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Stability of Human Blood Mitochondrial DNA under Different Degrees of Temperature

A Research

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بسم الله الرحمن الرحيم

اللَّهُ لاَ إِلَهَ إِلاَّ هُوَ الحَيُّ القَيُّومُ لاَ تَأْخُذُهُ سِنَةٌ وَلاَ نَوْمٌ لَّهُ مَا فِي السَّمَوَاتِ وَمَا فِي الأَرْضِ مَن ذَا الَّذِي يَشْفَعُ عِندَهُ إِلاَّ بِإِذْنِهِ يَعْلَمُ مَا بَيْنَ أَيْدِيهِمْ وَمَا خَلْفَهُمْ وَلاَ يُحِيطُونَ بِشَيْءٍ مِّنْ عِلْمِهِ إِلاَّ بِمَا شَاءَ وَسِعَ كُرْسِيُّهُ السَّمَوَاتِ وَالأَرْضَ وَلاَ يَئُودُهُ حِفْظُهُمَا وَهُوَ العَلِيُّ العَظِيمُ

صَدَقَ الله الْعَلِيُّ الْعَظِيمُ

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Dedication

To my Lord, my Supporter……

To Prophet Muhammad and the pure infallible Imams,

my ultimate guide…

To my dearest lost (father and brother) …

The kind heart my mother, the secret of my existence……

I dedicate this work

Ansaf

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Ansaf

Summary

Human identification is an important field of study and research in forensic science and that aimed to diagnose human identity. Human mitochondrial DNA has become a useful tool in forensic investigations. Its polymorphic nature and maternal inheritance are characteristics that have, combined with its sequence information, enabled investigators to identify missing persons, war casualties and individuals involved in mass disasters and criminal cases.

Twelve blood samples from patients were collected in EDTA-tubes, three samples obtained of as a control and nine samples was divided in to three groups each group contain 3 repeats. This study continued for four months from 23/4/2021 to 22/8/2021. This study was carried out at University of Babylon - Faculty of Science- Biology department-DNA laboratory.

The aim of this study was to evaluate the stability of Mitochondrial DNA under different temperatures in forensic application by exposed the sample to different degrees of temperature (100°C,200°C and300°C) through 60 min, then DNA was extracted and PCR amplification of Mitochondrial high variable region (HV1a).

The results of this study showed the effect of temperature on the stability of DNA, after exposed to heat, it was found that the color of the human blood was change and become dark when exposed to 100 °C,200°C while when exposed to 300°C then human blood color become black, then genomic DNA is extracted from white blood cells the result show that control group appeared clear in gel electrophoresis, only six sample appeared which exposed to 100°C,200°C for 60 min ,while group 3 which exposed to 300°C not appeared in gel electrophoresis because the DNA was degradation.

After DNA extraction, polymerase chain reaction of specific mitochondrial gene (HV1a), was performed for all samples, to certain that mitochondrial genome was not destroy due to exposed to high temperature and it is present and used it in forensic evidence, the product size was 280 bp, the result of PCR shows present of nine specific monoconidial (HV1a) band the product size was 280bp.

The results of measuring the concentration and purity of the DNA showed that the concentration decreases when the DNA is exposed to heat, as it showed the highest concentration when exposed to 100 degrees and the lowest concentration when exposed to 300 degrees, while the purity of the DNA decreased as the exposed period increased and there was significant different between three period .

In forensic science the exposure DNA to different temperature above 100°C degradation of DNA and decrease of purity and concentration level.

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List of abbreviations

abbreviations	Mean
ATP	adenosine triphosphate
Bp	Base pair
BPA	Blood Stain Pattern Analysis
D-loop	displacement loop
HSP	Hevey single promoter
HV	hypervariable region
LSP	Light single promoter
mtDNA	mitochondrial DNA
mtGenome	mitochondrial genome
OXPHOS	oxidative phosphorylation
STR	short-tandem repeat
USA	United states American
UV	Ultra-violate light

Chapter One

Introduction

1. Introduction

In the forensic field, one of the main problems is the limited amount of sample available, as well as its degraded state. The maximum information from any biological remains could be obtained. Additionally, microbiome typification could be an interesting application to study for crime scene characterization (Alvarez cubero *et al*.,2017).

Mitochondria are cytoplasmic organelles with adouble phospholipid membrane and are present in almost all eukaryotic cells. Mitochondria are necessary for cell form and function. Their best recognized role is to generate energy by oxidative phosphorylation. The number of mitochondria in the cell varies and depends on the cell type and energy requirement, where cells with greater energy needs have more mitochondria than cells with smaller needs (Hudson and Chinnery., 2006).

Several features make mitochondrial DNA unique, for example in mammals it is maternally inherited. Moreover, there are up to thousands of mtDNA copies in each cell. When all the mtDNA molecules have the same sequence (wild or mutated) it is called homoplasmy while heteroplasmy implies the mixture of two or more types of mtDNA (for example wild type and mutant). The heteroplasmy level of pathogenic variants correlates with the phenotype to some extent (Chen *et al*., 2010).

Human mitochondrial DNA (mtDNA) has long been a useful tool to identify war casualties and victims of mass disasters, the sources of biological samples derived from crime scenes or to confirm matrilineal relatedness. The mitochondrial genome (mitogenome) has multiple copies per cell, allowing better recovery of sequence information from degraded samples. Some biological samples such as fingernails, old bones, teeth and hair have mtDNA but little or heavily degraded autosomal DNA. It has now become widely feasible to sequence all 16,569 sites of the mitochondrial genome (or 10 mitogenome) as part of a forensic investigation. The variation in mitogenomes in any extant population is greatly restricted 16 compared with what is potentially available given the genome length, this variation has important implications for the use of mtDNA to help identify individuals or establish relatedness. Mitogenome was at the time unobserved in the available databases, its observation in the skeleton meant that it was expected to exist in hundreds and perhaps thousands of others (Mikkel and David, 2018).

Human mitochondrial DNA has become a useful tool in forensic investigations. Its polymorphic nature and maternal inheritance are characteristics that have, combined with its sequence information, enabled investigators to identify missing persons, war casualties and individuals involved in mass disasters and criminal cases. Various screening procedures have been developed to reduce the need to sequence samples that do not match, but DNA-sequence information is still necessary to verify a match. Even though several challenges remain before mitochondrial-DNA-sequence information can be used unambiguously,

Comparative mitochondrial-DNA-sequence analysis appears to be a reliable and powerful means for human identification (Budowle *et al.*,2003).

1.2 Aim of this study

This study is aimed to estimate the stability of Mitochondrial DNA under different temperatures in forensic application. This performed by following objective:

1- Blood Sample collection

2- Blood Samples exposing to different temperature 100,200, and 300 °C

3- DNA extraction from blood samples and control.

4- Estimation of purity and concentration of DNA.

5- Polymerase chain reaction (PCR) for detection of mitochondrial DNA by using special primer HV1a (control region).

6- Detection the PCR product by electrophoresis.

Chapter Two

Literature Reviews

2. Review of Literatures

2.1 Forensic Blood Importance

Blood is one of body fluid that found in criminal scene. Identification human blood to distinguish it from other substances that like blood very important in investigation. Identification of human blood may be useful for solving many problems in criminal investigation. Blood evidence uses in DNA analysis and DNA fingerprint (Advenier *et al.*,2018).

The human blood has high resistance to degrative agent (sun light, UV, chemicals and high temperature) more than other body fluid. Blood has large amount of DNA that useful when evidence that found in criminal science very small amount. Most criminal case include blood evidence (Al-Yasari, 2017).There are many tests applied on blood evidence such as DNA analysis, colorimetric assays and serological assays. Blood evidence can be used in homicide, suicide and sexual assault to distinguish between guilty from innocent person. also, blood test can be performed at criminal scene (Edelman, 2014; Marizzi *et al*., 2018).

Blood Stain Pattern Analysis (BPA) can be provided useful information about crime and how crime accrues. Blood stain age estimation helpful to determination time elapsed after the crime also used for determination injury age that contributes to solve many problems that facing investigator (Bernstein, 2005).

2.2 Mitochondrial DNA and human evolution

2.2.1 Mitochondrial DNA Profiling

Forensic mitochondrial DNA (mtDNA) analysis is an important tool for human identification and is especially useful for identifying victims such as missing persons and individuals in mass fatality cases. Because mitochondrial DNA is maternally inherited, the mitochondrial DNA profiles of these individuals can be

compared to those of their maternal relatives, and thus these individuals can be identified. Additionally, cells contain a much higher copy number of the mitochondrial DNA genome than that of the nuclear genome. Therefore, mitochondrial DNA testing is frequently used to analyze evidence samples, such as hair shafts, that contain low amounts of nuclear DNA. Furthermore, buried bones and decomposed tissues, in which nuclear DNA may be degraded, can be tested with mitochondrial DNA analysis (Adachi *et al*.,2014).

2.2.2 The Mitochondria

Mitochondria are often referred to as the molecular power house of the cell, as they are responsible for the majority of adenosine triphosphate (ATP) synthesis in the body. In addition to supplying cellular energy, mitochondria are involved in a multitude of other processes, including cellular respiration, steroid synthesis, elongation of fatty acids, apoptosis, and heat production, mitochondria are cellular compartments present in every cell of the human body (Except red blood cells) and are responsible for generating almost all of the energy needed to sustain life and to grow (Neupert et al .,2015).

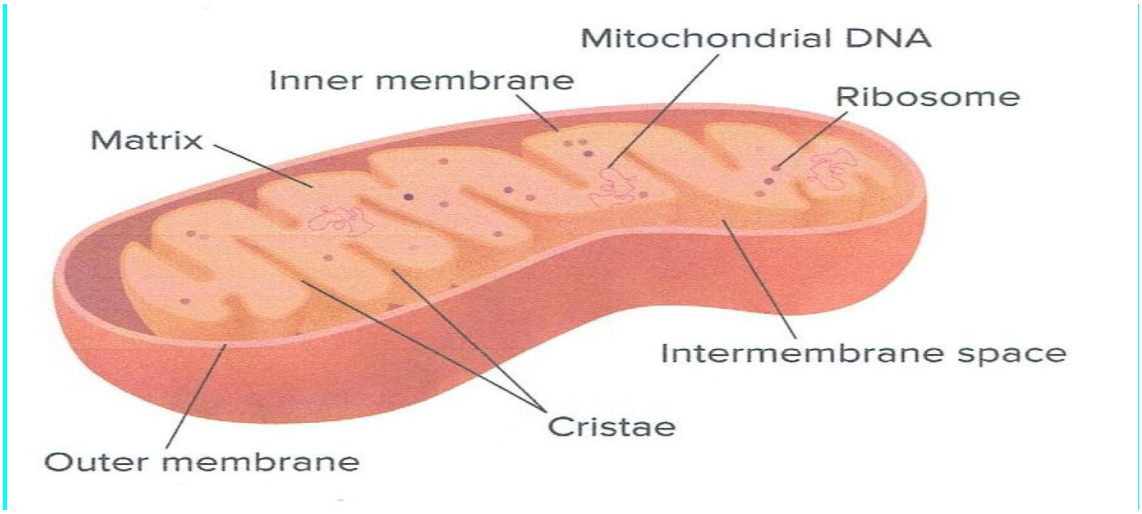

Figure (2 – 1) Mitochondria (Richard , 2015)

It may be appeared as sphere, rods or filamentous bodies, their numbers are different depending to requirements of tissue to amount of energy for example cells of skeletal muscle and kidney are contain abundant number of mitochondria this with their physiological function involved in production of energy, in mammalian cells, approximately there are 800 to 2.500 mitochondria per cell (Miles,2003) figure (2 – 1).

In mitochondria, energy is produced in the process of oxidative phosphorylation (OXPHOS), the biogenesis of the OXPHOS system entails assembly of approximately 90 proteins into five complexes. Most of these proteins are encoded by DNA that is contained within the cell nucleus (nDNA). However, 13 of these are encoded within a small, circular genome inside mitochondria Recent research has identified defects in mitochondrial DNA (mtDNA) expression that are associated with a diverse group of human disorders characterized by impaired mitochondrial respiration (Boczonadi and Horvath,2014).

Mitochondria consist of two membranes and two aqueous compartments; The surface area of the mitochondrial inner membrane is several- fold larger than that of the outer membrane, it therefore forms invaginations known as cristae, which contain the oxidative phosphorylation system, comprising the respiratory complexes I to IV and the F1F0-ATP synthase for ATP production. Only a small set of proteins are encoded by the mitochondrial genome, and these are typically hydrophobic membrane proteins that form core parts of the oxidative phosphorylation complexes of the mitochondrial inner membrane. Approximately 99% of mitochondrial proteins are encoded by nuclear genes and depend on specific targeting signals that direct them from the cytosol, where they are synthesized, to mitochondrial surface receptors and then into the proper mitochondrial sub compartments (Neupert., 2015; Wiedemann and Pfanner., 2017).

The number of mitochondrial DNA molecules per cell varies between different types of cells and tissues. It has been reported that each cell has on average 107 mitochondria and that each mitochondrion has between 1 to 15 mtDNA molecules with an average of 4.6. Consequently, each cell has approximately 500 copies of mtDNA, compared to two copies of nuclear DNA. However, even taking the higher copy number into account, 24 mtDNA only comprises approximately 0.25% of total DNA in a cell. This is due to the significantly smaller size of the mitochondrial genome, consisting of 16,569 bp compared to the 3 billion bp of DNA in the nucleus (Sadikovic *et al*., 2010).

2.2.3 Human Mitochondrial Genome

The mitochondrial genome consists of multiple copies of 16,569 bp, double stranded mitochondrial DNA (mtDNA) molecules and located adjacent to the

OXPHOS system in the matrix. Only thirty-seven genes (22 transfer RNAs, 2 ribosomal RNAs and 13 polypeptides that form structural subunits of OXPHOS system) are encoded by mtDNA (Pohjoismaki and Goffart., 2011). There are no introns in mtDNA, the only major non- coding region in the molecule is the displacement loop (D-loop), which is a 1.1kb region that contains elements of mtDNA transcription and replication (Greaves and Taylor ,2006), The individual strands of the mtDNA molecules are denoted heavy (H) and light (L) strand because of their different buoyant densities in a cesium chloride gradient. L-strand transcription is initiated from one single promoter (LSP), whereas H-strand transcription is initiated from two specific and differentially regulated sites, HSP1 (H1) and HSP2 (H2) (DiMauro and Schon, 2003).

Inside mitochondria, mtDNA is organized in nucleoprotein particles called nucleoids, the nucleoid, considered a heritable unit of mtDNA, may contain several copies of the mitochondrial genome as well as several different proteins (Wang and Bogenhagen ,2006).

The distribution of nucleoids during mitochondrial Fission and fusion events and during cytokinesis affects the segregation, Transmission and complementation of mitochondrial genomes. This has particular importance in the context of primary mtDNA diseases, in which heteroplasmic cells bear a mixture of healthy and mutated mtDNA molecules, cell fusion experiments have indeed demonstrated that mitochondrial nucleoids and the respiratory complexes are mobile and diffuse efficiently into mitochondria previously devoid of mtDNA (Legros *et al*., 2001).

Mitochondrial DNA is maternally inherited, and paternal mtDNA are destroyed during fertilization of sperm so that mtDNA is only inherited by females, at fertilization, all mtDNA derives from the ovum. Therefore, the mode of transmission of mtDNA and of mtDNA point mutations (single deletions of

mtDNA are usually sporadic events) differs from Mendelian inheritance. A mother carrying a mtDNA point mutation will pass it on to all her children (males and females), but only her daughters will transmit it to their progeny females (Elson *et al.*, 2004) figure (2-2).

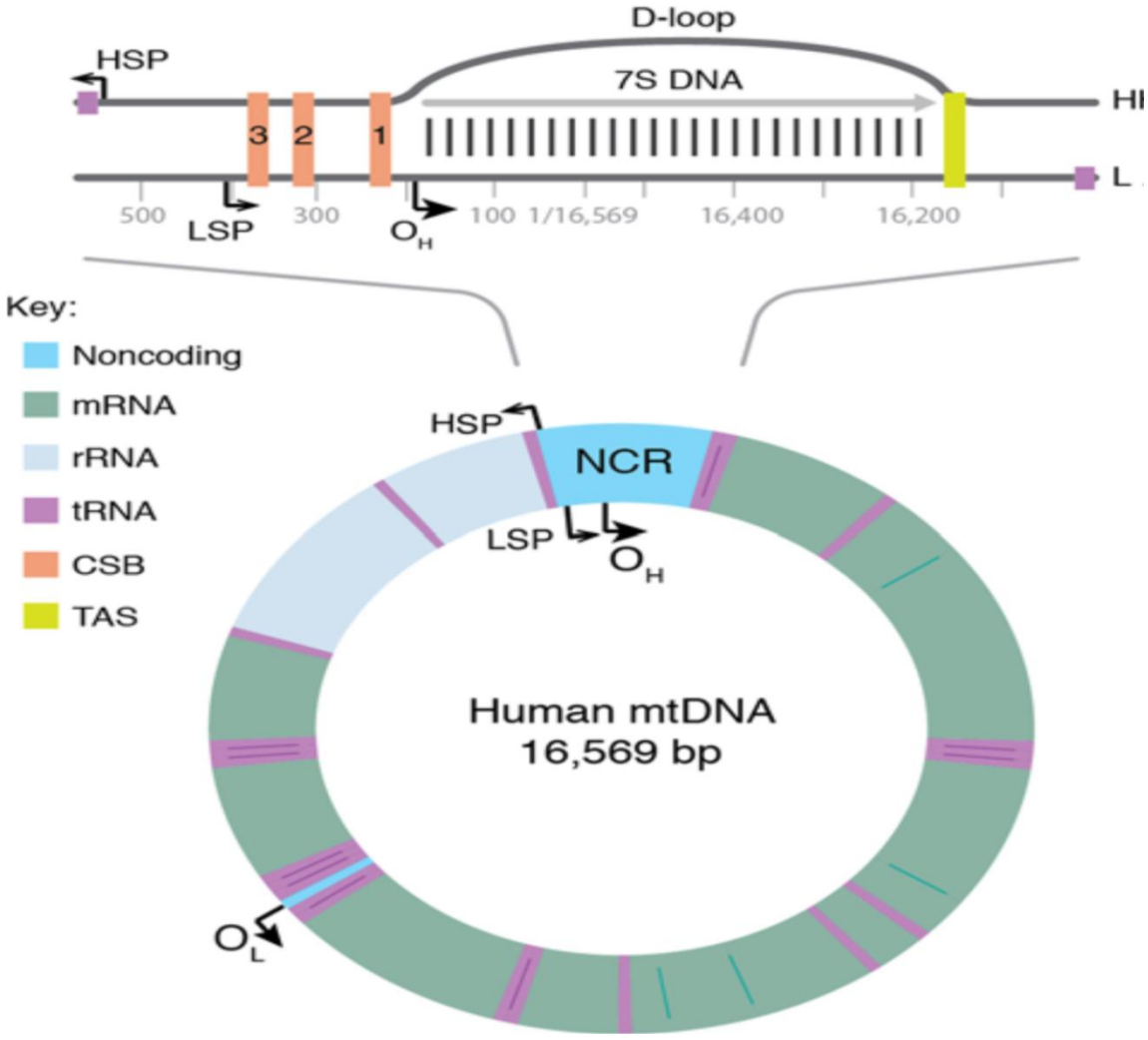

Figure (2-2) Map of human mtDNA (Holt and Reyes, 2012)

2.2.4 mtDNA Polymorphic Regions

2.2.4.1 Hypervariable Regions

Control region of mtDNA is called D-loop which is highly polymorphic and hence is used for forensic purpose in criminal investigations. The length of this loci is 1100 bp and has two regions called (hypervariable regions HV1 and HV2). There are three hypervariable regions in the D-loop are designated hypervariable region I (HV1: 16,024–16,365; 342 bp), hypervariable region II (HV2: 73–340; 268 bp), and hypervariable region III (HV3: 438–574; 137 bp). The most common polymorphic regions of the human mtDNA genome analyzed for forensic purposes are HV1 and HV2 (Andrews *et al.*, 1999).

Mitochondrial have been used as a tool for forensic identification since 1993, Mitochondria contain 2-10 copies of mtDNA, and there can be as many as 1000 mitochondria per somatic cell. In common, blood epithelial cells are preferentially used in forensic casework as a result; detection becomes extremely sensitive even in low amount samples. Some regions of the mtDNA genome appear to evolve at a rate of 5–10 times higher than that of single-copy nuclear genes (Jobling *et al.*,2004).

2.2.4.2 Heteroplasmy and Homoplasmy

Homoplasmy implies all mtDNA are identical which could be all wild type or mutated. Heteroplasmy is a mixture of mutated and wild type mtDNA, Heteroplasmy occurs when an individual carries more than one mtDNA haplotype Heteroplasmy may be observed with one kind of tissue and may be absent in other kinds of tissues; for example, mtDNA heteroplasmy is commonly observed in hair samples, additionally, an individual may exhibit one mitotype in one tissue and a different mitotype in another. Thus, it is necessary to obtain and process additional samples to confirm the heteroplasmy when it is observed in a questioned sample

but not in a known sample or vice versa. The two types of heteroplasmies are sequence and length heteroplasmy (Gazi *et al*.,2018).

In the presence of heteroplasmy there is a threshold effect and clinical expression can vary between different tissues and mtDNA mutations, in women with heteroplasmic mtDNA mutations there is a bottleneck in the female germline which means that the transmission of heteroplasmy level from mother to offspring is often random and unpredictable (Schon *et al*., 2012).This explains the heterogeneity in heteroplasmy level, clinical phenotype and severity frequently observed within the same pedigree Multiple mtDNA deletions and mtDNA depletion (reduced copy number of mtDNA) are secondary changes in mtDNA due to mutations in the mtDNA replication and/or maintenance genes such as POLG(Sharma *et al*.,2005).

2.2.5 Characteristics of mitochondrial DNA

2.2.5.1 Location and structure of mtDNA

The vast majority of the human genome is located within the nucleus of each cell. However, there is a small, circular genome found within the mitochondria, the energy-producing cellular organelle residing in the cytoplasm. The number of mitochondrial DNA (mtDNA) molecules within a cell can vary tremendously. On average there are 4–5 copies of mitochondrial DNA molecules per mitochondrion with a measured range of 1–15 (Satoh and Kuroiwa., 1991). Because each cell can contain hundreds of mitochondria, mathematically there can be up to several thousand mitochondrial DNA molecules in each cell as in the case of ovum or egg cells. However, the average has been estimated at about 500 in most cells, this amplified number of mitochondrial DNA molecules in each cell enables greater success (relative to nuclear DNA markers) with biological samples that may have been damaged with heat or humidity. Consider though that mitochondrial DNA

makes up less than one percent (about 0.25%) of the total DNA content of a cell if we assume that there are 1000 copies of mitochondrial DNA (16 569 bp) in a cell and two copies of nuclear DNA (3.2 billion bp) (Shutt *et al*.,2010)

Mitochondrial DNA has approximately 16569 base pairs and possesses 37 'genes' that code for products used in the oxidative phosphorylation process or cellular energy production. There is also a 1122 bp 'control' region that contains the origin of replication for one of the mitochondrial DNA strands but does not code for any gene products and is therefore referred to sometimes as the'non-coding' region. The total number of nucleotides in a mitochondrial genome (mtGenome) can vary due to small mutations that are either insertions or deletions. For example, there is a dinucleotide repeat at positions 514–524, which in most individuals is ACACACACAC or (AC)5 but has been observed to vary from (AC)3 to (AC)7 (Bodenteich *et al.*, 1992; Szibor *et al.* 1997).

The 37 transcribed 'genes' of mtDNA found in the 'coding region' include 13 proteins, two ribosomal RNAs (rRNA), and 22 transfer RNAs (tRNA). The nucleotide positions for each coding and non-coding segment of the MT genome are indicated. Note that the genes are very tightly packed with only 55 nucleotides in the 15 447 bp of the coding region *not* being used to transcribe a protein, rRNA, or tRNA molecule. Thus, the genes within mitochondrial DNA are economically packaged with no introns and none or only a few noncoding nucleotides between the coding regions. An asymmetric distribution of nucleotides gives rise to 'light' and 'heavy 'strands when mitochondrial DNA molecules are separated in alkaline CsCl gradients (Sutovsky., 1999).

The 'heavy' or H-strand contains a greater number of guanine nucleotides, which have the largest molecular weight of the four possible nucleotides, than the 'light' or L-strand. Replication of mitochondrial DNA begins with the H-strand in the non-coding 'control region', also known as the displacement loop or D-loop. A

total of 28 gene products are encoded from the H-strand while the L-strand transcribes eight transfer RNAs (tRNAs) and an enzyme called ND6 Since the D-loop does not code for gene products, the constraints are less for nucleotide variability and polymorphisms between individuals are more abundant than in similar sized portions of the coding region. More simply, there can be differences in the D-loop region because the sequences do not code for any substances necessary for the cell's function. Most of the focus in forensic DNA studies to date has involved two hypervariable regions within the control region commonly referred to as HV1 and HV2, which will be described in more detail below (Scholpa, and Schnellmann, 2017).

2.2.5.2 Lack of Recombination

Another tenet of molecular anthropology that was shaken a few years ago is that mitochondrial DNA does not undergo recombination. This was regarded as an established fact (Wallace DC etal.,1999.) until 1999–2000, when four papers claimed evidence for recombination in human mitochondrial DNA (Awadalla *et al*.,1999a; Hagelberg *et al*., 1999. Awadalla *etal*.,2000b) Three of these studies were based on phylogenetic and statistical analyses of mitochondrial DNA sequences, with the authors arguing that the excess of homoplasmic sites observed in phylogenetic trees of mitochondrial DNA sequences (Eyre *et al*., 1999).

The correlation of linkage disequilibrium with distance across the mitochondrial DNA genome provided evidence for recombination. The studies claimed to have direct evidence for recombination in Melanesia. However, it was subsequently shown that the phylogenetic/statistical studies used faulty data and/or questionable statistical methods, with reanalysis giving no significant results (Eyre-Walker, 2000; Kivisild and Villems 2000), and the claim for direct observation of recombination in Melanesian mtDNAs was based on an alignment error and had to

be retracted (Hagelberg, *et al*.2000). Three subsequent studies of the correlation of linkage disequilibrium and distance in large data sets of complete mitochondrial DNA sequences (Elson *et al* .,2001 ; Ing man *et al*., 2000, Piganeau and Eyre-Walker.,2004) found no evidence for recombination, although again an excess of homoplasmic sites was detected (Piganeau and Eyre-Walker., 2004) this, however, is generally attributed to heterogeneous mutation rates in human mitochondrial DNA (Excoffier and Yang.,1999; Howell and Smejkal., 2003) .

Recently, however, a case of observed recombination in human mitochondrial DNA was reported in the only known human with both maternal and paternal mitochondrial DNA(Schwartz and Vissing,2002;Kraytsberg *et al*., 2004). Here, recombination between the maternal and the paternal mitochondrial DNA occurred in approximately 0.7% of the total mitochondrial DNA in the patient's muscle tissue. This finding underlines that recombination is possible because mitochondria possess a functional recombinase, although it is still unclear to what extent mitochondria within a cell are able to fuse and exchange contents (Enriquez, *et al*., 2000; Legros etal.,2002).

However, because leakage of paternal mitochondrial DNA is a very rare phenomenon , is not the major issue that it is sometimes made out to recombination be in the absence of heteroplasmic DNA molecules, any recombination events would result in mtDNA that do not differ from the original (Hagelberg,2003).

2.3 Forensic applications of mitochondrial DNA

Human mitochondrial DNA has become a useful tool in forensic investigations. Its polymorphic nature and maternal inheritance are characteristics that have, combined with its sequence information, enabled investigators to identify missing persons, war casualties and individuals involved in mass disasters and criminal

cases. Various screening procedures have been developedto reduce the need to sequence samples that do not match, but DNA-sequence information is still necessary to verify a match. Even though several challenges remain before mitochondrial-DNA-sequenc information can be used unambiguously, comparative mitochondrial-DNA-sequence analysis appears to be a reliable and powerful means for human identification (Parson *et al*.,2014).

The use of mtDNA sequence information to identify the remains of the Romanov family recently received considerable public attention, although their bones were in the ground for over 70 years, an exact mtDNA sequence match of the D-loop region was made between the Tsarina, her three daughters and the (living) Prince Philip of the UK, whose maternal grandmother was the Tsarina's sister. Tsar Nicholas II was identified by a comparison of the mtDNA sequence from the bones in the grave with that of the great-great-great granddaughter and great-great grandson of the grandmother of Tsar Nicholas II, Louise of Hesse Cassel (Gill *et al.*,1994**).**

The match was exact except for one heteroplasmic site (16169) at which the putative Tsar's mtDNA had both C and T bases. To confirm the match, mtDNA from bone samples from Georgij Romanov, the Tsar's brother, was sequenced and showed the same heteroplasmy15. This information and the parentage information obtained from short-tandem repeat (STR) analysis enabled the forensic investigators to be 98.5% certain that the bones were those of the Romanovs, in a similar fashion, the mtDNA information from Anna Anderson Manahan, who has claimed to be the missing Royal Duchess Anastasia, showed that the claim was false (Stoneking *et al*.,1995).

Today, mtDNA typing is utilized primarily in cases in which the nuclear DNA is too degraded or cannot be recovered in sufficient quantities to be typed. Several

laboratories, including the Federal Bureau of Investigation (FBI) Laboratory (Washington, DC, USA), the Armed Forces DNA Identification Laboratory (Rockville, MD, USA) and the Forensic Science Service (Birmingham, UK), are actively involved in using mtDNA for forensic identifications. Human skeletal remains from the Vietnam War and other wars are routinely being analyzed and identified by mtDNA typing. The success of the Armed Forces DNA Identification Laboratory17 in this endeavor has led to the formation of a repository for blood samples from all those entering the US Armed Forces, so that, if necessary, in the future, the DNA in the stored blood samples can be used to identify war casualties. Hopefully, this approach will result in no more 'unknown soldiers. Currently, however, because no blood repository existed for past soldiers, family reference samples from siblings or maternal relatives are used to confirm the identity of the human remains. Skeletons recovered from two ten-year-old Guatemalan mass graves have also been successfully analyzed by mtDNA testing (Tezel*et al.*,2016).

Chapter Three
Materials and Methods

3. Materials and Methods

3. 1. Chemicals and Instrument

The chemicals and instrument which were used in this study are listed in table (3-1), (3-2) and (3-3) with their producing companies and countries

Table (3-1): Chemicals used during this study.

Chemical name	Supplying company
Agarose	**Thermofisher / USA**
DNA Extraction kit	**Favorgen**
DNA Ladder	**Bioneer**
EDTA	**Himedia(India)**
Ethanol	**Biosolve company/USA**
Ethidium bromide	**Promega – USA**
Loading dye	**Thermofisher / USA**
Master mix	**Intron / USA**
Proteinase K	**Biolabs (England)**
TBE buffer	**Bio-Basic / (England)**

Table (3-2): Instrument used during this study

Instruments	Supplying company
Autoclave	**Haramaya/ Japan**
Centrifuge, Cooling centrifuge	**Hettich – Germany**
Distillater	**GFL – Germany**
Gel electrophoresis unite	**Cleaver scientific – Japan**
Photo documentation, UV source	**Cleaver Scientific / UK**
Spectrophotometer	**Shemadzu/ Japan**
Thermocycler	**Cleaver Scientific (Japane)**
Vortex	**Bioneer**
Oven	**Hettich – Germany**
Water Path	**GFL/ Germany**

Table (3-3): Disposable used during this study

Item	Manufacturer
Aerosol Resistant Micropipette tips(1000µl)	**Promega / USA**
Aerosol Resistant Micropipette tips (10-100µl)	**Bio-Basic / Canada**
Aerosol Resistant Micropipette tips (10µl)	**Bio-Basic / Canada**
Aerosol Resistant Micropipette tips (100-200µl)	**Promega / USA**
Collecting tube (2ml)	**FAVORGEN**
Eppendorf tube (1.5ml)	**China**
FABG column	**FAVORGEN**
Gel Loading Tips	**Promega / USA**
PCR tube (0.2ml)	**Bio-Basic / Canada**
Syringe 3,5,10 ml	**TG / Malaysia**

3. 2. Subjects and Methods

3. 2. 1. Study Design, Setting and Data Collection

Blood samples were obtained from twelve peoples 23-45 years, blood samples were drawn by a trained clinic nurse working in the main blood bank in Babylon Governorate / Morjan city Hospital and the samples were placed into EDTA containing tubes stored at - 20˚C, the study lasted for four months from 23/4 to 22/8/2021. This study was carried out in the DNA laboratory, College of Science, University of Babylon.

3.3 Experimental Design

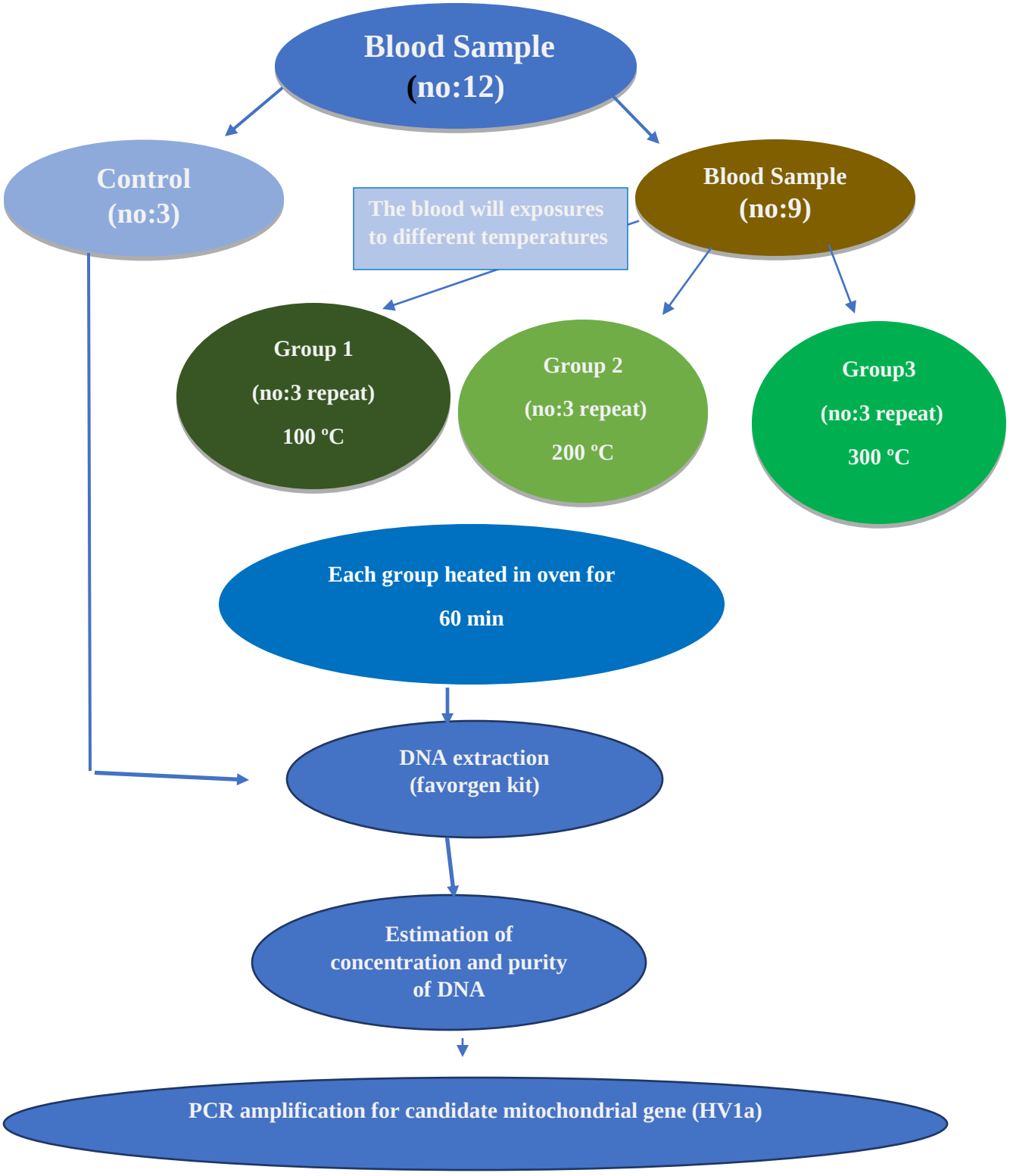

Figure (3-1) Experimental design of the current study

3. 4. Prepared Samples

The stored blood was placed at room temperature to dissolved, about 5 ml of blood was transferred to the Eppendorf tube and marked from 1 to 12, three blood samples were isolated to be considered as a control group for comparison ,while the remaining blood samples were 9 samples. It was divided into three groups, the first group contains 3 replicates, the second group contains 3 replicates, and finally the last group contains three replicates.

The temperature of the oven was set at 100 ˚C, the first group was placed at this temperature for period of time 60 minute, the second group was incubated at a temperature of 200 ˚C for period of time 60 minute, the third group was incubated at 300 ˚C for the same Time period, after treating the three groups with different temperatures including 100, 200 and˚300C, all samples were transferred until DNA was extracted. The control group not treated with any heat and DNA was extraction directly

3. 5. Genetic Analysis

3.5. 1. DNA Extraction

Genomic DNA from white blood cells (WBCs) for all groups and control group were extracted by using DNA extraction kit (Favorgen) table (3-4) according to the following:

A. Kit Contents

Table (3-4) Genomic DNA Kit contents

Cat. No. / preps	FABGK 100 (100 Preps)	FABGK 300 (300 Preps)
RBC Lysis Buffer	135 ml	405 ml
FATG Buffer	30 ml	75 ml
FABG Buffer	40 ml	100 ml
W1 Buffer	45 ml	130 ml
Wash Buffer	25 ml	50 ml
Elution Buffer	30 ml	75 ml
FABG Column	100 pcs	300 pcs
2 ml Collection Tube	200 pcs	600 pcs

3.5.1.B Special Protocol of DNA extraction (for frozen blood)

Step 1- Sample preparation

1. A 200µl blood was transferred up to a 1.5ml microcentrifuge tube.
2. A 40µl proteinase k (10 mg / ml) was added to the sample and briefly mixed. Then incubate for 15 minutes at 60°C.

Step 2- Cell Lyses

1. A 200µl FABG Buffer was added to the sample and mixed by vortex.
2. Incubated in 70 °C water bath for 15 minutes to lyses the sample. During incubation, invert the sample every 3minutes.
3. Elution buffer was preheated (for step 5 DNA Elution) in a 70 °C water bath.
4. (Optional step): If RNA –free genomic DNA is required, 5µl of 10 mg/ml RNAase was added to the sample and mix by vortex. Then incubate for 5 minutes at the room temperature

Step 3- Binding

1. A 200µl ethanol (96-100%) was added to the sample and vortex for 10 seconds. (Pipetting if there is any precipitate).
2. A FABG Column was Placed to a 2ml collection tube. Transfer the sample mixture (including any precipitate) carefully to FABG Column. Centrifuge for 5 minutes at full speed (14000 rpm) and discard the 2ml collection tube. Place the FABG Column in a new 2ml collection tube.

Step 4 – Washing

1. FABG Column with 400µl W1 Buffer was washed Centrifuge for 30 seconds at full speed (14000 rpm) and discard the flow-through.
2. The FABG Column was placed back in the 2ml collection tube. Wash FABG Column with 600µl Wash buffer (ethanol added). Centrifuge for 30 seconds at full speed (14000 rpm) and discard the flow-through.
3. The FABG Column was placed back into the 2ml collection tube. Centrifuge for an additional 3 min at full speed (14000 rpm) to dry the column.

Step 5 – Elution

1. The dry FABG Column was placed to a new 1.5ml microcentrifuge tube.
2. A 100µl of preheated Elution buffer or TE was added to the membrane center of FABG Column. Stand FABG Column for 3-5 min or until the buffer is absorbed by the membrane.
3. Centrifuge for 30 seconds at full speed (14000 rpm) to elute the DNA.

Step Final – Pure DNA

The DNA fragment was stored at -20 °C.

3. 5. 2. Estimation DNA Concentration and Purity

The DNA concentration of samples were estimated by using spectrophotometer (Nanodrop) as the following:

1- A 1µl of TE solution was added on the lens for empty- apparatus, be careful do not touch th elens.

2- A 1µl of DNA samples were added into the machine to detect concentration in ng/µl, the concentration of samples was 20-50 ng/µl, and the purity detected by observed the ratio of optical density (OD) 260/280 nm.

3.5.3 Gel electrophoresis protocol for DNA of blood.

3.5.3.1. Tris Borate EDTA Buffer preparation (1X TBE)

This solution was Prepared by adding 900 ml Distill water to 100 ml 10X TBE (Promiga/ Germany), with a modification in dilution by adding 25 µl TBE to 475 ml D.W. to get 0.5 X (Sambrook and Russel, 2001).

3.5.3.2. Preparation of Agarose

1- One hundred ml of 1X /or 0.5X TBE buffer was powered in conical flask.

2- Volume of 1.2 mg agarose powder (Biostatic) was added to the buffer.

3- The solution was heated to boiling using a heater until all gelparticles were melted.

4- The solution was left to cool down to 55-60 °C.

5- One µl of the Ethidium bromide (10 mg/ml) was added to agarose solution, and mixed. The mixture was casted in a horizontal tray (Sambrook and Russel et al. 2001).

3.5.3.3 Preparation of Horizontal Agarose Gel

1. The comb in 1 cm was fixed away from one edge of a gel tray,the agarose solution was poured into the gel tray.
2. The agarose was allowed to solidify at room temperature for 45 min.
3. The fixed comb was carefully removed, the wells of gel filled with the DNA, and tray was placed in the gel tank, which was filled with 1X TBE buffer until the buffer reached 3-5 mm over the surface of the gel (Sambrook & Russel et al. 2001).

3.5.4. PCR Amplification of mitochondrial DNA

After exposing the samples to different temperatures, the DNA of each sample was extracted using a special extraction kit, and then the presence of DNA was detected using electrophoresis, to ensure the stability of the mitochondrial DNA. After exposing the samples to different temperatures, the initiator sequence is shown in the table below

Table (3 -5) : The primer of detection mitochondrial genes

DNA Primer	Sequence	Amplicon size (kb)
HV1a	F -5- CAC CAT TAG CAC CCA AAG CT-3 R-5-GGC TTT GGA GTT GCA GTT GAT-3 F (A1) (L15997) R (B2)(H16237)	280bp

3.5.4.1 Primers Preparation

The primers were supplied by Bioneer (Korea) Company as a lyophilized product of different Picomole concentrations. The Applied Biosystems company protocol was adopted for primer resuspension, by bringing the final concentration of primers to 100 pmol/µl of deionized distilled water, stored at -20ºC until use as leaflet mentioned for every primer preparation. Primers were used by making working solution, 10µl from the stock solution plus 90µl deionized distill water to obtain 100µl working solution, stored at -20ºC until used.

3.5.4.2 PCR product electrophoresis

Table (3.6) Master mix components

Item	Concentration
Top DNA polymerase	1 U/ µl
Each: dNTP (dATP, dCTP, dGTP, dTTP)	250 Mm
Tris-HCl (pH 9.0)	10 mM
KCl	30 mM
$MgCl_2$	1.5 mM
Stabilizer and tracking dye	

Table (3-7): Explain condition work of lyophilized PCR 25 µl (as leaflet kit)

Component	50 µl reaction
Template DNA	1-1.5 ng
Forwarded primer	1-1.5 µl
Reverse primer PCR	1-1.5 µl
PCR grad water	Variable

3.5.4.3Amplification reaction mixture

HV1a region genes segments were (3-8) as follow.

Table (3-8): Cycling parameters for duplex PCR of HV1a amplification

Cycle No.	Stage	Temperature	Time
1	Initial denaturation	94	5min
29	Denaturation	94	1min
29	Annealing	58.8	30 sec
29	Elongation	72	30 sec
1	Final extension	72 ºC	5min.

3.5.4.4 Detection of PCR products by agarose gel electrophoresis.

Five microliters of amplified products were analyzed by electrophoresis in 1.2% agarose gel stained with 1µl (10mg /ml) Ethidium Bromide, at 75 V for 1.5/ and or 2 hours using 1X/or 0.5X TBE buffer, then visualized under UV light using ultraviolet Gel documentation. DNA ladder (100 bps) BIONEER marker DNA and (100 bps) VIOGENE were used as a comparative and the gel was photographed by a digital camera.

1- The gel was removed from the tank, and the excess liquid was drained.
2- The gel was placed in the dark room, of the gel documentation system Alpha In notech visualized at UV beam at 480 nanometers

Image for the gel were captured by digital camera connected to bio spectrum multispectral imaging system provided by UVP /USA. (Sambrook and Russel *et al*., 2001).

Chapter Four
Results and Discussion

4. Results and Discussion

The objective e of this investigation was to study the effect of different temperature on the degradation of whole human blood DNA. DNA degrades rapidly when exposed to environmental factors like high temperature, the extent of damage done to human DNA in relation to time of exposure to high temperature above 500°C that make blood samples unsuitable for forensic analysis has not yet been determined, and using two methods for identification first extraction DNA by using kit method and then detection of DNA, the gel electrophoresis was used to appear the bands and second amplification for specific mitochondrial gene (HV1a) by polymerase chain reaction PCR.

4.1 Effect of temperature

After exposure to heat, it was found that the color of the human blood was change and become dark when exposure to 100 ,200 while when exposure to 300 then human blood color become dark, and results that appear similar results were noted by (Mohite *et al.*, 2011).The exposure human blood to high temperature case destroyed antigen due to heating causes physical changes within the blood stain. degradation of biological trace evidence can be caused by heat; high temperature causes oxidation of Fe2+to Fe3+ that mean destroy hemoglobin. similar results were noted by (Sutthapodjanarux, 2009).

Genomic DNA is extracted from white blood cells following the procedure prescribed in Favorgen kit, the obtained gDNA eluted from the silica membrane is suspended with the equivalent volume of 6x loading dye, to yield 1x concentration, before running in 1% Agarose gel for 60 minutes at 85 V.

The electrophoresis photo Figure (1-4) showed clear bands of gDNA for all the tested samples including control and sample exposure to different temperatures (100 ,200 .300). This study was carried out with the main objective of isolation of DNA from blood after subjecting them to different temperatures. The mtDNA were extracted from blood samples using G-spinTM (silica column) total DNA extraction kit, it is silica column-based method, and it is the best duet high purity of the extracted mtDNA. Figure (4-1) demonstrated agarose gel electrophoresis 1% of the extracted samples. Out of the 12 samples taken for the study, DNA was extracted from eight samples (3 sample control ,3 group1 and 2 group2), and only group 3 samples subjected to a temperature of 300°C had not yielded DNA, the Genomic or Nuclear DNA, which is found in the nucleus of each cell and represents the DNA source for most forensic applications, was obtained in six samples subjected to temperatures below 300°C, the same inference was given in a study done by Vemuri *et al*.,2012).

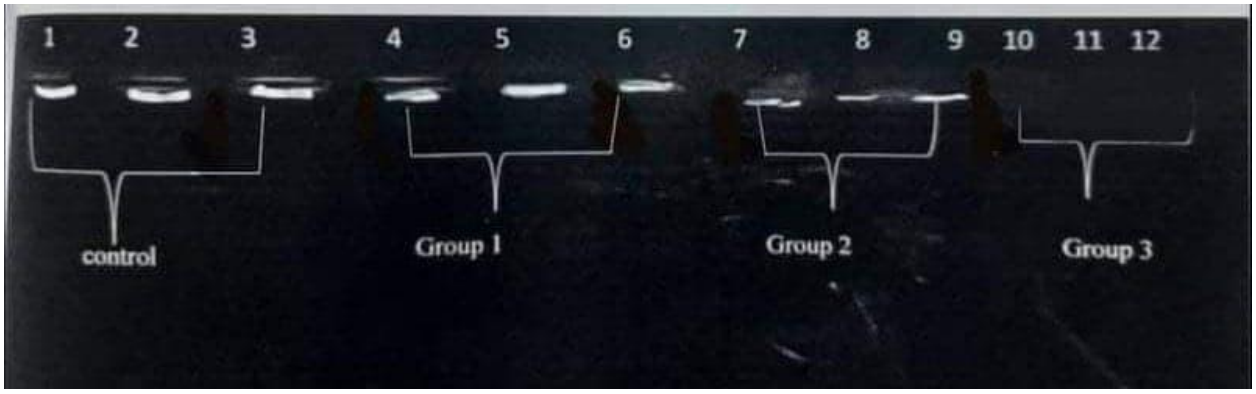

Figure (4-1) Gel electrophoresis of extracted DNA sample from human blood exposure to different temperature (100°C 200°C and300°C), lane 1-3 control, lane 4-6 group1(100°C), lane 7-9 group2 (200°C), lane 10-12 group3 (300°C),1% agarose, 20 mA, 70 v. for 40 min.

.

This result disagrees with the study of the effect of fire on DNA and extreme heat on blood, no DNA were extracted from samples exposed to 100°C and the yield of DNA was significantly higher for all the samples that were not in direct contact of fire (Kumar*et al*.,2019).The DNA recovery from remains is very important to robust analysis, some forensic studies still improvement new methods have short time-consuming low cost with high recovery of DNA (Cheng *et al*., 2019).

The helical structure of double-stranded DNA is destabilized by increasing temperature. Above a critical temperature (the melting temperature), the two strands in duplex DNA become fully separated. Below 100°C temperature, the structural effects are localized. It is likely that the flexibility and bend ability of DNA affect spatial genome folding and functioning. Indeed, it has been shown in biochemical ensemble measurements that temperature directly affects DNA structure by changing its persistence length, besides such a direct effect on DNA structure, temperature might also influence the interactions between DNA and architectural proteins, and hence chromatin structure, and also that temperature not only affects the intrinsic properties of DNA but also influences protein–DNA interactions of DNA-bending proteins (Driessen *et al*.,2014).

Our result similar to the study conducted Kundu *et al*.,2012, that explain that the high temperature effect on the DNA structure by effect on the hydrogen bonds between bases on opposite strands and caused break due and if it continually which cause complete denaturation.

Table (4-1): The relationship between period time that's exposure blood and time that effect purity and concentration of DNA, all results of experiments were recorded as mean for three replicates P value = 0.05.

Period Of exposure / Temperatures	Concentration ng/ul	purity
Control	13.487 ± 2.767	1.88 ± 0.53
100°C	8.41 ± 2.728	1.80 ± 0.51
200°C	5.150 ±1.077	1.63 ± 0.46
300°C	4,17 ± 0.148	1.49 ± 0.39
Sig.	0.030*	0.048*

* P≤ 0.05

After DNA extraction polymerase chain reaction of specific mitochondrial gene (HV2a), was performed for all sample to certain that monoconidial genome was not destroy due to exposure to different temperature and it is present and use it in forensic evidence, the product size was 280 bp, the result show (Figure 4 -2).

Figure (4-2) Electrophoreses pattern of PCR product of HV1a gene, sample from human blood, the amplification product was 280 pb, lane1-3 DNA from control, lane 4-6 Group1, lane 7-9Group 2 ,lane 10-12 Group3 1% agarose, 20 mA, 70 v. for 40 min.

In addition to genomic DNA, cells contain mtDNA, which can be used for identification. While analyzing samples containing no nuclear DNA, mtDNA analysis was performed in twelve sample by subjecting to temperatures of 100°C,200°C and 300°C for different period. We could able to retrieve mtDNA from six sample when subjected to a temperature from 100 to 200°C, but no mtDNA was detected in the sample subjected to 300°C (Budowle*et al*.,2005).in humans, each cell contains up to 2,000 mitochondria. Thus, mtDNA samples are often easier to obtain than nuclear DNA, this is particularly true in forensic

science, where investigators may be working with largely decayed bodies where only the teeth, bones, or hair is available. mtDNA was used as evidence for the first time in US courts in 1998, and it has since become a staple in many cases where DNA evidence is presented (Parsons and Coble,2001).

This infers that as the temperature increases, the amount of DNA obtained decreases significantly, which is not sufficient for identification. The reason for detection of mtDNA in blood exposed to even higher temperatures could be due the presence of mtDNA in more quantity and more robustness to decomposition, due to its cellular location when compared to the nuclear DNA. Thus, it is more likely to be preserved in highly degraded tissues (Foran, 2006) and is especially valuable in missing persons cases, (Alvarez-Cubero*et al.*,2012) wherein the DNA can be compared with even distant relatives (Budowle *et al.*,2003).

In Current study mtDNA testing involves polymerase chain reaction (PCR) amplification of one hypervariable region (HV1) in the control region. The control region is the only significant portion of mtDNA that does not code for genes, HV1 encompass roughly 610 bases of information. These regions are highly variable in the population, due to a very high evolutionary rate of mtDNA, and the fact that the non- coding regions are subject to diminished functional constraint. The evolutionary rate of the control region is approximately ten times that of the gene-coding region, so variation is very much concentrated in HV1. However, a point that has until now been ignored by forensics is that the coding region is fifteen times larger than the control region, so the greatest portion of total mtDNA variation occurs in the coding region (Just *et al*.,2009).

Mitochondrial DNA typing does not provide definitive identification. Firstly, an individual is expected to match maternal relatives. Secondly, mtDNA is a single linked molecule, so one cannot multiply the probabilities of individual

polymorphisms along the mtDNA molecule – the product rule does not apply to frequencies of individual polymorphisms in the mtDNA sequence. Hence, with mtDNA there is an appreciable chance for a random match in the population, although many mtDNA types are so rare that they have been seen only once in large databases (Sultana and Sultan, 2018).

The present study clarified some problems combined with mtDNA application like low concentration of samples because exposure sample to high temperature, this can be overcome using high sensitivity tools like RT-PCR or NGS. Furthermore, the highly fragment DNA also didn't give optimum results and this can be overcome by using shorter DNA amplification target segments by changes primer design flanking mtDNA motif repetition.

Finally,some concerns still remain regarding admissibility of mtDNA analysis in court especially related with the issue of heteroplasmy and, more recently, with the possibility of biparental in heritage. The complete elucidation of molecular mechanisms driving biparental in heritage of mtDNA, the ability to determine the situations where this is likely to occur, and the ability to identify and characterize heteroplasmy with high accuracy, are important issues that need to be addressed in order to ensure the robustness of mtDNA as an important and alternative tool in for forensic human identification (Amorim et al.,2019).

Conclusions and Recommendations

Conclusions:

1- The DNA will be degraded and denatured in forensic after exposure to high temperature above 100°C .

2- DNA concentration and purity was decrease over the time of exposure.

3- The use of Polymers Chain Reaction was specific to identified Mt DNA.

Recommendations

1- Studying and amplification another region in mitochondrial DNA. DNA recovering from positive samples.

2- Application of important comparisons of the mitochondrial DNA series to a large sample of Iraqi society members and their mothers in order to determine the genetic relationship in proving the motherhood.

3- Application sequences of mitochondrial DNA which is great importance in criminal analysis in determining the link of maternal proportions in Iraqi society based on PCR and the sequence analysis

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الخلاصة

يعد تحديد الهوية البشرية مجالًا مهمًا للدراسة والبحث في علوم الطب العدلي والذي يهدف الى تشخيص الهوية البشرية. أصبح الحمض النووي البشري في الميتوكوندريا أداة مفيدة في تحقيقات الطب العدلي. إن طبيعتها المتعددة الأشكال وميراثها من الأمهات ، إلى جانب معلوماتها التسلسلية، مكنت المحققين من تحديد الأشخاص المفقودين وضحايا الحرب والأفراد المتورطين في الكوارث الجماعية والقضايا الجنائية.

تم جمع أثنى عشر عينة دم من المرضى في أنابيبEDTA ، تم الحصول على ثلاث عينات كعنصر سيطرة وتسع عينات تم تقسيمها إلى ثلاث مجموعات تحتوي كل مجموعة على 3 مكررات. استمرت هذه الدراسة أربعة أشهر من22/8/2021 -23/4/2021. أجريت هذه الدراسة في جامعة بابل - كلية العلوم - قسم علوم الحياة - مختبر الحمض النووي.

كان الهدف من هذه الدراسة هو تقييم تقدير ثبات الحمض النووي للميتوكوندريا تحت درجات حرارة مختلفة في تطبيق الطب العدلي بواسطة تعريض العينات لدرجات حرارة مختلفة (100 درجة مئوية،200 درجة مئوية،300 درجة مئوية) خلال فترة زمنية (60) دقيقة، ثم تم استخلاص الحمض النووي وتضخيم PCR للمنطقة عالية المتغير (HV1a).

أظهرت نتيجة هذه الدراسة تأثير درجة الحرارة على ثبات الحمض النووي، بعد التعرض للحرارة، وجد أن لون دم الإنسان قد تغير وأصبح داكنًا عند التعرض لـ 100 درجة مئوية 200 درجة مئوية وأثناء التعرض لدرجة حرارة 300 درجة مئوية يصبح لون دم الإنسان أسود، ثم تم استخلاص الحمض النووي الجيني من خلايا الدم البيضاء، وأظهرت النتيجة أنDNA مجموعة السيطرة ظهرت واضحة في الترحيل الكهربائي للهلام، وظهرت ستة عينات فقط والتي تعرضت لدرجة حرارة 100 درجة مئوية،200 درجة مئوية لمدة (60) دقيقة، بينما المجموعة 3 التي تعرضت لـ 300 درجة مئوية لم تظهر في الترحيل الكهربائي بسبب ان الحمض النووي تحلل بس تعرضه للحرارة العالية.

بعد استخلاص الحمض النووي، تم إجراء عملية التضخيم بواسطة تفاعل البلمرة المتسلسل لجين الميتوكوندريا (HV1a) لجميع العينات للتأكد من أن جينوم الميتوكوندريا لم يتم تدميره بسبب التعرض لدرجة حرارة عالية وأنه موجود ويستخدم في أدلة الطب العدلي، كان حجم المنتج 280، ظهرت نتيجة تفاعل البوليميراز المتسلسل لتسعة عينات .كما وأظهرت نتائج قياس تركيز ونقاوة الحامض النووي ان التركيز يقل عند تعرض الحامض النووي للحرارة حيث اظهر اعلي تركيز عند تعرض لل 100 درجة مئوية

واقل تركيز عند التعرض لل 200 درجة مئوية، بينما كانت نقاوة الحامض النووي تتناقص كلما زادت فتره التعريض للحرارة.

في علم الطب العدلي، يؤدي تعرض الحمض النووي إلى درجة حرارة عالية أعلى من 100 درجة مئوية إلى تغيير طبيعة الحمض النووي وتقليل مستوى النقاء والتركيز له.

وزارة التعليم العالي والبحث العلمي
جامعة بابل- كلية العلوم
قسم علوم الحياة

تحليل ثباتية دنا الميتوكوندريا في الدم البشري تحت تأثير درجات حرارة مختلفة

بحث مقدم إلى كلية العلوم /جامعة بابل

وهو جزء من متطلبات نيل درجة الدبلوم العالي في العلوم/ الأدلة الجنائية

من قبل

إنصاف نصيف جاسم محمد

(بكالوريوس علوم حياة-١٩٩٦- جامعة بابل)

أشراف

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1443ه 2021 م