Isolation of epithelial cells from tooth brush and gender identification by amplification of *SRY* gene

Vikram Simha Reddy A,
Sriram G[1], Saraswathi TR[1],
Sivapathasundharam B[2]
*Department of Oral Pathology,
G. Pullareddy Dental College,
Kurnool, Andhra Pradesh,
[1]Vishnu Dental College,
Bhimavaram, Andhra Pradesh,
[2]Meenakshi Ammal Dental
College, Alappakkam Main Road,
Madhuravoyal, Chennai, India*

Address for correspondence:
*Dr. Vikram Simha Reddy A.
Department of Oral Pathology,
G. Pullareddy Dental College,
Kurnool, Andhrapradesh, India.
E-mail: drvicky999@gmail.com*

Abstract

Introduction: This study determines the importance of tooth brush from which DNA can be isolated and used for sex determination in forensic analysis. **Materials and Methods:** A total of 30 samples were collected and stored at room temperature for different periods of time interval. The epithelial cells adhered to the bristles of tooth brush were collected and genomic DNA was extracted and quantified using Nanodrop 1000 spectrophotometer. **Results:** Gender identification was done by amplification of sex determining region on Y chromosome (*SRY*) gene using real-time polymerase chain reaction and minimal amount of DNA (in pico grams) with 100% sensitivity and 73.3% specificity, i.e., all male samples showed positive results and out of 15 female samples 4 showed false positive results, i.e wrongly identified as males. **Conclusion:** With this study, we conclude that PCR is a valuable and sensitive procedure where minute contamination may cause alteration in the result, i.e, 4 females showed false positive result. Minute amount of DNA in picograms, which was collected at different intervals is enough for amplification of *SRY* gene and tooth brush can be used as one of the very valuable sources of gender identification.

Key words: Epithelial cells, gender identification, real time PCR, tooth brush

Introduction

Identification of an individual with usual conventional modalities like dermal ridge finger print, radiological and forensic pathological methods becomes difficult in mass disasters like natural, accidental or man made events such as earthquakes, floods, airplane crashes and terrorist attacks that result in multiple human fatalities. At this juncture, dental evidence emerges as one of the reliable identification modalities based upon its unique treatment procedures like restorations where they are resistant in badly burnt, traumatized, decomposed, and skeletonized remains. The conventional method of dental identification is done by comparing the post-mortem records with ante-mortem records. When ante-mortem record is not available, the DNA isolated from the hard or soft tissues helps in identification. Even a highly degraded DNA from post-mortem tooth sample can be amplified by polymerase chain reaction (PCR) and compared to suspected biological relative, i.e., to a parent or to a sibling. Even in the absence of biological relatives, the identification is made by comparing with known biological samples of decedent, like hair from a hair brush, epithelial cells from a tooth brush or a biopsy specimen.[1]

Identification of unknown bodies of a mass fatality incident by DNA analysis is the newest of several methods or techniques in the field of forensic medicine.[2] The usual sources of DNA from oral and maxillofacial region are soft tissues, pulp tissues, odontoblastic processes, and

cellular cementum. But saliva-stained stamps, human skin, cigarette butts, tooth brush, oral prosthesis, and other inanimate objects can also be used as sources of DNA, as saliva contains many epithelial cells. Oral epithelium undergoes exfoliation, which is replaced by new crop of epithelial cells. Even firm pressure can dislodge these cells like in case of tooth brushing. While brushing, tooth brush can harbor cells from the oral cavity and these cells can be isolated and retrieved for analysis of DNA.[3] Based on these findings, we used tooth brush as a source of DNA for sex determining region on Y chromosome (*SRY*) and its use in gender identification.

Materials and Methods

The present study was carried out in the Department of Oral and Maxillofacial Pathology, Meenakshi Ammal Dental College, Chennai, and in Shrimpex Biotech Lab, Chennai. Tooth brushes were given for 30 individuals and the subjects were instructed to use it for one week with their regular brushing habits and to return them. The samples were kept at room temperature. Care was taken to avoid any kind of contamination. To assess the possibility of any genetic abnormality and chimerism, information regarding any diagnosed syndromes, family history, bone marrow transplantation, and family details of female subjects were also recorded through personal interview using a questionnaire. Ten samples were subjected to analysis immediately, with no storage time, 10 after 1 month, and 10 after 2 months of storage time [Table 1].

DNA extraction and quantifcation
DNA extraction was performed using Real Genomics YGB 100 (Real Biotech Corporation, Taiwan) DNA extraction kit. Quantification of extracted DNA from each sample was done using Spectrophotometer, Nanodrop ND-1000. DNA in the solution was quantified by the absorbance of light (260 nm) in spectrophotometer.

Amplifcation and detection of *SRY* gene
The DNA samples were amplified for *SRY* gene using real-time PCR and Taq PCR master mix (Qiagen, India) with the following primers and probe.

Primers for *SRY* gene
Forward- GCG ACC CAT GAA CGC ATT.
Reverse-AGT TTC GCA TTC TGG GAT TCT CT.
Probe - FAM- TGG TCT CGC GAT CAG AGG CGC- TAMR

The PCR cycle consisted of initial denaturation at 95°C for 7 min, followed by 40 cycles of annealing at 95°C for 15 sec., and extension at 60°C for 1 min. The presence of target gene *SRY* is automatically detected by the Real Plex Master Cycler (Eppendorf, Japan) and result is plotted in the form of a visualization chart with red color indicating positive and green color as negative and as a FAM assay table.

Table 1: Distribution of subjects based on storage time

Storage time	Males	Females
Nil	5	5
30 days	5	5
60 days	5	5

Results

Quantification of DNA extracted using Nanodrop 1000 Spectrophotometer revealed that most of the samples yielded very low values and certain with negative values. The sensitivity of Nanodrop 1000 spectrophotometer as specified by the manufacturer is 2 ng/µl ± 2 ng/µl. Hence, values below 4 ng/µl indicate the presence of DNA at picogram levels. Only four subjects 1, 4, 8, and 22 showed the presence of DNA above 4 ng/µl, i.e., 29.32, 10.34, 5.19, and 4.74 ng/µl and the respective purity of DNA was 2.01, 1.6, 3.28, and 0.121 [Table 2].

Real-time PCR analysis of *SRY* gene identified all the 15 males as males based on the presence of *SRY* gene. Among the 15 female subjects, 4 females were wrongly identified as males by the presence of *SRY* gene in their tooth brush samples [Table 3].

In the present study, true positive indicates those males who were identified as males; true negative indicates those females who were identified as females; false positive indicates those females who were wrongly identified as males; and false negative indicates those males who were wrongly identified as females by the presence or absence of *SRY* gene.
- True positive = 15 - those males who were identified as males
- True negative = 11 - those females who were identified as females
- False positive = 4 - those females who were wrongly identified as males
- False negative = 0 - those males who were wrongly identified as females

Hence, the sensitivity and specificity of SRY gene in gender identification was 100% and 73.33%, respectively.

Discussion

Identification is the establishment of a person's individuality. Proper identification of the dead is required both for legal and humanitarian reasons. Identification of an individual includes the conventional methods like the birth of fingerprints in 1892, followed by serological typing with various blood group markers such as ABO, visual, personal effects, skeletal remains, autopsy findings, dental characteristics along with several special methods like bite marks, lip prints, cytology, histology, photography,

Table 2: Quantification of DNA extracted from tooth brush samples stored for various time period

Sample no.	Storage time	Gender	DNA yield (ng/µl)
1	Nil	Female	29.32*
2	Nil	Female	0.36
3	Nil	Female	1.12
4	Nil	Female	10.34*
5	Nil	Female	0.5
6	Nil	Male	1.37
7	Nil	Male	1.5
8	Nil	Male	5.19*
9	Nil	Male	-0.6
10	Nil	Male	0.71
11	30 days	Female	1.34
12	30 days	Female	2.23
13	30 days	Female	0.1
14	30 days	Female	1.89
15	30 days	Female	0.08
16	30 days	Male	0.67
17	30 days	Male	0.22
18	30 days	Male	-0.31
19	30 days	Male	0.69
20	30 days	Male	1.11
21	60 days	Female	-0.46
22	60 days	Female	4.74*
23	60 days	Female	-1.23
24	60 days	Female	-1.46
25	60 days	Female	2.32
26	60 days	Male	-0.86
27	60 days	Male	-1.19
28	60 days	Male	0.48
29	60 days	Male	0.63
30	60 days	Male	-0.01

As the sensitivity of Nanodrop 1000 spectrophotometer was 2 ng/µl ± 2 ng/µl, values below 4 ng/µl were not considered, *DNA above 4ng/ul

Table 3: Gender identification based on presence of *SRY* gene

Sample no.	Actual gender	Gender based on *SRY* Gene
1	Female	Female
2	Female	Female
3	Female	Female
4	Female	Female
5	Female	Male*
6	Male	Male
7	Male	Male
8	Male	Male
9	Male	Male
10	Male	Male
11	Female	Female
12	Female	Female
13	Female	Female
14	Female	Female
15	Female	Female
16	Male	Male
17	Male	Male
18	Male	Male
19	Male	Male
20	Male	Male
21	Male	Male
22	Male	Male
23	Male	Male
24	Male	Male
25	Male	Male
26	Female	Female
27	Female	Male*
28	Female	Male*
29	Female	Female
30	Female	Male*

*indicate false positive results

forensic radiography, and now the next evolution is DNA typing.[4]

Determination of sex using skeletal remains presents a great problem to forensic experts especially when only fragments of the body are recovered. At this point, the teeth that are resistant to all these conditions play an important role as they show particular differences in their tooth size like mesio distal width and canine dimorphism between males and females. Sex is also determined by observing barr bodies and f bodies in X and Y chromosomes respectively under microscope. All these methods are having limitations and the percentage of accuracy is not 100%. Advanced methods like extraction of the DNA and PCR amplification will assist accurately in determining the sex of the remains.[5]

The analysis of DNA has revolutionized the field of sex determination, and using which identification of an individual has become much more easier. The requirement for only a small amount of sample, stability of the molecule and the high degree of assay accuracy and precision has contributed to the application of this technology for forensic, parentage testing, population studies, medical analysis, and agricultural and animal genetic applications.[4]

Various sex typing markers used for identification are Amelogenin, centromeric alphoid repeats, ZFX/ZFY zinc finger genes, *SRY* gene, DXYS156, and DYZ1.[6]

Kastelic *et al*, Kashyap *et al*, Thangaraj *et al*, Chang *et al*, Steinlechner *et al*, LI-GI Kao *et al*, Drobnic *et al* in their

studies have observed deletion of amelogenin gene on Y chromosome and the males have been wrongly identified as females. They are called as amelogenin-deleted males. In these situations of unambiguous gender identifications, Y chromosomal STR markers and *SRY* gene are used for identification of gender of amelogenin-deleted males.[7-13]

The *SRY* gene provides instructions for making a transcription factor called the sex-determining region Y protein. A transcription factor is a protein that attaches (binds) to specific regions of DNA and helps control the activity of particular genes. The sex-determining region Y protein causes a fetus to develop as a male. It is the Y-chromosomal gene that acts as a trigger for male development in mammalian embryos.[14]

In the present study, we isolated epithelial cells from tooth brushes and analyzed the DNA for sex determining region Y (SRY) and its use in determining the sex of the individual. Similarly, Bandhaya *et al.* and Tanaka *et al.* also used tooth brushes for extraction of DNA.[15,16]

Various sources were used for extraction of DNA in different studies, like - Hanaoka *et al.* conducted a study to determine sex from blood and teeth.[17] Sivagami and co-workers prepared DNA from teeth by ultrasonication, and subsequent PCR amplification, they obtained 100% success in determining the sex of the individual.[18] Loreille *et al.* extracted DNA from bone.[19] Marchand *et al.* extracted DNA by isolating epithelial cells from buccal swabs, rinses and cyto brushes.[20] Thomson *et al.* in their study, extracted DNA from hair roots and buccal cells for PCR DNA analysis and stated them to be the first choice than invasive blood samples.[21] Herber *et al.* observed study subjects and crime cases by taking the dandruff as the material of choice for extraction of DNA.[22] Allen *et al.* in their study proved that saliva from envelops and stamps can be used for PCR- based DNA typing.[23] Graffy *et al.* in their study explained simple methods for mitochondrial DNA extraction from head hair shafts.[24]

In the present study, the quantity and purity of DNA extracted from tooth brush samples were not analyzed as only four samples have shown quantification as per the sensitivity of Nanodrop 1000 spectrophotometer specified according to the manufacturer as 2 ng/µl ± 2 ng/µl, i.e., 4 ng/ul. Hence, values below 4 ng/µl indicate the presence of DNA at picogram levels. This shows that minute traces of human DNA are sufficient to amplify a specific gene target using PCR. Real-time PCR offers an added advantage over the conventional PCR in that the amplification of the specified gene target is shown as a graph representing the amount of fluorescence emitted by PCR products after each cycle [Figure 1].
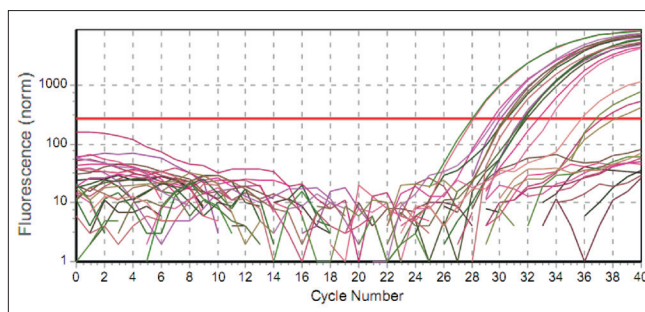
Figure 1: Real-time PCR amplification plot for *SRY* gene. The horizontal red line indicates the threshold value of fluorescence. All the samples positive for *SRY* gene show amplification of the target sequence that attains the threshold fluorescence at varying cycles of amplification. Those samples negative for the *SRY* gene show the fluorescence to be below the threshold value (red line)

Bandhaya *et al.* in their study have concluded that limited number of bundle of bristles give enough quality of DNA than the entire bundle of bristles even though they give more genetic material, as they contain PCR inhibitors in the tooth paste residue. They have yielded 0.025 to 2.355 ng/ul.[25] Tanaka *et al.* in their study used entire bundle of bristles and were able to retrieve 10 to 430 ng/µl of DNA. Similarly, Jiang *et al*, Mannucci *et al*, and Sullivan *et al* in their studies for sex determination extracted very minimal amount of DNA; 50 pg, 20 pg, and less than 1 ng, respectively.[16,26-28]

In the present study, gender determination of all 30 samples was done by *SRY* gene with 100% sensitivity and 73.3% specificity.

Sensitivity and specificity of *SRY* gene in gender identification was performed using the following formula
Sensitivity = Number of true positives / (Number of true positive + Number of false negative)
Specificity = Number of true negatives / (Number of true negatives + Number of false positives)

Real-time PCR analysis of SRY gene identified all the 15 males as males based on the presence of SRY gene. Among the 15 female subjects, 4 females were wrongly identified as males by the presence of *SRY* gene in their tooth brush samples.

Here, false positive results were observed in only married woman. This result might be due to
- Close proximity with male brush or used by a male either child or husband.
- Handling contamination, i.e., from other male samples or from the user.

Riley in his article states that in the close proximity or in contact of two samples, the genomic DNA from one sample can contaminate the other. During PCR, contaminants may be amplified up to a billion times their original

concentration. Contamination can influence PCR results, particularly in the absence of proper handling techniques and proper controls.[29]

SRY gene is a reliable sex typing marker for unambiguous sex typing in forensic samples. But in certain conditions like Turner syndrome (46,X0), Klinefelter syndrome (46,XXY), Swyer syndrome - caused by mutations in the SRY gene (46, XY disorder of sex development, or partial gonadal dysgenesis), 46, XX testicular disorder of sex development - associated with the *SRY* gene, Congenital adrenal hyperplasia,5-Alpha-reductase deficiency, Androgen Insensitivity Syndrome (AIS) and 47, XYY syndrome there may exist a discrepancy between the genotype and phenotype of the person.[30]

Identification and gender determination problem arises in case of chimerism (bone marrow transplants) or micro chimerism (pregnant women carrying male fetuses) like in case of false detection (or non-detection) of amelogenin specific fragments.

In case of chimerism, after successful transplantation, the patient shows similar short tandem repeats of that of donor and which are completely different from his or her own hair roots.

Microchimerism is the presence of a small number of cells that originate from another individual and therefore genetically distinct from the cells of the host individual. Immune cells (T and B lymphocytes, monocytes, macrophages, and Natural killer cells) from a fetus pass through the placenta and establish cell lineages within the mother. Fetal cells have been documented to persist and multiply in the mother for several decades. After giving birth, about 50–75 % of women carry fetal immune cell lines. Maternal immune cells are also found in the offspring yielding in maternal fetal microchimerism. Microchimerism also showed to exist after blood transfusions to a severely immunocompromised population of patients who suffered trauma. So, in case of different sex between donor and recipient, PCR-based sex testing gives wrong conclusions in forensic casework with regard to the gender.[31] Costa *et al* in their study using *SRY* gene proved that the maternal serum of the pregnant woman with male baby gives false positive result (microchimerism). And this was used to identify the gender of the fetus in first trimester.[32]

In our study, no subjects with chimerism and microchimerism were observed. Out of three married women, two were found to have female child and the other none.

There is no single gene that can be used as a sole marker for gender determination with 100% accuracy in all the cases like amelogenin-deleted males, certain mutations and syndromes. Hence, it is advised to use a combination of Y specific and X specific genetic marker for gender identification, which will be more useful in conditions like mutations.

Conclusion

In the present study, *SRY* gene showed 100% sensitivity and 73.3% specificity in gender identification. We also found that PCR is a valuable and sensitive procedure where minute contamination may cause alteration in the result, i.e., four females showed false positive result.

In this study, 86.7% (26) showed the amount of DNA below 4 ng/ul, i.e., below the lower limit of Nanodrop 1000 spectrophotometer. This indicates that even the minute amount of DNA, i.e., 1 pg/ul is enough for typing.

Based on these results, we finally conclude that tooth brush can be used as one of the very valuable sources for gender identification.

References

1. Rajendran R, Sivapathasundharam B, editors. Shafer's Text Book of Oral Pathology. 6th ed. Noida, India: Elsevier Publication; 2009. p. 871.
2. Available form: http://www.trinitydna.com [Last accessed on 2011 Apr 04].
3. Inoue M, Hanaoka Y, Minaguchi K. Personal identification by DNA analysis of samples from dental prosthesis made of acrylic resin. Bull Tokyo Dent Coll 2000; 41:175-85.
4. Fourney RM. Forensic Reality and the Practical Experience of DNA typing update national DNA data bank of Canada 2002. www.isrcl.org/paper/fourney.pdf, (international society for the reform of criminal law, Ottawa, Canada.)
5. Hemanth M, Vidya M, Nandaprasad, Bhavana VK. Sex determination using dental tissue. Medico-Legal Update 2008;8:7-12.
6. Bashamboo A, Giran HM, Asim Azfer MD, ALI S. Genomics of the Human Y Chromosome and Molecular Diagnosis Proc. Indian Natn Sci Acad 2003;4:525-38.
7. Kastelic V, Budowle B, Drobnic K. Validation of SRY marker for forensic casework analysis. J Forensic Sci 2009;54:551-5.
8. Kashyap VK, Sahoo S, Sitalaximi T, Trivedi R. Deletions in the Y-derived amelogenin gene fragment in the Indian population. BMC Med Genet 2006;7:37.
9. Chang YM, Burgoyne LA, Both K. Higher failures of amelogenin sex test in an Indian population group. J Forensic Sci 2003;48;1309-13.
10. Thangaraj K, Reddy AG, Singh L. Is the amelogenin gene reliable for gender identification in forensic casework and prenatal diagnosis? Int J Legal Med 2002;116:121-3.
11. Steinlechner M, Berger B, Niederstitter H, Parson W. Rare failures in the amelogenin sex test. Int J Legal Med 2002;116:117-20.
12. Kao LG, Tsai LC, James Chun-I Lee, Hsieh HM. Controversial cases of human gender identification by amelogenin test. Forensic Sci J 2007;6:69-71.
13. Drobnic KA. New primer set in a SRY gene for sex identification. Int Congr Ser 2006;1288:268-70.
14. Available from: http://ghr.nlm.nih.gov/gene=sry [Last accessed on 2011 Apr 04].
15. Bandhaya A, Panvisavas N. Optimization of DNA recovery from toothbrushes Forensic Science International. Genet Suppl Ser

2008;1:9-10.

16. Tanaka M, Yoshimoto T, Nozawa H, Ohtaki H, Kato Y, Sato K, *et al.* Usefulness of a toothbrush as a source of evidential DNA for typing. J Forensic Sci 2000;45:674-6.

17. Hanaoka Y, Minaguchi K. Sex determination from blood and teeth by PCR amplification of the alphoid satellite family. J Forensic Sci 1996;41:855-8.

18. Sivagami AV, Rao AR, Varshney UA. Simple and cost-effective method for preparing DNA from the hard tooth tissue, and its use in polymerase chain reaction amplification of amelogenin gene segment for sex determination in an Indian population. Forensic Sci Int 2000;110:107-15.

19. Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet 2007;1:191-5.

20. Le Marchand L, Lum-Jones A, Saltzman B, Visaya V, Nomura AM, Kolonel LN. Feasibility of collecting buccal cell DNA by mail in a chort study. Cancer Epidemiol Biomarkers Prev 2001;10:701-3.

21. Thomson DM, Brown NN, Clague AE. Routine use of hair root or buccal swab specimens for PCR analysis: advantages over using blood. Clin Chim Acta 1992;20:169-74.

22. Herber B, Herold K. DNA typing of human dandruff. J Forensic Sci 1998;43:648-56.

23. Allen M, Saldeen T, Gyllensten U. PCR-based DNA typing of saliva on stamps and envelopes. Biotechniques 1994;17:546-52.

24. Graffy EA, Foram DR. A simple method for mitochondrial DNA extraction from head hair shafts. J Forensic Sci 2005;50:1119-22.

25. Bandhaya A, Panvisavas N. Optimization of DNA recovery from toothbrushes Forensic Science International. Genet Suppl Ser 2008;1:9-10.

26. Jiang B, Guo JY. Determination of sex using PCR of the X-Y homologous gene. Amelogenin. Fa Yi Xue Za Zhi 1997;13:68-70,128.

27. Mannucci A, Sullivan KM, Ivanov PL, Gill P. Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homologous gene amelogenin. Int J Legal Med 1994;106:190-3.

28. Sullivan KM, Mannucci A, Kimpton CP, Gill P. A rapid and quantitative DNA sex test: fluorescence-based PCR analysis of X-Y homologous gene amelogenin. Biotechniques 1993;15:636-8,640-1.

29. Riley DE. DNA Testing: An introduction for non-scientists an illustrated explanation. University of Washington 2005.

30. Available from: http://ghr.nlm.nih.gov/gene=sry [Last accessed on 2011 Apr 04].

31. Von Wurmb-Schwark N, Bosinski H, Ritz-Timme S. What do the X and Y chromosomes tell us about sex and gender in forensic case analysis? J Forensic Leg Med 2007;14:27-30.

32. Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P, Dumez Y. First-trimester fetal sex determination in maternal serum using real-time PCR. Prenat Diagn 2000;21:1070-4.

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