

Hypersensitive PCR, Ancient Human mtDNA, and Contamination

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Abstract When highly efficient polymerase was used with high cycle numbers (50–60), strong amplifications were observed, but negative controls were also unexpectedly amplified in a study of ancient human mtDNA from 2000-year-old skeletons. The results of a series of tests revealed that the hypersensitive polymerase chain reaction (PCR) generated by higher cycles and the presence of contaminant DNA (though at extremely low levels) should be responsible for the amplification of negative controls. We suggest that PCR sensitivity be optimized to take advantage of highly efficient polymerase and at the same time prevent “background DNA” from becoming “contaminant DNA” and obscuring the analysis of authentic ancient DNA. We propose the use of multiple positive controls when amplifying ancient human mtDNA samples to indicate the sensitivity of individual PCR amplifications and to monitor the contamination levels of modern human DNA. This study provides some suggestions as to how to amplify and analyze ancient human mtDNA when unavoidable and extremely tiny amounts of modern human DNA exist.

Because DNA is highly degraded in ancient remains and only small amounts of short fragments are preserved, the analysis of ancient DNA was not practical until the polymerase chain reaction (PCR) technique became available (Pääbo 1990). However, damaged DNA templates and associated impurities often result in a low efficiency or even a failure of PCR amplification (Herrmann and Hummel 1994). To overcome this problem, a logical solution would be to increase PCR amplification efficiency. The availability of highly efficient polymerase such as AmpliTaq Gold™ has made the goal more achievable. For example, AmpliTaq Gold™ has functions of “hot-start” and “time-release” PCR amplifications that can generate considerably higher specificity and efficiency (Kebelmann-Betzing et al. 1998). We used AmpliTaq Gold™ to amplify ancient mitochondrial DNA (mtDNA) from 2000-year-old human skeletal remains from an Imperial Roman

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necropolis. We observed a strong boost of PCR amplification when using higher numbers of cycles (45–60), but also noted the unexpected appearance of positive amplifications in blank extracts and PCR negative controls. However, contamination was not apparent from previous PCR amplifications when using regular polymerase.

In order to examine how the positive amplification of negative controls occurred, PCR amplifications were set up with varying numbers of cycles. It was hoped that the results would also be useful in understanding the relationship between PCR sensitivity and the appearance of contamination, and answering the question of whether it is valid to analyze ancient DNA samples when minimal amounts of contaminant DNA exist in the background.

Materials and Methods

Skeletal Remains. The bone samples used in this study come from an archaeological excavation of the Isola Sacra necropolis, an Imperial Roman site dating from the first to the third centuries A.D. Approximately 2000 individuals have been recovered from the site since excavations began in the 1980s (Baldassarre 1990). The chronological relationships between different parts of the site are not currently known, although all of the individuals in this study come from in-ground burials or burial structures between monumental tombs (Baldassarre 1990). The bone samples of more than 30 individuals were chosen to study the genetics of this skeletal population.

DNA Extraction. The silica-spin column method (Yang et al. 1998) was used to extract DNA from the bone samples. Approximately 1 gram of cortical bone was ground from each individual using a liquid nitrogen grinding mill. The bone powders were incubated overnight with lysis buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 0.5 mg/mL proteinase K) in a rotating hybridization oven at 55°C. After centrifugation, 1.5–2.0 mL of supernatant was concentrated using Centricon™ 30 microconcentrators (Amicon Division, Danvers, MA). Approximately 100–150 µL of concentrated supernatant was passed through QIAquick columns (Yang et al. 1998). Finally, 100 µL of DNA was eluted from the column for PCR amplification.

PCR Amplification. Two primers were used to amplify a 176-base-pair (bp) HV1 fragment (np 16045-16220) of the D-loop area of human mtDNA (Anderson et al. 1981): primer A 5'-TGGGTACCACCCAAGTATTGAC-3' and primer B 5'-GTTGATTGCTGTACTTGCTTGTAAG-3'. PCR amplification was carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk, CT) in a 50 µL reaction volume containing 50 mM KCl and 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1.0 mg/mL BSA, 0.3 µM each primer, 5 µL ancient DNA sample and 1.25 U AmpliTaq Gold™ polymerase. PCR was run using

different cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with an initial denaturing at 95°C for 3 or 12 minutes. Five μL of PCR product was separated by electrophoresis onto an 8% nondenaturing polyacrylamide gel. PCR products were purified using a QIAquick™ purification kit. The samples were sequenced on an ABI 373 using primer A from one DNA strand or both primers A and B from both DNA strands. Sequencing was performed at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

Contamination Controls. All DNA extractions and PCR setups were carried out in a dedicated ancient DNA laboratory following the suggested protocols for contamination controls and detections (Herrmann and Hummel 1994). All bone samples and extraction reagents were exposed to UV irradiation in an ultraviolet cross-linker for 20–30 minutes at an intensity of $1500 \times 100 \text{ J/cm}^2$. Multiple blanks and negative controls were used with DNA extractions and PCR setups. Two extractions were prepared for each bone sample by two researchers to test reproducibility.

Results

PCR Amplification. Strong amplifications were observed when the number of amplification cycles was increased from 35 to 50. Figure 1 shows the result of 50 cycles for three ancient DNA samples. There is no indication of contamination in this amplification. This particular PCR amplification shows a high efficiency because 0.5 picograms (pg) of modern DNA sample (K562) produced a strong band. Unfortunately, this “perfect” result was only observed once out of more than ten amplifications at 45–60 cycles.

Positive Amplifications of Negative Controls. Generally, when there were strong PCR amplifications of ancient DNA samples at higher cycles, blank extracts and even PCR negative controls produced amplifications. Figure 2 presents the result of PCR amplification at 60 cycles. Although the PCR negative controls did not generate any detectable amplification, three blank extracts produced strong amplifications. Figure 1 and Figure 2 represent two extremes: no positive amplifications from blank extracts (Figure 1) and positive amplifications from all blank extracts (Figure 2). Quite often, the positive amplification appears randomly in blank extracts and even PCR negative controls.

Sequencing PCR Products. All PCR products from positive amplifications were sequenced. The ancient DNA samples were observed to have distinctive mtDNA sequences, indicating that at least not all ancient DNA samples were due to detected contamination. A “unique” DNA sequence could be identified from most positive amplifications of negative controls, although it was cosequenced

Marker 50pg 5pg 0.5pg N S1-C S2-C S3-C C-B1 C-B2 S1-Y S2-Y S3-Y Y-B1 Y-B2

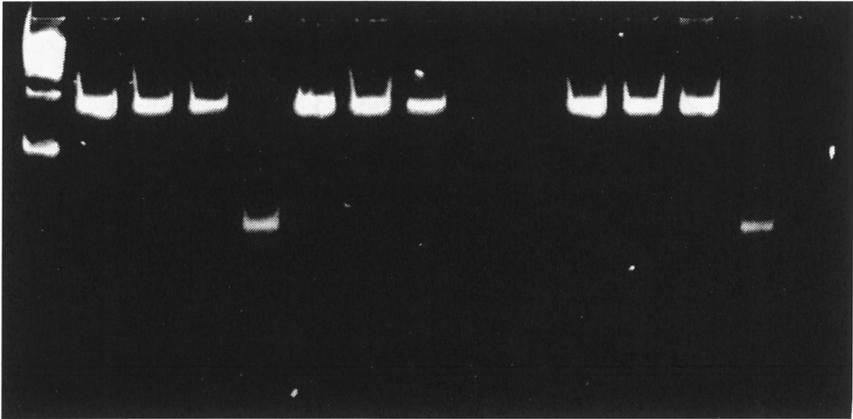


Figure 1. Ethidium-bromide-stained gel of PCR products of 50 cycles from three 2000-year-old specimens, S1, S2, and S3. Fifty picograms, 5 pg, and 0.5 pg: positive controls (K562). N: PCR negative controls. S1-C, S2-C, and S3-C: DNA examples extracted by researcher 1 from S1, S2, and S3. C-B1 and C-B2: blank extracts by researcher 1. S1-Y, S2-Y, S3-Y, Y-B1, and Y-B2 by researcher 2 for DNA samples (S1, S2, and S3) and blank extracts, respectively. Marker: 100 bp (250 ng).

Marker N1 Column C-B1 C-B2 S1-C S2-C S3-C 50pg 5pg S1-Y S2-Y S3-Y Y-B N2

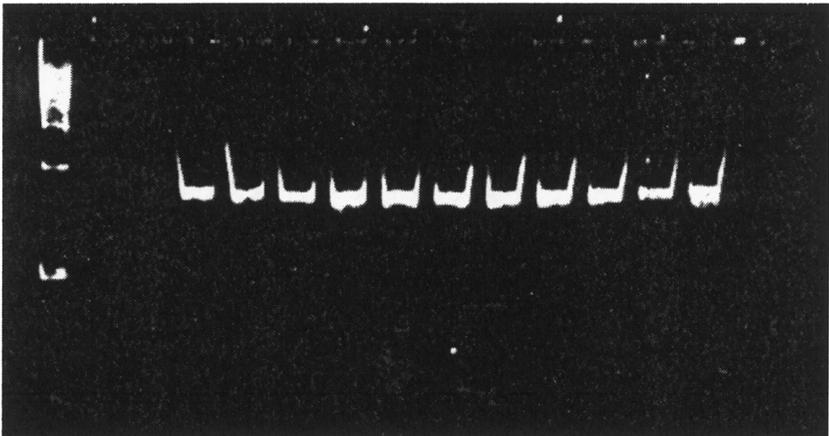


Figure 2. Ethidium-bromide-stained gel of PCR products 60 cycles from three 2000-year-old specimens, S1, S2, and S3. N1 and N2: PCR negative controls, Column: QIAquick column control. C-B1 and C-B2: blank extracts by researcher 1. S1-C, S2-C, and S3-C: DNA examples extracted by researcher 1 from S1, S2, and S3. Fifty picograms and 5 pg: positive controls (K562). S1-Y, S2-Y, S3-Y, and Y-B by researcher 2 for DNA samples (S1, S2, and S3) and blank extracts, respectively. Marker: 100 bp (250 ng).

with others in some cases. This result indicates that the contaminants may have come from different sources, but one was dominant. This dominant contaminant DNA sequence was different from all ancient DNA sequences extracted previously in the laboratory and from those of two DNA laboratory workers.

The Positive Amplification Disappeared When the Number of Cycles Dropped. To test how the number of cycles affects the outcome of amplification, PCR was set up and run at different cycle numbers, from 35 to 40, 45, 50, 55, and others. The results were clear: in general, no positive amplifications of negative controls were observed at 35 cycles but strong false bands were seen at 45–50 cycles or beyond. However, the amplification of ancient DNA samples was much weaker at 35 cycles than those at 45 cycles or beyond. It was also observed that positive amplifications of negative controls did not appear below 40 cycles. Ancient DNA samples could be subsequently reamplified with no contamination resulting.

Discussion

Contamination and Levels of Contamination. There is no doubt that the positive amplification of negative controls in this study was caused by contaminant DNA. From the random appearance of amplifications of blank extracts and negative PCR controls, the contamination seems to be sporadic in nature. However, the sequencing result suggests a systematic contamination because a dominant contaminant human mtDNA sequence was detected. This paradox is probably derived from a combination of a much lower level of contamination and a much higher level of sensitivity of the PCR amplification. The amount of contaminant DNA was so low that it could not be detected at lower cycles (and when using regular polymerase). Even at higher cycles, not all PCR negative and blank extracts appeared positive. Due to extremely low amounts of contaminant DNA, the actual number of templates participating in PCR amplification can fluctuate in individual PCR setups, resulting in inconsistencies in the appearance of positive amplifications of negative controls. That is, DNA templates might not be picked up during the first few cycles and would not be amplified into the sufficient amounts needed to indicate a positive amplification on electrophoresis gels unless at higher cycles. However, if the number of cycles continues to increase, the amplification can reach an amplification plateau (plateau effect), under which all samples will produce the same strong amplification regardless of the original amounts of DNA template (Figure 2). If contaminant DNA templates are introduced and the plateau is reached, although there are only a few molecules, their amplification may be as strong as that from authentic ancient DNA samples with 100 or 1000 times more original template. One might come to the false conclusion of mass contamination, and all samples including DNA extracts would be wrongly discarded.

In this study, many positive PCR results of ancient DNA samples were probably amplified from authentic ancient DNA templates, even though some positive amplifications of negative controls appeared in the same PCR setup. For example, two laboratory workers successfully reproduced their results for some of the ancient DNA samples. This is probably because authentic ancient DNA templates outnumbered contaminant templates in PCR amplifications. It seems reasonable to assume that ancient DNA analysis can still be carried out even when there are low (insignificant) amounts of contaminant DNA mixed with authentic ancient DNA templates.

Indication of Sensitivity of PCR Amplification. We propose the setup of multiple, quantified modern human DNA samples as positive controls when amplifying ancient human DNA samples. The positive controls can roughly indicate the sensitivity of individual PCR amplifications and can also show the level of contamination if it occurs. In most ancient DNA studies, blank and negative controls are used to indicate whether contamination occurs. However, usually only one positive control is used to indicate whether PCR amplification is properly set up (Herrmann and Hummel 1994). Studies show that the efficiency of PCR amplification varies between individual setups. It is not uncommon for the same DNA samples to produce good results in one setup but totally fail in another (Innis et al. 1990). A series of multiple positive controls can act as a simple monitoring system to show the true sensitivity of amplifications of individual PCR setups and the level of contamination if positive amplifications of negative controls appear. If the sensitivity of PCR amplification is too high and all samples including positive controls reach the plateau (Figure 2), no useful information should be drawn about the level of contamination (Figure 3A). If the sensitivity of PCR amplification is optimized to such a degree that it amplifies DNA samples proportionally to the amounts of original DNA template (Figure 3B and 3C), in other words, the plateau effect is avoided, then the contamination level can be more accurately estimated.

For human mtDNA, based on an empirical estimation, we propose the use of 50 pg, 5 pg, and 0.5 pg as positive controls. If amplifications of negative controls occur and the intensities indicate equivalence of 50 pg or above when compared to the amplification of positive controls, DNA extraction and amplification should be redone. However, if the amplification indicates less than 0.5 pg while ancient DNA samples are more than 5 pg or 50 pg when compared to positive controls, one can continue the experiment with "caution." In this study, based on the observation of amplifications of negative controls, the level of contaminant DNA can be estimated to be much less than 0.5 pg per setup. This can explain why, when the number of cycles was dropped below 40 cycles in this study, amplifications of negative controls disappeared while ancient DNA samples still produced amplifications. We believe that the PCR efficiency can be roughly indicated using three positive controls for each individual PCR setup. The making of a master-mix for PCR setup should be used to reduce the efficiency variation among individual PCR tubes.

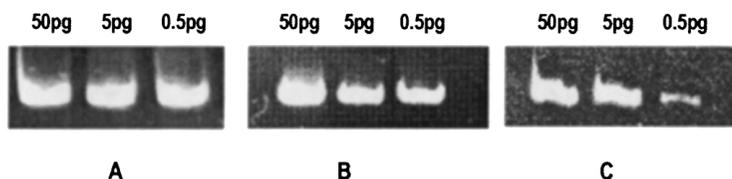


Figure 3. Ethidium-bromide-stained gels of PCR products from three quantified positive controls at different amplification efficiencies. **A:** amplification plateau was reached. **B:** amplification plateau was not reached. **C:** amplification plateau was not reached.

Although more than 40 cycles seems too high for the 176-bp mtDNA fragment in this study, it is not our intention to propose a “universal” optimal cycle number. Primers, length of amplified fragments, reagent concentrations, and other factors can all significantly affect amplification efficiency (Innis and Gelfand 1990). In principle, the number of cycles should be determined by the starting amount of template. For example, in modern DNA, 40 to 45 cycles should be sufficient for amplification from 50 templates (Innis and Gelfand 1990). However, the number of cycles should definitely go up for ancient DNA templates due to the damaged condition of the molecules and the presence of associated PCR inhibitors (Rameckers et al. 1997). A more hypersensitive PCR should be welcome for some types of ancient DNA, such as ancient human nuclear DNA or nonhuman ancient DNA studies. However, this is not necessarily true for human mtDNA amplifications, because there is always some “background human mtDNA” existing on ancient remains or in the laboratory due to its high copy number per cell; a super-hypersensitive PCR can easily convert them into “contaminant mtDNA.”

There may be a legitimate concern over the use of positive controls in ancient DNA studies (Cooper and Poinar 2000; Raoult et al. 2000). This is particularly true when one uses ancient DNA fragments that are identical to modern DNA counterparts such as some pathogen DNA used to diagnose ancient diseases. However, for ancient human mtDNA, this concern is easily addressed by the fact that most ancient DNA samples should have a different D-loop sequence from the positive control used in the laboratory. We suggest using one of the most commonly available modern DNA samples, K562, as the positive control, or intentionally synthesized DNA molecules that are incorporated with some specifically changed DNA sequences such as restriction sites (MacHugh et al. 2000). The same laboratory should continue to use the same positive control. Once contamination occurs, sequencing results can easily confirm or refute the positive control as the contamination source.

Ubiquitous Presence of Contaminant Human mtDNA. Gill and colleagues note that for forensic DNA analysis such as STRs, “laboratory-based contamination cannot be completely avoided, even when analysis is carried out under strin-

gent conditions of cleanliness” (Gill et al. 2000). This is even truer for ancient human mtDNA; an absolutely contamination-free situation cannot be obtained in reality. Human contaminant DNA can be introduced into the process at any point from archaeological excavation, to DNA extraction, to PCR setup. Contaminant DNA can be directly mixed with ancient DNA samples or can be found in buffers and other reagent solutions. One can exercise strict contamination controls in a dedicated laboratory for ancient DNA extraction and PCR setup, but one does not have much control over some processes that have to take place outside of the laboratory such as manufacturing chemicals, reagents, columns, tubes, and buffers (Schmidt et al. 1995; Eshleman and Smith 2001). One can choose products with the highest standards from brand-name companies. They may be the best for modern DNA studies but not necessarily meet requirements for ancient human mtDNA studies. If a shed skin cell drops into a reagent solution, there will be only two nuclear DNA fragments but perhaps one thousand mtDNA molecules. If the contaminated solution is aliquoted into small volumes, some of these small volumes may not contain any copy of a particular nuclear DNA fragment but more likely several mtDNA molecules. If PCR systems are extremely sensitive and there are no other DNA templates available such as authentic ancient DNA, these contaminant DNA templates can be picked up and amplified. The study of ancient human nuclear and nonhuman DNA samples indicates that they may not be affected as badly as ancient human mtDNA. We have tried to amplify the single-copy nuclear amelogenin gene for sex determination on the same set of DNA extractions, and no amplifications of negative controls were observed at high cycles (data not shown). In another ancient DNA study in our laboratory, strong amplifications of animal mtDNA were obtained at high cycles but no false positives were seen (data not shown).

Regular Polymerases and Highly Efficient Polymerases. As demonstrated in this study, highly efficient polymerases such as AmpliTaq Gold™ should be used in ancient DNA studies, since they significantly increase amplification efficiency, making nested PCR unnecessary in many cases.

Conclusions

The amount of contaminant DNA in this study was probably not significant. But this study has reflected a significant issue in ancient DNA studies: due to its high copy number and ubiquitous presence, contaminant human mtDNA cannot be totally prevented from entering the process of ancient human mtDNA investigation. This is an unfortunate reality for all ancient human mtDNA studies. However, ancient DNA analysis can still be carried out even when there are low (insignificant) amounts of contaminant DNA mixed with authentic ancient DNA templates. We propose the set-up of multiple positive and negative controls to indicate the sensitivity of individual PCR amplifications and to monitor the levels of

contamination. Highly efficient polymerase should be used for ancient DNA samples to increase amplification efficiency, but optimal efficiency must be determined. By optimizing the sensitivity of PCR amplifications, insignificant amounts of contaminant “background DNA” will be kept from obscuring the analysis of authentic, ancient DNA templates.

This study does not invalidate the notion that contamination must be strictly controlled and false results must be removed from ancient DNA studies. Instead, this study clearly demonstrates how tiny amounts of modern human DNA may become a major problem in ancient DNA studies. The greatest efforts should still be made to minimize contamination levels.

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