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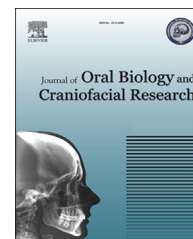
Original Article

Mitochondrial DNA from archived tissue samples kept in formalin for forensic odontology studies

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ABSTRACT

Background: Samples used for DNA isolation to be used for forensic odontology studies are often limited. The possibility to use tissue samples stored in formalin for a prolonged period, which contains nucleic acids of questionable quality, opens exciting possibilities for genetic and molecular biology studies useful in speciality of forensic odontology.

Aim: The present study defines substantial modification of existing protocols for total genomic isolation including mitochondrial DNA and proves the utility of such obtained mitochondrial DNA in microsatellite analyses.

Methods: 50 dental tissue samples which were kept in neutral buffered formalin liquid bottles were taken for DNA isolation and subsequent analysis. For the isolation of total genomic DNA from tissue samples, a new protocol with substantial modifications from routine ones was adopted by us. Total genomic DNA from matched blood samples were extracted using standard phenol-chloroform extraction method.

Results: Polymerase Chain Reaction and Sequencing of such extracted DNA samples for mitochondrial D loop region were successful and the results were comparable with DNA extracted from normal sources of samples.

Conclusion: The present study reports for the first time that nucleic acids extracted from human dental tissue samples under prolonged formalin fixation times can be used for forensic odontology studies using the described methodology.

1. Introduction

The discipline of forensic odontology has grabbed recent attention across forensic experts and dental fraternity alike.[1] Forensic odontology involves use of dental tissue remains in identification of humans. It has wide application from mass disasters to crime cases.[2] During mass disaster, victim identification becomes difficult and then only the DNA profiling systems can reveal the true identity of a person.[3] Similarly in criminal cases the identity of either the victim or criminal can be established through DNA identification.[3] DNA analyses require expertise in molecular biology techniques.[4] Hence, there have been calls for dentists in the discipline to get more

expertise with the molecular biology methodologies. Sources for genomic DNA from dental field are very useful and but often are not taken properly leading to a loss of potentially useful data.

With the expansion of molecular biology techniques of nucleic acid analysis, knowledge about the utility of the preserved DNA is increasingly important.[5] Fixed tissues are a huge resource of DNA in forensic odontology studies. These samples are often available at oral pathology centres or departments. Oral tissues during routine surgical procedures can also be easily obtained and put to future use. But, often, their collection and storage results in tissue fixation in formalin-containing solutions, ranging from few hours, days, months to years. Formalin treatment results in stiffening due to protein fixation at structural level and of DNA at molecular level, making DNA extraction difficult and its further use in molecular biology techniques unsuitable, though not impossible.

Use of mitochondrial microsatellites in forensic studies is well established.[6] C tract region is one such region. The idea of extracting total genomic DNA including mitochondrial DNA (mtDNA) from tissue samples kept in formalin solution for long and using them in forensic odontology studies has not been tried to date.

2.   Method & materials

50 dental tissue samples which were kept in neutral buffered formalin liquid bottles in the department of Oral & Maxillo-facial Surgery were taken for DNA isolation and subsequent analysis. Due consent from concerned subjects were taken and ethical approval from institutional ethics committee was taken prior to the study. Total genomic DNA from matched blood samples were extracted using standard phenol––chloroform extraction method. From tissue samples the isolation of total genomic DNA, including mtDNA following protocol was adopted (List & Composition Reagents and Solutions used in the protocol are given in Table 1).

2.1.   Protocol

1. Cut thin slices of Formalin-fixed non-paraffin-embedded tissue of about 10 mg with a sterile scalpel blade.
2. Briefly blot excess formalin from tissue.
3. Place tissue in 0.5 mL of the 1× GTE buffer in a 2-mL centrifuge tube. This buffer acts a binding agent for excess formalin.
4. Wash tissue six times, once every 12-h period for a total of 72 h. Additional washes may be needed until tissue becomes softened in texture.
5. Air-dry or place tissue at 55 °C until dry. Do not over dry tissue.
6. Cut tissue with sterile razor blade into 3–4 small pieces.
7. Place the pieces of tissue into 1.5 ml microcentrifuge tube containing 300 ul Cell Lysis Solution. Incubate the mixture at 55 °C in a water bath for 1 h.
8. Add 10 ul Proteinase K (10 mg/ml) to the mixture at intervals of 24 h for total period of 48 h. Incubate the mixture at 55 °C for 48 h. If the tissue is still not digested after 48 h, add further 10 ul of Proteinase K at 10 mg/ml for another 24 h.
9. Once all tissue is in solution, cool sample to room temperature. Add equal volume (300 ul) of equilibrated phenol (pH 8.0) to the mixture. Invert tube several times mixing it well for 10 min
10. Centrifuge for 10 min at 12,000 ×g to separate the layers.
11. Carefully remove the upper aqueous layer discarding the lower phenol layer and add to a new tube containing equal volumes i.e 150 ul each of phenol and 24:1 chloroform:isoamyl alcohol. Be careful not to remove the protein-containing interface. Invert tube several times mixing it well for 10 min.
12. Centrifuge for 10 min at 12,000 ×g to separate the layers.
13. Transfer the supernatant i.e. upper aqueous layer to a fresh tube containing equal volume (300 ul) of 24:1 chloroform:isoamyl alcohol avoiding the interface material. Invert tube several times mixing it well for 10 min.
14. Centrifuge for 10 min at 12,000 ×g to separate the layers.
15. Remove the upper aqueous layer and place in a new 1.5 mL Eppendorf tube. Discard the lower chloroform:isoamyl alcohol layer.
16. Add 2 volumes (600 ul) of absolute alcohol and invert gently a few times. The DNA precipitates as a thread like structure.
17. Incubate the tube at −20 °C overnight to allow complete DNA precipitation.
18. Centrifuge at high speed (>10,000 ×g) for 30 min at room temperature. Immediately following centrifugation, decant the liquid from the tube carefully. The DNA will

Table 1 – Reagents and solutions used in DNA isolation.	
List of reagents used	Solution preparation[a]
Glycine (Merck Catalogue# 3570)	1X GTE buffer: 100 mM glycine
Tris (Qualigens Catalogue# 10645)	10 mM Tris-HCL pH 8.0
EDTA (Qualigens Catalogue# 118445000)	1 mM EDTA
Phenol (Sisco Research Laboratories Catalogue# 1649142)	Cell Lysis Solution: 10 mM Tris–HCl pH 8.0
Chloroform (Qualigens Catalogue# 12307)	0.1 mM EDTA pH 8.0
Isoamyl alcohol (Sisco Research Laboratories Catalogue# 092945)	0.5% SDS
100% absolute Alcohol (Merck Emsure Catalogue# 100983)	24:1 v/v Chloroform and Isoamyl alcohol
SDS (Life Technologies Catalog# 15525017)	10 mg of Proteinase K in 1 ml of MiliQ (10 mg/ml)
Proteinase K (Sigma–Aldrich Catalog#P2308)	
[a] HINT: Reconstitute for the first time, aliquot it in 250 ul tubes, for uses, then store at −20 °C and reuse.	

Table 2 – Details of PCR primers & conditions.		
Primer	Sequence	Amplification conditions
Forward	5′ ACAATTGAATGTCTGCACAGCCACTT 3′	94 °C for 5 min, 35 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s,
Reverse	5′ GGCAGAGATGTGTTTAAGTGCTG 3′	followed by 72 °C for 10 min

Fig. 1 – 1% Agarose gel picture showing total genomic DNA.

have precipitated into a pellet at the bottom of the tube and may not be visible

19. Give two washes with 70% alcohol and air-dry the pellet.
20. Dissolve the DNA in 200 ul TE buffer for 4–6 h at 50 °C.

2.2. Mitochondrial DNA C tract amplification and sequencing

The mtDNA C tract amplification was done using a set of primer [Table 2] yielding a 109 bp fragment for all blood and tissue samples. Bidirectional direct capillary was done using BigDye® Direct Cycle Sequencing, Applied Biosystems, USA using manufacturer's recommended protocol. Both Forward and Reverse primers were used to analyse and confirm the sequences in the region of interest with the sequences.

2.3. Statistical analyses

Mitochondrial DNA sequences from tissue and blood samples were compared and difference if any was scored. Student 't' test was used to see significance of any such difference.

3. Results

Total genomic DNA from tissue samples kept in formalin solution for long periods (all > 1 Year) was isolated and visualized on 1% Agarose gel to check the quality and the quantity was confirmed by nanodrop readings with 260/280 and 260/230 values (Fig. 1). All the samples yielded DNA suitable for the running PCR (average 200 ng/ul from approximately 10 mg tissue sections; A260:A280 = 1.55) (Fig. 2). Upon mtDNA C tract amplification of all DNA samples, PCR products were of good quality as visualized by electrophoresis on 2% Agarose gels in 1× TBE (89 mM Tris–Borate, 89 mM boric acid, 2 mM EDTA) (Fig. 2). Further mtDNA C tract sequencing for 109 bp PCR products yielded good quality sequencing results which could be read well and compared with sequences obtained from

corresponding blood samples. 37/50 paired samples perfectly matched ($p > 0.05$) (Figs. 3 and 4).

4. Discussion

Formaldehyde as a 10% neutral buffered formalin is the most widely used universal fixative because it preserves a wide range of tissues and tissue components. Formalin preserved samples are quite common in dental practise. Routine day to day teeth extractions often yield tissues that are lacerated from the site and are waste hence thrown away. Preservation of such tissues in a dental office can put them to future use in forensic odontology analyses. However use of formalin is often frowned upon by molecular biologists. Formalin treatment results in cross-linking of biomolecules[7,8] which not only complicates isolation of nucleic acid but also introduces polymerase "blocks" during PCR and this is directly proportional to the time spent in formalin. Few methods exists for the recovery of DNA from archival tissues[9–13] and only 3 studies have analysed the ability to extract and study nucleic acids from samples after prolonged fixation and storage times.[14–16] These all studies were limited by the fact for they started DNA extraction from paraffinized tissue. Therefore till date no study has been done to isolate DNA directly from
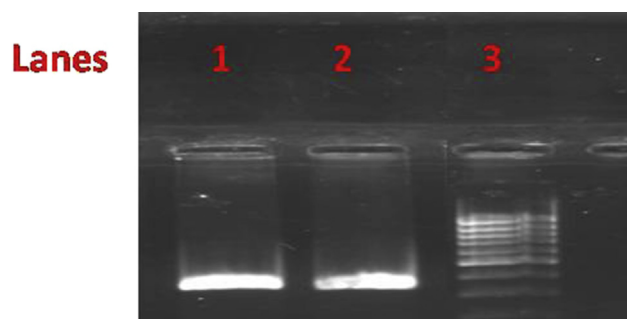
Fig. 2 – Lanes 1, 2 show mtDNA C tract PCR product while lane 3 shows 100 bp ladder.
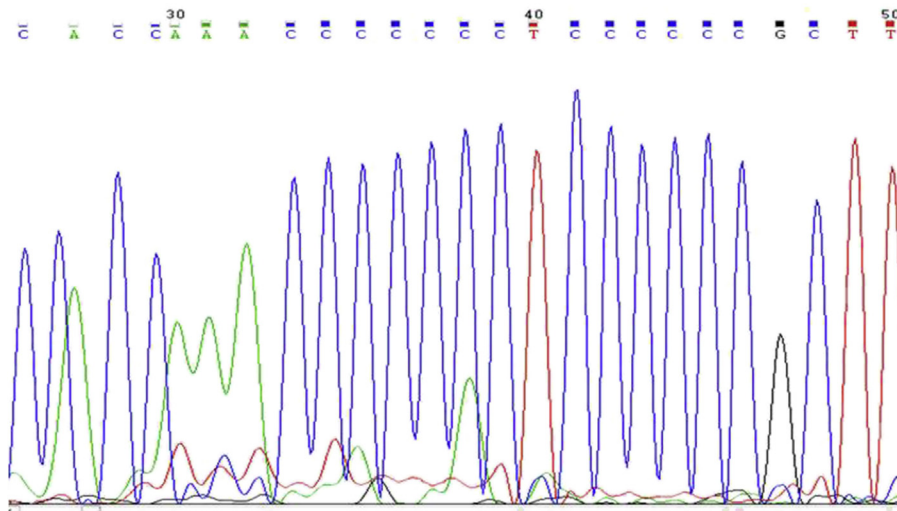
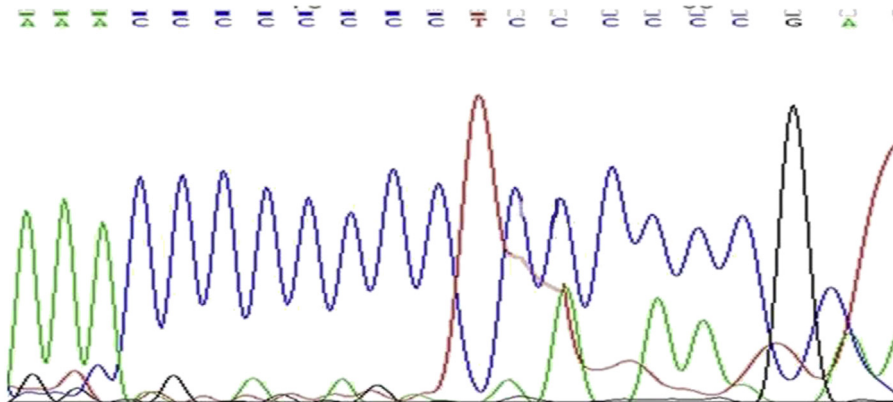**Fig. 3** – **Tissue sample mitochondrial C tract sequence (7CT6C).**

tissues stored in formalin solution and checking its suitability for downstream applications such as PCR and sequencing.

It is currently not impossible to reverse the effects of DNA fragmentation[17–19] and in our opinion, the key step of successful DNA extraction is the one at which there is reversal of the DNA–protein cross-links and the neutralization of the excess formalin in the sample. Formaldehyde reacts reversibly with water to form methylene glycol. Tissues are rapidly penetrated by the methylene glycol[20] but it has no role in fixation. The actual covalent chemical fixation depends on the fraction of the formaldehyde forming bonds with the tissue components and this formaldehyde comes from dissociation of methylene glycol. Interestingly, the equilibrium of this reaction strongly favours methylene glycol. Hence, formaldehyde though penetrates tissues rapidly as methylene glycol but fixes slowly as carbonyl formaldehyde. In our method we exploited this mechanism by using GTE (Glycine, Tris, EDTA) buffer which is glycine rich and alkaline in nature. Glycine reacts with formaldehyde to form either N-Methyleneglycine or Dimethylglycine and shifts the equilibrium towards formaldehyde thereby depleting the store of methylene glycol, the source of formaldehyde, in the tissue. Alkalinity of the buffer

further aids in reversing the cross-linking of DNA and proteins. EDTA neutralizes DNAse activity preventing further DNA degradation. In this paper, we therefore report a substantial modification of methods for DNA extraction, fit for molecular biology work in forensic odontology studies, from long duration formalin stored tissues.

Subsequently the method involves heat treatment and proteinase K action[21] which digests the denatured proteins and protein–protein cross-links followed by organic purification of the nucleic acids (Protocol). The method results in similar total yields of DNA per sample (as measured in ug/mL) compared to other extraction methods such as commercial kits marketed with the specific aim of processing formalin-fixed or paraffin-embedded (FFPE) material.

Use of mtDNA has been advocated as it possesses high copy number, maternal inheritance, and high degree of sequence variability.[6,22] Moreover, nuclear DNA tends to degrade over long period of time and hence mtDNA offers an alternative. Mitochondrial microsatellite studies have long been done in forensic samples due to their polymorphic nature.[23,24] In investigations involving missing persons, comparing the mtDNA profile of unidentified remains with

Fig. 4 – **Blood sample mitochondrial C tract sequence (7CT6C).**

the profile of a potential maternal relative is utilized. Nowadays, mtDNA samples from an individual are taken and compared either with sequence from crime site for identification or to available sequences on internet to confirm ethnicity or phylogeny. Our results provided an advancement in the field of forensic odontology by showing that DNA can be reliably amplified from tissues that have been maintained in formalin for considerable period of time (>1 Year). The sequencing results of 37 tissue samples matched with corresponding blood samples ($p > 0.05$). For rest of samples, difference can be attributed to the fact that C tract region is highly polymorphic site and resides in mitochondria which is in itself highly prone to mutation from external and internal stimuli. Thus there may be slight variability but this was not statistically in our study significant. Therefore, this simple, cost effective and non-laborious method should facilitate the molecular analysis of a large number of specimens fixed for long periods of time in retrospective studies.

5.   Conclusion

In summary, we report for the first time that nucleic acids extracted from human dental tissue samples under prolonged formalin fixation times can be used for forensic odontology studies using the described methodology. The quality of DNA fragments is sufficient to study polymorphisms involving deletion/insertion or single nucleotide mutations.

Conflicts of interest

All authors have none to declare.

Acknowledgements

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