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Original Article

Analysis of Amelogenin and Sex-determining Region on Y Chromosome Genes Obtained from Pulpal Tissue for Sex Estimation by Using Multiplex Polymerase Chain Reaction

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Abstract: In forensic odontology, Amelogenin and Sex-Determining Region on Y chromosome genes are commonly used for sex identification. Nevertheless, the amplification of both of these genes encounters limitations in determining sex, primarily due to deletions observed in certain racial groups and the influence of genetic disorders on these genes. This research aims to assess the precision of simultaneously amplifying both genes using multiplex polymerase chain reaction on samples derived from teeth that have been subjected to various forensic conditions. In this study, a total of 70 teeth, comprising 35 males and 35 females, were utilized, and all samples underwent genetic analysis to determine sex following exposure to different forensic scenarios. The findings revealed that both the quantity and quality of genetic material were reduced when exposed to harsh environmental conditions. Notably, the amplification of the sex-determining region on the Y chromosome gene outperformed the amplification of Amelogenin and achieved a sex-determination accuracy rate of 100%. In conclusion, dental pulp serves as a valuable source of genetic material even after exposure to diverse environmental conditions, and it can effectively be used for sex estimation purposes.

1. Introduction

In traditional forensic investigations, the antemortem information from the deceased person is compared with the post-mortem data of that person for identification [1]. However, in events (such as mass graves, earthquakes, tsunamis, airplane crashes, train and road accidents, bomb blasts, and wars) where a high number of extremely damaged and dismembered dead bodies are recovered, human identification would be very challenging because investigators struggle with obtaining proper antemortem records [2]. To narrow the search for identity, forensic experts prepare biological profiling for

unidentified human remains, which includes basic demographic information such as sex, age, stature, and ethnicity. Sex recognition is the first and essential step in the biological profiling of unidentified corpses, as it will narrow the search for the deceased person's identity by excluding nearly one-half of the cases [3,4].

In forensic dentistry, different methods have been used for sex determination [5], however, the most precise techniques for human identification are done through deoxyribonucleic acid (DNA) profiling or fingerprinting, which is a very useful and sensitive tool for the identification of human remains. This technique brought about a huge revolution in the field of forensic science [6-8].

From a forensic standpoint, the dental pulp is a rich source of DNA, because of its unique anatomical position which is situated deep within the tooth, encased by layers of enamel and dentin; in addition, the teeth are also well protected by facial bones and muscles [9-11]. This anatomical positioning shields the pulp and creates a protective barrier from environmental factors that can degrade DNA, such as high temperature, UV radiation, chemicals, moisture, and microbial activity. This reduced exposure contributes to the preservation of DNA integrity over time [12,13].

Furthermore, unlike some tissues in the body that undergo frequent cellular turnover, dental pulp contains a relatively stable population of cells which helps to maintain the integrity of genetic material [14]. DNA extraction from dental pulp after exposure to various environmental factors is a subject of great interest to forensic researchers; several studies concluded that genetic materials can be successfully extracted from dental pulp after being exposed to harsh environments such as high temperature, humidity, soil, and time [15].

Advanced molecular biology technique plays an important role in forensic odontology, with PCR offering sensitive and efficient methods for sex identification by analysis of sex-specific genes. In addition, this technique enables investigators to amplify even small amounts of DNA present in forensic samples. Due to its high sensitivity, and specificity, PCR-based sex determination is widely used in criminal cases, mass disasters, missing persons cases, and anthropological identification [16].

The most frequently used gene for sex estimation is the Sex Determining Region Y gene (SRY), located at position 11.3 of the (p) arm on the Y chromosomes. This gene marker is considered by scholars to be the signature gene that differentiates the Y chromosome from the X chromosome[17]. SRY gene can be detected from samples such as teeth, saliva, blood, and epithelial cells. The results of studies conducted to determine sex by amplifying SRY showed that the specificity and sensitivity for sex identification were 73.3% and 100%, respectively [18]. In some conditions using the SRY gene detection method is not applicable for the estimation of sex, such as Turner syndrome (46, X0), Klinefelter syndrome (46, XXY), SRY gene mutations like Swyer syndrome (46, XY), partial or pure gonadal dysgenesis, the testicular disorder of sex development (46, XX), XYY syndrome, androgen insensitivity syndrome [19].

Another drawback of this method is that it gives false-positive results in certain conditions such as microchimerism (maternal-fetal) and chimerism between different sex recipients and donors during organ transplantation [6].

Another widely used gene for sex identification is Amelogenin (AMEL), which encodes a group of proteins (amelogenins) that are involved in enamel formation during tooth development. AMEL gene has a different size, location, and nucleotide sequence pattern on both X and Y chromosomes. AMEL gene is located at the p22.1-p22.3 region on the X chromosome (AMELX), while it is located at p11.2 on the Y chromosome (AMELY); therefore, females have two identical pairs of AMEL genes, while males have two different pairs of AMEL genes [20].

In PCR amplification of the AMEL gene, the primers used to amplify the AMEL gene will generate a larger fragment of AMELY and a smaller fragment of AMELX (by 6 base pairs), if the sample showed only a short fragment (AMELX), indicating the sample contains only the X chromosome (female phenotype), while if both short and long fragments were observed, it is proposed that the sample will contain both X and Y chromosomes (male phenotype) [21].

The practicality of using the AMEL marker for sex identification is unsettled in forensics, based on failure to correctly estimate the sex of the samples due to deletion of the AMELY gene in some races [22].

Multiplex PCR is a method that allows for the simultaneous amplification of multiple target DNA sequences within a single reaction [23]. When it comes to forensic applications, including sex estimation, multiplex PCR offers several advantages, such as reducing sample material usage, improving efficiency, and providing more information in a single analysis [24].
To overcome the limitations of amplifying each gene (SRY and AMEL) separately for sex identification, numerous researchers have employed multiplex PCR to simultaneously target both genes [25, 26]. Nevertheless, this approach has yet to be applied to dental pulp samples subjected to various forensic environments.

The present study was conducted to evaluate the efficacy of multiplex PCR in sex determination using dental pulp samples that were subjected to different forensic scenarios.

2. Materials and Methods

2.1. Study Design

The samples of this study were collected at Shorsh Dental Center from September 2021 to April 2022. Seventy persons participated in this study (35 males and 35 females). This research received ethical approval from the Ethics Committee within the College of Dentistry at the University of Sulaimani, under reference number 36/21, dated August 11, 2021, in accordance with the prescribed ethical protocols, informed consent was taken from all participants, and a case sheet was filled out for each participant. For each person one extracted third molar was collected.

2.2. Exclusion Criteria

1. Teeth with necrotic pulp or that contained pulp stone.
2. Any abnormally formed teeth or teeth fractured during extraction.
3. Endodontically treated teeth and teeth with restoration.
4. Teeth with internal or external resorption.

2.3. Study Groups

The sample size was calculated using GPower software version 3.1.9.6 developed by Heinrich Heine University Dusseldorf, North Rhine-Westphalia, Germany. The study sample was stratified into seven distinct groups, each comprising ten teeth specimens, with an equal distribution of five males and five females within each group. This stratification aimed to emulate a variety of forensic scenarios. To simulate diverse forensic conditions, three of these sample groups were subjected to elevated temperatures for 15 minutes through a dental ceramic furnace: the first group was exposed to 100 °C (EHT1), the second group was exposed to 200 °C (ETH2), while the third group exposed to 300 °C (EHT3). The other three sample groups were interred at a depth of one meter beneath the earth's surface for different periods: 1 month (BUE1), 3 months (BUE2), and 6 months (BUE3) [27]. The last group was unexposed to any specific forensic conditions (FET), serving as the control group for comparative analysis. The distribution of the study groups is illustrated in figure 1.

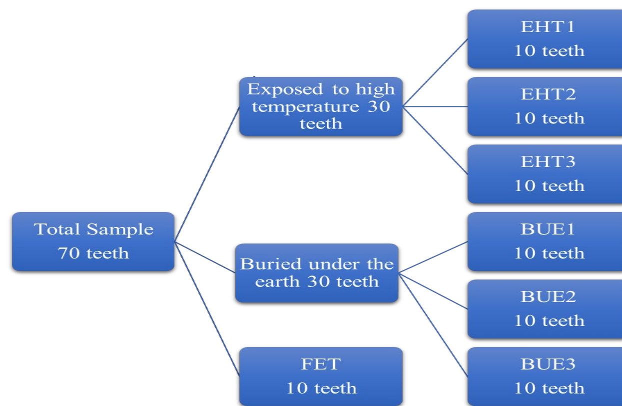

Figure 1: A diagram showing the distribution of the study groups.

2.4. *Sampling*

All teeth were washed with normal saline 0.9%, and soaked in 5% decontamination solution decon90®, manufactured by Decon Laboratories Limited, Hove, UK, for 2 hours to decontaminate the teeth from foreign DNA. Then the teeth were rinsed with DNA-free water to remove the residue of the decontamination solution on the tooth surface.

All teeth were sectioned longitudinally in mesiodistal direction using a sterile carborundum disc attached to a dental micromotor pr-s600strongdrill (Fuzhou Li Wang Electronic Technology Co., Ltd., Fuzhou, China), as shown in Figure 2. The teeth were irrigated with DNA-free water to overcome temperature elevation during the process of sectioning, The pulpal tissue was recovered by using a sterilized spoon excavator. Then the obtained samples were stored in a labeled DNA/RNA-free 2 ml Eppendorf tube (Kirgen Bioscience Co., Ltd., Shanghai, China) that contained 1 ml DNA-free water and the sample was stored at −20 °C till further analysis. All molecular analyses for this study were conducted at Biolab, a private laboratory in Sulaymaniyah, Iraq, with the exception of gene sequencing, which was carried out by Macrogen Inc. in Seoul, Republic of Korea.

Figure 2: (A) Shows mesiodistal tooth sectioning of the teeth. (B) Displays the dental pulp stored in Eppendorf tube containing DNA-free water.

2.5. DNA Extraction

The DNA extraction was performed by using the AddPrep Genomic DNA Extraction commercial kit, manufactured by ADD BIO INC, Daejeon, Republic of Korea, using the protocol recommended by the manufacturer, as shown in figure 3. After the extraction, the extracted DNA samples were stored at -20 °C for further analysis.

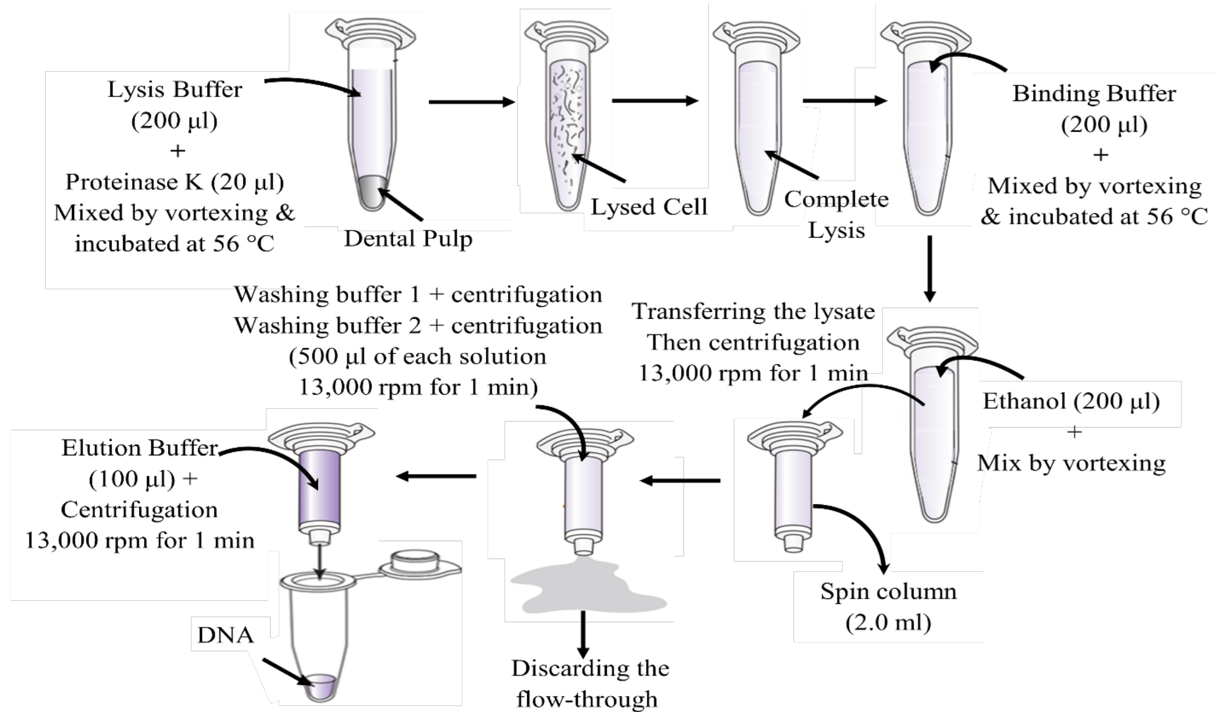

Figure 3: A diagram illustrating the procedure for DNA extraction from the dental pulp.

2.6. DNA Quantification

To quantify and compare the amounts of DNA that had been extracted from dental pulps of teeth from different groups, a nanoPlus nanodrop spectrophotometer, manufactured by MAAN LAB AB, Växjö, Sweden, was connected to a personal computer, the elution buffer was used as blank and the readings were obtained by using Spectrophotometer version 2.0 software developed by MAAN LAB AB, Växjö, Sweden.

2.7. PCR Primer Design

The full genomic sequences of AMELX, AMELY, and SRY genes were downloaded from the NCBI GenBank website developed by the National Library of Medicine, Washington, D.C., USA. Both genomic sequences of AMELX and AMELY were compared by using A Plasmid Editor software version v3.1.3, developed by M. Wayne Davis. This comparison revealed that AMELY had 184 base pair (bp) deletion compared to AMELX at a location between 8588 – 8772 and AMELX had 6bp deletion in the same region compared to AMELY, which resulted in 178bp difference between AMELX and AMELY in this region. A PRC primer was designed by using the Primer-BLAST website developed by the National Library of Medicine, Washington, D.C., USA, the sequence of all primers and the expected PCR product's length are shown in table 1.

Table 1: The primer sequences and the PCR product length of SRY, AMELX, and AMELY.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product length (bp)
SRY	CATGAACGCATTCATCGTGTGGTC	CTGCGGGGAAGCAAACTGCAATTCTT	265
AMELX	GGTTTCTGCTTCCAGGTCTCC	CCTTGCTCATATTATACTTGACAAAGCA	1022
AMELY			844

The primers for both AMEL and SRY genes were produced by Macrogen Inc., Seoul, Republic of Korea and they were sent back in bio freeze form and stored till further analysis.

2.8. Gradient PCR

The gradient PCR was performed to determine the best annealing temperature for the primers. The PCR procedure was performed following the multiplex PCR protocol which is explained in detail below, except that different annealing temperatures were used which were selected automatically by the thermal cycler model Bio-Rad C1000 Series, manufactured by Bio-Rad Laboratories, Hercules, CA, USA (58 °C,59.2 °C,60.5 °C, 61.9 °C, 63.2 °C,64.5 °C,65.8 °C, and 67 °C for the same male sample).

2.9. Multiplex PCR

The DNA samples were defrosted at room temperature and for each sample 1 µl of extracted DNA was pipetted and transferred to a labeled PCR tube, then 1 µl of forward and 1 µl of reverse primers of SRY primer and 1 µl of forward and 1 µl of reversed primers of AMEL primer to be amplified were added to the PCR tube, 10 µl of the Add multi Taq Master mix, produced by ADD BIO INC., Daejeon, Republic of Korea, and 5 µl of PCR water were added to the PCR tube; therefore, the final volume PCR cocktail for each sample was 20 µl. The PCR tubes were placed into the thermal cycler with the following settings:

Initial DNA denaturation step 5 min. at 95 °C.
DNA denaturation step 30 sec. at 95 °C.
Annealing step 30 sec. at 60.5 °C.
Extension step 40 sec. at 72 °C.
Final extension step 5 min. at 72 °C.

At the end of the above-mentioned cycles, the PCR products were ready to be analyzed using gel electrophoresis.

2.10. Gel Electrophoresis

To prepare 1% agarose gel, 1 g of agarose powder (GeneDireX, Inc., Taoyuan, Taiwan) was added to 100 ml of 1x TAEbuffer (Inno-train Diagnostik GmbH, Kronberg, Germany), and the mixture was mixed for a few seconds. Afterward, the glass container was covered with plastic wrap with a small hole to allow the solution to vent. Then the solution was heated in the microwave for 2 min.
The container was left to cool down until it reached 55 °C then 1 drop of Ethidium bromide (Inno-train Diagnostik GmbH, Kronberg, Germany) was added to the mixture and stirred for a few seconds. Soon after the edges of the electrophoresis rack were sealed with tape and the comb was secured at one end of the rack, then the rack was placed on a flat surface and the mixture was cast on it. After the gel was cooled the comb was removed by pulling it gently straight up to form the wells. Afterward, the tapes were removed from the rack, and the rack was placed into the electrophoresis device and soaked in a Tri-acetate-EDTA buffer (Inno-train Diagnostik GmbH), Kronberg, Germany), 10 µl of DNA ladder (GeneDireX, Inc., Taoyuan, Taiwan) was loaded into the 1st well, and then 20 µl of the PCR products were loaded into the other wells and one well was uploaded with negative control, then the electrophoresis device BIOBASE BEP-600I (BIOBASE GROUP, Shandong, China) was turned on with the following setting: 240 mA, 90 V, for 40 min.

Later, the agarose gel was pulled out from the electrophoresis device and uploaded into the gel documentation device, the Automatic Gel Imaging and Analysis System Biobase BK-AG100 (BIOBASE GROUP, Shandong, China), and the images of the gel were captured using Biobase software developed by BIOBASE GROUP, Shandong, China.

2.11 Gel Extraction

After the amplification of the AMEL gene of the male samples, the PCR product contained both AMELX and AMELY, therefore gel electrophoresis procedure was conducted to separate the 2 PCR products (AMELX and AMELY). Then the gel was transferred onto the gel documentation device. Each band was separated by using a surgical blade N° 15, then the separated gel was transferred to a micro-centrifuge tube. The gel extraction procedure was performed by using the AddPrep Gel Purification Kit (ADD BIO INC. Daejeon, Republic of Korea); the gel purification procedure is illustrated in figure 4.

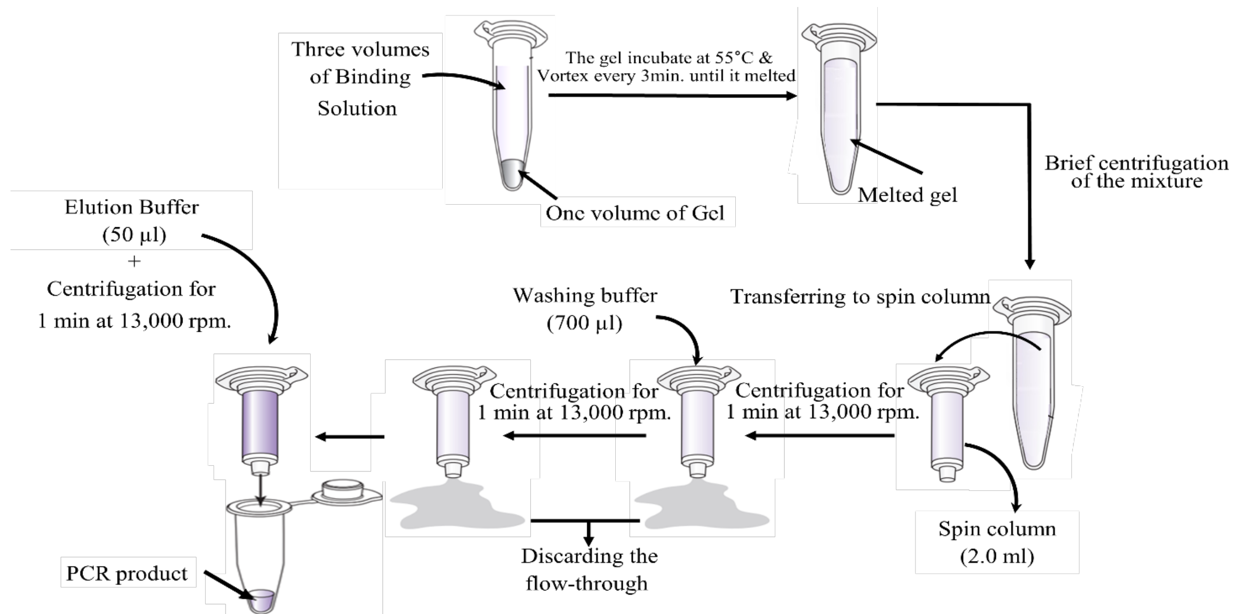

Figure 4: A diagram illustrating the procedure for PCR product extraction from agarose gel.

After the extraction, the extracted PCR product was stored. The PCR products of all targeted genes (SRY, AMELY, and AMELX) that belonged to 10 samples (5 males and 5 females) were sent to Macrogen Inc., Seoul, Republic of Korea for Sanger genomic sequencing.

2.12. Statistical Analysis

The analysis of all data was conducted employing GraphPad Prism version 9.0.0 (121), developed by GraphPad Software, Boston, MA, USA. After obtaining descriptive statistics, the normality of the data distribution was assessed through the Shapiro–Wilk test. To compare the mean age between male and female participants, an Independent T-test was employed, while the comparison of DNA quantity among various study groups was performed by using the Kruskal Wallis test, and the Mann–Whitney U test.

3. Results

A total of 70 persons participated in this study (35 males and 35 females), their ages ranging between 20 and 67 years. The mean ± standard deviation (SD) age of all participants was 28.93 ± 9.4, the mean ± SD age of male samples was 28.6 ± 9.92, while the mean ± SD age of female samples was 29.26 ± 8.97. The results of the independent T-test showed that there was no significant difference between the mean age of male and female samples ($p = 0.745$).

The results of the Shapiro-Wilk test revealed that the data of the following groups: FET, BUE2, BUE3, EHT1, EHT2, and EHT3, were normally distributed. Meanwhile, the data obtained from the BUE1 group were nonparametric ($p = 0.02$).

The study revealed that the median (interquartile range, IQR) DNA concentration extracted from the tooth pulp of the female samples measured 4.72 (with an IQR of 11.65) ng/µl. In contrast, the DNA concentration from the dental pulp of the male participants' samples exhibited a higher median (IQR) value of 5.84 (with an IQR of 16.35) ng/µl. The median (IQR) concentration of DNA of all study samples is shown in figure 5 and the median (IQR) concentration of DNA obtained from both sexes with the results of the Mann–Whitney U test is shown in table 2.

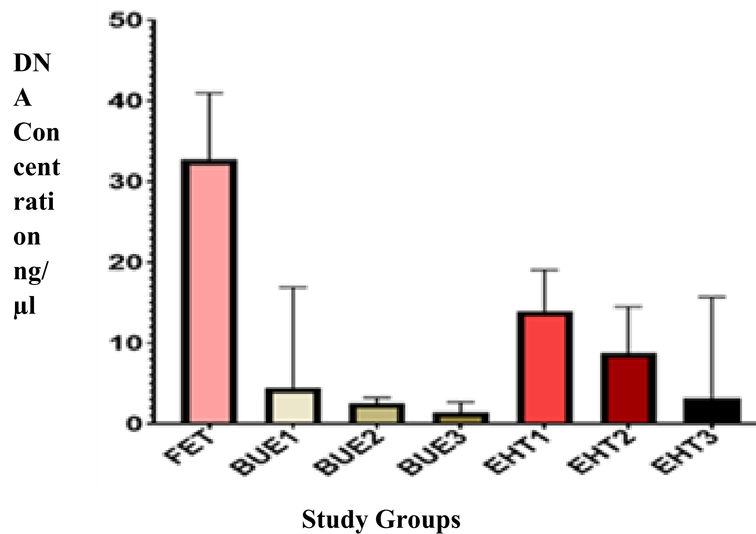

Figure 5: The bar chart presents the median and IQR of all groups.

Table 2: Shows the median (IQR) of DNA concentration of all study groups along with the outcome of the statistical comparison between male and female subjects using the Mann–Whitney U test.

Study group	Sex	Median (IQR)	P value
FET	Male	36.41 (21.84)	0.548
	Female	28.99 (22.22)	
BUE1	Male	8.76 (15.51)	0.556
	Female	4.83 (15.47)	
BUE2	Male	2.06 (1.49)	0.151
	Female	3.08 (1.43)	
BUE3	Male	2.49 (2.49)	0.008
	Female	0.51 (0.73)	
EHT1	Male	14.51 (10.86)	0.31
	Female	8.44 (11.32)	
EHT2	Male	13.12 (14.05)	0.151
	Female	6.81(8.53)	
EHT3	Male	2.68 (4.53)	0.548
	Female	3.58 (9.65)	

The Kruskal-Wallis test was employed in this study to assess the variations in DNA quantity among different groups. The findings indicated that the DNA concentration derived from samples in the FET group exhibited a statistically significant difference, with higher concentrations compared to all other groups, except for the EHT1 group. However, when comparing the remaining groups, excluding the FET group, there were no statistically significant differences in DNA concentrations among them, except in the case of the comparison between the EHT1 and BUE3 groups. In this comparison, a statistical difference was observed, with the EHT1 group showing a higher DNA concentration compared to the BUE3 group. Table 3 demonstrates the mean ranks, mean rank differences, and the results of the Kruskal-Wallis test for DNA concentration obtained from all study groups.

Table 3: Shows the mean ranks, and mean rank differences of DNA quantity ng/μl obtained from all groups and the result of Kruskal-Wallis test.

Study Groups	Mean rank 1	Mean rank 2	Mean rank differences	P Value
BUE1 vs. FET	33.00	63.80	-30.80	0.015
BUE2 vs. FET	21.20	63.80	-42.60	0.001
BUE3 vs. FET	13.10	63.80	-50.70	0.001
EHT1 vs. FET	45.30	63.80	-18.50	0.884
EHT2 vs. FET	40.60	63.80	-23.20	0.227
EHT3 vs. FET	31.50	63.80	-32.30	0.008
BUE2 vs. BUE1	21.20	33.00	-11.80	>0.999
BUE3 vs. BUE1	13.10	33.00	-19.90	0.604
EHT1 vs. BUE1	45.30	33.00	12.30	>0.999
EHT2 vs. BUE1	40.60	33.00	7.600	>0.999
EHT3 vs. BUE1	31.50	33.00	-1.500	>0.999
BUE3 vs. BUE2	13.10	21.20	-8.100	>0.999
EHT1 vs. BUE2	45.30	21.20	24.10	0.17
EHT2 vs. BUE2	40.60	21.20	19.40	0.694
EHT3 vs. BUE2	31.50	21.20	10.30	>0.999
EHT1 vs. BUE3	45.30	13.10	32.20	0.009
EHT2 vs. BUE3	40.60	13.10	27.50	0.053
EHT3 vs. BUE3	31.50	13.10	18.40	0.907
EHT2 vs. EHT1	40.60	45.30	-4.700	>0.999
EHT3 vs. EHT1	31.50	45.30	-13.80	>0.999
EHT3 vs. EHT2	31.50	40.60	-9.100	>0.999

The Kruskal-Wallis test was also employed to compare the amount of DNA extracted from upper right 3rd molar (URM), upper left 3rd molar (ULM), lower right 3rd molar (LRM), and lower left 3rd molar (LLM). The results showed no statistical differences in DNA concentrations obtained from all 3rd molars (P= 0.934). Figure 6 shows the median DNA concentrations ng/μl obtained from different 3rd molars of all groups.

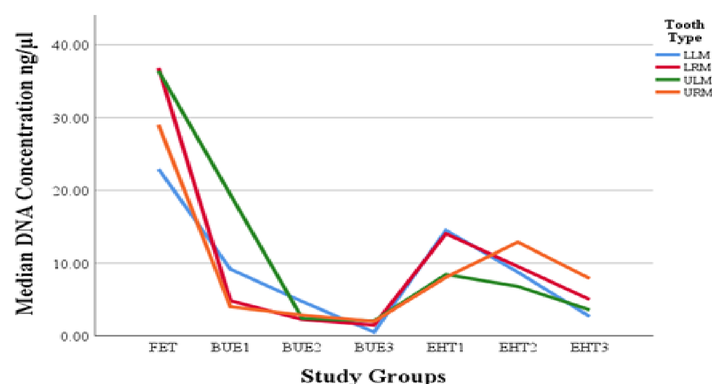

Figure 6: Shows the median DNA concentrations of different tooth samples in all study groups.

Gradient PCR was used to find the best annealing temperature for both primers, the result showed that 60.5 °C is the optimum annealing for the primers of this study. The Sanger genomic sequencing was performed on a set of 10 samples, comprising an equal distribution of 5 males and 5 females. Subsequently, Geneious Prime software was employed to align the sequencing data obtained from the samples of this study with genomic sequences SRY, AMELX, and AMELY sourced from the NCBI GenBank database. The outcome of this alignment revealed that all PCR products from the

sampled individuals exhibited complete concordance with the sequences archived in the NCBI GenBank database.

The findings of multiplex PCR analysis revealed that the SRY gene was successfully amplified in all male samples across all experimental groups. Additionally, the AMEL gene exhibited successful amplification in all samples acquired from the FET, BUE1, and BU2 groups, while no AMEL bands were detected in samples from BUE3, EHT1, and EHT2. Notably, the AMEL gene bands were only observed in 80% of the samples from the EHT1 group. Figure 7 illustrates the multiplex PCR results for the FET group, while Figure 8 presents the results for all other groups. In the present study, the sex of all samples was accurately determined with a 100% success rate based on the presence of the SRY gene. The sex of all samples collected from FET, BUE1, and BUE2 groups was estimated correctly, deepening on the AMEL gene, while the sex of 80% of the samples obtained from EHT1 was identified; however, the sex of samples obtained from BUE2, EHT2, EHT3 couldn't be determined by depending on AMEL gene amplification.

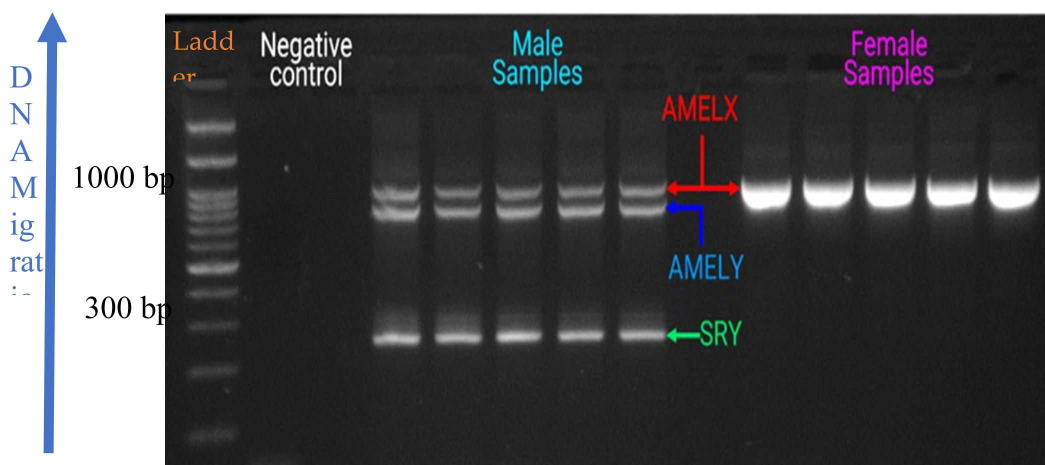

Figure 7: The result of multiplex PCR of the pulp sample obtained from freshly extracted teeth. Each line of the ladder represents 100 bp, AMELX at 1022 bp, AMELY at 844 bp, and SRY at 265 bp.

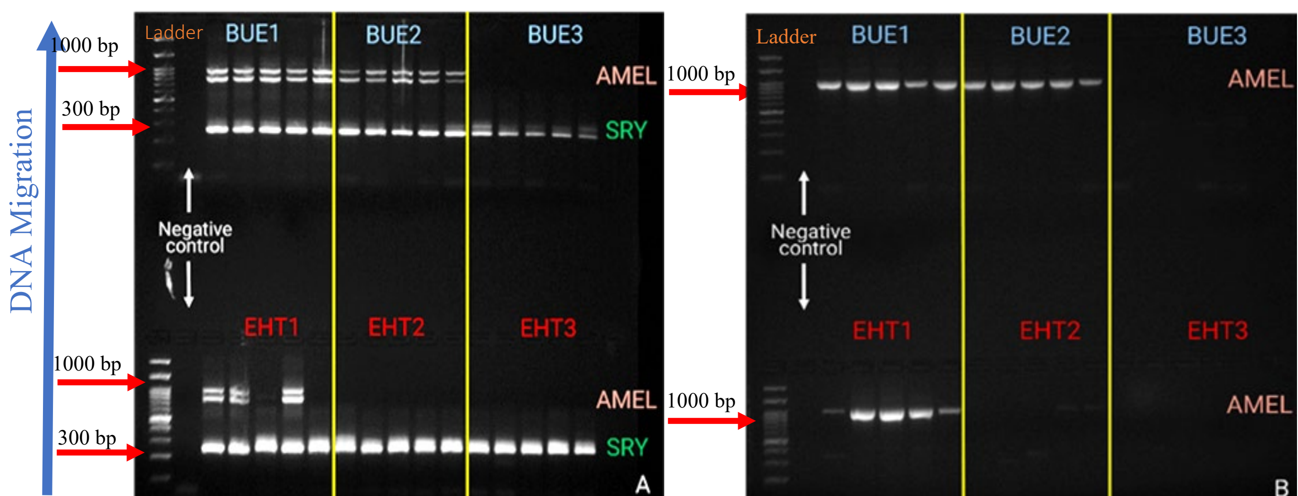

Figure 8: The findings of multiplex PCR of male (A) and Female (B) samples were obtained after burial under the earth for 1 month (BUE1), 3 months (BUE2), and 6 months (BUE3), andexposure to high temperatures of 100 °C (EHT1), 200 °C (EHT2), and 300 °C (EHT3). Each line of the ladder represents 100 bp, AMELX at 1022 bp, AMELY at 844 bp, and SRY at 265 bp.

4. Discussion

The process of discovering the identity of unidentified human remains holds significant importance for legal and social closure [28]. In situations where conventional means of identification are inadequate, forensic experts employ the creation of a biological profile as a method to narrow the search for potential identities. The initial and key step in constructing this biological profile involves determining the individual's sex [29]. In the field of forensic odontology, multiple techniques have been used for sex determination, including the examination of bone structure[30], soft tissue characteristics[31], and dental morphometry [32]. Nevertheless, molecular analysis emerges as the most precise technique for estimating an individual's sex [16].

This research has studied 70 third molars to evaluate the accuracy of SRY and Amelogenin genes obtained from dental pulp in sex determination, which were extracted because they were partially impacted or had periodontal diseases. The dental pulp's anatomical location affords exceptional protection against various environmental factors, preserving genetic material within human remains. This makes dental pulp an invaluable source of genetic material suitable for identification purposes [33]. Several techniques have been used for the collection of dental pulp samples for molecular analysis, which included endodontic access, grinding of the teeth, horizontal, and vertical sectioning of the teeth. In this study vertical section of the teeth has been used; Stavrianos *et al.* [34]suggested vertical sectioning for sound teeth, as it gains greater access to the pulp chamber which provides better dental pulp sampling than other techniques.

Several genes have been used for sex determination, such as SRY, AMEL, DYS14, and Y-encoded testis-specific protein genes, while the most widely used genes by scholars for sex identification are SRY and AMEL gene. However, the amplification of these genes for sex estimation faces certain limitations, primarily arising from gene translocations and deletions observed in several races. To overcome these challenges, multiplex PCR has been applied, enabling the simultaneous amplification of two or more genes at the same time.

Accordingly, the present study aimed to assess the efficiency of multiplex PCR-based amplification of SRY and AMEL genes in dental pulp obtained from tooth samples that were subjected to various forensic scenarios.

In the current study, the amount of DNA extracted from the dental pulp of teeth buried under soil for 1 month, 3 months, and 6 months significantly decreased compared to the amount of DNA obtained from the pulp of freshly extracted teeth. These findings align with prior research by Chowdhury *et al*. [35], where a decrease in DNA content was reported in their samples when assessing teeth subjected to burial for extended periods of 30, 60, and 90 days. This reduction in the amount of DNA is linked to the activation of diverse categories of intracellular enzymes, such as lipases, nucleases, and various classes of proteases that occur after cell death, which plays a crucial role in facilitating the DNA cleavage process [36].

The findings of the Kruskal-Wallis test indicate a statistically significant reduction in the DNA concentration within samples obtained from the EHT3 group in comparison to those belonging to the FET group. This observed decrease in DNA concentration can be attributed to the elevation in temperature, which causes the vaporization of water molecules associated with DNA. This vaporization, in turn, initiates hydrolytic reactions within the DNA molecule, resulting in the destabilization and gradual breakage of hydrogen bonds. Such bond disruption leads to the fragmentation of DNA when exposed to temperatures exceeding 110 °C. Moreover, the hydrolytic attack on DNA weakens the N-glycosyl bond, consequently promoting depurination and further diminishing the integrity of the DNA strand, ultimately resulting in its fragmentation. Additionally, high temperatures also induce hydrolytic deamination, with cytosine residues being the most frequently affected, resulting in the formation of uracil [37].

In this research, the SRY gene was amplified in all male samples of all groups. While this result was not detected in the female samples, based on SRY gene amplification the sex of all samples was determined with 100% sensitivity and specificity. Kholief *et al.* [38] and Khan *et al*. [17] found the same result as this study when they amplified the SRY gene from dental pulp samples. However, Reddy *et al.* [18] determined sex with 100% sensitivity and 73.3% specificity by amplification of the SRY gene

from a sample collected from oral epithelium, as four female samples showed false positive results. Reddy *et al.* attributed the low specificity in determining sex by using their methodology to the genetic disorders found in their study samples or the possibility of contamination of the female samples with male genetic material.

Regarding AMEL gene amplification, AMEL bands were detected in all samples collected from FET, BUE1, and BU2 groups and in 80% of the samples of the EHT1 group, while no AMEL could be noticed in the samples obtained from BUE3, EHT2, and EHT3. Lozano-Peral *et al*. [39], who conducted a study on thirty teeth that were exposed to temperatures ranging from 100 – 400 °C, found that DNA degrades between 100 °C and 200, and the molecular weight of the DNA will reduce to ≤ 200 bp. Their finding is similar to the result obtained from EHT2 and EHT3 groups of this study in which SRY gene (short length sequence) was amplified, while AMEL (AMELX 1022 bp and 844 bp for AMELY) were not detected. However, when Chowdhury *et al.* [28] subjected their samples to different environments (high temperatures between 150 °C, 250 °C, and 350 °C, and burial in soil for a period between 30 and 90 days), they successfully amplified the AMEL gene in all their samples except those subjected to 350 °C. This difference could be due to targeting the large sequence length of the AMEL gene in this study (1022bp for AMELY and 844bp for AMELX), as DNA quantity and quality are highly dependent on the temperature and duration [39,40].

This study is constrained by certain limitations. One limitation pertains to the challenge of replicating precise forensic scenarios, as naturally the teeth are covered by facial bones and soft tissue. Furthermore, an additional limitation was the necessity for an extended period of teeth burial over six months.

5. Conclusions

This study has demonstrated that dental pulp is a valuable source of DNA even after exposure of the teeth to harsh environments; furthermore, the amplification of the SRY gene shows 100% sensitivity and specificity in sex identification across all study groups. Even though amplification of the AMEL gene determined the sex of the samples belonging to FET, BUE1, BU2, and EHT1 groups with high sensitivity and specificity, it was not possible to amplify the AMEL gene in teeth samples that had been exposed to extreme temperature and burial in soil for more than 3 months due to the long length of the DNA sequence that was amplified.

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