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AMPLIFICATION AND SEQUENCING OF MITOCHONDRIAL DNA (HVR-I) EXTRACTED FROM 1200 YEARS OLD HUMAN BONE SPECIMEN

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ABSTRACT: Ancient DNA help in better understanding the evolution and migration pattern of humans throughout the world. The present work is a preliminary effort, to extract and amplify the ancient DNA from human bone samples provided by the Anthropological survey of India collected from the Himalayan region in 1952. Many technical difficulties arise during DNA extraction, PCR amplification and sequencing which make it difficult to work and interpret the analyses. Our preliminary result is encouraging, as we have successfully amplified 456 base pair PCR product of mitochondrial DNA hypervariable region I (HVR1) starting from position 15976 to 16431. Our results confirm that the ancient bone specimens harbours M haplogroup signature with considerable similarities to M21ab sub-group specific to East and Southeast Asia. Absence of negative amplification confirms that our ancient mitochondrial DNA is contamination free.

INTRODUCTION: An ancient DNA technology provides new insight for anthropologist and archaeologist to better understand the past happenings and interpret it with the future possibilities. The first ancient DNA study was done in 1984, on museum specimen of the Quagga [1] but the role of ancient DNA in humans comes into play after the successful study on 2400-year old Egyptian mummified material by Paabo and colleagues in 1985 [2].

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Ancient DNA has generated new opportunities for archaeological and anthropological investigators like contemporary population history, their migration patterns, attraction domestication of plant, solving the historical mysteries and much [3, 4]. It is always a tough task to work with ancient DNA, the two main hurdles which make it difficult is degradation of DNA and contamination of ancient DNA with contemporary DNA, the DNA molecule present in the ancient remains may also get destroyed during extraction due to physical and chemical treatment. In 1989, the invention of the polymerase chain reaction (PCR) [5] made it possible to amplify ancient DNA and study even single existing molecule which allows the number and assortment of ancient DNA studies to expand rapidly [6, 7, 8].

This technique is extremely sensitive to amplify a low copy number of DNA present in ancient remains in a matter of hours [9, 10]. Many researchers are trying to develop more reliable techniques for ancient human DNA analyses [11, 12, 13]. The main problem with the PCR amplification of ancient DNA is presence of the inhibitors and damaged DNA [14, 15]. In order to overcome this problem researchers have used Ampli Taq Gold TM Polymerase to increase the efficiency of PCR amplification which is highly expensive and low cost Taq polymerase (Promega), high number of PCR cycles, addition of BSA, etc are also used in some studies . Ariffin et al (2006) [16] and Yang et al (1998)[17] were able to amplify mitochondrial DNA from 400years old and 2000 years old human skeletal remains and are the best examples of amplifying the ancient DNA with high efficiency.

In the present study, the human bone samples were excavated from the Himalayan regions by Anthropological survey of India field team in the 1950s and carbon dating analysis and morphological analyses showed that these samples are 1200 years old and are human (Unpublished report 1959, Department of anthropology, Kolkata) [18]. Since the excavated bone samples were placed constantly at 4˚C after excavation, they seem to be well preserved.

In this report, we follow a simple procedure for mitochondrial DNA extraction and subsequent PCR amplification using AmpliTaq Gold (Applied Biosystems) polymerase followed by DNA sequencing from 1200 year old human bone sample. All ancient DNA research is carried out in a dedicated laboratory that undergoes regular decontamination process to remove the chance of contamination of modern DNA or PCR products. The DNA extractions were carried out on powdered bone and were subjected to DNA extraction by phenol-chloroform and ethanol precipitated purification procedure followed by PCR amplification.

MATERIALS AND METHODS

Ancient specimens: Ancient bone samples (**Figure 1**) were provided by the Anthropological Survey of India and all experiments were performed at the ancient DNA lab facility of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad. These samples were excavated from Himalayan region, India by the Anthropological Survey of India under the leadership of its Director, D.N. Dutta-Mazumdar (1956) and carbon dating was revealed that these samples are around 1200 years old (Unpublished report 1959, Department of anthropology, Kolkata) [18].

FIGURE 1: ANCIENT BONE SPECIMENS CONSIDER FOR PRESENT STUDY PROVIDED BY ANTHROPOLOGICAL SURVEY OF INDIA

Contamination controls: All ancient DNA extractions and PCR set ups should be conducted in a dedicated laboratory that undergoes regular decontamination [19]. We have a dedicated Ancient DNA laboratory where all rooms have ultraviolet lamps fixed to ceilings for general room irradiation. Glasses used in making doors and windows are ultraviolet proof and fitted airtight. Clean filtered air generated from an exclusive hepa-filter/AC unit is flushed into the room finally with positive pressure and restricted entry of people.

Personnel wear laboratory coats and face masks and changes gloves regularly every time. All general equipment and apparatus (e.g., centrifuges, pipettes, gel electrophoresis) were dedicated for this lab room for pre-PCR work (there is no shared equipment). Protective clothing from a post-PCR laboratory was never taken into the clean-room facility. All solutions, PCR reagents, and primers were kept in small carefully labelled aliquots dedicated solely for work with one ancient DNA collection.

Pre-PCR activities were spatially separated in the laboratory and post PCR sequencing performed in a different laboratory. Extraction of bone DNA was performed using dedicated chamber that were decontaminated by bleach followed by 70% ethanol prior to the procedure so as to minimize cross contamination with modern DNA as cited in Kemp and Smith 2005 [11].

Extraction of ancient bone DNA: The bone surface was shaved by sterile scalpel blade to remove dirt, soil, other foreign material and then wiped with alcohol. The bone samples were then exposed under UV lamp in the aspiration hood for 2hrs. About 4 mm square of cortical bone surface was removed with a sterile scalpel from all samples, followed by immersion of fragmented bones in 10% bleach solution for 10 min and washing with 70% alcohol [11]. The cleaned bone fragments were mechanically pulverized into a fine powder in sterile pestle-mortar.

Approximately 1 milligram (mg) of powdered bone was taken for DNA extraction. In this study, due to the importance of the respective samples, we were allowed to extract the mtDNA from a total of 1 grams of powdered bone. The powdered bone was UV irradiated prior to DNA extraction. The samples (500mg) were then soaked in 5ml of 0.5M EDTA (pH 8.0) along with 200µl of 20mg/ml Proteinase K and 120µl of 10% SLS (Sodium Lauryl Sulphate) followed by incubation at 55˚C for overnight (24 hours).

Two different methods were employed for the extraction of DNA from the powdered samples. In the first method decalcified samples were processed with Banglore genei kit as follows

Extraction using Bangalore Genei Kit: After overnight incubation, pellet was washed twice with sterile water and centrifuged for 15min at 4000 rpm. After discarding the supernatant, lysis buffer 1 and proteinas K were added as mentioned in Bangalore geni manual then incubated at 50C for 24-48 hours. On the third day lysis buffer was added and centrifuged at 5000 rpm for 10min. Supernatant was collected into fresh tube in addition with binding buffer and passed it through the column provided in kit by centrifuging at 10,000rpm for 1min. Column was washed by wash buffer I followed by wash buffer II . Finally the DNA is eluted from the column by adding 40 µl of elution buffer.

The extraction was also done with Phenol-chloroform method.

Extraction using Phenol-chloroform method: In this method decalcified samples were lysed in 2ml of Lysis buffer (10mM tris HCl ph 8.0, 1mM NaCl) at 60˚C for 24 hrs, which was later subjected to phenol-chloroform, chloroform-isoamyl alcohol extraction (Kalmar et al., 2000 with modifications) [20]. At last, the aqueous phase was concentrated by centrifugation driven dialysis using Centricon-30 micro concentrators from Amicon.

The concentrate, which was about 0.7ml, purified by Gene Clean Spin Kit and later eluted in 0.1m Tris-EDTA. Approximately 40µl of DNA extract was obtained.

Amplification of ancient bone mtDNA: The overall success of the extraction procedure was assessed based on the ability to amplify a long stretch of 456 base pairs target sequence at Hypervariable region I of mtDNA which is not usual case with ancient DNA samples [20]. This could be possible because of proper purification of DNA, which removed prominent PCR inhibitors efficiently. The following primers were designed and synthesized (Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India) for PCR amplification: F15976 5'CTCCACCATTAGCACCCAAAGC3' as the forward primer and R16431 5'GCGGGATATTGATTTCACGG 3' as the reverse primer.

PCR amplification was carried out using the Applied biosystem Thermocycler Model 2720 (ABI) in a 20 µl reaction volume containing 3µL of re-precipitated template, 100µM each of dNTPs, 4 pM of each primer, 1X PCR buffer [100mM Tris-HCL, pH 8.3 (at 25^0C), 500mM KCl, 15 mM $MgCl_2$, 0.01%(w/v) gelatin, bovine serum albumin (BSA, 10 mg/ml, New England Bio Labs) and 0.5 units of AmpliTaq Gold (Applied Biosystems). The PCR reaction was carried out in an ABI_2720 under the following conditions: 96˚C for 10 minutes, 35 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.

Finally 10 µl PCR products of ancient DNA were electrophoresed at 120V in 2% agarose gel. Agarose gel was prepared in 1X Tris-acetate-EDTA (TAE) buffer containing ethidium bromide (EtBr). After 30 Min. of run halfway the gel was observed under ultraviolet light of UV trans-illuminator and photographed (**Figure 2**).

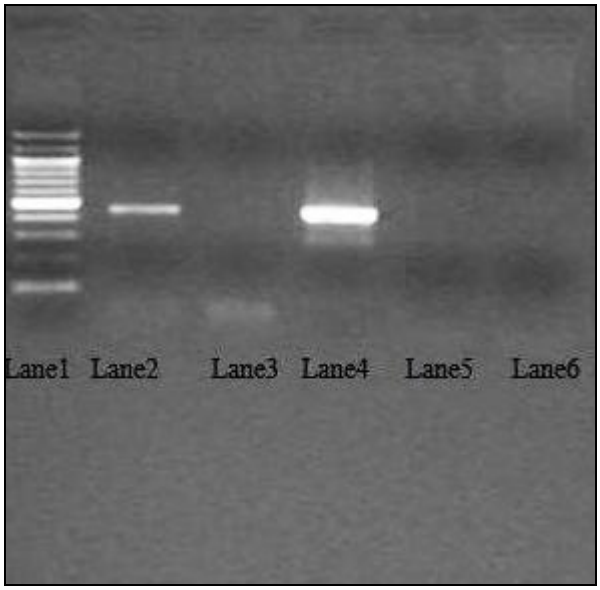

FIGURE 2: AGAROSE DNA ELECTROPHORESIS (2%) OF PCR REACTION PRODUCTS [Lane1: λ phage DNA marker (100bp), Lane 2: contain Ancient DNA extracted by Phenol- Chloroform method (single faint band), Lane 3: contain Ancient DNA extracted by Bangalore Genei Kit (no amplification), Lane 4: Positive Control, Lane 5: Negative control, and Lane 6: Blank**]**

ExoSAP treatment: In order to sequence, the amplicons were treated with exonuclease-1 and Shrimp Alkaline Phosphatase (ExoSAP-IT®; USB Corporation, Cleveland, Ohio, USA). 5µl of PCR Product is properly mixed with 2µl ExoSAP-IT and incubate (Thermocycler machine) at 37˚C for 15min (Activation of enzyme) followed by 80˚C for 30min (Inactivation of enzyme) to remove the extra nucleotides and primers (dimer) without loss of amplified PCR product.

Sequencing of ancient bone mitochondrial DNA (HVR-I): ExoSAP treated PCR products were directly sequenced twice using the ABI Prism 3700 DNA analyzer (Applied Biosystems).The sequencing PCR was carried out in GeneAmp 9600 thermocycler (Perkin-Elmer) for 3hours using the BigDye Terminator v3.1 ready reaction kit (Applied Biosystems, Foster City, CA, USA) and analyzed in an ABI 3730xl automated DNA Analyzer (Applied Biosystems). Sequences of ancient DNA were carefully edited and aligned with revised Cambridge reference sequence (rCRS) and compared with the mitochondrial HVR-I region using sequence analysis ,AutoAssembler tool-version 2.1 (Perkin-Elmer, Foster City,CA, USA) and Codon Code Aligner version 2.0.5 (Codon Code Corporation, Dedham, MA, USA) (**Figure 3**).

The variations detected in the Ancient DNA were checked in the mitochondrial databases such as mitochondria (http://www.mitochondria.org/ mitologial.php) for their significance.

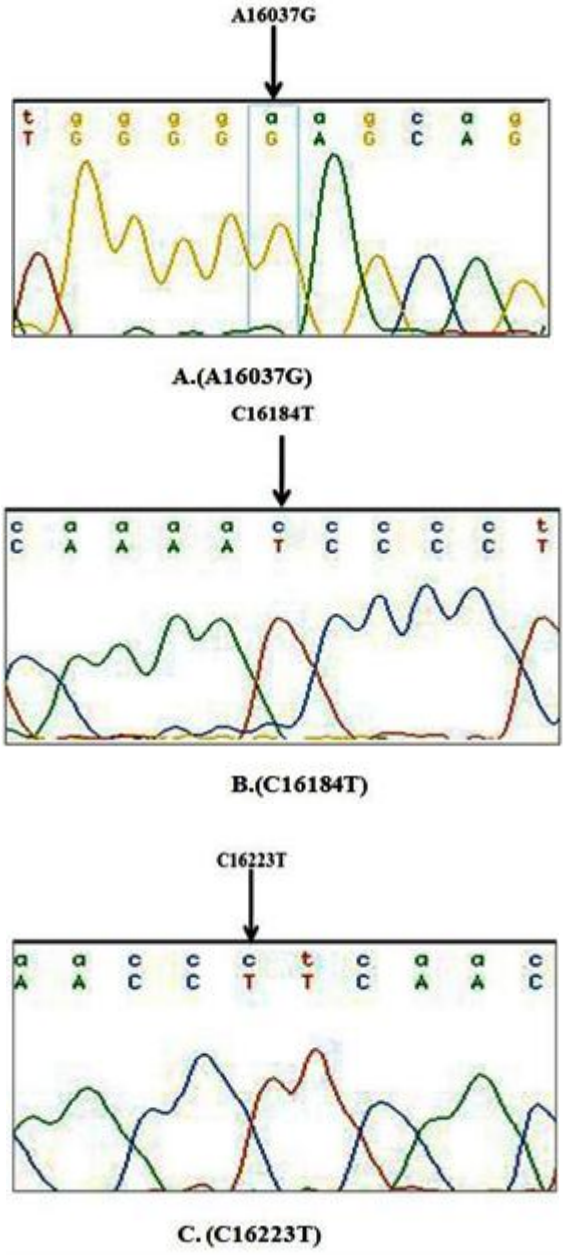

FIGURE 3: THE SEQUENCE ELECTROPHEROGRAM OF mtDNA POLYMORPHISMS OBSERVED. A. The wild type sequence showing (arrow) 'A' at the nucleotide position 16037. B. Sequence of an ancient DNA showing polymorphic allele 'C'at the nucleotide position 16184. C. Sequence of an ancient DNA showing polymorphic allele 'C'at the nucleotide position 16223.

RESULTS: Primers were specially designed to amplify mitochondrial DNA hypervariable region I on position 15976 to 16431 for 456 base pairs PCR product. A band approximately 456 base pairs was amplified in the samples containing ancient bone

(Figure 2, Lane 2) and positive control (Figure 2, Lane 4) whereas negative and blank controls were unable to amplify the 456 base pairs band (Figure 2, Lane 5, 6). However, Ancient DNA and positive control (+ve) samples showed the presence of primer-dimers (Figure 2, Lanes 2-4) during PCR amplification as expected due to the sequences of the primers [30].

In Figure 2 Lane 2, After 35 amplification cycles a single weak band of 456 bp provides an evidence of a successful amplification of DNA from an ancient bone. PCR products from successful amplifications were subjected to DNA sequencing after exosap treatment. The sequence electro-pherogram of mtDNA shows polymorphisms at the nucleotide position A16037G (Figure-3, A), C16184T (Figure-3, B), C16223T (Figure-3, C) in ancient DNA. BLAST analysis confirms that ancient DNA was 99% identical to the human mitochondrial displacement loop (D-loop) sequence.

The phylogenetic analysis revealed haplogroup M to be the major haplogroup and considerable similarities with M21ab sub-group from region of India, China, Pakistan, Japan population for our ancient DNA sample. The haplogroup nomenclature was based on published information [21]. The samples were not showing any heteroplasmy which also confirms that our ancient mitochondrial DNA is not contaminated.

Control amplification: The present study included control amplification in each step to assess any possible contamination. Negative control amplification, to check the purity of the PCR reagents with no DNA added (Figure 2, Lane6). Blank control amplification is used to check the purity of the extraction reagents with no bone added followed by PCR amplifications (Figure 2, Lane5) [15, 17, 22, 23].

Positive control amplification used to monitor the success of Thermocycler reactions and PCR components (Figure 2, Lane4).

DISCUSSION: Working with ancient DNA is very expensive, time-consuming, needs more amount of sample (approximately 5g of bone powder) and destructive undertaking. Every time the results were distrustful to be a contaminant free.

The reliability of ancient DNA is always questionable due to several reasons. Furthermore, the ancient DNA loses its integrity and decomposes with an irreversible loss of nucleotide sequence information relative to the modern DNA. From available studies it was shown that Svante Paabo (Nature news) [24] on 38,000 to 44,000 years old bones samples and Rasmussen on 4,000-year-old hair Palaeo-Eskimo samples were able to yield DNA that could be enzymatically amplified and sequenced [25].

In our earlier study we are able to increase the efficiency of PCR amplification from old bone specimens by optimizing PCR component as well as PCR condition for amplify HVR1 region[26].We choose slightly lower number of PCR cycles to avoid the unspecific amplification with use of standard DNA polymerase i.e AmpliTaq Gold polymerase (studies by other groups showed that a strong polymerase to amplify the ancient DNA) and addition of BSA. In this present study, absence of amplification in negative control confirmed that there is no modern DNA contamination during extraction and PCR procedures.

Although negative control shows primer-dimer but does not proves modern DNA contamination. From the gel picture, it was clear that amplified product similar in size to ancient D-loop mitochondrial hypervariable region I. This indicates that the procedure used for DNA extraction is able to produce a pure DNA from ancient bones that can be amplified and sequenced. Contamination of ancient samples by modern DNA molecules is a serious problem. Human remains are particularly difficult to work with; it gets easily contaminated at any step of excavation, extraction and finally PCR setup [10, 17].

For working with ancient DNA we have to follow the strict rule to avoid contamination. In many studies it was shown that researcher have the dedicated laboratory for ancient DNA extraction but they use the reagents, buffers, tubes and chemicals that take place outside of the laboratory can be contaminated by modern DNA [22]. In this study, we have strictly followed all decontaminated procedures to minimize or eliminate modern DNA contamination in the sample. There is no amplification product found in the negative control (Figure 2, Lane 6) and also sample processed with

Bangalore Genei kit not showed any amplification (Figure 2, Lane3). For our study, we have used the dedicated laboratory as well as laminar chamber to minimize the contamination. In addition, we have prepared all reagents in this dedicated laboratory only and also consumable items and equipments needed for ancient DNA work were double autoclaved and exposed to UV irradiation. Furthermore, Fumigation and disinfectants were regularly done in the lab to eliminate any surface contamination. In this study, we also performed different extraction method with some modification based on different reports. The good preservation of the samples and contamination free protocols have been key factors for the success of this study. The analysis of mitochondrial DNA (mtDNA) has been proven to be a powerful tool in the understanding of human evolution and a study by Hagelberg had confirmed that this is a potent tool for identification of variations in mitochondrial DNA from human remains for chronological investigations. The main reason for recovering mitochondrial DNA (mtDNA) from ancient Human bone specimen is its lack of recombination, contains high copy number, shows maternal mode of inheritance and help to calculate the divergence time elapsed. Finally small size and simple genome organization make it easier to study. The high copy number of mitochondria per cell increases the probability to amplify the ancient DNA. There are enough evidences that show successful mitochondrial DNA analyses on very old human remains [4, 27, 28]. In our study, we have chosen the mitochondrial Hypervariable segments, HVRI (positions 15976 - 16431) region as targets for PCR amplification (Figure 2, Lane 2). Best combination of DNA polymerase (Ampligold Taq) & other PCR components (BSA) provides sufficient amount of PCR product for DNA sequencing. For our smaller quantities of ancient DNA extracted from bone specimen, we used higher number of cycles to produce high number of copies [15, 17, 22]. Almost all studies of human evolution based on mtDNA sequencing have been confined to the control region also called the D-loop or the displacement loop. HVRI and HVRII data can provide useful insights about inter and intra-specific population variation. Displacement loop (D-loop) is non-coding regions of mitochondrial DNA which constitutes about 7% of the mitochondrial genome. The sequence and size of HVRI (D-loop) varies from species to species[29]. So on this basis expected sizes of ancient DNA PCR product showed that the DNA originated from human. The sequence generated by the amplified ancient DNA showed 99% identical with the hypervariable region I of D-loop region of the mitochondria with the absence of recombination. Furthermore, sequences were analysed using NCBI databases showed that ancient DNA was from human DNA only.

In summary, we managed to extract mitochondrial DNA from approximately 1200 year old bone specimen recovered by the Anthropological survey of India from the Himalayan region. The carbon dating conducted as reported showed that these samples are 1200 years old (unpublished data). Since, we were able to extract enough amount of DNA from these samples which is sufficient for amplifying 4-5 PCR reactions, the DNA sequences gave 99% homology with human mitochondrial HVR-I DNA.

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