Application of Two Sex Markers by Nested PCR for Gender Determination

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Abstract

Sex determination is one of the basic components in victim identification. There are many available methods, namely forensic anthropology method and conventional DNA typing method. In this study, nested PCR technique was employed in sex typing of burnt teeth through amelogenin (AMEL) and sex-determining region Y (SRY) markers. In this study, 17 teeth samples were burnt at temperatures that ranged from 100°C to 500°C for 2 min - 10 min. The whole tooth was used for DNA extraction by phenol-chloroform method. Accurate sex determination was achieved in 13 samples by both AMEL and SRY markers. The SRY marker achieved higher sensitivity as compared to AMEL marker. The sensitivity of both markers was improved consequent to nested PCR. Factors such as degraded DNA materials and the presence of tooth caries greatly affect sex typing results. Results showed that nested PCR proved to be a good method to amplify highly degraded DNA material as it greatly increased the DNA copy, and thus increased the possibility of sex typing.

Keywords*: amelogenin gene; burnt teeth; nested PCR; sex determination; SRY gene*

Introduction

In the victim profiling process, especially cases of mass disaster, sex determination is the basic tenet of forensic science with methods that include direct observation of genitals, forensic anthropology and conventional DNA typing. However, identification of victims is not always applicable in cases of explosion, burnt corpse or comingled remains when a complete body or skeletal remain is no longer intact. Therefore, an alternative method needs to be employed to determine the identity and sex of victims.

Conventional forensic DNA analysis utilises short tandem repeat (STR) to determine the sex based on the homologous amelogenin genes on X and Y chromosomes (AMELX and AMELY), which is incorporated in STR typing kit[1]. Previous study of a burnt corpse failed to produce result when STR typing was used to amplify due to degraded DNA materials[2].

Amelogenin gene is used as a sex marker for forensic samples since there is a length variation of amplified fragments between X and Y amelogenin[3]. This advantage of AMEL gene enables amplify by using one set of primers which target the X chromosome and Y chromosome. However, a study by Akane et al.[3] showed that this marker was unable to determine the sex of a drowned victim who was dead for more than a month. Based on a study by Zagga et al.[4], the success rate for sex identification by using amelogenin gene was only 33.3% for samples subjected to heating between 100°C to 300°C.

Besides, mistyping of male subjects as female was also reported due to AMELY deletion in the Y chromosome[5]. Primer site mutation of both AMELX and AMELY genes was theorised as one of the reasons for sex typing failure, causing the lack of an amplicon which caused confusion while interpreting DNA mixtures, especially in sexual assault cases[1]. A study conducted by Chang et al.[6] showed a significant number of AMELY

null individuals amongst Malay males (0.6%) and Indian males (3.2%), and none of Chinese male individual. A similar study also suggested that sex typing based solely on amelogenin is insufficient for use in routine forensic work[6].

As stated by previous studies, AMEL gene alone has high possibility of producing inaccurate results, thus it is important to utilise other sex markers with higher accuracy[5, 7]. In this study, sex-determining region Y (SRY) gene was chosen as it is the most closely linked to the male sexual phenotype and activation of SRY is required for testis formation[8]. This alternative sex marker is located at p11.31 on the Y chromosome[1], which is a part of the testis-determining factor (TDF) gene. The mutation of this gene has caused a sex reversal in XY females[8]. Currently, SRY gene is the only sex marker that has products which directly affects sex development in males. The strong association of SRY products and male phenotype suggests that SRY gene is the most accurate marker for male phenotype appearance when unreliable results were obtained from AMEL analysis[1]. According to Naik et al.[7], sex determination of human dental pulp through SRY gene showed 100% accuracy for all 20 tooth samples.

Forensic samples from the crime scene are always received in different conditions from normal samples, and thus application of nested polymerase chain reaction (PCR) is very helpful as compared to conventional PCR. Nested PCR is a modification of PCR which is designed to improve sensitivity and specificity of the amplified product through two primer sets and two successive PCR reactions[9]. The advantages of nested PCR are it reduces amplification error, consumes less time and involves a minimal cost.

In the present paper, two sex markers, namely amelogenin and SRY genes, were used to identify the sex of teeth samples treated at various temperatures and durations by employing the nested PCR amplification technique.

Material and Methods

Sampling

A total of 17 extracted teeth samples were obtained from School of Dental Sciences, Health Campus, Universiti Sains Malaysia and stored in dry condition. Prior to collection of teeth samples, ethic approval was obtained from the Human Research Ethics Committee USM (USM/JEPeM/16020081). A blind study was adapted, whereby the researcher had no information about the sex of tooth owner prior to analysis.

Then, all tooth samples were immersed in 10% bleach solution for 5 min, and followed by soaking them in 70% ethanol for 5 min. The teeth were later exposed to ultraviolet (UV) light for 15 min to eliminate exogenous DNA. All tooth samples were grouped based on designed experiment. Table 1 shows the specific temperature and exposure time applied for each tooth. The burning process was carried out in a burnout furnace. Teeth samples were ground into powder by using pestle and mortar prior usage for DNA extraction.

Genomic DNA Extraction

In this study, the phenol-chloroform organic method was employed for DNA extraction procedure, as suggested by Presecki et al.[10]. Approximately 0.15 g – 0.20 g of tooth powder was used as the starting material for DNA extraction. To increase the DNA extraction efficiency, 0.01 M of DTT (dithiothreitol) was added to the tooth powder mixture. The extracted DNA samples were then quantified by Thermo Scientific NanoDrop™ 2000 Spectrophotometer and stored at -20 °C for subsequent analysis.

PCR Amplification

In this study, the published primers were used for PCR amplification of amelogenin gene[1, 11] (Table 2). The PCR reaction mix was prepared in 20 µL which consisted of 1 µL of 10 pmol of each forward and reverse primer (Sigma, USA), 0.32 µL of 10 mM dNTPs (Bioline, USA Inc), 1X PCR buffer (Bioline, USA Inc), 2 µL of 25 mM $MgCl_2$ (Bioline, USA Inc), 1 U of Taq DNA polymerase (Bioline, USA Inc) and added with ddH_2O to make up to 20 µL of total reaction mixture. The external and internal amplifications have the same components in the PCR mix, with difference in volume of DNA template. The external PCR mix contained 2 µL of template while internal PCR mix contained 10 µL of DNA templates. The PCR applied for external and internal amplifications was as follows: 95°C for 7 min followed by 40 cycles of 95°C for 1 min; 56°C /58°C /60°C /66°C for 30 s; 72°C

for 45 s and the final elongation at 72°C for 7 min. For monitoring of contaminations, negative (without DNA) and positive (male and female DNA) controls were simultaneously amplified with samples.

Then, all amplified products were analysed by using 2% agarose gel and pre-stained with ethidium bromide solution (10 mg/mL). A total of 5 µL of PCR products was mixed with 2 µL Orange G dye and loaded for analysis. The electrophoresis was performed at 100 V for 1 h 30 min.

Results and Discussion

Genomic DNA Extraction

The analysis of extracted genomic DNA showed clear bands of high molecular weight DNA which were visualised in 6 out of 17 samples, with concentration that ranged from 27.3 ng/µL to 130.6 ng/µL after quantitating by using NanoDrop spectrophotometer. Other samples were undetected due to low concentration of DNA extracted and was supported by quantitation results which showed lower value of nucleic acid concentration. The purity value of $A_{260/280}$ reading ranged from 1.30 to 1.88, which indicated slightly lower than pure DNA

Table 1. Teeth sample information with respective exposure temperature and duration.

Sample ID	FDI Notation	Temperature (°C)	Duration (min)
A1	18	100	2
A2	13	200	2
A3	17	300	2
A4	47	400	2
A5	37	500	2
B1	12	100	4
B2	45	100	6
B3	23	100	8
B4	13	100	10
C1	17	200	4
C2	36	200	6
C3	26	200	8
C4	38	200	10
D1	25	300	4
D2	47	300	6
D3	37	300	8
D4	38	300	10

Table 2. Details of primer sequences.

Locus	Amplification	Primer sequences (5' – 3')	PCR products (bp)
AMEL	External	Forward (AMEF) ACC ACC TCA TCC TGG GCA C	281 (X) and 287 (Y)
		Reverse (AMER) TTA CGG CCA TAT TTA GGA	
	Internal	Forward (AMIF) ACC TCA TCC TGG GCA CCC TGG	212 (X) and 218 (Y)
		Reverse (AMIR) AGG CTT GAG GCC AAC CAT CAG	
SRY	External	Forward (SRYEF) CGG AGA AGC TCT TCC TTC CT	198
		Reverse (SRYER) CAG CTG CTT GCT GAT CTC TG	
	Internal	Forward (SRYIF) AGG CAA CGT CCA GGA TAG AG	85
		Reverse (SRYIR) TTT CGC ATT CTG GGA TTC TC	

Recommendation, although a few samples were within the suggested value. This may suggest that the phenol-chloroform method is still reliable and can produce sufficient DNA from the burnt and degraded tooth samples. As stated by previous studies, the DNA yielded from tooth can be affected by condition of tooth and chronological age of individual[12, 13]. Chronological age has positive effect on DNA yield because mature teeth are more mineralised and less porous, contributing to higher resistance to decomposition and more protection for endogenous DNA[13].

Comparison of Sex from DNA Typing and Known Sex

The results of PCR amplification were compared with the known sex of the tooth owner to verify sex of the teeth sample. Amplification products of single band indicated a female origin sample while double bands showed a male origin sample when amplified with AMEL primers. It was found that only seven samples were successfully amplified with AMEL external primer due to the degraded DNA condition. Second amplification by using AMEL internal primer showed that most samples were typed because smaller product size may increase the chances of amplification. Result showed that the accuracy of amelogenin typing was 76.47%. Application of SRY primers could distinguish male from female samples since the amplification only detect male samples. The accuracy of sex determination by using SRY primer was found to be higher than AMEL primer, with 88.24%accuracy. Therefore, it proved that the SRY gene is a suitable alternative marker for sex identification in situation when an individual cannot be resolved through AMEL gene.

Sensitivity of both AMEL and SRY genes were increased when nested PCR was applied (Table 3). A previously reported study showed that a negative result via classical PCR can be solved through nested PCR technique[11]. In addition, SRY marker showed higher sensitivity as compared to AMEL marker, which indicated a better sex marker. A study by Morikawa et al.[5] showed all samples were accurately typed by using

SRY gene marker, including AMELY-deleted male samples. Another research found that with the SRY gene in the pulp, 100 percent of the specificity was noticed, indicating that this gene may be a confirmatory test for male gender identity[14].

Discrepancy Upon Comparison

The findings showed that three male samples of AMELY gene had failed to be identified. However, typing was successful when SRY gene was used. This could be samples of those who were AMELY-deleted individuals, which required further verification by using an alternative sex marker for identification. Therefore, the situation of mistyped could happen if one sex marker was used rather than two sex markers. It was reported that the frequency of the AMELY-deleted amongst race of Malaysian population was 3.2%[6]. However, this study cannot be further investigated as the information on race of tooth owner was not collected. According to Shadrach et al.[15], lack amplification of AMELY product can be caused by mutation at the primer binding site in the amelogenin gene. A study showed that five out of six samples have primer site mutation, which was not amplified by amelogenin primer but amplified when Y-STR kit was used[16].

In this study, three out of ten male samples were identified as female, based on the amplification product of SRY external primer, but was corrected with internal amplification product. This shows that a false negative result can be obtained if internal positive control was not employed in amplifying SRY marker. Since presence of inhibitor or failure of amplification in the sample can also cause negative amplification, it cannot accurately define a sample with negative amplification which belong to a female sample. A study by Morikawa et al.[5] stated that the SRY primers were designed to amplify SRY marker on Y chromosome.

Table 3. Sensitivity results of each primer pair

Primer pair	Sensitivity (%)
AMEF-AMER	6.67
AME2F-AME2R	63.64
SRYEF-SRYER	70.00
SRYEIF-SRYIR	100.00

and two other homologous sequences in chromosome 7 and X chromosome which acted as internal positive controls by producing products of different lengths. This method found to eliminate the possibility of false negative while employing the SRY marker.

The absence of PCR product may suggest the possibility of PCR inhibitor in samples such as collagen and calcium in a great quantity, especially in tooth samples[17]. The incomplete removal of these PCR inhibitors during DNA extraction was found as one of the factors that contributed to false negative, which could interfere with the amplification process. Apart from that, the condition of highly degraded DNA as well as fragmented into small size can also cause false negative amplification. A study has shown that the highly fragmented DNA template was difficult to be amplified for product longer than 150 bp[18].

Conclusion

In conclusion, it was proven that genetic material can be typed by using sex markers AMEL and SRY from samples subjected to burning at 300 °C for 10 min. Apart from that, the nested PCR is found to be a useful technique in amplifying a minute and degraded genetic material of teeth samples. The findings also showed that the sex markers with a shorter length of PCR products could be considered when amplifying a compromised sample, especially from burnt samples.

Application of SRY gene to confirm the sex of individual can be proposed as alternative sex marker to avoid misinterpretation in situation when the amelogenin gene failed to discriminate an individual to provide a more conclusive result in sex determination.

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