Original Article

Sex determination from mesiodens of Indian children by amelogenin gene

Mohit Srivastava,
Swati Tripathi[1],
Madhusudan Astekar[2],
Deepa Singal[3],
Aditi Srivastava,
Pallavi Vashisth[1]

Department of Pedodontics and Preventive Dentistry, Karnavathi School of Dentistry, Gandhinagar, Gujarat, Departments of [1]Pedodontics and Preventive Dentistry and [2]Oral and Maxillofacial Pathology, Institute of Dental Sciences, Mahatma Jyotiba Phule Rohilkhand University, Bareilly, Uttar Pradesh, [3]Singla Dental and Eye Care Center, Moga, Punjab, India

Address for correspondence:
Dr. Mohit Srivastava, Department of Pedodontics and Preventive Dentistry, Karnavati School of Dentistry, Gandhinagar, Gujarat, India. E-mail: drmohitsrivastava@gmail.com

Abstract

Context: The identification of sex is the first and the foremost step in forensic science. Teeth consist of enamel which is the hardest tissue available in the body, protector of DNA presents in pulp tissue at the time of exposure of tooth to adverse conditions. Teeth can be stated as a sealed box of mystery as it contains various human and bacterial DNA for molecular utilization. **Aim and Objective:** The aim is to determine sex from mesiodens on the basis of gene identification by the polymerase chain reaction (PCR). **Settings and Design:** Total number of sample was 8 human-extracted mesiodens. DNA was isolated and was subjected to PCR analysis with use of predesigned primers for amelogenin (AMEL) X and AMEL Y genes. **Results:** Isolation of genomic and mitochondrial DNA from mesiodens was successful in six samples (75%). In samples, quantity of DNA present was also calculated. **Conclusion:** Mesiodens are a good source of DNA and are a very useful tool in identification of sex using PCR analysis which was simple and effective. Hence, the procedure presented in the present study can be applied for extraction of DNA and identification of sex for forensic purpose.

Key words: Amelogenin gene, mesiodens, polymerase chain reaction analysis, sex determination

Introduction

The identification of sex is the first and the foremost step in forensic science. Forensic odontology is the most emerging branch in the field of dentistry as well as forensics.[1] Various method have been used in the past to determine sex on the basis of morphological differentiation in tooth structure and by isolation of genomic and mitochondrial DNA from tooth.[2-4] There are various morphological variations in the human dentition. Mesiodens is also a kind of supernumerary tooth varying in shape and cannot be used for sex determination based on its morphology. In case of mass disaster, where only fragments of tissues are available and even in medico-legal cases where teeth can be found on site, the forensic investigators have to fully rely on the molecular techniques for sex determination.[5] These supernumerary teeth (mesiodens) if found at those sites can serve as a great tool for identification of sex through DNA analysis.

Teeth consist of enamel that is the hardest tissue available in the body and protector of DNA present in pulp tissue, at the time of exposure of tooth to adverse conditions along with these teeth have got dentin and cementum.[6,7] Tooth can

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Website: www.jfds.org

DOI: 10.4103/jfo.jfds_72_16

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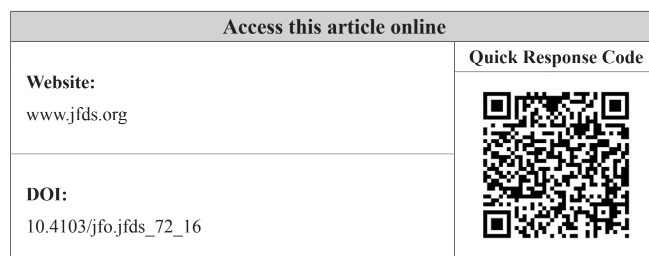

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How to cite this article: Srivastava M, Tripathi S, Astekar M, Singal D, Srivastava A, Vashisth P. Sex determination from mesiodens of Indian children by amelogenin gene. J Forensic Dent Sci 2017;9:125-9.

be stated as a sealed box of mystery as it contains various human and bacterial DNA for molecular utilization.[8] Amelogenin (AMEL) is one of the major matrix proteins secreted by the ameloblasts of the enamel. Different isoforms of AMEL also have been found in the dentin matrix and in odontoblasts as well as in long bone cells (osteocytes, osteoblasts, and osteoclasts).[9,10] Using AMEL gene which is highly conserved, protein located on X and Y chromosome identification of males having (XY) two nonidentical genes and females having (XX) two identical genes can be done.[9-11] To the best of our knowledge, the use of AMEL gene as a marker for sex determination by utilizing mesiodens in the Indian population has not been reported so far. Hence, in the present research, we describe a method to isolate DNA from mesiodens and the use of AMEL gene as a marker for sex determination in Indian population.

Subjects and Methods

A total of eight extracted human mesiodens teeth obtained from the outpatient Department of Pedodontics and Preventive Dentistry and from various local dental clinics. The study was conducted after obtaining consent from the Institutional Ethical Review Board. Signed informed consent was obtained from all the individuals before the study.

The present study was conducted by grouping the sample into two groups:

A. Study group: The control group comprised six mesiodens obtained from above-mentioned sources, whose data and essential variables, namely, sex, age, and location of the tooth in the arch were not known. The teeth samples were labeled according and used in the study. The blood sample was used as a positive control during each reaction
B. Control group: The control group comprised two mesiodens, samples obtained from sources whose data were known at the time of the study. This group served as the internal control group for the reaction and this study was done to ascertain the reproducibility of the method. The blood sample was used as a positive control during each reaction.

Inclusion criteria: mesiodens were selected randomly from healthy individual. Only those mesiodens that were indicated for extraction were considered.

Exclusion criteria: teeth other than mesiodens were not considered for the study.

Extraction of DNA from tooth

Mesiodens were extracted under local anesthesia and were stored at normal room temperature. Samples were kept in natural environmental conditions and were not stored in any medium before the beginning of DNA extraction procedure. During the procedure, mesiodens were cleaned in sterile distilled water and were dried at room temperature and were kept in ultraviolet (UV) chamber (Unicorn Dent Mart, India) for clearing exogenous contamination. Each sample was powdered using micromotor and straight handpiece (Unicorn Dent Mart, India) at different levels at slow speed.[3] The powder was collected in a buffer solution of Tris-EDTA (Sigma-Aldrich, USA) up to 0.5 ml in Eppendorf Tube (Eppendorf, Germany) and stored at −20°C. Two drops of blood from a female were collected in the Tris-EDTA and stored at the same temperature. This sample containing blood was used as a positive control in each reaction.

Sample volume was made up to 1 ml using Tris-EDTA with pH 8.0. The following reagents were added to the buffer containing tooth powder: 200 µl of 5 M NaCl (Rankem, India) (final concentration of 1 M) 50 µl of 10% sodium dodecyl sulfate (final concentration of 0.5%) and 50 µl of proteinase K (Sigma-Aldrich, USA) (20 mg/ml) and were mixed uniformly by repeated pipetting. Incubation was done overnight at 37°C in a dry incubator (Thermo Fisher Scientific, USA) followed by incubation at 56°C in a water bath for 5–6 h. Equal volumes of phenol: chloroform: isoamyl alcohol solution (Thermo Fisher Scientific, USA) was added to the tooth sample and was mixed gently and centrifuged (Eppendorf, Germany) at 12,000 rpm for 10 min at 4°C. The supernatant aqueous layer was collected in the fresh Eppendorf Tube (Eppendorf, Germany) and the DNA was precipitated by adding 3 M sodium acetate (Arihant Chemicals, India) to the final concentration of 0.3 M and twice the volume of chilled 100% ethanol (Sigma-Aldrich, USA). Incubation (Thermo Fisher Scientific, USA) was done for ½ h at −20°C and centrifuged at 13,000 rpm at 4°C for 15 min to get the DNA pellet. The supernatant was discarded and the pellet was washed with 70% ethanol, dried and suspended in 25–30 µl of (molarity) Tris (pH 8) elution buffer. DNA sample was checked on 1% agarose gel electrophoresis. Quantification of DNA was done by BioPhotometer (Eppendorf, Germany).

Amplification of AMEL gene by polymerase chain reaction (PCR): the reactions were conducted in a final volume of 25 µl, containing approximately 5–50 ng of genomic DNA, 5 pmol of forward primer (SigmaAldrich, USA) 2.5 pmol each of the X and Y chromosome-specific reverse primer (Sigma-Aldrich, USA), 250 µM deoxynucleotide triphosphates, and 10 units of Dream Taq DNA polymerase (Sigma-Aldrich, USA) in the 1X Dream Taq PCR buffer (consisting of 50 mM KCl, 10 mM Tris–HCl, pH 9.0, at 258°C, 0.1% Triton X-100, 1.5 mM MgCl). PCR (Bio-Rad, USA) amplifications was carried out by initial denaturation of the template at 94°C for 5 min, followed by forty cycles of incubations at 94°C for 1 min, 55°C for 2 min, and 70°C for 2 min. Final incubation was done at 70°C for 10 min according to Sivagami *et al*.[4]

Agarose gel electrophoresis: the 5 µl of PCR product was mixed with 1 µl of 6X loading dye and analyzed on 2.0% agarose gel (Thermo Fisher Scientific, USA) containing 0.5 µg/ml ethidium bromide (Sisco Research Laboratories Pvt. Ltd., India) along with 100 base pair (bp) DNA ladder at 100 volts using 1X TAE Electrophoretic buffer. The gel was visualized and photographed under UV-gel documentation system (Bio-Rad, USA).

Results

The results on the agarose gel showed the presence of X-specific bands at 330 base pair (bp) and Y-specific bands at 236 bp; males (XY) were distinguished from females (XX) by the presence of two bands whereas female samples showed only one, i.e., X-specific bands on the agarose gel.

Control group: DNA was obtained in both the mesiodens of control group [Table 1] along with sex identification was also possible and found to be accurate on the basis of PCR analysis [Figure 1].

Study group: DNA was obtained in three samples out of six mesiodens and sex identification was also possible on the basis of PCR analysis [Table 2]. In two samples, neither DNA was obtained nor was sex identification possible even after PCR analysis. In one sample, DNA was not obtained on agarose gel, but when it was amplified by means of PCR reaction, sex identification was possible [Figure 2].

Out of total eight evaluated samples (both control group and study group), six samples (75%) were successfully identified as male/female. In three samples out of eight (37.5%), identification of DNA was not possible on 1% agarose gel. The probable reason for this could be that primer binding site mutations or deletions of sections of the Y-chromosome have given incongruous result.[12] In one sample, out of above-mentioned three samples showed sex chromosomal bands when amplified by PCR. The data from control group were correlated with the original sex information of the samples. In two samples (100%) of control group, sex determination was possible and was accurate.

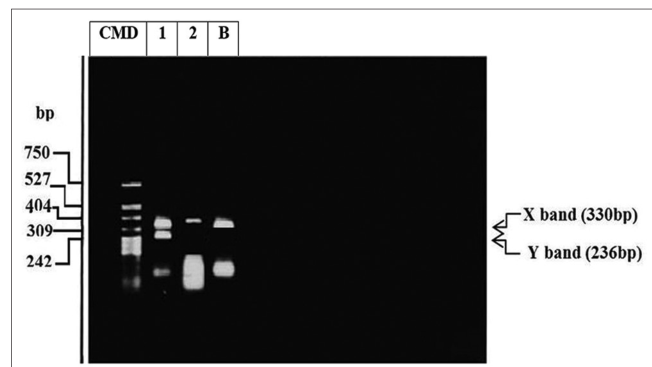

Figure 1: Column named Control group mesiodens (CMD) indicates ladder for counting base pairs. CMD 1 shows two bands one of X at 330 bp and another of Y at 236 bp indicating sample of male. CMD 2 shows a single band of X at 330 bp which indicates sample of female. B shows single band X at 330 bp which indicates sample is of female

In samples [Table 3], quantity of DNA present was calculated by means of BioPhotometer (Eppendorf, Germany). The minimum quantity of DNA that can be

Table 1: Control group mesiodens

DNA isolation on 1% Agarose gel	PCR analysis for amelogenin gene
Yes	XY (male)
Yes	XX (female)

PCR: Polymerase chain reaction

Table 2: Study group mesiodens

DNA isolation on 1% Agarose gel	PCR analysis for Amelogenin gene
No	Not obtained
Yes	XX (female)
No	XX (female)
Yes	X Y (male)
Yes	XX (female)
No	Not obtained

PCR: Polymerase chain reaction

Table 3: Quantity of DNA measured in sample (mesiodens)

Group type	Sample code	Quantity of DNA present
Control group	CMD1	14 ng/µl
Control group	CMD2	8 ng/µl
Study group	SMD1	Below detection level
Study group	SMD2	6 ng/µl
Study group	SMD3	9 ng/µl
Study group	SMD4	7 ng/µl
Study group	SMD5	4 ng/µl
Study group	SMD6	Below detection level

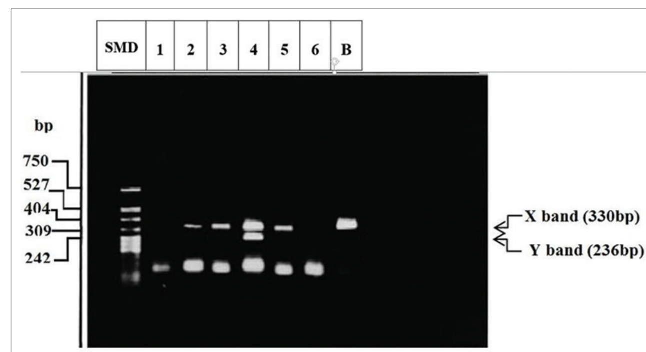

Figure 2: Sample group mesiodens (SMD) column indicates the ladder for calculating the base pairs. SMD 1 shows no bands at 330 bp and 236 bp showing that identification of sex not done. SMD 2 shows a X band at 330 bp indicates sample is of female. SMD 3 shows a single X band at 330 bp indicates sample is of female. SMD 4 shows two bands on at 330 bp and another at 236 bp indicating the sample is of a male. SMD 5 shows a single band at 330 bp indicating sample is of a female. SMD 6 shows no bands indicating identification not possible. B shows a single band at 330 bp indicating sample is of a female

calculated was 4 ng/μl and the maximum quantity was 14 ng/μl. One sample of study group showed undetectable amount of DNA from mesiodens.

Discussion

In the 21st century, society is being faced with new challenges in every possible area. Regardless of leaps in modern technology, medical breakthroughs, and the geographical changes that the last century has brought, crime still persists in all aspects of our lives. Violent and atrocious activities that blow apart the lives of victims, their friends, and families occur daily. Often, little can be done to repair such damage. The trepidation and subsequent prosecution of the perpetrator(s) is essential to maintain law and order. Through the specialty of forensic odontology, dentistry plays a minute but significant role in this process. By identifying the victims of crime and disaster through dental records, dentists assist those involved in crime investigation. Always part of a bigger team, such personnel is dedicated to the common principles of all those involved in forensic casework: the rights of the dead and those who survive them.

The study results demonstrated that it was possible to determine a person's sex by analyzing the dental tissues of pulp, dentin, and cementum. The determination of individual sex from his/her remains is a prime stage in identification process. Information obtained from DNA offers the chance to ascertain the sex of person and hence establishes a positive identification. Odontological examination has been a critical determinant in the search for identification of human remains where positive identification was not sufficient due to decomposition of the soft tissue.[5] Teeth generally resist post mortem degradation and hence act as possible vehicle for valuable forensic data.

AMEL is an important matrix protein found in the human enamel essential for normal tooth development. Two AMEL-specific AMEL genes are present, one on X chromosome and another on Y chromosome. The female has two identical AMEL genes or alleles, whereas the male has two different AMEL genes. The X and Y copies of AMEL do not undergo homologous recombination, so it is preferred by modern forensics for sex determination. Thus, by amplifying X and Y specific AMEL sequences, sex can be determined from very small samples of DNA using PCR technique.[12,13] Gurgul *et al*. 2010 has shown that AMEL gene-based primer can be successfully used for identification of sex on Indian population.[11] In the present study too, for identification of sex from mesiodens AMEL gene-based primers were used.

In this study, micromotor with a straight handpiece drill was used at slow speed to prevent denaturation of DNA. For extraction of DNA, we followed phenol/chloroform/isoamyl alcohol extraction technique which is a simple and cost effective.[7,11]

The mesiodens were kept in normal environment to mimic the same conditions that would be present during a forensic investigation. In this steps, involving cleaning teeth with chemicals was omitted as it can lead to possible degradation of DNA.[14] Previous studies have also shown cleaning of tooth samples for the purpose of DNA isolation with sterile water and UV irradiation.[5] The standard protocols which were followed in this study helped us successfully isolate DNA and identification of sex. This finding is in concurrent to previous studies which have shown isolation of DNA and identification of sex even after 6 months of keeping samples in normal environmental condition.[5,6,8]

The present study was successful in identifying DNA in 75% samples and sex determination with success rate of 100% in control group and 66% in the study group. In the study, 100% results were not obtained in the study sample. The probable reasons could be bacterial growths, which lead to contamination during storage process after extraction of DNA, and in another sample, it was not possible due to human error.

Typically, 0.1–1 ng/μl of mammalian genomic DNA is utilized per PCR analysis.[15,16] In the present study, DNA quantification showed presence of significant amount of DNA in the mesiodens. Hence, it can be said that mesiodens can be used to carry out molecular analysis.

In the literature review from the past 18 years, premolars and molars have been routinely used for the quantification and sex determination of an individual [Table 4].[17-25] The present study is a first of its kind where the mesiodens has been proved for DNA isolation and sex determination in the human population.

Conclusion

Each practitioner has a responsibility to understand the forensic implications associated with the practice of his other profession. In this study, we have found that mesiodens could serve as a reliable source of DNA for amplification-based forensic methods in sex determination. DNA extraction was possible irrespective of the storage of tooth sample in any environmental conditions before DNA extraction procedure. PCR-based analysis is being used these days extensively in forensic sciences. It serves a very important tenacity in identification of sex for various medico-legal conditions and in cases of mass disaster. Hence, in conclusion, any supernumerary teeth like mesiodens can be used as a good source in personal identification of human remains. Appreciation of the forensic field should give the dental clinician another reason to maintain legible

Table 4: Illustrates the literature review using teeth sample for identifying the sex of an individual

Author	Year	Sample size	Sample tooth	Gene expression
Pouchkarev *et al*.[17]	1998	-	Teeth	X-Y homologous gene amelogenin
Sivagami *et al*.[4]	2000	10	Teeth	Amelogenin gene
Das *et al*.[18]	2004	10	Teeth	Barr body
Galdames *et al*.[19]	2010	40	Premolars and third molars	Barr body
Veeraraghavan *et al*.[20]	2010	60	Premolars and molars	F-body, seen in the Y chromosome
Álvarez-Sandoval *et al*.[21]	2014	-	Bone and tooth samples	Amelogenin gene AMELX-allele
Khorate *et al*.[22]	2014	100	Teeth	X (Barr body) and Y (F body)
Nayar *et al*.[23]	2014	50	Maxillary and mandibular teeth	Fluorescent staining of the Y chromosome
Khanna[24]	2015	90	Premolars and molars	Barr body analysis
Praveen Kumar and Aswath[25]	2016	40	Teeth	Amelogenin gene
The present study	2016	08	Mesiodens	Amelogenin gene

and legally acceptable records and assist legal authorities in the identification of victims and suspects.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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