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# In Vitro Molecular Characterization of X and Y Gene From Formaldehyde Exposed Old Human Bones.

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**SUMMARY**

Gender Identification from old bones, excavated solid tissues can be an essential asset for the exploration of sex differences in unidentified cadavers and forensic sciences. Traditional Osteometry/Morphometric analysis flops in identification of incomplete remains of skeleton and fragmentary sample details of an individual. In this existing work, we have tried to establish a reliable and sensitive method, grounded for amplification of single copy of Amelogenin-encoding gene [AMG]. The small deletion in Y allele of first intron assisting the design of specific X- and Y- polymerase chain reactions. Amplification of three primers, out of three two primers are of distinctive allele, allows instant identification of both X- and Y- chromosome signals in a reaction particular and a core control, for further confirmation and analysis the reactions are conducted in isolated tubes for each allele. Using this technique, the sex determination was done for the long bone remains of 50 individuals collected from Department of Anatomy, the condition of these samples is varied from poor to best. Long bones [Tibia and Fibula] found likely to have a profound spring of DNA for extraction, the preservation techniques and purification methods are complex as it depends on the sample adulteration. Manual method of extraction used in this research gave the best results. This study shown the validity of the sex identification trial on the excavated remains of abandoned samples of different times.

## I. INTRODUCTION

Analysis of sex determination plays a significance in building social profoundness of past, present and future. Sex determination in forensic sciences or in medical sciences is generally identified through the whole bone morphology, though for fragmentary remain analysis this procedure found to be unrealistic and falsified. The fragmentary analysis with best molecular tools and reliable genetic methods can provide an ideal methodology that can be trusted. Still Several difficulties and methodological problems in evaluating these unidentified fragmented, left out samples.

PCR is an extremely sensitive reaction and thus susceptible to the hinderance by irrelevant genomic DNA amplification during PCR reaction.[i]

Sex determination based on Genomic DNA analysis from skeletal fragments was first performed by amplification of Y- chromosome definite sequences (Hummel and Herrmann,1991). On the other hand, negative result may be depicted as female, the finding is still questionable, the article from Elsevier sciences for sex determination through amplification of X- and Y- allele proposed the same negative findings, in which they preferably took it as the poor preservation of DNA, but still, it is questionable that poor preservation is not gender specific.

There could be possibilities of degradation and other factors too can hinder the PCR reactions and the reaction particular is mot amplified.

AMG often have an advantage of core control (Akane et al., 1991). The Y-allele carry sequence variation of intron specifically a deletion in 189 bp (Nakahori et al, 1991).

This procedure requires relatively large amount of immaculate DNA.

For old and ancient DNA, PCR that is based on amplifying smaller section of AMG gene can be considered. (Sullivan et al.,1993).

We are now improvising and developing new methods based on amplification of Intron 1 of amelogenin based sex determining gene, using 3 primers and also separate analysis of unknown samples.

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## II. EXPERIMENTS AND DISCUSSION

**Development of PCR reaction primer analysis for sex identification.**

The chief aim of this research is to determine the dependability of the method and to inspect several parameters that work as stimulus, and an ideal method of extraction. It is interesting to determine whether it could be possible for a successful DNA analysis by the examination of the poor to best preservation.

The process developed for sex determination in the fragmented bone samples. In order to amplify the specimens, the primer period, short DNA fragments from of the gene sequence (Nakahori et. al., 1991)

An additional specificity of the three primers is applied:

The 5`primer (Forward Primer) [M4, 5`-CAGCTTCCCAGTTTAAGCTCT] is going to be mutual for both the X- and Y- Alleles, while 3` primers are allele specific.

3` X- specific primer:

[M5, 5`-TCTCCTATACCACTTAGTCACT]

Extracted from the sequence deleted from the Y-chromosome locus

3` Y-specific primer: [ M6, 5`-GCCCAAAGT TAGTAATTTTACCT]

This deletion covers the deletion break points. The 320-bp and 218- bp PCR products of X-/Y-chromosome respectively, sensed by agarose gel electrophoresis, for a known sample all three primer are used in a single tube reaction, Ailment of this PCR reaction were established and stated high sensitivity, The extent for female with some faded bands is 25pg while for males it is 5-10pg, equivalent to DNA obtained from a diploid cell (Maria Faerman et. al.

**Accuracy of the DNA analysis for sex determination in fragmented bones.**

The sample is analyzed and listed with reference to the result and amplification 40 samples of which are unknown samples, 10 are known male and female samples with reference to which the genetic and molecular analysis is reliable.

DNA was extracted from bones by decalcification method, 400 mg of bone soaked overnight in 10-12 ml .5 M EDTA for 4º C that is susceptible to chelate $Ca^{2+}$ ion, which is further follow up by Lysis through Proteinase- K and cell lysis buffer and nucleus lysis buffer, also known as Maceration and lysis, Step 2 is precipitation and purification which involve NaCl and chloroform precipitation [salted chloroform precipitation] follow up by sodium acetate treatment, further for purification purpose ethanol (C2H5OH) is considered in the next step.

To distinguish that the band that is visible at 218 bp is Y- allele and not some other random amplification, X- and Y- specific primers.

The result demonstrates Y- specific PCR product and not specifically X-primer. which is one forward primer and two [M5 and M6] are allele specific reverse primer.

| S, No. | SampleName | identity | ExtractionMethod | sex |
|---|---|---|---|---|
| 1. | Sample1 | unknown | salted chloroform | NB /F |
| 2. | Sample2 | unknown | SaltedChloroform | M |
| 3. | Sample3 | unknown | Salted Chloroform | M |
| 4. | Sample4 | unknown | SaltedChloroform | M |
| 5. | Sample5 | unknown | Salted Chloroform | M |
| 6. | Sample6 | unknown | SaltedChloroform | M |
| 7. | Sample7 | unknown | Salted Chloroform | M |
| 8. | Sample8 | unknown | SaltedChloroform | M |

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| | | | | |
|---|---|---|---|---|
| 9. | Sample9 | unknown | Salted Chloroform | M |
| 10. | Sample10 | unknown | SaltedChloroform | NA /F |
| 11 | Sample11 | unknown | Salted Chloroform | NA /F |
| 12 | Sample12 | unknown | SaltedChloroform | NA /F |
| 13 | Sample13 | unknown | Salted Chloroform | NA /F |
| 14 | Sample14 | unknown | SaltedChloroform | M |
| 15 | Sample15 | unknown | Salted Chloroform | NA /F |
| 16 | Sample16 | unknown | SaltedChloroform | M |
| 17 | Sample17 | unknown | Salted Chloroform | M |
| 18 | Sample18 | unknown | SaltedChloroform | M |
| 19 | Sample19 | unknown | Salted Chloroform | M |
| 20 | Sample20 | unknown | SaltedChloroform | M |
| 21 | Sample21 | unknown | Salted Chloroform | M |
| 22 | Sample22 | unknown | SaltedChloroform | M |
| 23 | Sample23 | unknown | SaltedChloroform | M |
| 24 | Sample24 | unknown | saltedchloroform | M |
| 25 | Sample25 | unknown | SaltedChloroform | M |
| 26 | Sample26 | unknown | Saltedchloroform | M |
| 27 | Sample27 | unknown | Salted chloroform | NA /F |
| 28 | Sample28 | unknown | Saltedchloroform | M |
| 29 | Sample29 | unknown | Salted chloroform | M |
| 30 | Sample30 | unknown | Saltedchloroform | M |
| 31 | Sample31 | unknown | Saltedchloroform | NA /F |
| 32 | Sample32 | unknown | Saltedchloroform | M |

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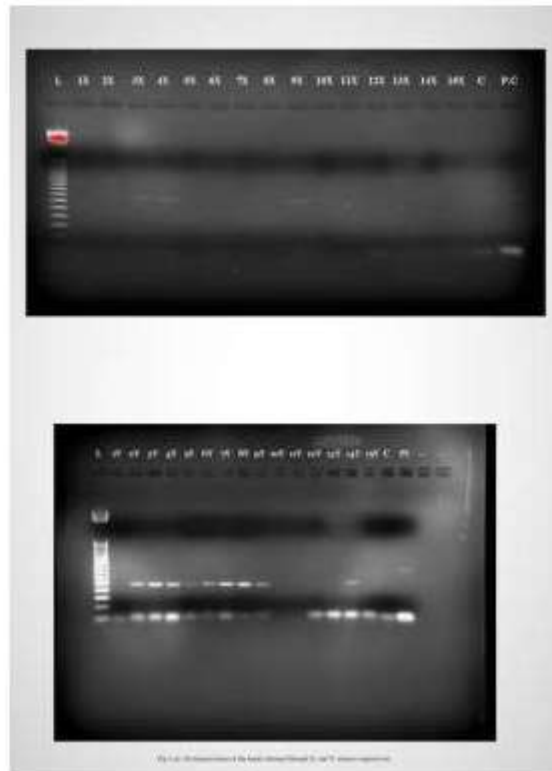
| | | | | |
|---|---|---|---|---|
| 33 | Sample33 | unknown | Saltedchloroform | NA /F |
| 34 | Sample34 | unknown | Saltedchloroform | NA /F |
| 35 | Sample35 | unknown | Saltedchloroform | M |
| 36 | Sample36 | unknown | Saltedchloroform | M |
| 37 | Sample37 | unknown | Saltedchloroform | M |
| 38 | Sample38 | unknown | Saltedchloroform | M |
| 39 | Sample39 | unknown | Saltedchloroform | M |
| 40 | Sample40 | unknown | Saltedchloroform | M |
| 41 | Sample41 | male | Saltedchloroform | M |
| 42 | Sample42 | male | Saltedchloroform | M |
| 43 | Sample43 | male | Saltedchloroform | M |
| 44 | Sample44 | male | Saltedchloroform | M |
| 45 | Sample45 | female | Saltedchloroform | NA /F |
| 46 | Sample46 | female | Saltedchloroform | NA /F |
| 47 | Sample47 | female | Saltedchloroform | NA /F |
| 48 | Sample48 | female | Saltedchloroform | NA /F |
| 49 | Sample49 | female | Saltedchloroform | NA /F |
| 50 | Sample50 | female | Saltedchloroform | NA /F |

Table1. sex determination of collected fragment remains using DNA extraction [ M, male; F, female; NA, not amplified]



[Fig.1 [a.] [b.] Top left (1-15); Sex determination in Skeletal remains through X- and Y- primer respectively]

[Fig.2 [a.] [b.] top right (16-30); sex determination in fragmented bones through x-and y- primer respectively].

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Fig.3 [a.] [b.] bottom right suggest (31-40); governed with single specific X- and Y- primer while from [40-50 are known, in which (41, 42, 43, 44 are males while (45, 46, 47, 48, 49, 50) are specifically females.] performed for M4, M5 and M6 primer in a single reaction.

The picture suggests the consistency and reliability of the results as these samples consist some known samples too, the ladder is of 50bp DNA ladder; C~ control without DNA
PC~ Positive Control with DNA.

For DNA extracted with NaCl, chloroform, sodium acetate and the amplified product were analyzed in the gel with EtBr. On analyzing different known and unknown sample present in our present research, separate amplification of Y- and X- alleles. DNA of unknown specimen no. 1 to no. 40 amplified with specific Y- primers [M4 and M6] and specific X – primers which is [M4, M5]. Our present research is based on the primer used by [Marina Faerman et. al.,1995]. The sample was extracted by various sources such as Femur, ulna, Tibia, Fibula, using a single method of extraction which is sensitive and reliable.

**DNA extraction and PCR procedure and material.**

The dependency of PCR reaction, 0.5 mg- 1.0mg of prepared bone powder, obtained from various specimen. Polymerase chain reaction with 2-4$\mu l$ per PCR reaction. The method of DNA extraction involves the decalcification of 400mg of bone powder which is crushed in Mortar and Pestle with EDTA [ pH~7.6, 0.5M]. 1mg of bone powder is enough to accomplished the DNA extraction for each bone specimen with a positive control [ bone sample] and a "no positive control" [without DNA]. The process of DNA extraction using salted chloroform extraction [ Lahari et. al.]. Extreme precaution should be taken to avoid contamination. The samples were handled using precaution measure such as Face masks, gloves, and laboratory aprons, the work should be handled carefully and at sterile location i.e., portable laminar workstation and should not have another DNA contamination. The extraction and PCR product were performed in two separate hoods in differently irradiated Ultraviolets, the disposable filtered tips, tubes and aliquots of sterile reagent and solution, Different pipettes were used for extraction of DNA.

The PCR was accomplished in 20-25µl using 10.5-12.5µl of Master mix (Taq polymerase, KCl, DNA polymerase, buffer, dNTPs), 0.3 to 0.5µl of forward and reverse primer (M4, M5 X-Specific), (M4, M6 Y- Specific), 0.5µl of MgCl2 for 40 cycles (1 min. in 94° C, 2 min in 54°C and 2 min. in 72° C. 16µl aliquots is prepared for 18 reactions in a batch to avoid pipetting error which is performed under 2% Merck agarose gel for Genei electrophoretic unit, analyzedunder EtBr

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staining, documentation under Bio Rad-Chemi DOC. The long-term DNA storage for future studies can be probably stored in the bones. Most proofs in this research suggested that Y- gene is amplified more than X- allelic gene. The Y-specific band was still visible when the primer concentration is diluted, the higher sensitivity of detection in Y-allele is because of short amplification product.

Although this study suggest that these primers are not prone to detect the female sample rather than male. The more proficient amplification suggests that in y-allele the presence of band at 320bp is not a false negative result*. The 4 known males (41, 42, 43, 44), confirm the other DNA analysis result for gender identification. Some samples three of them are not amplified. There found no such discrepancies between these two (known and unknown) molecular analyses.

## III. RESULT AND ANALYSIS

The optimizing amount of DNA in a reaction is a most important factor, increasing the amount of DNA, can improve PCR analysis.

No PCR reactions contain too little or too much DNA. the importance of analyzing the PCR reaction through gradient PCR, in which the result suggests that this primer which we are using is optimized to give result between the 50-60º C for each specimen which is 55ºC.

In this reaction analysis we have also concluded that this analysis is barely have to do with the concentration of DNA concentration because those primer are prone to detect the samples which is given in picograms.

Moreover, it is also observed that on observing the physical morphology, the bones which are tinted and comparatively more yellow than that of other bones, has slightly less amount of DNA concentration.

These result and observation suggest that out of 50 samples 34 are readily observed as males while rest of the observation suggest the female samples present 3 of these are not amplified but on illustrating the result and observation the 6 known sample of female (45-50), it can be assumed that the rest of others can be female too.

## IV. CONCLUSION

1. method of selective amplification of X and Y with three different primers are sensitive and reliable, when compared to known analysis of sample,
2. Sex was successfully determined on 33 samples out of 50 which provides that the observed sample is a male. This provides an important tool for forensic medicine and the gender differences.
3. Manual DNA extraction and purification of amplified DNA, minimize the damage and degradation
4. DNA is better preserved in long bones
5. Morphological appearance of bone cannot indicate the preservation.

6. DNA preservation is greatly depended on the local environment and condition on which those are kept.

## V. ACKNOWLEDGEMENT

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