CHAPTER I

INTRODUCTION

Forensic Science-It is the application of science and technology to the detection and investigation of crime and administration of justice system. It also known as application of science to criminal and civil laws mainly on criminal side during criminal investigations. As governed by legal standards of admissible evidence and criminal procedure. Brenner, J. C. (2017). *Forensic science glossary*. CRC Press.

Forensic science involves several divisions like forensic medicine, forensic biology, forensic serology, forensic pathology, forensic ballistics, forensic toxicology, forensic archeology, forensic anthropology, mobile forensics, forensic psychology, forensic podiatry, forensic optometry, forensic odontology, forensic linguistics, forensic geology, forensic entomology, forensic engineering, forensic botany, criminalistics, documentation, ballistics, cyber forensics, forensic chemistry, wildlife forensics and many more.

The history of Forensic science dates back thousand of years. Fingerprinting was one of its first applications (1892). Sir Francis Galton established the first system for classification. The Henry classification became the standard for criminal fingerprinting techniques worldwide. Tewari, R. K., & Ravikumar, K. V. (2000). History and development of forensic science in India. *Journal of postgraduate medicine*, *46*(4), 303.

In India, the first State Forensic Laboratory was established in the year 1952 at Calcutta. On the recommendations of the Royal Police Commission of 1902-1903, the first Central Finger Print Bureau in India was established at 1905 at Shimla. The Father of forensic science, Sir Edmond Locard, he formulated the basic principle of forensic science. "Every Contact Leaves Traces''.

There are about 7 laws in Forensic Science:

- Law of Individuality
- Principle of Exchange
- Law of Progressive change
- Law of Comparison
- Law of Analysis
- Law of Probability
- Law of Circumstantial facts

Wildlife in broad sense, includes whole Fauna and Flora found in wilderness zone.

According to Indian Board for Wildlife (1970), Wildlife is the whole native and uncultivated fauna and flora of a particular country.

According to **Wildlife (Protection) Act 1972**, wildlife includes any Animals, Bees, Butterflies, Crustacea, Fish and Moths And Aquatic or Land Vegetation which form part of any habitat.

Wildlife Forensic Science is forensic science applied to legal issues involving wildlife, using scientific procedures to examine, identify, and compare evidence from crime scenes, and to link the evidence with a suspect and a victim. Cooper, J. E., & Cooper, M. E. (2013). Wildlife forensic investigation: principles and practice. CRC press.

While animals and plants are the victims in the crimes of illegal wildlife trade and animal abuse, society also pays a heavy price when those crimes are used to fund illegal drugs, weapons and terrorism.

History of wildlife forensics: The international organization that monitors trade in wild animals and plants is the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which was established in 1963 and, as of now it includes 188 member countries. Wildlife Protection Act, was enacted in 1972 in India to protect the wildlife species in India.

Forensics in wildlife plays a vital role in scientific collection and preservation of evidences found at the crime scene. It helps in proper examination of evidences without

contamination. As Forensic science includes scientific procedures such as crime scene search methods which helps in careful search of evidences in scene of crime, collection of evidences which helps to collect the evidences with no contaminants through collection kits. Packaging the collected evidences is the major task to ensure no leakage of evidences, Post packaging, sealing of the evidences are of paramount importance that no intruders can circumspect the sample of forensic evidence. Preservation of the evidences is a prerequisite, for instance, biological evidences must be stored with necessary preservatives in accordance to the type of sample. As technology improves many techniques to preserve the evidences are been adapted such as FTA cards, EDTA tubes etc. And examination of evidences in the laboratory following the scientific procedures helps the evidence to be valid in the court of law.

WILDLIFE PROTECTION ACT ,1972-

An act to provide for the protection of wild animals, birds and plants and for matters connected there with or ancillary or incidental thereto with a view to ensuring the ecological and environmental security of the country

This act includes 6 chapters, as follows;

Chapter 1 Tells about the definitions under this act

Chapter 2 Tells about the authorities to be appointed or constituted under the act

Chapter 3 Tells about hunting of wild animals

Chapter 3A Includes protection of specified plants

Chapter 4 Tells about the protected areas such as sanctuaries and national parks

Chapter 4A Tells about central zoo authority and recognition of zoos

Chapter 4B National tiger conservation authority

Chapter 4C Tells about tiger and other endangered species crime control bureau

Chapter 5 Tells about the trade or commerce in wild animals, animal article and trophies

Chapter 5A Tells about prohibition of trade or commerce in trophies, animal articles etc derived from certain animals

Chapter 6 tells about prevention and detection of offences

Chapter 6A tells about forfeiture of property derived from illegal hunting and trade

Chapter 7 tells about miscellaneous

Mohanraj, P., & Veenakumari, K. (1996). Nomenclature, classification and the basis of the Schedules in the Indian Wildlife (Protection) Act, 1972. Current Science, 70(6), 428-432.

This Act includes 6 schedules within the above listed chapters, they are:

Schedule 1 to 4 includes Mammals, Amphibian and Reptiles, Fishes, Birds, Crustacea and Insects, Coelenterates, Mollusca, Echinodermata.

Schedule 5 - Vermin

Schedule 6 – Plants

- Scat sample as evidence helps in gathering information such as diet, distribution, abundance, and community dynamics. Molecular Scatology techniques have been developed to extract DNA from the donor cells present on the outer lining of the scat samples.
- Blood as evidence helps in identification of species such as it of humans or animal blood this compatibility is determined by several major antigens such as the ABO and Rhesus blood groups.
- Tissue sample as evidence helps in identification of species and its origin by molecular analysis.
- Bone as evidence is best source for DNA extraction as it contains huge amount of DNA

Molecular tools in Wildlife Forensics play a vital role in identification of species, such as assaying/ testing, DNA profiling, and determining its sex, and also to identify the geographical origin of species. are nuclear DNA, mt DNA, DNA fingerprinting, which helps to law enforce of wildlife conservation. Genetic methods are also been used to determine the geographic origin. Alacs, E. A., Georges, A., FitzSimmons, N. N., & Robertson, J. (2010). DNA detective: a review of molecular approaches to wildlife forensics. Forensic science, medicine, and pathology, 6(3), 180-194.And the latest technologies include PCR based cloning and sequencing techniques, construction of mutant and chimeric DNA using PCR, quantification of mRNA using PCR, approaches to tissue analysis. Although the techniques used are many the primary step is to isolate the DNA from any type of the sample.

Identification of Species- The most widely used markers to identify species are mitochondrial Cytochrome C Oxidase Locus (COL) …Multi locus markers such as Ribosomal Internal Transcribed Spacers (ITS DNA) along with mat K, rbcL, trnH, 12s ,16s, cytb

The latest technologies include

- PCR based cloning and sequencing methods
- Construction of mutant and chimeric DNA using PCR
- Quantification of m-RNA using PCR
- Approaches to tissue analysis

CHAPTER II

LITERATURE REVIEW

Alice Laguardia et al…2015 studied Many ecological studies and conservation management plans employ noninvasive scat sampling based on the assumption that species' scats can be correctly identified in the field. However, in habitats with sympatric similarly sized carnivores, misidentification of scats is frequent and can lead to bias in research results. To address the scat identification dilemma, molecular scatology techniques have been developed to extract DNA from the donor cells present on the outer lining of the scat samples. A total of 100 samples were collected in the winter of 2009 and 2011 in Taxkorgan region of Xinjiang, China. DNA was extracted successfully from 88% of samples and genetic species identification showed that more than half the scats identified in the field as snow leopard (*Panthera uncia*) actually belonged to fox (*Vulpes vulpes*). Correlation between scat characteristics and species were investigated, showing that diameter and dry weight of the scat were significantly different between the species. (Alice Laguardia et al…2015) However it was not possible to define a precise range of values for each species because of extensive overlap between the morphological values. This preliminary study confirms that identification of snow leopard feces in the field is misleading. Research that relies upon scat samples to assess distribution or diet of the snow leopard should therefore employ molecular scatology techniques. These methods are financially accessible and employ relatively simple laboratory procedures that can give an indisputable response to species identification from scats.

Rozieyati Mohamed saleh et al…2018 studied Transfusion procedures are always complicated by potential genetic mismatching between donor and recipient. Compatibility is determined by several major antigens, such as the ABO and Rhesus blood groups. Matching for other blood groups (Kell, Kidd, Duffy, and MNS), human

platelet antigens, and human leukocyte antigens (HLAs) also contributes toward the successful transfusion outcomes, especially in multi transfused or highly immunized patients. All these antigens of tissue identity are highly polymorphic and thus present great challenges for finding suitable donors for transfusion patients. The ABO blood group and HLA markers are also the determinants of transplant compatibility, and mismatched antigens will cause graft rejection or graft-versus-host disease. Thus, a single and comprehensive registry covering all of the significant transfusion and transplantation antigens is expected to become an important tool in providing an efficient service capable of delivering safe blood and quickly locating matching organs/stem cells.(Rozieyati Mohamed saleh et al…2018) This review article is intended as an accessible guide for physicians who care for transfusion-dependent patients. In particular, it serves to introduce the new molecular screening methods together with the biology of these systems, which underlies the tests.

Teerapong siriboonpiputtana et al…2017studied DNA typing from degraded human remains is still challenging forensic DNA scientists not only in the prospective of DNA purification but also in the interpretation of established DNA profiles and data manipulation, especially in mass fatalities. In this report, we presented DNA typing protocol to investigate many skeletal remains in different degrees of decomposing. In addition, we established the grading system aiming for prior determination of the association between levels of decomposing and overall STR amplification efficacy. A total of 80 bone samples were subjected to DNA isolation using the modified DNA IQ™ System (Promega, USA) for bone extraction following with STR analysis using the AmpFLSTR Identifiler® (Thermo Fisher Scientific, USA). In low destruction group, complete STR profiles were observed as 84.4% whereas partial profiles and non-amplified were found as 9.4% and 6.2%, respectively. Moreover, in medium destruction group, both complete and partial STR profiles were observed as 31.2% while 37.5% of this group was unable to amplify. Nevertheless, we could not purify DNA and were unable to generate STR profile in any sample from the high destroyed bone samples.

Compact bones such as femur and humerus have high successful amplification rate superior than loose/spongy bones. (Teerapong siriboonpiputtana et al…2017). Furthermore, costal cartilage could be a designate specimen for DNA isolation in a case of the body that was discovered approximately to 3 days after death which enabled to isolate high quality and quantity of DNA, reduce time and cost, and do not require special tools such as freezer mill.

James Fetzner Jr 1999 studied molecular studies involving reptiles often overlook shed skins as a source for high-quality DNA. In most cases, tissues or blood samples are preferred by researchers, but the process of sampling for these tissue types can be harmful or otherwise adversely affect the animals involved. While reptile breeders or zoological institutions are a potential source of specimens, most will likely decline requests for samples if the sampling will harm their prized animals. (James Fetzner Jr 1999) Generally, breeders and curators would be much more amenable to part with a shed skin—which is something they usually discard anyway.

E. Hagelberg et al..1991 studied the analysis of DNA from ancient bone has numerous applications in archaeology and molecular evolution. Significant amounts of genetic information can be recovered from ancient bone: mitochondrial DNA sequences of 800 base pairs have been amplified from a 750-year-old human femur by using the polymerase chain reaction. DNA recovery varies considerably between bone samples and is not dependent on the age of the specimen. (E. Hagelberg et al..1991) We present the results of a study on a small number of bones from a mediaeval and a 17th-century cemetery in Abingdon showing the relation between gross preservation, microscopic preservation and DNA recovery.

Claire Bellis et al…2003 studied This study investigated potential markers within chromosomal, mitochondrial DNA (mt-DNA) and ribosomal RNA (rRNA) with the aim of developing a DNA based method to allow differentiation between animal species. Such discrimination tests may have important applications in the forensic science,

agriculture, quarantine and customs fields. DNA samples from five different animal individuals within the same species for 10 species of animal (including human) were analysed. DNA extraction and quantitation followed by PCR amplification and GeneScan visualisation formed the basis of the experimental analysis. Five gene markers from three different types of genes were investigated. (Claire Bellis et al…2003). These included genomic markers for the β-actin and *TP53* tumor suppressor gene. Mitochondrial DNA markers, designed by Bataille et al. [Forensic Sci. Int. 99 (1999) 165], examined the Cytochrome *b* gene and Hypervariable Displacement Loop (D-Loop) region. Finally, a ribosomal RNA marker for the 28S rRNA gene optimised by Naito et al. [J. Forensic Sci. 37 (1992) 396] was used as a possible marker for speciation. Results showed a difference of only several base pairs between all species for the β-actin and 28S markers, with the exception of *Sus scrofa* (pig) β-actin fragment length, which produced a significantly smaller fragment. Multiplexing of Cytochrome *b* and D-Loop markers gave limited species information, although positive discrimination of human DNA was evident. The most specific and discriminatory results were shown using the *TP53* gene since this marker produced greatest fragment size differences between animal species studied. Sample differentiation for all species was possible following *TP53* amplification, suggesting that this gene could be used as a potential animal species identifier.

Tomáš Minarovič et al….2010 studied an alternative DNA detection system is based on the polymerase chain reaction (PCR) amplification of a segment of the mitochondrial cytochrome b gene. Subsequent cleavage by a restriction enzyme gives rise to a specie-specific pattern on an agarose gel. We used five animal species (*Mustela vison*, *Mustela putorius furo*, *Sus scrofa domesticus*, *Oryctolagus cuninculus*, *Anser anser*). Length of PCR product was 359 bp and we used universal primers. Restriction fragment length polymorphism was analyzed by using the restriction endonuclease *Alu*I. Results of cleavage were visualized by using electrophoresis and UV transiluminator. Every animal species has a unique combination of restriction fragments i.e. *Mustela vison* 81 bp, 109

bp and 169 bp, *Mustela putorius furo* 169 bp and 190 bp, *Sus scrofa domesticus* 115 bp and 244 bp, *Oryctolagus cunninculus* is not cleaved by *Alu*I so it has whole 359 bp fragment on agarose gel, *Anser anser* 130 bp and 229 bp. (*Tomáš Minarovič et al….2010),* The results suggest that the method of PCR - RFLP is rapid and simple method for identification of species. PCR – RFLP can reliably identify chosen species. Application of genetic methods is very useful for breeding of livestock and protection of biodiversity.

Gurdeep Rastogi et al…2007 studied We evaluated and compared the utility of mitochondrial markers *viz.* 16S rDNA and NADH dehydrogenase subunit 4 (*ND4*) and a nuclear marker *viz.* the actin gene to identify the specimens of animal origin for forensic identification, food regulatory control and to prevent illegal trading, poaching and conservation of endangered species. We also tested PCR fingerprinting methods like RAPD and actin barcoding to generate species-specific "fingerprints". Our results suggested that mitochondrial markers are more efficient than nuclear markers for the purpose of species identification and authentication. Among PCR fingerprinting approaches, RAPD was proved to be more discriminatory, accurate and efficient than actin fingerprinting.(Gurdeep Rastogi et al…2007) Considering the present scenario in trading of vertebrate animal tissues like buffalo, cow, pig, goat, chicken, frogs, fishes and snakes etc., mitogenomics based technology proved to be efficient and reliable in resolving problems like meat adulteration and smuggling across countries.

Guglich, E. et al… 1994 studied Highly repetitive DNA markers have been used for determining the species origin of animal tissues in cases of illegal commercialization and poaching of game animals. This approach has been used in cases involving white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*) and black bear (*Ursus americanus*). Digesting the DNA with various restriction enzymes, Agarose Electrophoresis and staining with ethidium bromide revealed unique banding patterns for each species. (Guglich, E. et al…1994) These patterns have been used to distinguish meat from game animal species from commercial sources of meat and organs. Data are

presented from two Ontario court cases that demonstrate the application of the procedure.

Bhawna Dubey et al…2011 studied Illegal trade of snake skin and uncontrolled hunting have instigated the extermination of many endangered snake species. Efforts to check illegal trade are often impeded due to lack of proper species identification methods. Hence, conservation strategies demand for authentic and quick identification techniques to trace the origin of the seized samples. This study employs DNA mini-barcoding as a method to identify some endangered snake species of India. We have designed two sets of novel primers for targeting regions within the mitochondrial Cytochrome Oxidase I gene to produce 175 bp and 245 bp amplicons. 175 bp fragment was amplified in all 11 snake species studied while the 245 bp amplicon was obtained in 10 species. DNA mini-barcodes recovered from these amplicons enabled the identification of snake species by retrieving the sequences available in public databases. The similarity scores ranging from 98 to 100% (98% taken as threshold value for species identification) signify the consistency of these mini-barcodes in snake species identification.(Bhawna Dubey et al…2011) Moreover, the results of the validation study confirm the effectiveness of the technique in forensic perspective, where the diagnostic morphological features of the seized sample are often missing

Janice A. Nicklas and Eric Buel 2003 studied Quantification of DNA in a forensic sample is of major importance for proper DNA amplification and STR profiling. Several methods have been developed to quantify DNA, from basic UV spectrometry, through gel-based techniques, to dye staining, blotting techniques, and, very recently, DNA amplification methods (polymerase chain reaction, PCR). Early techniques simply measured total DNA, but newer techniques can specifically measure human DNA while excluding non-human DNA (foodstuff, animal, or bacterial contamination). These newer assays can be faster and less expensive than traditional methods, making them ideal for the busy forensic laboratory. (Janice A. Nicklas and Eric Buel 2003) This paper reviews classic and newer quantification techniques and presents methods recently developed by the authors on the basis of PCR of *Alu* sequences.

CHAPTER III

AIM & OBJECTIVES

AIM:

To do Purification and Separation of DNA From Wildlife Species

OBJECTIVES

- To isolate DNA from Blood, Tissue, Scat, Bone of Wildlife species (Porcupine, Spotted Deer, Elephant, Leopard & Tiger)
- To analyze and quantify the isolated gDNA (genomic DNA)

CHAPTER IV

MATERIALS AND METHODOLOGY

MATERIALS REQUIRED

1. Proteinase k -50uL,1M DTT-100Ul,20%SDS-100uL, lysis buffer for soft tissue-750uL, TE buffer (10mM tris HCL, 1mM EDTA, ph-8,
2. Reagents- Lysis solution
3. Stool lysis buffer
4. Inhibitor removal solution
5. Wash solution concentrate (Dilute it by using 3ml of wash concentrate solution in 7ml of Ethanol)
6. Binding solution
7. Elution buffer (10mM tris C1 pH -8.5)
8. Proteinase k (20mg/ml)
9. RNAse A (20mg/ml)
10. 1XTAE buffer, conical flask, gel powder, gel casting trays which are available in a variety of sizes and composed of UV transparent plastic, sample comb, electrophoresis buffer, loading buffer, ethidium bromide, trans illuminator (an ultra violet light box

DNA ISOLATION:

PROTOCOL

100 mg to 150 mg of the scat (sample) is scrapped, in a 2ml tube. Add 50uL of proteinase k (stock of 20mg/ml), then add 100uL of 1M DTT+20%SDS and finally add 750uL of Lysis buffer. (Lysis buffer contains -50mM tris -HCL ,10Mm EDTA, 100mM NaCl, pH -8). Then incubate at 56 degree centigrade for overnight and centrifuge at 12000rpm for 5 minutes and add 1 volume of phenol and again centrifuge it at the same. Transfer the supernatant to the fresh tube and add 1 volume of Phenol: Chloroform: Isoamyl alcohol

at -20 degree centigrade, then centrifuge it at 12000rpm for 5 minutes. Wash the pellet for 2 times. Then discard the supernatant and allow the pellet to dry at room temperature and resuspend in nucleus free water.

Fig. 4.1 Isolation of DNA

DNA ISOLATION USING STOOL PURIFICATION KIT

PROTOCOL:

1. Resuspension-Take 250 mg of sample and add 1ml of TE buffer, vortex it for 15 seconds. Then centrifuge at 10000rpm for 3 minutes, flow through is discarded and the pellet is resuspended with 500uL of lysis solution. Now 200uL of solution is pipetted out into new 2ml capped collection tube.
2. Lysis- 200uL resuspended solution is taken and to that 20uL of proteinase k is added and vortexed and incubated for 30 minutes at 55 ℃ and centrifuge at 12000rpm for 5 minutes.
3. RNAase A treatment (optional): 25uL of RNAase A solution is added incubated for 5 minutes at 15 to 25℃

4. Lysis -200uL of stool lysis buffer is added and vortexed it for 15 seconds and incubated at 70℃ for 10 minutes.
5. Inhibitor removal- 250uL of inhibitor removal solution is added and vortexed it and incubated at 4℃ for 5 minutes. And centrifuged at 12000rpm at room temperature for 1 minute, then pellet is discarded.
6. Binding – The supernatant is transferred to clean fresh tube and 200uL of binding solution is added and vortexed it for 15 seconds, then the sample is loaded into Hi elute miniprep column and centrifuged at 12000rpm for 1 minute at room temperature.
7. Wash- 500uL of diluted wash solution concentrate is added and centrifuged at 12000rpm for 1 minute. Then the flow through is discarded. The wash is repeated, and centrifuged at 12000rpm for 4 minutes, then the flow through is discarded and centrifuged at 12000rpm at room temperature for additional 1 minute (to remove the residual ethanol).
8. DNA elution -transfer the column into fresh collection tube (1.5ml) and 200uL of elution buffer is added to it. And incubated for 5 minutes and centrifuged at 12000rpm for 2 minutes, transfer the elute to fresh capped 2ml tube.

DNA ISOLATION FROM BLOOD

Take 300uL of blood and then add 1200uL of reagent A and then mix it by rolling for 3-4 minutes. Then centrifuge it at 3000rpm for 5 minutes and then the supernatant is discarded. The obtained pellet was dried on paper towel. After it is dried,150uL of reagent B is added and vortexed it for 10 seconds. Then 37.5uL of reagent C is added and it is mixed by inverting the tubes for several times and then incubate at 65℃ for 15 to 20 minutes. After cooling the tubes at room temperature, 200uL ice-cold chloroform was added and mixed by rotating for 60 minutes. Then centrifuge at 2500rpm for 2 minutes and the supernatant is transferred to fresh tube and 200uL of IPA is added to get DNA precipitate at 2500 rpm for 5 minutes. Then it is washed twice with 70% Ethanol and air dry the pellet and resuspend the same with 50uL of nucleus free water.

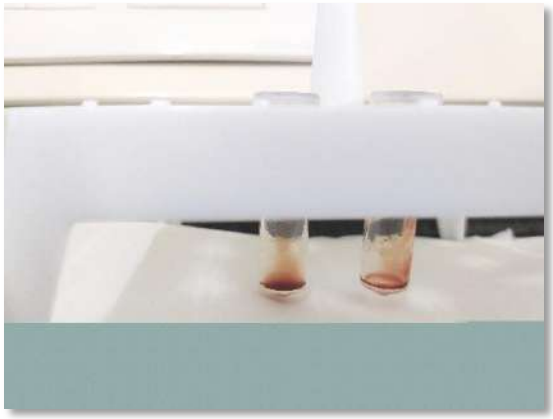


Fig 4.2 Tube 1 (left)-Animal blood(INDIAN ELEPHANT)

Tube 2 (right)-Human blood

Fig 4.3 Drying the samples at room tempertaure

ISOLATION OF DNA FROM TISSUE

To the sample, 875uL of Lysis buffer is added (for soft tissue) and then add 100uL of 20% SDS and 25uL (20mg/ml) Proteinase k. Incubate at 56℃ for 3 hours to ensure complete lysis and then centrifuge at 12000 rpm for 5 minutes at 4℃ , the supernatant is then transferred to the fresh tube and stored at -20℉ ,lysate thawed and equal volume of phenol is added. It is mixed by inversion for 10 minutes and centrifuge at 12000rpm at 5 minutes. Then supernatant is transferred to fresh tube, equal volume of Phenol:Chloroform:Isoamylalcohol is added and mix it by inversion for 10 minutes and centrifuge at 12000rpm for 5 minutes . Then aqueous phase is transferred to fresh tube and equal volume of chloroform is added , and it is mixed by inversion for 10 minutes and centrifuge at 12000rpm at 5 minutes ,the aqueous phase is transferred to fresh tube and 2 volume of ice cold IPA is added, then incubate at room temperature for 5 minutes (pellet /ppt is observed) and centrifuge at 12000rpm for 5 minutes ,then the supernatant is discarded and to the pellet 500uL of 70% ethanol is added and centrifuge at 12000rpm for 5 minutes, twice. Pellet is air dried and resuspended in 50uL nucleus free water ,3uL of sample +1uL of novel juice satin is loaded on the 0.8% gel and visualized.

DNA ISOLATION FROM BONE

Take 250-300mg of bone scrapings and incubate for overnight at room temperature with 0.5M EDTA at pH 8.0 and then centrifuge at 12000rpm for 5 minutes to remove EDTA Supernatant and 2ml distilled water was added to each tube and then vortex and centrifuge at 12000rpm for 5 minutes to remove excess EDTA. Supernatant is removed, then 875µL of lysis buffer (Total volume – 100mL, pH – 8.0, Tris-HCL – 30mM, EDTA – 5mM and NaCl – 50mM) is added for bone after EDTA wash and 100µL of 20% SDS and 25µL (20mg/ml) Proteinase k is added. And incubate at 56℃ for 4-6 hours and then centrifuge at 12000rpm for 5 minutes. Supernatant is transferred to fresh tube. 1 volume of phenol is added, mix by inversion and centrifuge at 12000rpm for 5 minutes. Supernatant (aqueous phase) is removed and transferred to fresh tube. To that add 1 volume of Phenol:Chloroform:Isoamylalcohol is added, mix by inversion and centrifuge at 12000rpm for 5 minutes. Supernatant is transferred to fresh tube, to that add 1 volume of chloroform then mix by inversion and centrifuge at 12000rpm for 5 minutes. Supernatant is transferred to fresh tube and 2 volumes of isopropyl alcohol is added and incubate for overnight at -20°F. Then centrifuge at 12000rpm for 5 minutes and the supernatant is discarded. Pellet is washed twice with 500µL of 70% ethanol and the pellet is air dried and resuspended in suitable volume of nuclease free water.

ANALYSIS AND QUANTIFICATION OF ISOLATED DNA

Isolated DNA samples were visualized on 0.8% agarose gel prepared by dissolving 0.24g of 1X TAE buffer and stained using novel juice stain (sigma). The agarose gel picture is documented using BioRad XR+ gel documentation system. The quantity and quality of the DNA is analysed using Thermo Scientific Nanodrop One spectrophotometer, to determine the 260/280 and 260/230 ratios.

GEL ELECTROPHORESIS

PROTOCOL-

Take 0.24g of gel powder weighed. Pour the powder into the conical flask and add 30ml of 1X TAE buffer, mix it and keep in the oven for around 1 minute 15 seconds (make sure that while boiling, it should not come out of the flask). Repeat the process until solution becomes transparent. Allow it cool and pour the solution into the container ,(make sure that no bubbles were formed while pouring the solution in to the container) wait until it gets solidifies ,then remove the comb (be careful while taking the comb that you are not disturbing the wells), and take the sample of 3uL and 1uL of novel juice stain (this stain is having 3 tracking dyes -bromo phenol blue-400bp, xylene cyanol ff – 8000bp, orange g 50 bp) ,with the help of pipette mix all the contents twice or thrice and then load sample in to the gel , add 1kb ladder (for genomic DNA). After loading all the samples, allow it to run. After its run. take out the gel and keep in gel doc to observe the bands formed and to capture the image.

POLYMERASE CHAIN REACTION USING 12S MARKERS

Polymerase chain reaction is carried out to amplify partial fragments of 12S markers as described by (Kocher et al..1989). The reactions were carried out in 10µL volume containing 1X KAPA Taq Buffer B, 2.5 µMdNTPs, 2.5 mM mgcl2, 1µM reverse primer,0.5 U KAPA Taq Polymerase and 50-100 ng of template DNA. The PCR reaction is carried out with an initial denaturation at 95^0 C for 5 mins, followed by 35 cycles of 95^0 C for 1 min., 50^0 C for 1 min. and 72^0 C for 1.5 mins. Final extension step is carried out at 72^0 C for 10 mins and the PCR products were visualized on a 2% agarose gel and stain it by novel juice stain. The agarose gel picture is documented using a BioRad XR+ gel documentation system.

Fig. 4.4 Preparation of Master Mix for PCR amplification

TABLE 4.1: SHOWS THE PCR REACTION CONDITIONS FOR 12S GENE REGION

STEP	TEMPERATURE (° C)	TIME (minutes)
Initial Denaturation	94° C	5’
Denaturation	94° C	1’
Annealing	50° C	1’
Extension	74° C	1.5’
x 35 cycles		
Final Extension	74° C	10’

TABLE 4.2: PCR COMPONENTS WITH RESPECTIVE CONCENTRATION AND VOLUME

PCR COMPONENT	VOLUME	FINAL CONCENTRATION
10 X KAPA Taq Buffer B (with 1.5 mM $MgCl_2$)	1 µL	1 X
10 mM dNTPs	1 µL	1 mM
Forward Primer (10 µM)	1 µL	1 µM
Reverse Primer (10 µM)	1 µL	1 µM
KAPA Taq Polymerase (5 U/ µL)	0.1 µL	0.5 U
Nuclease-free water	4.5 µL	
DNA template (~ 40 to 100 ng/ µL)	1 µL	
Total volume of reaction	10 µL	

GEL ELECTROPHORESIS-

Take 1.6g of gel powder weighed , pour the powder in to the conical flask and add 30ml of 1X TAE buffer ,mix it and keep in the oven for around 1 minute 15 seconds (make sure that while boiling it should not come out of the flask), repeat the process until solution becomes transparent .And allow it cool and pour the solution in to the container,(make sure that no bubbles were formed while pouring the solution in to the container), wait until it gets solidifies ,then remove the comb (without disturbing the

wells). take the sample of 3uL and 1uL of novel juice stain (This Stain was having 3 tracking dyes -Bromo Phenol Blue-400bp, Xylene Cyanol ff – 8000bp, orange g 50 bp) ,with the help of pipette. Mix all the contents twice or thrice and then load sample into the gel, add 100bp ladder (for pcr products). After loading all the samples allow it to run. After take the gel and keep in gel doc to observe the bands formed and to capture the image.

Fig. 4.5 Loading of samples in Gel Electrophoresis

GEL PURIFICATION OF PCR AMPLICON AND PREPARATION OF SAMPLE FOR SEQUENCING

The PCR products obtained using 12S primers were amplified in higher volume and run on 2% agarose gel and visualized using GeneiBlook illumination system to excise the gel piece containing the DNA band corresponding to a size of 300-400 bp. The agarose gel piece containing the band of DNA is transferred to a fresh centrifuge tube and DNA is purified from it using Qiagen Gel Purification kit. The DNA is eluted using elution buffer provided in the kit and its quantity is determined using Thermo Scientific Nanodrop One spectrophotometer. The sample was stored at (-20 ^{0} C) and thus prepared for sequencing.

CHAPTER V

RESULT AND CONCLUSION

DNA ISOLATION - QUANTIFICATION

1.Nanodrop Spectrophotometry

TABLE 5.1: Spectrophotometric quantification of samples in microliters using a nanodrop 2000 spectrophotometer showing concentration and purity ratios.

S.No	Species name	Conc(ng/µL)	A260/280	A260/230
1	Spotted deer tissue sample 1	2110.0	2.14	2.29
2	Spotted deer tissue sample 2	2042.7	2.14	2.22
3	Elephant blood sample	66.2	1.63	0.22
4	Human blood	177.4	1.86	0.59
5	Elephant bone marrow	112	1.65	0.8

DISCUSSION:

- The isolated DNA samples shows good concentration of DNA
- The purity ratios A260/280 of 1.8 and A260/230 of 2.0 is required

2. AGAROSE GEL ELECTROPHORESIS – POST DNA ISOLATION

A. DNA Isolated samples

L1 L2 L3 L4 L5 L6 L7

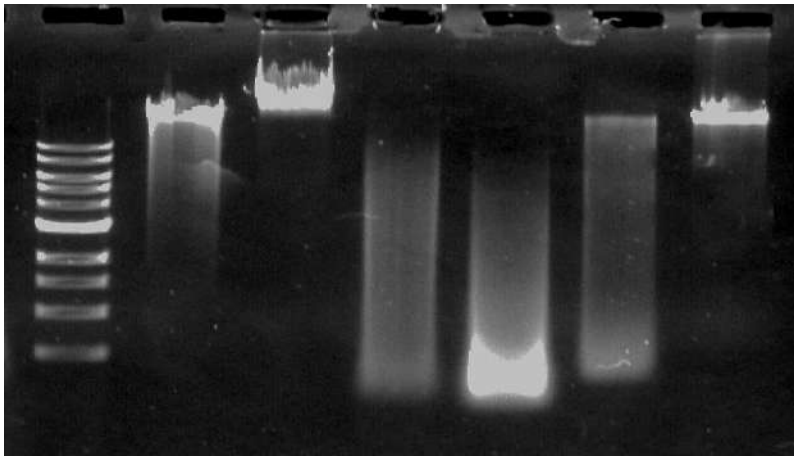


Fig 5.1 : DNA isolated from tissue, blood, scat, bone from spotted deer, elephant, tiger, human species were run on 0.8% agarose gel. Each sample of 3 μL was dyed using 1 μL of Novel juice stain.

L1 - 1Kb DNA ladder

L2 - Spotted deer tissue sample

L3 - Elephant blood sample

L4 - Tiger scat sample 1

L5 - Tiger scat sample 2

L6 - Human blood

L7 - Elephant bone marrow

DISCUSSION:

- The genomic DNA all the different species were been isolated successfully using manual method of isolation and run on an agarose gel of 0.8%
- Lane 2 of Spotted deer tissue sample appears with a crisp band of genomic DNA, which implies that DNA has been isolated with precision in handling without fragmentation of DNA molecules
- Similarly, Lane 3 of elephant blood and Lane 7 of elephant bone marrow has obtained crisp thick bands of genomic DNA
- Whereas, Lane 4 & 5 of tiger scat samples shows that the isolated DNA from the scat samples were sheared lacking intact DNA. Hence, kit-based method of isolation can be adopted to give better results without any inhibitors and contaminants

POST PCR QUANTIFIATION - AGAROSE GEL ELECTROPHORESIS

1. 12S PCR – TIGER SCAT

L1 L2 L3 L4 L5 L6

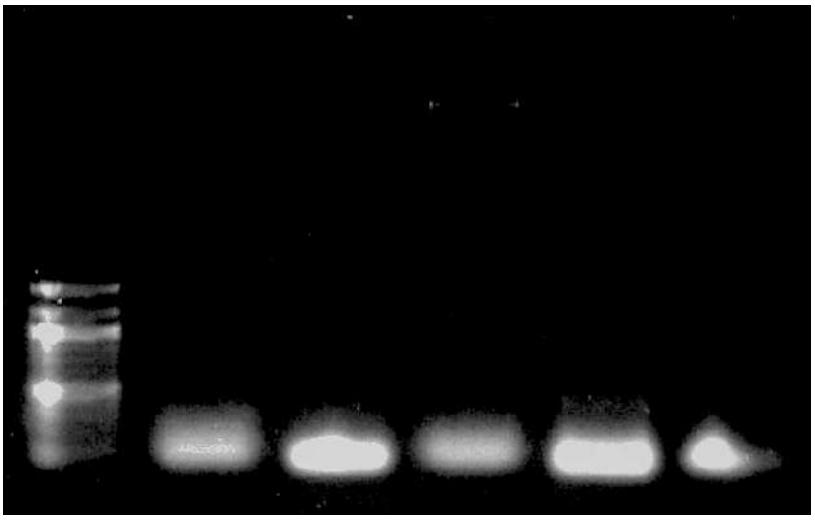


Fig 5.2: 12S PCR amplification products from Tiger Scat samples were run on 2 % agarose gel. Each sample of 3 μL was dyed using 1 μL of Novel juice stain

L1 - 100bp DNA ladder

L2 – Tiger scat 1 (no dilution)

L3 – Tiger scat 1 (1/2 dilution)

L4 – Tiger scat 2 (no dilution)

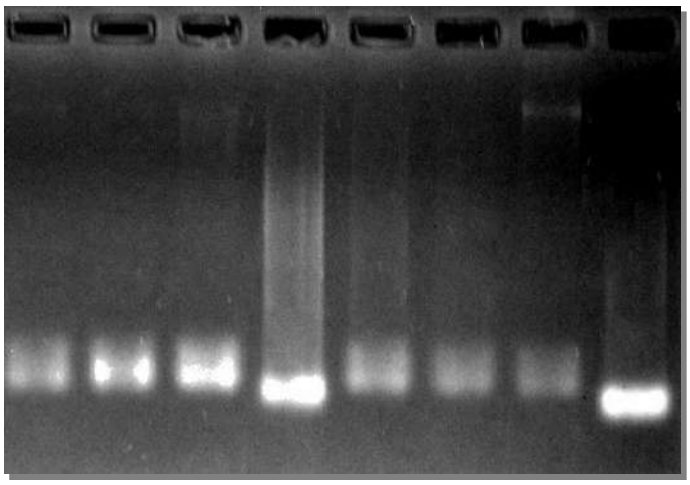
L5 – Tiger scat 2 (1/2 dilution)

L6 – Negative Control

DISCUSSION:

The above gel shows that PCR amplification of 12S gene region on tiger scat samples did not take place

2. PCR – SCAT SAMPLES OF TIGER AND PORCUPINE



L1 L2 L3 L4 L5 L6 L7 L8

L1 – Tiger scat 1

L2 – Tiger scat 2

L3 - Tiger scat 3

L4 - Tiger scat 4

L5 - Porcupine scat 1

L6 – Porcupine scat 2

L7- Positive control

L8 – Negative control

Fig.5.3: 12S PCR amplification products from tiger scat and porcupine scat samples were run on 2 % agarose gel. Each sample of 3 µL was dyed using 1 µL of Novel juice stain

DISCUSSION:

- The above gel shows that PCR amplification of 12S gene region on tiger scat and porcupine scat were sheared and having faint bands

3. PCR

L1 L2 L3 L4 L5 L6 L7 L8

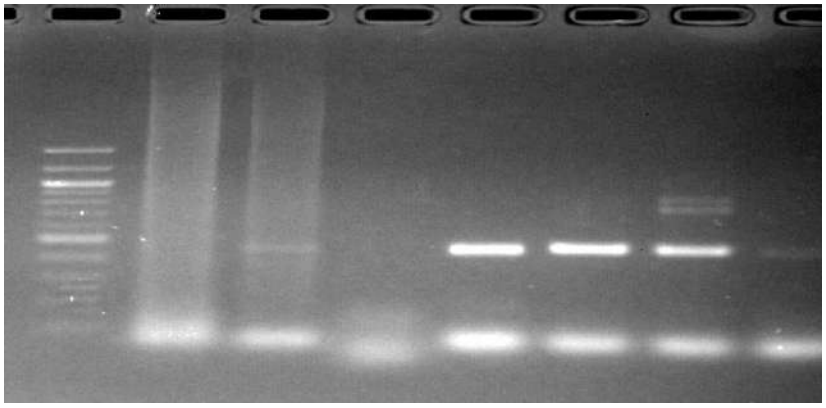


Fig. 5.4: 12S PCR amplification products from tiger scat and spotted deer tissue samples were run on 2 % agarose gel. Each sample of 3 μL was dyed using 1 μL of Novel juice stain

L1- 100bp DNA ladder

L2 – Spotted deer tissue 1 (no dil)

L3 - Spotted deer tissue 2 (1/2 dil)

L4 – Tiger scat 1 (no dil)

L5 - Tiger scat 1 (1/2 dil)

L6 - Tiger scat 2 (no dil)

L7 – Positive control

L8 – Negative control

DISCUSSION:

The above gel shows that PCR amplification of 12S gene region on spotted deer tissue and tiger scat has obtained crisp thick bands of DNA.

CONCLUSION

This study concludes that, DNA isolation and PCR with various forensic samples, like scat of Tiger, Porcupine, and Tissue of Spotted deer, Blood of human and animal (Elephant), Bone of Elephant. For all the samples DNA isolation and PCR was done, for bone I need to do PCR.

Further studies can be done on determining the amplicons for sequencing to identify species using bioinformatics tools.

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