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# Mitochondrial 16S rRNA Gene-Dependent Blood Typing as a Forensic Tool

*Hussein O.M. Al-Dahmoshi and Hayder J. Al-Nayili*

## Abstract

Mitochondrial DNA is an important tool for human identification and is used to differentiate between human and animal blood at the crime scene, because in extreme conditions nuclear DNA is severely destroyed while Mitochondrial DNA contains multiple copies (200–2000) per cell and resists harsh and more stable conditions. Seventy-two blood samples were collected from humans (*Homo sapiens*), sheep (*Ovis aries*), goats (*Capra hircus*), and cows (*Bos taurus*) (18 blood samples for each). All blood samples were withdrawn by a technician and 5 ml were aspirated using an aseptic technique and transferred to EDTA-Na<sub>2</sub> tubes. They were mixed well and stored in a refrigerator. The collection took 2 weeks (May 15, 2019–May 30, 2019). All samples were collected from Al-Diwanyia city. The results of PCR testing revealed that the primer pairs were specific and non-specific products did not appear for all samples. The amplification of *Homo sapiens* mitochondrial DNA with primer pairs of other (*Ovis aries*, *Capra hircus*, and *Bos taurus*) and amplification of each with primer pairs of another genus gave negative results, and this is primary evidence for primer pair specificity. The amplicon of 16S rRNA gene of *Homo sapiens* was 1200 bp, *Ovis aries* was 1060 bp, *Capra hircus* was 820 bp, and *Bos taurus* was 1300 bp. The sequencing revealed that 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. There is sensitivity, specificity, and accuracy in the designed species-specific primer pairs and applicability of the designed primer pairs in forensics to investigate blood spots or evidence belonging for human, sheep, goat, and cow.

**Keywords:** *Homo sapiens*, *Ovis aries*, *Capra hircus*, *Bos taurus*, forensic

## 1. Introduction

Mitochondria possess a small spherical genome, mtDNA, which encodes for the 13 important subunits of the electron transport chain and ATP synthase together with 22 tRNAs and 2 rRNAs necessary for mitochondrial protein synthesis [1, 2]. Mitochondrial DNA presents several characteristics which have the potential to be valuable for forensic studies, especially attendant to the absence of recombination, to a large copy number, and to matrilineal inheritance. Mitochondrial DNA typing founded on sequences of the control region otherwise filled genomic sequence is used to examine a variation of forensic mtDNA

profiling methods used for human proof of identity and present their use in the chief cases of human identification from non-human [3–5]. Mitochondrial markers that are used for species identification are as follows: cytb gene, cytochrome c oxidase subunit I gene, 12S and 16S rRNA segment, and control region in wildlife [6–8]. A short fragment of the 12S rDNA was employed for DNA amplification leading to species identification. The mitochondrial DNA 16S rRNA gene is an advanced genetic marker for animal genetic diversity. Polymorphism sites, nucleotide variation, and haplotype variety were determined using whole sequences of the mitochondrial DNA 16S rDNA gene [9, 10]. Animal mitochondrial DNA (mtDNA) is commonly described as a small, circular molecule that is conserved in size, gene content, and organization [11]. The aim of this study is to design valuable species-specific-PCR tool to discriminate blood of humans from non-human using a species-specific primer design.

## 2. Methodology

### 2.1 Study design

The study design was experimental to design species-specific primer pairs for typing the blood samples and their assignment to human (*Homo sapiens*), sheep (*Ovis aries*), goat (*Capra hircus*), and cow (*Bos taurus*).

### 2.2 Blood sample collection

Seventy-two blood samples were collected from humans, sheep, goats, and cows (18 blood samples for each). All blood samples were withdrawn by technicians and 5 ml were aspirated using an aseptic technique and transferred to EDTA-Na<sub>2</sub> tubes and mixed well and stored in a refrigerator. The collection took 2 weeks (May 15, 2019–May 30, 2019). All samples were collected from Al-Diwanya city.

### 2.3 Primer design

The gene selected for this study is the mitochondrial 16S rRNA gene. The NCBI data base was used to recover the sequences chosen for a primer design. The sequence ID of *Homo sapiens* (NC\_012920.1); sequence ID of *Ovis aries* (NC\_001941.1); sequence ID of *Capra hircus* (NC\_005044.2); sequence ID of *Bos taurus* (NC\_006853.1). Primer 3 software [12] was used to design the specific primer using the sequence of above-mentioned sequence IDs. The generated primers were as follows: Homo 16S-F: GCCTGGTGATAGCTGGTTGT, Homo 16S-R: ATCATTTACGGGGGAAGGCG (1200 bp); Ovis 16S-F: AGGCCTAAAAGCAGCCATCA, Ovis 16S-R: GCCCTTTTCTAGGGCAGGTT (1060 bp); Capra 16S-F: GCCTGGTGATAGCTGGTTGT, Capra 16S-R: TCACCCCAACCAAACTGCT (820 bp); and Bos 16S-F: CTAAGCAGCCCGAAACCAGA, Bos 16S-R: GGGCAGGGTTTTGTGTTGTC (1300 bp).

### 2.4 Mitochondrial DNA extraction

G-spin™ Total DNA Extraction Kit (50 Preps) (REF: 17045) was used to extract mitochondrial DNA from blood of different species according to the manufacturer's protocol instructions.

Primer		Conditions		References
Homo 16S-F Homo 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		59.3 °C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	
Ovis 16S-F Ovis 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		58.3 °C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	
Capra 16S-F Capra 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		58.3°C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	
Bos 16S-F Bos 16S-R	1	95°C	2 min	This study
	30	95°C	30 sec	
		59.3°C	30 sec	
		72°C	130 sec	
	1	72°C	5 min	

**Table 1.**  
PCR conditions.

2.5 Agarose gel electrophoresis

Agarose gel was prepared by dissolving agarose powder in 1X TBE buffer. The amount of agarose can be dissolved depending upon the purpose in which agarose sheet used. About 0.7% agarose gel was used for visualization of the DNA after extraction while 1.5–2% agarose sheet was used for visualization of PCR product (amplicon). RedSafe (alternative for ethidium bromide) stock solution concentration was 10 mg/ml. Only 5 µl of RedSafe stock solution were added to 100 ml of melted agarose gel to get the final concentration of 0.5 µg/ml [13, 14].

2.6 Primer pairs preparation and PCR conditions

The primers were synthesized at Macrogen/Korea, were provided in a lyophilized form, which were re-dissolved with 300 nuclease-free water according to the institution of the manufacture company to reach to the final concentration (100 pmoles/µl). The working solution will be 10 pmoles/µl to be used directly in PCR [15, 16]. The PCR conditions were calculated using online Protocol Optimize writer software. The conditions were illustrated in **Table 1**.

3. Result and discussion

The four sets of designed primer pairs were submitted to specificity using Primer-Blast and the results revealed that, they are specific to amplify the 16S rRNA gene of humans (*Homo sapiens*), sheep (*Ovis aries*), goats (*Capra hircus*), and cows

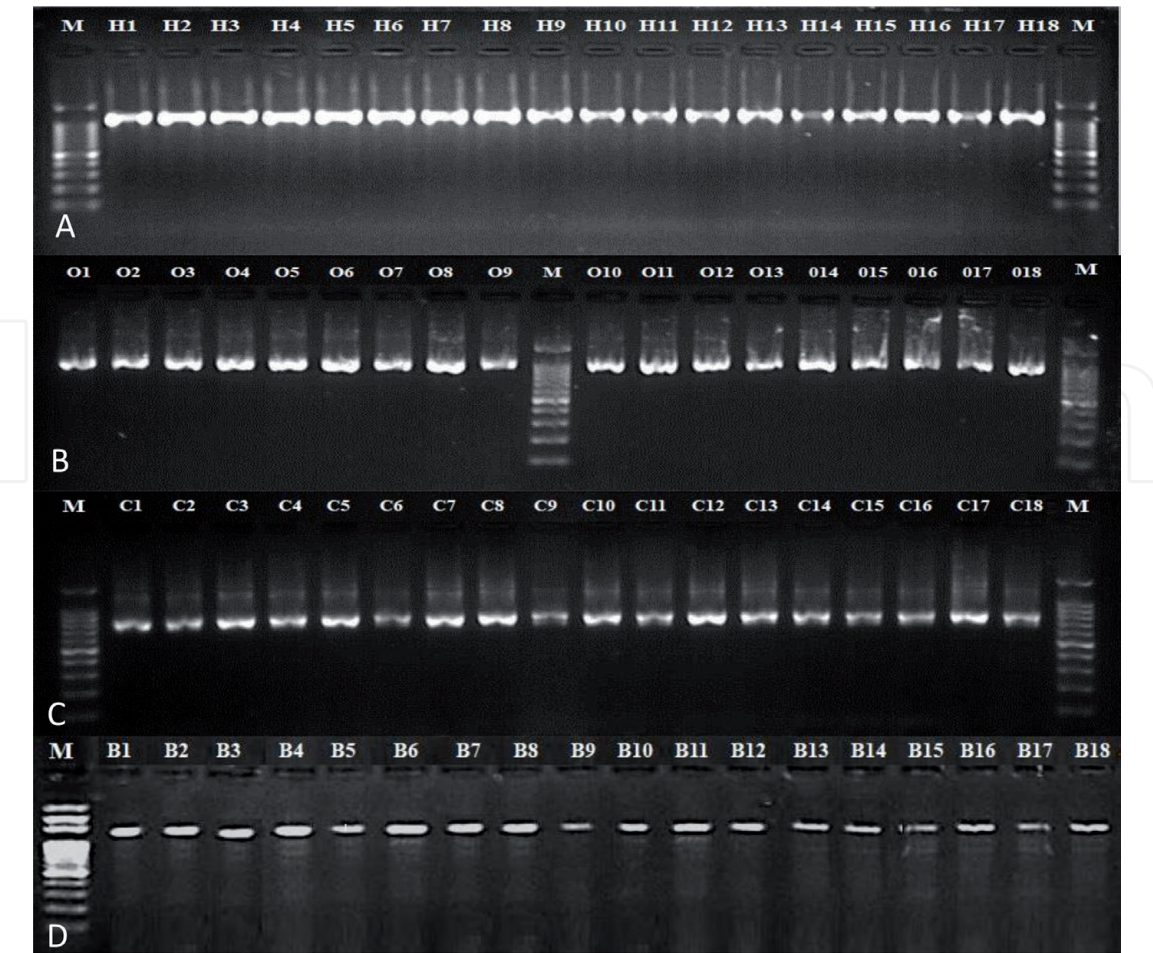
(*Bos taurus*) (**Table 2**). The 16S rDNA region is a highly conserved region among mtDNA [17]. mtDNA can be easier to retrieve from low-quantity and/or degraded DNA samples, as it is present at many copies per cell, thus providing a clear advantage over nuclear genome-based methods of species identification [18–20].

The results of PCR testing revealed that the primer pairs were specific and non-specific products did not appear for all samples. The amplification of *Homo sapiens* mtDNA with primer pairs of other (*Ovis aries*, *Capra hircus*, and *Bos taurus*) and amplification of each with primer pairs of another genus gave negative results. This was primary evidence for primer pair specificity. The amplicon of 16S rRNA gene of *Homo sapiens* was 1200 bp (**Figure 1A**), *Ovis aries* was 1060 bp (**Figure 1B**), *Capra hircus* was 820 bp (**Figure 1C**), and *Bos taurus* was 1300 bp (**Figure 1D**). PCR amplification and sequence analysis of the mitochondrial 16S rRNA gene was utilized for differentiation/identification and subsequently evaluation of their application in

Gene	Primer sequence 5' to 3'	Sequence ID of isolate	Identity
<i>Homo sapiens</i> 16S rRNA	F:GCCTGGTGATAGCTGGTTGT R:ATCATTTACGGGGAAGGCG	MN115376.1	100%
		MN053904.1	
		MN125706.1	
		MN163828.1	
		MN163832.1	
		MN125705.1	
		MN163282.1	
		MN125704.1	
		MN124446.1	
		MK069579.1	
<i>Ovis aries</i> 16S rRNA	F:AGGCCTAAAAGCAGCCATCA R:GCCCTTTTCTAGGGCAGGTT	KP998473.1	100%
		KP998472.1	
		KP998470.1	
		KP702285.1	
		MH841968.1	
		MH841967.1	
		MH841966.1	
		MG837554.1	
		MG837553.1	
		KU681224.1	
<i>Capra hircus</i> 16S rRNA	F:GCCTGGTGATAGCTGGTTGT R:TCACCCCAACCAAACTGCT	LS992662.1	100%
		LS992661.1	
		LS992659.1	
		LS992658.1	
		LS992656.1	
		LS992655.1	
		LS992654.1	
		LS992653.1	
		LS992652.1	
		LS992651.1	
<i>Bos taurus</i> 16S rRNA	F:CTAAGCAGCCCGAAACCAGA R:GGGCAGGGTTTGTGTTGTC	EU177866.1	100%
		EU177865.1	
		EU177864.1	
		EU177863.1	
		EU177862.1	
		EU177861.1	
		EU177860.1	
		EU177859.1	
		EU177858.1	
		EU177856.1	

**Table 2.**  
Primer-blast of designed primer pairs.





**Figure 1.** Agarose gel electrophoresis 1.5% for: (A) 1200 bp amplicon of *Homo sapiens* 16S rRNA gene. Lanes H1–H18 represent samples. (B) 1060 bp amplicon of *Ovis aries* 16S rRNA gene. Lanes H1–H18 represent samples. (C) 820 bp amplicon of *Capra hircus* 16S rRNA gene. Lanes H1–H18 represent samples. (D) 1300 bp amplicon of *Bos taurus* 16S rRNA gene. Lanes H1–H18 represent samples. M represents 100 bp DNA ladder.

solving forensic cases [21]. Mitochondrial 16S is suitable for the differentiation of 300 mammalian species. The 16S rDNA gene is a common mitochondrial gene for detection of blend mutton and pork at high sensitivity. The mitochondrial 16S rRNA genes have been used as molecular markers to identify mammals, birds, shrimps, and other species using species-specific primers that amplify the 12S rRNA or 16S rRNA gene regions from mtDNA [17, 22]. Gene loci on the mitochondrial genome have been used in species identification. These include the 12S and 16S rRNA loci. The D-loop (displacement loop) has been used less in species identification but more in intraspecies 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 [23, 24].

The secondary and confirmatory assay for specificity of primer pairs used in the study was sequences of PCR products. Eight amplicons from each were sent for sequencing using the Sanger technique (MacroGen/Korea). The retrieved sequences firstly must be trimmed to remove unwanted sequences before submitting them for BLASTN. The trimming performed by Bioedit was utilized to obtain the finally processed sequences. Abbreviations of *Homo sapiens* sequences were used as (HIS-1 to HIS-8), *Ovis aries* sequences be (IOA-1–IOA-8), *Capra hircus* sequences (IBCH-1–IBCH-8), and *Bos taurus* sequences (IBT-1–IBT-8).

The identity percentage and alignment results of the amplified 16S rRNA gene of *Homo sapiens*, *Ovis aries*, *Capra hircus*, and *Bos taurus* with database were illustrated in **Tables 3–6**, respectively.

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IHS-1	MH444415.1	0.0	98.98%	0/885(0%)	Plus/Plus
IHS-2	MK069579.1	0.0	99.74%	0/771(0%)	Plus/Plus
IHS-3	MK069579.1	0.0	99.57%	2/697(0%)	Plus/Plus
IHS-4	MK069579.1	0.0	99.08%	0/654(0%)	Plus/Plus
IHS-5	MK059695.1	0.0	99.86%	0/701(0%)	Plus/Plus
IHS-6	MK069579.1	0.0	99.39%	0/657(0%)	Plus/Plus
IHS-7	MK295855.1	0.0	99.50%	0/599(0%)	Plus/Plus
IHS-8	MK069579.1	0.0	99.69%	0/653(0%)	Plus/Plus

**Table 3.**  
*Identity of blasted isolates (IHS-1–IHS-8) with reference sequences of highest identity percentage.*

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IOA-1	MG489885.1	0.0	98.70%	0/769(0%)	Plus/Plus
IOA-2	MG489885.1	4e-170	100.00%	0/329(0%)	Plus/Plus
IOA-3	MG489885.1	0.0	98.98%	0/586(0%)	Plus/Plus
IOA-4	MG489885.1	0.0	99.73%	0/749(0%)	Plus/Plus
IOA-5	MG489885.1	0.0	99.63%	0/542(0%)	Plus/Plus
IOA-6	MG489885.1	2e-157	99.36%	0/312(0%)	Plus/Plus
IOA-7	MG489885.1	0.0	99.17%	2/483(0%)	Plus/Plus
IOA-8	MG489885.1	0.0	99.80%	0/489(0%)	Plus/Plus

**Table 4.**  
*Identity of blasted isolates (IOA-1–IOA-8) with reference sequences of highest identity percentage.*

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IBCH-1	KP271023.1	0.0	99.02%	0/614(0%)	Plus/Plus
IBCH-2	KP271023.1	0.0	98.58%	0/633(0%)	Plus/Plus
IBCH-3	KP271023.1	2e-174	99.74%	0/378(0%)	Plus/Plus
IBCH-4	KP271023.1	0.0	100.00%	0/729(0%)	Plus/Plus
IBCH-5	KP271023.1	0.0	99.83%	0/595(0%)	Plus/Plus
IBCH-6	KP271023.1	0.0	100.00%	0/480(0%)	Plus/Plus
IBCH-7	KP271023.1	0.0	99.84%	0/618(0%)	Plus/Plus
IBCH-8	KP271023.1	0.0	99.17%	0/481(0%)	Plus/Plus

**Table 5.**  
*Identity of blasted isolates (IBCH-1–IBCH-8) with reference sequences of highest identity percentage.*

The sequencing of the 16S rRNA has revolutionized the study and identification of human and non-human samples in forensic science. A simple method was developed using universal primers for species identification based on direct PCR sequencing using primer sets that were designed based on the conserved regions of the 16S rRNA loci detected by the comprehensive sequence comparison among 30 animals whole [25]. The mitochondrial DNA method could be a dominant tool for mammalian species identification, especially in forensic cases in which many unidentified biological samples need to be analyzed such as blood spots [25].

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IBT-1	MF169214.1	0.0	99.50%	3/601(0%)	Plus/Plus
IBT-2	KT184455.1	7e-139	100.00%	0/273(0%)	Plus/Plus
IBT-3	KT184466.1	0.0	99.32%	7/1177(0%)	Plus/Plus
IBT-4	KT184466.1	0.0	99.90%	0/979(0%)	Plus/Plus
IBT-5	KT184466.1	0.0	99.90%	0/965(0%)	Plus/Plus
IBT-6	KT184466.1	0.0	100.00%	0/512(0%)	Plus/Plus
IBT-7	KT184466.1	0.0	99.81%	1/1077(0%)	Plus/Plus
IBT-8	KT184466.1	0.0	100.00%	0/1092(0%)	Plus/Plus

**Table 6.**  
*Identity of blasted isolates (IBT-1–IBT-8) with reference sequences of highest identity percentage.*

The 16S and 12S sequences allow identification of most species to the genus level. Faster-evolving DNA regions are required to identify closely related animal species [26]. The successfully used forensically informative nucleotide sequencing analysis of the 16S rRNA mitochondrial DNA were very valuable to identify before unknown biological specimens of human and animals [27]. The mitochondrial 12S rRNA and 16S rRNA genes, including those from fish and amphibians to mammals including human beings. Therefore, universal primers were designed to amplify sequences in the fast-evolving animal mtDNA [17]. The PCR amplifications of mitochondrial 16S rRNA followed by sequencing and analysis were demonstrated to be very efficient for identification of species origin [21]. The 12S rRNA and 16S rRNA gene sequences of animals reveal the fitting level of interspecific variation but the great level of intraspecific homogeneity [7]. 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.

**3.1 Strength of PCR-dependent 16S mtDNA gene**

The extraction of mtDNA and amplification of mtDNA genes seem to be accessible, very easy, and cheap. Additionally when the sequences of amplified genes analyzed the results were very clear and no confusion with other genes. The accuracy is very high due to no cross-amplification between species-specific primers that were observed. The sensitivity is also high due to that the mixed blood at a very small amount (10%) can be detected. Their strength over another technique like real-time PCR was, post real time, cannot perform the sequencing when needed for verification.

**3.2 Weakness of PCR-dependent 16S mtDNA gene**

The only weakness in the technique is requirement for more time, preparation, possibility of contamination, and more machine when compared with real-time PCR.

**4. Conclusions**

There is sensitivity, specificity, and accuracy of the designed species-specific primer pairs and applicability of the designed primer pairs in forensics to investigate blood spots or evidence belonging for human, sheep, goat, and cow.



## 5. Registration of sequences in GenBank

All the 32 sequences of the 16S rRNA gene were submitted to GenBank for registration. After checking and revision, the following accession numbers were donated:

16S rRNA homo sapiens (Human): MN192057, MN192058, MN192059, MN192060, MN192061, MN192062, MN192063, MN192064 (Appendix 4–69–4–76).

16S rRNA Ovis *aries* (Sheep): MN173528, MN173529, MN173530, MN173531, MN173532, MN173533, MN173534, MN173535 (Appendix 4–77–4–84).

16S rRNA Capra hircus (Goat): MN173285, MN173286, MN173287, MN173288, MN173289, MN173290, MN173291, MN173292 (Appendix 4–85–4–92).

16S rRNA Bos taurus (Cow): MN197611, MN197612, MN197613, MN197614, MN197615, MN197616, MN197617, MN197618 (Appendix 4–93–4–100).

## Conflict of interest

There is no 'conflict of interest' for this work.

## Author details


Hussein O.M. Al-Dahmoshi<sup>1\*</sup> and Hayder J. Al-Nayili<sup>2</sup>

<sup>1</sup> Department of Biology, College of Science, University of Babylon, Hilla, Iraq

<sup>2</sup> Al-Diwaniyah Hospital for Maternity and Children, Al-Diwaniyah Health Directorate, Ministry of Health, Al-Diwaniyah, Iraq

\*Address all correspondence to: dr.dahmoshi83@gmail.com

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