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Storage of Second World War bone samples: Bone fragments versus bone powder

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ABSTRACT

Bone samples may yield low-quality and low-quantity DNA and duplicated analyses of different genetic markers have to be performed for identification of missing persons. Mostly no DNA extract is left after analyses and efficient storage of bones is needed to ensure the stability of the sample over time for retesting using new markers and new technologies. Usually not all of the bone powder prepared in grinder is used for extraction and rest can be stored for future analyses. After molecular genetic analyses of 88 victims of Second World War (WWII) Konfin I mass grave in Slovenia (performed in 2009), fragments of femurs and bone powder that were left were stored at -20 °C. Some authors reported that long-term storage of powder results in the reduction of DNA preservation and its degradation (even at low temperature), explained by an increase in oxidative damage as a result of the enormous increase in exposed surface area. Consequently, grinding of bones as shortly prior to DNA extraction was recommended. The goal of our study was to explore the difference in DNA yield between bone fragment and bone powder frozen for 10 years. 57 WWII femurs were examined and DNA extracted from each of them using bone fragment (piece sampled next to the one used in 2009) and bone powder obtained in 2009, both taken out of freezer after 10 years of storage. Half gram of bone powder was decalcified using full demineralization extraction method. The DNA was purified in a Biorobot EZ1 (Qiagen) and quantified with PowerQuant kit (Promega). Statistical analysis showed significant difference at the 0.05 level in DNA yield comparing fragments of bones and bone powder stored at -20 °C for 10 years. The results show there is more DNA stored in the bone powder than in the bone fragments. Because of time - consuming powdering procedure we recommend to store not only the fragment of the bone, but obtained bone powder as well.

1. Introduction

In missing persons identification cases efficient storage of bones is needed to ensure stability of sample over time for retesting using new markers and new technologies. Routinely fragments of bones are stored at -20 °C after identification of missing person. Usually not all bone powder is used for extraction and because of time - consuming powdering procedure we wondered whether storage of bone powder would be of use for future analyses. Hummel [1] suggest that homogenizing of bone samples should be done as shortly prior to DNA extraction and long-term storage of bone powder may lead to DNA degradation. Scientists agree that storage of bone samples plays a crucial role, since DNA preservation depends primarily on environmental conditions [2,3]. According to Hummel [1] powdering of bones increases exposed surface area and consequently oxidative damage of DNA. Considering above, the goal of our study was to determine DNA yield in WWII bone fragments and compare it with DNA yield from bone powder stored in the same conditions at -20 °C for 10 years. Samples analyzed were left after molecular genetic identification of 88 Slovenian victims of WWII Konfin I mass grave performed in 2009 [4].

2. Materials and methods

In this study, we compared the quantity of DNA obtained from 57 bone fragments with the quantity of DNA obtained from bone powder of the same bones that were both frozen for the same duration of time. We used the same extraction method and quantification kit for both analyses. All bones included in the study were femurs. Since we were working with aged DNA all precautions to avoid contamination were considered. Cleaning, grinding, decalcification and purification of DNA were performed following Zupanič Pajnič [5]. Briefly, the bones were cleaned mechanically and chemically. With a circular diamond saw,

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2 cm long bone samples were created. To avoid overheating, liquid nitrogen was used in all phases. Bones were grinded in powder using TissuLyser homogenizer (Retsch). Total demineralization was performed, adding 0.5 M EDTA to 0.5 *g* bone powder. The samples were incubated at 37 °C overnight in a Thermomixer comfort (Eppendorf), shaken at 950 rpm. After centrifugation, the supernatant was discharged 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 µl of DNA was obtained from each bone sample. DNA quantity was determined with real-time PCR (qPCR) method using the PowerQuant kit (Promega). All samples were analyzed in duplicate and average PQ results are reported. Descriptive statistics were computed and the research hypothesis was tested using the paired samples *t*-test using the computer program SPSS Statistics for Windows, version 25.0 (Statistical Package for the Social Sciences Inc., Illinois, USA).

3. Results

For the purpose of the paper, the following research hypothesis was tested: the quantity of DNA obtained from bone powder is higher than the quantity of DNA obtained from bone fragments. After deleting the outliers (in the case of PowerQuant (PQ) powder values higher than 0.245 and in the case of PQ bone fragment values higher than 0.09 were deleted) the distribution shown in Supplementary Figure 1 can be detected (see Supplementary Figure 1) and for most of the samples the quantity of DNA extracted from the bone powder is higher than the quantity of DNA extracted from the bone fragments. Descriptive statistics shows that the mean for the quantity of DNA extracted from bone powder is higher (M = 0.062; SD = 0.053) than the quantity of DNA extracted from the bone fragment (M = 0.03; SD = 0.022). The minimum value for DNA quantity is 0.0034 for bone powder and 0.0026 for the bone fragment, while the maximum value is 0.2435 for the bone powder and 0.088 for the bone fragment. Skewness (1.374 and 0.958 respectively) and Kurtosis (1.859 and 0.071 respectively) show a distribution close to a normal one. The results of the statistical test (t = 3.812, sig = 0.001) support the research hypothesis. Thus, there can be asserted there is higher amount of the DNA extracted from the bone powder than from the bone fragments.

4. Discussion

Results of the study shows there is statistically significant difference between DNA yield from bone fragments versus bone powder and higher quantity was obtained from bone powder. DNA extraction from skeletal remains is time consuming, because of long powdering procedure. Since frequent freeze-thaw cycles increase DNA degradation because of surface bleaching, organic component breakdown and humidity changes, one step freezing is recommend for long-term storage [6,7]. Our results in contrast to experience of Hummel [1] indicate that long-term storage of powdered bone does not necessarily mean lower quantity of extracted DNA from frozen bone powder in comparison to frozen bone fragment.

5. Conclusion

Taking into account the results of our study, we suggest long-term storage of bone powder, left from the first DNA extraction, for future investigations along with bone fragments.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsigss.2019.09.068.

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