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Molecular study of time dependent changes in DNA stability in soil buried skeletal residues

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Abstract

In the past years, many publications about identification and sex-determination of dry human bones by means of DNA analysis have been published. However, few studies exist that investigate the potential use of DNA technique to determine the postmortem interval (PMI). In the present study we analyzed the rate of increasingly smaller fragments of chromosomal DNA and PMI.

We examined DNA degradation in human bones with postmortem intervals ranging between 1 and more than 200 years that had been kept under comparable conditions concerning weather and soil. Following bone separation into the three different zones of interest of inner/middle/outer segments the quantity of total DNA was determined in each region. Subsequently, the degree of DNA fragmentation was estimated by searching for PCR products of defined size (150, 507 and 763 bp) with primers of the human-specific multicopy β-actin-gene.

Concerning DNA quantity we detected a significant correlation between the zone of interest and the amount of DNA. However, there was no correlation between the amount of DNA and PMI. In contrast to this, analyzing DNA using PCR showed a significant inverse correlation between fragment length and PMI. Thus, postmortem DNA degradation into increasingly smaller fragments reveals a time-dependent process. It has the potential to be used as a predictor of PMI in human bone findings, provided that environmental conditions are known.

Keywords: Bone; Forensic; DNA degradation; Time since death; Postmortem interval (PMI)

1. Introduction

The postmortem interval (PMI) is a crucial piece of information that leads to solving the puzzle of an unexplained death. For the determination of the early PMI several methods provide fairly precise information. After longer periods of time or when dry bones are the only remaining biological material, the current estimation of the PMI is vague or may even be impossible. Previously, many publications have been published about identification and sex-determination of human bones by DNA technology (see review [1]). However, few studies exist about the determination of the PMI using this technology. One reason for this is the fact that postmortal DNA degradation is incompletely understood. It is known that after death oxidative processes, cellular nucleases and other hydrolytic enzymes cause chromosomal DNA degradation into increasingly smaller fragments over time. Hydrolytic enzymes are released when cellular and subcellular membranes decay as a consequence of autolysis. Therefore, we raised the hypothesis that it is possible to determine the PMI by determination of (ancient) DNA fragment length.

A recently published study demonstrated a correlation between PMI and DNA fragmentation using single-cell gel electrophoresis. The authors revealed DNA fragmentation as an organ- and time-dependent process and concluded that this piece of information can be used to predict the PMI in the early postmortem period [2]. According to numerous studies, DNA in human bones can remain stable for a long period of time. Even

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after decades, bones have been shown to contain enough DNA for molecular analysis [3–9]. Holland et al. [10] demonstrated successful amplification of DNA despite extreme environmental conditions, small amounts and poor quality of the DNA. They were able to show that 2 g of bone can be sufficient for successful amplification.

In our present study, we focused on correlating postmortem DNA degradation and fragmentation of human bones to PMIs ranging between 1 and more than 200 years. For this purpose, different PCRs using primer-pairs with amplicon lengths of 150, 507 and 763 bp were applied. We hypothesized that the anatomical localization of the samples within the bone may play a role in the complex process of tissue degradation. Several other studies have shown that different bones seem to degrade in different ways and therefore contain variable amounts of DNA [11–13]. For our experiments we analyzed 14 samples of 11 human long bones (femurs) coming from comparable burial/recovery sites. It is widely known that all autolytic processes including degradation of DNA are highly dependent on environmental conditions like temperature and humidity [11,13–16]. In order to have comparable environmental conditions, the bones used in this study were retrieved from exhumations at local Munich cemeteries only. For comparison with this soil-embedded material, one femur from a South German ossuary was additionally analyzed.

2. Material/methods

2.1. Bone material

In this study 14 samples of 11 human long bones (femurs) were analyzed. These came predominantly from exhumations from local Munich cemeteries with recorded time periods since death, ranging between 1 and 34 years. Because of a quite close geographic relation of the habitats, conditions concerning climate and soil were very similar. In addition to the soil-embedded material, we included one femur from a South German ossuary with a PMI of more than 200 years (inhumation between 1400 and 1800 AD) (Fig. 1).

2.2. DNA extraction

To reduce the risk of contamination, the bones were first superficially and thoroughly cleaned with sodium hypochlorite 0.5% solution. Then, the outer surface of the bone was removed mechanically using a sterile dental blade. Subsequently, a complete cross section of 2 cm thickness was removed exactly from the middle of the diaphysis of every femur using an electric saw with sterile blades. These cross sections were further divided into three circular areas covering the inner third (which is facing the bone marrow), the middle third or the outer third (which is in contact to the surrounding environment) (Fig. 2). Every sample was then separately pulverised with a mixer mill (Retsch MM200, Haan, Germany).

Fig. 1. Example of the analytical setting (bone from a South German ossuary with a PMI of >200 years).

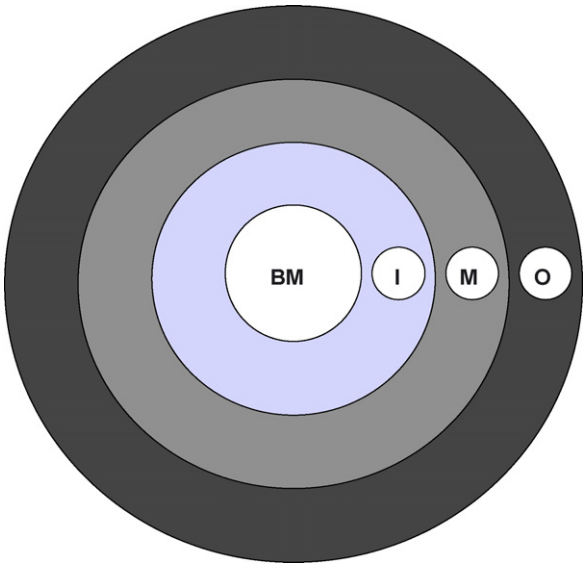

Fig. 2. Schematic presentation of the sample preparation: The bone slices were divided into three ring-like zones representing the inner (I), middle (M) and outer (O) region. In the centre there is contact to the bone marrow (BM), the outer zone is adjacent to the soil.

For further analytic processing, two different methods of DNA extraction were used to optimize the efficacy of DNA preparation. First, the method of Boom et al. in a modification of Haas et al. was used [17,18]: Every sample of the pulverised material (1 g) was incubated with 2 ml of 0.5 M EDTA solution containing proteinase K 20 mg/ml at room temperature for 72 h on a rotatory mixer. Following centrifugation for 15 min at 4000 g, 1 ml of the supernate was removed and 1 ml of GSCN solution and 50 μl diatomaceous earth were added. After incubation in a rotatory mixer for another 2 h, the diatomaceous earth was pelleted by centrifugation and washed twice with ethanol 70% and once with acetone. The DNA was eluted with 120 μl of sterile water. Finally, another washing and concentration step was performed with Microcon-30 filters (Millipore, Bedford, MA, USA) and the final DNA solution was diluted to 20 μl with sterile water. In parallel, we used a commercial First-DNA Kit of GEN-IAL (Troisdorf, Germany): every sample of the pulverised material (1 g) was incubated with 40 ml of 0.5 M EDTA solution at room temperature for 72 h on a rotatory mixer. Following centrifugation for 10 min at 4000 g, the pellets were washed three times with sterile water. Then 1000 μl of Lysis 1 (GEN-IAL), 100 μl of Lysis 2 (GEN-IAL), 25 μl of 0.8 M DTT solution (Sigma-Aldrich, Steinheim, Germany) and 50 μl of enzyme solution (GEN-IAL) were added. After incubation for another 24 h at 56 °C, 750 μl of Lysis 3 (GEN-IAL) were added. Following centrifugation for 20 min at 13,000 rpm, the pellet was washed with 70% ethanol. Finally, the DNA solution was dried and diluted in 30 μl of sterile water.

2.3. Precautions to prevent contamination

Several precautions were taken to avoid contamination during the analytical steps. The extraction, PCR and post-PCR analyses were all conducted in separate rooms of the building. Disposable gloves were worn during all different procedures and changed frequently. Two extraction blanks were always included in the same procedure and an additional PCR blank was included in each PCR reaction, containing all reagents, but no DNA template.

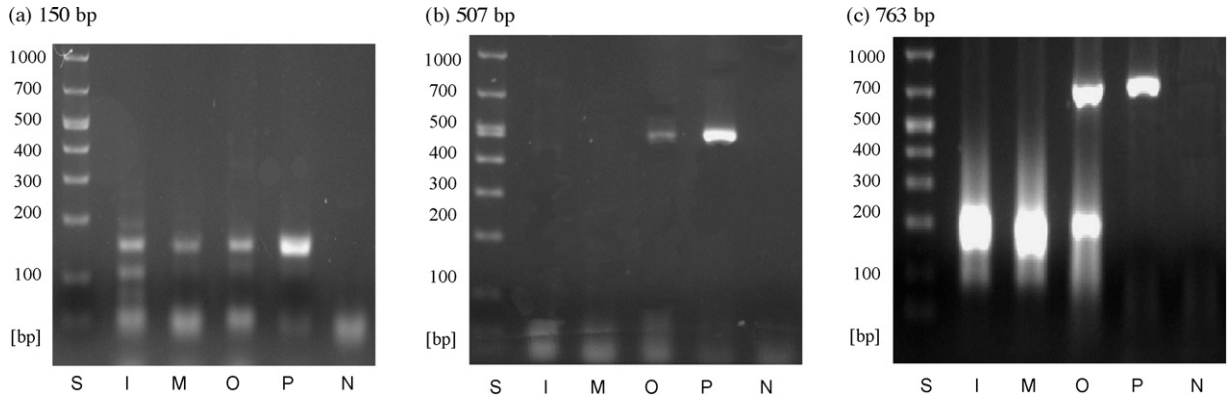

Fig. 3. PCR agarose gels of bone #11 with a PMI of 1 year. Specific amplicons of 150 bp length in all sections (Fig. 3a), positive specific amplicons of 507 bp and of 763 bp length in the outer part of the bone (Fig. 3b and c). (S) standard, (I) inner zone, (M) middle zone, (O) outer zone, (P) positive control, (N) negative control.

2.4. Quantitative DNA measurement

The amount of DNA was determined photometrically by a RNA/DNA-calculator. The measurement was done at 230, 260, 280 and 320 nm.

2.5. Amplification of human DNA

By using the known genomic DNA-sequences (published in GenBank, NCBI), appropriate primers of the human-specific multicopy β-actin-gene were selected. As this gene does not exist in the genome of bacteria or fungi, false positive results concerning this matter could be avoided. The chosen primer-pairs produced amplicons of different length: 150, 507 and 763 bp.

The PCR was carried out in a total reaction volume of 25 μl, containing 1 μl of each primer (Interactiva, Thermo Hybaid, Ulm, Germany), 200 ng DNA template, 0.3 μl of dNTP solution (Amersham Pharmacia Biotech, Upsala, Sweden), 0.4 μl of PANScript polymerase (PAN Biotech, Aidenbach, Germany) and 0.5 μl, respectively 0.6 μl $MgCl_2$ (PAN Biotech, Aidenbach, Germany) in 2.5 μl of a 10× buffer (PAN Biotech, Aidenbach, Germany). PCR conditions were as follows: 40 cycles, denaturation at 94 °C for 30 s, hybridization at 60 °C (primer 2)/62 °C (primers 1 and 3) for 30 s and synthesis at 72 °C for 30 s (primer 1)/60 s (primers 2 and 3).

2.6. Detection of PCR products

The PCR products were electrophoretically separated on an agarose 2% gel, visualized on a UV screen after staining with ethidium bromide and on a polyacryamide 14% gel, visualized by silver staining (see Fig. 3; case #11).

3. Results

3.1. Quantitative analysis of DNA

The quantification of the DNA from the three different segments revealed a correlation between the zone of interest and the amount of DNA. The outer parts of the bones showed the highest and the middle parts the lowest DNA concentrations. In 8 of 14 samples the highest values were detected in the outer zone, whereas 9 of the 14 samples showed the lowest concentration in the middle region. Moreover, the mean value of the DNA concentration was notably higher in the outer parts (180.4 ng/μl) than in the middle (121.6 ng/μl) and inner parts (142.3 ng/μl) (Table 1). Despite this association, we were not able to show a correlation between amount of DNA and PMI ($p > 0.5$).

3.2. Comparison of the two different extraction methods

3.2.1. Quantitative analysis of DNA

It turned out that every PCR required a minimum amount of 200 ng DNA. Also, it became clear that extraction method one (Boom et al. in a modification of Haas et al. [17,18]) was less efficient than extraction method two (First-DNA Kit of GENIAL), which was used for all extractions. It was more effective and showed better results for all measured values (see also Table 1).

3.2.2. Amplification of DNA (PCR)

Moreover, not only did extraction method two yield more DNA, the DNA was also of significantly better quality. Using method two, the amplification of a 150-bp-fragment in the outer parts of the bones #9 and #13 and the amplification of a 507-bp-fragment in the outer part of bone #13 were possible. Using method one, the outer parts of both bones remained negative.

Table 1
Results of the quantitative analysis of DNA [ng/μl] using the two extraction methods (E1) and (E2) described in the text. (E1) is highlighted in gray in this table. The lowest values in each case are marked in bold type, the highest values in italic type. The columns (I), (M), (O) demonstrate the test results for the inner (I), the middle (M) and the outer (O) zone of the analyzed bones

PMI (bone number)	**DNA [ng/μl]**		
	I	M	O
1 year (#11)	31,5	**25,9**	*51,8*
4 years (Exhum.)	56,0	**43,4**	*163,1*
8 years (#12)	**56,0**	61,6	*86,1*
8 years (#12)	**68,6**	*206,5*	162,4
8 ½ years (#3)	*338,8*	**120,4**	309,4
15 years (#9)	*71,4*	**49,0**	49,7
15 years (#9)	224,7	*251,3*	**123,2**
18 years (#19)	268,1	**175,7**	*512,4*
21 years (#10)	139,9	**86,8**	*198,0*
23 years (#37)	74,2	**67,9**	*205,6*
30 years (#8)	209,3	*312,9*	**140,0**
34 years (#13)	*100,8*	*100,8*	**83,3**
34 years (#13)	271,6	**157,5**	*338,8*
>200 years (R/L27)	81,2	**42,7**	*101,5*

Table 2
Results for the various PMIs using the two extraction methods (E1) and (E2) described in the text. (E1) is highlighted in gray in this table. (+) is used for a positive test result and (−) for a negative result. The columns (I), (M), (O) within the column for each base pair (150, 507, 763) demonstrate the test results for the inner (I), the middle (M) and the outer (O) zone of the analyzed bones

PMI (bone number)	150bp			507bp			763bp		
	I	M	O	I	M	O	I	M	O
1 year (#11)	+	+	+	-	-	+	-	-	+
4 years (Exhum.)	+	+	+	+	+	+	-	+	+
8 years (#12)	+	+	+	-	+	-	-	+	-
8 years (#12)	+	+	+	+	+	-	+	-	-
8 ½ years (#3)	+	+	+	+	-	+	-	-	-
15 years (#9)	-	+	-	-	+	-	-	-	-
15 years (#9)	-	+	+	-	-	-	-	-	-
18 years (#19)	-	+	-	-	-	-	-	-	-
21 years (#10)	+	+	+	-	-	-	-	-	-
23 years (#37)	-	-	-	-	-	-	-	-	-
30 years (#8)	+	+	+	-	-	+	-	-	-
34 years (#13)	+	+	-	-	-	-	-	-	-
34 years (#13)	+	+	+	-	-	+	-	-	-
>200 years (R/L27)	-	-	-	-	-	-	-	-	-

For bone #12, using method two allowed us to get an additional specific amplicon at 507 bp for the inner part (Table 2).

3.3. Amplification of DNA (PCR)

3.3.1. Extraction method one

A total of four bones extracted with method one contained enough DNA for further investigation by means of PCR. We found that the middle part of the bones provided more specific amplicons than the inner and outer parts. This finding can be seen especially definite for the bones with a PMI of 8 years (#12 E1) and 15 years (#9 E1).

Furthermore, the results revealed a correlation between fragment length and time since death. Whereas the 150-bp-fragment of the β-actin-gene was detectable in all four samples (1, 8, 15 and 34 years), a specific band of 507 bp was only seen in the bones with a PMI of 1, 8 and 15 years. The 763-bp-fragment was only positive for the two ''youngest'' bones (1 and 8 years) (Table 2, see also Fig. 3).

3.3.2. Extraction method two

The results obtained with method one could be confirmed with method two. Specific PCR-products were found most frequently in the middle parts of the bones as well. This result is most definite for the amplification of the 150-bp-fragment.

Furthermore, we were able to demonstrate an association between presence of specific DNA-products and the PMI. The 150-bp-fragment was detectable in all samples with the exception of one 23 and the >200 years old bone, whereas the 507-bp-fragment could only be amplified up to a PMI of 15 years. The bone #13 E2 (PMI of 34 years) was the only exception in this case as it showed a positive band at 507 bp. By using primer-pair 4 (763 bp), specific PCR-products were only obtained for the two youngest bones with a PMI of 4 and 8 years (Exhum. E2, #12 E2) (Table 2).

4. Discussion

The aim of this study was to determine the application of DNA analysis for the estimation of the PMI. Our results show that there exists a time-dependent process of nuclear DNA degradation due to autolytic processes and decomposition. The analysis of this process with respect to quantity and quality of human DNA can be used for a novel, more precise method to determine the PMI. This approach may especially be helpful in very long PMIs, where dry bones are the only remaining biologic material.

Normally, environmental influences that promote DNA degradation act on the DNA over a long period of time and may lead to poor quality and reduced quantity of ancient DNA. Usually, these environmental factors cannot be controlled by the investigator. However, an avoidable problem is contamination with recent DNA, e.g., coming from the investigators. Principally, this may occur during every step of the process. Therefore, every measure has to be taken to avoid contamination. Furthermore, it is crucial to use an effective extraction method in order to obtain enough uncontaminated human DNA and to optimize the standard procedure for the subsequent PCR. To date, several commercially available extraction kits are available as recently reviewed by MacHugh et al. [13]. In the present study, a conventional extraction method (Boom et al. in a modification of Haas et al. [17,18]) was compared with a commercially available extraction kit (''First-DNA Kit'' of GEN-IAL). The commercial kit provided better results in all subsequent investigations. This confirms the conclusion of Cattaneo et al. [6] that the extraction method can significantly influence PCR results. Moreover, only results obtained with identical extraction methods can be used for a comparative study, as done here. Regardless of any previous recommendation, the best extraction method is the one that is routinely performed in the lab. Finally, it is pivotal to standardize the amount of DNA applied in every PCR. In our study the quantity of the extracted DNA solutions was determined photometrically by a RNA/DNA-calculator.

In the present study, we followed a dual approach with quantitative and qualitative aspects: first, we measured the amount of total ancient DNA in the three regions of interest. In a second set of analyses, we determined the fragment length of the human multicopy gene β-actin as a frequently used nuclear gene in ancient DNA studies (e.g., Ref. [18]). In order to evaluate whether there exists a correlation between anatomical localization and amount of retrievable DNA, a 2-cm slice was removed from the exact middle of the diaphysis of every femur. Subsequently, these cross sections were divided into three regions of analysis: the inner zone facing the bone marrow, the middle region and the outer zone with contact to the environment.

We were able to show that the localization within the bone slice influences its degree of degradation. Obviously, this is due to a different degree of protection against environmental

influences. Likewise, we conclude that the outer zone is significantly more influenced by the direct contact to the environmental soil; the inner zone also is more affected via the bone marrow cave; the middle zone provides the best preserved bone with adequate amounts of retrievable DNA.

On a quantitative basis, in 8 of 14 samples the highest amounts were detected in the outer part, whereas 9 of these 14 samples showed the lowest concentration in the middle part (outer vs. middle vs. inner = 180.4 ng/μl vs. 121.6 ng/μl vs. 142.3 ng/μl). The explanation for this is that extrinsic DNA, such as bacterial DNA, was included in the photometrical measurement of the amount of DNA. The high amount of extrinsic DNA and the high degree of DNA degradation in the outer part led to the conclusion that the protected middle part of the bone is the most useful localization to obtain DNA for the determination of the PMI by DNA technology. Previously, it has been shown that the type of bone (e.g., rib, clavicle) can influence the DNA degradation rate [11–13]. To our knowledge, no previous study has focused on DNA content with respect to the localization within the respective bone.

Finally, we used our optimized extraction and PCR methods to answer the question whether there is a correlation between the amount of DNA and PMI. Some others have tried to answer this question previously. Consistent with Fisher et al. [19] our results negate this. The ''younger'' bones did not contain more DNA than the ''older'' ones—and vice versa. But again, quantity of DNA correlated with the localization within a single bone. However, as mentioned earlier, the middle parts of the bones more often provided positive specific amplicons than the inner and outer parts. This strongly suggests that the DNA in the middle part of the bone is of smaller quantity, but higher quality. Although we were able to extract the highest amount of DNA from the outer part of the bone, this high amount of DNA is either degraded in a stronger degree or it is contaminated with extrinsic (bacterial) DNA.

We can conclude from our data that the determination of PMI using dry bone analysis and the analysis of DNA fragment length may provide relevant data. First of all, falsification of the results due to bacterial or fungal DNA can be avoided by using human-specific primers (e.g., β-actin-gene). Secondly, DNA degradation can be assessed by analysis of differently long fragments of a specific gene (150, 507 and 763 bp in the present case). Our results revealed an inverse correlation between fragment length and PMI. Large fragments of 763 bp could be detected within the first 8 years postmortem, smaller fragments of 507 bp fragment up to a PMI of 15 years, whereas the 150-bp fragment could be amplified in all bones with exception of one 30 and one >200 years old bone. Thus, in accordance with other studies [6,11,21], our results indicate an inverse correlation between fragment length and PMI in human bones. We were able to show that DNA degradation and fragmentation proceeds rapidly and causes the loss of high-molecular DNA already within the first year postmortem. Furthermore, we were able to show differences concerning DNA degradation and fragmentation within the inner, middle and outer zone of a single bone. In order to use the measurement of DNA fragmentation for the determination of PMI, further investigation is necessary to understand more about the complex DNA degradation postmortem, its dependence on environmental conditions, and DNA analysis technology.

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