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Research Article

Determination of Gender using Enamel, Dentin and Pulp of Primary Teeth- a Comparative Study

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- Primary teeth; Enamel; Dentin; Pulp; AMEL gene; Polymerize chain reaction

Abstract

Objective: To determine gender using enamel and dentin and compare it with that of the pulp of primary teeth.

Materials and method: Twenty freshly extracted caries free primary teeth were collected from children. The gender of the patient was recorded, followed by coding and double coding of the samples in a double blind manner. The teeth were cleaned and decoronated. Pulp tissue from the crown portions were removed and stored in normal saline. The enamel and dentin samples were then obtained by removing dentin from enamel, using a straight diamond dental bur of size 008. The extracted enamel, dentin and pulp samples were stored in sterile containers, coded and immediately carried to the laboratory for extraction and quantification of DNA from samples of enamel, dentin and pulp tissue. The DNA was subjected to Polymerase Chain Reaction (PCR) analysis for gender determination. Data obtained was decoded and compared with actual gender of the child. Data was tabulated and subjected to statistical analysis.

Results: Gender was successfully determined from sixteen enamel samples and from twenty samples of dentin and pulp. Gender determined from enamel was found to be 20% successful and that from dentin and pulp was 100% successful, which was highly significant.

Conclusion: In comparison to enamel of primary teeth, dentin and pulp were more reliable in determination of gender of an individual.

INTRODUCTION

Dental identification has long been considered a reliable method for gender identification when other methods fail because of critical body conditions or unavailability of body parts. The oral cavity is a rich and noninvasive source of deoxyribonucleic acid (DNA) [1]. In forensic cases involving unidentified bodies, often the only source of DNA for identification are the calcified tissues- bones and teeth [2]. Teeth can survive long after soft and skeletal tissues have been destroyed. Tooth enamel is a unique entity among all mineralized tissues because of the presence of high mineral content in it [3]. As a result, teeth provide an ideal source of both nuclear and mitochondrial DNA [4,5].

Amelogenin (AMEL) is a major protein constituent of developing enamel matrix. The AMEL gene, a coding for a highly conserved protein amelogenin, is located on X and Y chromosomes in humans. The length of the base pairs of AMEL gene varies on X and Y chromosomes. Therefore, this gene is suitable for gender determination in forensic identification.

Polymerized chain reaction (PCR) offers an efficient and sensitive method for sex determination by amplifying a gender-specific sequences. Information from DNA offers the potential to establish the sex of the person from fragments and to contribute to positive identification [3]. Extraction of DNA from teeth samples yields sufficient amounts of good quality DNA useful for PCR-based forensic methods in sex determination [6].

Previous studies have used dental pulp or the entire tooth for gender determination [7,8]. However, there is a paucity of studies to determine gender using specific tooth tissue such as enamel and dentin. Thus, the aim of the study was to determine gender using enamel and dentin and compare it with that of the pulp of primary teeth.

MATERIALS AND METHODS

The present *in vitro* study was carried out in the department of Pedodontics and Preventive Dentistry, The study protocol was approved by the Ethics Review Committee of our institution. The nature of the study was described to the parents of the children. Prior written permission was taken from the parents for extraction of their child's primary teeth and its subsequent use for the study.

Twenty caries free, freshly extracted primary teeth were selected for the study. These primary teeth were indicated for extraction in children visiting the department of Pedodontics and Preventive dentistry. The gender of the patient was recorded. Coding and double coding of the samples was done in a double blind manner.

The inclusion criteria for the study were: teeth indicated

for extraction, teeth showing pre shedding mobility, retained primary teeth and children undergoing serial extraction.

Teeth with extensive decay involving pulp, teeth with developmental defects and endodontically treated teeth were excluded from the study.

Each tooth was cleaned with a soft tooth-brush under running sterile distilled water to remove any contaminants. Decoronation of the teeth was done using a diamond-cutting disc of diameter 0.5 mm and water spray. The pulp tissue from the crown portion was removed using a spoon excavator and stored in normal saline. The enamel samples were obtained after removing dentin using a straight diamond dental bur of size 008. From another tooth, dentin samples were obtained after removing enamel using a straight diamond dental bur of size 008.

These enamel, dentin and pulp samples were stored in sterile containers, coded and immediately carried to the laboratory for extraction of DNA. The following procedures were performed in the laboratory: DNA extraction (phenol/chloroform (organic) extraction), amplification of the extracted DNA by polymerase chain reaction (PCR) and product analysis.

The enamel and dentin were immersed in liquid nitrogen and pulverized to a fine powder using an autoclaved mortar and pestle.

Phenol/chloroform (organic) extraction [2]

Organic extraction of DNA was done by the following procedure: 40-60 mg of tooth powder was added to 500µl of Ethylene diamine tetra acetic acid (EDTA) and incubated at 37°C for 3 days To this, 1 ml of TRIS NaCl EDTA (TAE) buffer with sodium dodecyl sulfate (SDS) was and 10µl of proteinase K was added and mixed by pulse vortexing for 15 seconds and the sample was incubated at 56°C for 24 hours.

It was then transferred into 2 ml eppendorf tubes where, 1 ml of lysate was added with 1 ml of phenol chloroform isoamyl alcohol and pulse vortexing was done for 15 seconds. It was then incubated for 5 minutes at room temperature and centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge. The supernatant formed was collected into a 1.5 ml fresh tube and the collection tube containing the flow through was discarded.

To this supernatant, an equal volume of isopropanol was added and then incubated for 30 minutes to 1 hour at room temperature. It was further centrifuged at 10,000 rpm for 15 minutes in a refrigerated centrifuge and then the supernatant was discarded. The DNA pellets formed were washed twice with 70% ethanol. And then dissolved in 20µl nuclease free water (NFW) and stored at –4°C until the PCR procedure.

Polymerized chain reaction [4]

The oligodeoxyribonucleotides used in this study were designed and nucleotide sequences of the common 22 mer forward primer (AMEL-fp) and the two allele-specific reverse primers (AMEL-Xrp, 22 mer; AMEL-Yrp, 24 mer) were as follows:

AMEL-fp: 59-CAG CTT CCC AGT TTA ACT TCT G-39;

AMEL-Xrp: 59-CTC TCC TAT ACC ACT TAG TCA G-39;

AMEL-Yrp: 59-TGC CCA AAG TTA GTA ATT TTA CCT-39.

The PCR amplification involves three basic steps

Denaturation: It was done to promote the single stranded DNA formation by heating the template to 94°C for 1 minute.

Annealing: It was done by lowering the temperature significantly to promote binding of base pairs of template and the primer, at 54°C for 2 minutes.

Extension: The temperature was shifted to optimum temperature for DNA polymerase to synthesize sequences complimentary to the template using annealed primer as a starting point for extension of a newly synthesized single strand. Extension temperature used was 74°C for 1 minute.

These three steps constitute a single cycle of the reaction. The apparatus was fed with this temperature program after the reaction mixtures were in place and a total of 40 cycles were performed per reaction, with a ramping time of less than a minute between the cycles. Samples were then held at a temperature of 4°C before the product analysis.

Product analysis: (1)

The products of the polymerase chain were then run on a 1.5 percent agarose gel with ethidium bromide staining on gel documentation system (GDS) for one hour at 100 volts. This GDS consists of an ultra violet visualiser which is connected to a computer, for visualization, analysis and storage of the results.

Data obtained was decoded and compared with the actual gender of the child from the dental records. The data was subjected to statistical analysis using Fischer Exact test to find the significance of failures between the two groups. Significance was considered as $p<0.001$.

The Statistical software namely; SAS 9.2, SPSS 15.0, Stata 10.1 and MedCalc 9.0.1 were used for the analysis of the data.

RESULTS

Of the 20 samples, the DNA retrieved from 4 samples was below detection level. The quantity of DNA obtained from 16 enamel samples ranged from 1.8 to 10.2 ng/ml. Five samples showed the gender to be male and 11 samples were female. Gender determined from only 5 samples (31.25%) of enamel matched with that of the true gender (Table 1).

The quantity of DNA obtained from dentin samples ranged from 22.5 to 65.4ng/ml. Of the 20 dentin samples, 11 samples showed the gender to be male and 9 samples were female (Table 2). The quantity of DNA obtained from pulp samples ranged from 28.2 to 57ng/ml. Similarly, of the 20 pulp samples, 11 samples showed the gender to be male and 9 samples were female (Table 3). Gender determined from all 20 samples each of dentin (100%) and pulp (100%) matched with that of the true gender and was found to be highly significant ($p<0.001$) (Tables 2&3). Dentin and pulp samples were more reliable than enamel samples in determination of gender ($p<0.001$) (Table 4).

DISCUSSION

Gender determination is an important element in the analysis

Table 1: Comparison of gender determined from enamel samples and true gender.

Coded sample	DNA quantity (ng/ml)	Determined gender	True gender	Correct/ Incorrect (C/I)	p value
1.	8.5	M	M	C	0.0001*
2.	6.3	F	M	I	1
3.	Below detection level	--	M	--	--
4.	5.4	F	F	C	0.0001*
5.	4.9	F	F	C	0.0001*
6.	2.7	F	M	I	1
7.	9.6	F	M	I	1
8.	3.8	M	F	I	1
9.	4.6	F	M	I	1
10.	2.2	F	M	I	1
11.	7.4	M	M	C	0.0001*
12.	10.2	F	M	I	1
13.	Below detection level	--	M	--	--
14.	6.5	F	M	I	1
15.	Below detection level	--	F	--	--
16.	8.4	F	F	C	0.0001*
17.	3.1	F	M	I	1
18.	1.8	M	F	I	1
19.	2.5	M	F	I	1
20.	Below detection level	--	M	--	--

*p<0.001 is highly significant

Table 2: Comparison of gender determined from dentin samples and true gender.

Coded sample	DNA quantity (ng/ml)	Determined gender	True gender	Correct/ Incorrect (C/I)	p value
1.	25.9	M	M	C	
2.	41.0	M	M	C	
3.	29.6	M	M	C	
4.	29.3	F	F	C	
5.	27.4	F	F	C	
6.	30.8	M	M	C	
7.	38.6	F	F	C	
8.	38.5	F	F	C	
9.	49.5	M	M	C	
10.	65.4	M	M	C	
11.	48.0	M	M	C	
12.	47.2	M	M	C	
13.	56.3	M	M	C	
14.	24.9	F	F	C	0.0001*
15.	31.0	F	F	C	
16.	28.8	F	F	C	
17.	22.5	M	M	C	
18.	38.7	F	F	C	
19.	49.1	F	F	C	
20.	39.0	M	M	C	

*p<0.001 is highly significant

Table 3: Comparison of gender determined from pulp samples and true gender.

Coded sample	DNA quantity (ng/ml)	Determined gender	True gender	Correct/ Incorrect (C/I)	p value
1.	28.2	M	M	C	
2.	44.8	M	M	C	
3.	48.4	M	M	C	
4.	32.3	F	F	C	
5.	34.7	F	F	C	

6.	52.0	M	M	C	
7.	51.0	F	F	C	
8.	57.0	F	F	C	
9.	37.6	M	M	C	
10.	36.6	M	M	C	
11.	42.0	M	M	C	
12.	44.3	M	M	C	
13.	62.0	M	M	C	0.0001*
14.	53.1	F	F	C	
15.	41.9	F	F	C	
16.	48.8	F	F	C	
17.	39.6	M	M	C	
18.	54.9	F	F	C	
19.	48.3	F	F	C	
20.	42.7	M	M	C	
*p<0.001 is highly significant					

Table 4: Comparison of gender determined from enamel, dentin and pulp samples.

Dental tissue		Z value	p value
Enamel	Dentin	-4.3885	0*
	Pulp	-4.3885	0*
Dentin	Pulp	0	---
*p<0.001 is highly significant			

of biological evidence submitted to forensic laboratories. Determination of gender from teeth can provide an important means of personal identification in mutilated bodies, skeletal remains and mass disasters [9]. A difficulty of gender determination occurs with the skeletal remains of children and preadolescents owing to the lack of sufficiently developed sex characteristics [10,11]. Dental surgeon or forensic dentists are presented with remains of a children and adolescents for gender identification. During the primary or mixed dentition stages, the teeth could be the only available source for gender identification.

Role of DNA in gender identification is invaluable. The oral cavity is an extremely useful source of DNA. The latter can be obtained from saliva, oral mucosal cells and teeth. Dental tissues such as enamel, dentin, pulp and cementum, have an advantage of being resistant to physical and environmental degradation such as incineration, immersion, trauma or decomposition. They are ideal sources of DNA for PCR-based post-mortem investigations [12]. Therefore, in this study, tissues from primary teeth were taken for gender determination.

In situations involving fragmentary remains, the tooth could be partially destroyed. As a result, it is important to determine whether we can identify the person's sex from a partial tooth [2].

DNA within teeth is well preserved because of their mineralized structures. Thus, these tissues provide ideal sources for DNA for PCR-based post-mortem investigations [4].

Dental pulp tissue is an excellent source of DNA. It is the preferred tissue for DNA compared with that of other dental tissues [13]. However, in many cases the pulp tissue is sometimes decomposed or becomes rapidly depleted despite being protected by hard tissues it also may be contaminated by microorganisms or by non-human DNA. Therefore, in this study, freshly extracted non carious teeth with adequate amount of pulp tissue were selected.

In certain circumstances, teeth may lack pulp tissue or may have been endodontically obturated. In these conditions, other parts of teeth such as enamel and dentin are used for DNA extraction [14].

In primary teeth, there is continuous physiologic resorption of the tooth taking place and obtaining cementum is not always possible. Thus, cementum was not included in this study.

The efficiency of DNA isolation from dentin depends more on the type of tooth analyzed than on the extraction methodology [2]. The quantity of nuclear DNA available from dentin is negatively affected by age of the individual and dental disease, suggesting a strong relationship between the presence/absence of pulp and recovery of DNA from dentin [9].

Tooth enamel is a highly mineralized tissue, which does not undergo resorption and remodeling [6], Amelogenins constitute about 90% of the total enamel matrix proteins [15]. This information can provide us a strong distinction between male and female amelogenins as well as highlights this fact that females have two identical amelogenin genes present on X-chromosome whereas, males have two different genes, present on both the sex chromosomes. The difference in male and female genotypes can be utilized as an indispensable tool in gender determination, having good sensitivity and specificity [6]. Amelogenin gene present on X-chromosome has 106 base pairs in length whereas this gene present on Y-chromosome has 112 base pairs.

In this study, the tooth samples were stored and transported to the laboratory in normal saline, which is shown to have no effect on the DNA. Prior to preparation of the dental tissue

samples, teeth were subjected to decontamination processes to remove exogenous DNA, environmental contaminants and micro-organisms. These decontamination techniques are mostly designed to destroy exogenous DNA, which have an unknown effect on endogenous DNA [16]. The recovery of DNA from teeth is complicated by mineralization of the tissues requiring specialized sampling equipment, additional dedicated laboratory space and modified DNA extraction protocols.

Gender determination can be done by different methods like fluorescence Y chromosome test, polymerase chain reaction (PCR) and southern blot hybridization. PCR is a preferred method since it has a high rate of sensitivity and specificity [4,17,18]. According to Witt and Erickson, PCR is a rapid and reliable means for gender determination [19]. The protein, alphoid satellite family (ASF) is located in the pericentromeric regions of human chromosomes and has a higher repetition pattern which can be better detected by PCR method [20].

A major drawback in the preparation of DNA from the hard tissues relates to the difficulty of disrupting the cells. In this study, pulverization of the dental hard tissues alone does not yield any DNA from the tooth samples but it sufficiently disrupts the cells of these hard tissues to allow extraction of DNA [4],

During the extraction of DNA, using PCR, co-extraction of calcium and collagen was minimized as they are inhibitors of polymerase chain reaction (PCR) amplification. There is an intimate relationship between DNA and hydroxyapatite which necessitated demineralization of these tissues for maximum recovery of DNA in our study. Further, EDTA acid was used to improve DNA recovery. However, EDTA is also a PCR inhibitor and therefore, it was also removed along with calcium and collagen prior to PCR analysis.

The PCR technique is extremely sensitive and is prone to contamination from extraneous DNA, which could lead to false positive results. Non-specific binding of primers and primer–primer dimmer formation are other possible reasons for unexpected results.

Amelogenins have a distinct difference in size and pattern of nucleotide sequence in male and female enamel. This difference between the two enamel phenotypes is a sensitive sex determinant for very minute DNA samples produced from unknown human skeletal/ dental remains.

In the present study, quantity of DNA obtained from enamel samples ranged from 1.8 to 10.2ng/ml. DNA could not be quantified from four samples of enamel. This could be attributed to a low target concentration of the gene in the clinical specimen and the target not being recognized because of mutations and inhibitions of the gene expression [21].

Eleven enamel samples showed incorrect gender which could have been due to amplification/detection and contamination. Very low temperature during annealing and the presence of long primers or high salt concentration in reaction buffer could have been potential causes for the results obtained.

Various authors have found dentin and pulp to be very accurate in determining the gender [21-26]. This is in complete accordance with our results, wherein, dentin and pulp were found to show 100% accuracy in determining the true gender of an individual. The quantity of DNA obtained from dentin and pulp ranged from 22.5 to 65.4ng/ml and 28.2 to 57.0ng/ml, respectively. This quantity of DNA provided an adequate amount for successful detection of AMEL gene to determine the true gender of an individual.

From this study, it is seen that dentin and pulp tissues of primary are a good source of DNA and can be utilized for gender determination in forensic dentistry. Although PCR is a relatively expensive method, it is a reliable method for gender determination.

CONCLUSION

1. Sufficient quantity of DNA was available from primary teeth for gender determination.

2. Gender determination from dentin and pulp tissue of primary teeth was found to be 100% correct.

3. Dentin and pulp were found to be 100% accurate and more reliable for gender determination than enamel.

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