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Comparison of DNA yield after long-term storage of Second World War bone samples

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ABSTRACT

Sample storage is of paramount importance in forensic genetics laboratories since only optimal storage enables successful recovery of DNA from old bones that contain very low amount of severely degraded DNA. When identification of missing persons from skeletal remains is completed, bone sample is routinely stored at -20 °C for long-term storage for retesting in future, if necessary. After molecular genetic analyses of Slovenian Second World War (WWII) victims, small fragments of femurs were stored at -20 °C. Reduction in DNA recovery has been observed in frozen liquid DNA extracts by some authors and the goal of our study was to explore how freezing of bone samples affects the preservation of DNA. To achieve this goal, the difference in DNA yield in extracts obtained from WWII bones analyzed in 2009 (data from published paper) and DNA yield in extracts obtained from the same bones (piece sampled next to the one used in 2009) taken out of the freezer after long-term storage on -20 °C for 10 years was examined, using the same extraction method and the same quantification kit. Up to 100 ng DNA/g of bone powder was obtained from 57 WWII femurs and up to 31 ng DNA/g of bone powder from the same femurs investigated after long-term storage in this study. 0,5 g of bone powder was decalcified using full demineralization extraction method. The DNA was purified in a Biorobot EZ1 device (Qiagen) and DNA quantity determined with the Human Quantifiler kit (TFS). Statistical analysis showed significant difference in DNA yield in extracts obtained from WWII bones in 2009 and extracts obtained from the same bones stored at -20 °C after 10 years. As reported for frozen liquid DNA extracts, reduction in recovery of DNA was confirmed for frozen bone samples as well.

1. Introduction

Optimal sample storage is of paramount importance in forensic genetics laboratories since it enables successful recovery of DNA from forensic samples, especially from old bones that contain very low amount of severely degraded DNA [1]. In skeletonized missing person identification cases, bone sample is routinely stored at -20 °C for long-term storage [2]. The main purpose of freezing the samples is to minimize DNA degradation, resulting in loss of cell integrity, quality and quantity of genomic DNA [1]. Due to previous studies, reduction in DNA yield occurs with refrigerated liquid DNA [1], with changes in temperature in the process of freezing and thawing the samples [1,3] and in the samples stored in various microcentrifuge tubes [4]. Additionally, the spontaneous decay with progressive molecular damage of the DNA was reported [5]. This brief report is intended to add to our knowledge regarding the quantity of DNA loss after long-term storage of bone samples in its original form at -20 °C.

2. Materials and methods

In this study, we compared the quantity of DNA obtained from 57 femur bones of WWII victims in the study performed in 2009 (data published in [6]), with the quantity of DNA obtained from the same bones after 10 years of storage in freezer, with the same extraction method and the same quantification kit. The piece of bone sampled in this study was next to the one used in 2009. The bones were stored in its original form at -20 °C for 10 years. The laboratory procedure consisted of three stages: preparation of bone samples, isolation of DNA and determination of DNA quantity. Cleaning, grinding, decalcification, and purification of DNA were performed following Zupanič Pajnič [7]. Briefly, the bones were cleaned, both, mechanically and chemically and dried over night. With a circular diamond saw, 2 cm long bone samples were created. Bones were grinded in bone powder, using TissuLyser (Retsch) homogenizer. Total demineralization was performed, adding 0.5 M EDTA to 0.5 g bone powder. The samples were incubated at 37 °C

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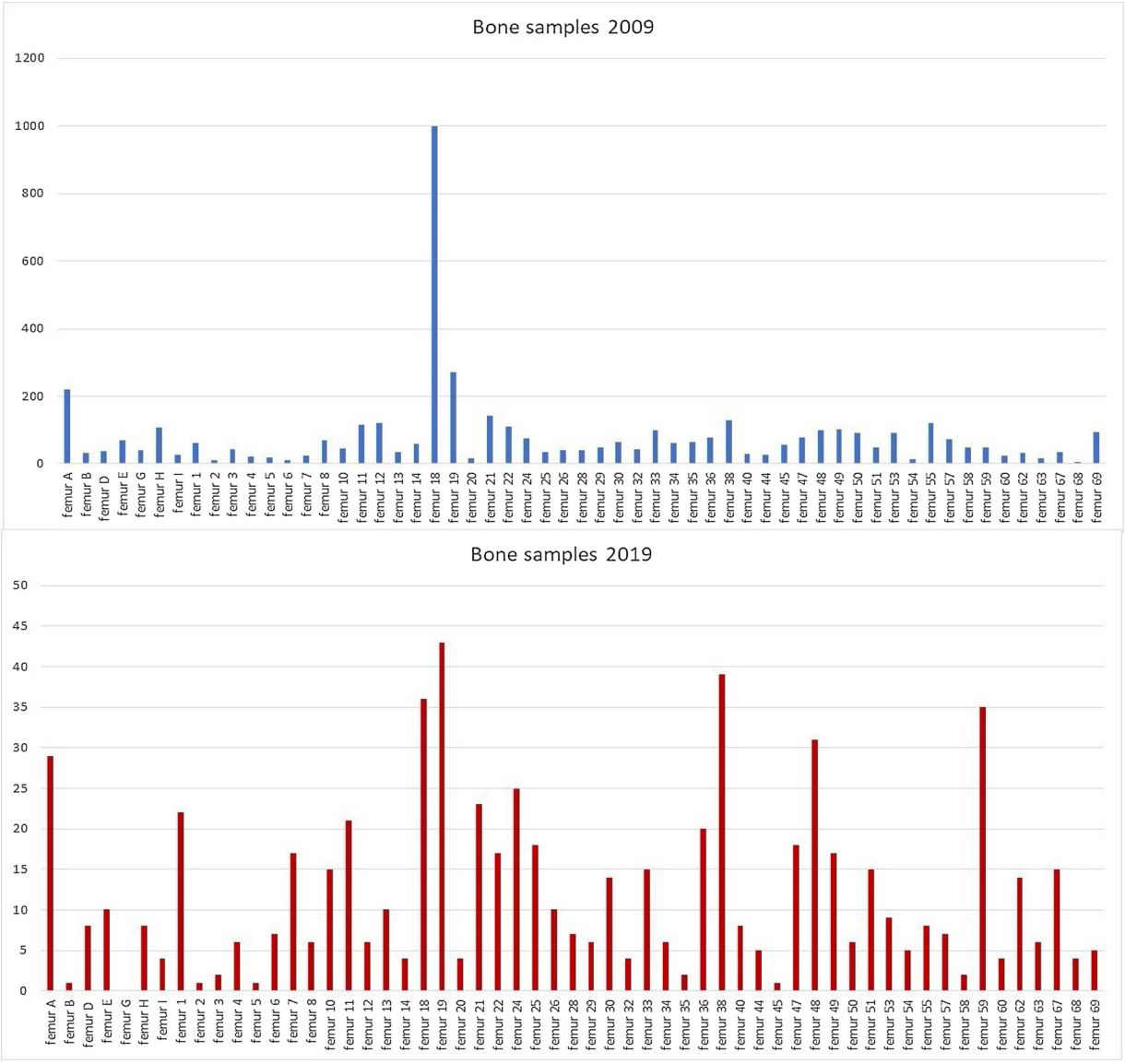

Fig. 1. Distribution of the DNA quantity expressed in pg DNA/μl of isolate in WWII bone samples analyzed in 2009 (blue) and 2019 (red) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

over night in a Thermomixer comfort (Eppendorf)t, shaken at 950 rpm. After centrifugation, the supernatant was discarged and the precipitate was washed with bidistilled water, centrifuged and G2 buffer, proteinase K and DTT added to precipitate and incubated at 56 °C. Finally, the DNA was purified in a Biorobot EZ1 device (Qiagen) using the EZ1 DNA Investigator Kit (Qiagen) and 50 microliters of DNA obtained from each bone sample. DNA quantification was performed by quantitative PCR (qPCR) and two microliters of each sample was assessed by the Human Quantifiler Kit (ThemoFisher) on a 7500 Real-Time PCR System (Applied Biosystems) with the HID Real-Time PCR Analysis Software, version 1.1 (Applied Biosystems).

3. Results

For the purpose of the paper, the following research hypothesis was elaborated: the bone fragment DNA deteriorates after 10 years of freezing. After deleting the outliers (bone fragment values higher than 145) the distribution of the variables shown in Fig. 1 can be seen. Quantity of DNA is expressed as pg of DNA per microliter of sample.

Fig. 1 shows there is a higher amount of the DNA extracted from bone fragments in 2009 than in 2019. In the latter, descriptive statistics were computed and the research hypothesis was tested using the paired samples *t*-test. The statistical analysis was carried out with the computer program SPSS Statistics for Windows, version 25.0 (Statistical Package for the Social Sciences Inc., Illinois, USA). The quantity of DNA extracted from bone fragments in 2009 is higher (M = 57.7; SD = 34.984) than the one extracted from bone fragments in 2019 (M = 12.18; SD = 10.354). The minimum amount of the DNA obtained from bone fragments in 2009 is 5 and 1 for 2019, while the maximum amount of the DNA obtained in 2009 is 142 and 43 for 2019. The results from the paired samples *t*-test (t = 10.723, sig = 0.001) support the research hypothesis. Thus, there can be concluded that the quantity of DNA extracted from bone fragments in 10 years difference is statistically significant. It can be seen that the quantity of the DNA extracted from bone fragments decreased significantly with time in freezer.

4. Discussion

The results proved reduction in recovery of DNA for frozen bone samples. Forensic DNA research efforts have been focusing on developing new methods of long-term sample storage, since the current standard has proved to be less than optimal [1]. There are several

factors that must be taken under consideration. The humidity plays an important role, since dry storage on a solid matrix prevents movement on a molecular level, and therefore chemical reactions are less probable [8]. However if the moisture is reintroduced to the sample or the temperature is varied, chemical reactions can occur again [8]. Temperature in which the sample is kept affects DNA preservation as well, with changes in crystallinity values of bone mineral, visible only after one freezing and greater decrease in DNA yield after each freeze-thawing cycle [3]. The effect of light, initial contamination, efficiency of the seal and intrinsic factors of the bone, such as bone type and size, must be taken under consideration as well [1].

5. Conclusion

As reported for frozen liquid DNA extracts [1,8], reduction in recovery of DNA was confirmed for frozen bone samples as well, with significant decrease in DNA yield after 10 years of freezing. Additional studies are required to increase our understanding of long-term storage of forensic DNA samples methodologies and their effect on DNA quantity.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

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