A simple method for extracting DNA from old skeletal material

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Received 10 October 1994; revision received 14 March 1995; accepted 30 March 1995

Abstract

Extraction of DNA from old skeletal material is of great importance in the identification of human remains, but is particularly difficult because the methods currently employed, especially those using phenol/chloroform, are not always satisfactory. A simple technique based on the removal of non-nucleic acid material by salting out (precipitation) with saturated sodium acetate is described; the presence of DNA in the extract being confirmed by amplification of selected sequences of the \textit{HLA-DRB1} gene using the polymerase chain reaction (PCR). The method was applied to fresh bone (five femoral heads and six vertebral bodies) and to bone from two forensic cases, 3 and 9 months post-mortem, respectively. Parallel extractions using the phenol/chloroform technique were performed on all samples in order to compare the efficiency of the two methods. Using sodium acetate precipitation, amplifiable DNA was consistently extracted from fresh bone, as well as from the two forensic cases. With the phenol/chloroform method, amplification was successful in only 7 out of 11 instances with the fresh bone samples and failed in both forensic cases. The studies also showed that an effective way of removing PCR inhibitors is to subject the extract to agarose gel electrophoresis, isolate the high molecular weight area and re-extract the DNA from the gel by boiling. It was concluded that the sodium acetate method is a valid alternative to established techniques for extracting DNA from bone and that it offers the advantages of being simple, quick, inexpensive and avoids using hazardous reagents.

Keywords: DNA extraction; Bone; PCR; Forensic sciences; Archaeology; Inhibitors

1. Introduction

DNA analysis of human skeletal material can provide important information to

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forensic and archaeological specialists, such as an individual's identity [1] or sex
[2,3], when morphological methods cannot be applied, or it can provide details of
the genetic make-up of ancient populations [4,5]. Although the mechanisms of survi-
val of DNA in bone are virtually unexplored [6,7], there are now many reports of
its successful extraction. In the forensic field, these include the extraction of DNA
from the bones of Josef Mengele [8], the Romanov family [9], a murder victim [1],
and a few cases of missing people [10,11]. Similar successes are recorded in the ar-
chaeological literature, for example, mitochondrial DNA has been amplified from
human [12–15], and animal bone [16], and there have been occasional reports of
genomic DNA being found in ancient human bone [4,17]. However, it is generally
agreed that current extraction methods are not consistently satisfactory and more
and more alternatives (particularly to phenol/chloroform) are being sought [18,19].
Other methods for extracting skeletal DNA, for example, water elution [20] and
chelation [21], may be equally effective. In fact, chelating is the method of routine
choice in many laboratories, and recently, DNA has been successfully isolated from
ancient bone using silica-based techniques [22–24].

In this article, a further method for extracting skeletal DNA is assessed, based on
the removal of non-nucleic acid material by salting-out (precipitation) [25] with sat-
urated sodium acetate; it is routinely used at the Blood Transfusion Centre in Shef-
field on fresh blood samples, but has not previously been applied to osseous tissue.
PCR amplification for the second exon of the HLA-DRBl gene [26] was performed
to confirm that DNA had been effectively extracted. This sequence was chosen as
a marker for DNA because it is both anthropologically informative [5] and also
small enough at 274bp to escape substantial degradation.

2. Materials and methods

Samples of fresh human bone (five femoral heads and six vertebral bodies) were
obtained from 14 adults undergoing either orthopaedic surgery or autopsy. Two
forensic cases from the Institute of Legal Medicine in Milan were also examined. The
first was a completely skeletonized individual discovered on the edge of a railway
track, the second was a partially skeletonized body found in woods. It was estimated
that the first subject had been lying in the open for a period of at least 9 months,
whereas the second had been reported missing 3 months prior to the retrieval of his
remains. Bone samples were taken from lumbar vertebrae in the first case and from
a femoral head in the second. All specimens were obtained with patient's, relatives'
or Magistrates' consent and after Ethical Committee approval for the study had
been granted.

Using a previously sterilised hand-saw, fragments of bone ~ 1 cm³ were cut from
areas which seemed least impregnated with blood and pulverised with a mortar and
pestle after freezing in liquid nitrogen. Fragments of bovine bone were prepared in
the same way as a negative control.

First, 1.5 g of the bone powder were incubated in a polypropylene tube containing
3 ml of White Cell Lysis Buffer (10 mM Tris–HCl (pH 7.6), 10 mM Na₂EDTA, 50
mM NaCl), with the addition of 25 μl of proteinase K (20 mg/ml) and 50 μl of 10%
sodium dodecyl sulphate (SDS), in a water bath overnight at 42°C. One milliliter of
saturated sodium acetate was added and the tube shaken manually for 30 s and then centrifuged at 4000 × g for 10 min. The supernatant containing the DNA was transferred to a new polypropylene tube, 4 ml of 100% isopropanol were added and mixed for at least 10 min to precipitate the DNA before centrifugation at 4000 × g for 10 min. The supernatant was discarded; the remaining whitish-yellow pellet of DNA was suspended in 250 μl of 70% ethanol, transferred to a 1.5 ml microcentrifuge tube and centrifuged at 13 000 × g for 10 min. The pellet was dried in an incubator at 37°C, reconstituted in 250 μl of sterile distilled water and the resulting DNA solution divided into 20 μl aliquots which could be stored at −80°C until required for testing.

The quality and quantity of the extracted DNA were assessed subjectively using agarose gel electrophoresis. DNA (4 μl) were added to 1 μl of × 5 gel loading solution (40% sucrose, 0.1% bromophenol blue, 50 mM Na₂EDTA, 50 mM Tris–HCl (pH 7.6) and 5% SDS) and electrophoresed on a 1.5% agarose gel containing ethidium bromide at 90 V for 20 min. The resulting pattern gave an approximate (but satisfactory) estimate of the quality and quantity of the DNA: a good quality extract gave a compact (as opposed to extended) smear and the amount was judged by comparing the intensity to that given by known quantities of DNA.

PCR was performed in a Coy Tempcycler to amplify selected sequences of the HLA-DRBI gene, recommended precautions [27,28] being taken to avoid contamination. Here, 40 μl volumes were used, each containing Tris–HCl pH 8.3 (10 mM), KCl (50 mM), MgCl₂·6H₂O (1.5 mM), gelatin (100 μg/ml), dNTPs (200 μm), oligonucleotide primers specific for the second exon of the HLA-DRBI gene (0.5 μM), Taq polymerase (1 U) and varying quantities (1–5 μl) of DNA to ensure that the optimum amount for amplification would be present. The positive control was 1 μl of DNA extracted from fresh blood, negative controls consisting of reactions with either bovine extract or distilled water (no DNA added). The primers used were, DRBAMP-A: C CCC ACA GCA CGT TTC TTG and DRBAMP-B: CCG CTG CAC TGT GAA GCT CT [26]. The reaction profile consisted of an initial step at 95°C for 5 min followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, and a final step at 72°C for 5 min. The 274bp amplification product of the HLA-DRBI sequence [26] was visualised by electrophoresis in 1.5% agarose gel containing ethidium bromide, a molecular weight marker (PBR 328 BglI + HinfI) was always included in the first lane.

In order to compare the sodium acetate method with an established technique, parallel extractions using phenol/chloroform [29], followed by amplification of the HLA-DRBI sequences, were performed on all samples using the same amount of bone. Briefly, the method consisted of a pre-incubation phase in proteinase K/SDS, two extractions using phenol/chloroform and DNA precipitation with ethanol.

3. Results

The amount of DNA in the individual extracts (both sodium acetate and phenol/chloroform) is shown in Table 1, together with the results of PCR amplification of the HLA-DRBI sequences. In nearly all cases, the quality and quantity of
Table 1
Comparison of sodium acetate and phenol/chloroform methods of extracting DNA and their ability to produce an amplifiable product

<table>
<thead>
<tr>
<th>Bone sample</th>
<th>Amounts of DNA in extract*</th>
<th>Successful amplification of extracted DNA</th>
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<tbody>
<tr>
<td></td>
<td>Sodium acetate</td>
<td>Phenol/chloroform</td>
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<tr>
<td>Femoral heads</td>
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<td>1</td>
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<td>5</td>
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<td>Vertebrae bodies</td>
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<td>6</td>
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<td>Forensic cases</td>
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<td>2</td>
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</table>

*From comparison with electrophoresis of known quantities of DNA.
++Equate approximately to > 5 ng of DNA.
+Equate to 1–5 ng of DNA.
0, no DNA visible in gel.

DNA extracted were judged to be better with the sodium acetate method — these differences are illustrated in Fig. 1.

Amplification of DNA from the fresh bone samples was always successful when testing sodium acetate extracts. Fig. 2 shows the results from three femoral heads

![Fig. 1. Extraction of DNA comparing sodium acetate and phenol/chloroform methods.](image)

The superiority of the saturated sodium acetate technique is shown by the greater staining intensity and more compact smear in lane 2 indicating (respectively) a larger amount of better quality DNA compared with the phenol/chloroform extract in lane 3.
and the control reactions; the amplified \textit{HLA-DRB1} bands are very clear. The \textit{HLA-DRB1} sequence was also amplified from the extracts in the first forensic case (9 months post-mortem), though the band was rather weakly stained. In the second forensic case (3 months post-mortem), the presence of an inhibitor was shown by the failure of the positive control to amplify after being mixed with 1 \textmu{l} of the extract. The DNA was, therefore, purified by electrophoresing the extract on agarose gel, cutting out the area of the smear which represented the higher molecular weight DNA, crushing the piece of gel in 0.5 ml of sterile distilled water and boiling for 10 min. Then, 5\textmu{l} of this solution were used in a repeat PCR; the inhibition was eliminated and good amplification resulted (Fig. 3). Control extracts of DNA-free gel did not amplify.

The extractions using phenol/chloroform gave successful amplification in only 7 of the 11 samples of fresh bone, and the electrophoretic patterns were diffuse and of low intensity; in both forensic cases, phenol/chloroform extraction failed to produce amplifiable DNA (Table 1).

The positive and negative control samples gave the expected results consistently throughout all the tests.

4. Discussion

The present findings (Table 1) suggest that simple techniques are equally or even more efficient in the extraction of DNA from bone than the traditional
phenol/chloroform method and can lead to better PCR amplification. The procedure described here using saturated sodium acetate to remove non-nucleic acid material before precipitating with isopropanol provides an effective and quick method for extracting reasonable quantities of good quality DNA from fresh and relatively old (9 months post-mortem) bone. Amplifiable DNA was extracted from all the fresh femoral heads and vertebral bodies as well as from the forensic specimens (Figs. 2 and 3). The phenol/chloroform technique on the other hand, where the extracts yielded less DNA and showed greater degradation, produced amplifiable DNA with the same fresh bone samples in only 7 out of 11 instances and failed in both the forensic cases (Table 1).

The present work may also shed some light on PCR inhibitors and the means to remove them. PCR inhibition was evident in extracts of 3 month old bone, but not in those from fresh and 9 month post-mortem samples. Because the forensic cases represent two different stages of degradation of human skeletal material, it is possible that certain products of decomposition present at an earlier stage of degradation act as inhibitors and then disappear when complete skeletonisation has occurred. Although research on the nature of inhibitors [30–32] must continue, our studies have shown that one simple and effective method for removing some of them is to
subject the extracts to agarose gel electrophoresis, isolate the high molecular weight fraction and re-extract the DNA by boiling. Gel filtration has also been found successful in this respect [31].

The importance of having control specimens in every test run cannot be stressed enough. Their results guaranteed, as far as possible, the reliability of the findings and ensured that all the comparisons made were valid. They were instrumental in demonstrating the PCR inhibitor in the second forensic case and it is suggested that including a mixture of test material and positive control is considered whenever amplification of an extract of non-fresh bone is carried out.

In conclusion, the procedure described here seems to be a valid alternative to the traditional phenol/chloroform method of extracting DNA from bone. It has advantages in that it is rapid, has few steps (and hence lower risks of losing precious material), is inexpensive and avoids using hazardous reagents.

Acknowledgements

We thank Mrs. A. Steward and Medical Photography at the Northern General Hospital in Sheffield for their help, and Profs. A. Farneti and M. Grandi and Drs. A. Bogoni, T. Costa, E. Mangili and R. Zoja of the Medico-Legal Institute in Milan for granting access to the forensic cases.

References


