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Standardization of PCR conditions for an Ancient DNA Amplification

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Abstract
An ancient DNA provides us a powerful tool to study the miniscule amounts of DNA present in hundreds of thousands of years old archaeological remains. Since the advent of the PCR, it became possible for the population biologists to use this scarce and rare genetic material (aDNA) to understand prehistoric population histories. Working with ancient DNA is challenging in itself as it needs a manifold attention in order to maintain the archaeological sample free from contemporary DNA contamination. Apart from that, there are several other complications associated with ancient DNA work such as the preservation of DNA itself that is in degraded state and low copy number, DNA isolation and its successful PCR amplification. Despite the critical role of PCR in this field of research, till date no study has comprehensively evaluated ancient DNA amplification. In this paper, we have reported our results to optimize PCR component as well as PCR condition to amplify HVR1 region in 600 years old biological samples.
Keywords: Ancient DNA, Polymerase Chain Reaction, Optimization, Contamination

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Introduction

In order to elucidate human population and human prehistory scientists have long used the archaeological and fossil records. Even though the archaeological record provides us with clues about prehistoric events, but molecular genetics analyses may provide a further tool in helping us understand the past. Ancient DNA provides numerous possibilities to investigate ancient populations, their movements, diseases and much more. Investigation on ancient DNA initiated in the mid-1980s from molecular evolutionary research, with the plan of extending phylogenetics (studies of evolutionary relationships) and population genetics to died out species and populations. Possibly the first ancient DNA study was in 1984, with a publication by Russ Higuchi and colleagues at Berkeley that was to reform the capacity of molecular biology. Traces of DNA from a museum specimen of the Quagga, which remained in the specimen over 150 years after the death of the individual were extracted and sequenced (Higuchi et al, 1984). As ancient DNA is a potential tool to access temporal data in order to delve deep and elucidate the hidden past. Currently aDNA is playing a crucial role in the field of population genetics, homonids, sediments, diet and behaviour, medical molecular archaeology, origins of domestication and solving the mysteries, which are unsolved through the ages (Paabo et al, 2004). The two main problems with the ancient DNA research are, (i) the amount of endogenous DNA available in the sample is often limited and (ii) contemporary DNA contamination which is almost always associated with ancient remains. A wide range of technique on extraction and amplification has been published to date, all of them aiming to improvise the quality and quantity of DNA yields. (Hofreiter et al, 2004; Hoss et al, 1993; Kalmar et al, 2000; Leonard et al, 2000; Yang et al, 1998; Hanni et al,1995). Although many studied were made in comparison of extraction methods (Bouwman et al, 2002; Hummel et al, 2003; Prado et al, 2002) but till date no study is done in order to optimize the PCR conditions. We are reporting here a PCR standardization performed on approximately 600 years old ancient DNA sample. The optimizations were tried at three different points, the first; an efficient Taq Polymerase, second; to overcome PCR inhibitors (Pabbo et al, 1988)and third is to check an effect of final extension time on successful PCR amplification

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Materials and Methods:

Ancient specimens:

Ancient bone samples were kindly provided by the Anthropological Survey of India and all experiment performed at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad. These samples were collected from Himalayan region, India and carbon dating was conducted by Anthropological Survey of India which tells us that these samples are 600 years old (unpublished data)

Contamination Precautions:

Standard contamination precautions were strictly followed in ancient DNA studies during the experiments (Paabo et al, 2004; Kalmar et al, 2000; Willerslev et al, 2005; Hebsgaard et al, 2005; Kaestle and Horsburgh, 2002; Cappellini et al, 2004; Shinoda et al, 2006; Thomas et al, 2004; Kemp and Smith et al, 2005; Willerslev et al, 2004)

DNA Extraction:

About 3 mm of cortical bone surfaces is removed with a sterile scalpel from all samples, followed by immersion in 10% bleach solution for 10 min and washing with 70% alcohol. The cleaned bone fragments were mechanically pulverized into a fine meal in sterile pestle-mortar. The samples (500mg) were then soaked in 5ml of 0.5M EDTA (pH 8.0) with 200µl of 20mg/ml Proteinase K and 120µl of 10% SLS (Sodium Lauryl Sulphate) at 55˚C for overnight. Followed by the overnight incubation of bone powder in EDTA, approximate volumes of 2ml of Lysis buffer, 200µl of SDS (Sodium dodecyl Sulphate), 50µl of 20mg/ml Proteinase K was added to the pellet and again subjected to overnight incubation at 60˚C. Next day Centrifuge at 3000rpm for 15min, after adding equal volume of phenol to the supernatant, centrifuge it for 5min at 3000rpm. Supernatant were collected in chloroform /isoamyl alcohol and mixed by inverting for 10min and centrifuge 5min. The supernatant collected was added on Centricon filter and centrifuged at 3000rpm till the remaining volume is 200µl. Add equal volume of Isopropanol and 20µl of sodium acetate to the remain and keep it on -20C for precipitation for 2hours and centrifuge at 14,000rpm for 20min.

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After discarding the supernatant 1ml of fresh 70% Ethanol added to wash the pellet. Dry it till the ethanol removes completely from the pellet and dissolve the pellet in 30 to 50µl of T.E buffer.

PCR Performance

For performing PCR, we designed and custom synthesized (Sigma-Proligo; Proligo Singapore Pty Ltd, Singapore, Singapore) primers to amplify Hypervariable Region-I (HVS region-I). The sequences of the primers used were F159965'CTCCACCATTAGCACCC AAAG 3' & R16420 5' TGATTTCACGGAGGATGGTG 3' which amplified polymerase chain reaction (PCR) products of 447 bp. PCR for each sample was performed in 0.2-mL, thin-walled tubes using 4.0 µL of template, 4 pm of each primer, 200 mM dinucleotide triphosphates, 10× PCR buffer, 1.5 mM MgCl2, and 0.5 units of AmpliTaq Gold (Applied Biosystems). Bovine serum albumin (BSA) was used as it can bind to various types of PCR inhibitors that might have carried during extraction. The PCR reaction was carried out in a GeneAmp 9700 Thermal Cycler (Applied Biosystems) under the following conditions: 96˚C for 10 minutes, 30 cycles at 96˚C for 45 seconds, 57˚C for 1 minute, and 72˚C for 2 minutes, and a final extension at 72˚C for 20 minutes. PCR products of HVR1 were electrophoresed at 120V in 2% agarose gel. The PCR products were then visualized under UV light in trans illuminator. Following optimizations were done on above mentioned PCR conditions performed on the ancient DNA extract.

Optimization of DNA polymerase

We compared the amplification efficiency of three commercially available DNA polymerase with one home made DNA polymerase (CCMB made). We found that the PCR amplification achieved with AmpliTaq Gold (Applied Biosystems) was maximum in comparison to Platinum Taq (Invitrogen), Bioron Taq, homemade polymerase, which yielded either no amplification or faint bands. (Fig1)

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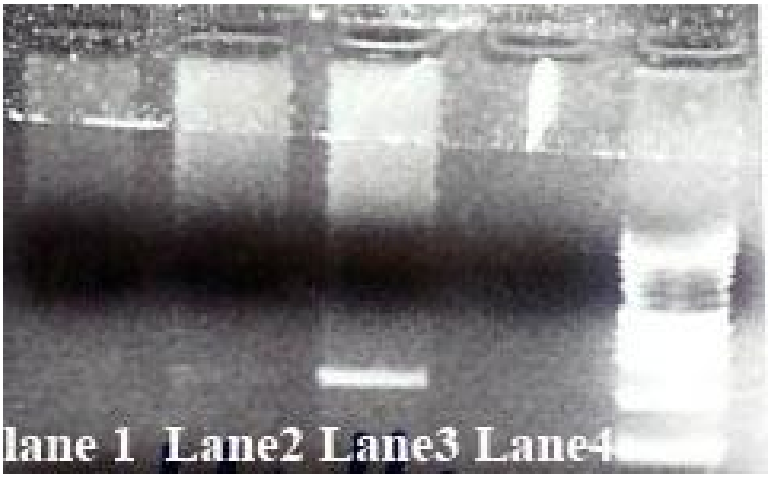

Figure1. Agarose DNA electrophoresis (2%) of PCR reaction products for Optimization of DNA polymerase, [lanes 1-4: lane 1 Bioron Taq polymerase (no amplification), lane 2 Platinum Taq, Invitrogen (faint band), lane 3 AmpliTaq Gold (maximum yeild), lane 4 home made Taq (no amplification), and lane 5: λ phage DNA marker].

Optimization of PCR inhibitors

To investigate the effect of BSA, we compared the amplification success of AmpliTaq Gold polymerase in both the presence and absence of BSA. We used 5 mg/ml BSA final in 20-50 microlitre PCR assay to compare with the PCR without BSA. We found a striking difference between the PCR without using BSA (lane 1, Fig 2), which was unable to yield any product, whereas with BSA the amplification was achieved (lane 4, Fig 2). Hence, BSA plays an important role in binding to inhibitors and enhancing the amplification of PCR products.

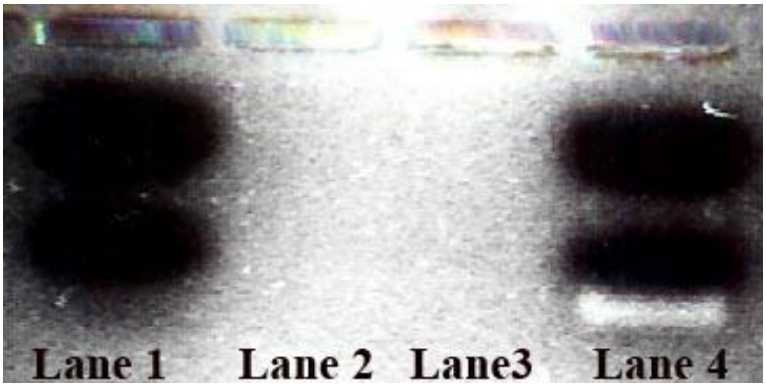

Figure2. Agarose DNA electrophoresis (2%) of PCR reaction products for Optimization of PCR inhibitors, [Lane 1 without BSA (no amplification), lane 4 with BSA (High intensity of amplification), and lanes 2-3: blank].

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Optimization of Extension Time

We also tried to check an effect of final extension time on the efficiency of PCR carried out on ancient DNA sample. We tried three different extension times; 30mins, 25mins and 20mins, on 3 different PCRs derived from the same PCR master mix. We observed that after 30 cycles of PCR a final extension time of 20 mins yeilds the maximum amplification (Fig 3).

Figure3. Agarose DNA electrophoresis (2%) of PCR reaction products for Optimization of Extension Time, [lane 1: 30 minute's final extension (dimer formation), lane 2: 25 minutes final extension (no dimer formation and faint amplification, lane 3: 20 minutes final extension (no dimer formation with high intensity band) and lane 4: contain λ phage DNA marker.]

Result and Discussion:

We successfully investigated the positive effect of BSA on the amplification of ancient DNA in the present study. In addition to this, we have also demonstrated that the gradual increase in final extension time also affects the amplification. Finally on comparing the amplification efficiency of various available Taq Polymerase, our study showed that AmpliTaq Gold polymerase is more effective than others. It is also noticed that gradual increasing of extension time yields unspecific bands (Fig.3). In India, the present work is a preliminary effort on the standardization and amplification of ancient DNA from human bone samples excavated from the Himalayan regions by anthropological survey of India. Attempts on Indian specimens have been few but initial results are encouraging, however, such studies need adequate attention in order to interpret the analyses. Our proposed method of amplification could be helpful in successful examination of excavated materials which will further provide a deeper insight. A wide range of literature concerning extraction and

amplification of ancient DNA is available but according to our knowledge, till date, no study had been done on standardization of PCR condition. In this paper, we have shown the combination of technical methods and alternative ways of already known processing methods by optimizing each step of the Polymerase Chain Reaction. This experimental attempt is providing a systematic method of ancient DNA amplification and expected to give a significant impetus to this subject which might be useful for other researchers from different institutions. Taken altogether, our results provide a consolidated protocol to amplify the ancient DNA. At the same time, we hope that the current study will encourage further research in this field to solve migration pattern as well as mysteries using different ancient specimens.

Acknowledgment

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