Article

Forensic DNA Typing From Demineralized Human Bone Sections

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Abstract

Historically, protocols for genetic analyses in postmortem human bone shave included sample pulverization. There are only a few studies that describe protocols without a pulverization step. We sought to validate the DNA extraction of previously demineralized bone sections through a protocol that does not involve pulverization of the sample. We analyzed 16 bone samples from 16 unidentified human bodies exposed to the tropical climate. We performed organic DNA extraction, real time PCR quantitation, amplification of STRs using a commercial kit and capillary electrophoresis on the samples. Results showed that it was possible to extract DNA in a concentration above 35ng/μL (median=0.052), generating 50% of full genetic profiles. For the first time in the forensic science field the demineralization of large bone fragments is described as a pre-treatment step. The volume of reagents utilized during extraction is drastically reduced when compared to a total demineralization protocol, while maintaining good quality genotyping.

Introduction

For more than 20 years, scientists have been performing genetic analyses on post-mortem human bone samples [1]. Human identification through genetic analyses, in the forensic sciences field, has contributed substantially in cases of mass disasters [2], identification of victims of armed conflicts such as the second world war [3], historical characters [4], among others.

Although generally showing lower DNA yield than other biological tissues [5], bones are ubiquitous in cases of human identification [6] and, usually, the most viable sources, since soft tissue tends to decompose more rapidly.

Historically, the vast majority of DNA extraction protocols of bone samples have involved pulverizing/powdering the sample [7,8], including that the protocols that are based on total demineralization have been proven to be more efficient than the ones based on partial demineralization [7,21], which happens when undigested bone powder remains after digestion and ends up being discarded.

However, to achieve total demineralization, it is mandatory to add an extremely high number of EDTA molecules to the extraction buffer. Loreille et al [7] suggest 15mL of 0.5M EDTA solution for every 1gram of bone powder. Hence, large volumes of reagents are used, tubes of at least 15mL, and exhaustive concentration steps to reach an elution volume of less than 200μL.

Accordingly, the extraction cost of each sample is raised by the need to use several commercial kits and consumables (buffers, membranes and columns) as well as secondary equipment (Mill [6], Freezer mill [10], High speed rotation device [6], Thermo-mixer [11], and Vacuum pump [10] among others) not always available at forensic laboratories of developing countries.

In this article we describe an alternative protocol for DNA extraction from bones that does not involve powdering the sample. This method composes a recent trend of articles that report DNA extraction from bones and teeth using powder-free methods [8,11-14].

Materials and Methods

We analyzed 16 bone samples (13 femurs, 1 tibia, 1 ulna, 1 skull) collected from 16 unidentified human bodies, sent to the Forensic DNA Laboratory at POLITEC (Official Forensics and Technical

Table 1: History of samples.

No	Sample	History
1	Femur	Caraccidentwithcarbonization
2	Femur	exhumednewborn
3	Femur	firegunprojectilevictim
4	Femur	Human skeleton found in rural area
5	Femur	Human skeleton found in rural area
6	Tibia	Human skeleton found on the side of a road
7	Femur	Human body found in advanced decomposition stage in rural area
8	Femur	Human skeleton found in rural area
9	Femur	Human skeleton found in rural area
10	Ulna	Human skeleton found next to a river
11	Femur	Unidentifiedhumanbody
12	Femur	Unidentifiedhumanbody
13	Femur	Semi-skeletonized human body found in commercial building
14	Femur	Unidentifiedhumanbody
15	Femur	Unidentifiedhumanbody
16	Skull	Unidentified human body found buried

Identification) in Cuiabá, Mato Grosso, Midwest Brazil. The Forensic DNA Laboratory comprises separate rooms for preparation and pre-treatment of bone samples, questioned samples extraction, known samples extraction, pre-PCR, PCR, post-PCR among others. Equipment and consumables appropriated for molecular biology laboratories were used and the manufacturers' recommendations were followed unless stated otherwise.

Table 1 describes the history for each sample. Minimum postmortem age of the samples was among 0.3 and 9 years. We randomly selected 10 cases (8 femurs, 1 tibia and 1 skull) among those currently awaiting analysis and 6 cases (5 femurs, 1 ulna) considered technically challenging, since they had already failed using many other extraction techniques.

Only the samples from challenging cases had been previously sanded externally and washed in 2% sodium hypochlorite and 70% ethanol. All samples were sectioned transversally with the use of decontaminated bows, yielding sections in the form of a ring, of approximately 1cm in thickness. The skull was received by the laboratory already fragmented in pieces of approximately 5cm of diameter.

The samples were placed in their respective plastic containers of 45mL capacity, following an addition of approximately 20mL of 0.5M EDTA solution, pH8. The samples were incubated at room temperature and the solution was changed daily. After 5-7 days, we excised about 150 to 250 mg of each demineralized sample, with the aid of disposable scalpel blades, and placed them in 1.5mL micro-tubes.

After that, we followed a conventional organic extraction protocol, briefly: 350µL of extraction buffer and 20µL of proteinase-K, incubated overnight at 56°C. To each batch of extraction a reagent blank was included. 370µL of phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma Aldrich) was added to the lysate, mixed and centrifuged. The aqueous phase was transferred to a Microcon® YM-100 (Merck) membrane. The membrane was washed with 500µL of water and the elution volume was 50µL.

Real time PCR quantification was carried out in a 7500 (Applied Biosystems) equipment and Quantifiler™ Duo (Thermo Fisher) kit. Dilutions were made when indicated by the quantification results; otherwise we performed PCR amplification with 10µL of DNA extract and NGMSElect™ (Thermo Fisher) kit in a Veriti™ (Thermo Fisher) thermal cycler. Amplification controls were added.

Table 2: Quantification and capillary electrophoresis results.

No	Sample	Postmortemage[a]	Mass (g)	DNA Concentration (ng/uL)	DNA in Demineralized Samples (ng/g)	STR Loci obtained	Genetic profile
1	Femur	2.4	0,211	35,551	8424,41	16 of 16	Full
2	Femur	2	0,156	0,734	235,26	16 of 16	Full
3	Femur	2.2	0,202	0,153	37,87	16 of 16	Full
4	Femur	4.3	0,254	0,108	21,26	0of 16	Not obtained
5	Femur	3.5	0,199	0,086	21,61	16 of 16	Full
6	Tibia	1.2	0,226	0,050	11,06	0of 16	Not obtained
7	Femur	0.5	0,199	0,049	12,31	16 of 16	Full
8	Femur	1.7	0,236	0,026	5,51	7of 16	Incomplete
9	Femur	4	0,188	0,015	3,99	16 of 16	Full
10	Ulna	1.3	0,176	0,008	2,27	1of 16	Incomplete
11	Femur	9	0,204	0,011	2,70	8of 16	Incomplete
12	Femur	2.5	0,206	0,219	53,16	12 of 16	Incomplete
13	Femur	2.5	0,200	0,036	9,00	16 of 16	Full
14	Femur	6	0,193	0,054	13,99	16 of 16	Full
15	Femur	9	0,221	0,097	21,95	12 of 16	Incomplete
16	Skull	0.3	0,200	Undetermined	Undetermined	0of 16	Not obtained

[a]=minimum post-mortem age in years.

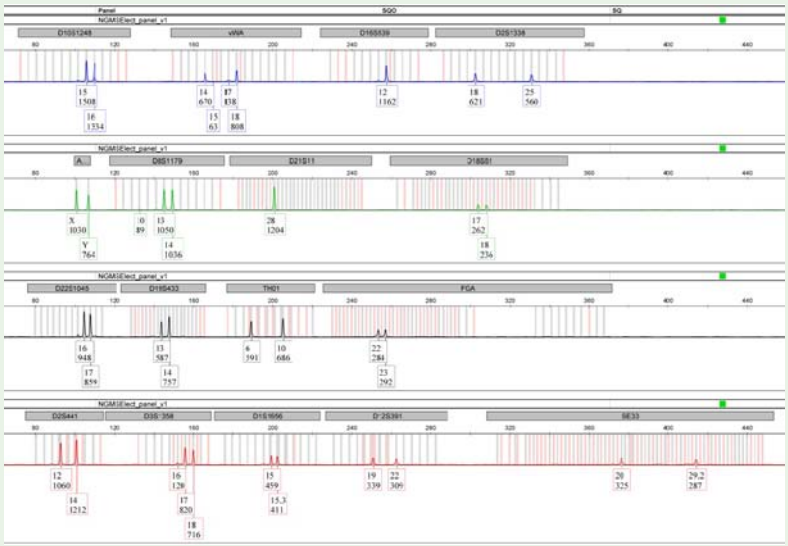

Figure 1: Electropherogram of sample-04, which presented a small drop in on D8S1179.

Capillary electrophoresis was performed in a3130 (Applied Biosystems) genetic analyzer and the data interpreted with Gene Mapper® ID v.3.2 (Thermo Fisher) software.

Results

Quantification data were analyzed and the results were within acceptable parameters (R2=0.995 and 0.998; slope=-3.462 and -3.142).Quantification results did not show signs of inhibition for any of the analyzed samples. In Table 2 we can see that the Ct values for the IPC of the bone samples and the standards (mean of the IPC Cts for the included standards) were always under 30 cycles, with delta IPCCt always well below 1.

Real time PCR quantifications showed DNA concentrations between 8pg/μL and more than 35ng/μL (median= 0.052ng/μL). Sample 16 (skull) showed DNA concentration under the detection limit (undetermined).

Generally, the genetic profiles obtained showed good quality and balanced Peak Height Ratio (PHR). However, some artifacts were observed, including a small drop in 2 samples (Figures 1 and 2).

In Table 2 we present the number of STR markers obtained from each sample. According to the ICMP (International Commission on Missing Persons) classification [15], full profiles were obtained for 50% of the samples; submittable profiles (11 to 15 STR loci) were obtained for 12.50% of the samples and non submittable profiles (0 to 10 STRloci) for 37.50% of the samples.

To this moment, the Forensic DNA Laboratory at POLITEC has concluded 7 cases (43.75%), confirming the identity of 6 victims. After the end of this research, through further extraction attempts based on different protocols but always using demineralized samples, the Forensic DNA Laboratory has concluded 5 more cases (favoring kinship in 3 cases, excluding kinship in 2 cases).

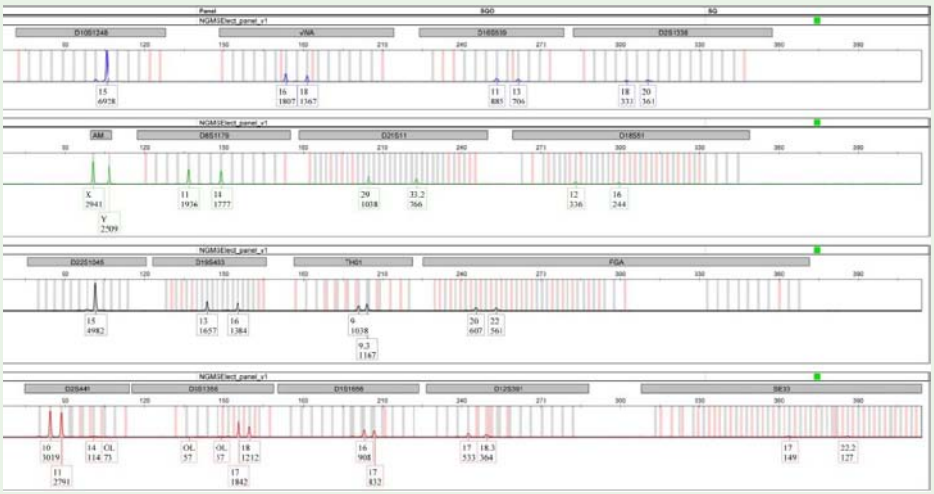

Figure 2: Electropherogram of sample-01, which presented high DNA quantity but also presented evidence of degradation, with decreasing peak height as the DNA fragment size increases.

Discussion

Most scientific articles regarding DNA extraction from bones represent temperate and continental climate countries. There are only a few studies of this kind conducted with samples from tropical climate countries [16]. In Mato Grosso state, where the bodies included in this study were found, the climate is mostly tropical with a dry season with the rest of the state having a tropical monsoon climate [17].

One of the major difficulties in ancient and forensic DNA typing is the presence of PCR inhibiting substances [1]. In this study there were no observed signs of inhibition in agreement with the IPC Ct values of the samples, always lower than 30, and delta IPC Ct always well below 1. Marshall et al. [18], found IPC Ct values well below 1 as well, both for the silica membrane extraction and organic extraction protocols. Although Davoren et al. [19] did not find evidence of inhibiting substances in their extracts using a silica column based protocol, their organic protocol showed delta IPC Ct equal to 3, on average. It is worth noting that these researches involved extensive purification and concentration steps while in the present study only one 500µL wash with ultrapure water was performed.

Effective removal of most PCR inhibitory compounds can also be attributed to the EDTA solution pre-treatment. It was noticed a brown color in the solution, with varying intensity specific to each bone sample, that turned more clear after each change of the solution. We believe that the EDTA solution is capable of extracting humic compounds as well as other PCR inhibitory substances present in the bones.

This protocol was capable of extracting 0.0520ng/µL of DNA (median), whereas Soler et al. [16] extracted 0.0016ng/µL (median) of DNA. Both studies included bone samples exposed to the tropical climate in Brazil, suggesting that our extraction technique might be more suitable for working with samples subjected to this type of climate.

According to other researches, bones from the arms and skull [6,20,21] are amongst the most challenging in forensic DNA typing. In this study, these types of samples also provided the worst quantification and genotyping results.

In accordance to what Amory et al. [15] observed, we also noticed two cases where unknown PCR inhibiting substances did not inhibit the quantification reaction, but impaired or even precluded the generation of a genetic profile. Sample-04 (0.108ng/µL) yielded a blank profile and sample-08 (0.236ng/µL) yielded a partial profile even though it quantified more than double the required DNA input.

Although EDTA demineralization takes a long time, there are several ways to mitigate this problem. It is not necessary to wait for the complete demineralization of the bone section, since sampling can occur as the demineralization progresses from outside to inside. Besides, thinner bone slices are demineralized faster [22], as well as performing better in the DNA extraction when compared with powdered bone of the same mass [11].

Although this protocol is based on organic extraction, the main focus of this study is the demineralization as a pre-treatment step for bone sections to be used with different extraction protocols, similarly to another recent publication [14]. After demineralization and sampling, DNA can be extracted through commercial kits or automated platforms, following protocols for tissue samples such as muscle (our unpublished data).

In order for this protocol to be temporally viable, we suggest practitioners to obtain two sections of bone. While one is immediately processed through grinding, the other can be demineralized for a second extraction attempt in case the first attempt is not successful.

Conclusion

Bone section demineralization is presented as a pre-treatment step for forensic DNA analysis from bones. It was shown that the samples still retain endogenous DNA in high enough quantity and quality for generating full STR profiles, as well as it significantly reduces the volume of reagents during DNA extraction when compared to an equivalent total demineralization protocol.

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