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Molecular Markers for Human Sex Determination in Forensic Genetics Analysis

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ABSTRACT

Sex determination is indispensable in forensic anthropology, sexual disorder, and also as part of large-scale genetic population studies. The purpose of this investigation is to determine the human sex from whole blood using multiplex PCR analysis. Blood samples from 75 male and 70 female healthy volunteers were taken from Tikrit city, Iraq. Our study identified a reliable set of three primer locus, namely SRY, ALT1 (internal control) and amelogenin locus. The SRY primer on the Y chromosome showed a 254 bp of PCR product, with 100% accuracy for human male identification. Thus, the pair of SRY primers was considered a strong genetic marker for human sex identification. Amelogenin regions in the Y chromosome showed a true positive band (236 bp) with 100% accuracy on sex identification. Amelogenin regions in X chromosome also showed positive bands (330 bp) in female samples and positive band in male samples except for two samples showed a negative band (null bands). The most obvious finding from this study is that multiplex PCR of ALT1 and SRY is consider as a reliable genetic marker for human sex identification. The research has also shown that amelogenin is good genetic marker for human sex identification.

Keywords- Sex determination, Forensic DNA, SRY, Amelogenin.

I. INTRODUCTION

Forensic genetic analysis is a most powerful laboratory technique that has become a vital tool, in various forms, the optimal standard by which other fields of forensic sciences are compared [1]. In recovering human remains, sex determination is an important step in addition to estimating age and Ethnicity. Determination of sex is beneficial for guessing of age and stature and can decrease the probabilities of forensic cases being matched according to the results of gender [2, 3]. Blood samples are the ideal form for biological evidences that are collected from different kinds of crimes i.e. murder, rape-sexual homicide, hit and run, road accident cases, tool marks, or other types of a heinous crime [4]. Genomic DNA isolation from whole blood samples afford high quantity and quality DNA compared to any other biological source [5,6]. The advanced technological and molecular biology development in the field of forensic medicine, especially the Polymerase Chain Reaction (PCR) offers the most sensitive, accurate and rapid technique for sex determination across DNA analysis of gender-specific sequences on X and Y chromosomes [7]. The genetic markers for sex determination, based on PCR analysis, included various loci especially SRY (sex-determining region Y) and amelogenin [8, 9].

SRY is also known as the sex-determining region of Y chromosome; even though several genes are involved in human sex determines, SRY is the switch responsible for the development of the male, while individuals lacking SRY will develop into females [10]. The SRY gene is found in the short arms of the human Y chromosome at p11-31 [11], exactly, between nucleotides no. (2,786,854 - 2,787,740) [12]. Amelogenin or AMEL gene is presently the most common genetic marker for sex-typing in forensic investigation and prenatal sex determination. A single copy of the amelogenin gene has existed in both the X and Y chromosome (sex chromosomes) [13]. The AMEL gene is different in size and arrangement in both the Y and X chromosomes. Two homologous AMEL genes have existed in humans (AMELX and AMELY): AMEL X size is 2872 base pairs, which is located in the p22. 1 → p22 region of the distal short arm of the X chromosome, and AMEL Y size is 3272 base pairs, which is located in p11.2 region near the centromere of the Y chromosome [14].

The challenge of forensic DNA analysis often caused by partial DNA degradation. In such cases, degraded DNA may offer partial or no results or definite conclusion [15]. On the other hands, there exist several clinical cases, the people patients with absence of the SRY gene has a fully female phenotype despite the genetically that they have the Y chromosome [16,17], the disorder cases with 46, XX maleness and in rare cases 46, XX true hermaphrodites (both fertile men and women) caused by the found of SRY on the X chromosome [18,19]. On the contrary, the patient has a male phenotype even though carrying two X chromosomes, these types of sexual disorders caused by translocation of the SRY gene to an autosomal chromosome [20]. Other challenges, the mutations or deletions in DNA sequencing may result in no product of amplification. Previous research has shown rare failures in an amelogenin test, the failure test of the amelogenin sex was 0.018% as earlier mention in the Austrian National DNA database [21], a variable frequency of

amelogenin failure from 0.018% to 8% in populations of the Indian subcontinent [22], and 0.6% sex test was failure in unrelated Sri Lankan people [23].

Despite the importance of sex determination in various field, there no previous study has investigated the efficiency of SRY and AMEL gene in sex determination of Tikrit population. The aim of this research was to assess an efficient and sensitive human sex determination in samples of the Tikrit population by using polymerase chain reaction amplification of SRY and amelogenin genes.

II. MATERIALS AND METHODS

2.1 Population study

A random sample of volunteers was recruited from the Tikrit population who have descended from Tikriti paternity for at least the two previous generations, Iraq. The samples was divided into two groups according to gender, half the sample was male (75 male, 52%), while 48 % (70 female) were female.

2.2 Samples collection

About 3 ml of whole blood were collected for genetic analysis in EDTA tubes. The sample tubes were barcoded as M1, M2, M3 to M65 for Male and the female samples were barcoded as F1, F2, F3, to F60. All samples were kept at −20°C until further use.

2.3 DNA extraction

The volume of whole blood used in DNA extraction was 200 µl, using the procedure described by [24]. DNA was stored at -20 °C for short period and -80 °C for long period. DNA quantity and quality were estimated through the NanoDrop spectrophotometer (Thermo Fisher Scientific).

2.4 Amplification of SRY and AMEL genes by PCR

In the current research, primers used for PCR amplification of SRY was a modification method was described by [25], while primer sequence used for ATL1gene was described by [26]. Primers for the ATL1 gene amplification was used as an internal control. Additionally, a set of primers to amplify the AMEL gene was used as described by [14, 27]. Table 1 shows the detailed sequences of primers used in the current study.

Table 1: Primers sequences to amplify the SRY, ATL1, and AMEL gene by PCR with amplicon size, respectively.

Gene	Forward Primer (5')	Reverse Primer (3')	Product size bp
SRY	CATGAACGCATTCATCGTGTGGTC	CTGCGGGAAGCAAACTGCAATTCTT	254
ATL1	CCCTGATGAAGAACTTGTATCTC	GAAATTACACACATAGGTGGCACT	301
AMEL	CAG CTT CCCAGT TTA ACT TCT G	CTC TCC TAT ACC ACT TAG TCA G	330
		TGCCCAAAGTTAGTAATTTTACCT	263

In PCR reaction, the total volume is 25 µL comprising 12.5 µL Go Taq Green Master Mix (Promega, USA), 10µM of each upstream and downstream primers (2 µL), containing about 30–40 ng of genomic DNA (2 µL), and Nuclease-Free Water to final volume. For SRY and ATL1, PCR cycling parameter is repeated of heating and cooling consisted of predenaturation at 95 °C for 4 min, followed by 35 repetitive cycles of denaturation at 95 C for 45 s, annealing at 57 C for 60 s, and extension at 72 C for 60 s, and post extension at 72 °C for 10 min. PCR products were electrophoresed on 2.5 % agarose gels prestained with RedSafe stain to visualize amplification bands under UV light. The AMEL gene was amplified by only one denaturation step at 94 °C for 4 min, followed by repetitive 35 cycles of denaturation at 94 °C for 45 s, annealing step for 60 s, and synthesis step at 72 °C for 60 s. An extra extension step of 10 min at 72°C. Staining PCR product by RedSafe allows to visualization of DNA bands in the agarose gel (1.5%) under UV light.

2.5 Estimation of sensitivity and specificity

Sensitivity test was used to determine the proportion of positives results that are correctly diagnosed. Whereas the specificity was referred to the percentage of negatives samples which were correctly diagnosed. The following equation is used to calculate the sensitivity and specificity:

Sensitivity (%) = [TP / (TP + FN)] × 100

Specificity (%) = [TN / (TN + FP)] × 100

Note:

- TP = number of true positives.
- FN = number of false negatives.
- TN = number of true negatives.
- FP = number of false positives.

III. RESULTS

PCR of the SRY and ATL1 genes successfully amplified specific bands at 254bp and 301bp respectively.

SRY gene is a gene located on the Y chromosome. Therefore, the PCR product band sized 254 bp for the SRY gene was used as a genetic marker to distinguish between males and females. DNA from male samples produced in two bands of 254bp and 301bp as a result of PCR amplification from Y and X chromosomes respectively, whilst DNA from female samples produced only a single band, 301bp as a result of PCR amplification for the X chromosome (Figure 1).

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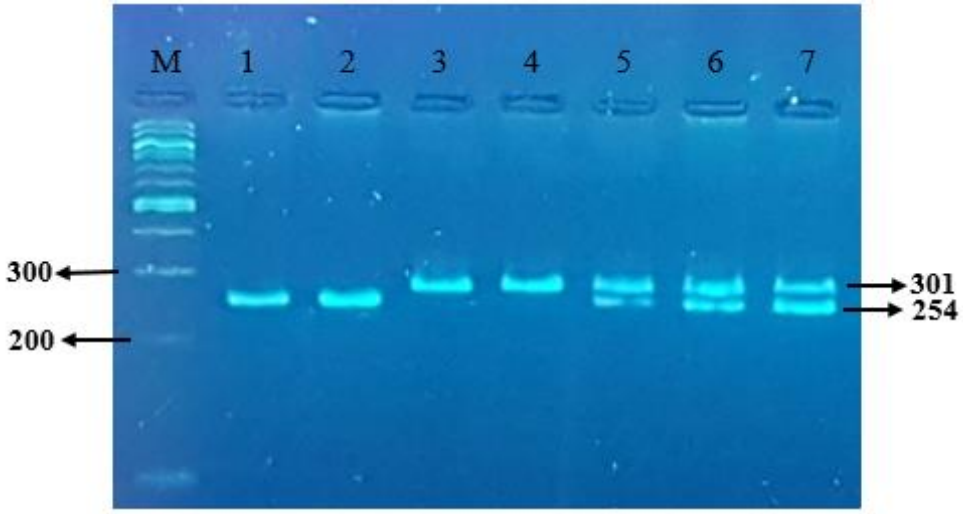

Figure 1: PCR product of SRY and ATL1 genes (M= marker, lanes 1 and 2 single PCR for male samples with only SRY primer, lanes 3 and 4 multiplex PCR for female samples with SRY and ATL1 primers, lanes 5, 6 and 7 multiplex PCR for male samples with SRY and ATL1 primers)

The male DNA samples in lanes 1 and 2 show PCR results with only an SRY gene primer. The samples from 3 to 7 contain specific bands at 301bp were amplified from the ATL1 gene, confirming that the ATL1 gene could be considered as reliable reference gene (internal control). A 301 bp band representing female samples with the multiplex PCR (ATL1/ positive, SRY/ negative) in the third and fourth lanes, whilst the fifth, sixth and seventh lanes, show the male samples with the multiplex PCR (ATL1/ positive, SRY/ positive).

As shown in Figure 2, a specific band with 330 bp was obtained from X-chromosome-AMEL genes whilst a specific band with 236 bp was obtained from Y-chromosome-AMEL genes.

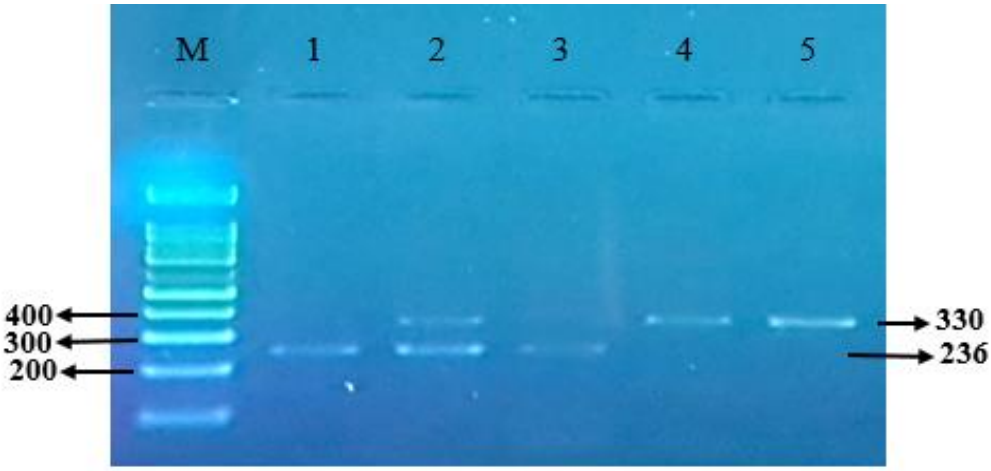

Figure 2: PCR product of AMEL genes (M= marker, lanes 1, 2 and 3 multiplex PCR for male samples, lanes 4 and 5 multiplex PCR for female samples).

From the amelogenin locus, 2 bands were shown in the male human sample. The fragments derived from two loci (AMEL -X and AMEL -Y) showed recognizable differences between male and female samples. All male samples showed two-band (330- AMEL -X and 236 AMEL -Y bp) except two samples showed AMEL -Y/ positive and AMEL -X negative (Fig. 2, lane 1and 3). As anticipated, only one band (330- AMEL -X) for all 70 females' sample was identified when using multiplex PCR (Fig. 4, and 5 lanes).

The sensitivity and specificity of these genes using the 145 DNA samples were 100% (Table 2), with false-negative or false-positive results were not detected. Although the current study recorded negative results in the AMEL -X locus of two male samples (AMEL -Y/ positive and AMEL -X negative), the current study has correctly identified human sex for all DNA samples with 100% exactitudes.

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Table 2: Interpretation of the sensitivity and specificity conclusion for both SRY and AMEL genes

Gene	Gender		Total
	Male	Female	
SRY (positive)	75	0	65
SRY (negative)	0	70	60
Total	75	70	125
Sensitivity (%) = 100 %			
Specificity (%) = 100 %			
AMEL -Y (positive)	75	0	125
AMEL-Y (negative)	0	70	60
Total	75	70	125
Sensitivity (%) = 100 %			
Specificity (%) = 100 %			

IV. DISCUSSION

The determination of gender in human populations played a key role in evolutionary processes, prenatal sex determination, Sex-Linked (X and Y chromosome) and sex determination in forensic cases. There are several advanced methods in molecular biology using for sex identification with more reliable and reproducible results [28]. The data from our study verified the usefulness of multiplex PCR of X and Y chromosomes in determining the sex of individuals by using SRY and AMEL genes.

The ATL1 (X-chromosome-specific) gene and the SRY gene (Y-chromosome-specific) have efficiently identified the gender of all samples with 100% sensitivity and specificity of human sex determination. Previous studies have demonstrated that the ATL1-specific sequences were to be a dependable internal control (reference control) for X-chromosome determination in all samples [29, 30, 31]. From Fig. 1, the SRY gene can be utilized as a reliable genetic marker to distinguish between male and female samples according to the results of the population of study. SRY is a powerful sex-typing marker in forensic genetic analysis because the gene product of SRY has connected with male sexual development [3]. This makes SRY gene an accurate biological predictor in the current population of study for the male sex [32]. These result also accords with our earlier observations, which showed that SRY is a potential molecular marker for detect male sex [33, 34,35].

The AMEL gene is located on sex chromosomes (the X and the Y chromosomes). Whilst, previous research indicated there were differences in gene size between the X and the Y chromosomes, which have been used as a genetic marker for sex-typing in prenatal testing and forensic genetic casework [36]. From the 70 female samples (X chromosomes), this procedure produced presentable results, 70 (100% of positive bands) samples with sex identification. Whilst 75 X chromosomes in male samples produced 73 positive bands and only two negative bands. On other hands, the 75 male samples (Y chromosome), produced perfect results (100% of positive bands) with 100% sensitivity and specificity of human sex determination. From the two male samples, only the amelogenin Y allele amplicon was observed. These cases may result from the polymorphism that occurs on primer binding regions of the X amelogenin gene [37,38]. Therefore, the primer of the AMEL X- chromosome did not bind to the target DNA, resulting with no amplification. These results seem to be consistent with a few research which found rare mistyped sex identification by the amelogenin sex test in various human populations [24,39,40,41]. Although our results approved the reliability of the AMEL gene as sex determined markers, further work is required to establish the viability of the AMEL gene as sex determination markers especially in forensic investigations.

V. CONCLUSION

The present study was designed to determine the SRY and AMEL gene accuracy in sex determination for samples from the Tikrit population. Our experiments confirmed those genes were highly reliable for human sex identification with no positive or negative false results. Nevertheless, AMEL gene Y was absent from the two male samples which makes these findings less acceptable. Therefore, further research is required to establish the efficiency of the AMEL gene as a forensic genetic marker for sex determination. Finally, the findings make an important contribution to the field of DNA database in the Tikrit population, with relevant results from other previous studies [42,43].

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