Forensic Human and Non-human Blood Discrimination using Mitochondrial 12S rRNA gene

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Abstract

Background: The blood samples of spots typing regards most important step in forensic analysis. Determining the blood affiliation as human on non-human is not enough as a forensic tool leading to the truth . The mitochondrial 12S rRNA gene are suitable for taxonomy and species identification, especially for the discrimination of even closely related species. The current study aimed to showed the validity of designed 12S rRNA species specific primer pairs for human and nonhuman animal blood typing as a forensic tool. **Methodology:** Seventy two blood samples were collected from *Homo sapiens*, *Ovis aries*, *Capra hircus* and *Bos taurus* (18 blood samples from each). Five milliliter were withdrawn and placed in EDTA-tube and stored in refrigerator for further processing. Four sets of species specific primer pairs targeting mitochondrial 12S rRNA were designed and checked. PCR and sequencing were performed and sequences were analyzed and register in GenBank. **Results:** the results revealed that, the amplicon of 12S rRNA gene of Homo sapiens were 800bp, *Ovis aries* were 560bp, *Capra hircus* were 460bp, and *Bos taurus* were 600bp The first confirmatory test for validity and specificity of designed primer pairs is no amplification for intraspecies primer pairs (i.e. using homo12S rRNA primer pairs to amplify *Ovis aries*, *Capra hircus*, and *Bos taurus* 12S rRNA will not give product and vice versa). The second confirmatory test is the results of sequencing. The identity percentage and alignment of sequences results of amplified 12S rRNA gene of *homo sapiens*, *Ovis aries*, *Capra hircus* and *Bos taurus* revealed that, the similarity percentage ranged from 98.03% to 100%.**Conclusion:** The current study conclude that, validity and accuracy of designed 12S rRNA species specific primer pairs for human and nonhuman animal blood typing as a forensic tool and there is no intraspecies cross amplification.

Keywords: *Homo sapiens*, *Ovis aries*, *Capra hircus* and *Bos taurus*, 12S rRNA, Sequencing

Introduction

The assignment of blood samples is a fundamental aspect of forensic investigations, mainly for vehement crimes. The important step in forensic that is often skipped or unnoticed is the determination that a bloodstain is of human or nonhuman origin at level of species[1]. The mitochondrial genome consists of multiple copies of 116,569 bp, double stranded mitochondrial DNA (mtDNA) molecules and located adjacent to the OXPHOS system in the matrix. It is haploid in countryside, and thus should evolve four times faster than the average nuclear gene. Hence, mtDNA can be used to track divergence in very closely related taxa and even within species[2-4]. Mitochondrial 12SrrRNA has proven to be a useful molecular marker for better conservation and management of the endangered species. Mitochondrial DNA present in high copy numbers, survive for prolonged periods and absence of recombination when compared with nuclear DNA making it suitable for forensic investigations [5,6]. The mitochondrial 12S rRNA gene are suitable for taxonomy and species identification, especially for the discrimination of even closely related species. It is widely used to differentiate between human from non-human species. Genotyping using 12S rRNA gene

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provide cost and time effective results, and yielded information not only for species identification but also for individualization[7,8]. The current study aimed to showed the validity of designed 12S rRNA species specific primer pairs for human and nonhuman animal blood typing as a forensic tool.

Methodology

Sampling:

Seventy two blood samples were collected from *Homo sapiens*, *Ovis aries*, *Capra hircus* and *Bos taurus* (18 blood samples from each). Five milliliter were withdrawn and placed in EDTA-tube and stored in refrigerator for further processing.

Polymerase Chain Reaction:

G-spinTM Total DNA Extraction Kit (was used to extract mitochondrial DNA from blood of different species according to the manufacturer's protocol instructions (Intronbio/Korea). All extracted DNA samples were submitted for Nanodrop to ensure adequate purity and concentration required for PCR. Setting done by selection dsDNA measuring mode for 2 µl of sample. Blanking were performed using 2 µl microliter of elution buffer and then measuring the DNA samples concentration and purity[9].

The primer pairs were designed using the following reference sequences: *Homo sapiens* (NC_012920.1,), *Ovis aries*: (NC_001941.1), *Capra hircus* (NC_005044.2) and *Bos taurus* (NC_006853.1). Primer 3 software was used to design the specific primer pairs: Homo12S-F: CATCCCCGTTCCAGTGAGTT, Homo12SR: TACTTGAGGAGGGTGACGGG (800bp); Ovis 12S-F: GCCTGGTGATAGCTGGTTGT, Ovis 12S-R: ACGTTTAGTCACTGGGCAGG (560bp); Capra 12S-F: GGTGAGTAACGCCCTCCAAA, Capra 12S-R: ATAAAGCACCGCCAAGTCCT (460bp); Bos 12S-F: TAAATCTCGTGCCAGCCACC, Bos 12S-R: TGCGTGCTTCATGGCCTAAT (600bp). The specificity of designed primer pairs were checked using online NCBI/primer blast[10,11]. The PCR conditions were calculated using online Protocol Optimize writer software. The conditions were illustrated in table (1).

Sequence Analysis and Submission

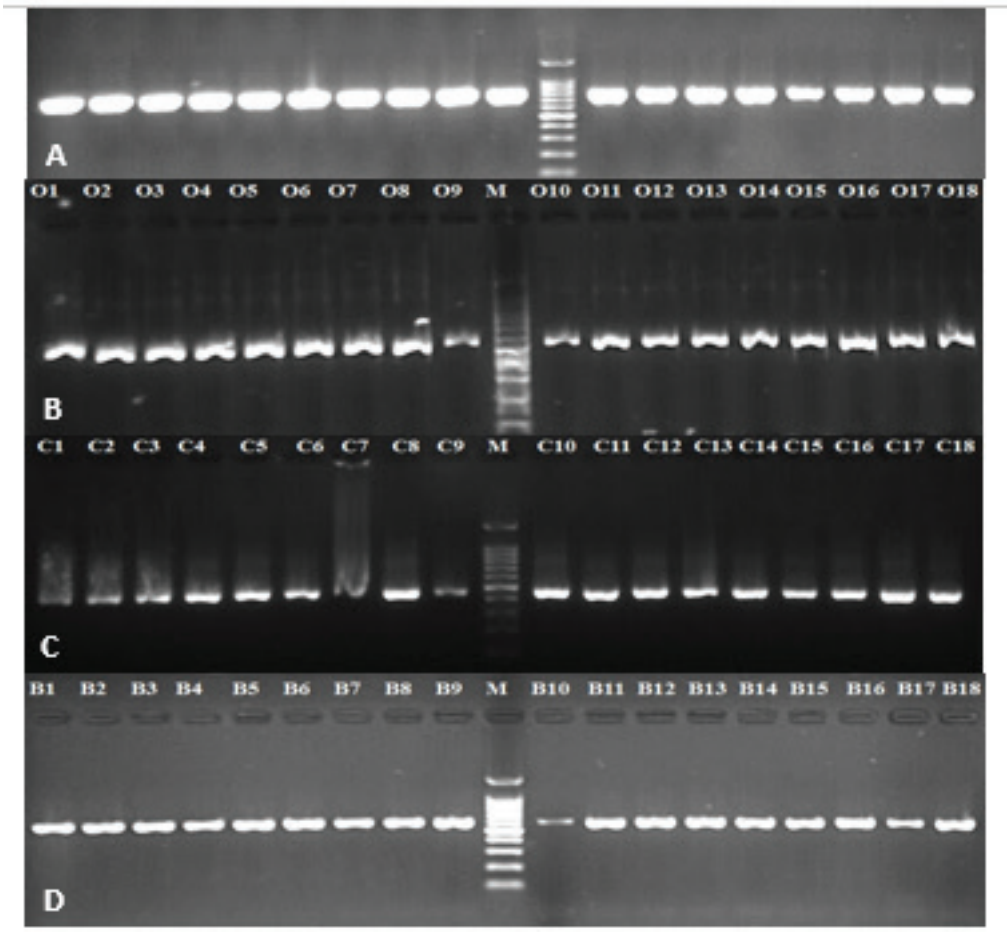
Trimming of sequences were perform by FinchTV and then submitted to NCBI-BLASTN to see the identity of sequences with reference sequences within NCBI data base. All trimmed and confirmed sequences then submitted for GenBank to register all sequences within the database. The gathered accession no. after submission verified the assignment of the sequences samples to the intended species.

Table (1): PCR Conditions

Primer	Conditions			References
Homo 12S-F Homo 12S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		60.3°C	30sec.	
		72°C	80sec.	
	1	72°C	5min.	
Ovis 12S-F Ovis 12S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		59.3°C	30sec.	
		72°C	130sec.	
	1	72°C	5min.	

Cont.. **Table (1): PCR Conditions**

Capra 12S-F Capra 12S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		58.3°C	30sec.	
		72°C	130sec.	
	1	72°C	5min.	
Bos 12S-F Bos 12S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		58.3°C	30sec.	
		72°C	80sec.	
	1	72°C	5min.	



Results and Discussion

The results of PCR reveal that, the amplicon of 12S rRNA gene of Homo sapiens were 800bp, *Ovis aries* were 560bp, *Capra hircus* were 460bp, and *Bos taurus* were 600bp (Figure 1: A,B,C,D respectively). The mitochondrial genes have been used as molecular markers to identify mammals, birds, shrimp, and other

species using species-specific primers that amplify the 12S rRNA or 16S rRNA gene regions from mtDNA[12,13]. Gene loci on the mitochondrial genome have been used in species identification due to the greater sequence variation at this non-coding locus, it is now being used as a tool for identifying the presence of particular species within mixture of many species[14,15].

Figure (1): 1.5% Agarose gel electrophoresis for: A: (800bp) amplicon of Homo sapiens 12S rRNA gene. lane H1-H18 represent samples. B: (560bp) amplicon of Ovis aries 12S rRNA gene. lane O1-O18 represent samples. C: (460bp) amplicon of Capra hircus 12S rRNA gene. lane C1-C18 represent samples. D:(600bp) amplicon of Bos taurus 12S rRNA gene. lane B1-B18 represent samples. M represent 100bp DNA ladder.

The first confirmatory test for validity and specificity of designed primer pairs is no amplification for intraspecies primer pairs (i.e. using homo12S rRNA primer pairs to amplify *Ovis aries*, *Capra hircus*, and *Bos taurus* 12S rRNA will not give product and vice versa). The second confirmatory test is the results of sequencing. The identity percentage and alignment of sequences results of amplified 12S rRNA gene of *homo sapiens*, *Ovis aries*, *Capra hircus* and *Bos taurus* with database were illustrated in tables (2,3,4,5) respectively. The PCR amplification and sequencing of mitochondrial DNA 12S rRNA gene could be used for unambiguous species identification of mammalian . The technique has the advantage that only a small amount of material is required. The method could be used even for biological material stored at ambient temperature for prolonged periods of time[16]. Mitochondrial gene12S rRNA the specificity of the primers was tested against 29 animal species including mammals, birds, and fish, as well as 8 plant species. Analysis of experimental feedstuffs demonstrated the detection of each target species in the range of 0.1 to 100%[17] .Frezza *et al.,* (2003)[18] Polymerase chain reaction allows direct species identification, but high temperatures involved in rendering animal products (133°C for 20 min) cause fragmentation of DNA. Amplifying and sequencing a fragment of mitochondrial DNA that codes for 12S ribosomal RNA, to identify the species origin of non-human casework samples thus mtDNA results are obtained and is especially useful degraded samples[19] .

Table (2): Identity of blasted isolates (IHS12-1 to IHS12-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Identities	No. of Aligned Base
IHS12-1	MH444415.1	100.00%	731
IHS12-2	MK069579.1	99.86%	771
IHS12-3	MK069579.1	100.00%	697
IHS12-4	MK069579.1	99.86%	654
IHS12-5	MK059695.1	100.00%	701
IHS12-6	MK069579.1	100.00%	657
IHS12-7	MK295855.1	100.00%	599
IHS12-8	MK069579.1	100.00%	653

Table (3): Identity of blasted isolates (IOA12-1 to IOA12-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Identities	No. of Aligned Base
IOA12-1	MG489885.1	99.78%	464
IOA12-2	MG489885.1	98.63%	291
IOA12-3	MG489885.1	99.32%	442
IOA12-4	MG489885.1	99.13%	459
IOA12-5	MG489885.1	98.03%	458
IOA12-6	MG489885.1	100.00%	283
IOA12-7	MG489885.1	99.38%	486
IOA12-8	KU681201.1	98.64%	367

Table (4): Identity of blasted isolates (ICH12-1 to ICH12-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Identities	No. of Aligned Base
ICH12-1	LR025741.1	100.00%	408
ICH12-2	LR025741.1	99.75%	408
ICH12-3	LS992659.1	100.00%	408
ICH12-4	LR025741.1	99.75%	408
ICH12-5	LS992662.1	99.75%	407
ICH12-6	LR025741.1	100.00%	406
ICH12-7	LR025741.1	99.75%	408
ICH12-8	HQ996553.1	100.00%	324

Table (5): Identity of blasted isolates (IBT12-1 to IBT12-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Identities	No. of Aligned Base
IBT12-1	EU177870.1	100.00%	544
IBT12-2	MK028750.1	100.00%	537
IBT12-3	EU177870.1	100.00%	545
IBT12-4	EU177870.1	100.00%	538
IBT12-5	MK028750.1	100.00%	543
IBT12-6	MK028750.1	100.00%	262
IBT12-7	EU177870.1	100.00%	544
IBT12-8	EU177869.1	100.00%	543

Sensitivity, specificity and accuracy of designed primer pairs can be achieved using following equation depending upon sequencing as gold standard [20]. (Table 6).

Table (6): Sensitivity, specificity and accuracy of designed primer pairs

Test	PCR of 12S rRNA gene			
	Homo sapiens	Ovis aries	Capra hircus	Bos taurus
True Positive	8	8	8	8
False Positive	0	0	0	0
True Negative	0	0	0	0
False Negative	0	0	0	0
Sensitivity	1(100%)	1(100%)	1(100%)	1(100%)
Specificity	1(100%)	1(100%)	1(100%)	1(100%)
Accuracy	1(100%)	1(100%)	1(100%)	1(100%)

The results showed no cross-reactivity of designed primer pairs and the PCR assay based on the designed primer pairs will be simple, fast, sensitive, specific, and cost-effective.

Registration of Sequences in GenBank:

All the 32 sequence of 12S rRNA gene were submitted to GenBank for registration. After checking and revision the following accession numbers were donated:

12S rRNA *homo sapiens* (Human): MN203699, MN203700, MN203701, MN203702, MN203703, MN203704, MN203705, MN203706.

12S rRNA *Ovis aires* (Sheep): MN202782, MN202783, MN202784, MN202785, MN202786, MN202787, MN202788, MN202789.

12S rRNA *Capra hircus* (Goat): MN197657, MN197658, MN197659, MN197660, MN197661, MN197662, MN197663, MN197664.

12S rRNA *Bos taurus* (Cow): MN197785, MN197786, MN197787, MN197788, MN197789, MN197790, MN197791, MN197792.

Conclusion

The current study conclude that, validity and accuracy of designed 12S rRNA species specific primer pairs for human and nonhuman animal blood typing as a forensic tool and there is no intraspecies cross amplification.

Ethical Clearance: The project plan displayed on the scientific committee and scientific ethical committee of the department of Biology-college of science at university of Babylon and get approval

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Conflict of Interest: There is no conflict of interest

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