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Comparison across grinding and broaching methods of DNA extraction from dental pulp

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ABSTRACT

Introduction

Various methods are available for obtaining yield of High Molecular Weight DNA for subsequent Multiplex PCR from teeth sample. However, a proper validation and comparison of these methods for obtaining maximum output with quantifiable output not clearly established. The primary objective of this study was to measure the effectiveness across the grinding and broaching methods of DNA extraction in freshly extracted tooth pulp , itsquality check using Real Time-Polymerase Chain Reaction (RT-PCR) and subsequent multiplex STR typing.

Method

A total of 40 freshly extracted normal teeth were randomly collected. Isolation and extraction of DNA was done by organic extraction method. Precipitation of samples was done using 100% chilled ethyl alcohol followed by concentration and washing of DNA via column- based technique using DNA binding buffer and DNA wash buffer. Agarose gel electrophoresis was done to roughly estimate the DNA content while exact quantity of DNA was estimated by RT-PCR technique.

Result

Independent sample t test analysis revealed that the mean quantity of DNA (in µg/l) was significantly higher in broaching method (M=29.91, SD=0.65) than grinding (M=9.71, SD=0.45), t (38) =114.19, p<0.000. Similarly, quality of DNA was analyzed using smear quality and it was found that the quality of DNA for broaching (M=4.55, SD=0.51) was significantly higher that the grinding method (M=2.55, SD =0.6), t (38) =11.3, p<0.000. As far as multiplex STR typing was concerned, a more clear, sharp and balanced genotype plot was obtained from the DNA obtained via broaching method in comparison to grinding method.

Conclusion

The significant quantitative and qualitative loss of DNA was observed in tooth samples processed via grinding method compared to broaching method which was further supported by the fact that DNA profiles generated from the DNA extracted using broaching method provided adequate resolution of the autosomal markers and sex identification marker (amelogenin marker) in the present study which is valuable for human identification and the gender identification finally leading to individualization.

Keywords: Amelogenin, DNA, Hydroxyapetite, Gender Identification, Electrophoresis, Forensic Investigation

INTRODUCTION

The samples of DNA have been widely used for genotyping and subsequent identification of human bodies. It also proves to be a cornerstone in unveiling the mystery of migration of population and origin of modern man [4]. Tooth is consideredto be a very good source of DNA which is resistantto damage. The property of tooth which makes it resistant to unfavorable environmental conditions owes to highly mineralized structure. The DNA in tooth is principally present in pulp that can be collected by crushing, sectioning and trepanation. Anatomically tooth comprises of coronal and radicular sections in which crown or coronal section is covered by enamel and root or radicular portion is covered by cementum. The crown portion is emerging into oral cavity while radicular portion remains embedded into alveolar ridge [14].

Histologically the tooth is classified into four tissues like enamel, dentin, cementum and pulp. The odontoblasts are highly differentiated columnar cells which forms dentin throughout the life [5]. These columnar cells are peripherally arranged with an odontoblastic process extending through the thickness of the dentin. The enamel is composed of highly mineralized hydroxyapetite which is acellular in nature while the cementum of root is both cellular and a cellular in nature. The cementum is synthesized by cemetocytes [6]. The indestructible property even under extreme testing conditions is commonly used in forensic investigations for human identification [9]. Based on the morphological characteristics, the tooth can serve as an identification tool. Teeth can serve as source of human tissue in the form of pulp and human cell in form of embedded cells in the calciferous material of the teeth for DNA extraction [13]. The recent advancement in molecular biology based on DNA amplification and sequencing of nucleotide have revolutionized the Forensic Odontology by allowing identifications in missing persons, mass disasters, terroristic acts or crimes scenes [10]. Tooth pulp is assumed to be thepotential source for DNA extraction and validated for forensic identification purpose [8].

Several methods are used to extract DNA from tooth which facilitates forensic identification [6]. It includes aspartic acid racemization for age estimation, racial origin estimation by analysis of occlusal surface of tooth or by presence of accessory cusp or supernumerary cusp [9]. Cusp of carrebelli in posterior tooth or shovel shaped incisors is one of the examples [11]. The pulp containing central cavity of tooth constitutes the coronal portion called as pulp chamber and pulp canals is radicular portion of tooth [2]. The pulp is soft connective tissue & has nutritive, formative, sensory and defense functions. The coronal and radicular pulp comprises of odontoblasts, fibroblasts, endothelial cells, peripheral nerve cells, undifferentiated mesenchymal cells and nucleated blood cells. The highly developed vascular network enters through apical orifice present at the tip of root with ramifications into sub-odontoblastic region [12]. The objective of our study is to compare pulp retrieval techniques and its outcome on DNA quantitation by agarose gel electrophoresis and real time polymerase chain reaction. The qualitative and quantitative DNA outcome was compared across the broaching and grinding methods of pulp extraction.

MATERIALS AND METHODS

Participant/sample

A total of 40 teeth were randomly selected from various dental clinics of Delhi-NCR region as sample for this study. Consent from the person who was scheduled for dental extraction was obtained. The extracted teeth collected from the patients had history of some periodontal pathology/ wasting disease/ moderately carious tooth. The samples

were collected based on the criteria of mild to moderate carious tooth, tooth undergoing orthodontic extraction, extraction performed by the registered dental practitioner, participants of the age range of 20 to 60 years and permanent premolar and molar. Since premolar and molar teeth are voluminous thus they were assumed to be a rich source of DNA compared to the incisors and canines. However, grossly carious tooth, anterior tooth like central incisor, lateral incisor, canines & primary teeth as well as permanent tooth under root canal treatment was excluded from the study. Each tooth of the collected sample is considered asexhibit in this study. Sample preparation was done by washing extracted tooth with the solution of ethyl alcohol prior to grinding. Tissue lyzer machine was used for grinding the tooth. All the laboratory work was performed in the Central Forensic Science Laboratory, CBI, New Delhi, an NABL accredited laboratory strictly as per the DNA standards.

Materials

The chemicals used for Phenol-Chloroform-Isoamyl Alcohol (PCI) based organic extraction method were-

Proteinase K (20mg/ml), EDTA (0.5 M; pH 8.0),

- Sodium Dodecyl Sulfate (SDS),
- Sodium Chloride (NaCl),
- Tris Saturated Phenol: Chloroform: Isoamyl alcohol i.e. PCI (25:24:1 v/v),
- Absolute Ethyl alcohol,
- 70% Ethyl Alcohol,

DNA Clean & Concentrator Kit (M/S Zymo Research) was used to clean &concentrate the DNA for further downstream processing. The chemicals used for Quantitation of DNA done using both Agarose Gel Electrophoresis method as well as RT- PCR method were:

- Bromophenol blue loading dye,
- Interchelating Ethidium bromide solution
- Tris-Acetic acid-EDTA (TAE) running buffer.
- Quantifiler Human DNA quantification kit

PROCEDURE

The outcome of the study was achieved by using following seven step procedure-

Step 1

Each tooth sample was pulverized in Tissue Lyzer machine after washing it with ethyl alcoholas well as MilliQ water thoroughly and collected in 1.5 ml Eppendorf tube. After each step of crushing of tooth, jar was washed with warm water to avoid contamination.

Step 2

300 µl Stain Extraction Buffer + DTT (0.006gm/ml of SEB) was added to each Eppendorf tube containing the tooth sample. Each sample was vortexes for 15 seconds to facilitate homogenization. 20 µl Proteinase-K was added to each sample and they were incubated for 48hours at 56°C in Hot Water Bath Shaker. The samples were vortexes followed by centrifugation for 5min at 5000rpm. The supernatant was scooped out carefully using micropipette into fresh Eppendorf tubes.

Step 3

300µl PCI was added to each tube and centrifugation was done at 15000 rpm for 15 minutes. Top layer was scooped out into fresh Eppendorf tubes.

Step 4

Then precipitation was done with 100% chilled ethyl alcohol. The tubes were in Deep Freezer for 20 minutes at -20°C. It is followed by centrifugation at 10,000 rpm for 10 min. The supernatant was removed from each tube. Washingof the pellet was done with 70% Ethyl alcohol.

Step 5

DNA purification and concentration wasachieved by DNA binding buffer and DNA wash buffer (supplied with DNA Clean & Concentrator Kit, M/S Zymo Research).

Step 6

Basic Quality and quantity of DNA was obtained by Agarose gel electrophoresis technique and accurate Quantitation of DNA was evaluated by Real-time PCR technique. The gel electrophoresis technique is a semi-quantitative /qualitative assay that allows for the estimation ofthe concentration and quality of DNA present in a specimen. The method consists ofthe electrophoresis of DNA in an agarose gel matrix by incorporating a fluorescent intercalating

dye such as Ethidium bromide (EtBr). The agarose gel based quantitation has two step procedures. It comprises of preparation of gel followed by Electrophoresis.

The same samples were analyzed by Quantitative PCR (Real Time PCR). It is one of the most accurate, precise and efficient method currently available for human DNA quantitation. The rate of progression of amplification process is detected and measured by the accumulation of fluorescent dyes. The initial quantity of DNA in the sample was detected by monitoring the exponential growth phase of the reaction. It is also measured by the cycle number at which the fluorescent intensity of the sample overcomes the background noise orthreshold. This cycle number is directly proportional to the quantity of DNA in the reaction. Analysis of the quantity of DNA in the sample is performed using software that compares the unknowns with the best fit regression line constructed from the standards. The principle behind the method is that as the PCR amplification process progresses, there is an increase in fluorescence from SYBR Green dye. As the SYBR Green dye binds to double-stranded amplicon, it undergoes a conformational change and emits fluorescence at a greater intensity. The TaqMan probe principle relies on the 5´–3´ nuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophores-based detection. As in real-time PCR methods, the resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR.

Step 7

After quantitation of DNA, STR profiling was done for determining the sex of an individual. Commercially available kits, STR kits supply allelic ladders containing common STR alleles that have been previously characterized for the numberof repeat units via DNA sequencing.

RESULTS

The mean quantity of DNA (in µg/l) was compared across two retrieval methods used in this study which were broaching and grinding. It was assumed that qualitatively and quantitatively, both way, DNA retrieved using broaching method will yield better outcomes. Level of significance was tested using independent sample t test at $p<0.05$ using two-tailed assumptions. A significant difference was observed between broaching ($M=39.80\pm0.47$) and grinding ($M=29.91\pm0.65$), $t(38) = 54.93$, $p<0.000$. The outcome of the result suggested that mean quantity of DNA (in µg/l) obtained from broaching method was significantly more than the grinding method. Refer figure 1.

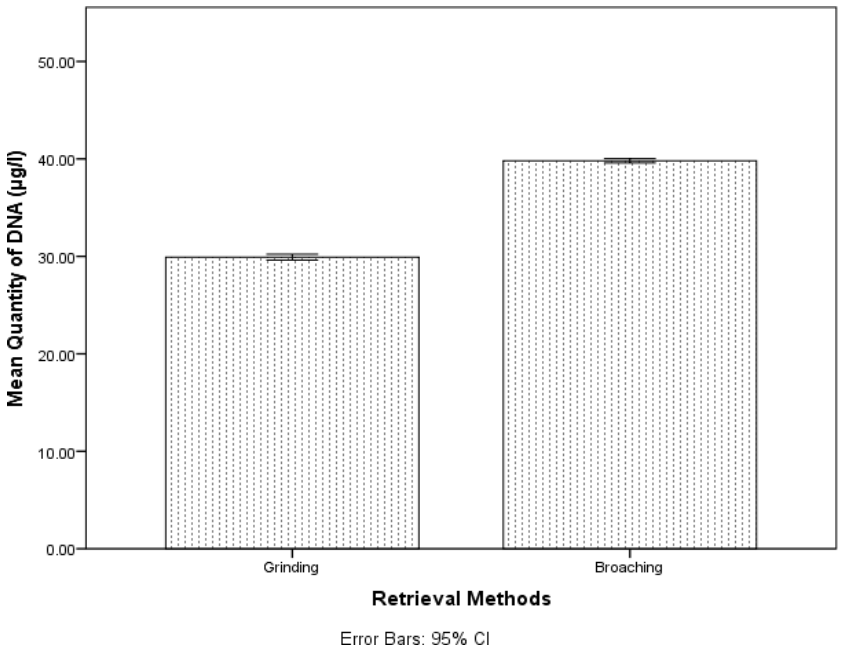

Figure 1: Mean quantity of DNA across two retrieval methods

Quality of DNA was also examined across two retrieval methods i.e. Grinding and Broaching by Agarose gel electrophoresis technique. The smear quality/bands obtained were compared when the DNA samples extracted using broaching techniques of pulp retrieval as well as grinding or crushing technique were run on Agarose gel. The quality of DNA based on the smear quality was categorized into five subcategories, extremely good, good, average, below average and poor qualities. Level of significance was tested using independent sample t test at $p<0.05$ using two-tailed assumptions. Asignificant difference was observed between Grinding (M=4.55, SD=0.51) and Broaching methods (M=5.29, SD=0.68), $t(38)=11.3$, $p<0.000$.

The outcome of the result suggested that median quality of DNA obtained from grinding method was significantly lesser than the Broaching method. Refer figure 2.

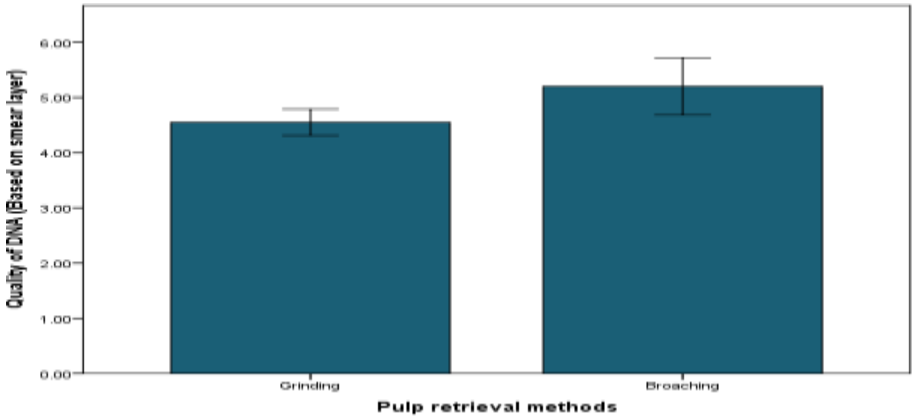

Figure 2: Quality of DNA obtained based on the smear quality by Agarose Gel electrophoresis across two retrieval methods.

DNA Profile generated using Promega powerplex fusion DNA amplification kit which includes 23 Autosomal STR markers and Amelogenin marker from grinding method of tooth.

The STR image for the Amelogenin marker obtained from grinding method is depicted below. Refer figure 3. Similarly figure 4 denotes the STR image obtained for broaching method.

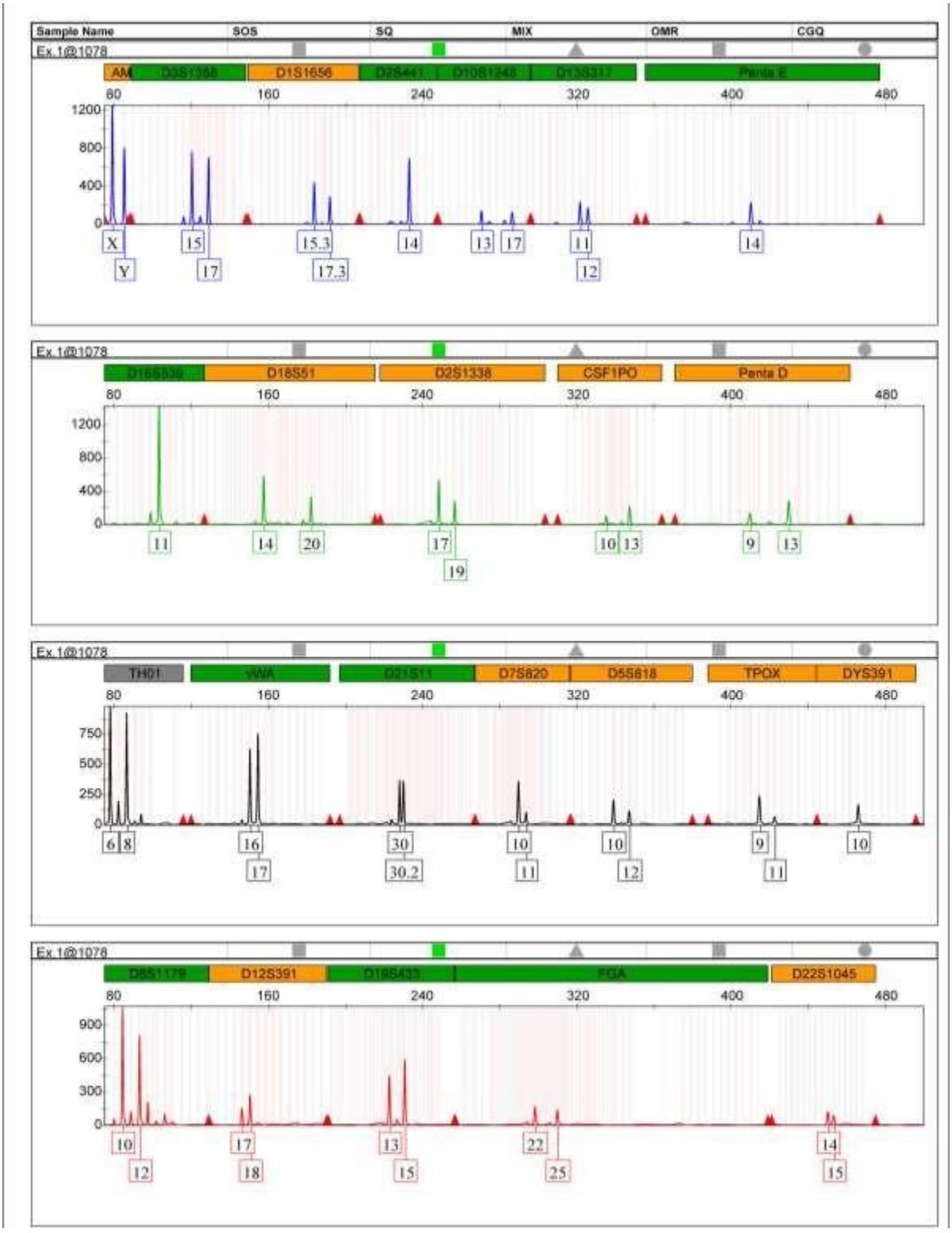

Figure 3: DNA Profile generated using Promega powerplex fusion DNA amplification kit which includes 23 Autosomal STR markers and Amelogenin marker from grinding method of tooth.

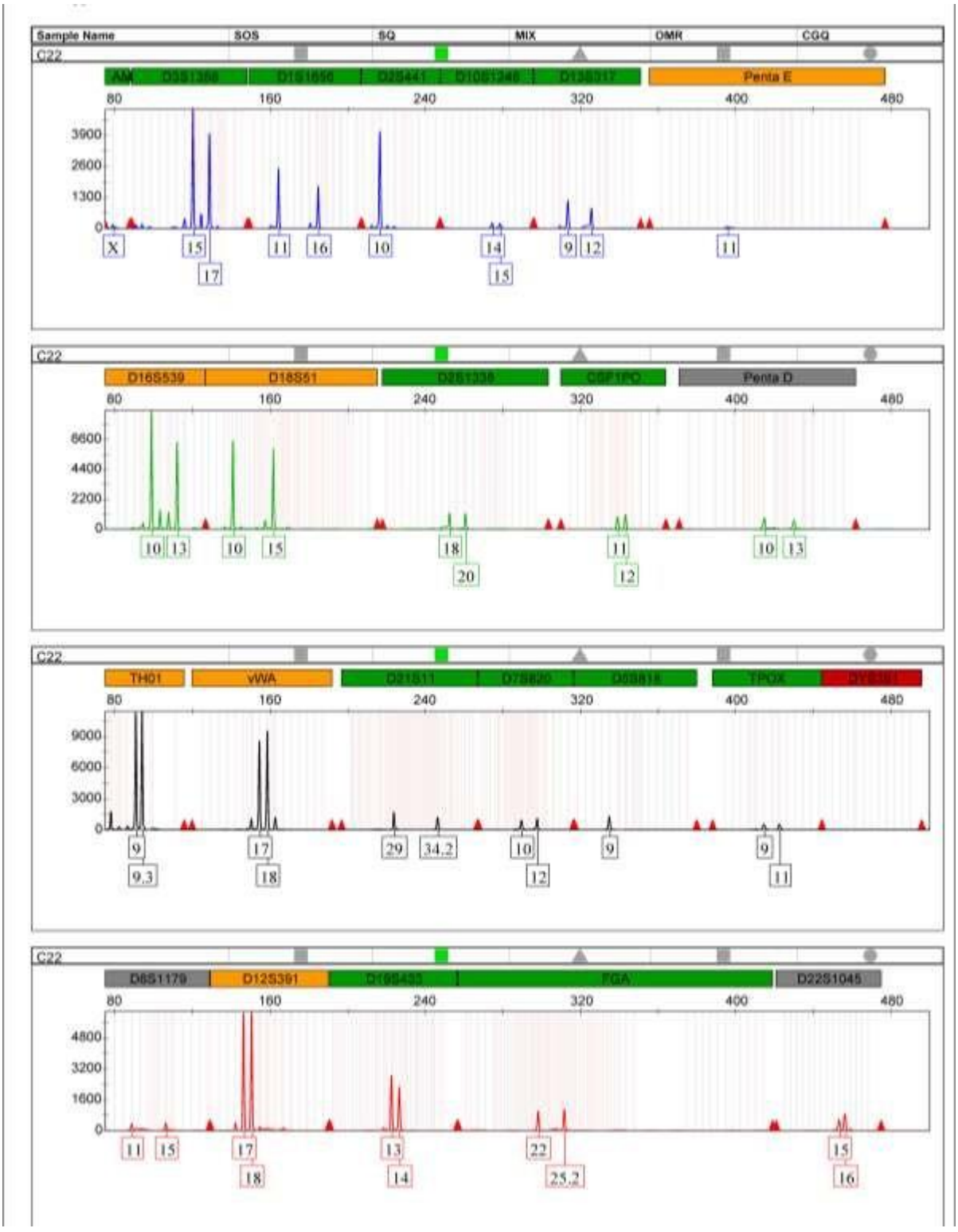

Figure 4: DNA Profile generated using Promega powerplex fusion DNA amplification kit which includes 23 Autosomal STR markers and Amelogenin marker from broaching technique of pulp retrieval

DISCUSSION

The preparation technique for pulp retrieval has its crucial role in access of dental DNA. With the grinding or crushing technique both mitochondrial DNA and nuclear DNA is obtained which is simple to perform and used in all types of tooth. The main

disadvantage associated with this technique is thatit compromises the evidentiary value of tooth thus preventing it from further radiographic, anatomic or biochemical analysis. Furtherance it limits the exact location of DNA thus obtaining poor DNA content by adding dilution factor to material.

Retrieval of DNA by radicular access opening is achieved by means of Hedstrom files or H-files and can be performed by dental practitioners. This technique can also be known as broaching technique as series of broaches or H-files are used from no 15 to 80 to gain access into radicular pulp through apical foramen. The series of files are usedin ascending order for opening the apices of root. The broaching technique is considered as more conservative as tooth is left in situ. The trepanation is performed by airotor under air and water spray to prevent the damage of genetic material by heat produced from airotor. This technique is applicable to all teeth and it also reduces the risk of contamination.

The other proposed techniques are vertical and horizontal sectioning of tooth performed by diamond bur however it is difficult to perform particularly in molars or posterior tooth. Several studies have been demonstrated that degradation of genetic material is time dependent also depends upon temperature, pH, moisture and presence of micro-organisms. Alvarez Gracia et al demonstrated that environmental conditions are the function of the loci however results were moderatefor the D1S80 locus but overall STR locus produce excellent results. It has also been demonstrated that hard tissues like dentine, enamel and cementum of tooth protects the genetic material as compared tobone. Hence it is possible to extract the DNA from pulp even after intense carbonization which is difficult from bone.

When we compared the DNA profile generated from a normal tooth to a profile generated from broaching method compared to grinding method the visible differences can be concluded in the following basic points. First, the peaks are sharp and uniformly distributed at all the loci throughoutthe plot. But the profile generated from a carious tooth shows the peak of less height on a graded, short towards the right side of the plot that is towards the loci of larger base pairs. This is mainly because of the less quantity of DNA extracted bythe grinding method. Secondly, the profile generated in the case of broaching was comparatively clear and clean, it was also devoid of any stutters and extra peaks. But in the profile generated from the grinding method, the profile shows certain small stutters and peak in the loci region of small base pairs. This is because of theextra amount of microbial DNA present in the extracted DNA which sometimes competes for binding of the primers in the reaction mix during amplification process. Besides above differences itcan be clearly opinioned that the both methods are capable of yielding sufficient amplifiable DNA that can generate a complete DNA profile for subsequent use in human identification and comparison. However, careful procedure of optimized DNA extraction and its purification are to be employed for its success in generation of readable genetic profile, thus, the broaching method had advantage over the grinding method. In current socioeconomic, cultural and diversified human society identification of an effective method of pulp extraction for human individualization is warranted in forensic investigation.

The present study shall be further exploredusing tooth sample under various demanding environmental condition, as in this study only freshly extracted normal tooth samples were considered. Considering all the factors together, the DNA integrity decreases considerably due to an effect of putrefaction. In this study the pulp material was taken into consideration for DNA extraction, although other parts of the tooth, mainly cementum may be accountable as the other source of DNA (Alia-García et al., 2015). Several methodologies have been validated for tooth sampling and DNA extraction because the method of processing a key determinant of the quality of the yield of DNA. As stated, all subjects included in this study belong to the same geographical area and differences between gender and age were matched with their dental pathology.

CONCLUSION

Based on the finding of present study, thebroaching technique can be considered as standard technique of pulp retrieval by gaining access through radicular pulp canal. The means of collection of pulp significantly reduces the risk of contamination of genetic material. Significantly complete genetic profiles are obtained with lesser loss of alleles hence gives comparatively better

qualitative and quantitative results. The advantage of broaching technique is that radicular morphology and coronal morphology is preserved which enables the sample for further analysis. Since the preparatory phase of pulp retrieval involves washing and asepsis during sampling hence good quantity of amplifiable DNA is obtained.

Conflict of Interest

There is no conflict of interest associated with this research.

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Ethical approval

Hence extracted tooth were the sample for this study, no institutional ethical approval was required.

Abbreviations

Phenol Chloroform Isoamyl alcohol (PCI),Short Tandem Repeat (STR), Real Time Polymerase chain reaction (RT-PCR)

Contributions

Conceptualization of research idea: LK, TD, RD; Hypothesis generation: LK, TD RD; Review of literature: LK, VK, KC, BM; Sample collection and analysis: LK, KC, BM, RD; Statisticalanalysis: LK, VK, KC, BM, Result writing: LK, VK, KC, BM, Discussion: LK, TD, RD, VK, KC, BM; Manuscript preparation: LK, TD, VK, KC, BM Final proofreading and editing: LK, TD,RD, VK, KC, BM; Citation and references: LK, VK, KC, BM; Journal selection and manuscript submission: LK, VK.

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