Archives of Orofacial Sciences (2011), **6**(1): 9-14.

ORIGINAL ARTICLE

Extraction of mitochondrial DNA from tooth dentin: application of two techniques

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(Received 17 February 2011; accepted 6 June 2011)

Keywords

Dentin,
extraction,
mitochondrial DNA,
tooth.

Abstract Mitochondrial DNA (mtDNA) is a hereditary material located in mitochondria and is normally maternally inherited. Mutational analysis performed on mtDNA proved that the mutations are closely related with a number of genetic illnesses, besides being exploitable for forensic identification. Those findings imply the importance of mtDNA in the scientific field. MtDNA can be found in abundance in tooth dentin where it is kept protected by the enamel, the hardest outer part of the tooth. In this study, two techniques of mtDNA extraction were compared to determine the efficacy between the two techniques. Teeth used for the study was collected from Dental Clinic, Hospital Universiti Sains Malaysia. After the removal of tooth from the tooth socket of the patient, the tooth was kept at -20°C until use. Later, pulp tissue and enamel was excised using dental bur and only the root dentin was utilized for the isolation of mtDNA by crushing it mechanically into powdered form. MtDNA was extracted using the two published methods, Pfeifer and Budowle and then subjected to spectrophotometry DNA quantification and purity, Polymerase chain reaction (PCR) amplification of hypervariable-two region of mtDNA, followed by DNA sequencing to analyze the reliability of the extraction techniques. In conclusion, both techniques proved to be efficient and capable for the extraction of mtDNA from tooth dentin.

Introduction

Mitochondria produce cellular energy, a byproduct of the oxidative phosphorylation processes. The proteins involved in the processes are encoded from both mitochondrial and nuclear DNA (Calvo *et al*., 2006). The human mitochondrial DNA (mtDNA) exists as a double stranded circle containing 16.6 kb DNA which encodes 13 protein subunits of oxidative phosphorylation complexes, 2 ribosomal RNAs and 22 transfer RNAs essential for mitochondrial protein synthesis (Galeano, 2008). It contains a hypervariable region of about 500 bp in its D-loop (Higuchi *et al*., 1988), and the polymorphism of this region has been extensively analyzed in the fields of human genetics (Aquadro and Greenberg, 1983; Di Rienzo and Wilson, 1991) and archaeology (Gill *et al*., 1994). This polymorphism also facilitates in forensic identification, particularly in maternally inherited cases (Smeitink *et al*., 2001; DiMauro, 2004). Mitochondrial-associated-diseases are usually associated with mutations of the mtDNA and it functions as energy producing organelles, where the

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defects will eventually affect the organ systems and tissues in need of high energy, such as muscle and nerve (Wallace *et al*., 1988; Weber *et al*., 1997) but other system is no exception (Wong, 2010). Unlike nuclear DNA, mtDNA is present at levels of more than 103 or 104 copies/cell and can be amplified from highly degraded samples (Ivanov *et al*., 1996) using the polymerase chain reaction (PCR) technique.

A tooth is made up of 3 major tissues; enamel, dentin and dental pulp. Dental pulp cavity contains blood capillaries whereas the dentin contains a network of tubules where the odontoblastic processes rich in mitochondria occur (Mörnstad *et al*., 1999). Since dentin and pulp is covered with enamel; the outer layer as well as the hardest part of tooth, both are resistant to incompatible conditions for DNA (García *et al*., 1996; Ginther *et al*., 1992). Thus, in extreme conditions such as exposure to the bacterial infection, dentin remains as a better source of DNA, particularly mtDNA.

Understanding the importance of mtDNA in studying the genetic-based-diseases, anthropology, forensic and population genetics provides the necessity to reveal a better extraction technique of the mtDNA from dentin of tooth. This is particularly true for Malaysian scientific environment which, to the best of our knowledge there is no research conducted using tooth dentin especially in those above mentioned areas. On the other hand, looking at the international scientific community, a large number of reports, especially on the protocols of preparing the mtDNA from tooth is basically from forensic studies and anthropology research areas (Trivedi *et al*., 2002; Pfeiffer *et al*., 1998; Rohland *et al*., 2004; Budowle *et al*., 2000; Reich *et al*., 2010) but not on mtDNA-related-diseases. As for the extraction technique of extracting mtDNA from tooth, there are always discrepancy on the quality of the extracted mtDNA and its authenticity since those two factors are significance to be dealt in molecular biology analysis and that was one of the reasons many articles on the mtDNA extraction from tooth dentin were published (Trivedi *et al*., 2002; Pfeiffer *et al*., 1998; Rohland *et al*., 2004; Budowle *et al*., 2000). Hence, in this study, two techniques of mtDNA extraction were used to determine its reliability and effectiveness in producing a good quality of mtDNA for the downstream molecular analysis with an intention to use the suitable technique for related genetic study of our group.

Materials and methods

Preparation of teeth samples

This study was approved by the Universiti Sains Malaysia Human Ethical Committee (USM/PPSP®/Ethics Com/2004/(129.3[2]) dated 1st August 2004. Teeth samples were collected from 4 human subjects having intact dentin root, who came to Dental Clinic, Hospital Universiti Sains Malaysia for tooth extraction of other reasons, after getting their consent. The teeth were kept at -20°C before use. The teeth were cleaned mechanically with brush and rinsed with sterile distilled water, and then 10% concentrated commercial bleaching solution and followed by rinsing with 95% ethanol. Then, a dental bur was used to separate the tooth crown from the root and the pulp tissue was cleaned from the pulp cavity by drilling inside the cavity. This was to ascertain that the mtDNA extracted was from the dentin. The root dentin was then cut into smaller pieces with the bur before being rinsed with distilled water and air dried. The dentin was finally crushed into powder using pestle and mortar and kept at -20°C until use for mtDNA extraction. Two powdered dentin samples were used for each mtDNA extraction technique.

MtDNA extraction techniques

Two techniques were employed to extract mtDNA in this study. The first one, based on Pfeiffer *et al*. (1998), 700 µL lysis buffer (10mM Tris, pH 8.0, 100mM NaCl, 50 mM EDTA, pH 8.0, 0.5% SDS pH 8.0 and 20 µL proteinase K (20 mg/ mL) were added to 0.2 g sample, vortexed and incubated at 56°C, overnight. Next, 720 µL of extraction buffer (phenol:chloroform:isoamyl alcohol of 25:24:1 ratio) was added to the sample, vortexed and then centrifuged for 2 min at 15000 rpm. The upper aqueous layer was transferred to a sterile microcentrifuge tube. This process of extraction buffer was repeated twice. Later, 720 µL of isobutanol was added to the aqueous layer, vortexed, and centrifuged (2 min) at 15000 rpm. At this stage, lower aqueous layer was used and transferred to the reservoir column of Centricon-100 concentrator (Millipore). Then, 1 mL of Tris-EDTA buffer for washing was added into the same reservoir column,

followed by centrifugation at 3000 rpm for 20 min or until the sample had spun through. This washing step was repeated twice. Finally, the sample in the collection column which contained the mtDNA was transferred to a sterile microcentrifuge tube and stored at -20°C.

The second method was based on Budowle *et al*. (2000) where lysis buffer, 300 µL (10mM Tris, 100mM NaCl, 39 mM Dithiothreinol (DTT), 10mM EDTA, 2% SDS) and 2 µL of 600 U/ mL proteinase K were added to the 0.2 g sample in a microcentrifuge tube. Briefly, the tube was vortexed to mix the sample and the buffer, followed with a short spin. The mixture was incubated overnight at 56°C. Afterwards, the extraction buffer consisting of phenol/ chloroform/ isoamyl alcohol (25:24:1) was added to the sample, vortexed, and centrifuged at 15000 rpm for 3 min. Prior to the usage of the Microcon 100 concentrator (Millipore) that was used to filter the mtDNA, 100 µL of distilled deionized water was added into the sample reservoir of the concentrator. This was followed by adding the upper aqueous layer of the sample mixture (approximately 300 µL) into the reservoir and centrifugation at 15000 rpm for 5 min. The retentate vial was then separated from the sample reservoir, and the eluted buffer in the vial was discarded before the reservoir was again returned to the vial. This washing step was repeated once. After that, 60 µL of hot (80-90°C) distilled deionized water was added to the sample reservoir and vortexed. The sample reservoir was then taken out and placed upside down into the new retentate vial before centrifugation at 15000 rpm for 3 min. The sample reservoir was discarded and the new vial containing the eluted mtDNA was kept at -20°C until further use for PCR amplification.

Determination of mtDNA concentration and purity using spectrophotometer

Optical density (OD) absorption of wavelength 260 nm and 280 nm is usually used for quantitation and purity evidence of nucleic acids (DNA and RNA). The blank sterile distilled water was placed in the cuvette and used as a blank for the spectrophotometer (Biophotometer, Eppendorf). Later, the original concentrated mtDNA was diluted 50X in the sterile distilled water and placed into the cuvette before being read at wavelength of 260 nm and 280 nm, respectively. The calculation of the mtDNA concentration was based on the fact that OD_{260} that is equal to 1.0 contains a 50 µg/ mL solution of double stranded DNA multiple with dilution factor, which in this case is 50 (Barbas *et al*., 2007). The same calculation was used because mtDNA is a double stranded circular DNA. The ratio of OD_{260}/ OD_{280} is an indicator of nucleic acid purity where the ratio of 1.8 to 2.0 indicates highly purified nucleic acid (Ausubel *et al*., 2002).

MtDNA HV2 region amplification by PCR

The mtDNA was then subjected to PCR analysis (Saiki *et al*., 1985). Amplification of hypervariable-two (HV2) segment of non-coding region of human mtDNA (Stoneking, 2000) was done to demonstrate the efficacy of mtDNA extracted by both methods. The HV2 gene was amplified using Qiagen Multiplex PCR kit (Qiagen, Germany) where the reaction mixtures contained a 25 µL of 2x QIAGEN Multiplex PCR Master Mix, 1 µg/ µL DNA template and 2 µM of each primers; forward primer, 5'-CACCCTATTAACCACTCACG-3' and reverse primer, 5'-ATGAGATTAGTATGGGAG-3' in a final volume of 50 µL. The amplified product size was 431 bp. The temperature for thermal cycling used in the study was 95°C (15 min) for hot-start PCR activation, followed by 30 cycles of 94°C (30 sec), then 59°C (1 min), finally 72°C (1 min) and for final extension, 72°C (10 min). The PCR was performed on the PCR machine PTC-200 (MJ research, USA). In order to analyze the efficiency of mtDNA extracted from each technique to be amplified by the PCR technique, the same amount of mtDNA concentration was used for each PCR reaction.

MtDNA sequencing

The purified PCR products amounting 10 ng of each samples were then sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (AppliedBiosystem, USA). The cycle sequencing process was carried out as suggested by the manufacturer with addition of 10% DMSO (v/v) of final volume and analyzed on an ABI Prism 3100. Data were analyzed by Sequencing Analysis 3.3 Software (AppliedBiosystem, USA) and aligned with the GenBank AY963582.2 mtDNA sequences based on suggestion of Human Mitochondrial Genome Database (http://www.mtdb.igp.uu.se/) using BioEdit (version 7.04) software.

Results

MtDNA concentration and purity using spectrophotometer

Based on the OD reading of the samples from the two groups, the concentration of the mtDNA extracted using Budowle technique was higher than the one using Pfeiffer (Table 1). Nevertheless, both the techniques yielded sufficient amount of mtDNA. However, the quality of mtDNA extracted using both techniques showed lower purity where the ratio OD_{260}:OD_{280} of all samples was less than 1.8, which was suggestive of contamination.

HV2 gene amplification and sequencing of mtDNA extracted from tooth

All the samples were successfully amplified and yielded the right product size (Fig. 1). The amplified mtDNA of HV2 was also successfully sequenced using the automated sequencer. The sequencing results were then aligned with the GenBank sequence using ClustalW application (Chenna *et al.*, 2003) of BioEdit software, which revealed few polymorphisms (Fig. 2). This however was anticipated, since HV2 region is one of highly mutable regions of mtDNA D-loop.

Table 1 Concentration and purity of mitochondrial DNA extracted using two different methods

	Mitochondrial DNA extraction methods			
	Pfeiffer		**Budowle**	
Sample	1	2	3	4
mtDNA concentration per powdered dentin weight (µg/ µl per g)	1.94	0.5	4.76	12.44
Purity (OD_{260}:OD_{280})	1.29	1.39	1.35	1.48

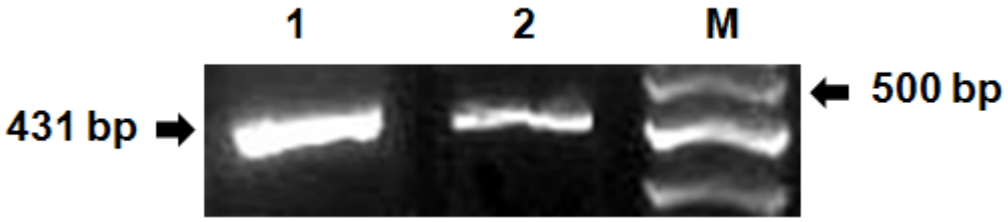

Figure 1 Amplification of HV2 region of mitochondrial DNA (mtDNA) by Polymerase Chain Reaction (PCR). (1) PCR product of mtDNA extracted using Budowle technique. (2) PCR product of mtDNA extracted using Pfeiffer technique. (M) 100 bp DNA ladder.

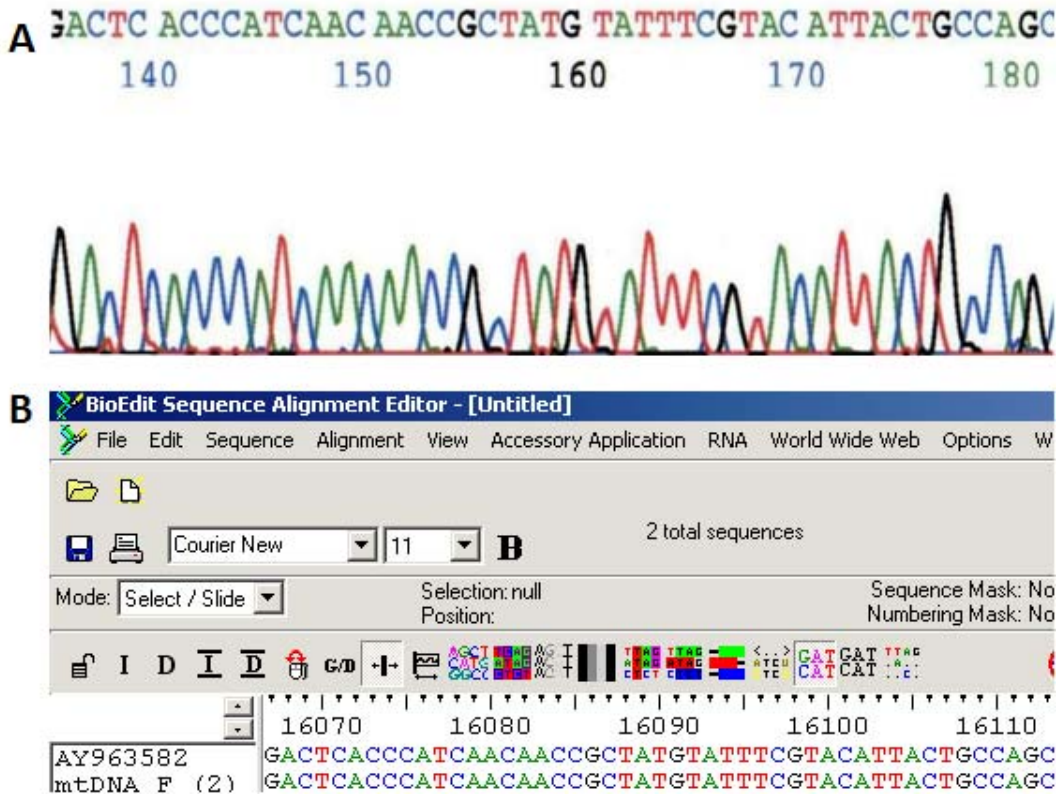

Figure 2 Sequencing and alignment analysis of amplified HV2 region of mtDNA. (A) Electropherogram of the mtDNA sequencing. (B) Alignment reading of mtDNA using ClustalW application of BioEdit software where the sample (mtDNA-F) was compared to the GenBank sequence (AY963582).

Discussion

Both methods are reliable to be used for mtDNA extraction from tooth dentin. Although this study was not able to statistically demonstrate that Budowle method produced good quantity of mtDNA due to inadequacy of sample size, yet, based on the mtDNA concentration per gram sample (Table 1), the Budowle technique seemed to produce more concentration of mtDNA per gram of dentin sample. Besides, the small amount of powdered dentin also made it difficult to use the same tooth dentin for both methods which could be the limitation in proving which of the two is better. It is because the tooth collected for the study had always had the dentin portion affected.

Basically, both methods of mtDNA extraction work on the same principle which starts with lysing the dentin of tooth, then mtDNA extraction using phenol buffer followed by filtration. Most chemicals used in both methods were similar besides addition of DTT in Budowle lysis buffer. Therefore, DTT could possibly play a strong role in the efficiency of mtDNA extraction. DTT is a strong reducing agent which is capable of breaking disulfide bonds while sustaining monothiols in the reduced state (Cleland, 1964). Thus, when used in lysis buffer it could protect the sulfhydryl group of DNA from forming the dimer and as a result increase the effectiveness of mtDNA extraction. On top of that, since dentinal tubules of tooth dentin contain disulfide groups (Steinman, 1960; Sasaki and Garant, 1996), addition of DTT could disrupt the sulfur bond of dentinal tubules and hence increase the efficacy of releasing the mtDNA into the lysis buffer for extraction. Regarding the low purity of all samples, this probably could be contributed by the calcification of the dentin, but, since it did not affect the HV2 amplification; the mtDNA extraction methods are dependable. One step of the DNA sequencing processes is cycle sequencing, where it was performed to bind the fluorescent dye with the single DNA template while at the same time amplifying the template. In sequencing the

difficult-to-sequence DNA template, addition of an additive such as DMSO in cycle sequencing preparation should be able to improve the cycle sequencing and thus increase the sequencing signal when analyzed by the machine (Choi *et al*., 1999).

Both the techniques (Budowle and Pfeiffer) employed in this study proved to be effective in extracting the mtDNA from dentin despite its low purity. However, the analysis of polymorphisms needs to be carried out in larger samples to come to a conclusion.

Acknowledgements

Authors would like to acknowledge the funding from USM short term grant 304/PPSG/6131356 and staff from Dental Clinic, HUSM and Human Genome Centre, USM for their support.

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