Comparative forensic analysis of reverse root canal filing and conventional method for DNA isolation from extracted teeth under different environmental conditions: A prospective study

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ABSTRACT

The grinding of a whole tooth specimen has been considered the conventional method to extract genomic deoxyribonucleic acid (DNA) in forensic science. However, we have tried the less destructive reverse root canal filing (RRCF) method without disturbing the morphology of the tooth to achieve competent amplifiable DNA. A total of 27 pairs of bilateral intact extracted teeth from the same subject were used in three different simulated environmental conditions for the respective RRCF and conventional methods: (a) soil burial for six months, (b) incineration at 200° C for four minutes, and (c) immersion in water for two months. Qualitative agarose gel electrophoresis assessment and downstream amplification were performed. The results showed significantly higher mean DNA concentration for the RRCF method in all three environmental conditions (p value = 0.008) in comparison to the conventional method. However, comparable qualitative results were found in both methods for the mean DNA concentration for incinerated (159.49 ng/ml), soil (119.52 ng/ml), and water (108.60 ng/ml) samples. It was concluded that the RRCF method is better quantitively (ng/ml) and comparable in terms of quality with respect to the conventional method, with the added advantage of preservation of the tooth morphology.

INTRODUCTION

Calcified tissues like teeth and bone are a major source for forensic research, which are accessible and useful even after exposure to extreme conditions (e.g., mass disaster, fire, blast, decomposed body). Teeth are most likely to be contaminated when exposed to the environment.[1] Gaytmenn and Sweet showed that DNA quantity extracted from the root is 10 times greater than that retrieved from the crown.[2] The dental tissues comprising the dentine and pulp contain the majority of the cells, as the enamel and cementum are mostly acellular and contain relatively small amounts of DNA. The complete powdering of the tooth specimen has remained the gold standard for DNA extraction from samples of teeth.[3,4]

A tooth, when chosen as forensic evidence, needs to be preserved for reference as well as for further analysis. But the standard method of complete pulverisation of the tooth results in loss of the specimen. With the evolution of newer techniques,[5] alternative methods of DNA extraction have been tried and tested in order to prevent the loss of the

specimen.[6] Numerous techniques for sampling teeth are described in the literature, including pulverisation of the tooth[7] and root,[8] vertical sectioning of the tooth, [9, 10]horizontal sectioning at the enamel–cementum (crown–root) junction,[11] endodontic filling method,[12]and non-powdering digestion buffers method.[13] Harvella et al. proposed that occlusal perforation, cervical perforation, and the cervical cut method allowed preservation of the tooth sample. They found that a satisfactory amount of DNA was obtained from 30 different ancient samples.[14]

The amount of DNA in the tooth depends upon various factors like tooth type, age, and individual variations. Thus, bilateral teeth of the same individual were chosen to eliminate variation in the amount of DNA within the tooth.[15]Premolars were chosen among other teeth due to availability. Garriga et al. ascribed the use of premolars to the fact that bilateral tooth extraction was primarily for orthodontic purposes (23 pairs). The most common extracted teeth for orthodontic reasons are maxillary and mandibular first premolars.[16, 17] A female predilection was also noticed with a male/female ratio of 3:4.[16]

A method using a reverse root canal filing (RRCF) technique by endodontic files for retrieving dentine and pulp for DNA analysis was first demonstrated by J.C. Cobb.[18] This method used H files to retrieve the dentine and pulp tissue in powdered form and therefore allowed a substantial amount of preservation of the tooth structure. Cobb demonstrated the superior results of the RRCF method over the conventional method.

The current study aimed to compare the efficacy of DNA extraction from teeth sampled by two different methods. Teeth were subjected to different simulated environmental conditions to evaluate the effect on the sampling process. Comparison of DNA yield between the two sampling methods would allow for the evaluation of the RRCF method.

MATERIALS AND METHODS

A two year prospective study was conducted using 27 pairs of teeth from patients undergoing bilateral extraction of permanent teeth. Bilateral healthy intact teeth from the same individual of the same type, devoid of caries, restorations, and root canal treatment, were extracted. The samples were then categorised and subjected to three simulated environmental conditions: (a) soil burial for a duration of six months,[15] (b) incineration at 200 °C for four minutes,[16] and (c) immersion in fresh water for two months. Two teeth from the same arch of the same subject were divided to be assessed by the RRCF and conventional grinding methods, respectively. We obtained approval from the institute's ethics committee, along with informed consent from the volunteers.

Extracted teeth were physically cleaned from attached soft tissue using a BP blade with handle (Amkay, India). They were soaked in 2% sodium hypochlorite (Chemdent, India) for 5 minutes and then 100% ethanol for 5 minutes, followed by drying at 30 °C overnight. For samples buried in soil, plastic containers containing soil with teeth placed at a depth of 20 cm were used and labelled for each sample. These containers were then exposed to normal weather conditions for six months (soil and water). For incinerated samples, all the samples were heated to a standing temperature of 200° C in an electric furnace for four minutes. For samples exposed to water, plastic containers containing fresh water with teeth placed at a depth of 30 cm were used and labelled for each sample. Fresh water was changed weekly for a total duration of two months.

After completion of the different environmental exposures, the teeth went through physical cleaning, followed by chemical cleaning with 1% hypochlorite. Finally, UV irradiation was applied for 30 minutes to achieve maximum neutralisation of exogenous contamination.

Grinding of tooth samples:

A: Conventional method: The tooth was ground to a powder mechanically using a sterile mortar and pestle. This powder was then collected over a sterile sheet of foil and transferred to a microcentrifuge tube (Eppendorf, Germany) of 2 ml capacity.

B: RRCF method: Pulp cavity was accessed from the apical portion of the tooth after removing the apical root portion of 2 to 3 mm. Hedstrom endodontic files (Mani, Japan) (sizes 08–80) were used to powder the dentine and collected on sterile sheets of foil. Radiovisiography (RVG) of each tooth was performed before and after filing in order to assess the condition of the root and pulp chamber. (Figures 1 and 2).

Figure 1.

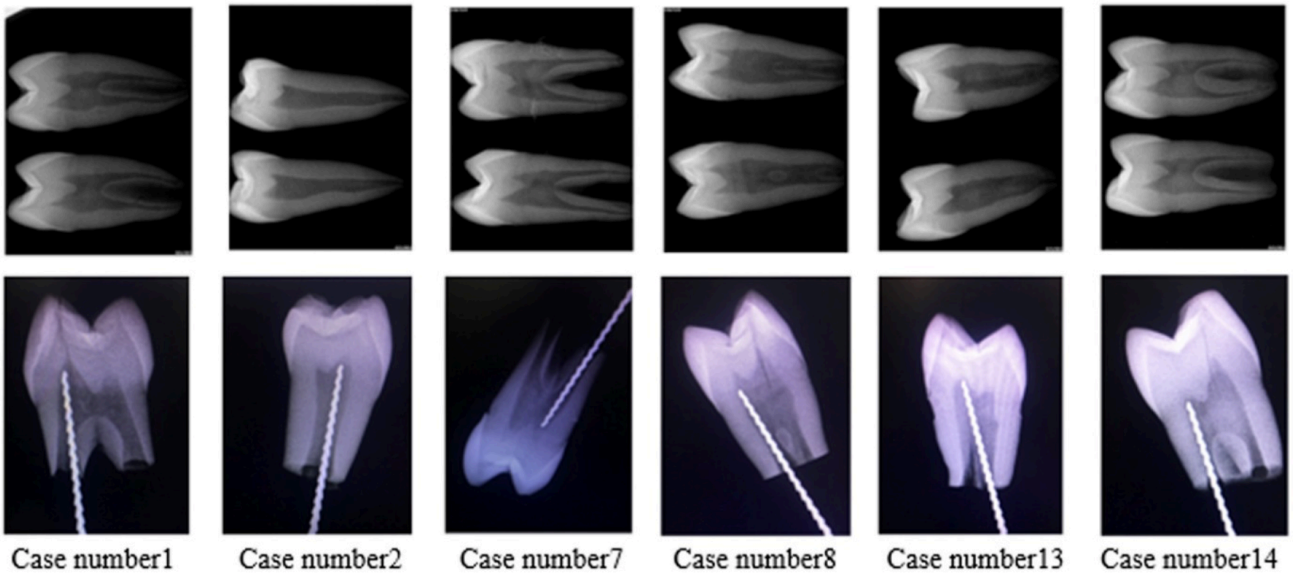

Figure 2.

Organic extraction by standard phenol-chloroform method (Fisher, BP17521-400) was used uniformly in both the groups and the three environmental conditions. The quantity of extracted DNA was evaluated by Nanodrop, BioPhotometer (Eppendorf, Germany). This involved the standardising of the cuvette with 2 µl of buffer (molecular grade water). After standardisation, 2 µl of extracted DNA was used to determine the concentration and optical density (OD) value of each sample. Quality assessment was performed by 1% agarose gel electrophoresis (Gel Electrophoresis unit, Vari-Gel Maxi system - Scie-Plas, England). One percent agarose was used in TAE buffer to cast the gel along with 5 µl of ethidium bromide. Then, 7 µl of DNA sample with 3 µl of loading dye was placed in the well. The gel was run at 200 kvh for thirty minutes. The image was captured using a gel documentation system (Gel-Doc™ EZ Imager - Bio Rad, California, USA). The same purified extracted product was amplified using a known primer to ascertain its reliability.

The primers used are as mentioned below:

ALU-STYa

Forward Primer 5'- CAT GTA TTT GAT GGG GATAGAGG-3'

Reverse primer 5'- CCT TTT CAT CCA ACT ACC ACTGA-3'

ALU-STXa

Forward primer 5'- TGA AGA AAT TCA GTT CAT AGCTTG T-3'

Reverse primer 5'- CAG GAG ATC CTG AGA TTA TGT GG-3'

STATISTICAL ANALYSIS

The mean values, standard deviation, and ranges (maximum and minimum) were calculated for each variable. The resulting data were analysed using SPSS software version 21. Data were expressed as mean ± standard deviation. Differences between different variables were analysed using Mann–Whitney test. Pearson's correlation coefficient was carried out to determine the level of correlation between the findings of the two methods. A p value ≤ 0.05 was considered statistically significant.

RESULTS

The mean weight of tooth powder used in our study was 73.56 ± 2.28 mg in the RRCF method

and 170.40 ± 7.04 mg in the conventional method (Table 1). For all the samples, the mean DNA concentration was 155.64 ± 89.82 ng/ml in the RRCF method and 102.77 ± 55.0 ng/ml in the conventional method (Table 2).
The mean DNA concentration per mg of tooth powder was 2.21 ng/m/ in the RRCF method and 0.60 ng/ml in the conventional method. The mean DNA concentration was 1.68 ng/ml in the group of incinerated samples, 1.24 ng/mlin samples buried in soil, and 1.14 ng/ml in samples submerged in water. Twenty one samples were amplified in both the RRCF method and the conventional method.

Table 1. Comparison of powder weights by Mann–Whitney U test

METHOD	MEAN WEIGHT	MEAN RANK
RRCF (N=27)	73.56 ± 2.28 mg	14
CONVENTIONAL (N=27)	170.40 ± 7.04 mg	41
p value	0.0001 (significant)	

Table 2. Groupwise comparison of mean DNA concentrations (A)

METHOD	MEAN CONC. SOIL ng/ml	MEAN CONC. INCINERATION ng/ml	MEAN CONC. WATER ng/ml	MEAN CONCENTRATION OVERALL ng/ml
RRCF METHOD	145.57	191.83	129.52	155.64
CONVENTIONAL METHOD	93.47	127.15	87.69	102.77
DIFFERENCE	52.1	64.68	41.83	52.87

METHOD	MEAN RANK SOIL (N=9)	MEAN RANK INCINERATION (N=9)	MEAN RANK WATER (N=9)	MEAN RANK (OVERALL) (N=27)
RRCF	11.67	11.33	11.89	33.20
CONVENTIONAL	7.33	7.67	7.11	21.80
p value	0.085	0.145	0.058	0.008

(B) Mann-Whitney U test

Table 3. Comparison of mean DNA concentrations by weight in Mann–Whitney U test

METHOD	MEAN RANK SOIL (N=9)	MEAN RANK INCINERATION (N=9)	MEAN RANK WATER (N=9)	MEAN RANK OVERALL N=27
RRCF	13.56	13.78	13.67	40.07
CONVENTIONAL	5.44	5.22	5.33	14.93
p value	0.001	0.001	0.001	0.001

DISCUSSION

The RRCF method is a relatively new method of tooth sampling, with published data limited to only two studies, one by Cobb in 2002 and the other by Hughes-Stamm et al. in 2016. Cobb suggested the RRCF method for the first time with the view that it concentrates on the dentine-pulp rich area for obtaining powder of the tooth. This method approaches the tooth from the apical portion of the root, thereby preserving the tooth architecture and morphology with good amount of concentration of DNA per mg of tooth powder.[18]In the present study, we included three environmental parameters (soil, incineration, water) with the RRCF method to evaluate the efficacy and advantages of this method over the conventional method in terms of DNA yield.

The RRCF method used a lesser amount of dentine powder compared to the conventional method. The results were comparable to the study conducted by Hughes-Stamm et al., where the amount of tooth powder in the conventional method was significantly higher than in the RRCF method (Table 1).[19] This can be related to the powdering of only dentine near the pulpal region in the RRCF method in comparison to the complete grinding of the tooth in the conventional method. This implies that the RRCF method preserved tooth samples by utilizing only a fraction of the tooth specimen as compared to the conventional method, in which all the powder is used at once.

Our results indicated that the RRCF method yielded more DNA as compared to the conventional method (Table 2 & 3), and the difference was statistically significant. This was in accordance with the study by Cobb.[18] Hughes-Stamm et al. also observed that the DNA concentration was greater in the RRCF method as compared to the conventional method in most samples.[19] The first possible explanation of these results could be the selective processing of a DNA-rich zone of the dentine–pulp complex of the tooth in the RRCF method as compared to the conventional method processing the whole tooth, including the acellular cementum and part of the enamel. Secondly, the non-cellular part of the tooth could result in decreased yield by reducing the action of the reagents during the DNA extraction procedure. These results suggest the possible advantage of the RRCF method over the conventional method in terms of DNA quantification. The RRCF method reduces the chance of exogenous contamination by targeting a specific portion of the tooth. Moreover, it excludes the non-cellular component of the tooth, thereby decreasing the quantity of polymerase chain reaction (PCR) inhibitors and contamination.

The environmental conditions were defined in a way that they included the maximum conditions for the forensic testing like accidental fire, drowning, or soil burial. The negative effect on DNA yield from teeth buried in soil for six months was shown by Schwartz et al.[15] Samsuwan et al. also showed that one year of tooth burial in soil is better in comparison to six months of burial. They hypothesised that in one year the specimen becomes dry and it is easy to extract the DNA.[20]The results of our study were partly not in accordance with the previous literature, wherein the soil-buried samples showed better results as compared to incinerated samples.[21] Garcia et al. found that samples exposed to soil yielded the maximum quantity, followed by incinerated samples, while samples submerged in water gave the minimum yield.[21] Chowdhury et al. and Kumar et al. also found similar results to those of Garcia et al. [22, 23]

Teeth immersed in water for more than one month have shown detrimental effects for DNA extraction.[19]The low value of mean DNA concentration in samples kept in water and soil might be related to the DNA degradation due to exposure to microorganisms. The results could not reach significant values, probably due to the small number of samples in each group.[24]

In samples buried in soil, the degradation of DNA depends upon the accessibility of the apical foramen. The apical foramen is not protected by alveolar bone and is a gateway for bacterial entry and exit to the pulpal region. Secondly, microorganisms from soil can act as a source of exogenous contamination. The exogenous contamination reduces the DNA yield, thereby posing difficulty in isolation of endogenous DNA. Thirdly, chemicals like fulvic acid and humic acid that are indigenous to the soil increase the DNA degradation by facilitating the growth of microorganisms and may act as a PCR inhibitory factor. [7, 22, 24]

Samples submerged in water showed the poorest result due to the dilution effect. Because of the presence of water, the rate of DNA hydrolysis

would increase, and the degradation in water would be more than in any other condition like soil burial or incineration. [22]

Incineration at a temperature of 200° C was performed to simulate fire incidents.[10] Total genomic DNA is comprising of nuclear (nDNA) and mitochondrial DNA (mt DNA).

It was interesting to note that the group of incinerated samples showed the best results, followed by those buried in soil and the samples submerged in water, showing the lowest concentration by both methods (Table 2). The possible reason for the better yield of incinerated samples could be the ability of enamel and cementum to withstand a higher temperature (>200° C for four minutes) for degradation and protecting the dentinal and pulpal portion of the tooth.[25]

The best possible explanation for the difference in results was the low incineration temperature. Garriga et al. showed that physical degradation of enamel and dentine was seen at a temperature of 200° C for ten minutes, along with a decrease in the quantity of DNA yielded. The fragmentation of the crown and fracture of the root occurred at a temperature greater than 300 °C for four minutes or more. Once the encapsulation of enamel and dentine was lost, there was tremendous decrease in the DNA yield.[16] In our study, the incineration temperature was 200° C for four minutes, and in all samples the macroscopic examination of the specimen showed no evidence of fracture or fragmentation. Thus, at this temperature the encapsulation of dental hard tissue was intact, and therefore the DNA-rich zone was preserved.

This inter-group comparison implies that for a lower temperature of incineration (<200°C), the degradation effects were lower as compared to the degradation in soil and water. Moreover, the samples immersed in water have the lowest DNA quantity compared to any other environmental exposures.

DNA quantification was also analysed in terms of DNA yield per mg of tooth powder of dentine. In all samples, the mean DNA yield per mg of dentine powder was significantly higher in the RRCF method as compared to the conventional method. It has been hypothesised that dentine and pulp have more cells with mtDNA and nDNA, whereas enamel is devoid of nuclear content.

Hughes-Stamm et al. found that the DNA yield per mg of tooth powder in the RRCF method was high in all samples.[19] It was hypothesised that the efficiency of DNA yield was due to the lower amount of powder utilised for DNA extraction in the RRCF method. Also, the powder generated in the conventional method was considered suboptimal due to the possibility of contamination from pulverisation of the complete sample. The utilisation of a relatively lower amount of powder for DNA isolation highlights the efficiency of the RRCF method.

The mean concentration per mg of DNA yield was analysed in three environmental conditions (Table 3). The incineration group showed the best yield per mg of dentine powder, followed by samples buried in soil and samples immersed in water. The efficiency in terms of concentration per mg of dentine powder was significantly higher in the RRCF method as compared to the conventional method in all three groups. The mean DNA concentration obtained in the incineration group was highest, followed by the soil group and the samples immersed in water. The reason for this high efficiency can be attributed to the utilisation of a low amount of powder in the RRCF method.

Hughes-Stamm et al. compared DNA quality by measuring the average number of alleles recovered. Overall, they recorded a higher amplification in the RRCF method.[18] It was postulated that the contamination due to suboptimal powder and delay in PCR cycles in the conventional method accounted for the difference in amplification.

The DNA amplification of the water-immersed group showed a positive result in comparison to the conventional group. DNA amplification depends upon many factors, such as purity, concentration of DNA, and presence of inhibitors. The grinding of the whole tooth has many inhibiting factors, which do not allow downstream amplification. [19]

Apart from better efficiency, the RRCF method has an added advantage of preservation of tooth samples by utilizing only an internal part of the tooth. Thus, if needed, samples could be further analysed, as required many times by forensic experts. In addition, due to the approach of tooth samples from the apical portion of the root, the crown portion remains intact. Thus, the morphology and anatomy of the tooth are also preserved. The major disadvantage of the RRCF

method is the laborious and time-consuming filing of tooth samples with H files.

CONCLUSIONS

A higher mean DNA concentration by the RRCF method in all three environmental conditions was indicative of better DNA yield in comparison to the conventional method. The RRCF method yielded better results in terms of DNA concentration per mg of tooth powder. Amongst different environmental conditions, the incinerated samples yielded the best results. However, the water-immersed samples showed comparable results in both methods. Further research with broader criteria of environmental exposures coupled with a larger sample size is required.

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