STUDY OF PRESERVATION SOLUTIONS BY COMPARING DIMETHYL SULFOXIDE – SODIUM CHLORIDE (DMSO-NaCl) AND ETHANOL AS STORAGE FOR SOFT TISSUE SAMPLES

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BY

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DEDICATION

This book is dedicated to my parents, my late father Mr. Ali Hersi, and my beloved mama Mrs. Warsan Jibril whose love, care and guidance has made my studies possible and enjoyable.

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All praise is due to almighty Allah, the creator, who makes it possible to complete my thesis easily and successfully. Many thanks due to Allah for all things, specifically for giving me the strength, health, and intellectual capability to accomplish this task on time. I would like to acknowledge with deep reverence, sincerity and feel the utmost pleasure in expressing my heartiest gratitude to the many people who helped me to make this thesis possible.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

ANOVA	: Analysis of Variance
A260	: Absorbance at wavelength 260nm
A280	: Absorbance at wavelength 280nm
A260/230	: Absorbance ratio of a wavelength at 260nm and 230nm
A260/280	: Absorbance ratio of a wavelength at 260nm and 280nm
U	: Activity units
A	: Adenine
Bp	: Base pairs
C	: Cytosine
°C	: Degree Celsius
ddH_2O	: Deionized Distilled Water
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic acid
dNTPS	: Deoxyribonucleotide triphosphates
Na_2EDTA	: Disodium ethylenediaminetetraacetic acid
dH_2O	: Distilled Water
EDTA	: Ethylenediaminetetraacetic acid
EtOH	: Ethanol
g	: Grams
G	: Guanine
Kbp	: Kilobase pair

L	: Litre
Mg	: Magnesium
$MgCl_2$	: Magnesium Chloride
µL	: Microliter
mg	: Milligram
mL	: Milliliter
MtDNA	: Mitochondrial Deoxyribonucleic acid
M	: Molarity
ng/µL	: Nanogram per microliter
NaCI	: Sodium Chloride
nm	: Nanometer
%	: Percentage
pmol	: Picomole
PCR	: Polymerase Chain Reaction
RT	: Room Temperature
RNA	: Ribonucleic acid
Rpm	: Revolution per minute
T	: Thymine
TBE	: Tris-borate EDTA
UV	: Ultraviolet
V	: Voltage
H_2O	: Water

STUDY OF PRESERVATION SOLUTIONS BY COMPARING DIMETHYL SULFOXIDE – SODIUM CHLORIDE (DMSO-NaCl) AND ETHANOL AS STORAGE FOR SOFT TISSUE SAMPLES

ABSTRACT

DNA profiling for victim identification is one of the most important aspects to consider in mass disaster identification. Proper collection, storage and preservation procedures are crucial to reduce the effect of the degradation. Therefore, the aim of this study is to assess the potential of 20%DMSO-NaCI in preserving the soft tissue sample by comparing with the ethanol solution. A total of 112 fresh beef meat were stored in 20% DMSO-NaCI, absolute ethanol, 35% ethanol and sterile deionized distilled water and incubated at ambient temperature (25-28°C) and 37°C until 42 days. DNA extraction of the soft muscle tissues was carried out based on the Phenol-chloroform DNA extraction method and DNA quantification was performed using a Nanodrop™ spectrophotometer. The statistical analysis of Variance (ANOVA) shows that there is no statistical significance between the average DNA concentration and the time of incubations (P=0.539756). The success of PCR amplification was seen in all extracted DNA for both incubation conditions and preservative solutions for up to 42 days by amplifying 120 bp of cytochrome b gene. In this study, the effect of DNA degradation cannot be determined because the use of short amplicon length increased the potential for successful amplification of the target gene. In summary, the findings show that 20% DMSO-NaCI can be proposed as an alternative preservative solution for the storage of tissue samples based on the capability to retain the DNA integrity as good as ethanol.

KAJIAN PERBANDINGAN CECAIR PENGAWET DIMETIL SULFOKSIDA – SODIUM KLORIDA (DMSO-NaCI) DAN ETANOL SEBAGAI PENYIMPANAN BAGI SAMPEL TISU LEMBUT.

ABSTRAK

Pemprofilan DNA untuk pengenalan mangsa merupakan salah satu aspek yang paling penting untuk dipertimbangkan dalam pengenalpastian bencana besar. Kaedah pengumpulan dan penyimpanan yang sesuai adalah penting untuk meminimumkan kesan kemerosotan DNA. Oleh itu, matlamat kajian ini adalah untuk menilai keupayaan 20%DMSO-NaCI dalam memelihara sampel tisu lembut dengan membandingkan dengan larutan etanol. Sebanyak 112 sampel daging segar disimpan dalam 20%DMSO-NaCI, etanol pekat, 35% etanol dan air suling ternyahion steril dan diinkubasi pada suhu bilik (25-28°C) dan 37°C selama 42 hari. Pengekstrakan DNA tisu lembut dijalankan berdasarkan kaedah pengekstrakan DNA Fenol-klorofom dan kuantifikasi DNA dengan menggunakan spektrofotometer Nanodrop^{TM}. Analisis statistik Varian (ANOVA) menunjukkan bahawa tiada signifikasi statistik antara purata kepekatan DNA dan masa pengeraman (P=0.539756). Kejayaan amplifikasi PCR dilihat untuk semua DNA yang diekstrak untuk kedua-dua keadaan inkubasi dan larutan-larutan pengawet sehingga hari ke 42 dengan pengamplifikasian 120 bp gen sitokrom b. Dalam kajian ini, kesan kemerosotan DNA tidak dapat ditentukan kerana penggunaan amplikon yang barsaiz pendek meningkatkan potensi untuk kejayaan ampilifikasi gen sasaran. Secara ringkasnya, hasil penemuan menunjukkan bahawa 20% DMSO-NaCI boleh dicadangkan sebagai larutan pengawet alternatif untuk penyimpanan sampel tisu berdasarkan keupayaan untuk mengekalkan integriti DNA sebaik etanol.

CHAPTER 1

INTRODUCTION

1.1 Research Study Background

Forensic Science is the practice of applying the methods of the natural and physical sciences to issues of criminal and civil law, in accordance with the rules of admissible evidence and criminal procedure in each country. Forensic science can not only be involved in the investigation and prosecution of criminal cases (e.g., rape & murder) but also involves matters in which a crime has not been committed but in which someone has been charged with a civil wrong. Recognizing, identifying, and evaluating physical evidence is the focus of forensic sciences, which draws from a wide range of scientific fields such as physics, chemistry, and biology. It has become an important aspect of the court system since it serves a wide range of scientific disciplines to aid the criminal investigation by providing information related to criminal and legal evidence (Baptista and Goodwin, 2017; Jay, 2020).

During mass fatality events or Disaster Victim Identification (DVI), a large number of bodies may quickly decompose in severe environmental situations. Time also accelerates the breakdown of the DNA present in those tissues, which may prevent successful DNA profiling (Sorensen *et al.*, 2016). Effective DNA analysis necessitates the proper collection, storage and preservation of biological evidence from the scene of a crime (Baptista and Goodwin, 2018). However, the use of a simple field preservative solution will be a valuable tool to immediately stop DNA damage and degradation and storing a large number of tissues at ambient temperatures

prior DNA typing, as well as preserving the morphology of the tissues, some tissue preservers can also be used to keep DNA safe for typing in the future (Michaud and Foran, 2011; McNevin, 2016).

According to International Criminal Police Organization (INTERPOL) (2018), forensic DNA analysis is one of the three fundamental methods of identification together with fingerprint and dental analysis and can therefore plays a critical part in identifying victims whether it involves a single individual or a large-scale disaster (Allen-Hall and McNevin, 2012; Bruce *et al*., 2021). However, according to one recent study conducted by Watherston *et al*., (2021) the advancement in technology has increased options for the collection, sampling, preservation, and DNA typing for DVI samples.

In the process of tissue preservation, there are multiple factors considered when evaluating tissue preservation protocols, cold storage by retarding the enzymatic or microbial degradation of DNA, the transportability of storage equipment, the ease and the cost of obtaining them and finally, the length of the time tissues may be stored and the ease of downstream DNA extraction and analysis should also be considered (Michaud and Foran, 2011; Watherston *et al*., 2021). There are three basic types of tissue storage and DNA preservation which are cold storage, desiccation, and storage in a preservative, each with its own set of benefits and disadvantages.

After cellular death, the cell's metabolic process will be disrupted and causes harmful endogenous enzymes to release and DNA become prone to damage and start to degrade (autolysis) by both nuclease effects and exogeneous factors leading to base changes, strand breaks and crosslinks. Consequently, the successful sample collection and optimal preservation of the tissue samples are irreplaceable (Moustafa, 2021). Optimizing the conditions of samples collection as

duration, temperature and preservation method is crucial as they can cause an impact on DNA quality and quantity (Michaud and Foran, 2011; Moustafa, 2021).

Tissue preservatives typically consist of sodium Chloride (NaCl) and other salts, detergents, chelating agents, and alcohols. However, NaCl is a common preservative that has been used for past many years. Sodium chloride in it is solid form, it desiccates the tissue sample by removing moisture, which inhibits endogenous nucleases and growth of microorganisms (Watherston *et al.*, 2021). In liquid form, salts also denature proteins. Detergents, such as Tween 20, lyse the cell membrane and aid in the release of DNA materials. Ethylenediaminetetraacetic acid (EDTA), which is chelating agents, prevents the nuclease activity by binding to metal ions that are required for normal function of nucleases. Soft tissue samples stored in alcohol prevent DNA from becoming cross-linked, which is useful for later nucleic acid extractions. Ethanol (EtOH) is a better preservative for soft tissue samples and it is the most commonly used medium for storage and tissue preservation, it is flammable and toxic but it has the capacity to remove water content of the tissue sample causing denaturation of enzymes and proteins in addition to it is an antimicrobial agent and will protect against bacterial decomposition (Moustafa, 2021).

Less common tissue storage solutions have the potential to be as easy to utilize as alcohols, while being more effective at DNA preservation. One of these, salt-saturated dimethyl sulfoxide (DMSO) is not only considered as a preservative agent but also, is considered as a well enhancing vehicle for the absorption of other preservatives across the biological membrane and into the cell owing its high tissue permeability and dehydrating effect through water displacement (Sorensen *et al.*, 2016; Baptista and Goodwin, 2017; Moustafa, 2021).

1.2 Statement of Problem

DNA Profiling using Short Tandem Repeats (STRs) is considered a gold standard for victims' identification of DVI. Highly degraded samples often result in partial STR profiles because of large loci (>250 loci) usually fail to amplify due to fragmentation of the DNA structure (Sharpe *et al*., 2020). Proper DNA preservation from the point of collection to the laboratory is very crucial to genetic studies because many genomic procedures and protocols require high quantity DNA samples (Moustafa, 2021). However, the growing importance of DNA based research has generated an growing demand for techniques that can preserve high quality DNA in biological specimens (Sharpe *et al.,* 2020). Consequently, it is suggested that tissue samples stored at 4°C or -20°C prevents more DNA damage and degradation.

Ethanol solution is a well-known preservative solution used to store a wide range of sample types. However, the use of DMSO-NaCI as an alternative preservative solution is limited to certain biological types and species. As reported by Moustafa., (2021) the performance of different preservation methods for tissue samples is yet undiscovered although there are many alternative methods developed for tissues samples preservation for forensic DNA analysis. Hence, in this field, there is still a certain lack of studies concerned with applicable methods for specific tissue sample storage. Therefore, the aim of this research is to study the effectiveness of DMSO-NaCI as a storage solution for soft tissue samples compared to ethanol to find a suitable preservative solution that later can be suggested to be implemented at remote field sites.

1.3 Study Objectives

1.3.1 General Objective

To study the preservation properties of dimethyl sulfoxide – sodium chloride (DMSO-NaCl) and ethanol as a storage solution for soft tissue samples.

1.3.2 Specific Objectives

1. To assess the DNA yield of soft tissue samples incubated with DMSO-NaCI and ethanol at two different temperatures (room temperature 25-28°C and 37°C) at interval time.
2. To profile DNA degradation of soft tissues samples incubated with DMSO-NaCl and ethanol using polymerase chain reaction (PCR).
3. To compare the effectiveness of DMSO-NaCl and ethanol a preservative for soft tissue sample storage.

1.4 Significance of Study

DNA typing is considered a gold standard for victim's identification from a mass casualty accident. However, successful DNA analysis id dependent on the proper samples collection, storage, and preservation of the biological evidence. In addition to that, insufficient preservation procedures can lead to DNA damage and degradation, which may reduce the effectiveness of DNA typing process (Moustafa, 2021). Hence, storage of samples became critical issue to avoid further DNA degradation which may affect DNA analysis later.

According to the DNA commission of the International Society for Forensic Genetics (ISFG) report, storing of soft tissue muscle samples in preservative solution at room temperature can be an alternative to cold storage. However, INTERPOL (2018), DVI guide also recommends preserving soft tissues in ethanol. Similarly, several previous studies have shown that absolute ethanol is effective at preserving DNA from fresh and partially degraded soft tissues (Baptista and Goodwin, 2017; Moustafa, 2021; Watherston *et al*., 2021). However, there is no such study available reported by comparing salt-saturated dimethyl sulfoxide (DMSO-NaCI) and ethanol as storage for soft tissue samples. Therefore, this study is aimed to evaluate the effectiveness of DMSO-NaCI as a storage preservative solution for soft tissue samples by comparing with ethanol.

1.5 Overview of Study

The methodology of the study is described in the flow chart below.

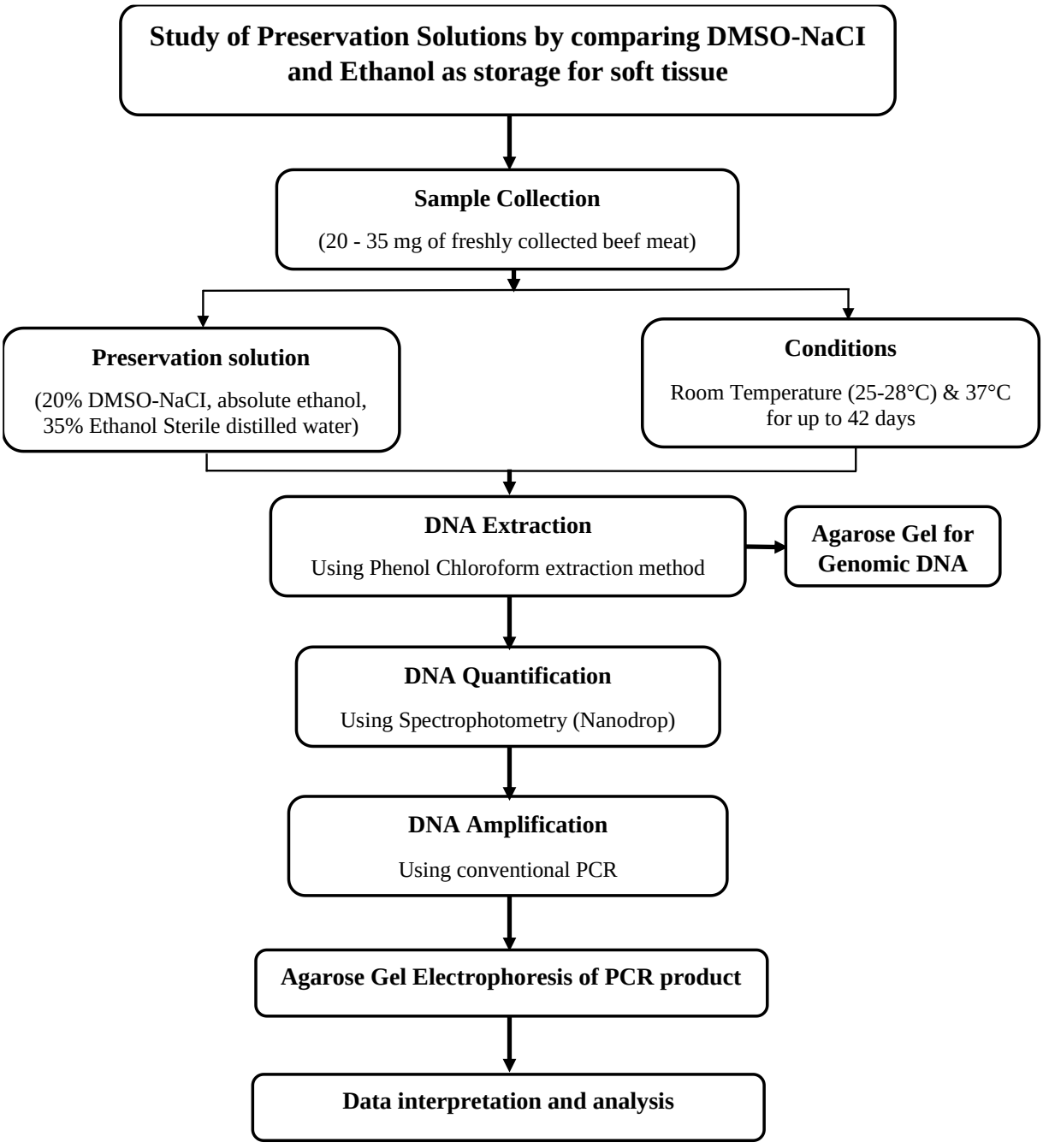

Figure 1.1:Showing the study flowchart

CHAPTER 2

LITERATURE REVIEW

2.1 Deoxyribonucleic-acid (DNA) structure and Genome

Deoxyribonucleic- acid (DNA) was first described by Watson and Crick in 1953, as a double-stranded molecule that adopts a helical arrangement. Each individual's genome contains a large amount of DNA that is a potential target for DNA profiling. DNA also known as the "blueprint of life" is a molecule which is inherited in humans because it contains all the information that an organism needs in function, development of life and reproduction (Malik *et al*., 2021). It is located in the nucleus of most organism cells which is protected by the nuclear envelope.

The model of the DNA double-helix structure was first proposed by Watson and Crick, which is a two stranded molecule that appears twisted and gave a unique structure (Haddrill, 2021). DNA molecule is a polymer of nucleotides and it also stated that DNA strands own a long sequence of nucleotides which made up of a phosphate molecule, a pentose sugar which is called deoxyribose and nitrogen containing region (Figure 2.1). According to Malik *et al*., (2021), there are four nitrogenous bases in DNA; two purines (adenine and guanine) and two pyrimidines (cytosine and thymine). Each base is attached as complimentary base: adenine base always pairs with thymine base whereas cytosine base always pairs with guanine base.

In recent years, a rapid improvement and development of many methods in molecular biology, especially in the study of DNA. The development of the Polymerase Chain Reaction (PCR) has enabled researchers to replicate and amplify a very small amount of DNA into millions of copies for subsequent DNA analysis (Iyavoo, 2014).

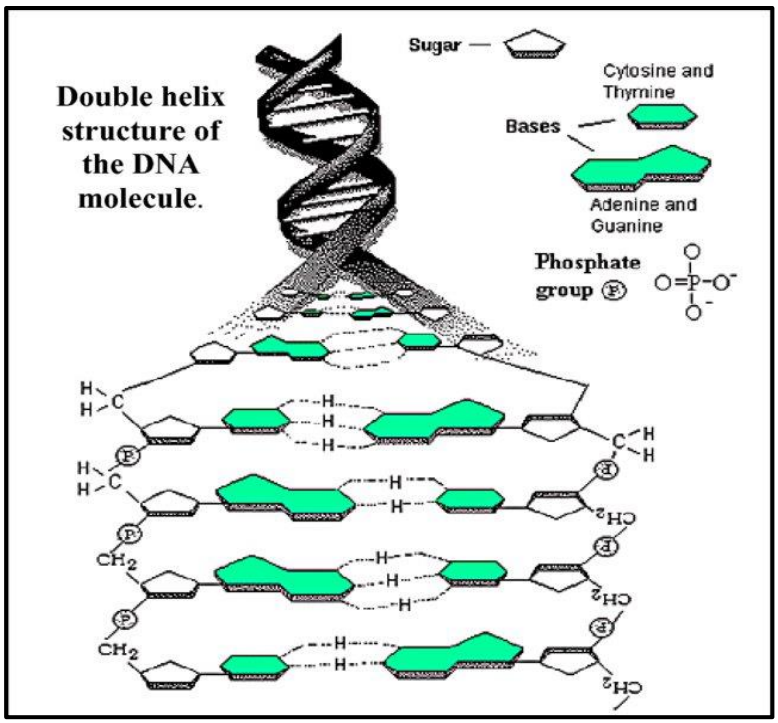

Figure 2.1: The structure of deoxyribose nucleic acid (DNA), showing the double helix structure of the double stranded molecule. The two strands are linked together by hydrogen bonding between the purine and pyrimidine base units. These are in turn linked together by the phosphodiester backbone of the DNA molecule. (Image source: http://astarte.csustan.edu/~tom/bioinfo- S03/frames/content-bioinfo.html).

2.2 DNA Profiling

In the field of forensic science, DNA profiling is one the main techniques and highly reliable tools that have been used in many cases especially homicides, sexual assaults, and human identification since each individual show a slightly different of DNA profile due to the mutations which arise as a result of an error in DNA replication or DNA repair process. Deoxyribonucleic acid (DNA) fingerprint was first developed by Sir Alec Jeffrey in 1985 and started with the DNA typing of minisatellites region (Jeffreys *et al*., 1985). Because it enabled the examination of smaller, more degraded DNA templates, the application of the PCR technique in DNA profiling

played an essential part in many forensic casework investigations. In addition, DNA profiling is widely utilized for the purpose of human identification following a variety of terrible occurrences, including but not limited to war, disasters that affect a large number of people, and other armed conflicts and terrorist acts. Because of its sensitivity, speed, and capacity to make many copies of target DNA sequences, PCR amplification, which was used to process these samples, improved the likelihood that the organism in question could be identified (Haddrill, 2021; Shrivastava *et al.*, 2021).

With PCR technology, Short Tandem Repeats (STRs) typing rapidly became the standard procedure for profiling DNA because only short fragments of DNA were required. The STR analysis has the advantages of allowing multiplexing, which is a system where concurrent amplify of several regions and highly polymorphic which contributes to high power of discrimination. The methods and procedures that used for DNA typing have evolved over the last 30 years and becoming more sensitive, powerful, and fast through a revolution in DNA technology. As stated by Shrivastava *et al*., (2021), all steps implemented in the DNA profiling must be as efficient as possible to avoid re-analysis because forensic samples are always limited in number and not good in condition.

2.3 DNA Storage and Preservation

Forensic methods of identifying victims, such as fingerprinting and dental examination, can be nearly impossible when human bodies have been dismembered, burned, or otherwise deteriorated during mass fatality situations or at particular crime scenes. DVI cases often occur in a remote location with extremes of temperatures and humidities. Access to mortuary facilities and

refrigeration are always not available. Therefore, robust DNA sampling, storage, and preservation procedure would increase the likelihood of effective DNA profiling and allows more rapidly repatriation of bodies and body parts (Sorensen *et al*., 2016). INTERPOL recommends fingerprinting, odontology and DNA typing as key techniques of identification (INTERPOL 2018). In such circumstances, DNA typing can perform a vital role; however, effective DNA recovery depend on strongly on the proper collection and preservation of biological materials (Sharpe *et al.*, 2020). DNA Commission of the International Society for Forensic Genetics reported that storing soft tissue samples in preservative at room temperature can be an alternative to cold storage.

According to study by McNevin.,(2016) had suggested four tissue preservation solutions such as solid salt, salt-saturated dimethyl sulfoxide (DMSO)-EDTA solution, ethanol solution, and ethanol-EDTA solution that preserved muscle tissue at 35°C for up to 1 month. Interestingly, full STR profiles were generated after preservation of muscle tissue by DMSO-EDTA and ethanol solution. He also found out that the salt saturated DMSO-EDTA solution yielded full STR profiles from aliquots of the liquid preservative surrounding the muscle tissue. Whereas formalin (formaldehyde solution) which is used extensively to preserve medical and museum specimens, irreparably damages DNA (Sharpe *et al*., 2020).

According to a study conducted by Moustafa, (2021) stated that tissue type is one of the factors affecting DNA preservation. In forensic science, tissue preservation is usually related to DVI (Watherston *et al.*, 2021) and the preservation technique should be capable to generate a DNA profile by using commercial kits. However, inadequate preservation techniques can cause DNA to degrade to the point that it cannot be used for victim identification. Successful preservation of

biological material can be achieved by a variety of physical and chemical methods (Baptista and Goodwin, 2017).

According to several studies conducted at different places around the world, the soft tissue samples have been preserved in the cell lysis solution for various times. (Allen-Hall and McNevin, 2012; Ali *et al*., 2016; Moustafa, 2021). Complete STR profiles were obtained from 5-100 mg of muscle tissue that had been kept at room temperature in lysis storage and transit buffer for up to 12 months (Graham *et al*., 2015). The liver tissue was preserved for up to two years using lysis buffer. After two years of storage, no DNA could be extracted from the tissue, but high molecular weight DNA was recovered from the buffer solution (Guo *et al*., 2018). However, alcohol storage is considered to be an excellent long-term tissue preservation method because it allows for effective DNA extraction from preserved samples (Baptista and Goodwin, 2017).

Ethanol (EtOH) has previously been used to keep specimens safe at room temperature. It is both inexpensive and easy to obtain, making it an appealing candidate for field use (Oosting *et al*., 2020; Moustafa, 2021). The impact of a range of alcohol concentrations on DNA preservation in ant specimens was studied by (Michaud and Foran, 2011), who found that the most effective to be 95-100 percent concentration. They found that ethanol was chosen over other alcohols because it penetrated cell membranes faster and deactivated deoxyribonuclease (DNase) activity more effectively.

DNA persistence on a soft tissue using vodka as a preservative solution showed similar ability to ethanol by successfully amplified up to 42 days (Baptista and Goodwin, 2017). Other study has used salt solution or DMSO solution to preserve marine animal, but the problem arises during DNA extraction process where salt was reacted with ethanol to form foam thus cause

damage to the DNA (McNevin, 2016). Oakenfull (1994) reported on the use of vodka to preserve zebra liver samples in the African bush over several days. The DNA extracted using this method was found to be of lower quality than that extracted from samples stored in ethanol, however, the result showed the amplified product was able to generate from the extracted DNA.

2.4 DNA Extraction

The first DNA extraction was performed by Friedrich Miescher in 1869. Since then, scientists have made progress in designing various DNA extraction methods that are easier, cost-effective, reliable, faster to perform, and produce a higher yield. According to study Xavier *et a*l. (2021), the traditional DNA typing and analysis in forensic laboratories workflow includes DNA extraction process as the initial stage to purify DNA from the cellular debris and eliminate PCR inhibitors that interfere with the downstream STR typing and can decrease the efficacy of the amplification. The selection of the method must adapt so that each sample is treated accordingly. At the present, there are numerous approaches of DNA extraction protocols namely; organic DNA extraction , Chelex® 100 extraction, FTA® paper, and silica-based methods that have been used in the forensic DNA society for a number of decades (McNevin, 2016).

2.4.1 FTA Paper DNA Extraction Method

The Flinders Technology Associates (FTA®) Whatman filter paper cards are based on a chemically treated cellulose membrane, which lyses cells, their nuclei, and organelles from variety of sources (for instance., blood, saliva, and plant tissue). FTA paper DNA extraction method is a easy method suitable for reference samples. Upon the immediate cell lysis, the released nucleic

acid is bound within the supporting material, the card fiber. The matrix protects the nucleic acids from damaging agents such as, nucleases, oxidative agents and bacterial growth which serves to reduce degradation (Elnagar *et al*., 2021). Additionally, FTA cards are impregnated with chaotropic agents that inactivate infectious agents and reduce the biohazard potential of the sample, thereby, minimizing risks of exposure to technical staff during the sample processing. Thus, this enables the storage of biological material on FTA card at room temperature for extended period without need of refrigerators or freezers. This extraction method is based on washing steps to remove non-DNA materials (GE Healthcare, 2010).

2.4.2 Chelex® 100 DNA Extraction Method

The Chelex® method is quite common for crime scene samples. This method is based on an ion-exchange principle, where the polar resin will bind to polar substances, and denatured non-polar DNA will remain in the solution (Patzoldi *et al*., 2020). Chelex® method is sometimes preferred as it is a quick method and does not require toxic chemicals, but this method does not efficiently remove PCR inhibitors in the sample (J Shetty, 2020). The Chelex® resin is itself a PCR inhibitor, capturing ions such as Magnesium (Mg^{2+}) ions that are required for the *Taq* polymerase during PCR amplification. Previous study has shown that cell free DNA obtained in many biological samples is lost in the supernatant during the Chelex® extraction process, resulting in a reduction of starting DNA material (Patzoldi *et al*., 2020).

2.4.3 Silica-based Extraction Method

This method is the most successful because it can remove PCR inhibitors and concentrating the DNA in a small volume (Rothe and Nagy, 2016). This method involves several steps of chemical additions, incubation, and centrifugation that make this DNA extraction protocol laborious and time consuming, particularly when hundreds of samples require processing. In addition, it has been reported that irreversible binding of DNA to the silica columns can lead to significant loss of sample, especially for samples already in low template amounts (Ali *et al*., 2017). Similarly, DNA purification using silica-coated magnetic beads has emerged as one of the most popular approaches for DNA extraction in forensic laboratories. This method is based on DNA binding to the silica-coated magnetic beads in a certain ionic charge, while unbound contaminants are removed. These DNA purification processes also allow for automation via various DNA extraction platforms, thereby reducing human manipulation and the risk of personnel error (Rothe and Nagy, 2016; J Shetty, 2020).

2.4.4 Phenol-Chloroform-isoamyl alcohol DNA Extraction Method

The phenol-chloroform has been used for the longest period and one of the most commonly used DNA extraction method in forensic laboratories (Dilhari *et al*., 2017). Phenol-chloroform-isoamyl alcohol extraction which referred as organic DNA extraction consist of serial additions of several chemicals. First, Proteinase K and a detergent like Sodium Dodecyl Sulfate (SDS) are first added to the cellular material in a tube to liquefy the membrane and denature the proteins that shield the DNA molecule (J Shetty, 2020). After adding the phenol-chloroform combination, the

proteins and DNA can be isolated from one another. The tube is centrifuged to separate the organic phenol-chloroform phase and the aqueous phase because the DNA is more stable in the aqueous phase (Xavier *et al.*, 2021). The denatured proteins form a pellicle at the interface of these phases. Then using ethanol precipitation or clean-up columns, the DNA in the aqueous phase can be purified. Even though is it a preferred DNA extraction protocol for high molecular weight DNA, phenol potentially causes health problems because it is toxic in nature (Dilhari *et al*., 2017; J Shetty, 2020).

Depending on the kind of tissue and the degree of decomposition, a different approach is optimal for DNA extraction from decayed samples. Multiple investigations have demonstrated that low retention rates of tiny fragments in extraction processes may be the root cause of the low copy number typically associated with degraded samples (Sorensen *et al*., 2016).

2.5 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is the most commonly used molecular diagnostic technique via in vitro amplification. PCR is usually performed after the isolation of DNA and the main ingredients of the reaction are *Taq* polymerase, oligonucleotides, DNA template and nucleotides which act as the DNA building blocks.

The basic PCR steps involves denaturation, annealing and extension (Figure 2.2). During the denaturation, the reaction is performed at 95°C to denature the DNA double strand into single stranded template for the next step. Consequently, in the annealing process, the template usually in the ranged of 55°C – 65°C to ensure at the specific condition so that the primer can bind

specifically to the complementary sequence of the single-stranded DNA template. Last step of the PCR operates at temperature of 72°C so that the *Taq* polymerase enzyme is able to extends the primers for the synthesizing a new DNA strand. This step was known as extension and the duration was decided based on the PCR size product.

The DNA strand that made in the first PCR round will be served as a template for the following DNA synthesis and therefore DNA molecules can roughly double each round of the cycling due to many copies of the primer and many molecules of *Taq* polymerase floating around the reaction. Overall, the cycle consists of denaturation, annealing and extension were repeated for 25- 40 times and will be completed in 2-3 hours depending on the PCR cycle number. Failure in PCR amplification process can be troubleshot by optimizing the reaction components such as primers, deoxyribonucleotide triphosphates (dNTPS), $MgCI_2$ concentration, PCR buffer concentrations, amount of template DNA and *Taq* polymerase that are used in the PCR mixture.

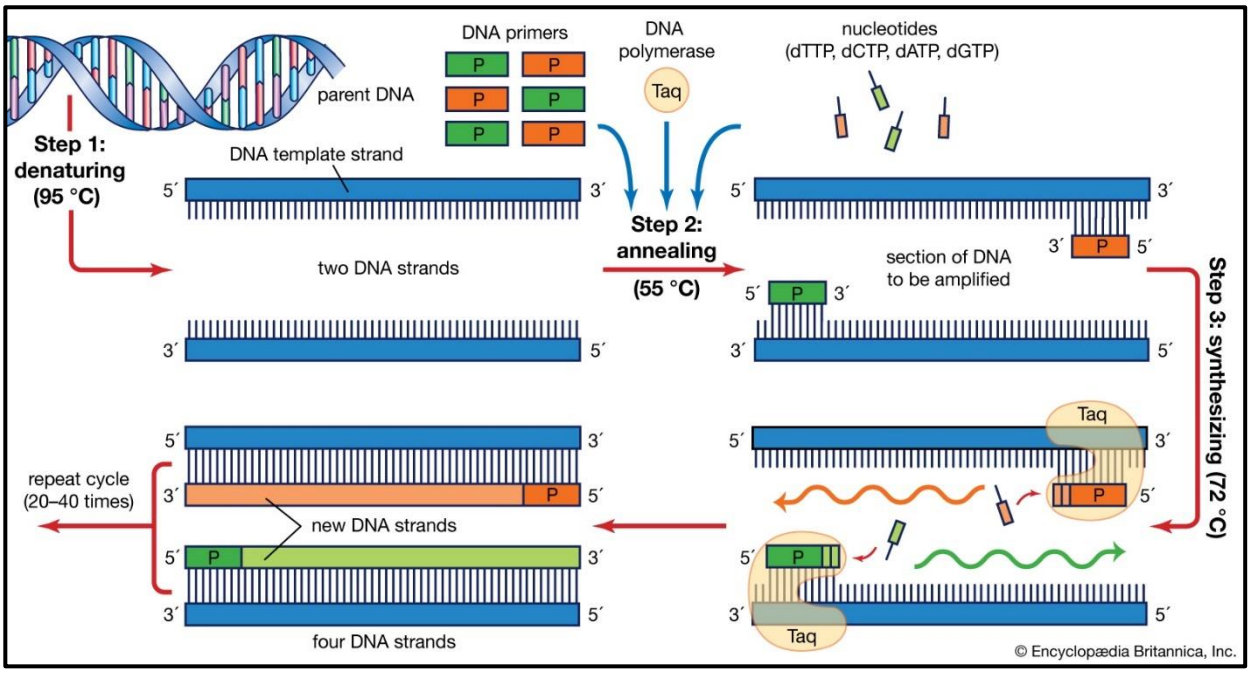

Figure 2.2**:** Polymerase Chain Reaction (PCR): The three-step process of the polymerase reaction. (Source: https://www.britannica.com/science/polymerase-chain-reaction#/media/1/468736/18071).

2.6 DNA Damage and Degradation

DNA degradation and damage occur all the time through enzymatic process, oxidative damage, ultraviolet (UV) radiations from direct sunlight can induce double-stranded DNA damage and form T-T dimers, and hydrolysis (Sorensen *et al*., 2016). However, living organism has repairing enzymes to fix it. When death occurs, cells and tissue become low of oxygen and the physiological process cease to work and decay begins. As sample DNA degradation progresses, DNA fragments become smaller, resulting a longer locus (>250base pairs) which insufficient to amplify during PCR, hence generate a partial STR profiles.

The rate of DNA damage and degradation is accelerated by prolonged exposure to heat, ultraviolet (UV) radiation, humidity, and microorganisms. In humid environments, microbial activity is common, makes DNA more vulnerable to degradation processes like hydrolytic damage (Alaeddini *et al*., 2010). Hot and high temperatures tend to degrade the DNA faster, while more chilly and low temperatures preserve DNA for longer period (Oosting *et al*., 2020). When a cell or organism dies, the DNA in each cell is vulnerable to damage and degradation caused by endogenous nucleases as well as exogenous insults such as microbial activity. Depending on several internal and external factors, cells would undergo one of two different patterns: apoptosis (programmed cell death) or necrosis. After cell death, cell changes include cytoplasm condensation, nuclear fragmentation, chromosomal DNA fragmentation (double strand breaks), and a reduction in cell volume. One of these factors is the level of intracellular adenosine triphosphate (ATP). Even if the intracellular ATP level does not change during the entire process of apoptosis, which is an energy-dependent form of programmed cell death.

On the other hand, necrosis is a passive energy independent degenerative phenomenon (Baptista and Goodwin, 2017). This pattern of cell death is associated with a temporary increase in cell volume, swelling of cytoplasmic organelles, and chromatin condensation, resulting in cell membrane rupturing, organelle breakdown, and lysosomal enzyme leakage. As a result, DNA is released into the environment, exposed to harmful enzymes and external insults, resulting in a random pattern of degradation.

Apoptosis usually results in DNA fragments of 180 base pairs (bp), with DNA breaks occurring between nucleosomes. While apoptosis produces the ladder-like pattern of oligonucleosomal-sized fragments in agarose electrophoresis and necrosis produces the smear associated with degraded DNA due to random digestion. Endonuclease-mediated DNA cleavage in early necrosis is characterized by the selective generation of 5' overhangs. Endonucleases cleave the DNA around the histone structure (the most vulnerable sections), resulting in 300 kb (rosette structure) or 50 kb fragments (loop structure). After the chromatin proteins have been digested, endonucleases will randomly digest them, with the rate of degradation varying depending on temperature, pH levels, and enzyme expression levels (Baptista and Goodwin, 2017; Oosting *et al*., 2020). Necrosis is typically induced by extremes in the external environmental conditions of the cell (such as hypoxia), or by the action of membrane active toxicants and respiratory poisons. On the other hand, non-enzymatic processes are also present and responsible for DNA breakdown, which occur more slowly but should not be overlooked. Some of them are more likely to occur in muscle tissue than others, but in an uncontrolled environment, DNA can be exposed to any of them (Alaeddini *et al.*, 2010).

Denaturation of DNA occurs when the hydrogen bonds that hold the DNA double strand together breaks, causing the double helix structure to unwind. Denaturation increases susceptibility to other types of chemical attack despite the nucleotide sequence remaining unchanged. Cross-linking occurs when one of the double helix strands forms chemical bonds with other molecules. Nucleotides remain unchanged, similar to denaturation, but cross-linking can cause problems with analyses (Sharpe *et al*., 2020).

The glycosidic base sugar bond is attacked by hydrolic reactions, and the presence of water accelerates DNA hydrolysis, resulting in strand breaks, base loss, and chemical modifications to nucleotide units. Chemical modifications on nucleotides include the addition, removal, or replacement of a chemical groups. These modifications have the potential to alter the entire nucleotide sequence. Strand breaks occur when the sugar phosphate backbone of the DNA is broken, causing the entire molecule become fragment (McNevin, 2016).

As a result of oxidative damage, it can change the sugar residues, remove bases, and cause the strand to break and cross-linking. At the end, factors such as UV radiation, oxidative, and hydrolytic damage can result in base modifications, strand breaks, crosslinks, and mismatches and later can complicate the DNA typing (Alaeddini *et al*., 2010). Graham *et al*. (2015) and Guo *et al*., (2018) had stated that the DNA degradation starts within minutes or hours after sampling from a live specimen and-continue to degrade depending on how the DNA is preserved. Enzyme activities such as endonucleases and exonucleases can lead to the rapid breakdown of DNA inside the cells. However, the DNA degradation process is reduced at lower temperature since the enzyme activity is sensitive to temperatures. Thus, keeping the samples cold will slow down the enzymatic degradation of DNA. In addition, oxidative damage by free radicals and hydrolysis through

interaction with water particularly acidic water compromises the DNA integrity. Therefore, tissue samples will always be, to some extent, subject to all the processes presented above during transportation.

Once the DNA is extracted, DNA will continue to degrade even while being stored under optimal conditions such as low temperature, buffered media, sterile environment and/or minimal manipulations. Storing extracted DNA in a buffer solution at certain pH (e.g., Tris-HCl pH 8) able to protects the tissue samples from oxidative damage and hydrolysis of phosphate bonds, increasing the chance of retaining good quality DNA (Haddrill, 2021).

A study conducted by Oosting *et al*. (2020) using two commonly used preservative solutions (DESS and ethanol >99.5%) over three months suggested that DNA stored in ethanol was significantly more degraded after one day, while DNA stored in DESS (20% DMSO, 0.25 M EDTA, NaCl saturated solution) appeared relatively stable over the first month. The drastic reduction of high molecular weight DNA in ethanol after one day suggested that enzymes were actively degrading the DNA. On the other hands, the DNA samples stored in DESS showed clear degradation after three months of storage in DESS. The exact processes that caused the observed degradation are unknown. However, it is possible that enzymes degrade DNA gradually over time, or that chemical processes (such as hydrolyzes) have become a contributing factor over time, or both.

Additionally, another recent study conducted by Moustafa., (2021), stated that there is a significant difference between ethanol (95%) and salt-saturated dimethyl sulfoxide (SSDMSO) preservatives in DNA concentrations which were extracted after one and two months at room temperature with the superiority to SSDMSO was retaining DNA concentrations a long period of

time. Similarly, the same study also demonstrated a significant decline in DNA concentration for samples preserved in ethanol 95% over two months while SSDMSO shown a minor decline. These results indicated that SSDMSO has a better capability to preserve DNA than ethanol. Therefore, effective DNA-based identification is dependent on timely sample collection after death to ensure optimal tissues are sampled, as well as adequate DNA sample is preserved. Preservation prior to processing.

2.7 DNA profiling of challenging samples

Because of the nature of crime samples, when samples are usually degraded or damaged, inhibited, or contains little amount of DNA its always challenging to generate a full profile. Since the reference samples contains is originated from the fresh DNA its always possible to obtain a full DNA profile (Haddrill, 2021). In the process of DNA analysis, there are several factors that can affect the DNA profiling process includes: the presence of inhibitors and trace amount or degraded DNA which might results an incomplete profile or possibly no profile.

Chemical contamination of DNA sample might also inhibit the downstream process and most of the inhibitors found in forensic samples are contributed by heme from blood, calcium from bones and humic acid from soils and other types of inhibitors (Watherston *et al*., 2021). Each inhibitor has its own inhibition pathway, and some inhibit multiple chemical reactions. Small amounts of DNA (less than 200pg) are usually categorized as low copy number or low template DNA. To analyse those samples, laboratories employ procedures designed to improve assay sensitivity, such as increasing PCR cycles, decreasing amplification volumes, concentrating

products prior to electrophoresis, and increasing injection times during electrophoresis (Watherston *et al*., 2021).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Laboratory structure and Overview

This study was conducted at Forensic Science and Molecular Biology laboratories located in Health Campus of University Science Malaysia (USM), Malaysia. These two laboratories are divided into pre-PCR and post-PCR amplification to minimize and prevent contamination that may occur during the research process. All pre-PCR works including preparation of soft tissue, preparation of preservation solutions and DNA extraction was performed in the dedicated forensic laboratory, following strict protocols including the use appropriate personal protective equipment's. In addition, prior to use, every working station was cleaned with 70% ethanol, all the tips were autoclaved to sterilize and dried for an overnight in the oven to completely remove any water condensed. In order to monitor either the potential materials or worker/personnel originated human DNA contamination, a negative control (reagent blanks) was included throughout the entire process.

3.2 Materials

3.2.1 Chemicals and reagents

All the chemicals, reagents and consumables used in this study was listed in the Appendix A.

3.2.2 Instruments and Apparatus

All the instruments and apparatus used in this study was listed in the Appendix B.