Original Article

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Dentistry Section

Amelogenin Gene - The Pioneer in Gender Determination from Forensic Dental Samples

PRIYANJALI DUTTA[1], SATISH BHOSALE[2], RAJESHWAR SINGH[3], PRIYANKA GUBRELLAY[4], JITENDRA PATIL[5], BHUMIKA SEHDEV[6], SACHIN BHAGAT[7], TAJINDER BANSAL[8]

ABSTRACT

Introduction: In the event of any mass fatality incident, DNA analysis plays a vital role in disaster victim identification. Teeth are one of the most resistant structures in the human body that resist decomposition hence making them prime choice for extracting DNA for identification of individuals. Polymerase Chain Reaction (PCR) analysis that target regions of Amelogenin gene have become the method of choice for sex determination of biological samples.

Aim: Determining the sex of a given DNA sample from either dental pulp or dentin of tooth and help in identification of missing persons and disaster victims.

Materials and Methods: In our study 50 teeth samples were studied and they were subjected to various environmental conditions along with freshly extracted teeth taken as control for duration of one month and three months. Pulpal tissue was retrieved from the teeth specimens by access opening of root canals and for incinerated samples, the specimens were crushed. From the DNA that was extracted from the dental pulp sample Amelogenin gene locus was used for sex determination by amplifying a segment of X-Y homologous gene locus through PCR analysis.

Results: ANOVA test and t-test proved to be statistically significant and 100% retrieval rate was observed in samples.

Conclusion: Pulpal tissue along with degenerating odontoblastic processes yield sufficient amount of DNA for gender determination when subjected to various forensic conditions with maximum accuracy.

Keywords: Incineration, Forensic odortology, Mass fatality, Polymerase chain reaction

INTRODUCTION

Forensic odontology is the branch of dentistry dealing with the correct handling followed by examination, evaluation and presentation of dental evidence in criminal or civil legal proceedings in the interest of justice [1]. Human teeth have been considered as a prime choice in determining the identity of an individual [2].

Jeffreys AJ et al., created radioactive molecular probes to identify highly variable regions of DNA, and established that there are specific patterns of each individual and these were named as DNA fingerprints [3]. In cases of paternity and human identification, DNA profile tests are totally reliable, and are accepted as legal proofs in courts. DNA can be obtained virtually from all human body tissues. Blood (more specifically WBC's) are considered to be the best source of DNA extraction from living tissues. Bone and teeth qualify as better sources of DNA from decomposed human remains [4].

In the event of any mass fatality incident, DNA plays a vital role in victim identification [5]. As teeth have rich supply of genetic information [6], they are the prime choice for extracting DNA for identification purposes [7]. Enamel and dentin of teeth are highly calcified structures in the body that resist decomposition thus, making teeth, a better source of DNA [8].

Determining the sex of a given DNA sample from either dental pulp or the dentin of a tooth can also provide criminal investigators with useful intelligence and can aid the identification of missing person and even of disaster victims whose physical identification may have been destroyed. PCR analysis that target regions of Amelogenin gene has become the method of choice for sex determination of biological samples [9].

In the present study gender determination from the DNA samples was done from either dental pulp or dentin of tooth using PCR technique.

MATERIALS AND METHODS

The present study was an in vitro study conducted in the Department of Oral Pathology and Microbiology in Krishnadevaraya College of Dental Sciences, Bengaluru, Karnataka, India. Dental pulp/dentine pulp complex were obtained from 50 teeth samples collected from Department of Oral and Maxillofacial Surgery. Mostly premolars and molars were used in the study as these teeth are seen to retain more quantity of pulpal tissue if subjected to adverse conditions also because of their sturdy teeth structure which provides a much more protective encasing for the pulp.

These 50 teeth specimens were then grouped into five different categories of forensic insults [Table/Fig-1].

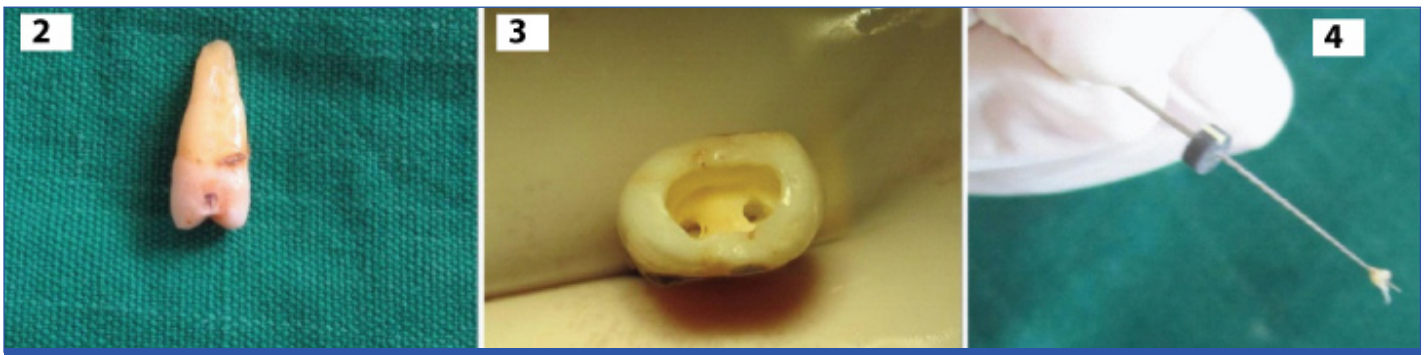
These samples were compared with the normal and freshly extracted carious teeth. Later pulpal tissue was excavated by routine endodontic procedure and put in a DNA extraction buffer followed by genomic DNA extraction as well as PCR amplification targeted for Amelogenin gene locus.

During incineration, a constant increase of temperature (i.e., a 100^0 variation between each sample) was considered for firing the teeth samples. Since with increasing temperatures above 1100°C, DNA extraction for gender analysis is not authenticated in literature because of plausible amount of genomic DNA remaining for retrieval [10], so a marginal raise of 50°C was considered above 1000°C. This was done to find out the finer nuances in DNA retrieval from degenerating odontoblastic processes at higher temperature.

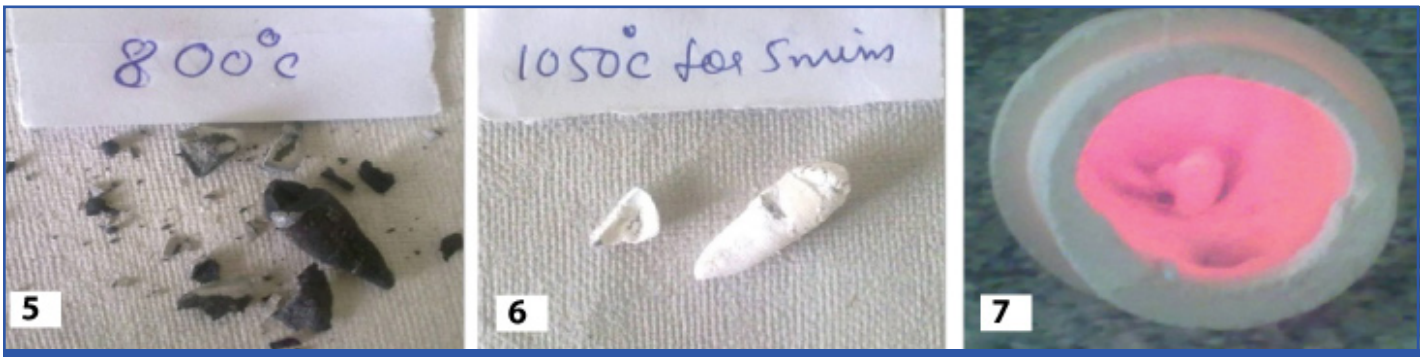
The teeth samples after being subjected to various environmental insults were decontaminated with 5.2% sodium hypochlorite solution. The teeth were then cleaned and washed again with sterile distilled water [Table/Fig-2]. Access opening was done with the help of an air rotor handpiece and pulp tissue was retrieved [Table/Fig-3,4]. For incinerated samples, after being subjected to the incineration

Category I	Category II	Category III	Category IV	Category V
Normal/ Control samples	Samples subjected to sea water condition (no agitation used)	Samples kept at normal room temperature for desiccation (air-dry, no medium was used)	Buried in garden soil about 10cm	Incinerated samples
I A- 5 normally extracted teeth without caries	II A- 5 teeth kept for 1 month	III A- 5 teeth kept for 1 month	IV A- 5 teeth kept for 1 month	V A- 8 samples heated to increasing temperatures- from 500-1050^{0}C for a constant 5 minutes
I B-5 Teeth with carious lesion	II B- 5 teeth kept for 3 months	III B- 5 teeth kept for 3 months	IV B- 5 teeth kept for 3 months	V B 2 samples heated at 500^{0}C and 600^{0}C for a constant 10 minutes

[Table/Fig-1]: Fifty teeth samples were subjected to various simulated forensic conditions based on above mentioned categories.



[Table/Fig-2-4]: Excavating dental pulpal tissue from freshly extracted non-carious teeth by access opening.



[Table/Fig-5,6]: Teeth samples subjected to incineration at 800^{0}C, 1050^{0}C. **[Table/Fig-7]:** Incinerated sample freshly taken out of the incinerator at 950^{0}C in a crucible.

the samples were crushed manually with the help of mortar and pestle to collect the degenerated odontoblastic processes [Table/Fig-5-7].

The pulp tissue/degenerated odontoblastic processes from the incinerated samples obtained were then put into a DNA extraction buffer that is Sodium Dodecyl Sulphate (SDS) for example. The genomic DNA from the above solution was extracted using phenol-chloroform method [2]. The acquired extract was concentrated by centrifugation and then incubated at 62^{0}C and the supernatant was taken in a 2 microlitre pipette. Genomic DNA was later visualized on 1% agarose gel. A set of specially designed primers prepared on a database from gene bank of Chromous Biotech Pvt. Ltd., Bengaluru was used to detect X and Y alleles of Amelogenin locus [11]. X specific and Y specific primers were stored in freezer temperature (-20^{0}C) separately to conduct the reaction [Table/Fig-8]. Conventional PCR reaction was carried out for amplification of DNA in the samples by using Taq DNA polymerase enzyme. PCR product was then finally visualized on a 0.8% agarose gel.

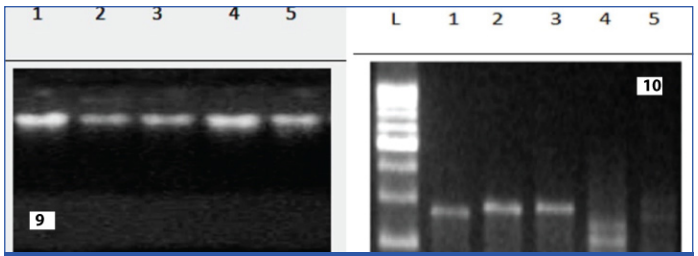
Later to compile the study inferences, statistical analysis like ANOVA and t-test as well as percentage analysis were carried out. Descriptive and inferential statistics were used to analyze the data. The SPSS software, version 20, was used for statistical analysis and the p-value <0.05 was considered significant in the study.

RESULTS

In our study, we were able to extract genomic DNA from all the teeth samples (100% retrieval of genomic DNA) [Table/Fig-9]. Expected size: ~ 1.3 kilobases (kb) from Y-form and ~ 1.5 kb from X-form.

Primers	Sequence (5'->3')
Forward primer	CTACCACCTCATCCTGGGCA
Reverse primer	GCTCTGGTACCACTTCA

[Table/Fig-8]: Primers designed for the study to help target Amelogenin gene locus.



[Table/Fig-9]: Genomic DNA isolated from dental pulp samples were loaded on 1% agarose gel. **[Table/Fig-10]:** Amelogenin PCR products were loaded on 0.8 % agarose gel. Male: two band at ~ 1.3 kilobase (kb) {Lane 4,5} and 1.5 kb. Female: Single band at 1.5 kb {Lane 1,2,3}.

Male: two band at ~ 1.3 kb and 1.5 kb. Female: Single band at 1.5 kb. Samples 1,2,3- showing amplified products from X-specific Amelogenin gene indicating that the samples were of a female origin. Samples 4 and 5 were showing X-specific and Y-specific amplified products. This indicates that the above samples were of a male origin [Table/Fig-10].

With the use of more advanced techniques of PCR analysis, the results were obtained in a shorter frame of time (approx.. 5 hours) and variations from normal number of PCR cycles (30-35 cycles) of amplification was noted in category II and category V where 45

Gender	n	Mean band width on X chromosome (kb)	SD	t-value	p-value
Male	22	1.3395	0.1404	1.7021	0.1007
Female	28	1.2475	0.0921		

[Table/Fig-11]: Comparison of mean band width (in kb) on X chromosome in male and female samples.
Test applied: t- test, SD: Standard deviation; p< 0.05- Significant

Category	Mean band width (kb)	SD
Category I A	1.37	0.12
Category I B	1.34	0.11
Category II A	1.22	0.03
Category II B	1.31	0.06
Category III A	1.37	0.13
Category III B	1.39	0.12
Category IV A	1.40	0.12
Category IV B	1.44	0.11
Category V	1.17	0.13
F-value	2.4155	
p-value	0.0648	

[Table/Fig-12]: Comparison of mean band width on X-chromosome among different categories using ANOVA test.
Test applied: ANOVA test, SD: Standard deviation; p< 0.05- Significant

Category	Mean band width (kb)	SD
Category I A	1.40	0.14
Category I B	1.38	0.13
Category II A	1.23	0.04
Category II B	1.31	0.06
Category III A	1.41	0.16
Category III B	1.43	0.15
Category IV A	1.44	0.15
Category IV B	1.44	0.11
Category V	1.19	0.15
F-value	1.3542	
p-value	0.3131	

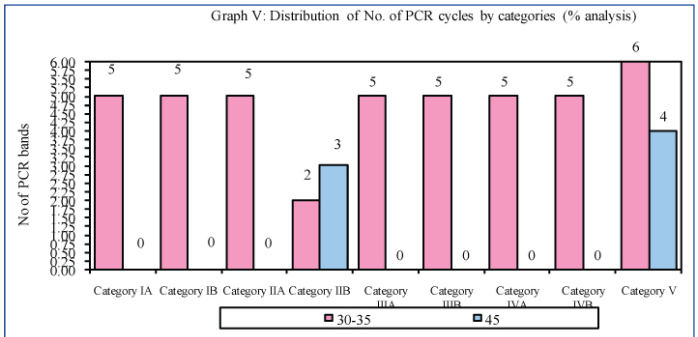
[Table/Fig-13]: Comparison of mean band width on Y-chromosome among different categories.
Test applied: ANOVA test, SD: Standard deviation; p< 0.05- Significant

cycles were needed for adequate amplification which was a 10 cycle raise. So, we could draw an inference that samples which were kept in sea water condition as well as those subjected to incineration (especially above 800^{0}C) required alterations of PCR cycles so that effective DNA retrieval was achieved in these cases.

Statistically ANOVA and t-tests proved to be significant. In our study, the mean band width values on X chromosome did not differ significantly between male and female samples (p=0.1007) as seen with t-test [Table/Fig-11]. With ANOVA analysis, variation of mean band width on X chromosome among different categories was not statistically significant inferring that the band width is similar in all male and female samples [Table/Fig-12]. Similar result was noticed where the variation of mean band width on Y chromosome among different categories was not statistically significant where conclusion was that the band width is similar in all male samples considered in the study [Table/Fig-13]. Variations were noticed in group II B as well as group V samples, where the number of PCR cycles had to be increased as compared to normal control and other samples to amplify minute amount of DNA to obtain gel electrophoresis positivity [Table/Fig-14].

DISCUSSION

Disasters either natural or man-made require identification of the postmortem remains due to severe mutilation. This process requires comparing hundreds, sometimes thousands of ante- and



[Table/Fig-14]: Distribution of No. of PCR cycles by categories (% analysis).

postmortem data [12]. Human remains in such cases may be highly fragmented, and hence only part of the body may be recovered which makes the identification a tough task [13].

Determination of gender of unknown human remains is one of the most important aspect of the triad of dental profiling, which also includes determination of ethnicity as well as age of the victim [13]. When comparing large amount of data for victim identification in mass calamities, gender identification gains prime importance to establish the individual identity with accuracy. It will also categorize the groups of victims so that further identification procedures including age assessment or personal identification procedures can be effectively carried out without delay and in a cost-effective manner. Gender determination can be carried out by various methods but targeting Amelogenin gene locus is one of the reliable methods in forensic science.

Amelogenin is a protein of dental enamel that is present on the human X and Y chromosomes, and deletion of this gene causes X-linked amelogenesis imperfecta, a genetic disorder affecting enamel formation [14]. Two copies of the Amelogenin genes were detected by Lau et al., in the human male genome by southern blot analysis. One copy was located on the distal short arm of the x-chromosome at p22.1-p22.3 region, while the second copy was localized near the centromere of the y-chromosome [15].

Fifty teeth samples in our study were exposed to various extreme environmental conditions roughly simulating mass disaster situations where human subjects are degraded beyond recognition by various forensic insults. So, it is imperative to analyze the effects of these conditions on human tissues especially using the dentition, as the latter is one of the prime choice for determining individual identity since other tissues deteriorate under extreme forensic conditions.

Even though the study yielded significant retrieval of DNA from samples, but to detect minute amount, the PCR cycles had to be raised for neat visualization of the amplification which was a prominent limitation of the study. PCR itself is a time consuming procedure especially where limited resources are available and time constraint is present.

Recently another method which is termed as Loop Mediated Amplification Method (LAMP reaction) which can give results in approximately half an hour time limit is recommended by the authors as an alternative to conventional PCR technique. One more advantage of the LAMP method is that, it works on isothermal condition which stops further denaturation of the DNA [16].

Through the present study, it can be hypothesized that the band width of Amelogenin gene on X-chromosome and Y-chromosome did not alter even in the presence of extreme environmental conditions or insults like incineration upto 1050^{0}C, thus facilitating accurate sex determination which will help especially in delineating the individual identity in case of mass disaster casualties. In future, Amelogenin gene analysis for gender determination should be collaborated with age estimation techniques so that more accurate protocol for individual identification can be devised.

CONCLUSION

DNA extracted from teeth samples yields sufficient amounts of genomic material useful for PCR- based diagnostic methods [2]. There are various extreme environmental conditions to which human body is exposed especially more so in mass fatalities. So, it is worthwhile to study the effects of extreme environment on the human dentition. Very few studies have been carried out in this field. Gender determination can be carried out by various methods but targeting Amelogenin gene locus is one of the reliable method in forensic science. In the present study for all the 50 samples, 100% retrieval of DNA along with gender determination had been successfully carried out in a short span of time with optimal technique sensitivity.

LIMITATION

1. Increased number of PCR cycles had to be performed to obtain sufficient DNA material for amplification.
2. Time consuming procedure for incinerated (800°C) and sea water samples.

DNA analysis by conventional PCR methods has its technical difficulties also for eg., long time to analyze the results as well as complicated work requirements.

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PARTICULARS OF CONTRIBUTORS:
1. Senior Lecturer, Department of Oral Pathology, R.R. Dental College and Hospital, Udaipur, Rajasthan, India.
2. Senior Lecturer, Department of Oral Pathology, R.R. Dental College and Hospital, Udaipur, Rajasthan, India.
3. Senior Lecturer, Department of Orthodontics, R.R. Dental College and Hospital, Udaipur, Rajasthan, India.
4. Senior Lecturer, Department of Prosthodontics, R.R. Dental College and Hospital, Udaipur, Rajasthan, India.
5. Senior Lecturer, Department of Oral Pathology, MGVKBH Dental College and Hospital, Nashik, Maharashtra, India.
6. Senior Lecturer, Department of Periodontics, R.R. Dental College and Hospital, Udaipur, Rajasthan, India.
7. Senior Lecturer, Department of Periodontics, Ajinknya D. Y. Patil Dental School, Pune, Maharashtra, India.
8. Reader, Department of Oral Medicine and Radiology, R.R. Dental College and Hospital, Udaipur, Rajasthan, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Priyanjali Dutta,
Senior Lecturer, Department of Oral Pathology, R.R. Dental College and Hospital, Udaipur-313001, Rajasthan, India.
E-mail: priyanjalirrdc@gmail.com

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