Republic of Iraq

Ministry of Higher Education and Scientific Research

University of Babylon

College of Science

Department of Biology

Estimation of the Stability of Mitochondrial DNA in Human Blood under Different Concentrations of Acid and Base

A Research

Submitted to the College of Sciences - University of Babylon

in Partial Fulfillment of the Requirements for the

Degree of Higher Diploma in Science/ Forensic Evidence

By

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2021 A.D **1443 A.H**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

الْحَمْدُ لِلَّهِ الَّذِي لَهُ مَا فِي السَّمَاوَاتِ وَمَا فِي الْأَرْضِ وَلَهُ الْحَمْدُ فِي الْآخِرَةِ وَهُوَ الْحَكِيمُ الْخَبِيرُ ﴿١﴾

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سورة سبأ-الآية(١)

Supervisors Certification

I certify that the research entitled ″**Estimation of the Stability of Mitochondrial DNA in Human Blood under Different Concentrations of Acid and Base**″ is Prepared under my Supervision at the Department of Biology College of Science University of Babylon in Partial Fulfillment of the Requirements for the Degree of Higher Diploma in Forensic Evidence.

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The Recommendation of the Department

In the view of the available recommendation, I forward this thesis for debate by the examining committee.

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We, the examining committee, certify that we have read the research entitled **(Estimation of the Stability of Mitochondrial DNA in Human Blood under Different Concentrations of Acid and Base)** and examined the student **(Mansoor Thamer Mashkoor Saud)** in its contents, and that in our opinion it is accepted as a research for the degree of High Diploma in Forensic Evidence with **(Excellent)** estimation.

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Dedication

To science loving

Acknowledgements

I wish to express my deep and sincere gratitude to my supervisor Prof. Dr. Ali Hmood AL-Saadi and Assist.Prof. Dr. Mona Najah AL-Terehi for their valuable guidance, assistance, and encouragement, which were the essential motive to continue this project. I extend my thanks to Ansaf Nsaif Jasim , Ali Ahmed Nayyef and the staff in the DNA laboratory and department of biology, College of Science, University of Babylon.

Summary

Blood is one of the most common evidence at crime scenes, so it is necessary to conduct blood tests, which are carried out by locating blood spots, then performing virtual tests, and finally confirmatory tests and when genetic and serological tests are important operations in the field of forensic medicine.

The study took five months for the period from 23/3/2021 to 22/8/2021, this study was conducted at the University of Babylon - College of Science - Department of Life Sciences - DNA laboratory to estimate the stability of mitochondrial DNA (mtDNA) under exposure to different concentrations of acids and bases in the application of forensic medicine.

Two pieces of (30 cm x 15 cm) of fabric, one for the acid test and the other for the base test, were taken and sampled in an anticoagulant tube, distilled water , with hydrochloride acid in three concentrations (25%, 50% and 100%) and sodium hydroxide base in three concentrations (25%, 50% and 100%). Eight sites were numbered on the fabric. Five drops of blood were added to each site and left to dry for one hour. After that, five drops of distilled water were added to two sites as a control group, five drops of acid from each concentration (25%, 50% and 100%) on the respectively for three sites, five drops of base from each concentration (25%, 50% and 100%) respectively for the other three sites and left for one hour and then extracted DNA.

The result showed that immersion of blood in hydrochloric acid at a concentration of (50% and 100%) would lead to the complete decomposition of DNA during the mentioned time period, while the dilute concentration of acid (25%) did not affect DNA during the time period, and the result of immersion of blood in a base of

Low concentration (25% and 50%) sodium hydroxide did not affect the mtDNA during the period, while the higher concentration (100%) causes complete damage to the mtDNA during the same period. After DNA extraction, PCR technique was applied to all samples to ensure the stability of mtDNA, estimation of purity and concentration of mtDNA.

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List of Abbreviations

Abbreviation	Means
ATP	Adenosine triphosphate
bp	Base pairs
H-strand	Heavy strand
L-strand	Light strand
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial genome
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid

Chapter One
Introduction

1. Introduction

The forensic human identifier of deceased is set upon for different reasons included legal and humanitarian in lager scales of accidents, like plane crashes, to natural disasters and conflict between countries (Hagelberg *et al*., 1991). In some states, especially the complex cases and contexts, the genetic data is become one of the important tools used for identification. in the 1990s, the first using of it for the human remains detection, by recovering DNA from post- mortem samples, making highly informative profiles, and evaluating the DNA data (Jeffreys *et al*., 1992 and Clayton *et al*., 1995). 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 often refer to as the molecular powerhouse 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 (McBride *et al*., 2006). Mitochondria, present in almost all eukaryotic cells, were first discovered as distinct cytoplasmic organelles in 1840, and in 1963 it was determined that they in fact carry their own DNA (Robin, and Wong, 1988; Mounolou and Lacroute, 2005).

Human mitochondrial genome (mtDNA) is a double-stranded and circular DNA that contains approximately 16.569 nucleotide base pairs (bp), including the coding and non-coding regions (Andrews *et al*.,1999; Lee and Wei, 2005).

Each human cells contains several hundreds to one thousand of mitochondria, and each mitochondrion harbors 2 to 10 copies of mtDNA. The mtDNA copy number in a human tissue is dynamic and may vary widely with cell type and the physiological condition, the two strands of mtDNA differ significantly in their base composition. The heavy strand (H-strand) is purine rich, having a greater number of guanine nucleotides, whereas the light strand (L-strand) is pyrimidine rich and thus physically lighter (Schaefer *et al*., 2008).

The mtDNA is very useful characteristics to forensic studies, (Mascher, 2013) especially related to lack of recombination, a large number of copies, and matrilineal inheritance. The mtDNA based on control region sequences or total genomic sequence analysis is used for examining a range in forensic samples such (old bones, teeth, hair, and other biological samples low DNA material). Results of assessment and reporting include close consideration of biological issues as well as other concerns such as databases for the nomenclature and reference population, in addition, the major weaknesses are its lack of informative ness (as compared to short tandem repeats analysis) and the interpretational complications that arise from heteroplasmy. Therefore, in the context of forensic practice, human mitochondrial DNA analysis is currently limited to the kinds of samples that will not routinely work with short tandem repeat analysis (Andersen and Balding, 2018).

1.1. Aim of this Study

This study is aimed to estimate the stability of mtDNA under exposure to different concentration of Acid and Alkaline in forensic application. This performed by following objective:

1- Blood sample collection.

2- Blood sample exposing to different concentration of Acid (HCl) and Alkaline (NaOH) on fabric.

3- DNA extraction from blood samples and control from fabric.

Estimation of purity and concentration of DNA.

4- Application of PCR for detection of mtDNA by using special primer HV2a (control region).

Detection the PCR product by electrophoresis.

Chapter Two
Review of Literatures

2. Review of Literatures

2.1. Mitochondria

Mitochondria, with plastids in plants, are the only cytoplasmic organelles in the eukaryotic cell that carry genetic elements. In the last release of organelle section of the genome database of National Center for Biotechnology Information (NCBI) Mitochondria play a central role in variety of cellular processes including metabolism, ATP production and apoptosis (Tipirisetti *et al*., 2014). Mitochondria are specialized sub cellular organelles unique to the cells of animals, plants and fungi. They serve as power hub for various powering functions of the cells and organisms as a whole. Energy requiring cells have higher number of mitochondria and differ from cell to cell. They are situated close to the part of the cell that shows highest energy requirement (Figure:2-1) (Buckleton *et al*., 2011).

The number of mtDNA 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 mitochondria 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).

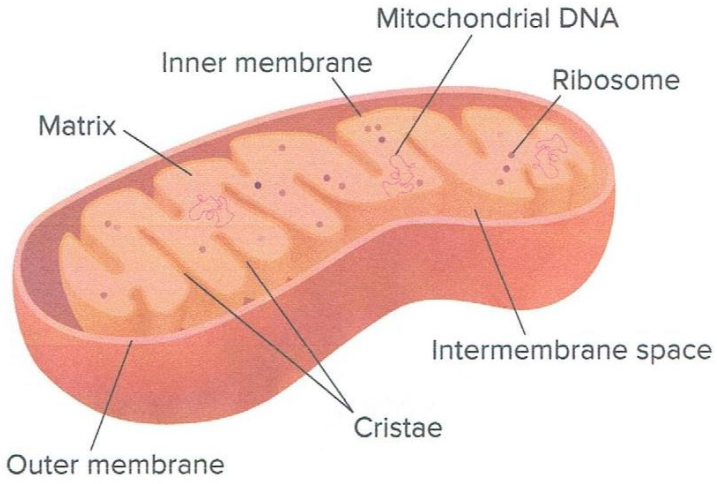

Figure (2-1): Mitochondria (Richard, 2015)

2.2. Structure of Mitochondria

Mitochondria are double-membrane cell organelles that form networks inside the cell. Depending on the energy needs of the cell, mitochondria are either in fusion or fission; this process happens continuously. This mitochondrial reticulum interacts with the cytoskeleton and the endoplasmic reticulum. The mitochondrion is composed of an outer membrane, inner boundary membrane, inter membrane space, cristal membranes, intracristal space and matrix. The cristal membrane forms cristae, which are folds inside the inter boundary membrane. They are tubular in shape and are connected to the inner boundary membrane via crista junctions. ATP synthase particles and ribosomes are situated on the cristal membrane. The folded cristal membranes provide a larger surface area for oxidative phosphorylation (Frey and Mannella 2000; Logan, 2006).

The matrix space between the inner boundary membrane and the cristal membranes holds the multiple copies of mtDNA molecules and stores NAD, NADH, ATP and ADP molecules, The number of crista junctions and the form of the intracristal space changes according to the metabolic state of the mitochondria . Large intracristal spaces have been linked to a reduction in ATP production and a condensation of cristae that creates high levels of ATP energy production (Mannella, 2006).

Contact sites are special regions where the outer and inner membranes are in close proximity. Sometimes a bridge-like structure is formed to keep the membranes apart while maintaining a required close distance. Solutes and small molecules pass between these contact sites from the cytosol into the matrix. It is also suggested that pre-cursor proteins are bound to these sites (Schwaiger et al., 1987). The electron transport chain is present in the inner mitochondrial membrane and is the final common pathway by which electrons derived from different fuels of the body flow to oxygen. Electron transport and ATP synthesis by oxidative phosphorylation proceed continuously in all tissues that contain mitochondria (Chandra and Singh, 2011).

2.3. Mitochondrial DNA Genome

The mtDNA an extra nuclear genome has certain features that make it desirable for forensics; high copy number, lack of recombination, matrilineal inheritance, heteroplasmy, expression variability, and mitotic segregation**.** The mtDNA is a double-stranded, small, circular molecule with 16.569 bp (Gazi *et al*., 2018).

The mtDNA encodes for 37 genes of which 13 are polypeptides that form the subunits of the OXPHOS system, 22 tRNAs, and 2 rRNAs. It is maternally inherited and present within cells in multiple copies depending on the energy demand of the particular tissue. When all mtDNA copies are identical (i.e., all are wild-type or all harbour mutation), the scenario is referred to as homoplasmy (Shiau *et al*., 2017).

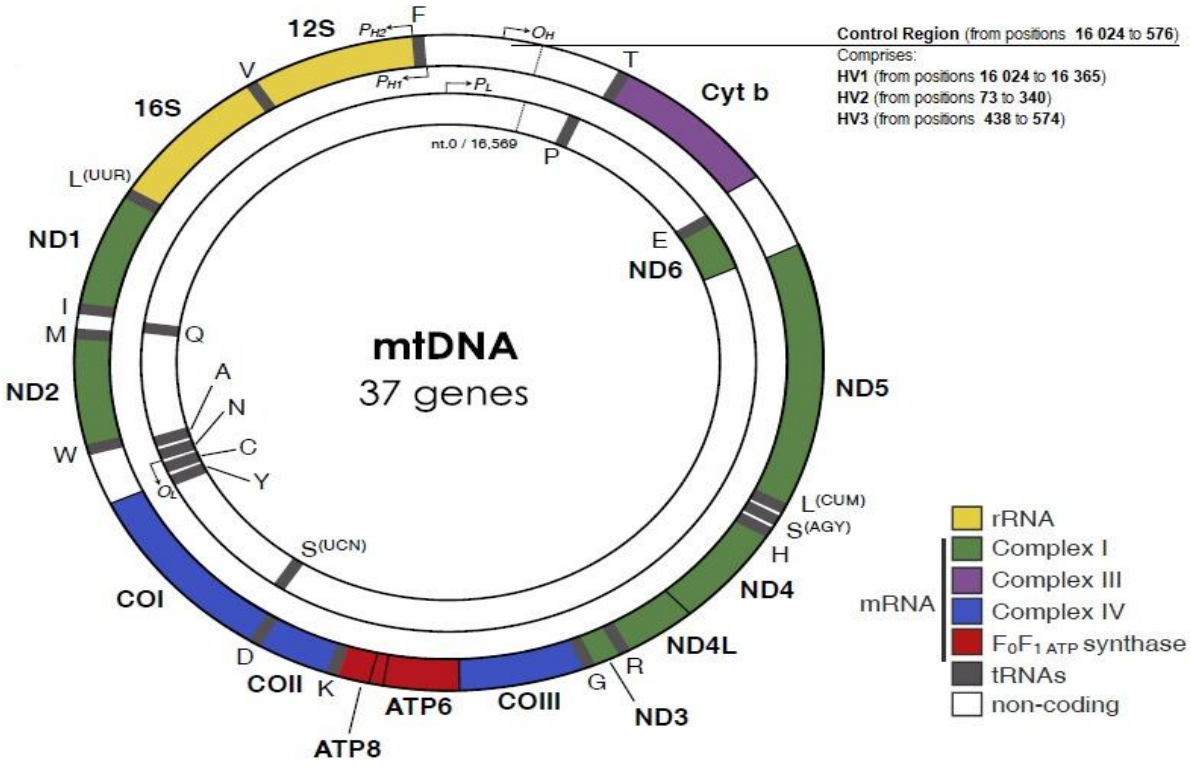

Figure (2.2): The human mitochondrial DNA genome

(Wallace and Burelle 2016)

There are no introns in The mtDNA. The major only non-coding region in the molecule is the displacement loop (D-loop), which is a 1.1 kb region that contains elements of mtDNA transcription and replication, 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 is 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, mutated mtDNA molecules, cell fusion experiments have indeed demonstrated that mitochondrial nucleoids, the respiratory complexes are mobile and diffuse efficiently into mitochondria previously devoid of mtDNA (Legros *et al*., 2001).

The mtDNA is maternally inherited, and paternal mtDNA is eliminated during early embryogenesis, both selection and genetic drift are thought to have effect on mtDNA evolution. The precise contributions of these phenomena are not clear and are debated (Elson *et al*., 2004).

The mtDNA undergoes frequent adaptive evolution, genetic draft is a result of the ″hitchhiking″ process associated with positive selection acting on beneficial mutations (McMenamin and Hadly 2012). The mitochondria and so mtDNA molecules are distributed randomly to oocytes, but there is strong selection against defective mtDNA during embryogenesis, in a study of primary oocytes from a woman who harbored them. 3243A>G mtDNA mutation the frequency distribution of mutation load indicated that random drift is the principal mechanism that determines the level of mutant mtDNA within individual oocytes (Brown *et al*., 2001).

Deleterious mtDNA mutations are selectively eliminated from the female germ line, a process which minimizes their impact on population fitness, a recent study presented direct experimental observations of the fate of random mtDNA mutations 16 in the mammalian germ line demonstrating that a purifying selection process shapes mitochondrial sequence diversity, the concept of a bottleneck mechanism governing segregation of mtDNA in the mammalian maternal germ line is at present considered well established. Most damaging mtDNA protein-coding gene mutations are removed by a process of purifying selection during oocyte development. The molecular mechanisms for rapid purifying selection and bottleneck segregation are, however, currently not fully understood (Stewart *et al*., 2010).

2.4. Control Region of mtDNA

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) HVR-I and HVR-II (Wilson *et al*., 1993). 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 and Gill, 2004 ; Gazi and Mohammad, 2018).

The control region (CR) of the mtDNA also includs displacement loop (D-loop) extends between nucleotide positions (np) 16024-576. The D-loop which extends from around OH. The mutation in the D-loop can modulate mtDNA copy number, subsequent OXPHOS dysfunction and increased reactive oxygen species (ROS). The CR region of the mtDNA is the hotspot for both germ line and somatic mtDNA alterations. In addition, the most frequent DNA alterations in the mitochondrial genome were reported in the hypervariable segment-1 (HSV1, np 16204-16383) and -2 (np 57-373) of the CR, which were mostly located in the D-loop, at various types of tumors such as head and neck, colorectal, lung, bladder, melanoma, uterine cervix and breast cancer (Cai et al., 2011; Ashtiani et al., 2012; Zhang et al., 2015).

Further associations were also established between single nucleotide polymorphisms (SNPs) of the CR and various complex diseases such as; metabolic syndrome, type II diabetes mellitus (DM), neurodegenerative diseases and aging in

literature (Tipirisetti et al., 2014; Zhang et al., 2015).

However, some of the findings are still debatable. Moreover, several studies reported that mtDNA polymorphisms and mitochondrial haplogroups have either predisposing or protective role in various cancer types (Cocos et al., 2017).

These regions are of interest for human identity testing because of their hypervariability consequent of the higher mutation rate. Both the coding and control regions of mtDNA play roles in the generation of diabetes (Gazi and Mohammad, 2018).

2.5. Heteroplasmy of mtDNA

Heteroplasmy is a problem for forensic investigators since a sample from a crime scene can differ from a sample from a suspect by one base pair and this difference may be interpreted as sufficient evidence to eliminate that individual as the suspect (Rebecca *et al*., 2015).

Human mtDNA heteroplasmy is common and heteroplasmy of cells from different tissues within a single individual has also been observed (Coa *et al*., 2006). The mtDNA heteroplasmy is one of the factors affecting the performance of forensic mitochondrial analysis. The detection of heteroplasmy at the whole mitochondrial genome level has been reported (Li et al., 2010), supporting the advantages of using next generation sequencing (NGS) to detect mitochondrial heteroplasmy, including high accuracy and sensitivity, high throughput, low cost, and simple operation (Tang *et al*., 2010) .

A high load of cells with heteroplasmic mitochondrial mutations at low to intermediate frequencies might increase the risk to an individual for mitochondrial disease. Thus, measuring heteroplasmy early on, when the mutant genome is a small portion of the whole, and tracking it over time, may be informative in

monitoring disease progression as well as treatment responsiveness (Cavelier et al., 2000; Poe et al., 2010;Reiner et al., 2010).

2.6. Maternal Inheritance.

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. A disease expressed in both sexes but with no evidence of paternal transmission is strongly suggestive of a mtDNA point mutation (Schon et al., 2012; Koopman *et a*l., 2013).

The standard model of mtDNA inheritance is that it is transmitted strictly through the maternal line (Payne *et a*l., 2013) and that mtDNA lineages are therefore clonal. However, this model has recently been challenged. Low levels of paternal transmission of mtDNA have been observed in crosses between mouse species, but not within species (Hirst, 2011), although further studies showed that this paternal mtDNA was not transmitted to the subsequent generation (Smith *et al*., 2012).

In addition, there is evidence that recombination has paid to the distribution of mtDNA polymorphisms within the human population (Angerer *et al*., 2011).

2.7. Differences between mitochondrial DNA and nuclear DNA

Mitochondrial DNA uses a different genetic code than nuclear DNA for example, the codon for mitochondrial-transcribed amino acid tryptophan is UGA while the universal (nuclear) genetic code for UGA is a stop codon. In the mitochondrial DNA genetic code, AUA codes for methionine instead of

isoleucine and AGA and AGG both code for stops rather than arginine (Scheffler., 1999).

Fewer DNA repair mechanisms exist in mitochondria thereby leading to higher mutation rates compared to nuclear DNA. In addition, lack of proofreading capabilities in the mitochondrial DNA polymerase increases mutations during replication. However, the 10-fold higher mutation rate (relative to nuclear DNA) helps introducing more variability in samples from identical maternal lineages that otherwise would not vary. This increasing variation is a good thing for most applications in human identity testing although mutations can sometimes be a hindrance when trying to definitely establish familial relationships (e.g., when comparing remains to reference samples from distant maternal relatives) (Bai *et al*., 2007).

The circular nature of mitochondrial DNA makes less susceptible to exonucleases that break down DNA molecules needed to survive until forensic DNA testing can be completed. The presence of an increased number of mitochondrial DNA molecules per cell relative to the nuclear DNA chromosomes also enhances the mitochondrial DNA survival rate, as does the fact that they are encapsulated in a two-walled organelle (Bogenhagen , 2012).

2.8. Overview of Mitotyping Protocol

Most laboratories providing mtDNA service have very similar protocols (Andereason *et al*., 2002). All samples including DNA abundant blood reference samples are handled very carefully from the beginning of sample collection to sequencing. The primary reason for the approach is to avoid cross contamination between samples. The analysis is mainly based upon the strategy of PCR amplifications that focuses upon control hyper variable regions HVR-I and HVR-

II. The various steps of the mtDNA analysis include primary visual analysis, sample preparation, DNA extraction, PCR amplification, post-amplification, quantification, purification and automated DNA sequencing and data analysis. The case samples require individualize attention during PCR and sequencing phase either because of damaged DNA or because of sequence specific variation, such as length heteroplasmy or more specifically rare site heteroplasmy. Previous report suggested that handling of single sample is much easier than batch samples analysis (Sreeshyla *et al*., 2014).

The mtDNA has a very high mutation rate, approximately 10-fold higher compared to nuclear DNA, probably as a result of poor repair mechanisms as well as a decreased proofreading efficiency of the mtDNA polymerase. When a mutation arises, a mixture of two types of mtDNA can be observed in an individual, a certain tissue, a single cell, or mitochondrion. This situation is known as heteroplasmy and is generally at such low level that it is normally not detected by standard methods. However, deleterious heteroplasmic missense mutations have been associated to disease. The proportion of normal and pathogenic types has been reported to affect the clinical outcome of the disease. Furthermore, the threshold for expression of disease by the same mtDNA mutation can differ among cell types (Kang, 2005).

2.9. Mitochondrial DNA as a Forensic Tool

Mitochondrial DNA has a number of characteristics which makes it an ideal choice for forensic use. First, it has been estimated that the mtDNA genome evolves at a rate that is up to ten times that of its chromosomal counterpart ,this is an important factor when considering that data consistently show that unrelated individuals are extremely likely to have different mtDNA haplotypes thus making mtDNA useful for purposes of human identity testing (Meyerand Chan 2017).

This higher mutation rate can be accounted for by such factors as DNA repair inefficiencies, oxidative damage, and the greater number of replicate cycles that mtDNA undergoes during cell growth, evidence also suggests that in spite of such an elevated mutation rate. The majority of mtDNA molecules within a given individual will still be represented by a single sequence (homoplasmy). Occasionally, however, a de novo mutation may occur and propagate, resulting in the phenomenon known as heteroplasmy. Heteroplasmy is a state in which two distinct mtDNA haplotypes coexist within a single individual. This is thought to be due to a mtDNA genome copy "bottleneck" during the early stages of oocyte development (Mambo *et al*., 2003).

The bottleneck theory purposes that the number of copies of mtDNA in each early oocyte is reduced to a small number of copies as compared to the mature oocyte. Thus, a small number of molecules are chosen as the founder population for all of the mtDNA molecules that are transmitted to the next generation. This set of molecules could contain a homogenous population of mtDNA, or perhaps a heterogeneous mixture due to mutations. Sometimes, such heteroplasmy may increase the discriminatory power of mtDNA identification by providing an additional inclusionary tool for the mitotype, such as situations where an evidentiary sample and a reference sample both exhibit heteroplasmy at the same nucleotide. Other times, it can lead to confusion when comparing two sequences that are assumed to be concordant, as it may be considered a mixture of mitotypes from more than one individual (Amorim *et al*., 2019).

Second, human mtDNA is thought to be almost completely maternally inherited this can be explained by the nearly 100.000 copies of the mitochondrial genome residing in the oocyte, and the fact that the few (possibly only two or three) mitochondria present in the spermatozoa are concentrated in the mid-piece and tail region, which are lost following fertilization. Additionally, if the sperm mitochondria do make it to the oocyte, they appear to be preferentially degraded. Despite this maternal preference, some research has reported a few incidences known as "paternal leakage" where some paternal inheritance of mtDNA and recombination has occurred. A single case of paternal co-inheritance of mtDNA in humans has been reported so far, in a male individual with a mitochondrial myopathy (Schwartz and Vissing, 2002; Bandelt *et al*., 2005).

In addition, such paternal inheritance of mtDNA has been reported in species ranging from mussels to sheep, although paternal leakage may occur in rare instances, the normal detectable inheritance pattern of mtDNA is maternal. The maternal inheritance pattern, barring multiple mutations, allows for forensic identifications to be made using reference samples from within the entire maternal lineage, including those that may be separated by several generations, when those of close relatives are no longer obtainable (Zhao *et al*., 2004).

Third, mtDNA is present in a high copy number within most cells. It is estimated that a single cell may contain hundreds of mtDNA genomes for every copy of nuclear DNA, depending on the needs of the particular cell type, the actual copy number present per cell can vary greatly among different tissue types. For instance, there are more mitochondria in muscle and brain cells than in skin cells (Parson *et al*., 2014).

The general abundance of mtDNA can prove vital in situations where the amount of sample may be limited or its quality may be degraded, which is often the case in

forensic DNA analyses. Samples that are typical candidates for mtDNA analysis include aged bloodstains, skeletal remains, fingernails, teeth, and hair shafts lacking root tissue. The use of mtDNA typing of skeletal remains is often essential in cases of missing persons or in events such as mass disasters where small bone fragments may be the only remaining source of DNA available. In addition, mtDNA testing of hair shafts is of particular importance because shed hairs are common sources of evidentiary material at crime scenes (Bär *et al*., 2000).

Chapter Three
Materials and Methods

3. Materials and Methods

3. 1. Chemicals and Instrument

The chemicals and instrument that 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**
Fabric	**Cotton**
HCl	**Himedia (India)**
Loading dye	**Thermofisher /USA**
Master mix	**Intron /USA**
NaOH	**Himedia (India)**
Proteinase K	**Biolabs (England)**
TBE buffer	**Bio-Basic /(England)**

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

Instruments	Suppling 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
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**
Plastic Pasteur Pipettes	**China**
Syringe 3,5,10 ml	**TG / Malaysia**

3. 2. Subjects and Methods

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

Preparation of blood sample in EDTA tube, Preparation of HCl (25%, 50% and 100%), Preparation of NaOH, detection the effect acid (HCl), base (NaOH) in different concentration (0.25%, 50% and 100%), their effect on the concentration and purity of mtDNA in human blood (on fibric), then identification of human mtDNA by using specific mitochondrial loci HV2b, estimation of purity and concentration of DNA.

The study lasted for five months from 23/3/2021 to 22/8/2021. This study was carried in the DNA laboratory, College of Science, University of Babylon.

3.3. Experimental Design of this study.

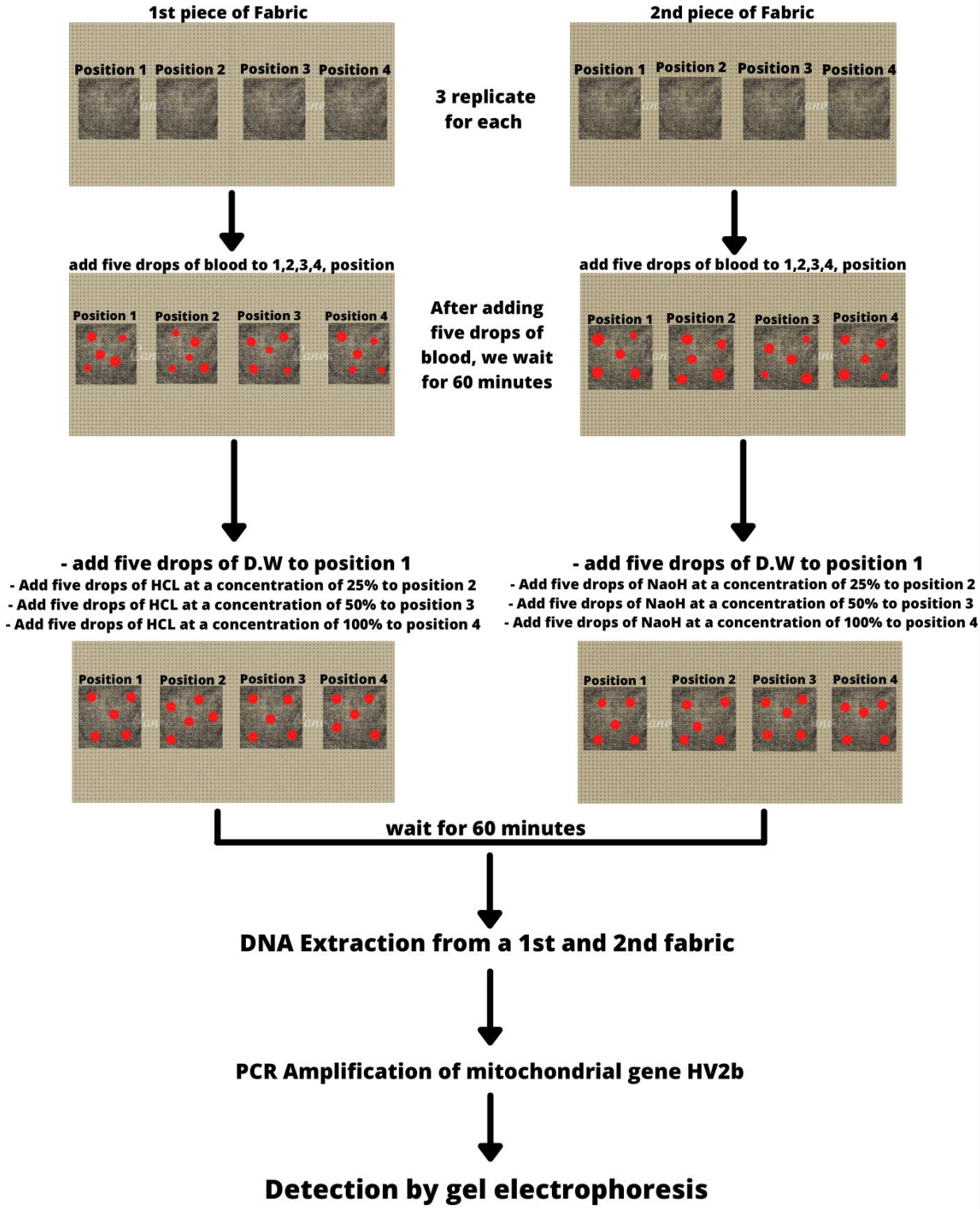

Figure (3-1): Experimental design of current study

3. 4. Methods:

3.4.1. Preparation of blood sample:

Blood sample placed at room temperature, about 5 ml of blood in EDTA tube.

3.4.2. Preparation of Acid (HCL)

The Tris-HCl buffer (pH 7.0) was prepared by adjusting a 1 M Trissolution to pH 7.0 with concentrated HCl.

Preparation HCl 25% :1 ml HCl 100% + 3 ml DW

Preparation HCl 50% :2 ml HCl 100% + 2 ml DW

3.4.3. Preparation of Base (NaOH)

By dissolved 40 grams of NaOH in 1 Littre of D.W. to prepare 1M NaOH solution.

Preparation NaOH 25% :1 ml NaOH 100% + 3 ml DW

Preparation NaOH 50% :2 ml NaOH 100% + 2 ml DW

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:

3.5.1.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
Collection Tube	200 pcs	600 pcs

3.5.1.B: Protocol of DNA extraction (From Fresh Human Blood)

Step 1- Sample preparation

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

Step 2- Cells Lysis

1. A 200 µl FABG Buffer was added to the sample and mixed by vortex.
2. Incubated in 70 °C water bath for 15 minutes to lyse the sample. During incubation, invert the sample every 3 minutes.
3. Elution buffer was preheated (for step 5 DNA Elution) in a 70 °C water bath.

Step 3- Binding

1. A 200 µl 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 2 ml 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 2 ml collection tube. Place the FABG Column in a new 2ml collection tube.

Step 4 – Washing

1. FABG Column with 400 µlW 1 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 2 ml collection tube. Wash FABG Column with 600 µl 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 2 ml 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 1.5 ml microcentrifuge tube.
2. A 100 µl 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 the lens.

2- A 1 µl 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.

3.5.3.1. 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 gel particles were melted.

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

3- 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

Fix the comb in 1 cm away from one edge of a gel tray, the agarose solution was poured into the gel tray.

1. The agarose was allowed to solidify at room temperature for 45 min.
2. 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)
HV2a	F-5-CTC ACG GGA GCT CTC CAT GC-3 R-5- GGG GTT TGG TGG AAA TTTTTTG-3 F (C1) (L 048) R (D2) (H 285)	278bp

2.5.4.1 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, 10 µl from the stock solution plus 90 µl deionized distill water to obtain 100 µl 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**

3.5.4.3. Amplification reaction mixture

HV1aregion genes segments were (3-٨) as follow.

Table (3-٨): Cycling parameters for duplex PCR of HV2a amplification

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

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 1 µl (10 mg /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 no tech 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. Result and Discussion

The present study aims to detection the effect acid (HCl) and base (NaOH) in different concentration (0.25%, 50% and 100%) and their effect on the concentration and purity of mtDNA in human blood (on fibric) and then identification of human mtDNA by using specific mitochondrial loci HV2b.

4.1. Effect of Acid (HCl)

Three different concentrations of HCl used in this study (25%, 50% and 100%) for period (60 min), after addition 5 drops of HCl to 5 drops of blood After adding the acid, it was noticed that the color of the blood is change to turned dark, and the viscosity of the blood changed and lumps appeared when blood sample immersed in acid, then DNA was extraction form (whole blood) show in figure (4-1). The mtDNA were extracted from blood samples (on fabric) using G-spin TM (silica column) total DNA extraction kit, it is silica column-based method, and it is the best duet high purity of the extracted mtDNA.

The results showed that the mtDNA extraction from samples of the Acid immersion human blood. All sample that treated with Acid were lysis and degradation and no DNA band appeared, while control sample not treated with acid was observed, when the blood samples were immersed in the acid or base, there were changes in the color of them, it turned dark and appeared of thrums, but when DNA was extracted from it and the electrophoresis was carried out , we got one clear band. For many previous studies have already assumed that the pH is different from the environment may have an important impact on speed and kind of decomposition (Turner and Salter, 2008).

Moreover, the environmental pH can play a key role in the specific forensic context of bodies immersed in essential acids or fluids, little is known the true effects of these fluids on human tissues, criminals are always looking for available and cheap acid Efficiency will work through the destructive ability of the body to be used to hide the victim's body Previous studies have shown that hydrochloric acid and sulfuric commercially easier than cost nitric acid and hydrochloric acid is cheaper than sulfuric and nitric acid, thus we conclude that hydrochloric acid 37% will most likely be used in such offenses. However, it is always recommended to conduct the usual biochemical tests to verify the acid used (Mazza *et al*., 2005).

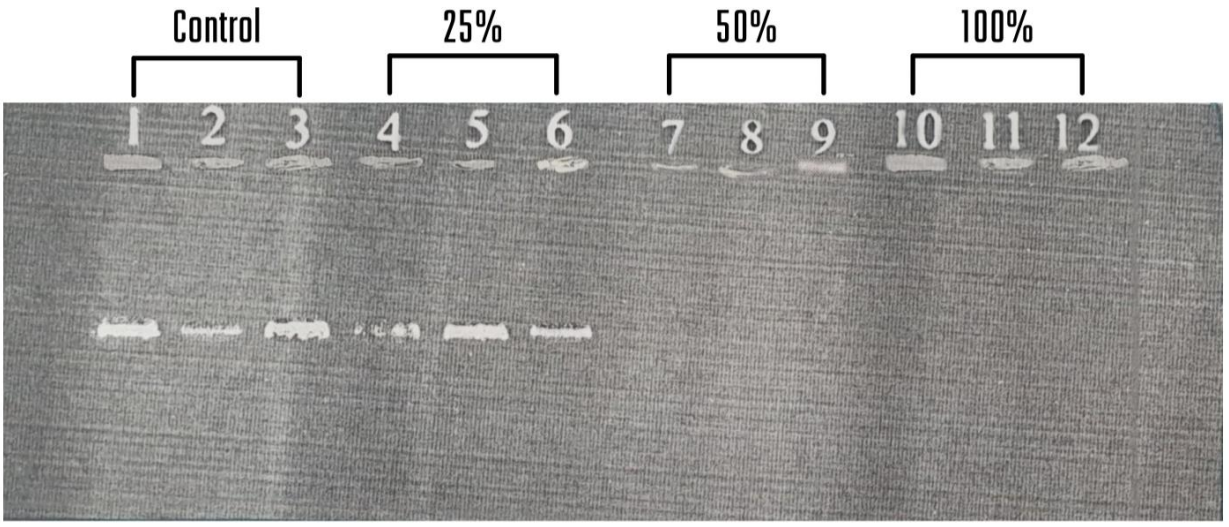

Figure (4-1): Gel electrophoresis of extracted genomic DNA from human blood, lane: 1-3 DNA from control, lane: 4-6 DNA extracted from blood treated with HCl: 25% , Lane: 7-9 DNA extracted from blood treated with HCl: 50%, lane: 10-12 DNA extracted from blood treated with HCl; 100%, 1% agarose, 20 mA, 70 v. for 60 min.

Table (4-1): Effect of HCl on the concentration and purity of DNA

Percentage %	Concentration (µg/ml)	Purity: A 260/280
Control	**12.63 ± 0.53**	**1.94 ± 0.20**
25 %	**5.71 ± 0.32**	**1.81 ± 0 15**
50 %	**---**	**---**
100 %	**---**	**---**
P	**0.031 ***	**0.051**

* **Significant ($P \leq 0.05$)**

The effect of 25%, 50% and 100% of HCL on the concentration and purity of DNA as show in table (4-1) showed that concentration and purity of DNA decrease.

The result showed that all DNA in acid immersed blood was destroyed due to effect of HCl on the blood and this agree with study conducted by (Mazza *et al*., 2005) that explained that acid immersion of victim's body is one of the methods employed to subvert identification of the victim, and hence of the perpetrator, The practice of destroying the human body by immersing in acid or some other caustic substance in order to avoid any personal identification is drawing a great deal of importance in forensic sciences (Mazza *et al*., 2005).

The effects of HCl on human red blood cells. The effect of HCl on the of red cell constituents resulted in decreased glutathione stability, and oxidation of hemoglobin and membrane protein components 1, 2, and 3, forming large molecular weight complexes. Membrane lipids were also per oxidized, because HCl has the potential of inhibiting ATPase, including both Na-K-dependent

ATPase and ouabain insensitive ATPase, at concentrations not inhibitory to other enzymes, the net sodium content increased, and potassium content decreased after incubation of red cells with HCl at high concentrations, there was greater oxidizing effect on glucose-6-phosphate dehydrogenase (G-6-PD) deficient than on normal erythrocytes (Sabolová *et al*., 2018).

DNA extraction, from soft tissues from bodies immersed in acid, has proven to be difficult, if no time possible and the DNA extraction procedure is an important step for the downstream molecular applications such as PCR or next generation sequencing. Low-yielded DNA or contaminated DNA with inhibitors may lead to unsuccessful results of these applications, so selecting the right DNA extraction method enhance the successfulness of the results of these applications (von Wurmb-Schwark *et al*., 2008).

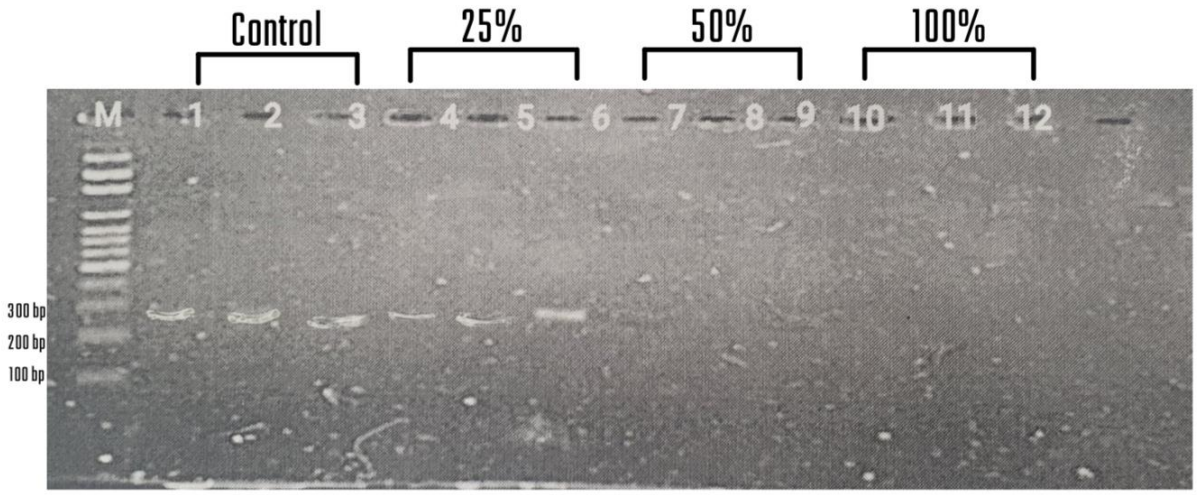

Figure (4-2): Electrophoreses pattern of PCR product of HV2a gene after treated with acid, the amplification product was 278 pb, lane 1-3 for controle, lane 4-6 for 25% HCl, lane 7-9 for 50% HCl, lane 10-12 for 100% HCl, 1% agarose, 20 mA, 70 v. for 60 min.

4.2. Identification of human mtDNA by specific loci HV2a

After blood treated with hydrochloride acid (HCl), no DNA bands were observed in all samples in electrophoresis this is because low concentrations of DNA that cannot be appeared in electrophoresis, so, Polymerase Chain Reaction (PCR) applied to detection of mitochondrial DNA by using specific gene HV2a, mtDNA loci were amplified for all samples, the size product of amplification were 278 bp as showed in Figure (4-2).

The result of PCR showed there are no PCR product in sample treated with different concentration of Acid HCl because there is no template in PCR mixture, the template is complete degradation because the effect of strong Acid HCl on the DNA, and this study agree with study conducted by (McCord *et al*., 2011) that showed the effect of inhibition, degradation

and low copy number in the recovery of information in Forensic DNA casework (McCord *et al*., 2011).

Many studies shared same above idea concerned silica-based extraction like (Nagy *et al*., 2016 and Silva *et al*., 2019) whose recommended the method as good method for PCR and sequencing due to their little or trace impurities that may be interfere with PCR and also can give very clear and readable sequence. Some forensic studies still improvement new methods have short time-consuming low cost with high recovery of DNA (Cheng *et al*., 2019*).*

Pervious study used these types of tissue for amplification system but with more modification for micro dissected tumor cells (Akalu and Reichardt, 1999) later amplification technologies are development to find other high efficiency methods. In present study because of COVID-19 we had not capabilities to used RT-PCR or PCR sequencing. New strategies are developed to overcome these problems that deal with low concentration and short segment of DNA for forensic application like next generation sequencing **(**Adams et al., 2009; Alvarez- Cubero *et al*., 2017).

4.3. Effect of Base (NaOH)

Three different concentrations of NaOH were used in this study (25%, 50% and 100%) for (60 min), after addition 5 drops from NaOH to blood spot, it was noticed that the color changed and lumps appeared when blood sample immersed in base, then mtDNA was extraction form fabric. The mtDNA were extracted from blood samples using G-spin TM (silica column) total DNA extraction kit, it is silica column-based method, and it is the best duet high purity of the extracted mtDNA.

The result of mtDNA extraction shows that the concentration of NaOH (25% and 50%) does not effect on the DNA and all treated sample appeared in gel

electrophoreses, while 100% concentration will strong effect on DNA sample not appeared in electrophoreses because DNA will complete degradation.

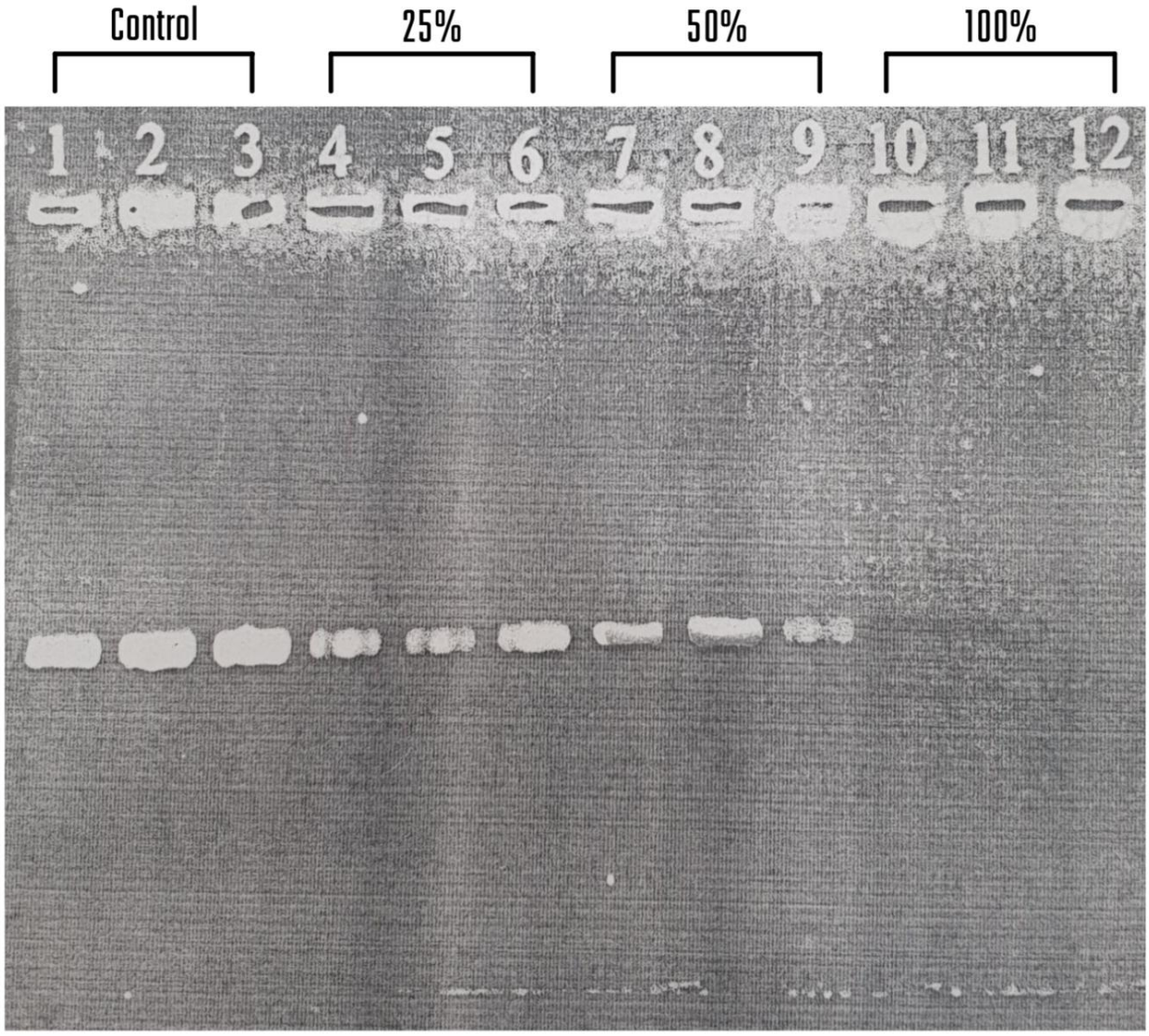

Figure (4-4): Gel electrophoresis of extracted genomic DNA from human blood, lane: 1-3 DNA from control, lane: 4-6 DNA extracted from blood treated with NaoH: 25%, Lane: 7-9 DNA extracted from blood treated with NaoH: 50%, lane: 10-12 DNA extracted from blood treated with NaoH; 100%, 1% agarose, 20 mA, 70 v. for 60 min.

Table (4-2): Effect of NaOH on the concentration and purity of DNA

Percentage %	Concentration (µg/ml)	Purity: A 260/280
Control	13.80 ± 0.61	1.97 ± 0043
25 %	10.62 ± 0. 55	1.89 ± 033
50 %	4.71 ± 0.22	1.80 ± 028
100 %	---	---
P	0.049 *	0.055

* **Significant ($P \leq 0.05$)**

The mtDNA is one of the important tools to human identifier which used to discriminate individuals among them, in present study we used it to estimate the stability of Mitochondrial DNA under exposure to different concentration of acid (HCl) and base (NaOH) in forensic application, mitochondrial genes used frequently in forensic studies due to the fact that, high copy number of mitochondria, small size, possessing small numbers of gene, about 37 introns less, un-parental inheritance, low rate of nucleotides substitutions, lack of recombination and very low errors of fast replicating mutations , so in this study we use one mitochondrial gene (HV2a), to esteemed the effect of base (NaOH) on the DNA ,the result of amplification showed one clear band, the size was 278 bp and appeared only eight sample and the remain sample was degradation , Figure (4-4).

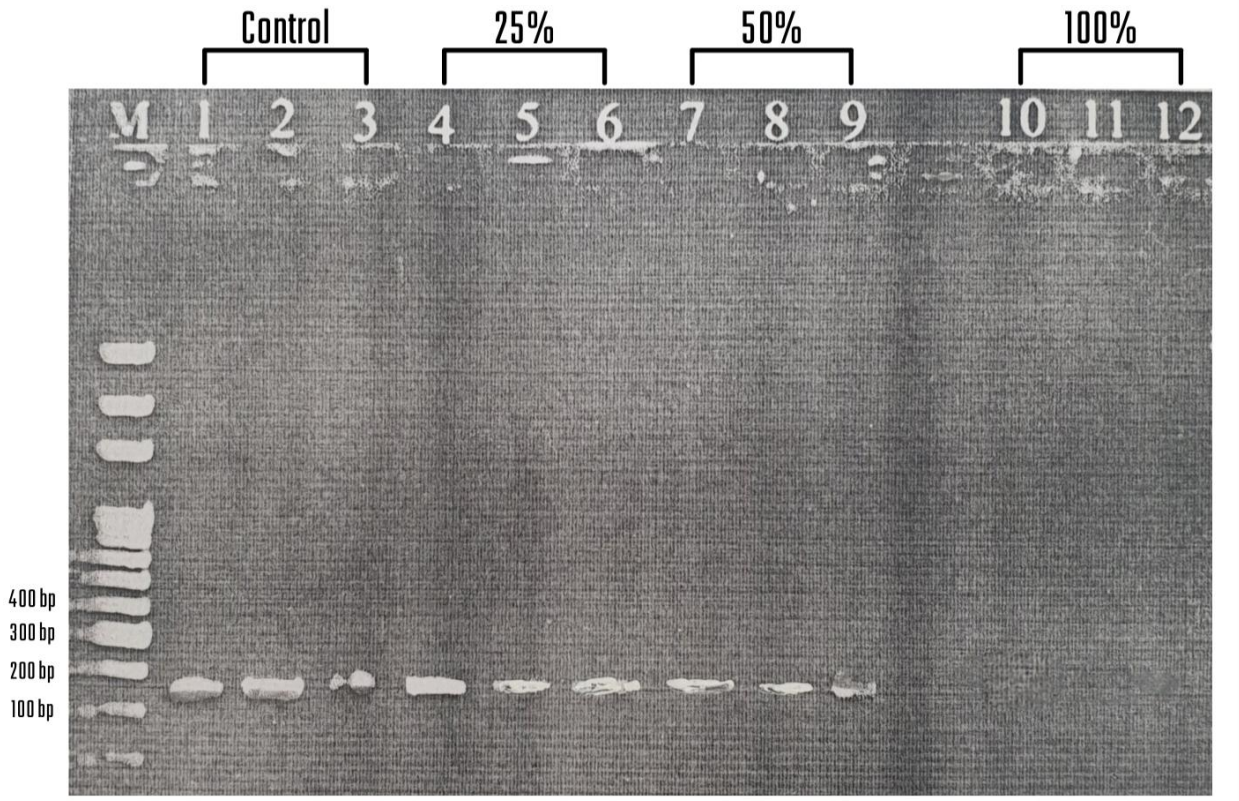

Figure (4-4): Electrophoreses pattern of PCR product of HV2a gene, sample from human blood, the amplification product was 278 pb lane 1-3 sample treated with NaOH (50%), lane 4-6 sample treated with NaOH (25%), lane 7-9 DNA from control, 1% agarose, 20 mA, 70 v. for 40 min.

The result of current study showed that the NaOH in high concentration and extreme time of immersion, completely effect DNA and denegation it, that will not appear in gel electrophoreses and also not appear in PCR, our result agrees with study conducted by (Wang *et al*.,2014), that showed that the chemical methods were applied to denature the DNA and their denaturation efficacies are presented, the highest concentration thoroughly denatured the DNA fragment in the early stage.

The denaturation by NaOH rapidly increased, the sodium hydroxide (NaOH) is a commonly used reagent to denature the DNA by increasing the pH at an alkaline pH, OH- groups are predominant. They remove the hydrogen- bonds-contributing protons from guanine and thymine, thus breaking the hydrogen bonds between the two oligonucleotides (Poltronieri *et al*., 2008).

Conclusions and Recommendations

Conclusions

1- mtDNA is very useful characteristics in forensic studies, especially related to lack of recombination, a large number of copies, and matrilineal inheritance.
2- Immeration of blood in HCl in concentration (25%) does not effect on mtDNA while concentration (50% and 100%) cause degradation of mtDNA.
3- Immeration of blood in NaOH in concentration (25% and 50%) does not affect on mtDNA while concentration (100%) cause degradation of mtDNA.

Recommendations

It is recommended that:

1- Used another mtDNA loci for human remains identification.
2- Using more investigation techniques about positive results, like DNA sequencing.

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الخلاصة

الدم هو أحد الأدلة الأكثر شيوعا في مسرح الجريمة. لذلك لابد من إجراء فحوصات الدم، والتي يتم إجراؤها عن طريق تحديد مكان بقع الدم، ثم إجراء الفحوصات الافتراضية، وأخيراً الفحوصات التأكيدية. وعدد الفحوصات الجينية والسيرولوجية من العمليات المهمة في مجال الطب العدلي.

استغرقت الدراسة خمسة أشهر للفترة من ٢٠٢١/٣/٢٣ - ٢٠٢١/٨/٢٢م وأجريت هذه الدراسة في جامعة بابل - كلية العلوم - قسم علوم الحياة - مختبر الحمض النووي لتقدير ثباتة الحمض النووي للمتقدرات تحت التعرض لتركيزات مختلفة من الأحماض والقلويات في تطبيق الطب العدلي .

تم أخذ قطعتين من القماش القطني ٣٠سم X 15سم واحدة لاختبار الحامض والأخرى للقاعدة وعينة دم في أنبوبة مضادة للتخثر وماء مقطر وحامض الهيدروكلوريك بثلاث تراكيز (٢٥٪، ٥٠٪، ١٠٠٪) وقاعدة هايدروكسيد الصوديوم بثلاث تراكيز (٢٥٪، ٥٠٪، ١٠٠٪)، تم ترقيم ثمانية مواقع على القماش ثم إضافة خمس قطرات من الدم على كل موقع وتركت لتجف لمدة ساعة واحدة بعد ذلك تم إضافة خمس قطرات ماء مقطر لموقعين كمجموعة سيطرة وخمس قطرات من الحامض من كل تركيز (٢٥٪، ٥٠٪، ١٠٠٪) على التوالي لثلاث مواقع وخمس قطرات من القاعدة من كل تركيز (٢٥٪، ٥٠٪، ١٠٠٪) على التوالي للمواقع الثلاثة الاخرى وتركت لمدة ساعة واحدة كررت هذه العملية ثلاث مرات. بعدها تم استخلاص الحمض النووي باستخدام (favorgen) ثم إجراء تفاعل البلمرة المتسلسل (PCR) لجين MtDNA (HV2a) ثم الكشف عن ناتج التضخيم بواسطة الترحيل الكهربائي. ثم قياس تركيز ونقاوة الحمض النووي للمتقدرات لكل العينات.

أظهرت النتيجة أن غمر الدم بالتراكيز (50٪، 100٪) من الحمض (HCl) سيؤدي إلى التحلل الكامل للحمض النووي في الفترة المذكورة بينما التركيز المخفف للحامض (25٪) لا يؤثر على الحمض النووي في كل الفترة وأظهرت نتيجة غمر الدم في القاعدة (NaOH) أن التركيز المنخفض للقاعدة (٢٥٪، ٥٠٪) لا يؤثر على الحمض النووي للمتقدرات في كل الفترة بينما التركيز الأعلى (١٠٠٪) يسبب تلف كامل للحمض النووي للمتقدرات بنفس الفترة.

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

تقدير ثباتية دنا الميتوكوندريا في الدم البشري تحت تأثير تراكيز مختلفة من الحامض والقاعدة

بحث مقدم الى كلية العلوم - جامعة بابل
وهو جزء من متطلبات نيل درجة الدبلوم العالي في العلوم /الأدلة الجنائية
من قبل

منصور ثامر مشكور سعود
علوم حياة - جامعة الكوفة ٢٠٠٥ - م

إشراف
الأستاذ الدكتور علي حمود محيسن عبد الله

٢٠٢١م ١٤٤٣ه