Sex Determination by Amplification of Amelogenin Gene from Dental Pulp Tissue by Polymerase Chain Reaction

Abstract

Introduction: Forensic odontology necessarily involves the application of dentistry along with various other branches of sciences which deals with proper handling, examination, evaluation, and presentation of dental evidences, that aids to investigate a crime and deliver justice. Sex determination is a part of forensic odontology and an essential priority when traditional identification of the deceased becomes impossible. Aim: To determine Sex by analysis of the Amelogenin gene using Polymerase Chain Reaction (PCR) method on Deoxyribose nucleic acid (DNA) isolated from dental pulp, which was exposed to various environmental conditions created artificially to mimic a forensic scenario. Materials and Method: This in-vitro study was conducted by subjecting extracted teeth to various conditions imitating a forensic scene, viz. desiccation at room temperatures, immersion in salt water, burial in soil and even exposing to extremes of temperatures. DNA was extracted from dental pulp tissue and sex determination was achieved by amplification of the amelogenin gene through AMEL gene based primers in PCR. Result: Among all the samples used in this study, DNA could be extracted from all, except from those that were subjected to a temperature of 350 °C. DNA amplification and sex determination of the samples were found to be accurate when compared to sex of the individual which was recorded initially, during collection of teeth samples. Conclusion: This study shows teeth to be a potent source of DNA even in extreme environmental conditions, barring high temperatures and determination of sex by PCR amplification of AMEL markers to be quite reliable.

Keywords: Amelogenin, dental pulp, DNA, environmental conditions, sex determination

Introduction

Forensic odontology (Dentistry) is a new and growing branch of forensic medicine. The journey started from the mother of Roman Emperor Nero, in 49 AD who was identified and discovered by her discolored front teeth, following her assassination.[1] The branch of forensics is associated with the application of science and its technological advancement to detect and investigate any criminal offense, thereby aiding in administration of justice, requiring the coordinated efforts of a multidisciplinary team.[2,3] In mass disasters, especially in fires, explosions, or in decomposed and skeletonized bodies where there is negligible material remaining for visual identification, forensic odontology plays a major and important role. Dental identification, by DNA analysis necessarily involves comparisons of antemortem and postmortem records and gains importance especially where exogenous factors leads to mutilation of the dead body, limiting the retrieval of DNA from other body remnants.[4]

Human dentition can survive several furies of nature during death and decomposition of the body, even when subjected to extreme forces and temperature. Dental pulp tissue is located inside the pulp chamber and is well protected by hard tissues such as enamel and dentine containing fibroblasts, odontoblasts, endothelial cells, peripheral nerves, undifferentiated mesenchymal cells and nucleated components of blood found in coronal and radicular pulp. These are rich sources of DNA and free from contamination by external factors.[5]

During the early stages of tooth development, cells of the inner enamel epithelium, differentiate to form ameloblasts synthesizing specific proteins, that form the enamel matrix. Amelogenin (AMEL) being one such protein is present in the dental pulp tissue. The presence of two AMEL genes was confirmed by studies of Lau et al. (1989).[6]

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Sasaki and Shimokawa (1989);[7] one on the X chromosome and the other on the Y chromosome. One copy was located on the distal short arm of the X chromosome in the p22.1–p22.3 region and the other was located near the centromere of the Y chromosome.[8,9] Therefore, amplification of the homologous X–Y AMEL gene by polymerase chain reaction (PCR) provides a suitable and effective method of sex determination.

In this study, we aim to determine sex using PCR on AMEL gene from DNA isolated in dental pulp that was subjected to various conditions created artificially to imitate a forensic environment.

Methodology

The study was conducted after necessary clearance from the Institutional Research Committee at the Department of Oral and Maxillofacial Pathology, Vyas Dental College and Hospital, Jodhpur. A total of 130 extracted premolars were used as samples for the study. These 130 teeth were then segregated into different categories, among which 10 immediately extracted teeth were used for pulp extirpation, whereas the remaining 120 extracted teeth were subjected to various environmental conditions mimicking a forensic environment, on similar lines to studies carried out by Murakami et al.[10] and Corte Real.[11] Sex of all individuals were recorded, before their extraction so that it could be tallied and verified later on sex determination by PCR analysis and gel electrophoresis.

Creating artificial forensic environment

Sampling

I. Teeth samples desiccated at room temperature for a period of 30–90 days (30 teeth)
   (a) 10 teeth – 30 days, (b) 10 teeth – 60 days, (c) 10 teeth – 90 days
II. Teeth submerged in salt water for a period between 30 days and 90 days (30 teeth)
   (a) 10 teeth – 30 days, (b) 10 teeth – 60 days, (c) 10 teeth – 90 days
III. Teeth samples buried in soil for a period between 30 days and 90 days (30 teeth)
   (a) 10 teeth – 30 days, (b) 10 teeth – 60 days, (c) 10 teeth – 90 days
IV. Teeth subjected to high temperatures between 150°C and 350°C (30 teeth)
   (a) 10 teeth – 150°C, (b) 10 teeth – 250°C, (c) 10 teeth – 350°C

Retrieval of pulp tissue and its storage

Teeth were either sectioned longitudinally using sterile carborundum disc or by splitting open the teeth using a hammer. Dental pulp was recovered by using sterile curette or a spoon excavator or with a barbed broach.

The samples of dental pulp procured were stored in 0.9% saline, in separate labeled vials at a temperature of −20°C till further analysis.[12,13]

DNA isolation and quantification

DNA was isolated from dental pulp by using conventional organic phenol chloroform method, and quantification of DNA was done by measuring its absorbance at a wavelength of 260 nm using a spectrophotometer.

Polymerase chain reaction

PCR amplification of the X–Y AMEL gene with 330 bp X chromosome-specific sequence and 236 bp Y chromosome-specific sequence was conducted. PCR was carried out in 100 μl tubes using 25 μl of reaction mixture comprising of 5–50 ng of DNA template from dental pulp tissue, 250 μM of dNTP mix, 10 pmol of Forward primer, 5 pmol of X Reverse primer, 5 pmol of Y Reverse primer, 2.5 units of Taq DNA polymerase, 1 × PCR buffer, and triple distilled, nuclease-free water.

The nucleotide sequence of primers 5'-3'

- AMEL forward primer – CAG CTT CCC AGT TTA ACT TCT G
- AMEL X reverse primer – CTC TCC TAT ACC ACT TAG TCA G
- AMEL Y reverse primer – TGC CCA AAG TTA GTA ATT TTA CCT.

40 PCR cycles were performed in FlexCycler2 [Figure 1] with denaturation of DNA at 94°C for 1 min, followed by annealing at 54°C for 30 s and extension at 72°C for 2 min.

Gel electrophoresis and sex determination

The products of PCR were subjected to agarose gel electrophoresis conducted with 0.8% agarose solution in 1X TAE buffer at 60 V for a maximum of 2 h. X- and Y-specific bands were visualized and examined under an ultraviolet illuminator. If both X- and Y-specific sequences...
were detected, the sample was considered as male whereas detection of only X-specific sequence was deemed to be a female.

Results

The mean amount of DNA obtained from 10 samples of freshly extracted teeth was determined to be 70.21667 µg/ml. The mean value of DNA obtained from dental pulp tissue after the teeth had been subjected to various artificial environmental conditions are tabulated in Tables 1-4. Graphs 1-3 show the relative comparison between DNA obtained in different environmental conditions for a particular time period. Data were entered into Statistical Package for Social Sciences (SPSS) (IBM) for Windows and then analyzed using Mann–Whitney U-test. The P value was set as 0.005.

The results showed a steady decrease in the amount of DNA obtained on increasing the time period at which teeth were exposed, irrespective of the environmental condition. The quantity of DNA obtained from teeth desiccated at room temperatures was healthy when compared to those immersed in salt water or buried in soil [Tables 1-3]. Teeth buried in soil [Table 3] yielded least amount of DNA over a period of time and no DNA could be obtained at high temperatures [Table 4]. The P values of all samples irrespective of the environmental condition was determined to be <0.00001, which was found to be statistically significant, except for those at 350ºC, as no DNA could be obtained.

To avoid individual bias, 3 samples from freshly extracted teeth and 3 samples from each of the conditions, i.e., a total of 39 samples (3 + 36 samples) were randomly picked and subjected to PCR and gel electrophoresis for sex determination. Figure 2 shows a gel picture for determination of sex. Table 5 deciphers sex determined by PCR and was found to be accurate when matched with that of a particular individual.

Discussion

The subject of Oral and Maxillofacial Pathology has come leaps and bounds, with research and technological

| Table 1: DNA quantification of from tooth pulp desiccated for varying time periods |
|-------------------------------|-------------------------------|
| No. of days of desiccation     | Mean, amount of DNA extracted |
| 30 days                        | 35.09 µg/ml                   |
| 60 days                        | 30.025 µg/ml                  |
| 90 days                        | 26.865 µg/ml                  |

| Table 2: DNA quantification of from tooth pulp submerged in salt water for varying time periods |
|-------------------------------|-------------------------------|
| No. of days of submerged in salt water | Mean, amount of DNA extracted |
| 30 days                        | 25.285 µg/ml                   |
| 60 days                        | 21.73 µg/ml                    |
| 90 days                        | 16.095 µg/ml                   |

| Table 3: DNA quantification of from tooth pulp buried in soil for varying time periods |
|-------------------------------|-------------------------------|
| No. of days of buried in soil | Mean, amount of DNA extracted |
| 30 days                        | 18.695 µg/ml                   |
| 60 days                        | 14.46 µg/ml                    |
| 90 days                        | 11.095 µg/ml                   |

| Table 4: DNA quantification of from tooth pulp subjected to various temperatures |
|-------------------------------|-------------------------------|
| Temperature at which heated   | Mean, amount of DNA extracted |
| 150 ºC                        | 26.19 µg/ml                   |
| 250 ºC                        | 13.995 µg/ml                  |
| 350 ºC                        | -                            |
advances in all branches of science. The amalgamation of newer technologies is imperative to expand the scope and add to our repertoire of knowledge and skills. Forensic Odontology, a branch of Oral and Maxillofacial Pathology is widely being accepted nowadays for its applications in identification of individuals for medicolegal cases, when bodies are decomposed, destroyed, or mutilated beyond recognition. In several cases of natural calamity or in cases of homicide, teeth are often the sole material recovered. In these cases, as also in cases of paternity disputes or mapping of family descendants, forensic odontology plays an important role.

DNA extraction from dental tissues both hard tissue and pulp has been reported. In this study, pulp was used as a source of DNA. Extirpation of pulp was performed using a suitable method and the pulp tissue was stored in saline, which served as an excellent storage media. It has been conclusively proven that DNA extraction is possible even in the most adverse circumstances such as conflagration scenes or mummified remains. Storage at room temperatures can be done for periods extending up to many years. Murakami et al. have reported the storage of up to 22 years.[10]

This study showed that DNA extraction with conventional phenol-chloroform method is effective in all forensic conditions. The results are presented in the Table 5:

Table 5: Sex determination by PCR, through DNA analysis of dental pulp tissue

| Sample in no* | Known age and Sex | Environmental Stress condition | Bands obtained gel electrophoresis |
|---------------|-------------------|-------------------------------|----------------------------------|
| 2             | 36 years, Female  | Freshly extracted teeth       | 1                                |
| 8             | 42 years, Male    | Freshly extracted teeth       | 2                                |
| 9             | 50 years, Male    | Freshly extracted teeth       | 2                                |
| 11            | 28 years, Male    | Room Temperature-30 days      | 2                                |
| 17            | 35 years, Female  | Room Temperature-30 days      | 1                                |
| 14            | 26 years, Female  | Room Temperature-30 days      | 1                                |
| 28            | 16 years, Male    | Room Temperature-60 days      | 2                                |
| 24            | 34 years, Male    | Room Temperature-60 days      | 2                                |
| 23            | 47 years, Female  | Room Temperature-60 days      | 1                                |
| 33            | 25 years, Male    | Room Temperature-90 days      | 2                                |
| 37            | 33 years, Male    | Room Temperature-90 days      | 2                                |
| 40            | 30 years, Male    | Room Temperature-90 days      | 2                                |
| 43            | 56, years Male    | Salt water-30 days            | 2                                |
| 47            | 43 years, Male    | Salt water-30 days            | 2                                |
| 41            | 51 years, Female  | Salt water-30 days            | 1                                |
| 53            | 38 years, Male    | Salt water-60 days            | 1                                |
| 56            | 54 years, Female  | Salt water-60 days            | 2                                |
| 57            | 27 years, Male    | Salt water-60 days            | 2                                |
| 67            | 23 years, Female  | Salt water-90 days            | 1                                |
| 69            | 46 years, Male    | Salt water-90 days            | 2                                |
| 64            | 35 years, Male    | Salt water-90 days            | 2                                |
| 71            | 29 years, Female  | Buried in soil-30 days        | 1                                |
| 73            | 21 years, Male    | Buried in soil-30 days        | 2                                |
| 77            | 34 years, Female  | Buried in soil-30 days        | 1                                |
| 86            | 35 years, Male    | Buried in soil-60 days        | 2                                |
| 90            | 40 years, Female  | Buried in soil-60 days        | 1                                |
| 82            | 32 years, Female  | Buried in soil-60 days        | 1                                |
| 99            | 18 years, Male    | Buried in soil-90 days        | 2                                |
| 95            | 25 years, Male    | Buried in soil-90 days        | 2                                |
| 92            | 43 years, Female  | Buried in soil-90 days        | 1                                |
| 105           | 29 years, Male    | Heated to 150º C              | 2                                |
| 107           | 21 years, Female  | Heated to 150º C              | 1                                |
| 102           | 52 years, Male    | Heated to 150º C              | 2                                |
| 114           | 20 years, Female  | Heated to 250º C              | 1                                |
| 119           | 37 years, Male    | Heated to 250º C              | 2                                |
| 117           | 26 years, Female  | Heated to 250º C              | 1                                |
| 124           | 34 years, Female  | Heated to 350º C              | -                                |
| 121           | 28 years, Female  | Heated to 350º C              | -                                |
| 127           | 30 years, Male    | Heated to 350º C              | -                                |

*3 samples were randomly chosen from each condition
conditions barring only at high temperatures of 350°C where no DNA could be extracted. These findings were in correlation with that of Sivagami et al.,[14] Murakami et al.,[10] Corte Real,[15] and Vemuri, et al.[12] In this study, we have used only a particular tooth type, i.e., premolars, to rule out any ambiguity between DNA obtained from various tooth types.

Although any tissue is amenable to extraction of DNA and its use in PCR, dental tissues offer us the advantage of being resistant to physical, environmental degradation and has proved to be a good source of genomic DNA. Dental pulp as a source for DNA has been reviewed extensively by Komuro et al.,[15] and Sweet and Sweet.[16] Pötsch et al.[17] in their study have found that DNA obtained from a single tooth varied from 6 to 50 μg. This very study showed that mean DNA obtained from a freshly extracted tooth was 70.217 μg/ml, using standard conditions of extraction. The DNA has proven to be of sufficient quantity and of a good quality for its use as a forensic evidence material.

The mean DNA obtained from teeth submerged in salt water were 25.285 μg/ml, 21.73 μg/ml, and 16.095 μg/ml with days of teeth being submerged as 30 days, 60 days, and 90 days, respectively. Our results showed correlation with Murakami et al.,[10] and Corte Real[15] in which they were able to determine sex from teeth samples immersed in seawater for 4 weeks. This suggests that the salt water wet condition does not cause autolytic degradation or decay of the DNA content inside the teeth.

The mean DNA obtained from dental pulp tissue after they were buried in soil for 30 days, 60 days, and 90 days were 18.695 μg/ml, 14.46 μg/ml, and 11.095 μg/ml, respectively, which was again in accordance with the studies of Murakami et al.,[10] and Corte Real.[15]

Similarly, this study also finds correlation with the one conducted by Urbani et al.,[18] wherein they investigated the efficacy of utilizing DNA retrieved from the pulp of human teeth which had been exposed to different temperatures for various lengths of time to assess the sex, by PCR. Their study showed PCR to be reliable in sex determination when teeth were subjected to a temperature of 100°C but less reliable when subjected to higher temperatures. On the same line, our study also shows that quality DNA with a mean value of 26.19 μg/ml and 13.995 μg/ml could be obtained from dental pulp when teeth were subjected to 150°C and 250°C, respectively, and hence was useful in sex determination by PCR, but no DNA was retrieved when teeth samples were subjected to heat of 350°C temperature.

In this study, it was also observed that there is almost a decline of around 50% of the DNA obtained in freshly extracted teeth to those obtained from teeth which had been desiccated at room temperature for a period of 1 month. DNA obtained from teeth desiccated for 60 days and 90 days showed some stability though there was decline in value. This is in accordance with the study conducted by Rubio et al.,[19]

Among known X–Y homologous genes, the AMEL gene is the most suitable for the sex test by PCR. Research conducted till date on AMEL shows that it has a particular difference in size and pattern of nucleotide sequence in male and female enamel. This phenotypic difference between male and female enamel is used in modern forensic sciences as a sensitive sex marker for negligible quantities DNA obtained from unknown human remains. The AMEL gene, coding for a highly-conserved protein, is located on the X and the Y chromosomes in humans. The two alleles are similar for the exonic sequences but differ in the intronic sequences. Thus, the females (XX) have two identical AMEL genes but the males (XY) have two nonidentical genes. The fact that the X- and Y-specific AMEL genes are 106 and 112 base pairs (bp) in length, respectively, provides a relatively direct procedure to discriminate between male and female AMEL.[12,20]

Kobayashi and Hecker performed gender determination using AMEL gene. Their study showed that PCR amplification of segments of XY homologous AMEL gene yields a specific base pair product for male and female samples. Although PCR products are analyzed by gel electrophoresis, in this study, the authors reported the use of ion-pair reversed-phase high performance liquid chromatography – DNA chromatography – using the WAVE Nucleic Acid Fragment Analysis System, for automated gender determination with AMEL specific PCR products.[21]

Gupta et al.[22] in their review article contemporary practice in forensic odontology states that forensic DNA analysis for sex determination yields highly accurate results. Sex can be
determined with very minute quantities of DNA (as little as 20 pg) and from very old specimens of teeth. AMEL is one of the major matrix proteins secreted by the ameloblasts of the enamel. Modern DNA extraction methods can isolate genomic DNA from dental cells and the gene AMEL, which is a sex-linked gene. In addition, microscopic examination of the cells from the pulp can also reveal the presence of Barr bodies in females.

Dutta et al. carried out a study of gender determination from the DNA samples which were obtained either from dental pulp or dentin of tooth, that had been exposed to extreme forensic environmental conditions using PCR technique targeting the AMEL gene locus. Results obtained from their study showed AMEL gene to be a potent sex-determining factor in trying conditions, even in temperatures of 1050°C. This study too reveals the same regarding the efficacy of AMEL gene although we could not retrieve DNA at temperatures of 350°C.[23]

Kholief et al. in their study conducted on Egyptians found that considerable amount of DNA could be obtained from dental pulp irrespective of the condition of teeth, whether sound or carious, and determining of gender was found to be accurate by analysis of DYS14 and SRY genes.[24]

Recent researches on amelogenin

For decades, it was regarded that AMEL is a protein of epithelial origin, present only in enamel. However, recently various isoforms of AMEL have been discovered in places other than enamel like dentin matrix, odontoblasts, in remnants of Hertwig’s root sheath and in periodontal ligament cells. The expression of ameloblasts has also been found in long bone cells such as osteocytes, osteoblasts, osteoclasts, some bone marrow cells, and articular cartilage, chondrocytes of the articular cartilage and in cell layers of the epiphyseal growth plate. Different functions of AMEL may be attributed to the following facts: first, the presence of a relatively large number of AMEL alternatively spliced mRNA translated polypeptides and second, it is expressed in different tissues (calcifying and soft tissues) and of different embryonic origin.[9]

AMEL gene provides a direct link to discriminate and identify sexes in extreme conditions. The method used in this study of extracting DNA from dental pulp by organic phenol chloroform though was quite cost-effective, but is quite tedious and time-consuming, and requires extreme precision, or else contamination of DNA is quite common. Newer DNA extraction methods Chelex 100™ (Medox Bio)[10] and QIA cube™ (Qiagen, Germany)[25] could be substituted for the traditional method.

Conclusion

DNA obtained from teeth samples yields sufficient amounts of genetic material useful for PCR-based analysis. There are various extreme forensic environmental conditions which a human body could encounter in mass destructions. Therefore, it is of utmost importance to study the effects of extreme conditions on the human teeth. Only a handful of studies have been carried out in this domain. Gender determination can be carried out by various methods but targeting AMEL gene locus is one of the reliable methods in forensic science. In the present study, determination of sex of all the samples was possible with complete sensitivity and specificity, except in temperatures of 350°C, where no DNA could be obtained, proving that AMEL gene (AMEL marker) to be definite and effective for gender identification.

Even though AMEL serves as a benchmark in sex determination for forensic cases, questions are raised in certain conditions. However, some studies have cast doubts on the reliability of the AMEL gene analysis due to reports of allele dropout of the X AMEL gene in males due to the polymorphism of primer binding sites. A high prevalence of deletions and other variations in the Y chromosome is quite common, some of which affect Yp11.2, the locus of the AMEL gene. This induces males to be wrongly identified as females because of similarity in AMEL profile. The ideal standard for gender determination is Y-STR and SRY (sex-determining regions of Y) testing and these tests have to be performed in conjunction with the AMEL testing.[26]

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Conflicts of interest

There are no conflicts of interest.

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