Advances in Forensic Biology and DNA Typing

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Advances in Forensic Biology and DNA Typing examines a broad range of forensic DNA applications and topics, based on internationally recognized best practices.

As a contributed volume that includes chapters by experts from around the world, the book covers a broad range of forensic DNA applications and topics. This includes current methods for DNA extraction and typing as well as other technologies and emergent techniques in the field such as Trace and Touch DNA, Forensic DNA Phenotyping (FDP), Forensic Investigative Genetic Genealogy (FIGG), Rapid DNA Biological Fluid Identification by epigenetics, and Pharmacogenomics. The book also explores the development and usage of forensic biology for the analysis of non-human samples and the relevance of DNA databases, management systems and quality certification in forensic.

Key features:

- Highlights sources of DNA (including biological fluids, hair, bones, teeth) detailing how to address the challenges of various sample types, quantities, and environmental factors
- Presents best practices in investigative and collection procedures, as well as evaluative and testing methods, of biological samples
- Addresses both human and non-human DNA analyses and applications for both criminal and wildlife investigations

Advances in Forensic Biology and DNA Typing is a highly illustrated guide that will serve as a useful reference for forensic laboratory professionals, investigators, and students, as well as legal professionals.

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Edited by Anna Barbaro and Amarnath Mishra

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2.1 Introduction

Human identification is a key area of study in forensic science, focusing on establishing a person's identity using their body. A major milestone in this field was the discovery of the DNA double-helix structure by Watson and Crick in 1953 (Ata-Ali & Ata-Ali, 2014). This breakthrough, which uncovered the basis of genetic inheritance, led to significant advancements across various scientific disciplines. The discovery paved the way for developing techniques that can determine each individual's unique genetic makeup based on their DNA sequence (Jeffreys et al., 1985).

Three decades later, in 1985, Jeffreys et al. developed radioactive molecular probes that could identify highly variable regions of DNA (mini-satellites in the human genome), allowing for the determination of unique DNA patterns for each individual, known as DNA fingerprints (Jeffreys et al., 1985). Today, DNA profiling tests are highly reliable and widely accepted as legal evidence in courts, particularly in cases involving paternity disputes and human identification (Yadav et al., 2024). DNA can be extracted from nearly all types of human tissues, such as bone tissue, hair follicles, biopsy samples, saliva, semen, blood, teeth, and various other body tissues, although the quantity and quality of DNA obtained may vary depending on the source (Singh et al., 2024) (Figure 2.1).

Teeth are considered the most valuable source of DNA in forensic investigations because they are encased in a hard, calcified structure that shields the DNA from harsh environmental conditions (Hanaoka et al., 1995). A single tooth, when analyzed, can provide crucial information about the individual to whom it belonged. Due to the durable nature of dental tissues, teeth are highly resistant to damage from incineration, immersion, trauma, and mutilation, making them an excellent source for obtaining DNA (Singh et al., 2024). Genomic DNA, found in the cell nuclei, can be extracted from the dentin, enamel, or pulp for forensic purposes. When genomic DNA is not

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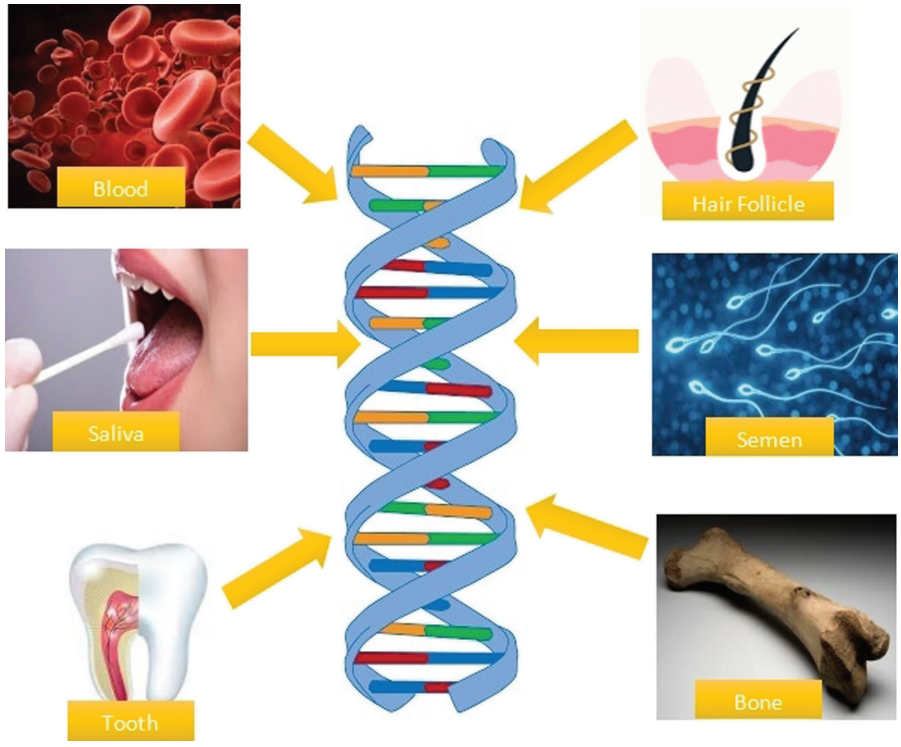

Figure 2.1 Example of various source for DNA extraction.

available, mitochondrial DNA (mtDNA), which is maternally inherited, can be used to identify siblings (Silva et al., 2007).

The primary external factors that can hinder the retrieval of information from the body remains and complicate human identification are elements associated with fire, including flames, heat, explosions, decomposition, or skeletonization (Ata-Ali & Ata-Ali, 2014). Forensic Dentistry has become increasingly significant in human identification, particularly in scenarios where limited material is available for identification (Singh et al., 2024).In such situations, teeth are crucial for identification and forensic investigations due to their highly distinctive dental characteristics and their substantial physical and chemical resistance. Because of their ability to withstand environmental changes, teeth are an excellent source of DNA, which can be invaluable for identifying an individual when traditional dental identification methods are unsuccessful. This has encouraged forensic dentists to familiarize themselves with advanced molecular biology techniques. DNA tests that are currently available have a high level of reliability and are widely accepted as legal evidence in courts (Ata-Ali & Ata-Ali, 2014), and Jeffreys et al. (1985) used

molecular typing of genetic material for the first time in England for the resolution of an immigration problem. This technique was used by them year after year to identify the rapist and murderer of two victims (Jeffreys et al., 1985).

Since then, criminalistics and forensic medicine have advanced and used DNA fingerprint molecular typing techniques as a powerful tool for solving thousands of crimes and identifying people.

Schwartz et al. (1991) isolated high-molecular-weight (HMW) proteins from teeth under various challenging conditions, including different pH levels, humidity, temperature, and storage methods. They demonstrated that these conditions did not affect the integrity of the protein, further confirming that teeth effectively protect DNA. DNA extracted from teeth can be amplified using the polymerase chain reaction (PCR) to increase the DNA sequence for identification purposes (Pretty & Sweet, 2001). Only about 2%–5% of the DNA is involved in coding for proteins, while the remaining 95%–98% is non-coding or "junk" DNA. Variations in DNA sequences, known as polymorphisms, can be used for individual identification. Radicular pulp tissue is a particularly good source of DNA (Schwartz et al., 1991).

Additionally, odontoblastic processes extending into the dentinal tubules, cellular cementum, soft tissues in accessory canals, and periodontal ligament fibers can also be utilized for DNA extraction (Malaver & Yunis, 2003). Various techniques have been developed to extract DNA from teeth, with the preferred method being a horizontal section through the cervical area (Ata-Ali& Ata-Ali, 2014). This approach allows the use of rotary instruments to obtain material from the inner dentine of the root canals while preserving the coronal structure, which is important for morphological identification.

2.2 Genomic and Mitochondrial DNA

Genomic DNA, located in the nucleus of every cell in the human body, serves as a primary source for most forensic applications (Silva et al., 2007). Teeth are particularly useful for extracting genomic DNA because PCR analysis enables comparison of collected post-mortem samples with known ante-mortem samples or parental DNA.

mtDNA is another type of material that can be extracted and be used to identify a body. Its primary advantage lies in its high copy number per cell, ranging from hundreds to thousands of organelles (Silva et al., 2007). In cases where DNA samples are minimal or degraded, such as those obtained from skeletonized remains, the chances of obtaining a DNA profile from mtDNA are higher than from any markers found in genomic DNA. The analysis of mtDNA for forensic purposes is typically limited to ancient tissues like bones, hair, and teeth, where nuclear DNA cannot be analyzed. However, this type

of examination involves direct sequencing of nitrogenous bases, making it a costly procedure due to the advanced technology required (Schwartz et al., 1991). Additionally, mtDNA is inherited solely from the mother, making it less informative, which is why it is not commonly used in all forensic laboratories for crime resolution and person identification.

For forensic samples, DNA analysis (both genomic and mitochondrial) is commonly performed using short tandem repeats (STRs), which are hypervariable regions of DNA characterized by consecutive repetitions of fragments consisting of two to seven base pairs (Silva et al., 2007). Variable number tandem repeats (VNTR) testing, which involves repeated sequences of intermediate length (15–65 base pairs), is rarely used in forensic analyses due to the poor quality of DNA typically obtained with this method (Silva et al., 2007). The most effective STRs for human identification are those that exhibit high polymorphism (a greater number of alleles), smaller size (in base pairs), a high frequency of heterozygotes (over 90%), and a low mutation rate.

2.3 Distribution of DNA in Teeth

To establish effective sampling protocols for DNA extraction from teeth, it is crucial to understand tooth morphology, the distribution of DNA within different tooth structures, and the impact of post-mortem changes. This knowledge helps select the optimal tooth for sampling and determine specific areas within a tooth to target for DNA extraction (Malaver & Yunis, 2003).

Human teeth are anatomically divided into the crown, which is visible in the mouth, and the roots, embedded in the jawbone. Roots, composed mainly of cementum and dentine/pulp, generally yield more DNA than the crown (Higgins & Austin, 2013). The crown, while containing some dentine/pulp, is mostly made of enamel, which is the hardest tissue in the human body and does not contain DNA. Enamel protects the inner tissues of the tooth from external conditions such as heat, UV light, and microbial contamination (Malaver & Yunis, 2003).

The dentine/pulp complex makes up the bulk of the tooth and is highly cellular. The pulp contains a variety of cells, including odontoblasts, fibroblasts, defense cells, nerve cells, and undifferentiated mesenchymal cells. Dental stem cells can be obtained from periodontal ligament, dental pulp, and apical papilla,making it a rich source of DNA (Jain et al., 2020). However, in older or diseased teeth, pulp tissue may be limited or absent. Dentine, which contains fewer nucleated cells, has a unique structure with tubules that house odontoblastic processes and nerve fibers rich in mtDNA (Higgins & Austin, 2013). Dentine is generally a poor source of nuclear DNA, except in cases where pulp remnants are present.

Cementum, which covers the roots, is an avascular mineralized tissue that contains cementocytes (cells similar to bone cells) within its matrix, making it another valuable source of nuclear DNA. Cementum can also include other sources of DNA, such as soft tissue inclusions and blood residues.

Overall, pulp and cementum are the most valuable sources of nuclear DNA, while both these tissues and dentine provide good mtDNA sources (Higgins & Austin, 2013).Enamel is important for protecting these tissues, but it lacks DNA. The presence of enamel can dilute DNA concentrations and complicate the extraction process. Optimizing tooth selection and targeted sub-sampling can enhance DNA profiling success rates, improving the effectiveness of forensic analyses (Malaver & Yunis, 2003).

2.4 Guidelines for Extracting DNA from Dental Samples

Sample Collection: If there is any soft tissue or blood on the tooth, it should be sampled.

Cleaning the Tooth: Remove any plaque or calculus using a curette, then thoroughly clean the tooth with hydrogen peroxide, followed by ethanol.

Accessing the Pulp: If the tooth is intact and recently extracted from the alveolus, standard endodontic access and instrumentation can be performed. Sectioning the tooth may allow better access to the pulp.

Collecting Pulp Tissue: After opening the tooth, the walls of the pulp chamber should be curetted or instrumented with a slow rotary burr. The collected pulp tissue should be placed in a sterile, wide-mouthed tube.

Handling Dried Specimens: For dried specimens, where the pulp may appear mummified or parchment-like, the chamber should be irrigated with a buffer solution after instrumentation. The liquid can then be ultra-filtered in the laboratory to extract the necessary cellular material for DNA analysis.

Tooth Crushing: In some cases, it may be necessary to crush the tooth to access the DNA but it destroys the morphology of teeth. However, crushing gives better results than sectioning as more DNA can be obtained (Chaudhary et al., 2020) and (Nandini & Joji, 2020).

Other methods are as follows:

- Conventional endodontic access
- Vertical splitting
- Horizontal section
- Cryogenic grinding.

2.5 DNA Extraction and PCR Amplification

For forensic purposes, an ideal DNA extraction method should maximize DNA yield, be cost-effective and time-efficient, involve minimal steps, and be suitable for automation. Fewer steps in the extraction process reduce the risk of contamination. When working with degraded skeletal remains, it is essential to recover trace amounts of DNA while removing potential inhibitors and minimizing contamination by external DNA (Stavrianos et al., 2010).

As previously stated, several protocols are used for DNA extraction and analysis, and no standard methodology exists. As a result, researchers must carefully assess the conditions of the material to be examined, particularly when dealing with forensic cases, where there is a higher risk of sample contamination and the influence of environmental factors, in addition to a limited amount of material available in most situations.The PCR technique has traditionally been used to investigate the frequencies of STRs (Chaudhary et al., 2020) (Figure 2.2). Teeth exposed to the environment may be contaminated with bacteria, fungi, and substances like humic acid, fulvic acid, and metals, which, if co-extracted with endogenous DNA, could inhibit PCR amplification (Ahmed et al., 2014). This method enables the amplification of restricted regions of the human genome, which is associated with genomic hybridization. Recent advancements in the technique of length amplification of polymorphic fragments have increased the potential for forensic sample analysis. PCR method enables the differentiation of an individual from another, with a high level of reliability and with about 1 ng (one one-billionth of a gram) of the target DNA (Chaudhary et al., 2020).

In practice, the adequacy of collection procedures, verification of the conditions of the collected material, selection of methodology for DNA extraction and analysis, and finally analysis of results are steps aimed at reliable results that may contribute to elucidating forensic cases (Stavrianos et al., 2010).

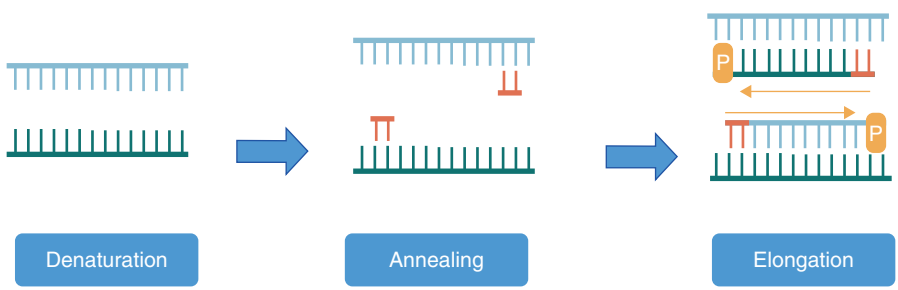

Figure 2.2 3 Main steps of each Polymerase Chain Reaction (PCR) cycle.

It should be noted that DNA extraction is a three-step process that includes the following:

- Cell rupture or lysis (which allows the use of several techniques for effective cell membrane rupture)
- Protein denaturation and inactivation (by chelating agents and proteinases to inactivate elements such as proteins)
- DNA extraction

Various DNA extraction techniques have been developed for mineralized tissues, including phenol–chloroform, chelex, silica, and magnetic bead systems. Before extraction, decalcifying mineralized tissues by soaking them in Ethylene Diamine Tetra-Acetic Acid (EDTA) for varying durations has been suggested. Decalcification helps release DNA by dissolving the mineral matrix and allowing enzymatic digestion of the organic matrix. Some studies suggest that retaining the EDTA phase during decalcification increases DNA yield, although this requires additional handling steps, which could increase contamination risk and make the process less suitable for automation (Stavrianos et al., 2010).

If pulp tissue is available, demineralization may not be necessary; if only hard tissues remain, it could be beneficial. However, adequate DNA can sometimes be extracted without prior decalcification, depending on the condition of the tooth.

The organic method (composed of phenol–chloroform and used for high-molecular-weight DNA, with a higher likelihood of errors due to the use of multiple tubes) is preferred for extracting DNA from human teeth and bone. Organic methods, while effective and less costly, involve hazardous chemicals, whereas silica methods are safer and more suitable for automation. Silica-binding methods use guanidinium-based salts to disrupt proteins and bind DNA to silica, effectively preventing co-extraction of inhibitors (Chaudhary et al., 2020).

However, these methods are sensitive to pH changes, which need to be carefully controlled. Chelex 100 (the fastest with the lowest risk of contamination, but very expensive); FTA paper (composed of absorbent cellulose paper with chemical substances, which speed up its use); as well as isopropyl alcohol (containing ammonium and isopropanol, which is less expensive and also an alternative to the organic method) (Nandini & Joji, 2020).

An important observation is that when degraded samples of ancient DNA are the only artifacts, techniques to overcome contamination and degradation of DNA samples are required. The factors that contribute to DNA degradation include time, temperature, humidity (which promotes the growth of microorganisms), light (both sunlight and UV light), and exposure to

various chemical substances. Combinations of these conditions are common in nature and tend to degrade samples into smaller fragments. As a result, depending on the type of biological material, a sample must be dried (or remain dry) after collection. It can also be frozen (if necessary), though this is less important for DNA than it is for conventional proteins and enzyme systems. The sample should not be subjected to fluctuations in either temperature or humidity (Ahmed et al., 2014).Grinding entire teeth for extraction requires extensive demineralization, increasing contamination risk and complexity. However, selecting specific tooth parts and sub-sampling, especially targeting pulp tissue, can simplify the process and minimize the need for demineralization, making the extraction more efficient and less prone to contamination (Chaudhary et al., 2020).

2.6 Applications of DNA in Forensic Dentistry

The applications of DNA from teeth in forensic odontology enhance the ability to solve crimes, identify unknown individuals, and provide critical evidence in legal proceedings. The pH (3.7 and 10.0), as well as the temperature (4°C, 25°C, 37°C, and tooth incineration), humidity (20%, 66%, and 98%), the type of soil in which the teeth were buried (sand, potting soil, garden soil, immersion in water, and outdoor burial) as well as periods of inhumation (one week to six months) effect the concentration of DNA obtained from the environment (Silva et al., 2007) and (Ahmed et al., 2014). It was determined that the examined environmental conditions did not impair the ability to obtain HMW human protein DNA extracted from dental pulp. DNA extracted from teeth can help identify unknown human remains, particularly in cases of mass disasters, fires, decomposed bodies, or skeletal remains.

Among the numerous cases of DNA isolation from teeth described in the literature (Sweet & Sweet, 1995) published a very important report in 1995. They described a case of human remains identification in which a murder victim was incinerated and her body was almost completely carbonized, reduced to approximately 25% of its original size, which then precluded DNA analysis using conventional methods (Saranya, 2014). A preserved unerupted third molar, on the other hand, allowed DNA extraction from the dental pulp (1.35 g), which provided an excellent source of high-molecular-weight genomic DNA.

DNA obtained from teeth can be used to establish familial relationships through genetic profiling (Malik et al., 2022). This application is particularly useful in cases where direct DNA samples from individuals are not available, and comparisons must be made with potential relatives to confirm the identity of the deceased. Analysis of mtDNA from teeth can provide

information on the maternal lineage of an individual (Silva et al., 2007). This type of analysis is useful in forensic investigations for determining ancestry or geographic origin, especially when the individual is of unknown descent (Shah et al., 2019). Teeth provide a stable DNA source for long-term storage, allowing for genetic analysis years after the crime or event.

Aside from human identification, another aspect of Forensic Dentistry that is related to molecular biology is the analysis of bite mark evidence (Sweet & Hildebrand, 1999). In cases of physical assault, such as sexual abuse, murders, sexual assault, child abuse, bite marks are frequently found on the skin (Ata-Ali & Ata-Ali, 2014). Genetic analysis of saliva or epithelial cells left in a bite mark can link a suspect to the crime, providing compelling evidence in court.

Nonliving objects like clothing, food items, cigarettes, cigars, other smoking devices, oral health tools, beverage containers, dental appliances, postage stamps, and envelopes can serve as accessible sources for saliva collection (Silva et al., 2006). During biting, kissing, or suction, the aggressor's saliva is usually deposited on the victim's skin. Extracting DNA from saliva on personal items often depends on the specific protocols of the laboratories handling the genetic material, especially when processing a bite mark for DNA analysis, (Sweet & Hildebrand, 1999). The buccal swab technique involves collecting exfoliated oral mucosal epithelial cells for DNA analysis. Saliva samples collected from crime scenes and subsequent DNA analysis can help investigators link the suspect to both the crime scene and the victim. The buccal swab offers a meticulous and less invasive alternative to blood samples for DNA collection (Sweet & Hildebrand, 1999). Several studies are currently underway to optimize the methodology of DNA extraction from saliva deposited on the skin for use as evidence in forensic cases, such as double-swab testing.

In cases where the sex of an unidentified individual is not apparent from the skeletal remains, DNA analysis of teeth can be used to determine sex, using markers on the X and Y chromosomes (Silva et al., 2007). This information can aid in narrowing down the identity of the deceased (Silva et al., 2007).Although DNA analysis itself does not provide direct age information, combining genetic analysis with dental histology and other forensic techniques can help estimate the age of an individual at the time of death, which is valuable in the identification process.

The ABO system can identify the aggressor's blood group in 90% of cases, but this method is not very informative and would not be used if DNA amplification techniques like STR profiling were available (Saranya, 2014). It is also possible to isolate DNA from these cells in order to identify the aggressor. Teeth can retain residues of drugs or toxins. DNA analysis, coupled with chemical analysis of teeth, can provide information on drug use, exposure to

toxins, or cause of death, which is crucial in forensic toxicology (Malik et al., 2022). The versatility and reliability of teeth as a DNA source underscore their importance in forensic science, helping to solve crimes, identify victims, and provide critical evidence in legal proceedings.

2.7 Limitations

Molecular methods are highly accurate, reproducible, and unique and are extremely reliable in forensic science. Drawbacks, however, do exist in this relatively new methodology. Errors may develop in sample collection, processing, and interpretation. The process of extracting DNA from teeth can be complex and time-consuming, often requiring specialized equipment and techniques. Some methods, such as those involving decalcification or multiple handling steps, increase the risk of DNA loss or contamination and are not easily automated. Any bacterial contamination and a second person's DNA can alter the interpretation. While processing, too little amount of DNA can produce less-intense bands which can cause misinterpretation of results. Also, degraded samples can produce a very scant amount of high-molecular-weight DNA. The process of extracting DNA from teeth can be complex and time-consuming, often requiring specialized equipment and techniques. Some methods, such as those involving decalcification or multiple handling steps, increase the risk of DNA loss or contamination and are not easily automated. Teeth contain natural substances such as calcium, collagen, and certain proteins that can inhibit PCR. Additionally, environmental contaminants like humic acids, metals, and other materials may also interfere with the extraction and analysis processes (Malik et al., 2022). The process of DNA extraction and analysis from teeth, particularly when advanced techniques are needed, can be expensive and resource-intensive. This can limit the feasibility of such analyses in resource-constrained settings or for routine applications.

Despite these limitations, DNA from teeth remains a valuable resource in forensic odontology. Continued advancements in technology and methodologies will help address some of these challenges, improving the reliability and efficiency of forensic DNA analysis.

2.8 Future Prospects

Although tooth tissues are increasingly being used in forensic investigations, there is limited reliable information about the decomposition of these mineralized tissues, the location of DNA after post-mortem changes, or the

effectiveness of various sampling techniques (Verma et al., 2014) and (Quadri et al., 2023). While research has been conducted on the interaction of DNA with minerals in bone, similar studies on teeth are lacking. Further investigation is needed to understand the interaction between tooth minerals and DNA as well as how this interaction changes in a post-mortem environment. Potential mechanisms for DNA preservation in teeth, such as encapsulation within the pulp chamber, binding to minerals, and binding to collagen, have not been thoroughly investigated (Verma et al., 2014). Techniques such as laser micro-dissection or the use of novel reagents that selectively extract DNA from surface cells may provide alternatives to traditional, more destructive methods (Quadri et al., 2023) and (Menon & Kumar, 2021). Understanding these mechanisms is crucial for developing effective sampling and extraction techniques. Evaluating the post-mortem changes in teeth over a period relevant to forensic investigations would enhance the selection of tissues for DNA extraction and inform the choice of extraction techniques, thereby improving the efficiency of the process. This knowledge would also aid in determining optimal decontamination methods.

Future protocols may integrate rapid DNA analysis technologies, allowing for quicker identification of victims, especially in disaster scenarios where timely identification is critical (Menon & Kumar, 2021).Innovations in computational algorithms and data analysis will enable more precise interpretation of DNA profiles, reducing the likelihood of errors and improving the accuracy of matches. It will increasingly be integrated with other forensic techniques, such as fingerprint analysis, facial recognition, and digital forensics, to provide a comprehensive approach to solving crimes (Harisha et al., 2023). These advancements will not only improve the accuracy and reliability of forensic investigations but also contribute to a more comprehensive understanding of human remains in various forensic contexts.

2.9 Summary

Violence and crimes against human life, such as bomb explosions, wars, or plane crashes, as well as cases of carbonized bodies or bodies in advanced stages of decomposition, highlight the need for ever faster and more accurate methods of identifying victims. In such cases, the findings of the several studies show that teeth are an excellent source of DNA, which is protected in case of incineration by epithelial, connective, muscular, and bone tissues. Enamel, dentin, and cementum hard dental tissues also protect the dental pulp cells.

As a result, dental professionals working in the field of Forensic Dentistry should incorporate these new technologies into their work, as there are several

methods available for DNA extraction from biological materials, yet standardization of the protocols adopted for such purpose has not been reached so far. For this reason, studies on molecular biology applied to human identification will probably further enhance DNA extraction with less material available and under increasingly adverse conditions.

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