

Pitfalls in DNA extraction from ancient bones found in Brazilian shell-mounds

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Resumo: Os autores descrevem suas dificuldades na extração de ácido desoxiribonucléico (DNA) de ossos encontrados em sambaquis nas praias da ilha de Sta. Carina, SC, Brasil. A presença de ácido húmico, do ácido fúlvico, radicais livres oxidantes e o efeito hidrolítico dos ambientes úmidos degradam o DNA em fragmentos da ordem de 20-30 pares de bases, impedindo qualquer utilização do DNA extraído. Além disso, o ácido húmico e o ácido fúlvico se ligam fortemente ao magnésio, necessário à atividade da Taq-polimerase, impedindo a reação da polimerase em cadeia (PCR). Neste trabalho são discutidos os protocolos utilizados mais eficientes de extração do DNA e de várias condições possíveis de ensaio da PCR.

Palavras-chave: Sambaquis - Ossos antigos - Extração de DNA de ossos antigos - PCR de DNA antigo.

Ancient DNA has been discovered in various types of biological material, including ancient bones, mummies, museum skins, amber-preserved insects, and plant fossils. Such DNA has provided precious information related to archaeology, conservation biology, and forensic science (Brown&Brown 1994; Kolman&Tuross 2000; Pääbo *et al.* 2004; Willerslev & Cooper 2005).

The advent of techniques for the enzymatic amplification of specific DNA sequences has allowed the collection of genetic data from thousands and even millions of years ago (Kaestle & Horsburgh 2002; Pusch *et al.* 2003; Mulligan 2005). However, most such studies have been severely criticised for failing to take measures to prevent the contamination of purportedly ancient DNA by recent material (Pääbo *et al.* 2004). Therefore, the authenticity of the DNA sequences obtained cannot be verified. Researchers dealing with ancient material, such as preserved bone, face tremendous difficulty in obtaining good DNA samples, since the quality of ancient DNA can be significantly affected by environmental factors.

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In this study, based on our experience in studying the ABO blood group in ancient bones of indigenous Brazilian peoples, we discuss the problems that can arise during the extraction of DNA from such bones. The skeletons in question were found in *sambaquis*, which are shell-mounds located on the beaches of the island of Santa Catarina, in the state of Santa Catarina, Brazil, and have been dated to approximately 100 A.D. (Schmitz *et al.* 1992; Schmitz *et al.* 1993; Schmitz *et al.* 1996). The skeletons were found by Röhr *et al.* (1977) at the archaeological sites Tapera, Laranjeiras I, Laranjeiras II, Pântano do Sul, Cabeçadas, and Armação do Sul (Table 1) (Schmitz *et al.* 1992; Schmitz *et al.* 1993; Schmitz *et al.* 1996). Access to the bones was kindly afforded by the Instituto Anchieta, São Leopoldo, RS, and the Museu do Homem do Sambaqui in Florianópolis city, SC, where they are very well stored in individual boxes. Circa 212 bone samples from 190 skeletons which were in good conditions were studied from the archaeological sites.

Table 1

Bone samples from the archaeological sites	
Archaeological sites	Sample sizes
Tapera	138
Armação	8
Cabeçadas	22
Pântano	21
Laranjeiras I	2
Laranjeiras II	1

The bones were carefully cleaned with 70% ethanol and submitted to UV irradiation for 10 min on both sides in order to destroy superficial contaminating DNA. Initially, we attempted to extract the DNA from the trabecular bones, in which remnants of haematopoietic cells should be found. However, the trabecular bones were filled with dirt and roots, and we therefore decided to work with the compact cortical bone. The cortical bone, although presenting fewer nucleated cells, might better protect these cells from environmental contaminants (Hochmeister *et*

al. 1991; Hochmeister *et al.* 1998). The bones were cut with a circular saw using sterilised saw blades at 150°C in an oven, after which they were scraped using a dental drill with a diamond burr (Ramos *et al.* 1995) in a laminar flow hood. The powder obtained was suspended in buffered extraction solution containing 10 mM TRIS-HCl, pH 8.0, 500 mM EDTA, 100 mM NaCl, 10% SDS, and 50 mL proteinase K (20 mg/ml), diluted to 50 mg:500 mL of buffer. The tubes were inverted and rotated (15 cycles/min) overnight at 56°C. The obtained suspension was centrifuged, and the supernatant was saved. The precipitate was then submitted to the same procedure, after which both supernatants were pooled and submitted to further purification steps. In attempts to extract DNA from these samples, various methods were used. Protocols described by Maniatis *et al.* (1989) and Boom *et al.* (1991) were employed, as were those involving DNAzol (Gibco BRL, Gaithersburg, MD, USA), the rapid genomic blood DNA amplification kit (Pharmacia Biotech, Piscataway, NJ, USA), Chelex-100 (Bio-Rad, Hercules, CA, USA), and the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) (Alves *et al.* 2000; Meijer *et al.* 1992; Cattaneo *et al.* 1995; Walsh *et al.* 1991; Yang *et al.* 1998; Malhi *et al.* 2003). The best results were obtained with the Maniatis *et al.* (1989) protocol. In brief, the suspension was added 1:1 to a phenol/chloroform/isoamyl alcohol mixture (25:24:1), mixed, and centrifuged at 5000 g for 10 min, after which the DNA obtained was submitted to a further precipitation step. For DNA precipitation, isopropyl alcohol, rather than ethanol, was chosen, as very small DNA molecules, such as those found in the present study, are not efficiently precipitated by ethanol (Maniatis *et al.* 1989). In addition, as has been the case in most DNA and RNA precipitation protocols (Evans *et al.* 1998), 3 M sodium acetate pH 5.2, was chosen over 2.5 M ammonium acetate. The DNA precipitate was washed twice with chloroform in order to remove the excess phenol. All subsequent steps were as previously described (Maniatis *et al.* 1989).

However, despite all of these precautions, the polymerase chain reactions (PCRs) failed to achieve satisfactory amplification, omitting DNA amplifications of 100- to 200-bp fragments, which were exactly those we were looking for. These findings led us to suppose the ancient DNA was degraded to very small fragments. This was, in fact, found to be the case, as evidenced by the finding that electrophoresis of the final DNA extract preparation on a 1% agarose gel revealed a dark band in the 15- to 30-bp region.

The bones with which we worked were found in wet ground along the shoreline. Reviewing the literature on ancient bones, we found that contaminating humic acid and fulvic acid, both of which are found in wet soil, can inhibit the Taq polymerase, the crucial factor in the PCR (Tsai *et al.* 1989; Goodyear *et al.* 1994). Humic and fulvic acid are strong magnesium chelating agents, and it is known that Taq polymerase is magnesium dependent. In addition, humic and fulvic acid are absorbed between 260 nm and 280 nm (Tuross *et al.* 1994), exactly the range in which DNA is absorbed (260 nm), and can alter the results of DNA concentration assays. Powered bone becomes darker in parallel with increasing degrees of contamination with humic substances. In order to prevent PCR inhibition, 2% bovine serum albumin was added (Herrmann *et al.* 1994).

In order to compensate for the chelating effect on magnesium, we performed some

PCRs with DNA samples obtained through the use of increasing concentrations (up to 4 mM) of magnesium chloride, bearing in mind that magnesium concentrations higher than 4 mM inhibit Taq polymerase. However, the results obtained were unsatisfactory.

The primers used were designed to amplify fragments of 128 bp and 200 bp (Franco *et al.* 1994). However, the extracts were found to be degraded (15-30 bp). Fragments of approximately 300 bp were occasionally found but were not reproduced. Such fragments were likely the result of contamination with bacteria or fungi (Pääbo 1990).

Table 2 displays strategies for optimising PCR amplification. Since humic and fulvic acids chelate magnesium chloride, which is a crucial factor in Taq polymerase activity, various magnesium chloride concentrations were employed (Tsai *et al.* 1992).

When no PCR products were obtained, primer concentrations were increased, as were nucleotide concentrations. By virtue of increasing the probability that the primers will identify the target sequences in DNA preparations, higher Taq concentrations might be desirable (Fisher *et al.* 1993). Various DNA sample dilutions were also employed in order to dilute the inhibitors. However, as the DNA in the preparations was found to be degraded or absent, it was decided that undiluted DNA extracts from bones would be used.

In order to improve the results, various procedures were conducted: increasing the number of PCR cycles (Hardy *et al.* 1994);

Table 2

Conditions that can be modified in order to optimise PCR (<i>apud</i> Hirata & Hirata 2000)		
Conditions	In case of absence of PCR products	In order to avoid non-specific bands
Mg Cl ₂ concentration*	↑	↓
Primers concentration	↑	↓
dNTP concentration**	↑	↓
Taq concentration	↑	↓
DNA concentration	↑	↓
Sample dilution	↓	↑

* MgCl₂: magnesium chloride; ** dNTP: deoxynucleoside triphosphate.

biphasic amplification (Holland *et al.* 1992; Hummel *et al.* 1992); partial amplification of the PCR product (Horai *et al.* 1989); and “heat soak” (Ruano *et al.* 1992), in which the extracted DNA was heated to 95°C for 15 min in order to improve the denaturation and hybridisation of the double-stranded DNA. This last procedure was employed in all experiments.

It has been frequently observed that oxidative free radicals and the hydrolytic effect of wet environments degrade ancient DNA (Handt *et al.* 1994; Schwartz *et al.* 1991; Lindhal *et al.* 1993, Höss *et al.* 1996), inducing structural changes in the DNA, thereby leading to poor and irreproducible results. These pitfalls can explain the enormous difficulty in performing the PCR with ancient bones, especially those unearthed from wet soil.

In addition to the role that humic and fulvic acid play in Taq polymerase inhibition, it has recently been shown that other environmental factors can affect the chances of finding DNA. Favourable conditions include low temperatures, dry environment, few micro-organisms (as micro-organisms and their metabolites can destroy DNA), and neutral or slightly alkaline pH (Burger *et al.* 1999; Lee *et al.* 1999). Storing DNA samples at temperatures below 20°C can also aid in preserving their integrity (Lindahl *et al.* 1993; Höss *et al.* 1996).

In order to ensure the authenticity of amplified DNA of ancient material, it is mandatory to avoid contaminating the DNA, which can greatly jeopardise the experiments

and undermine the reliability of the data obtained.

The most important experiment, which could indicate the presence of PCR inhibitors in the bone DNA extracts, was that in which those extracts were mixed 1:1 with leucocyte DNA extracts obtained from volunteers. Other DNA extracts from those same volunteers had previously afforded successful PCRs. In the present experiment, amplification was not achieved, suggesting that Taq polymerase inhibitors were present in the ancient bone DNA extracts, thereby precluding any amplification.

In conclusion, obtaining good DNA preparations from ancient bones depends on a multitude of factors, and various pitfalls can be encountered. These obstacles are primarily attributable to environmental factors, although skilful technicians, proper laboratory techniques employed in the extraction, and the quality of the reagents used are factors which together ensure good results.

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Abstract: The authors report their difficulties in DNA extraction from ancient bones found in shell-mounds of Sta. Catarina Island, SC, Brazil. The presence of humic acid, fulvic acid, free oxidant radicals and the hydrolytic effect of wet environment degrade the DNA to 20 to 30 base pairs, what jeopardizes any further utilization. The humic acid and the fulvic acid chelate magnesium ion necessary to Taq-polymerase activity, inhibiting the polymerase chain reaction (PCR). In this work the most efficient used protocols for DNA extraction, and the several PCR assay conditions are discussed.

Keywords: Shell mounds - Ancient bones - DNA extraction of ancient bones - PCR of ancient DNA.

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