

Amelogenin Gene Failure in Sex Determination of Dry Human Teeth Specimens from Sokoto, Northwestern Nigeria.

AD Zagga,¹ H Ahmed, OON,² AA Tadros¹ and SM Ismail.³

¹Department of Anatomy, College of Health Sciences, Usmanu Danfodiyo University, Sokoto, Sokoto State, Nigeria.

²Department of Paediatrics, College of Health Sciences, Usmanu Danfodiyo University, Sokoto, Sokoto State, Nigeria.

³Department of Medical Molecular Genetics, Division of Human Genetics and Genome Research, National Research Centre, Cairo, Egypt.

ABSTRACT: *The study of teeth reveals a lot concerning forensic medicine. Particularly it is useful in human identification. In addition to determination of age, sex can also be determined from teeth. The genetic difference between males and females is defined by the presence or absence of the Y-chromosome. The majority of the DNA of the sex chromosomes is specific to either the X or Y form. There are regions of homology between the two sex chromosomes that are also useful targets for genetic sex typing of samples. Materials and Methods: A single blind study for genetic sex identification using amelogenin gene multiplex primers on dry human teeth specimens from Sokoto, Northwestern Nigeria, was undertaken, from 2009 through 2011. No genetic sex identification was achieved in any of the dry human teeth samples. For each group of teeth, PCR Sensitivity = 0%, Specificity = 0%, Predictive value of positive test = 0%, Predictive value of negative test = 0%, False positive rate = 0%, False negative rate = 0%, Efficiency of test = 0%. Fisher's exact probability test $P = 1$. Z-test: z- and p values were invalid. No genetic sex identification was achieved in any of the dry human teeth samples. Therefore, amelogenin PCR for genetic sex identification should not, at least for now, completely replace traditional morphological methods of sex identification. This is the first known study that used amelogenin gene multiplex primers to determine the sex of human dry teeth specimens by means of PCR in Nigeria. There is need for further studies in Nigeria to complement the findings of this study.*

KEYWORDS: *Amelogenin, failure, sexing, teeth, Sokoto.*

I. INTRODUCTION

Determination of sex using skeletal remains presents a great problem to forensic experts especially when only fragments of the body are recovered. Forensic dentists can assist other experts to determine sex of the remains by using teeth and skull. [1] However, discrimination between male and female teeth is extremely difficult, morphologically. Although, male teeth are usually larger than female teeth, there is conflicting evidence as to the reliability of sex determination from tooth size. [2] Such measurements are unreliable in determining the sex of an individual. [3]

In instances where antemortem records are not available for comparison, or where destruction of the dentition by fire leaves too little dental data for comparison, alternative methods of identification are required. A valuable source of DNA evidence is contained within dentition, and this can be extracted and profiled to confirm identity by comparison to a known antemortem sample or to DNA profiles from relatives of the individual.[4] With the development of the polymerase chain reaction (PCR), it has become possible to analyze DNA that is severely degraded, of low copy number, and present only in minute quantities for accurate sex determination of the remains.[5] Alternative approaches to sex determination of DNA samples involve investigation of regions of the *amelogenin* gene. This is the gene that encodes tooth enamel and is present on both the X- (Xp22.1–Xp22.3) [6] and Y- (Yp11.2) chromosomes.[7] The rareness of failures in sex determination provides confidence in current techniques, but the *amelogenin* locus alone cannot anymore be considered as infallible. The phenotypic nature of the information provided by investigation of the *amelogenin* locus could potentially present massive problems if an error occurs that is not identified. The failure of a forensic DNA profiling technique to correctly resolve an issue as simple as sex could seriously misdirect the police investigation, as well as cast doubts on the techniques' reliability for criminal prosecution.[8]

The general objective of this study was therefore, to apply PCR-based method using *amelogenin* gene multiplex primers, to undertake genetic sex identification of dry human teeth specimens from Sokoto, Northwestern Nigeria.

II. MATERIALS AND METHODS

A total of nine dried human teeth specimens were grouped into three for this study, as follows;

- A. Three dry teeth samples from embalmed cadavers. They included; 1 canine and 2 molars. Very dark brown in colour. Dry, and mildly brittle. Although mildly eroded, contained a crown, root and closed pulp cavity.
- B. Three dry teeth specimens (for disposal) from adult patients. They included; 1 molar, 2 premolars. White in colour. Dry and hard. Contained a full crown and root with a closed pulp cavity.
- C. Three deciduous teeth specimens. All were incisors, white in colour. Dry and hard. Comprised of only the crown. No root. Empty, very shallow, dried pulp cavity.

This study was a single blind type. Information about the morphological sex of the teeth samples was withheld from me by my supervisors. Samples of embalmed cadaveric teeth specimens were collected by my colleagues from the remains of the dissected cadavers in the Department of Anatomy, College of Health Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria. Teeth samples extracted (for disposal) from patients were collected by my colleagues from the Dental Unit, Usmanu Danfodiyo University, Teaching Hospital, Sokoto, Nigeria. Deciduous teeth were provided by my supervisors, in Nigeria, and handed over to me (the investigator). All the samples were collected in 2009. The laboratory experiment was carried out at the Department of Medical Molecular Genetics, Division of Human Genetics and Genome Research, National Research Centre, Cairo, Egypt, from 2010 through 2011.

2.1 Precaution Against Contamination

In addition to the specific and stringent precautions against contamination recommended by Cooper and Poinar, (2000) [9] while handling ancient hard tissues for molecular analysis, other peculiar precautions were undertaken to handle the dry teeth samples before grinding. Samples of the dry teeth were initially placed in a freezer at -20°C (for minimum of 72 hours) to eliminate surface contamination from the depositional environment and post depositional handling. With gloved hands, each tooth was held with a sterile forceps (CE Stainless Pakistan) and washed under running tap. The surfaces of the tooth were brushed with an abrasive paper. The tooth was then cleaned with 4% hypochlorate bleach (4ml chlorex + 96ml distilled water). The surfaces of the tooth were further brushed with a tooth brush. The tooth was rinsed with distilled water, then placed on a piece of a sterile aluminium foil in a hood and exposed to UV light for 15-30 minutes, before grinding. The equipment and surface of the hood were cleaned with distilled water, sterilised with 70% ethanol (Art-Nr. K928.3 ROTH. Ethanol vergällt. ≥99.8%, mit Ca. 1% MEK. Carl Roth GmbH + Co. KG. 2.5L. Schoemperslenstr. 3-5, 76 185 Karlsruhe) and UV irradiated before and after grinding each sample. A sheet of aluminium foil (Helwan Aluminium Foil. 15M x 40CM. Made in Egypt) sterilized with 70% ethanol (Art-Nr. K928.3 ROTH. Ethanol vergällt. ≥99.8%, mit Ca. 1% MEK. Carl Roth GmbH + Co. KG. 2.5L. Schoemperslenstr. 3-5, 76 185 Karlsruhe) was placed on the surface of the hood. Samples were further sterilised with 70% ethanol and a sterile soft tissue was used to absorb excess ethanol from the sample (to dry the sample and minimize the PCR inhibitory effect of alcohol) before grinding. Each sample (a whole tooth at a time) was then placed in a sterile mortar and pestle (MN 100cl), for pulverization. Pulverization continued until the tooth turned into powder form. Aliquot of the ground tooth powder was then transferred into 1.5ml microtubes (Bio Basic Inc. (BBI). Cat. No. BT620NS – 100. Sterilized 1.5ml microcentrifuge certified RNase DNase and pyrogen – free. Lot. No. 08112) and stored in a refrigerator (Elite. Air Multi=flow. Freezer and Refrigerator. No frost) at -80°C, before DNA extraction.

2.2 DNA Extraction

The extraction of DNA from all the samples was done by standard phenol-chloroform method established by the Department of Medical Molecular Genetics, Division of Human Genetics and Genome Research, National Research Centre, Cairo, Egypt (2010) [10]. The samples consisted of about 0.5mg aliquot of the ground tooth powder. The equipment and surface of the DNA extraction hood were cleaned with distilled water, sterilised with 70% ethanol (Art-Nr. K928.3 ROTH. Ethanol vergällt. ≥99.8%, mit Ca. 1% MEK. Carl Roth GmbH + Co. KG. 2.5L. Schoemperslenstr. 3-5, 76 185 Karlsruhe) and UV irradiated. A sheet of aluminium foil (Helwan Aluminium Foil. 15M x 40CM. Made in Egypt) sterilised with 70% ethanol (Art-Nr. K928.3 ROTH. Ethanol vergällt. ≥99.8%, mit Ca. 1% MEK. Carl Roth GmbH + Co. KG. 2.5L. Schoemperslenstr. 3-5, 76 185 Karlsruhe) was placed on the surface of the hood. To each 0.5mg of tooth powder contained in 1.5ml microtubes (Bio Basic Inc. (BBI). Cat. No. BT620NS – 100. Sterilized 1.5ml microcentrifuge certified RNase DNase and pyrogen – free. Lot. No. 08112), 600 µl extraction buffer (8% D sucrose, 50mM EDTA, pH 8, 50mM Tris HCL, 5mM sodium acetate, 5mM ammonium acetate, 5.5% triton X-100) was added, using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 100-1000µl (Sr No:

712689). The tubes were shaken and vortexed using Retsch Mixer. 220V 25W, to ensure thorough mixing of the contents. To the above mixture, 600 µl phenol (BioFlux. Biozol-RNA/DNA extraction reagents for phenol, equilibrated, stabilized. Volume: 100ml. Storage: 2-8°C. Lot. No: 20090701. Cat. No: BSA02M1), taken from the lower layer was added, using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 100-1000µl (Sr No: 712689). The tubes were shaken, vortexed using Retsch Mixer, then sealed with Para film foil (PARRAFILM “M.” 4 W x 125 FT. ROLL. RECHINEY PLASTIC PACKAGING. MENASHA, WI 54952. CHICAGO, IL. 60631), to avoid spillage of the contents, before being mounted and left on a mixer (Rotator. Model No.:Simi/Rotator. S. No.:1003. Lot No.:800 Rot. 1H. Volt: 220. Wat: - 30. Made in Egypt), at its maximum speed, for 72 hours. The tubes were fixed on the rotator with a solutape (Nova premium quality. Made in UAE). The Para film seal was removed from the tubes. The tubes were then centrifuged in a microcentrifuge (SiGMA Laborzentrifugen, D -37520 Osterode am Harz, Germany. Model =1K15. Serial No =69422. Year =1999. Max. Speed (dependent on rotor) rpm =5300. Max. Capacity (density of material max 1.2kg/dm² =53ml. Kinetic energy Nm =4980. Total power consumption W =450. Electrical supply V/HZ =230/50 1Ph. Input fuse A =6, 3 AT. Interference suppression acc to EN 55011 cl. B. Made in Germany), at 14,000 rounds per minute (rpm) for 15 minutes. About 500 µl from the supernatant of each sample was transferred into a new 1.5ml microtube (Bio Basic Inc. (BBI). Cat. No. BT620NS – 100. Sterilized 1.5ml microcentrifuge certified RNase DNase and pyrogen – free. Lot. No. 08112), using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 100-1000µl (Sr No: 712689). To each supernatant 500 µl chloroform (HPLC grade. Rankem Chloroform. M. W. 119.38. Product code: CO580. Pack: 1 Litre. Batch no: R155C06. Ranbaxy, Fine Chemicals Limited, New Delhi, India) was added, using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 100-100µl (Sr No: 712689). The tubes were vortexed and centrifuged again as done previously. About 500 µl from the supernatant of each sample was transferred into a new 1.5 ml microtube (Bio Basic Inc. (BBI). Cat. No. BT620NS – 100. Sterilized 1.5ml microcentrifuge certified RNase DNase and pyrogen – free. Lot. No. 08112), using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 100-1000µl (Sr No: 712689). To each supernatant, 500 µl Isopropanol (LAB-SCAN analytical sciences. PROPAN-2-OL HPLC. 2.5L. Code No. C19C11X. Batch No. 0731/9. Manufacturing Date: Feb 2009. Expiration Date: Feb 2012. POCH SA. 44-101 Gliwice, ul. SowinsKiego 11, Poland) was added, using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 100-100µl (Sr No: 712689). The tubes were shaken and vortexed again, using Retsch Mixer, before being placed, in a freezer (Elite. Air Multi=flow. Freezer and Refrigerator. No frost) at -20°C for 24 hours. The tubes were then centrifuged in a microcentrifuge (SiGMA Laborzentrifugen, D -37520 Osterode am Harz, Germany. Model =1K15. Serial No =69422. Year =1999. Max. Speed (dependent on rotor) rpm =5300. Max. Capacity (density of material max 1.2kg/dm² =53ml. Kinetic energy Nm =4980. Total power consumption W =450. Electrical supply V/HZ =230/50 1Ph. Input fuse A =6, 3 AT. Interference suppression acc to EN 55011 cl. B. Made in Germany), at 14,000 rpm for 15 minutes. The supernatant was discarded by gently raising the bottom of the tube slightly higher than the mouth, and the pellets left at the side of the tube. The pellets were washed with 500 µl 70% ethanol (Art-Nr. K928.3 ROTH. Ethanol vergällt. ≥99.8%, mit Ca. 1% MEK. Carl Roth GmbH + Co. KG. 2.5L. Schoemperslenstr. 3-5, 76 185 Karlsruhe), taken using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 100-1000 µl (Sr No: 712689) and centrifuged in a microcentrifuge (SiGMA Laborzentrifugen, D -37520 Osterode am Harz, Germany. Model =1K15. Serial No =69422. Year =1999. Max. Speed (dependent on rotor) rpm =5300. Max. Capacity (density of material max 1.2kg/dm² =53ml. Kinetic energy Nm =4980. Total power consumption W =450. Electrical supply V/HZ =230/50 1Ph. Input fuse A =6, 3 AT. Interference suppression acc to EN 55011 cl. B. Made in Germany), at 14,000 rpm for 15 minutes. The supernatant was discarded, and the pellets in the tubes were left to dry at room temperature. The opened tubes were then sealed with parafilm foil (PARRAFILM “M.” 4 W x 125 FT. ROLL. RECHINEY PLASTIC PACKAGING. MENASHA, WI 54952. CHICAGO, IL. 60631) to minimize contamination. Separate sterile needles (Omeco 10ml 21G x 11/2”, single use syringe, sterilised, non-toxic, non pyrogenic, Lot 22543 Made in Egypt) were used to perforate the top of the seal of each tube, to enhance dryness. The pellets were dissolved in 40-50 µl ddH₂O (DEPC water) (Art. T143.1. ROTH. Wasser fur die. Molekular biologie. DEPC- bhandelt water. M 18.01. 250ml. Carl Roth GmbH + Co. KG. 76 185 Karlsruhe), by pipetting up and down with sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 10-100 µl (Sr No: 712689). The eluted DNA was stored in a refrigerator (Elite. Air Multi=flow. Freezer and Refrigerator. No frost) at -80°C before used for amplification.

2.3 Amplification of the Extracted DNA for Sex Determination Using *Amelogenin* Gene Multiplex Primers

PCR amplification of the extracted DNA for sex determination from dry human teeth specimens used previously prescribed *amelogenin* gene multiplex primers by Faerman *et al.*, 1995 [11] and Matheson and Loy, 2001[12]. The equipment and surface of the PCR hood were cleaned with distilled water, sterilized with 70% ethanol (Art-Nr. K928.3 ROTH. Ethanol vergällt. $\geq 99.8\%$, mit Ca. 1% MEK. Carl Roth GmbH + Co. KG. 2.5L. Schoemperslenstr. 3-5, 76 185 Karlsruhe) and UV irradiated. A sheet of aluminium foil (Helwan Aluminium Foil. 15M x 40CM. Made in Egypt) sterilized with 70% ethanol (Art-Nr. K928.3 ROTH. Ethanol vergällt. $\geq 99.8\%$, mit Ca. 1% MEK. Carl Roth GmbH + Co. KG. 2.5L. Schoemperslenstr. 3-5, 76 185 Karlsruhe) was placed on the surface of the hood. One 0.5ml PCR tube (Bio Basic Inc. Cat. No: Bp602-B. 0.5ml PCR thin wall PCR tubes flat blue cap with writing surface. Lot. No. 081114) for master mix, was labelled as MMX and nine 0.25ml PCR tubes (Bio Basic Inc. Cat. No: Bp602-B. 0.25ml PCR thin wall PCR tubes flat blue cap with writing surface. Lot. No. 081114) were labelled 1 to 9 with names of the respective samples and date on the flat blue cap writing surfaces and the sides with a permanent marker (STAEDTLER permanent Lumocolor Art. Nr. 313-3. EAN 40 07817 308677), and placed in a microtube rack (LP ITALIANA SPA – Milano/made in Italy). A PCR master mix for 10 tubes (one extra tube added to compensate for pipetting error) was prepared in a single 0.5ml PCR tube using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 0.5-10 μ l, 10-100 μ l (Sr No: 712689). The master mix was constituted from: **2.5 μ l of 10x Buffer** (F – 511. 10x buffer for DyNazyme. DNA Polymerase. Lot. 130. Finnzymes), **2.5 μ l of dNTPs (a. dATP; N001. dATP. Conc.: 100mM. Pack.: 5umoles. Lot. #24. SibiEnzyme. Store at -20°C. b. dCTP; N004. dCTP. Conc.: 100mM. Pack.: 25umoles. Lot. #28. SibiEnzyme. Store at -20°C. c. dGTP; N006. dGTP. Conc.: 100mM. Pack.: 25umoles. Lot. #28. SibiEnzyme. Store at -20°C. d. dTTP; N008. dTTP. Conc.: 100mM. Pack.: 25umoles. Lot. #25. SibiEnzyme. Store at -20°C), 2.5 μ l of MgCl₂** (25mM MgCl₂. 1.0ml. Fermentas. Lot. 00016395. Store at -20°C), **4 μ l of forward primer M4** (67 50-2235-35/381. Eurofins MWG Operon. SI-M4. 21 mer. 08-Dec-09), **2 μ l of X reverse primer M5** (62 50-2235-36/380. Eurofins MWG Operon. SI-M5. 22 mer. 08-Dec-09) and **2 μ l of Y reverse primer M6** (67 50-2235-37/381. Eurofins MWG Operon. SI-M6. 23 mer. 08-Dec-09), **1.25 μ l of Taq polymerase** (F – 501L. DyNazyme II. DNA polymerase. 2 U/ μ l 500ul. Lot. 134. Assayed 07/09. Finnzymes) and **6.25 μ l of DEPC water** (DEPC water (Art. T143.1. ROTH. Wasser fur die. Molekular biologie. DEPC-bhandelt water. M 18.01. 250ml. Carl Roth GmbH + Co. KG. 76 185 Karlsruhe). These, in the same tube, were mixed together by pipetting up and down using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 0.5-10 μ l, 10-100 μ l (Sr No: 712689), and then spun down in a mini centrifuge (COSTAR MINI CENTRIFUGE. Ser. No. 10MVSS – 12145. Made in USA. RPM x1000). A total volume of **23 μ l** from the master mix, was transferred into each of the already labelled 9 tubes. To each contents of the tubes, **1 μ l Paraffin oil** (Paraffin Oil Pure = 60ml. Philo Pharm Pharmaceuticals. Tenth of Ramadan – Egypt) was added to seal and avoid evaporation of the reaction mixture. A volume of **2 μ l DNA** (template) from the respective 9 samples was finally added to the 9 tubes (containing PCR reagents), to accomplish a reaction volume of **25 μ l** for each tube. The contents of each tube were mixed again, by pipetting up and down using sterile pipettes and disposable filtered tips (BioSTC. High Performance Micro volume Pipettors. Calibration Confirming. DN 12650 & ENISO 8655. Standards – Germany. 0.5-10 μ l, 10-100 μ l (Sr No: 712689), and then spun down in a mini centrifuge (COSTAR MINI CENTRIFUGE. Ser. No. 10MVSS – 12145. Made in USA. RPM x1000). Each filtered tip was used once and disposed off in a waste container.

Normal PCR was accomplished in a thermocycler (Minicycler™ SN: MC 009597. Model: PTC – 150. Rating: 100-240VAC, 50-60Hz, 220 Watts. Fuses: (2) F2.5A 250V. NRTL/C = LR97357. MJ RESARCH, INC, 149 Grove Street, Watertown, MA 02172, United States of America), in a **25 μ l** reaction volume, to amplify selected sequences of the *amelogenin* gene, as follows; Denaturation step: was at 94°C for 1 minute. Annealing step: was at 55°C for 2 minutes. Extension/elongation step: was at 72°C for 2 minutes. The above first three steps were repeated for 40 cycles. Final extension/elongation: was at 72°C for 15 minutes. Cooling of the reaction process: was at 4°C for 48hours. The protocol employed by Maniatis, *et al.*, (1982) [13] for preparation of Agarose gel electrophoresis was adopted. Amplification with *amelogenin* primers was at 330 base pair (bp) bands for X chromosome, and 218 base pair (bp) bands for Y chromosome, respectively. The expected amplification products of *amelogenin* gene sequences were visualised by electrophoresis in 1.5% agarose gel containing 4 μ l ethidium bromide. A molecular weight marker was always included in the first lanes (Figs. 1, 2 and 3).

2.4 Statistical Analysis

Data was initially sorted out manually and tabulated and then entered into the computer using Microsoft Excel and Minitab 15.1 statistical package. Statistical tests were employed for data analysis. Fisher's

exact test (probability) and Z-test were employed for comparison of values. [14] The sensitivity, specificity, efficiency, predictive value of positive tests, predictive value of negative tests, false positive rates and false negative rates of the PCR were determined according to the arithmetic definitions of these terms.

III. RESULTS

3.1 Results of Genetic Sex Identification of Adult Embalmed (from cadavers) Teeth Using *Amelogenin* Gene Primers

Table 1 shows the result of the genetic sex identification, with *amelognin* gene, of the human adult embalmed (from cadavers) teeth. Only smear was observed for both X and Y chromosomes in all the samples. No genetic sex identification was achieved in all the 3 samples.

Table 1: Results of Genetic Sex Identification of Adult Embalmed (from cadavers) Teeth Using *Amelogenin* Gene Primers

Tooth serial number	Tooth sampled	Morphological Sex	PCR results with <i>amelogenin</i> gene primers.		Genetic sex
			X	Y	
			chromosome amplification (330 bp)	chromosome amplification (218 bp)	
1	Canine	Female	Smear	Smear	Unidentified
2	Molar	Male	Smear	Smear	Unidentified
3	Molar	Male	Smear	Smear	Unidentified

Only smear was observed for both X and Y chromosomes in all the samples. No genetic sex identification was achieved in all the samples (see Table 1 and Fig. 1). PCR Sensitivity = 0%, Specificity = 0%, Predictive value of positive test = 0%, Predictive value of negative test = 0%, False positive rate = 0%, False negative rate = 0%, Efficiency of test = 0%.

Fisher's exact probability test $P = 1$. Z-test: z - and p values were invalid.

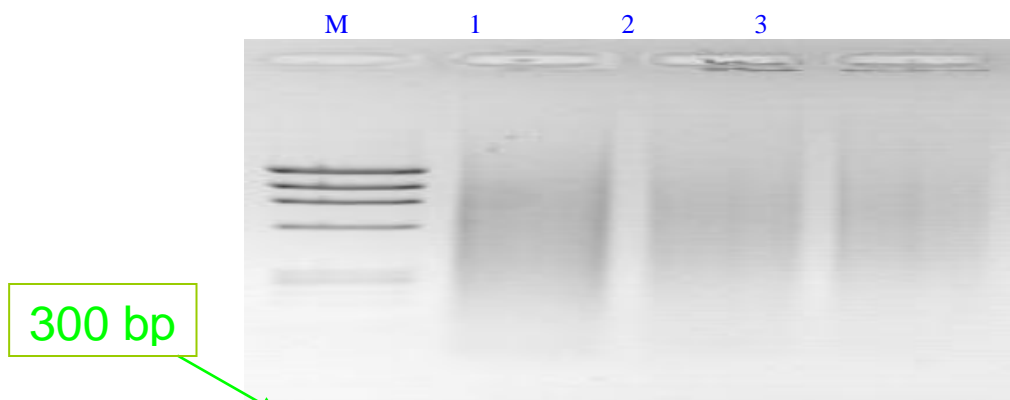


Figure 1: Amplification of the *amelogenin* gene X and Y primers from the DNA of the adult embalmed (from cadavers) teeth samples electrophoretically separated on 1.5% agarose gel (Reassay: 005/14).

Lanes: M = 1000 bp DNA ladder (Lot: 73020G3. Toyobo); 1= tooth sample1; 2 = tooth sample 2; 3 tooth sample 3.

3.2 Results of Genetic Sex Identification of Adult Unembalmed (from patients) Teeth Using *Amelogenin* Gene Primers.

Table 2 presents results of the genetic sex identification, with *amelognin* gene, of the human adult unembalmed teeth. Only smear was observed for both X and Y chromosomes in all the samples. No genetic sex identification was achieved in all the 3 samples.

Table 2: Results of Genetic Sex Identification of Adult Unembalmed Teeth Using Amelogenin Gene Primers.

Tooth serial number	Tooth sampled	Morphological Sex	PCR results with <i>amelogenin</i> gene primers.		Genetic sex
			X chromosome amplification (330 bp)	Y chromosome amplification (218 bp)	
1	Molar	Male	Smear	Smear	Unidentified
2	Premolar	Male	Smear	Smear	Unidentified
3	Premolar	Female	Smear	Smear	Unidentified

Only smear was observed for both X and Y chromosomes in all the samples. No genetic sex identification was achieved in all the samples (see Table 2 and Fig. 2). PCR Sensitivity = 0%, Specificity = 0%, Predictive value of positive test = 0%, Predictive value of negative test = 0%, False positive rate = 0%, False negative rate = 0%, Efficiency of test = 0%.

Fisher's exact probability test $P = 1$. Z-test: z - and p values were invalid.

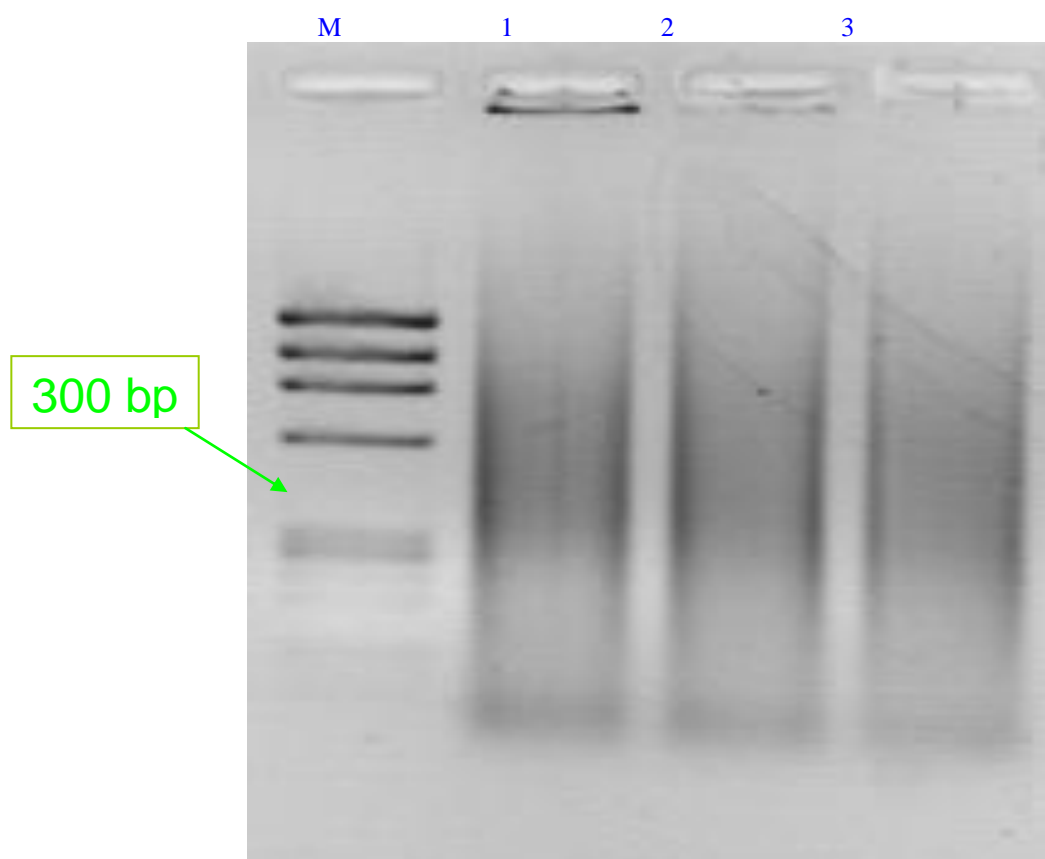


Figure 2: Amplification of the *amelogenin* gene X and Y primers from the DNA of the adult unembalmed (from patients) teeth samples electrophoretically separated on 1.5% agarose gel (Reassay: 005/14).

Lanes: M = 1000 bp DNA ladder (Lot: 73020G3. Toyobo); 1= tooth sample1; 2 = tooth sample 2; 3 tooth sample 3.

3.3 Results of Genetic Sex Identification of Deciduous Teeth Using *Amelogenin* Gene Primers.

In Table 3, the details of the result of the genetic sex identification, with *amelognin* gene, of the human deciduous teeth are shown. Only smear was observed for both X and Y chromosomes in all the 3 samples. No genetic sex identification was achieved in all the 3 samples.

Table 3: Results of Genetic Sex Identification of Deciduous Teeth Using *Amelogenin* Gene Primers.

Tooth serial number	Tooth sampled	Morphological Sex	PCR results with <i>amelogenin</i> gene primers.		Genetic sex
			X	Y	
			chromosome amplification (330 bp)	chromosome amplification (218 bp)	
1	Incisor	Male	Smear	Smear	Unidentified
2	Incisor	Male	Smear	Smear	Unidentified
3	Incisor	Female	Smear	Smear	Unidentified

Only smear was observed for both X and Y chromosomes in all the samples. No genetic sex identification was achieved in all the samples (see Table 3 and Fig. 3). PCR Sensitivity = 0%, Specificity = 0%, Predictive value of positive test = 0%, Predictive value of negative test = 0%, False positive rate = 0%, False negative rate = 0%, Efficiency of test = 0%. Fisher’s exact probability test **P = 1**. Z-test: *z*- and *p* values were invalid.

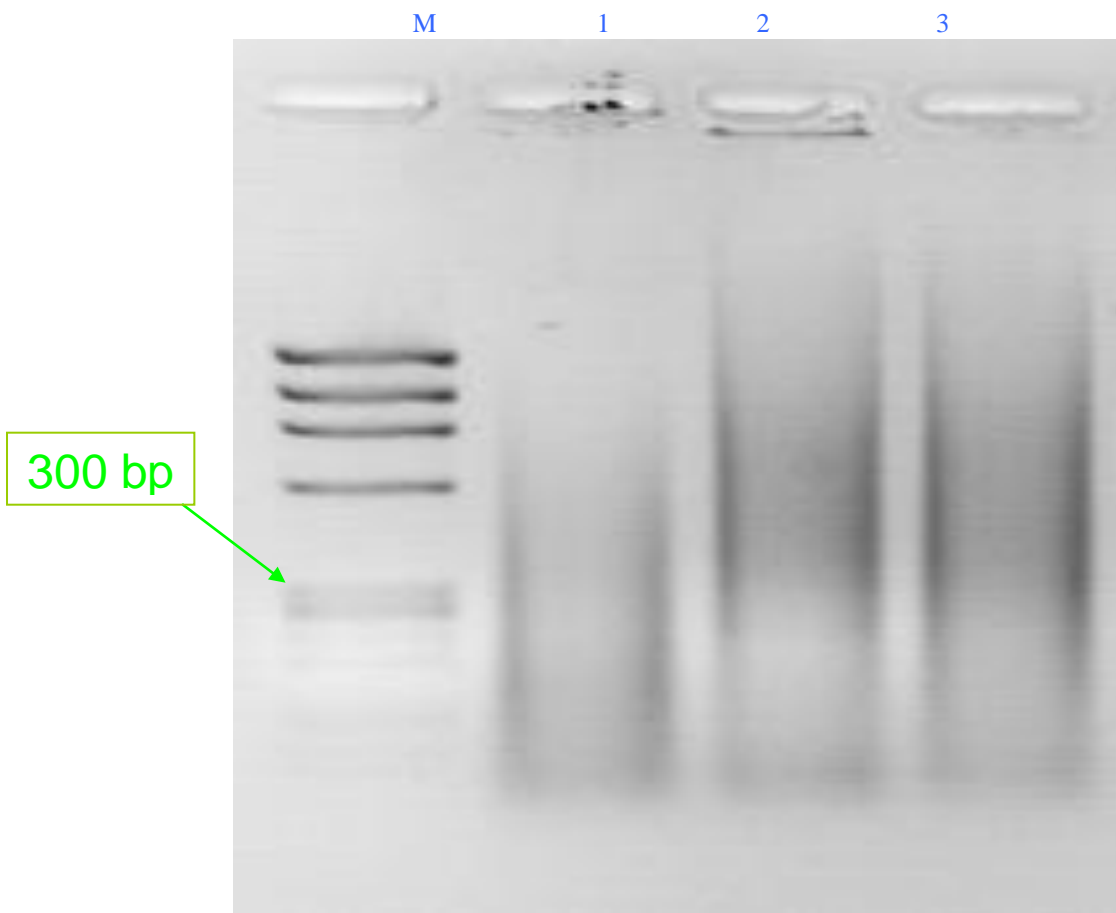


Figure 3: Amplification of the *amelogenin* gene X and Y primers from the DNA of the deciduous teeth samples electrophoretically separated on 1.5% agarose gel (Reassay: 005/14).

Lanes: M = 1000 bp DNA ladder (Lot: 73020G3. Toyobo); 1= tooth sample1; 2 = tooth sample 2; 3 tooth sample 3.

IV. DISCUSSION

New developments in molecular biology and especially in analysing DNA recovered from hard tissues have provided reliable methods for sex determination based on amplification of DNA sequences specific to the X and/or Y chromosomes, [15] a method that this study employed. In this study, primers that span short DNA fragments from the *amelogenin*-encoding gene were used for sex determination from dry human teeth

specimens. Only generalised smear was observed for both X and Y chromosomes in all the teeth samples (Tables 1, 2, 3 and Figs. 1, 2, and 3). Thus, there was no amplification observed in any of the teeth specimens. Therefore, no genetic sex identification was achieved in any of the teeth specimens. Failures of amplification could be due to factors such as severe DNA degradation, presence of PCR inhibitors (associated both in antemortem and postmortem conditions of the teeth samples) and poor/inappropriate PCR optimisation.

A generalised smear, as observed in all the samples of teeth with *amelogenin* gene, could completely obscure the desired product. Generalised smear of amplified product could result from amplification of primer dimers and/or too much template DNA. [16] The concentration of each primer was optimised independently, touchdown PCR was also employed and the amount of the template DNA was also reduced. However, the result shown was the best obtainable after applying all the above suggested remedies. Despite all these remedies that were undertaken to improve the yield of the results, the success rate for genetic sex identification for each group of teeth samples was 0%.

Considering the appearance of the smears in the three groups of teeth samples used in the study, the unblemished adult human teeth is the densest, followed by the deciduous teeth samples. Thus, the cadaveric teeth are the least dense. The higher the density of the smear, the more qualitative the DNA and vice-versa. For the fact that the embalmed cadaveric teeth samples were from embalmed cadavers, the fixative agents might have negative effects on both DNA and PCR of the samples. Formaldehyde treatment causes single-strand breaks in treated cells, [17] and that this fragmented DNA amplifies poorly in PCR reactions. [18] Thus, fixative agents might be the source of PCR inhibition in these samples. Cross-linking of histones on plasmid DNA by formaldehyde severely reduces its ability to be amplified by PCR. For the teeth samples from adult patients and children (deciduous teeth), their DNA might become degraded by endogenous nucleases. Furthermore, deamination, depurination and other hydrolytic processes will lead to destabilization and breaks in DNA molecules. All these processes create problems for the retrieval of DNA sequences, which block DNA polymerases and thus the PCR. [19] Exogenous agents, like microorganisms, humidity and many organic compounds, to which the corpses were exposed, also reduce the amount of informative DNA available, [20] this may be the probable reason for failure of amplification in PCR reactions of these samples.

As in any PCR technique, problems can be encountered if a nonlethal mutational event occurs in the recognition sequence of either primer. Mutational events of this kind are usually recognized, depending on the position and extent of the mutational change, by inefficient amplification of target sequence or failure of the PCR altogether. [21] The reliability of *amelogenin*-based sex testing was first questioned in 1998 with the observation of two phenotypically male individuals being classified as female after PCR analysis. [22] However, in 1996, Stone and his colleagues¹⁵, in a study of ancient human skeletal remains of twenty individuals from Germany, reported one case (5%) which was classified as a male based on morphology, but PCR products hybridized only with the X oligonucleotide. This may be the result of insufficient DNA, or mutations in the priming or oligonucleotide binding sites on the Y chromosome copy of the *amelogenin* gene. In a study on sex determination of hard tissues remains of 4000 year old children from the Czech Republic, Vanharova and Drozdova (2008), [23] reported seven cases in which the results were divergent between archaeological and genetic analyses.

V. CONCLUSION

No genetic sex identification was achieved in any of the dry human teeth samples. Therefore, *amelogenin* PCR for genetic sex identification should not, at least for now, completely replace traditional morphological methods of sex identification. This is the first known study determining the sex of human dry teeth specimens by means of PCR in Nigeria. There is need for further studies in Nigeria to complement the findings of this study.

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