

DNA repair enables sex identification in genetic material from human teeth

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Abstract

Background: The purpose of this study was to test the effectiveness of a DNA repair protocol in improving genetic testing in compromised samples, frequently encountered in Forensic Medicine.

Methods: In order to stretch the experiment conditions to the limits, as far as quality of samples and DNA is concerned, we tried the repair protocol on ten ancient human teeth obtained from an equal number of skeletons from a burial site in Lerna, Middle Helladic Greece (2100 - 1700 BC). For these samples, sex was previously determined morphologically, serving as a reference to compare our molecular data with. The samples were analysed using the DNA amelogenin sex test assay prior and after DNA polymerase repair. For every individual, two molecular sex determinations were obtained by visualising PCR products on an agarose gel.

Results: DNA repair enabled genetic testing in these samples. Successful amplification of the amelogenin gene was obtained only from the repaired DNA in eight out of ten samples. Prior to the repair treatment, none of these samples yielded any PCR products, thus attesting to the authenticity of the amplified sequence. The concordance between morphological and molecular analysis was in reasonable agreement (71%).

Conclusions: These results reveal the impact of the repair process in studying single copy genes from low quality DNA. This protocol could facilitate molecular analysis in compromised samples, encountered in forensic medicine, as well as enable genetic studies in ancient remnants. Hippokratia 2009; 13 (3): 165-168

Key words: DNA; repair; sex; amelogenin; forensics

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Molecular analysis in compromised samples is a challenge in Forensic Medicine. The basic problem in these cases is the low integrity of the DNA under study¹⁻³.

Recent advances in the field involve the introduction of repair methods for the amplification of cross-linked DNA, which are particularly suitable for sub-optimally preserved skeletal material⁴⁻⁶. This approach has recently been applied in ancient DNA studies⁴⁻⁶ and focuses on the state of preservation of the chemically altered DNA, consisting of nicked double strands due to hydrolysis, oxidation or enzymatic destruction. aDNA enzymatic repair can be performed successfully only for molecules presenting unmodified 3'OH and/or 5'P termini. Damaged DNA can be terminally elongated by DNA polymerase I, sealed by T4 DNA ligase, or filled in and then sealed by the concerted action of these two enzymes (Figure 1).

The goal of such manipulation is to obtain PCR results with maximum specificity, and to amplify templates that have been refractory to PCR-mediated multiplication without optimization strategies. Special care should be taken to ascertain that the sequence that is obtained is the correct, since this method could lead to the generation of amplification artifacts.

Molecular identification of sex by PCR amplification

of the amelogenin gene⁷⁻⁸ is standard practice in Molecular Forensics. In fact, the amelogenin gene is included in all the available multiplex short tandem repeat (STR) systems used in DNA fingerprinting⁹⁻¹⁰.

In the present study we applied a repair protocol on ten ancient human teeth obtained from an equal number of skeletons from a burial site in Lerna, Middle Helladic Greece (2100 - 1700 BC) in order to achieve molecular sex identification by PCR amplification of the amelogenin gene.

Human bones from Lerna have been previously mor-

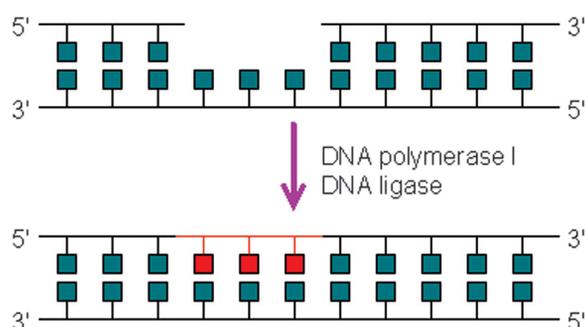


Figure 1: DNA repair mechanism

phologically studied by Angel¹¹ and recently re-examined by Triantaphyllou¹². In Triantaphyllou's re-examination, sex identification was based on pelvic and cranial morphology according to standards set by Buikstra and Ubelaker¹³.

In the present study we used the results of Triantaphyllou's morphological analysis as a reference for our molecular results.

Material and Methods

The choice of samples was based on the information derived from the morphological study in the way that the samples chosen would be the most appropriate for the goal of the study. Teeth were preferred to long bones. Due to the great importance of the material, it was impossible to acquire multiple samples from each individual, as we would desire.

In order to avoid surface contaminants prior to DNA extraction, the outer surface of each tooth was removed using the appropriate dental equipment (dental hand-piece) under aseptic conditions¹⁴. The teeth were then rinsed with water and alcohol and exposed to UV light (254 nm) at 2.5 cm from the light source for 30 minutes on each side. The teeth were finally pulverized and the pelleted bone powder was decalcified by incubation (at 56°C overnight) in a solution containing EDTA 0.5M, SDS 20% (at a final concentration of 0.5%) and Proteinase K (at a final concentration of 100 µg ml⁻¹)¹⁵. From the above solution, DNA was extracted using the phenol-chloroform method. The acquired extract was desalted and concentrated by centrifugation-driven dialysis at room temperature, using Centricon 30 microconcentrators¹⁶.

On these DNA extracts, we performed repair reactions according to previous reports⁴. This method promises an

increase of the number of informative PCR-based amplifications for single-copy DNA segments of the human nuclear genome. Escherichia coli DNA polymerase I was used to translate the nicks in the DNA and the remaining gaps were closed by the subsequent use of T4 DNA ligase (Figure 1). This strategy was applied to all our aDNA extracts from which no PCR products were obtained prior to repair. The repair reaction contained 2.5 U E.coli DNA polymerase I, 5 µl 10X nick translation buffer, 50µg/ml BSA, aDNA isolate, 0.4 mM each dNTP and H₂O to a total volume of 50 µl. The reaction was carried out for 90 min at 37°C, and terminated by incubating at 70°C for 20 min. Subsequently, 18 µl of the polymerase-treated aDNA were mixed with 2 µl of 10X ligase buffer and 200 U T4 DNA ligase. A 1 h ligation reaction was then performed with cycles between 10°C and 25°C, holding at each temperature for 10 sec.

Sex identification was based on the amplification of the amelogenin locus using the following primers¹⁷:

AmelA: 5'CCCTGGGCTCTGTAAAGAATAGTG3'

AmelB: 5'ATCAGAGCTTAAACTGGGAAGCTG3'

The amplification reaction consisted of 1.25 U Taq polymerase, 5µl 10X buffer, 2mM MgCl₂, 10mg/ml BSA, 250µM dNTPs, 200nM of each primer, 1-3 µl of the repaired DNA and H₂O to a total volume of 50 µl. The reaction was carried out for 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C with a final 30 min extension at 72°C.

The above PCR product was submitted to re-amplification under the same conditions and then visualised on a 4% metaphor gel containing ethidium bromide. A specific segment of the human X chromosome generates a 106 base pair (bp) product, while the corresponding human Y chromosomal DNA segment produces a 112 bp fragment.

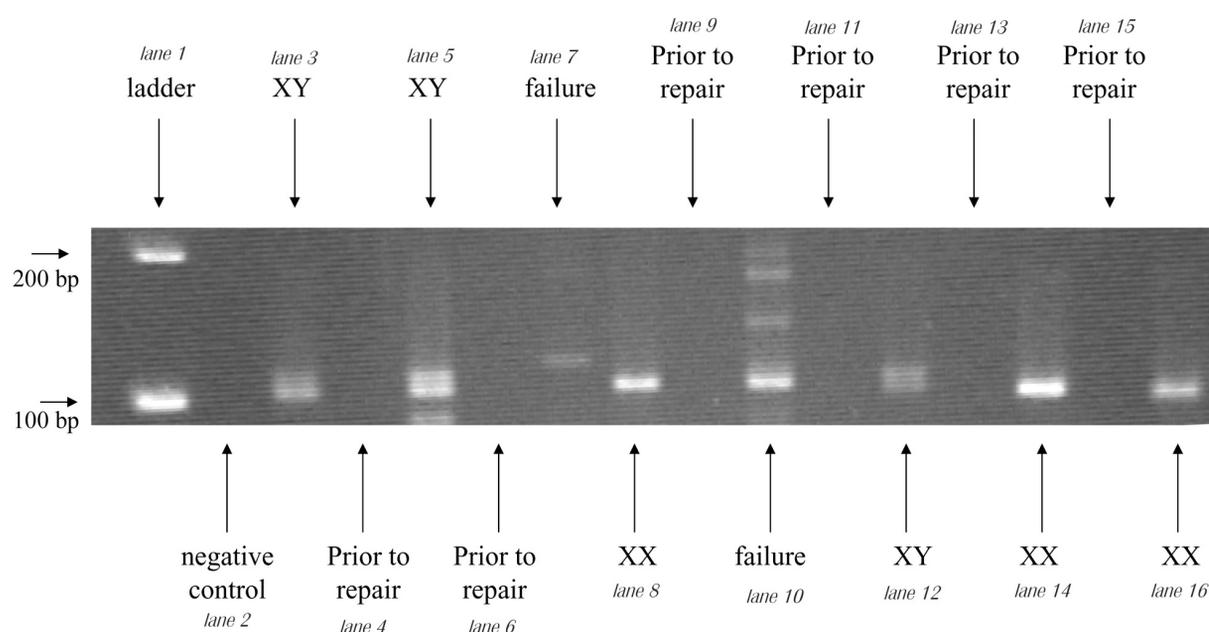


Figure 2: PCR amplification of 8 samples both prior and after DNA repair.

All possible precautions to exclude contamination were taken¹⁸. First of all, a separate laboratory space was dedicated to guard against contamination from other experiments. Nucleic acid extraction and PCR setup were conducted in different rooms. All labware and equipment were dedicated solely for use in this project and were sterilized by autoclaving or UV crosslinking. Positive PCRs were never performed. Benches and equipment were frequently treated with a 20% bleach solution. For each sample, contamination monitoring was performed by the use of two to three extraction blank controls and one water control. In addition, PCR amplifications were carried out upon all samples, always including negative controls.

Finally, in order to ensure that our results are genuine, the target sequences were amplified twice.

Results

The amplification of a single 106 bp segment of the amelogenin gene (Figure 2) corresponding to the X chromosome (lanes 8-14-16), indicates the female origin of those samples. The double PCR bands (106 bp and 112 bp) in lanes 3-5-12, corresponding to the X and Y chromosomes (Figure 2), indicate the male origin of the samples. It is interesting to point out the absence of any amplification product prior to DNA repair. In two cases (lanes 7-10) sex identification was not possible (Figure 2).

The amelogenin gene was amplified by PCR in 8 out of 10 DNA extracts (amplification success rate 80%) following DNA repair (Table 1), while prior to this repair treatment no DNA extract yielded PCR products for this gene. Four out of the ten teeth examined were found to belong to females, another four were found to belong to males and two could not be typed (Table 1).

A correlation of the DNA analysis and anthropometric data (Table 1) also reveals that in five out of the seven cases (where both anthropological and molecular data were available), the molecular data were in agreement with the anthropological analysis. In two cases molecular and anthropological data were not in agreement. Out

of these two cases, in one case the anthropological data pointed towards a male, when the molecular analysis revealed a female while in the other case, the reverse was observed.

Finally, in one case, where anthropological examination was inconsistent, molecular analysis provided sex identification and revealed a male.

Discussion

The results of the study presented in this paper reveal that the introduction of this DNA repair protocol prior to amplification, greatly enhances the amplification efficiency of DNA from compromised samples. The lack of amplification from extracts which were not subjected to the repair protocol indicates that the repair process does not enhance DNA contamination.

To our knowledge, there is no other work on human DNA utilizing repair as a means for increasing success in sex determination or other applications.

The skeletal material analysed in our study is very compromised and considered a challenge in molecular forensics. It is very old (2nd millennium BC) and preservation conditions were unfavourable as far as DNA is concerned (the graves were intramural, the burials were often disturbed, the temperature at the site can reach 40° C in the summer). In view of these limitations, it is evident that the repair protocol used in our study enhances the limits of DNA analysis, even in very difficult samples.

The results from DNA and morphological analysis were in reasonable agreement (five out of seven cases, 71%). In one of the two cases where inconsistent results were observed, molecular analysis revealed that the subject studied was female instead of male (as deduced from morphological analysis). Allelic drop-out of the 112 bp fragment could be responsible for the false identification of a male specimen as a female. Similar observations have previously been reported by other authors¹⁹.

In the second case where inconsistent results were observed, molecular analysis revealed that the subject studied was male instead of female (as deduced from morphological analysis). This could not be attributed to contamination with DNA from other sources since the only people that handled the specimens were both females. It should also be mentioned that morphological sex identification is not always straightforward since characteristics indicating sex can be influenced by genetics, disease and environmental factors²⁰.

Obtaining the expected PCR product sizes for alleles corresponding to both sexes (males and females) as well as the reasonable agreement between DNA and morphological analysis can safely rule out the possibility of false positive results and prove the credibility and usefulness of this repair protocol in the specific application.

We would finally like to point out that except from the field of Molecular Forensics, the field of Paleopathology could also benefit from this repair protocol.

Table 1: Correlation of sex identification results between morphological and molecular analysis.

Sample Number	Angel's Numbering	Grave number	Morphological analysis	Molecular analysis
1	123 Ler	BE 18	Inconsistent	M
2	129 Ler	BE 23	M	M
3	131 Ler	BE 25	M	M
4	137 Ler	BE 30	F	F
5	138 Ler	BE 30	F	F
6	139 Ler	BE 30	F	M
7	182 Ler	DE 36	F	F
8	196 Ler	DE 53	F	failure
9	198 Ler	DE 55	M	F
10	200 Ler	DE 59	F	failure

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