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Human DNA identification and sex determination from bloodstains by duplex PCR analysis

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Abstract

This study aimed to identify DNA of human origin and determine human sex from bloodstains by duplex PCR analysis. Two pairs of primers amplifying *Amelogenin* gene, AMEL(1) and AMEL(2) and a primer pair targeting *Sex Determining Region Y* gene [*SRY*] were compared at the preliminary stage to measure the accuracy of sex determination. Human specific region of *Cytochrome b* (*Cyt b*) gene sized 412 bp successfully targeted only human-origin DNA, while other animal DNAs (dog, cat, cow, chicken and pig) did not produce any PCR product. SRY primer showed 100% accuracy on male identification with 197 bp amplicon. Therefore, the SRY primer pair was chosen for sex identification. The optimal annealing temperature of duplex PCR analysis was 55°C, producing the most distinctive PCR products, determining male with 197 bp and 412 bp bands and female with only 412 bp. For sensitivity, the smallest blood sample that could be detected was 20 µl. For specificity, both human origin and sex determination could be detected in all ratios of mixed human and dog bloods, including using 100 fold less human blood to animal blood. Determination of male blood in mixed male and female blood samples was also investigated. The results showed that male blood could be determined in mixed female blood sample at ratios less than 10 fold. This study demonstrated that duplex PCR analysis of *Cyt b* and *SRY* is a reliable tool to investigate human DNA and sex of questioned bloodstains.

Keywords: Duplex PCR; Bloodstains; *Cytochrome b*; *SRY*

1. Introduction

In forensic DNA analysis, when a crime has occurred biological materials are mostly concerned because they contain nucleated cells which are the sources of DNA. Based on the transfer theory, biological evidence can be found on victims, perpetrators and objects [1]. Prior to processing DNA analysis, biological evidence needs to be presumptively examined in order to ensure its biological origin. For blood evidence, samples must have shown positive result when examined by phenolphthalein test. However, more information on blood evidence like species origin and sex are always necessary. For human origin examination of bloodstains, various methods have been studied. However, methods based on DNA analysis are more preferable than protein based analysis because the quantity and condition of evidence has little effect on the results. Human specific DNA regions are mostly the target for human origin examination. These regions are possibly located on gene such as *Myoglobin* [2], *Cytochrome b* [3] or non-gene e.g. D-loop [4].

For sex determination of human evidence, DNA sequence located on both X and Y chromosome with different sizes is of particular interest. Sex origin of forensic evidence has successfully been determined by the number of amplified products from alphoid repeat region [5], amelogenin [6] and zinc finger gene [7]. Later, sex determination errors occurred by deletion of Y copy of the amelogenin gene were reported. [8,9]. New primer set of SRY gene was then conducted for gender identification by coamplifying with STR kit [10]. Though, human origin and sex identification has been extensively studied. A single step examination of forensic evidence for both human origin and gender identification has rarely been demonstrated. In 1986, examination of both human origin and gender was introduced by a hybridization technique, but the results could not be obtained in a single step

because two DNA probes were hybridized [11]. If human origin and sex could be quickly determined by a simpler method like PCR, it would be very useful for forensic evidence investigation.

The objective of this study was to determine human origin and sex from bloodstains in a single run by duplex PCR analysis. Human specific *Cyt b* region was the target of interest to determine human origin. For sex identification, primers amplified *Amelogenin (AMEL*) and *Sex Determining Region Y* (*SRY*) genes were compared for their accuracies. Bloodstains samples were then analyzed by duplex PCR analysis using selected primers. Sensitivity, specificity and accuracy of duplex PCR analysis on human bloodstains and mixed bloodstains samples were also examined.

2. Materials and Methods

2.1 Sample preparation

Male and female blood from five individuals each were kindly provided by blood bank, Faculty of Medicine, Khon Kaen University with permission from the Khon Kaen University Ethics Committee in Human Research (Project no. HE581013). The animal blood samples, which were dog, cat, cow, chicken and pig, were supplied by Department of Veterinary Technology, Faculty of Technology, Udon Thani Rajabhat University. Samples were withdrawn at 3 ml and kept in blood collecting tubes, supplemented with anti-coagulants. Collecting tubes were then stored at 4°C. For bloodstain preparation, 200 µl of blood samples were placed on cotton swabs and left to dry at room temperature before preceding with DNA extraction.

2.2 DNA extraction

Nucleospin® (Macherey-Nagel, Germany) kit was used for DNA extraction from both fresh blood and bloodstains. The extraction procedure was performed according to the manufacturer's instructions. Fresh blood samples (200 µl) were added to the microcentrifuged tubes followed by proteinase K and extraction buffer. The solution was mixed thoroughly and incubated at 70°C for 15 min. After incubation, the ethanol was added, mixed and transferred to the column. The column was then centrifuged at 10,000 rpm for 1 min to remove the flow through solution. The washing buffer was then added to the column and spun down. This washing step was carried out twice. An elution buffer was subsequently added to the column to release the binding DNA. For DNA extraction from bloodstain samples, a cotton swab was cut into small pieces before being immersed in 1 ml of deionised water for 30 min at room temperature with vortexing every 5 min, and then centrifuged. The aliquots of 200 µl were taken for DNA extraction following the same procedure as DNA extraction from fresh blood described earlier. The extracted DNA solution was quantified and qualified by NanoDrop.

2.3 Simplex PCR analysis of human origin

Human *Cyt b* specific primer reported by [12] was chosen for this study. The DNA sequences of forward and reverse *Cyt b* primers are 5'-CTTCCTTCTCTCCTTAATG ACATTAAC-3' and 5'-TAGGGAGATAGTTGGTATTAGGAT-3', respectively. The PCR amplification of *Cyt b* on extracted DNA from human and fresh animal blood was examined to ensure that *Cyt b* primer pair is able to identify human DNA from those of other animals. The PCR reaction composing of 2 ng DNA template, 0.1 µM of each forward and reverse primer, 1x*Taq* PCR Master mix (Vivantis, Malaysia) was prepared. Deionised water was added to make a total volume up to 20 µl. After being mixed and quickly spun, the PCR reaction was placed in a Thermal Cycler. The program was set as follows; 2 min at 94°C for an initial denaturation step continued with 30 cycles of 30 sec at 94°C, 30 sec at 50°C and 1 min at 72°C. Then, the final extension step was established at 72°C for 2 min. When the PCR cycler terminated, 5 µl of each PCR product was examined on 1.2% agarose gel by electrophoresis. To visualize the DNA band, gel was illuminated under UV light after being stained in 0.5 µg/ml ethidium bromide solution for 10 min.

2.4 Comparison of sex determining primers

Three pairs of sex determining primers including AMEL(1), AMEL(2) and SRY were compared for the greatest accuracy in sex determination, before being chosen for duplex PCR in the next step. The details of these primers are listed in Table 1. These primers were used to amplify human DNA extracted from five male and five female fresh blood samples. The experiment was performed in three replicates of each sex (n=15). The PCR reaction of 20 µl contained 2 µg of DNA template, 10 µM of each forward and reverse primer, 1x *Taq* PCR Master Mix and deionised water. For amplification using SRY and AMEL(1) primer, the PCR conditions were 94°C for 1 min followed by 30 cycles of 94°C for 20 sec, 59°C for 30 sec and 72°C for 20 sec, and then continued at 72°C

for 7 min. For amplification using AMEL(2), the same PCR conditions were performed except for an annealing temperature of 52°C. The PCR product was then analyzed by 1.5% agarose gel electrophoresis and DNA bands were visualized as described earlier.

Table 1 Lists and details of sex determining primes used in this study

Primers	DNA sequences (5' to 3')	Expected size of amplicon (bp)		References
SRY	F: TCCAGGAGGCACAGAAATTA	Male	197	[13]
	R: TCTTGAGTGTGTGGCTTTCG	Female	-	
AMEL(1)	F: ATCAGAGCTTAAACTGGGAAGCTG	Male	106,112	[13]
	R: CCCTGGGCTCTGTAAAGAATAGTG	Female	106	
AMEL(2)	F: CTGATGGTTGGCCTCAAGCCTGTG	Male	788, 977	[14]
	R: TAAAGAGATTCATTAACTTGACTG	Female	977	

2.5 Optimal condition examination of duplex PCR analysis

This study was carried out to investigate the optimal duplex PCR condition for human origin and sex determination of bloodstains. Two pairs of primers from the previous result were chosen which target Cyt *b* and *SRY* genes. DNA extracted from male and female bloodstains were used as DNA template. PCR reaction of 20 µl contained 2 ng of DNA template, 10 µM of each primer, 1x *Taq* PCR Master Mix and deionised water. The PCR reaction was run at the same conditions as sex determining analysis, except for annealing temperatures that were separately set at different temperatures (51°C, 53°C, 55°C, 57°C and 59°C). The PCR product of each run was examined by gel electrophoresis. The optimal conditions of duplex PCR was indicated by the most clearly identified DNA bands with the correct sizes of amplicons as expected from the male and female samples.

2.6 Sensitivity, specificity and accuracy examination

Bloodstain samples were prepared. For sensitivity analysis, human bloodstains samples were prepared by dropping different amounts of human blood (5, 10, 20, 50, 80 and 100 µl) onto cotton swabs which were left to air dry. For specificity tests, aliquots of 200 µl was taken from the various ratios of mixed human and dog blood (1:1, 1:2, 1:5, 1:10, 1:20, 1:50 and 1:100) and dropped onto cotton swabs. The cotton swabs were left to dry at room temperature. Mixed bloodstains of male and female bloodstains samples at different ratios (1:1, 1:4, 1:9, 4:1 and 9:1) were carried out for accuracy analysis. To do this, 200 µl of mixed male and female blood was dripped onto cotton swabs and left to dry. DNA was then extracted from the bloodstain samples as described before. Duplex PCR analysis was subsequently performed. The running conditions of Thermal Cycler were set at 94°C for 1 min, followed by 30 cycles of 94°C 20 sec, 55°C 30 sec and 72°C 20 sec, and then continued at 72°C for 7 min.

The sensitivity of duplex PCR analysis on human origin and sex determination was determined by investigating the smallest amount of blood giving the correct amplified DNA products. The lowest ratio of human blood volume versus dog blood that gives correct result demonstrates the specificity of the test. The accuracy of duplex PCR analysis shows that the technique was able to amplify *Cyt b* and *SRY* of male samples even when mixed with female blood.

3. Results

3.1 Amplification of human specific Cyt b

Cyt b primers designed from human specific *Cyt b* region were used to amplify DNA extracted from human (male and female) and animal (dog, cat, cow, chicken and pig) fresh blood samples. The amplification results are shown in Figure 1. It can be seen that only the human sample produced the PCR product with an approximate size of 412 bp, as was expected. No amplification DNA product was observed with the animal samples.

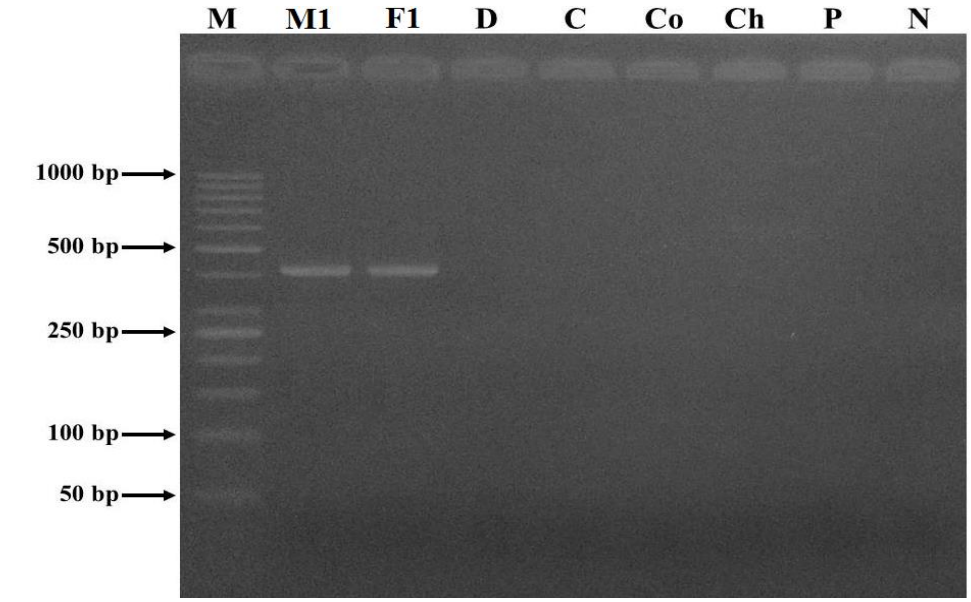

Figure 1 PCR amplification of *Cyt b* from male (M1) and female (F1) blood samples compared with those of dog (D), cat (C), cow (Co), chicken (Ch) and pig (P). The size of DNA product was compared with 100 bp DNA ladder (M). Negative control was represented by N.

3.2 Comparison of sex determining primers

For human sex determination from blood samples, three pairs of sex determining primers were used to amplify DNA extracted from five individual male and female blood samples. The experiments were conducted in three replicates each sex (n=15). Representative gels showing PCR products amplified by SRY, AMEL(1) and AMEL(2) primers were demonstrated in Figure 2, 3 and 4 consecutively. An amplicon of *SRY* (197 bp) was observed only in male blood samples. In contrast, amplification by AMEL(1) or AMEL(2) represented two PCR amplicons with male blood DNA and one amplicon with female blood DNA. For AMEL(1), the amplicons of 106 bp and 112 bp were observed in male samples while the 106 bp amplicon was seen in female samples. For AMEL(2) amplification, the product sizes of male (two bands, 788 and 977 bp) and female (one band, 977 bp) were amplified. These results confirmed that male and female blood samples were successfully differentiated by PCR analysis with these primers. The number of human blood samples that showed the correct results of sex determination was then investigated. The results showed that SRY primer had the most accurate sex determination results followed by AMEL(1) and AMEL(2), respectively (Table 2). Therefore, SRY primers were subsequently selected for duplex PCR analysis.

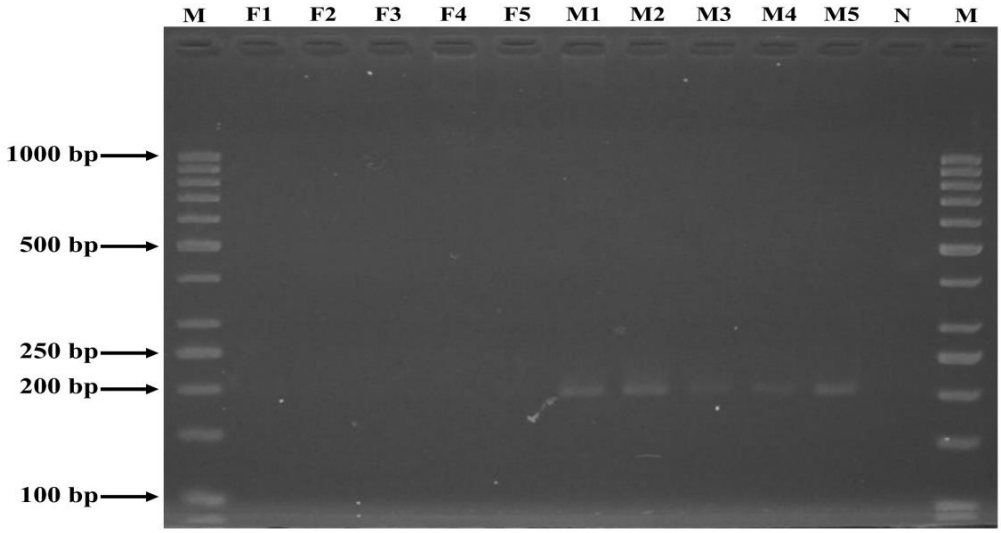

Figure 2 Agarose gel electrophoresis of amplified products from female (F1-F5) and male (M1-M5) blood samples by SRY primer. M and N represent 100 bp DNA ladder and negative control, respectively.

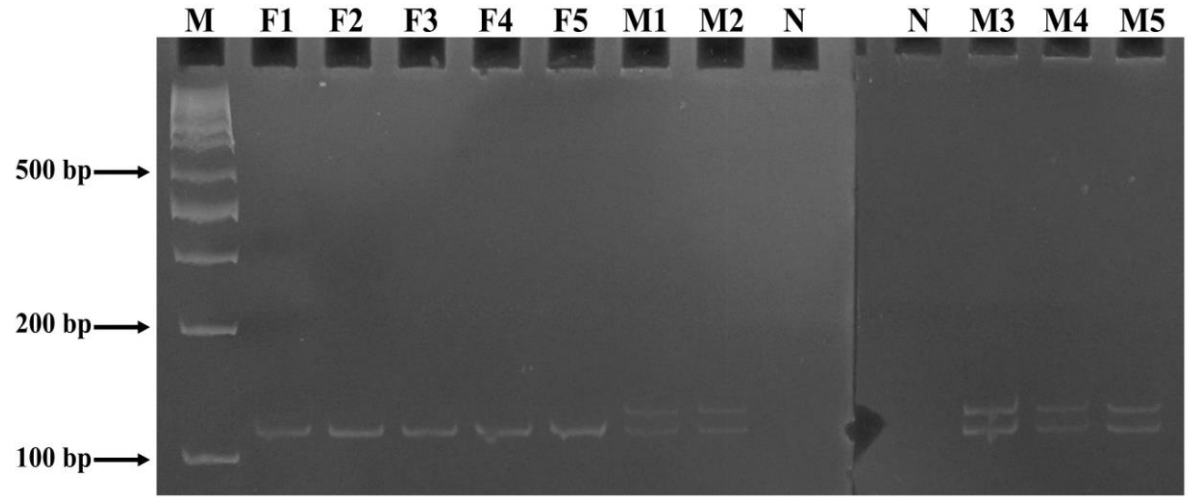

Figure 3 PCR products from AMEL(1) primer amplified on DNA extracted from female (F1-F5) and male (M1-M5) blood samples compared with 100 bp DNA ladder (M) and negative control (N).

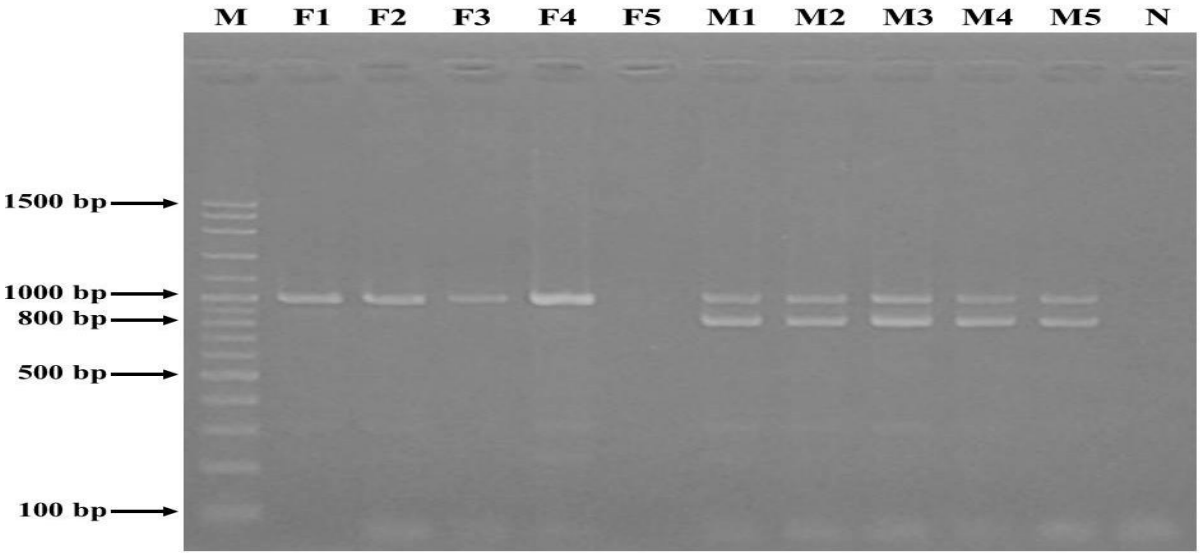

Figure 4 PCR products from AMEL(2) primer amplified on DNA extracted from female (F1-F5) and male (M1-M5) blood samples compared with 100 bp DNA ladder (M) and negative control (N).

Table 2. Sex determination results from male and female blood samples amplified by AMEL(1), AMEL(2) and SRY.

Primers	Male (n=15)		Female (n=15)		% Correct results
	Correct	Incorrect	Correct	Incorrect	
SRY	15	0	15	0	100
AMEL(1)	15	0	14	1	96.67
AMEL(2)	13	2	13	2	86.67

3.3 Optimal condition of duplex PCR analysis

Duplex PCR amplifications of *Cyt b* and *SRY* on human blood samples were performed at different annealing temperatures. The PCR products amplified at 55°C showed the most distinct bands with the correct sizes of amplicons (Figure 5). As a result, this temperature was chosen for duplex PCR condition. Duplex PCR analysis

of human bloodstains using these conditions was then investigated. Amplicons were clearly visible in male and female bloodstains samples, indicating suitable conditions for duplex PCR amplification (Figure 6).

3.4 Sensitivity

Duplex PCR was performed on different quantities of human bloodstain for sensitivity determination. Interestingly, the smallest amount of male bloodstain that clearly showed DNA products of *Cyt b* and *SRY* was 20 µl. The related results were also found in the female bloodstain sample, where an amplicon of *Cyt b* was detected (Figure 7). Therefore, the achievement of duplex PCR analysis on human origin and sex could be attained with a quantity of blood sample higher than 20 µl.

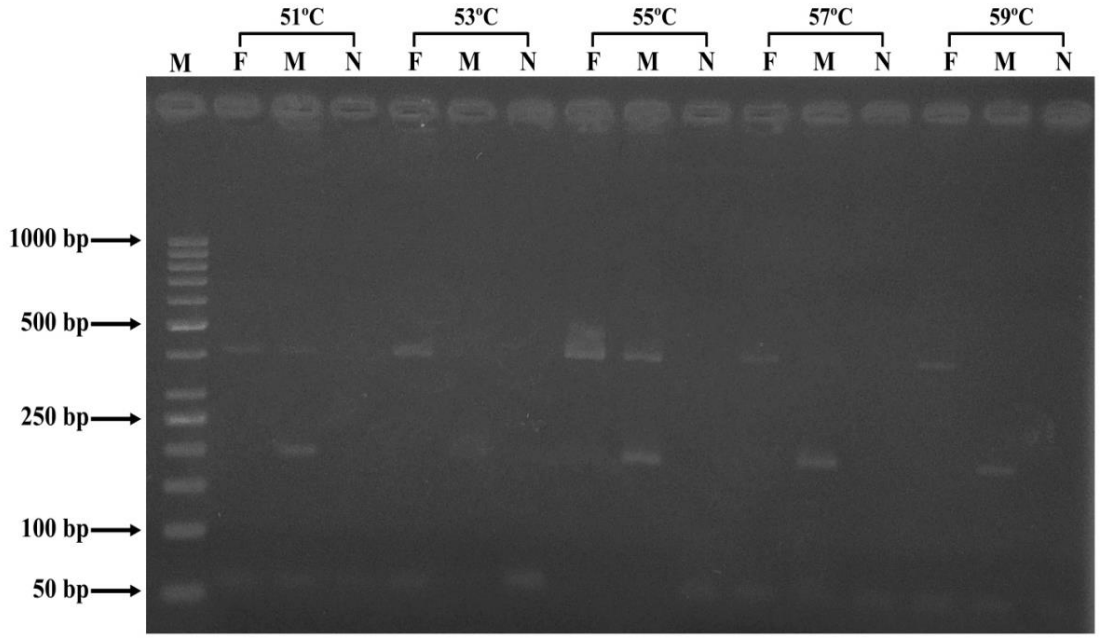

Figure 5 Duplex PCR amplification of female (F) and male (M) blood samples by using different annealing temperatures. A 100 bp DNA ladder was used as DNA marker (Lane M).

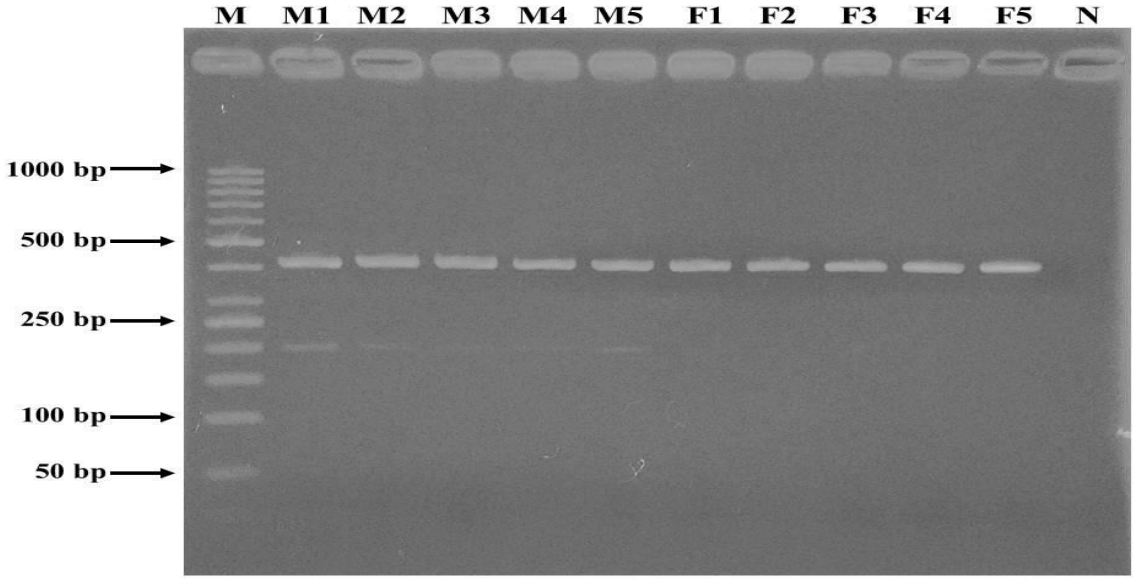

Figure 6 Duplex PCR analysis of male (M) and female (F) bloodstains. Negative control and 100 bp DNA ladder are denoted by N and M, respectively.

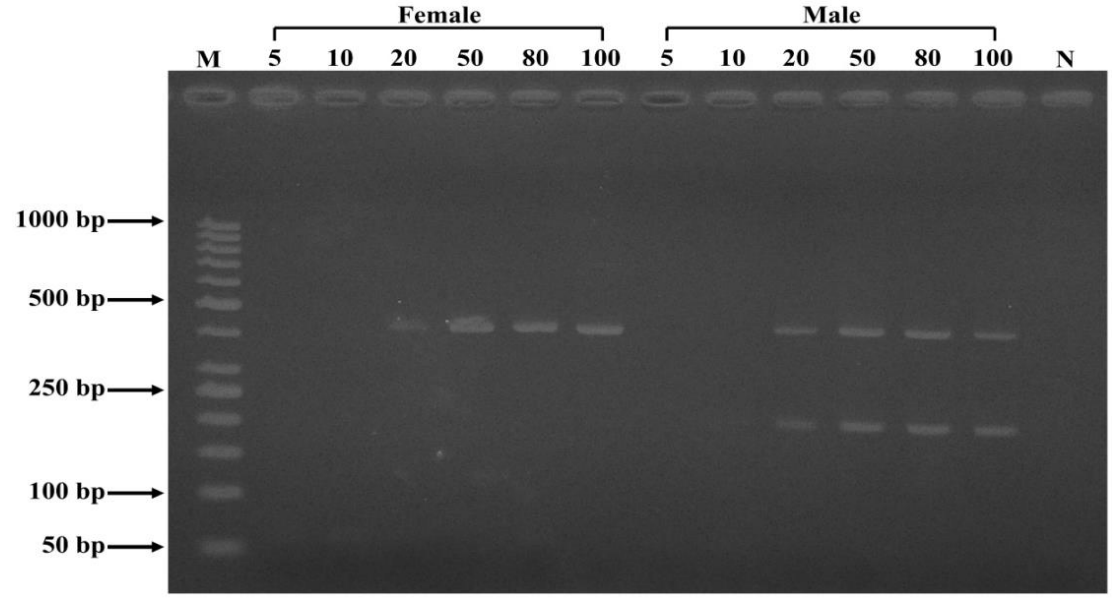

Figure 7 Duplex PCR analysis on different amounts of human bloodstain samples compared with negative control (N) and 100 bp DNA ladder (M).

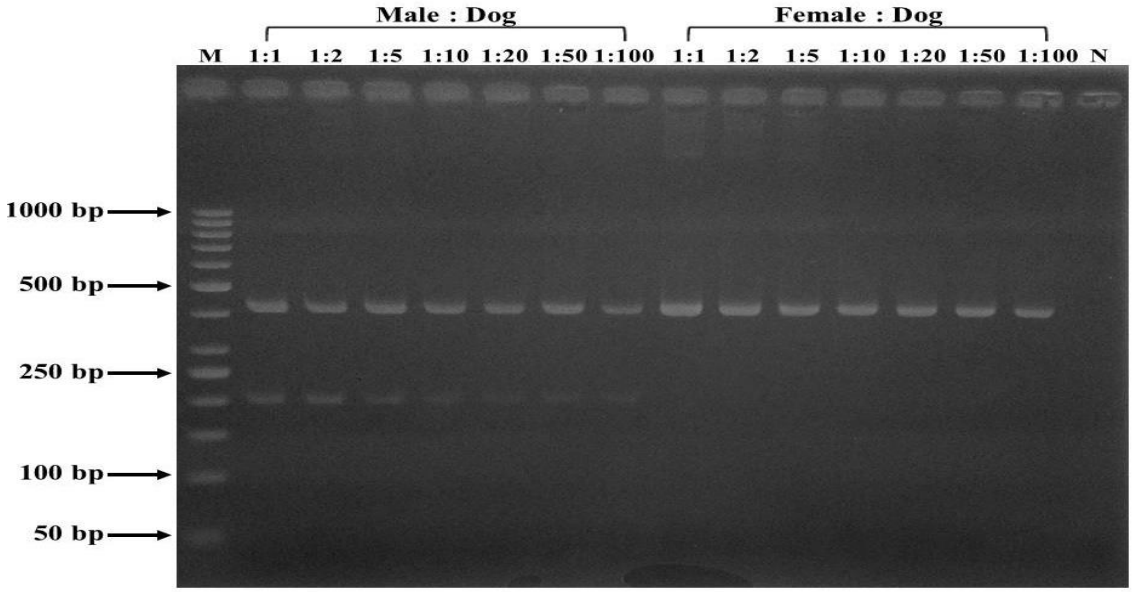

Figure 8 Duplex PCR analysis of mixed human and animal bloodstain samples at different ratios.

3.5 Specificity

Duplex PCR analysis of mixed human and dog bloodstains were conducted. Results showed that amplicons of *Cyt b* (412 bp) and *SRY* (197 bp) were detected in mixed blood at every ratio. However, the intensities of DNA bands were gradually reduced when the ratio of dog's blood was higher. This may have resulted from the reduction of human DNA template in the samples. Similar observations were also made in mixed female and dog blood where *Cyt b* amplicons (412 bp) was produced. These results indicated the ability of duplex PCR analysis to determine human origin and sex even where the proportion of human blood was less than 100 fold.

3.6 Accuracy

Different ratios of mixed male and female blood samples were analyzed by duplex PCR. It can be seen that amplified *Cyt b* and *SRY* were visible in all tested mixed blood ratios (Figure 9). However, the intensity of *SRY* amplicons was inversely proportionate to the increment of female blood. The lowest intensity of *SRY* amplicon was found in mixed male female blood at 1:9. For human origin determination of mixed male and female bloodstains samples, the intensities of the *Cyt b* amplicons were not different.

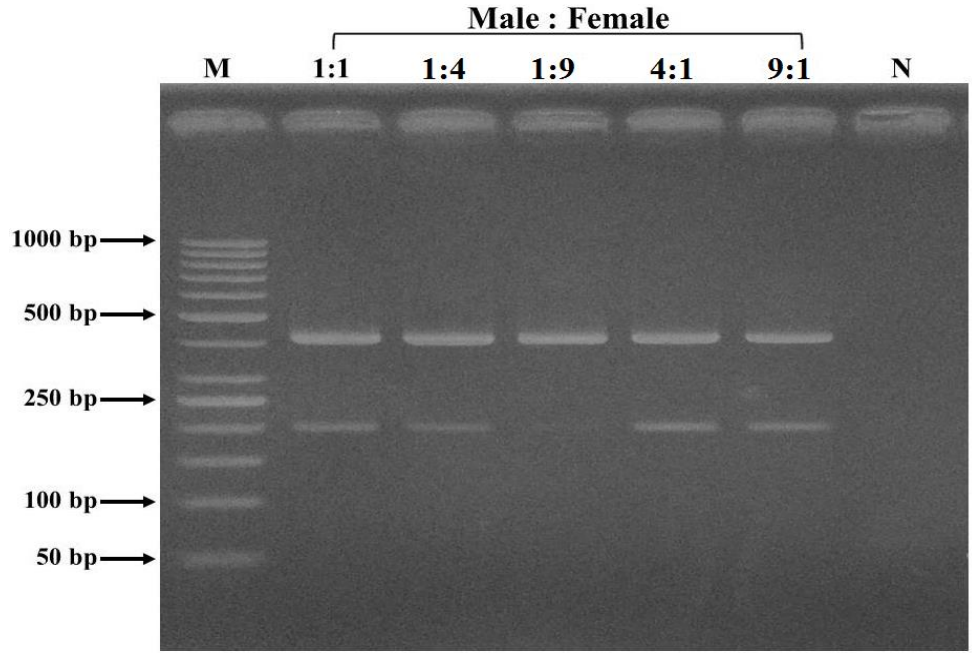

Figure 9 Duplex PCR analysis on different ratios of mixed male and female bloodstain samples.

4. Discussion

Forensic investigation of human origin and sex from bloodstains by duplex PCR analysis has been focused on in this study. Two genes demonstrating human species and sex were considered as target sites. For species identification, the sequence of *Cyt b* had been reported as species-specific [3]. Hence, Cyt b primer designed from human *Cyt b* specific region was chosen to amplify human blood origin. PCR analysis of human and animal blood by human specific Cyt b primer showed that this primer could determine human origin. Human identification of blood sample by amplification of *Cyt b* (157 bp) has been successfully reported previously [15] but the amplified target of Cyt b primers were reported differently compared to this study. For sex identification of blood samples, the efficiencies of AMEL(1), AMEL(2) and SRY primers on sex determination were compared because false interpretation of *AMEL* amplification had been reported earlier [16,17]. In addition, contamination of sample has previously caused incorrect interpretation of *SRY* analysis [18]. Therefore, new techniques for sex determination have been studied. Amplification of *AMEL* together with *SRY* was introduced to confirm the result of sex determination [19,20]. Sex determination by amplification of SRY together with 4 sites of miniX-STR called GenderPlex was used to confirm the results [21]. The advantage of this technique is that highly degraded DNA can be efficiently analyzed because the sequence of X-STR is short [22].

The duplex PCR amplification of *Cyt b* and *SRY* has shown that this technique could determine both human origin and sex differentiation from male (197 and 412 bp) and female (412 bp) bloodstain samples. However, the intensity of *Cyt b* amplicon was much higher than that of *SRY*. This phenomenon may have resulted from high copy number of mitochondrial DNA where *Cyt b* is located on. Y chromosome containing *SRY* has only two copies number per cell. Thus, the initial DNA template of *Cyt b* is higher than *SRY*. To determine nuclear and mitochondrial DNA copy number in blood samples, real-time PCR analysis should be conducted. This technique has successfully been used to quantify the copy number of DNA in forensic samples [23-25].

Sensitivity analysis of duplex PCR on determination of human origin and sex from bloodstains samples has shown that the minimum amount of bloodstains required for the successful detection is 20 μl. Previous study of human origin identification based on amplification of human-specific *Cyt b* found that 1 pg of DNA was the detection limit of the test [15]. However, to make comparison with our study, the quantity of DNA in 20 μl of bloodstains needs to be examined. The results of specificity and accuracy examination showed that duplex PCR analysis of *Cyt* and *SRY* is capable of determining the human origin and sex of male bloodstains mixed either with female or animal blood at more than 10 fold and 100 fold, respectively. The small volume of blood sample required is not the only reason for the success of duplex PCR analysis; our results give an estimation of successful duplex PCR analysis on human origin and sex in mixed blood samples.

From this study, it can be seen that duplex PCR analysis on *Cyt b* and *SRY* is reliable for human origin and sex determination of bloodstains. Human origin and sex determinations from other forensic evidences such as bone, hair and buccal cells using this technique require further study before forensic application. Although the study of human origin identification has been extensively reported, more highly efficient methods are needed. DNA and RNA are largely the targets of examination. Genome profiling has been introduced to discriminate human blood

from animal blood by comparing the DNA profiles in which DNAs were randomly amplified and separated by temperature gradient gel electrophoresis [26]. Identification of human samples by quantitative real-time PCR (qPCR) has also been reported [27]. Recently, pyrosequencing of 12S rRNA gene by only 12 bases has been used to accurately identify 17 species including human [28]. Though these methods are efficient, specialized instruments and expertise are needed in their application. Simpler methods such as PCR analysis remain favourable.

5. Conclusion

Human origin and sex of bloodstains samples could be determined by duplex PCR analysis of *Cyt b* and *SRY*. Male bloodstains samples produced two amplicon of *Cyt b* (412 bp) and *SRY* (197 bp) while female bloodstains samples gave only one amplicon of *Cyt b* (412 bp). For animal bloodstains samples, no amplicons were generated. The smallest amount of bloodstain sample determinable by duplex PCR analysis was 20 µl. Duplex PCR analyses could determine male bloodstains even when mixed with either female or dog blood in amounts of between 10 and 100-fold.

6. Acknowledgements

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