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The Integrity of Deoxyribonucleic Acid (DNA) Extracted from Whole Teeth Samples Burnt with Different Accelerants Using Two Extraction Protocols for Forensic Sex Determination

*[1]Onyekachi O. Iroanya, [2]Abdul-Warith O. Akinshipo, [1]Tochukwu F. Egwuatu, [3]Jamey P. Mairiga
[1]Department of Cell biology and Genetics, University of Lagos, Nigeria.
[2]Department of Oral and Maxillofacial Pathology, College of Medicine, University of Lagos, Nigeria.
[3]Department of Biochemistry and Molecular Biology, Nasarawa State University Keffi, Nigeria.
*Corresponding Author's email: oiroanya@unilag.edu.ng

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Abstract
Sex determination is usually the first step in forensic identification of victim(s) in disasters, jungle justice and arson scenarios for many medical and legal reasons. The use of DNA in forensic analysis offers a good method in sex determination and the quality of extracted DNA is very important for downstream PCR. The aim of this study is to investigate the viability of DNA obtained from burnt teeth for forensic sex-determination. Two DNA extraction methods consisting of silica based commercial kit and phenol-chloroform organic method, followed by polymerase chain amplification of amelogenin gene for sex determination were employed. Based on the DNA yield and optical density values, the kit extraction method performed better than the phenol-chloroform method with 100% and 85.71% success respectively. A Mann-Whitney U test of 260/280 absorbance showed no statistically significant difference in the median absorbance for aviation fuel (median = 1.34) and gasoline (median =1.32) burnt samples. Amplification of AMEL genes using the commercial kit and phenol-chloroform method were 52.38% and 22.22% positive respectively compared to pre-extraction sex determination. This study shows that the use of the silica based commercial kit technique yielded higher DNA quality and quantity from whole teeth burnt with gasoline and aviation fuel as accelerants for downstream PCR amplification of AMEL genes compared to organic phenol chloroform method.

Keywords: Deoxyribonucleic acid, Sex determination, Amelogenin gene, Forensic odontology, Arson

1. Introduction

Forensic identification of victims and/or perpetrators is crucial in criminal investigations as well as humanitarian or civil cases. Disaster Victim Identification, (DVI) often involves comparing the unique post-mortem data available with their ante mortem data or data taken from their home or close relations. When a corpse is burnt, skeletonized, putrefied, old or badly damaged from mass disaster, its identification can be difficult and complicated, impeding a conclusive result on the identification of the victim [1]. In these situations, DNA profiling becomes a reliable tool for identification [2]. Sex determination as a form of DNA profiling can be used to identify cases in forensics [3-7].

For sex determination, DNA from different tissue has been used though the quality and quantity extracted is of essence. The chances of getting a good DNA yield are dependent on the type of tissue available post mortem [8]. Most tissues of the body putrefy within hours of death. However, out of the several tissues in the body, the teeth can withstand many of nature's elements and has thus been very useful in identification of victims.

Furthermore, forensic scientists have turned to DNA from the tooth to identify victims especially in burnt or heavily degraded human remains [9-12]. In forensic dentistry, the commonly used methods are organic (phenol–chloroform) method and the silica-based method which extracts DNA using ion exchange columns. Both methods have been made into commercial DNA extraction kits which are designed to yield high DNA content from samples. These two methods are most probably considerable improvements of PCR amplification and can be useful in poorly preserved, PCR resistant, ancient samples [2].

Generally, the fires from homicidal and accidental causes tend to destroy evidence so much that investigators find little hope of getting any evidence from the fire incidence [13]. However, with the rise of DNA analysis in forensics sciences, many researchers are looking into the robustness of DNA from biological fluids and tissues as an evidence that can endure the destruction of fire incidences. No tissue other than the tooth provides a suitable biological source of DNA in these scenarios [14]. These tissues having been exposed to accelerants have their DNA highly degraded. Consequently, the quality and quantity of extracted DNA and the method of DNA extraction are important factors in the downstream application of human identification using PCR and sequencing methods.

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The focus of this study was to determine if high quality DNA can be extracted from teeth samples burnt with two common fire accelerants (gasoline and aviation fuel). Since DNA degradation decreases the odds of getting a complete profile, it would be helpful to understand how fire degrades DNA in teeth samples. The two DNA extraction methods; one with a Quick-DNA Mini Plus kit and the other, the commonly used phenol chloroform DNA extraction method of mixing buffers were employed. This was followed by polymerase chain amplification of amelogenin gene for sex determination. The teeth samples were exposed to two fires ignited using accelerants which introduced more impurities and probably degraded the DNA in the samples. Gasoline, was used because it is an item that is frequently used to cover up crimes in the form of arson and in execution of jungle justice while aviation fuel is commonly seen in mass air disasters and pipeline explosions in Nigeria. The aim of this study is to investigate the viability of DNA obtained from burnt teeth for forensic sex-determination.

2. Materials and Method

2.1 Ethical Approval

The aim of the research was explained to the participants and they willingly consented to participate in the study. Preceding sample collection, short questionnaires were completed by all the study participants and informed written consent was gotten. The study was approved by the Health Research Ethic Committee of the College of Medicine, University of Lagos, with approval number CMUL/HREC/11/19/684.

2.2 Experimental Design

This was an experimental prospective study which was carried out over a period of six months at the Department of Cell Biology and Genetics, Faculty of science, University of Lagos. For this study a total of 21 teeth were used. The teeth were obtained in collaboration with the Dental Clinics at Lagos University Teaching Hospital (LUTH), Lagos State University Teaching Hospital (LASUTH) and Lagos State General Hospital Randle, Surulere, Lagos, Nigeria. These hospitals, regularly perform therapeutic extractions in living subjects. In addition, the health personnel of these centres and the patients that participated in this study also provided us with information relevant to research such as the patient's age, sex, type of tooth and dental history if any.

2.3 Inclusion and Exclusion Criteria

Non-carious disease-free teeth planned for orthodontic treatment were used. Only posterior teeth were used due to their potential to yield more DNA than anterior teeth. Lastly, the samples chosen were identified by sex and tooth type. Teeth from the anterior quadrant (incisors and canines), and teeth with any sign of disease such as caries were excluded from this study.

2.4 Sampling Technique

Purposive and convenience sampling was used. This is due to the difficulty of obtaining sound teeth samples. Teeth were collected from patients undergoing extraction for orthodontic purposes. Non-carious premolars and molar teeth from the dental arch were used. Additional data including the patient gender, age and tooth notation were collected.

2.5 Sample Preparation and Purification

Following extraction each tooth was rinsed with saline water to remove blood deposits and salivary coating. The decontamination process was done using sequential washes of 5% hypochlorite and 96% ethanol based on the method from previous studies [15]. Briefly the specimen was debrided by gentle mechanical cleaning using a sterile blade, immersed in 5% hypochlorite for 15 minutes, followed by washing with 96% ethanol and finally rinsed in distilled water. Each specimen was subsequently stored in labelled sterile containers. Universal protective principles including the use of laboratory coats, gloves and face masks which were changed periodically was followed to prevent external DNA contamination of samples.

2.6 Environmental Conditions (Fire Trials) and Timeline

The cleaned teeth were air dried, grouped, labelled and exposed to different accelerants - gasoline and aviation fuel. The tooth was placed in the crucible then saturated with gasoline/aviation fuel and the fire was ignited using a lighter for 10 min. One tooth at a time was burnt. During this period, additional wood or accelerant was added as necessary until it was 10 min, after which the fire was put off and the tooth removed from the fire. It was allowed to cool and was placed in a sterile bottle. All of the teeth were frozen immediately in -20°C until the time for DNA extraction. Burnt teeth samples were pulverized and grounded in a metal mortar and pestle to a fine powder and placed in labelled test tube. Two DNA extraction methods were used.

2.7 Deoxyribonucleic Acid (DNA) Extraction Protocol

2.7.1 Commercial Kit Extraction of DNA Samples

The first DNA extraction method involved using a kit (Quick-DNA Mini prep Plus Kit, Zymo® Research, California USA), which is systematically used in a wide variety of biological samples including decayed tissues. The kit's technology utilizes the selective property of a silica gel membrane that binds the DNA and allows the purification of the initial sample. One of the benefits of utilizing the kit is the possibility to obtain a final elution volume variable between 20 and 100 µL, allowing a maximum concentration of DNA. In this study, DNA was eluted to a minimal volume of 20 µL and was measured by using a spectrophotometer. The extraction was done according to manufacturer's instruction. Approximately

25 mg of sample was measured on a scale and placed in a microcentrifuge tube to which 100 µL of nuclease free water was added, followed by100 µL of solid tissue buffer and 10 µL of proteinase K. The solution was mixed thoroughly and incubated at 55 °C for 3 h. The mixture was then centrifuged at 12,000 rpm for 1 min and the aqueous supernatant was carefully decanted into a clean tube. To the supernatant, 2 volumes of genomic binding buffer was added, depending on the volume obtained for each sample. For example, 400µL of genomic binding buffer was added to supernatant samples that were 200 µL. The mixture was mixed thoroughly and transferred to a Zymo® spin IIC-XL column in a collection tube. This was then centrifuged for 1 min. The liquid in the collection was discarded and a new collection tube was placed and 400µL of DNA free wash buffer was added to the column. The mixture was centrifuged for 1 min, collection tube emptied then 700µL of g-DNA wash buffer was added to the column and centrifuged for 1 min. After discarding the collection tube, a final 200µL of g-DNA wash buffer was added to the column and centrifuged for 1 min. The collection tube was discarded with the flow through. DNA obtained was transferred to a clean microcentrifuge for elution. This was done by adding 50µL DNA elution buffer to the tube, incubated for 5 min and centrifuged for 1 min. The final DNA product was stored at -20°C until it was used for spectrophotometry and polymerase chain reaction.

2.7.2 Phenol-chloroform Extraction of DNA Samples

The second DNA extraction method was Phenol-chloroform (organic) extraction. This was carried out using the protocol of Kumar and Aswath [16]. Approximately 40mg of pulverised teeth samples were incubated at 55°C for 3 days in a mixture containing 500 µl of 0.5M EDTA, 1 ml of TRIS NaCl EDTA (TAE) buffer with sodium dodecyl sulphate (SDS) and 10 µl of proteinase K. After incubation, the entire lysate was transferred into 2ml Eppendorf tubes. Phenol chloroform was added to each lysate in a ratio 1:1, pulse vortexed for 15 seconds, incubated at room temperature for 5 min then centrifuged at 10,000 rpm for 10 min in refrigerated centrifuge. The supernatant was collected into a fresh 1.5ml tube into which an equal volume of isopropanol was added. The mixture was incubated for 30 min at room temperature, centrifuged at 10,000 rpm for 15 min in refrigerated centrifuge. The supernatant was discarded and the pellet obtained was washed twice with 70% ethanol. The DNA pellet was thereafter dissolved in 20 µl nuclease free water (NFW) and stored at –4°C until the PCR procedure.

2.8 Determination of Concentration and Purity of Extracted DNA Samples

A quantitative spectrophotometric assay of genomic DNA samples was done to determine the concentration and purity of extracted DNA samples using Nanodrop 1000 spectrophotometer (ND-1000 Spectrophotometer; Nanodrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each DNA sample was used directly. Absorbance was measured at wavelengths of 260, 280 and 230 nm, which are maximum absorption peaks for bases in nucleic acids, proteins and polyphenols/polysaccharides, respectively. DNA was quantified by measuring absorbance at 260nm and 280nm (A260/A280) and 260nm and 230nm (A260/A230). The absorbance quotient (O.D260/O.D280) value of 1.7-2.0 was considered to be purified DNA. An OD260/OD280 ratio of <1.8 is indicative of protein contamination while an OD260/OD280 ratio of ≥2.0 indicates RNA contamination [17].

2.9 Polymerase Chain Reaction (PCR) Amplification

The primers described by Sullivan *et al.* [18] were used for downstream PCR reaction. These primers target a 6-bp insertion/deletion within an intron of the amelogenin gene on the X and Y chromosomes (AMELX and AMELY) and produce 106-bp and 112-bp amplicons for the X and Y chromosomes, respectively [18]. These consisted of: AMEL X gene: 5′-CCCTGGGCTCTGTAAAGAATAGTG-3′andAMEL Y gene: 5′ATCAGAGCTTAAACTGGGAAGCTG- 3. (Table 2.1)

The amplification reactions were made for a final volume of 50µL, containing 25µL of master mix, 1µL of forward primer, 1µL of reverse primer, 5µL of DNA, and 18µL of distilled nuclease free water. The DNA amplifications were done using ABI Prism 7900 Thermal Cycler under the following conditions: 3 min at 95°C (initial denaturation), followed by 40 cycles of 30 s at 95 °C (denaturation), 45 s at 60 °C (annealing) and 45 s at 72°C (extension), with final extension for 10 min at 72°C. The products from the amplifications were separated in 2% agarose gel, crowned with Syber gold (Invitrogen), using the 100 pb Ladder 50 µg (1.0 µg/µL) (Invitrogen) marker and observed under an ultra-violet light and registered in a digital UVP EpiChemi Blue Darkroom Gel Imager.

Table 2.1: List of Primers Used for this Study

Primer	Sequence	Product Size
Amelogenin X	CCCTGGGCTGTAAA GAATAGTG	X = 106 bp
Amelogenin Y	ATCAGAGCTTAAAC TGGGAAGCTG	Y = 112bp

2.10 Statistical analysis

All numeric data were subjected to SPSS software analysis, version 21.0. Continuous data was checked for normality using Shapiro-Wilk's methods [19]. All data are presented as mean ± standard deviation, unless where normality was violated, in which case data was summarized as median and interquartile range.

Independent T-test was used to find the differences between groups with normal gaussian distribution and Mann-Whitney U in cases where test of normality was violated. P values <0.05 were considered significant.

3. Results and Discussion

3.1 Demographics of the study population

The study population consisted of twenty-one teeth samples for both the kit and phenol/chloroform extractions. 10 of the samples were from 5 individuals, while the rest of the samples were from 10 different individuals. The samples consisted of 4 pairs of teeth from 4 males and 1 pair from a female. The remaining 11 samples consisted of teeth samples from6 males and 5 females. Furthermore, 14 teeth were premolars while 7 were molars (Table 3.1).

Table 3.1: Burnt Teeth Samples with Accelerants Used, Teeth Type and Sex

S/N	Tooth type	Gender	Accelerant used
1	Premolar	Male	Aviation
2	Premolar	Male	Aviation
3	Premolar	Male	Aviation
4	Premolar	Female	Aviation
5	Premolar	Male	Gasoline
6	Premolar	Female	Aviation
7	Premolar	Male	Gasoline
8	Premolar	Male	Gasoline
9	Premolar	Male	Gasoline
10	Molar	Female	Aviation
11	Molar	Male	Gasoline
12	Molar	Male	Aviation
13	Molar	Female	Aviation
14	Premolar	Female	Gasoline
15	Premolar	Male	Gasoline
16	Premolar	Male	Gasoline
17	Molar	Female	Gasoline
18	Molar	Male	Gasoline
19	Premolar	Male	Aviation
20	Premolar	Male	Gasoline
21	Molar	Female	Aviation

3.2 Deoxyribonucleic Acid (DNA) Extraction

DNA was successfully extracted in all samples using the commercial kit (Table 3.2) while the DNA extraction was successful in 18 out of 21 samples using the phenol/chloroform method (Table 3.2). The kit method is based on a Spin-column extraction method which is a solid phase extraction method, in which the target molecules bind to immobilized silica in the column [20]. Many studies have used the popular phenol/chloroform method to extract DNA from teeth [4, 5, 22], while some have used silica method for DNA extraction in teeth [23]. The wide spread use of phenol/chloroform is due to the fact that it is relatively less expensive than other methodologies and can be used for many hard/tough substances. However it involves the use of some potentially toxic substances and can contaminate proteins [24]. Using traditional methods, such as phenol-chloroform, teeth samples can be challenging to process, resulting in low-quantity and/or quality nuclear DNA and insufficient profiles for comparisons [25]. Both methods were used to evaluate the DNA yield in a resource limited setting.

The kit extraction method performed better than the phenol chloroform method based on the DNA yield and optical density values. The median DNA yield of kit extraction method was 9ng/µL while that for manual extraction was 3.45ng/µL. This finding is similar to some studies in which the silica-based extraction method yielded more DNA than the phenol/chloroform method [4,14]. The silica-based method has been shown to be a reliable alternative to the phenol/chloroform technique. It improves the yield because it does not require a DNA precipitation stage, there is decreased protein contamination and reduces the risk of exposure to hazardous chemicals [23]. The relatively lower yield of DNA that was obtained using the chloroform method in the present study is similar to that reported by Edson *et al.* [26] and Vemuri *et al.*[27] in which phenol-chloroform yielded a lower concentration compared to other methods used in their studies. The low yield of DNA in both methods used in this study maybe connected to the presence co-extracting impurities from accelerants and the high calcium content due to the use of the whole tooth which can act as PCR inhibitors [28]. In the study of Edson [25], which used teeth samples tested in AmpFlSTR_ MiniFiler, a silica-based extraction kit, 16/70 teeth tested positive for STR loci, of which only 4 loci were positively identified.

3.3 Quantitative Assessment of DNA in Commercial Kit and Phenol Chloroform Methods

A test of normality was done using Shapiro-Wilk's test since the samples were less than 50. In samples extracted via the kit method, absorbance at 260/280 was normally distributed for aviation fuel-based samples ($p = 0.24$) but not for gasoline-based samples ($p = 0.001$). However, absorbance at 260/230 was not normally distributed in both aviation and gasoline burnt sample ($p = 0.001$). DNA concentration was also not normally distributed in gasoline and aviation burnt samples respectively ($p =$ 0.027 and 0.047). For this reason, non- parametric statistical test, Mann-Whitney U test was used to determine if there were statistically significant differences in the absorbance and DNA concentration scores in the two groups of burnt samples (Table 3.3).

Table 3.2: Spectrophotometric Values of DNA Samples Obtained from the Teeth Samples Using Commercial Kit Extraction and Phenol-Chloroform Methods

S/N	Commercial kit values			Phenol chloroform extraction values		
	260/280	260/230	ng/µL	260/280	260/230	ng/µL
1	1.35	0.02	5.3	0.85	0.04	1.4
2	1.46	0.05	14.9	0.11	0.01	0.2
3	1.46	0.05	19.8	0.96	0.07	3.3
4	1.33	0.09	35.8	1.04	0.09	3.9
5	1.33	0.02	6.0	0.76	0.17	3.7
6	1.47	0.03	11.6	0.61	0.26	2.3
7	1.23	0.03	6.4	0.60	0.37	3.3
8	1.42	0.03	9.2	0.85	0.09	4.9
9	1.39	0.07	16.5	0.75	0.11	2.5
10	1.35	0.18	48.5	0.72	0.04	1.5
11	2.21	0.00	1.4	1	0.07	3.6
12	1.24	0.03	6.6	1.29	0.27	17.9
13	1.22	0.03	4.7	1.32	0.52	14.6
14	1.41	0.01	2.4	0.80	0.13	2.9
15	1.32	0.03	8.5	0.78	0.04	2.2
16	1.33	0.03	9.0	-	-	BDL
17	1.38	0.02	7.4	0.97	0.11	4.4
18	1.34	0.07	26.2	0.94	0.33	6.6
19	1.18	0.02	38	1.34	0.11	9.6
20	0.92	0.14	26.0	-	-	BDL
21	1.25	0.01	5.1	-	-	BDL

Key: BDL = below detection limit

A Mann-Whitney U test of 260/280 nm absorbance showed that there was no statistically significant difference in the median absorbance scores for aviation (median = 1.34) and gasoline (median 1.32) burnt samples. U = 47.50, p = 0.589. A Mann-Whitney U test was also used to compare absorbance score at 260/230 in burnt samples. In this instance, the mean rank scores 260/230 absorbance values were not statistically significant different between aviation (mean rank = 12.30) and gasoline (9.82) burnt samples. U = 42.00, p = 0.360. Lastly, the difference in the DNA concentration value of burnt samples was also done using the Mann-Whitney U test. There was no statistically significant difference between the median DNA concentration values of aviation (13.25) and gasoline (8.50). U= 58.00, p = 0.832 (Table 3.3).

The test of normality for the phenol chloroform manual method for DNA extraction was also done using Shapiro-Wilk's test since the samples were less than 50. Absorbance at 260/280 was normally distributed for both aviation and gasoline burnt samples. (p=0.35, 0.67). In this case an independent t-test was used to find the difference in mean absorbance values. Absorbance at 260/230 was not normally distributed in both aviation and gasoline burnt sample (p=0.000), while DNA concentration was also not normally distributed in gasoline and aviation burnt samples respectively (p= 0.038 and 0.047). For this reason, non- parametric statistical test, Mann-Whitney U test was used to determine if there were statistically significant differences in 260/230 absorbance and DNA concentration values in burnt samples. An independent t-test of 260/280 absorbance showed that absorbance scores were statistically significantly different between aviation and gasoline burnt samples. (F=6.207, p= 0.024). A Mann-Whitney U test for absorbance at 260/230 showed that the distribution of the absorbance score for aviation and gasoline burnt samples were similar as assessed by visual inspection. In this situation median absorbance scores were not statistically significantly different for aviation (median = 0.11) and gasoline (median = 0.13) burnt samples. U= 47.50, p = 0.605. A Mann-Whitney U test for DNA concentration showed that the distribution of DNA concentration was not similar as assessed by visual inspection. In this case, the mean rank scores of DNA concentration for aviation and gasoline burnt samples was evaluated. Mean rank scores of DNA concentration values were not statistically significantly different between aviation (mean rank = 9.17) and gasoline (mean rank = 9.83) burnt samples. U = 43.50, p = 0.796 (Table 3.4).

Table 3.3: Descriptive Statistics for Spectrophotometric Values of DNA Obtained Via Kit Extraction Method

Absorbance	Accelerant used	No. of samples	Min value	Max value	Mean (SD)	Median (interquartile range)	Shapiro-wilk's test of normality (p-value)	Test statistics (p-value)
@260/280	Aviation	10	1.18	1.47	1.33 (0.11)	1.34 (0.23)	0.904 (0.24)	47.50 A
	Gasoline	11	0.92	2.21	1.40 (0.30)	1.32 (0.09)	0.715 (0.001)	(0.589)
@260/230	Aviation	10	0.01	0.18	0.05 (0.05)	0.03 (0.05)	0.726 (0.002)	42.00 B
	Gasoline	11	0.00	0.14	0.04 (0.03)	0.03 (0.05)	0.796 (0.008)	(0.360)
DNA conc.	Aviation	10	4.7	48.5	19.03 (16.07)	13.25 (31.1)	0.842 (0.047)	58.00 C
ng/µL	Gasoline	11	1.4	26.2	10.81 (8.51)	8.5 (10.5)	0.835 (0.027)	(0.832)

Key: A= Independent t-test B= Mann Whitney U (Comparison of median)
C = Mann Whitney U (Comparison of mean Rank) * = Statistically significant

Table 3.4: Descriptive Statistics for Spectrophotometric Values of DNA Obtained Via Phenol-Chloroform Method

Absorbance	Accelerant used	No. of samples	Min. value	Max. value	Mean (SD)	Median (interquartile range)	Shapiro-Wilk'stest of normality (p-value)	Test statistics (p-value)
@260280	Aviation	9	0.11	1.34	0.92 (0.40)	0.96 (0.64)	0.916 (0.35)	6.207 ^{A}
	Gasoline	9	0.60	1.00	0.83 (0.12)	0.80 (0.20)	0.948 (0.67)	(0.024) *
@260230	Aviation	9	0.01	7.00	0.93 (2.28)	0.11 (0.36)	0.450 (0.000)	47.50 ^{B}
	Gasoline	9	0.04	7.00	0.93 (2.27)	0.13 (0.25)	0.432 (0.000)	(0.605)
DNA conc.	Aviation	9	0.20	17.80	6.06 (6.39)	3.30 (10.65)	0.823 (0.038)	43.50 ^{C}
ng/µL	Gasoline	9	2.20	6.60	3.79 (1.35)	3.60 (1.95)	0.929 (0.470)	(0.796)

3.4 Comparison of DNA quantity in burnt samples

With respect to the differences in DNA concentration between the two methods, the kit method produced a purer and higher concentration of DNA than the manual method (Tables 3.3 and 3.4). Based on the accelerants used, the median values of DNA quantity in aviation burnt samples obtained from the kit was more than that obtained from the gasoline samples, albeit not statistically significant (Tables 3.3). Also, the median values of DNA quantity in aviation burnt samples obtained from the phenol/chloroform method was more than that obtained from the gasoline samples (Tables 3.4). DNA extracted from aviation fuel burnt teeth was higher than those from gasoline in the kit method while gasoline burnt teeth had a slightly higher DNA content in the manual method. The reason for the higher DNA content in the aviation burnt samples might be connected to the fact that aviation fuel burns more slowly than gasoline fuel and this might reduce the deleterious effect on the DNA content of the teeth. In the study by Chowdhury *et al*. [7], ten teeth incinerated at 150°C yielded an average DNA content of 26.19µg/ml while those incinerated at 250 °C yielded an average of 13.99 µg/ml of DNA. DNA could not be obtained at higher temperatures. Vemuri *et al*. [27] did not obtain DNA from teeth samples incinerated above 250 °C. In the study, DNA extracted with the commercial kit method yielded as much as 48ng/µL with a median value of 13.25 ng/µL. While these past studies did not use accelerants in the same way that the current one did, the comparison is relevant because the teeth were subjected to high temperatures. It can be inferred that temperatures in the region of 100-200 °C will give enough DNA quantity for downstream applications, while higher temperature above 300 °C will not yield DNA. Urbani *et al*. [29] in 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.

3.4 Amplification of AMEL genes in the commercial kit samples

Estimation of sex from the burnt teeth samples was performed via a conventional polymerase chain reaction (PCR) process to determine the two regions of the AMEL genes. Positive male gender was identified in 5/21 cases while 15 were designated as females (Plates 3.1 and 3.2). In Plate 1, lane "L" shows DNA marker (100–500 bp) used for analysis. Lanes 1 – 21 shows PCR reaction in samples extracted with commercial kit method. Lanes 1,3,5,8,11 showed two (2) bands, one at approximately 112 bp and other at approximately 106 bp, thereby confirming the samples to be males, while lanes 2,4,6,7,9,10,13-16 showed only a single band, confirming the samples to be females. Sex determination was correct in (that is, those in agreement with the known sex of the donor) 12/21 cases, consisting of 5 males and 5 females (Table 3.5).

Plate 3.1: Gel Picture Showing Identification of Sex, Based on Polymerase Chain Reaction Analysis of Kit Extracted Samples.

In Plate 3.2, Lanes17 – 21 showed PCR reactions in samples extracted with kit method and each of the lanes shows only a single band, confirming the samples to be females. Lanes 22-32 (Plate 3.2) showed PCR reactions in samples extracted with the manual phenol chloroform method. Lane 25 appears as a male since it has a dual band. Lanes 22-24, 26, and 28-30 are all females.

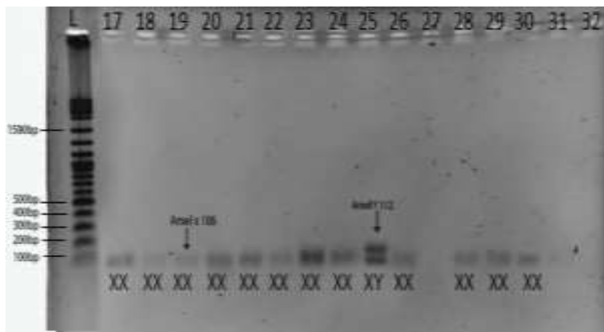

Plate 3.2: Gel picture showing identification of sex, based on polymerase chain reaction analysis.

3.5 Amplification of AMEL genes in the phenol-chloroform method

Four out of the 21 phenol chloroform extracted DNA samples appeared as males. Seven samples did not appear on the final agarose gel table and 10 out of the 21 samples were evaluated as females (Plates 3.2 and 3.3). Sex determination was correct in 3/21 cases, consisting of 2 males and 1 female (Table 3.4). In Plate 3.3, bands on lanes 33, 41 and 42 appeared as female while bands in lanes 37-39 appeared as males.

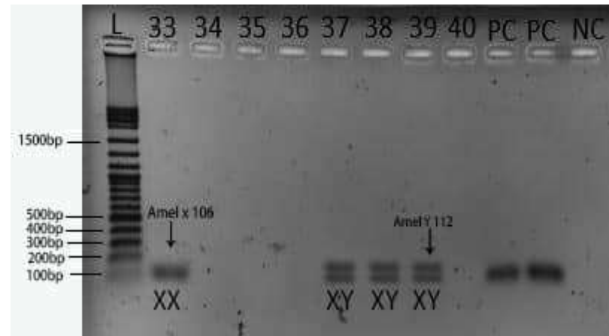

Plate 3.3: Gel Picture Showing Identification of Sex, Based on Polymerase Chain Reaction Analysis of Phenol Chloroform Extracted Samples.

Our study revealed two bands of 112bp and 106 bp corresponding to a positive male sample in the kit extraction. The two bands shown in this study corresponds to the bands gotten by Sullivan *et al.* [18] which is the commonest method employed to identify sex using amelogenin gene. Nayar *et al.* [30] was able to determine sex with an accuracy ranging from 86-100% from samples exposed to the environment with 96bp and 112bp bands on gel electrophoresis. In the study of Sivagami *et al*. [31] which extracted DNA from 10 single tooth, sex determination revealed two bands of 330 and 236bp in all six male samples and a single band of 330bp in all female samples. Thangaraj *et al.* [32] observed X-specific bands at 212 bp and Y-specific bands at 218 bp distinguishing males with both bands from females with the 212bp band.

3.6 Comparison of sex determination by PCR in extracted samples

Pre-DNA extraction sex status was compared to post DNA extraction sex status in Table 3.5. A concordance was observed in four samples with pre-DNA extraction sex status of participants. The discrepancy in uniformity of sexes before DNA extraction and after DNA extraction in our samples could be due to many factors, foremost of which is the introduction of impurities while burning and presence of calcium and other potential sources of PCR inhibitors. Sex of all individuals were recorded, before their extraction so that it could be tallied and verified after PCR analysis and gel electrophoresis. Twelve samples tallied overall in the kit extraction while 3 samples tallied with pre-extraction sex status of participants in the phenol chloroform extracted samples. Many studies have determined sex from teeth remains using molecular methods with varying degree of accuracy [4,5,16,30,33]. However, this study had a limitation; a control tooth was not included to check relative DNA quantity to burnt samples.

Table 3.5: Comparison of Sex Determination by PCR in Commercial Kit and Phenol-Chloroform Methods

Serial number kit	Pre- extraction sex determination (A)	Commercial kit sex determination (B)	Agreement between A and B	Serial number phenol/ Chloroform	Phenol-chloroform sex determination (C)	Agreement A and C
1	Male	Male	Yes	22	Female	No
2	Male	Female	No	23	Female	No
3	Male	Male	Yes	24	Female	No
4	Female	Female	Yes	25	Male	No
5	Male	Male	Yes	26	Female	No
6	Female	Male	No	27	Void	No
7	Male	Male	Yes	28	Female	No
8	Male	Female	No	29	Female	No
9	Male	Male	Yes	30	Female	No
10	Female	Male	No	31	Void	No
11	Male	Female	No	32	Void	No
12	Male	Male	No	33	DND	-
13	Female	Female	Yes	34	Female	Yes
14	Female	Female	Yes	35	Void	No
15	Male	Female	No	36	Void	No
16	Male	Female	No	37	DND	-
17	Female	Female	Yes	38	Void	No
18	Male	Female	No	39	Male	Yes
19	Male	Female	No	40	Male	Yes
20	Male	Male	Yes	41	Male	Yes
21	female	Female	Yes	42	Void	-

4. Conclusion

Amelogenin gene is a reliable means of identifying individuals even when samples are small as obtained in forensic cases. The use of a silica based commercial kit technique yields DNA of a higher quality and quantity for downstream PCR amplification than organic phenol chloroform method from teeth burnt with gasoline or aviation fuel as accelerants. From our study, it is advisable to use the kit in extracting the DNA from forensic samples, however, the phenol/chloroform technique is suitable in a resource limited setting - the use of which requires attention to decalcification stage in the protocol to yield enough DNA.

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