UNIVERSIDADE FERNANDO PESSOA

DETERMINAÇÃO DO DIMORFISMO SEXUAL ATRAVÉS DE MÉTODOS MOLECULARES: UMA REVISÃO DE ESCOPO

[Determination of sexual dimorphism trough molecular methods: a scoping review]

Dissertação de Mestrado

Mestrado Integrado em Medicina Dentária

Clarisse Marie Dupuis

Orientadora: Doutor Maria Inês Guimarães

Coorientadora: Doutor Inês Lopes Cardoso

Junho 2024

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I dedicate this thesis to my uncle, Thierry, and my grandfather, Marcel.

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Resumo

Introdução: O dimorfismo sexual é de importância fulcral nas investigações forenses. Vários métodos moleculares que utilizam a amelogina, uma proteína presente no esmalte dos dentes, podem ser utilizados para determinar o dimorfismo sexual, tais como: extração de ADN de dentes, amplificação por PCR do gene codificante da amelogenina e posterior análise do tamanho dos productos de PCR para identificar os cromossomas X e/ou Y.

Objetivo: O objetivo desta revisão de escopo foi explorar o trabalho científico que lida com a utilização do gene codificante da amelogenina na determinação do sexo aplicada a odontologia forense. Por conseguinte, o objetivo é responder à questão de investigação: Os métodos moleculares permitem a determinação do dimorfismo sexual para a identificação forense?

Material e Métodos: Foi efetuada uma revisão da literatura publicada entre 1996 e 2024, utilizando as bases de dados eletrónicas *PubMed, MEDLINE (via BVS)* e *CINAHL* (via EBSCO host). Foram aplicados critérios de inclusão e exclusão para selecionar as publicações mais relevantes, e esta seleção encontra-se resumida no fluxograma *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA). Adicionalmente, foi desenvolvida uma estratégia PCC (População-Conceito-Contexto) para formular a questão de investigação.

Resultados: De acordo com os critérios de inclusão e exclusão estabelecidos, foram selecionados para este estudo 10 artigos dos 1091 inicialmente considerados. Estes artigos exploram a ligação entre a medicina dentária forense e a determinação do sexo através da identificação da amelogenina. Todos estes artigos dizem respeito a investigação in vitro e são classificados de acordo com as categorias "com tratamento" (6 estudos) e "sem tratamento" (4 estudos).

Conclusão: Os métodos moleculares baseados na identificação do gene codificante da amelogenina, presente nos cromossomas X e Y, constituem uma abordagem precisa e fiável para determinar o sexo de um indivíduo.

Palavras-chave: medicina dentária forense; análises, determinação do sexo; análise, determinação do sexo; amelogenina.

Abstract

Introduction: Sexual dimorphism is of central importance in forensic investigations. Several molecular methods using amelogenin, a protein found in tooth enamel, can be used to determine sexual dimorphism such as: extraction of DNA from teeth, PCR amplification of the gene coding for amelogenin and subsequent analysis of the size of PCR products to identify the X and/or Y chromosomes.

Objective: The goal of this Scoping review was to explore the scientific work dealing with the use of the gene coding for amelogenin in sex determination applied to forensic dentistry. Therefore, the aim is to answer the research question: Do molecular methods allow the determination of sexual dimorphism for forensic identification?

Material and Methods: A review of literature published between 1996 and 2024 was conducted using the electronic databases PubMed, MEDLINE (via BVS) and CINAHL (via EBSCO host). Inclusion and exclusion criteria were applied to select the most relevant publications, and this selection is summarized in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart. Additionally, a PCC (Population-Concept-Context) strategy was developed to formulate the research question.

Results: According to the criteria stated for inclusion and exclusion, 10 articles out of the 1091 initially considered were selected for this study. These articles explore the connection between forensic dentistry and sex determination via amelogenin identification. All these articles are in vitro research and were classified according to the categories "with treatment" (6 studies) and "without treatment" (4 studies).

Conclusion: Molecular methods based on the identification of the gene coding for amelogenin, found in both the X and Y chromosomes, provide an accurate and reliable approach to determine the sex of an individual.

Key-words: forensic dentistry; forensic odontology; analyses, sex determination; analysis, sex determination; amelogenin.

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Lists of Abbreviations, Acronyms or Symbols

AMEL: Amelogenin

APLP: Amplified Product-Length Polymorphism

bp: Base pair, the unit of length in genetics representing the length of a DNA double helix segment

CGE: Capillary Gel Electrophoresis

CGTA: Canonical sequence of nucleotide bases (Cytosine, Guanine, Thymine, Adenine)

D1S80: DNA segment on chromosome 1

DNA: Deoxyribonucleic acid

DYS14: Y-chromosome-specific gene

DYZ3: Y-chromosome-specific alpha satellite DNA

HLADQA1: Human Leukocyte Antigen DQ Alpha 1 gene1

HUMFES/ FPS: Human Feline Sarcoma Oncogene homolog

HUMTH01: Human Tyrosine Hydroxylase gene

LAMP: Loop Mediated Isothermal Amplification

Mg^{2+}: Magnesium ion

PAGE: Polyacrylamide Gel Electrophoresis

PCR: Polymerase chain reaction

SNP: Single Nucleotide Polymorphism

SRY: Gene coding for the sex-determining region Y protein

STR: Short Tandem Repeats

UV: Ultraviolet

ZF: Zinc Finger proteins

1. INTRODUCTION

Forensic Dentistry represents an essential convergence between dentistry and legal imperatives, providing specialized expertise in the application of dental knowledge to solve forensic puzzles. At the heart of this discipline is the accurate identification of individuals, often based solely on their dental impressions. In fact, forensic odontology stands out as a specialized branch of forensic medicine that focuses on recognizing individuals from their unique dental structures, often when other means of identification, such as fingerprints or DNA (Deoxyribonucleic acid), are not available or are insufficient. Historically, digital fingerprints have been used for identification, but they deteriorate rapidly (Musse et al., 2008). In the modern world, facing a multitude of tragic events, such as acts of terrorism, bombings, earthquakes, hurricanes, tsunamis, train crashes, air crashes and other accidents of transport, bodies can suffer so severe damage that may become visually unrecognizable. In such circumstances, teeth prove to be an invaluable resource for the extraction of DNA, thanks to their remarkable resistance to multiple environmental attacks such as: cremation, immersion, trauma, mutilation and decomposition. In the absence of ante-mortem data, exact identification becomes even more complex. In these cases, DNA profiling systems become crucial in revealing the identity of individuals. Comparing DNA extracted from the teeth of an unidentified person with that collected from antemortem samples, such as stored blood, toothbrushes, clothing, cervical samples or biopsies, or even with samples from parents or siblings, is the standard method for analyzing DNA and making matches (Manjunath et al., 2011).

Teeth, being the strongest structures in the human body, often persist long after the destruction of soft and skeletal tissues by their inert and mineralized structures (Zapico et al., 2013). Their ability to withstand temperatures ranging from 150°C to 450°C makes them particularly robust due to their high content of inorganic substances (Urbani et al., 1999). Enamel and dentin are highly calcified, making them an excellent source of DNA (Dutta et al., 2017).

Dental enamel is distinguished from other mineralized tissues by its high mineral content, and unlike other tissues, it does not consist of collagen, nor does it undergo resorption or remodeling. Its formation is the result of a temporary network of enamel matrix proteins that regulate the growth and orientation of hydroxyapatite crystals. Similarly, enamel is

an equally interesting structure, since its matrix contains an essential protein, amelogenin, which plays a crucial role in enamel formation. It exists in various isoforms, generated by alternative splicing of the pre-RNA of amelogenin. These isoforms can combine to form spherical structures called nanospheres, with an average diameter of around 20 nm, composed of around 100 amelogenin molecules. There are significant differences in the size and nucleotide sequence pattern of amelogenins between male and female enamel, providing a useful sex marker for the identification of DNA samples from unknown human skeletal or dental remains. In fact, these differences make possible to distinguish males from females (Praveen Kumar et al., 1998).

Likewise, the pulp tissue, located inside the tooth and well protected by the hard tissues of enamel and dentin, which contain a diversity of cells and components, such as fibroblasts, odontoblasts, endothelial cells, peripheral nerves and undifferentiated mesenchymal cells, as well as nucleated elements of the blood present in the coronal and radicular pulp, also provides an abundant source of DNA preserved from any contamination (Chowdhury et al., 2018). Even after extreme conditions such as incineration, pulp DNA can be recovered and used for analysis, although the reliability of this method may vary depending on the circumstances (Williams et al., 2004).

Furthermore, permanent teeth are distinguished from primary teeth by several characteristics that could influence the stability of DNA in the event of exposure to adverse environmental conditions. Their morphological differences include higher levels of mineralization, reduced enamel permeability, lower organic matter content, and larger pulp chambers. These particularities contribute to a better preservation of DNA in permanent teeth in comparison to deciduous teeth (Williams et al., 2004).

Sex determination from fragmented or degraded DNA samples is a major challenge, as sample quality and quantity are crucial for DNA testing. Ancient samples, often broken down into small fragments and limited quantities, result from DNA degradation due to environmental factors and microbial attack. This fragmentation also makes forensic investigations difficult. Depurination, caused by the hydrolysis of N-glycosidic bonds, is a major mechanism of DNA fragmentation, inducing baseless sites that render DNA molecules unstable. Additionally, postmortem chemical reactions, such as deamination, can alter DNA, leading to unexpected base substitutions and pairings that compromise the reliability of DNA testing. Base substitutions due to deamination can also induce nitrogenic bases transitions, such as C → T and G → A, which are common in degraded

samples. These changes can lead to false or negative results in single nucleotide polymorphism (SNP) analyses, further complicating sex determination from fragmented DNA. To overcome these challenges, SNP analysis is preferable because SNP primers generate short amplicons, compatible with highly fragmented samples. Furthermore, a SNP primer design strategy suitable for highly degraded samples is crucial to obtain accurate results in sex determination from fragmented DNA (Masuyama et al., 2017).

Among the various methods available, amelogenin-based sex test is widely preferred in forensic applications (Masuyama et al., 2017). This gender typing system targets the gene coding for amelogenin, and thus amplifies only a short DNA sequence. It is particularly useful when biological samples are degraded or damaged, for example by fire or explosion. A significant portion of this DNA sequence is highly conserved and amplification of the amelogenin X-Y genes produces two different fragments that can be distinguished by their different sizes (Urbani et al., 1999). PCR analysis, specifically targeting regions of the *AMEL* gene, has become the preferred method for determining the sex of biological samples (Dutta et al., 2017). This technique offers an effective and sensitive approach, allowing a sex-specific sequence to be amplified and thus gender to be accurately determined (Praveen Kumar et al., 2016).

This work intends to carry out a scoping review to determine the sex of a person whose identity cannot be determined by other methods. To do this, molecular methods based on the *AMEL* gene, present on the X and Y chromosomes, will be evaluated.

2. DEVELOPMENT

2.1. State of the art

Sex refers to a set of biological attributes including anatomical, physiological, and genetic characteristics such as chromosomes, gene expressions, hormonal levels and reproductive anatomy. In contrast, gender concerns characteristics associated with femininity and masculinity, as well as socially constructed roles, behaviors, expressions, and identities. It is essential to note that chromosomal sex, determined at birth, is either male (XY) or female (XX), while gender is recognized as a spectrum. To understand the sexual dimorphism observed in human teeth and skeletons, it is necessary to understand the impact of chromosomal sex on human growth and development. Sexual differentiation occurs in several sequential stages: genetic, gonadal, hormonal, and phenotypic. At the genetic stage, chromosomal sex is determined now of fertilization, where XY indicates a male individual and XX indicates a female individual. The presence of the Y chromosome determines male sex, while its absence determines female sex. These chromosomal differences influence the development of the gonads, giving rise to testes in males and production of testosterone, or ovaries in females. As the sexual organs develop, hormonal and phenotypic differences start to develop in the human body, leading to a greater distinction between the male and female sexes (Heng et al., 2022).

Estimating sex from teeth relies on the detection of the Y chromosome in males or the X chromosome in females. These sex identification methods fit into a broader dental identification framework, including metric, non-metric and biochemical approaches, each with their advantages and limitations in terms of accuracy and applicability (Heng et al., 2022). It is possible to merge various approaches, such as metrics and biochemistry, to determine sex. Dental morphology analysis combined with the analysis of genomic and mitochondrial DNA extracted from teeth can be used for this purpose. Human teeth display significant morphological variability, but the presence of mesiodens, supernumerary teeth, can be particularly valuable for identifying sex through DNA analysis. Amelogenin (AMEL), a crucial protein secreted by enamel ameloblasts, exists in different isoforms in tooth matrix and bone cells. The *AMEL* gene, located in the X and Y chromosomes, allows the identification of sex chromosomes through the isolation of DNA from mesiodens and using the identification of the *AMEL* gene to determine sex (Srivastava et al., 2017). Traditionally, sex estimation from teeth involved the isolation of

dental pulp DNA and amplifying the alphoid repeat sequences specific of X and Y chromosomes by polymerase chain reaction (PCR), with subsequent analysis of the PCR products using polyacrylamide gel electrophoresis (PAGE) or denaturing PAGE. However, a newer method, capillary gel electrophoresis (CGE), has emerged, offering advantages such as automation of analyzes and direct recording of data without the need for staining or photography (Komuro et al., 1998). Moreover, different techniques are used to identify gender from DNA, such as visualization of restriction digestion patterns, hybridization with Y chromosome-specific probes, as well as a simple and ultra-sensitive sex test based on the amplification of part of the *AMEL* gene. This test can typify even small amounts of DNA from various samples such as blood, muscle and degraded bone. Additionally, it is possible to detect low amounts of male DNA in excess female DNA by labeling PCR products with a dye and measuring relative fluorescence. The development of a combined identification and gender test by co-amplification of HLA-DQA1 is also in discussion, opening the way to more complex analyses. Finally, different PCR-based methodologies offer advantages in terms of speed and sensitivity, including allowing the amplification of multiple or single copy sequences. However, quantifying DNA from the X and Y chromosomes remains complex due to differences in copy number and amplification efficiency. An alternative is to amplify single-copy X-Y homologous regions, such as amelogenin, for rapid sex analysis with sufficient sensitivity (Mannucci et al., 1994).

Furthermore, several PCR-based approaches have also been developed to determine sex by targeting specific Y chromosome sequences, such as *DYS14* and *DYZ3*. Key genes, such as the gene coding for the sex-determining region Y protein (*SRY*), are also used as genetic markers. Additionally, other DNA-based sex typing methods have been suggested, targeting homologous X-Y genes with insertions or deletions, such as centromeric alphoid repeats, short tandem repeats (STR), genes of zinc finger (ZF) proteins and amelogenin genes (Mannucci et al., 1994).

The loop-mediated isothermal amplification (LAMP) method can also be used to determine sex from difficult-to-analyze samples. It offers a faster and specific approach to amplify DNA under isothermal conditions. This method uses a DNA polymerase with template displacement activity and a set of 4 or 6 specific primers to recognize multiple sequences on the target DNA (Nogami et al., 2008). However, the determination of sex from biological samples from dental tissues has been considerably improved thanks to

the use of the PCR technique, which makes it possible to amplify small quantities of DNA, thus making the analysis of dental remains more reliable and efficient (Sivagami et al., 2000).

One of the genetic markers commonly used in this method is the amelogenin gene. The protein coded by this gene is a major constituent of developing tooth enamel and two distinct copies of this gene are located on the X and Y chromosomes in humans. The presence of these two genes allows accurate sex determination, as they have significant structural differences. The amelogenin gene homologs are located on the Xp22.1–Xp22.3 regions of the X chromosome and and the second copy was located near the centromere of the y chromosome (Dutta et al.,2017).

The PCR method for amelogenin gene amplification is widely used and has been shown to be effective in several studies. In the study of Zapico et al. (2013), most samples showed successful amplification and therefore reliable results for sex identification, even those containing low concentrations of DNA. Donor sex was correctly identified in all samples, regardless of type of tissue used, thanks to amelogenin markers. This highlights the reliability and usefulness of the method for forensic and genetic applications (Zapico et al., 2013).

In addition to amelogenin, other markers and genetic loci are explored to complement and confirm the sex determination results. For example, SNP analysis, particularly useful in complex cases where the *AMEL* gene alone may not provide conclusive results due to mutations or deletions. PCR-Amplified Product-Length Polymorphism (APLP) is an effective technique for SNP typing and sex determination, particularly useful in settings where DNA is fragmented or degraded. This method uses allele-specific primers and a counterprimer to detect SNP variations by amplicon length difference, providing a reliable solution for forensic and genetic applications (Masuyama et al., 2017).

In conclusion, the application of advanced PCR techniques and the exploration of additional genetic markers have significantly improved the reliability and accuracy of sex determination from dental tissues in forensic science. These technological advancements ensure that even the most difficult and degraded samples can be analyzed with a high degree of confidence, contributing to the resolution of forensic cases and the administration of justice.

2.2. Material and Methods

In this scoping review, the search was carried out in the following databases: PubMed, MEDLINE (via BVS) and CINAHL (via EBSCO host).

The descriptors used were: “amelogenin”, “analyses, sex determination”, “analysis, sex determination” and “human identification” with the Boolean operators AND/OR to form the combination: ("amelogenin"[Mesh] OR "amelogenin"[tiab]) AND (("Analyses, Sex Determination"[Mesh] OR "Analysis, Sex Determination"[Mesh] OR "Sex Determination Analyses"[Mesh] OR "Sex Determination"[tiab]) AND ("Human Identification"[Mesh] OR "Human Identifications"[Mesh] OR "Identification, Human"[Mesh] OR "Identifications, Human"[Mesh] OR "Human Identification"[tiab] OR "Human Identifications"[tiab] OR "Identification, Human"[tiab] OR "Identifications, Human"[tiab]).

Resulting from the search, 2704 results were obtained. However, after removing duplicates (47 duplicates), 2657 articles were identified. Of these, 853 articles were excluded after reading the title or abstract or because the full text was not available. Then, 1804 articles were selected for full reading and 1791 were excluded after complete reading, for not meeting the inclusion and exclusion criteria.

Thus, to carry out the scoping review, 12 articles were selected. Moreover, by citation search, authors found 12 more publications. From these, after applying inclusion and exclusion criteria 1 article was selected.

A total of 13 articles were selected for this scoping review, reflected on PRISMA 2000 flow diagram. The bibliographic search strategy carried out is presented in Table 1.

Table 1. Bibliographic search strategy carried out.

Database	Articulation of keywords	Number of articles
PubMed	("amelogenin"[Mesh] OR "amelogenin"[tiab]) AND (("Analyses, Sex Determination"[Mesh] OR "Analysis, Sex Determination"[Mesh] OR "Sex Determination Analyses"[Mesh] OR "Sex Determination"[tiab]) AND ("Human Identification"[Mesh] OR "Human Identifications"[Mesh] OR "Identification, Human"[Mesh] OR "Identifications, Human"[Mesh] OR "Human Identification"[tiab] OR "Human Identifications"[tiab] OR "Identification, Human"[tiab] OR "Identifications, Human"[tiab]))	2271
MEDLINE (via BVS)	("amelogenin"[Mesh] OR "amelogenin"[tiab]) AND (("Analyses, Sex Determination"[Mesh] OR "Analysis, Sex Determination"[Mesh] OR "Sex Determination Analyses"[Mesh] OR "Sex Determination"[tiab]) AND ("Human Identification"[Mesh] OR "Human Identifications"[Mesh] OR "Identification, Human"[Mesh] OR "Identifications, Human"[Mesh] OR "Human Identification"[tiab] OR "Human Identifications"[tiab] OR "Identification, Human"[tiab] OR "Identifications, Human"[tiab]))	301
CINAHL (via EBSCO host)	("amelogenin"[Mesh] OR "amelogenin"[tiab]) AND (("Analyses, Sex Determination"[Mesh] OR "Analysis, Sex Determination"[Mesh] OR "Sex Determination Analyses"[Mesh] OR "Sex Determination"[tiab]) AND ("Human Identification"[Mesh] OR "Human Identifications"[Mesh] OR "Identification, Human"[Mesh] OR "Identifications, Human"[Mesh] OR "Human Identification"[tiab] OR "Human Identifications"[tiab] OR "Identification, Human"[tiab] OR "Identifications, Human"[tiab]))	132

In this scoping review, the PCC criteria (Table 2) were considered, which define: Population (P), Concept (C) and Context (C) to formulate the question which this work aims to answer.

Table 2. PCC (Population-Concept-Context) strategy for formulating the research question.

Parameters	Assessment
Population (P)	Molecular methods
Concept (C)	Determination of sexual dimorphism
Context (C)	Forensic identification

Stage 1. Formulating the research question

A search protocol was developed according to the Joana Briggs Institute (JBI) model, which leads to the formulation of the following question: Do molecular methods allow the determination of sexual dimorphism for forensic identification?

Stage 2. Identifying relevant studies

Sources of information and search strategy: After several preliminary searches, the final search was carried out on April 16, 2024. The investigation was carried out using 3 bibliographic databases in the domain of health. The terms selected for the study as well as Boolean operators AND/OR are presented on Table 1. An additional article, identified through other sources, was also included.

The search strategy was developed to identify studies that have a direct correlation between "Forensic Dental Medicine" and "Sexual Dimorphism". The results of the electronic search were exported to Word and duplicates were eliminated.

i. Eligibility criteria:

Forensic dentistry and sexual dimorphism are distinct concepts, which are related due to the usefulness that the first may have in the second and are the focus of this review.

Therefore, articles were included if they met all the following eligibility criteria:

- Population: molecular methods

- Language: articles published in Portuguese, English, French or Spanish

- Timeline: no restrictions

- Inclusion criteria: all types of scientific work that addresses the topic of the relationship between forensic dentistry and sex determination through the identification of amelogenin. Articles that used teeth as a sample and in vitro studies.

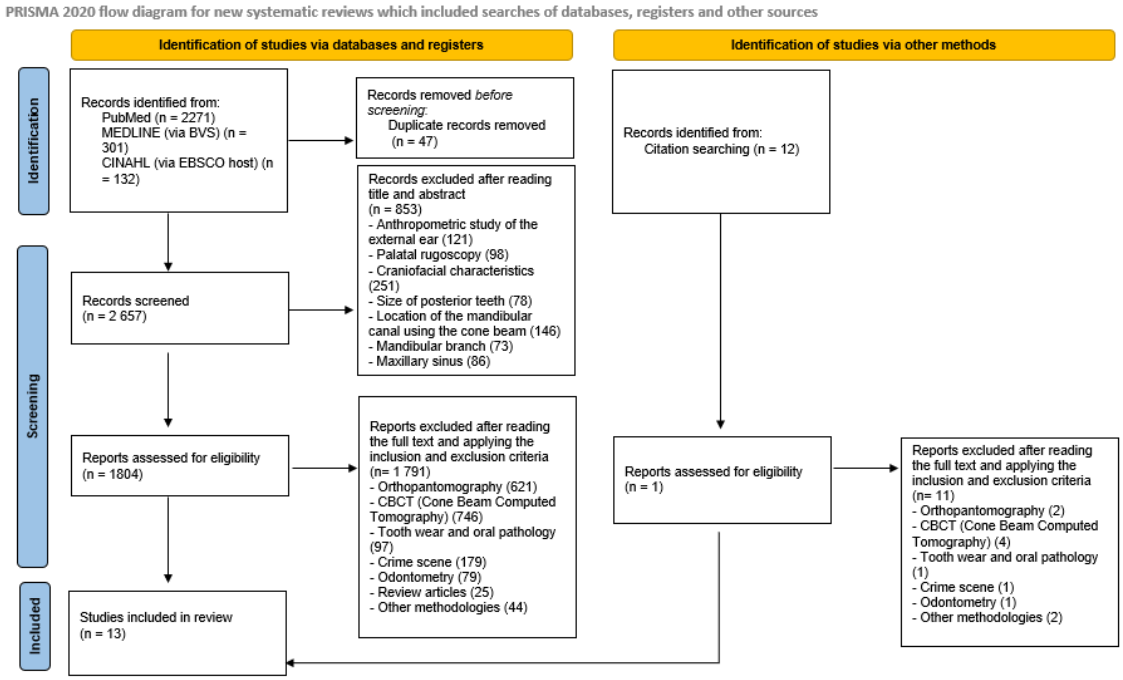
- Exclusion criteria: After fully reading the articles retained for eligibility purposes, 1791 articles were excluded for not meeting the inclusion criteria, that is, they did not make reference to amelogenin to determine the sex of unidentifiable cadavers and made reference to others criteria that were excluded from this study, such as: orthopantomography, CBCT (Cone Beam Computed Tomography), tooth wear and oral pathology, crime scene, odontometry, review articles, other methodologies (blood sample, bone and muscles samples, buccal swabs and PCR on different genes).

- Result: molecular methods based on the amelogenin gene present on the X and Y chromosomes to determine the sex of an individual.

Stage 3. Study selection

A total of 2704 articles were identified. After this identification, duplicates were removed. Next, the analysis was carried out first by title and summary, to select the articles that answered the research question. Secondly, by reading the full text of the selected articles including molecular methods used in dentistry. The PRISMA 2000 flow diagram that tracks progress is shown in figure 1.

Figure 1. Flowchart of the article's selection process, adapted from PRISMA 2000 flow diagram (Page et al., 2020).



Stage 4. Charting the data

The selected studies were independently reviewed with full text reading and the following information was extracted: name of the article, author, year and place of publication, methodology, main conclusions, and relevant final considerations. The data mapping is presented in Table 3.

Table 3. General characteristics of the selected studies.

	Author/Year	Article title	Type of study	Place of publication
E1	Nayar et al. (2017)	Determination of age, sex, and blood group from a single tooth	In vitro	Journal of Forensic Dental Sciences
E2	Williams et al. (2004)	Sex determination by PCR analysis of DNA extracted from incinerated, deciduous teeth	In vitro	Science & Justice
E3	Dutta et al. (2017)	Amelogenin Gene - The Pioneer in Gender Determination from Forensic Dental Samples	In vitro	Journal of Clinical and Diagnostic Research
E4	Chowdhury et al. (2018)	Sex Determination by Amplification of Amelogenin Gene from Dental Pulp Tissue by Polymerase Chain Reaction	In vitro	Indian Journal of Dental Research
E5	Alvarez García et al. (1996)	Effect of environmental factors on PCR-DNA analysis from dental pulp	In vitro	Internacional Journal of Legal Medicine
E6	Urbani et al. (1999)	The effect of temperature on sex determination using DNA-PCR analysis of dental pulp	In vitro	The Journal of Forensic Odonto-Stomatology.
E7	Zapico et al. (2013)	Sex determination from dentin and pulp in a medicolegal context	In vitro	Journal of the American Dental Association
E8	Praveen Kumar et al. (2016)	DNA isolation from teeth by organic extraction and identification of sex of the individual by analyzing the AMEL gene marker using PCR	In vitro	Journal of Forensic Dental Sciences
E9	Komuro et al. (1998)	Gender determination from dental pulp by using capillary gel electrophoresis of amelogenin locus	In vitro	The Journal of Forensic Odonto-Stomatology
E10	Srivastava et al. (2017)	Sex determination from mesiodens of Indian children by amelogenin gene	In vitro	Journal of Forensic Dental Sciences
E11	Musse et al. (2008)	Freshwater and salt-water influence in human identification by analysis of DNA: an epidemiologic and laboratory study	In vitro	Brazilian Journal of Oral Sciences

E12	Sivagami et al. (2000)	A simple and cost-effective method for preparing DNA from the hard tooth tissue, and its use in polymerase chain reaction amplification of amelogenin gene segment for sex determination in an Indian population	In vitro	Forensic Science International
E13	Nogami et al. (2008)	Rapid and simple sex determination method from dental pulp by loop-mediated isothermal amplification	In vitro	Forensic Science International: Genetics

Stage 5. Collection, summary, and consultation

PRISMA guided the collection, interpretation, and communication of results. Thus, with the search carried out in PubMed, MEDLINE (via BVS) e CINAHL (via EBSCO host), as well as other sources, several articles were analyzed to obtain only the relevant data for this scoping review. A total of 13 articles relevant to this study were found. Essential data taken from the selected publications is presented in Table 4.

2.3 Results

In this scooping review, a set of 13 articles has been explored from various aspects to enable sex determination from dental tissue in forensic contexts. Among these articles, papers E7, E8, E9, E10, E12 and E13 focused on standard DNA extraction methods and sex determination without controlled variations in environmental or storage conditions.

In contrast, references E1, E2, E3, E4, E5, E6 and E11 were selected specifically for their use of varied experimental treatments, aimed at simulating different environmental and preservation conditions. These studies involved submitting tooth samples to normal environmental conditions, salt water, immersion in salt water under different periods depending on the study, incineration varying time of exposure and temperature depending on the study, desiccation at room temperature, burying in soil and/or sand, river water and cadaver teeth embedded in bone and surrounded by soft tissue.

This approach enabled to compare results obtained under normal and extreme conditions, enriching our understanding on the challenges and possibilities of sex determination from dental tissue.

Work by Nayar et al. (2017) (E1) aimed to determine age, gender and blood group from a single tooth. This research involved the analysis of 60 teeth and was carried out in vitro and the method employed consisted of PCR performed directly on pulp tissue. Teeth were subjected to normal environmental conditions and saltwater treatment for 2 days and 6 weeks respectively. The results indicated high specificity for PCR sex determination, with no significant impact of time or environmental conditions.

Williams et al. (2004) (E2) aimed to determine sex by PCR analysis of DNA extracted from incinerated deciduous teeth. This in vitro study involved 84 primary decidual molars and 2 mandibular decidual teeth from a man who died in a fire. DNA was extracted using a commercial kit. Teeth were incinerated for 15 minutes at temperatures ranging from 100°C to 500°C, and the results showed that sex determination was possible for teeth incinerated at temperatures up to 200°C, but not beyond.

In 2017, Dutta et al. (E3) explored the use of the amelogenin gene for sex determination from dental samples in a forensic context. Carried out in vitro, this research involved 50 teeth (premolars and molars) with and without carious lesions. DNA was extracted using the phenol/chloroform method. Teeth were subjected to various treatments: immersion in salt water, desiccation at room temperature, burial in soil and incineration at temperatures ranging from 500°C to 1050°C. Results showed that it was possible to determine sex in most samples, however, samples exposed to seawater or incinerated at temperatures above 800°C required an increased number of PCR cycles to obtain adequate amplification.

The 2018 study by Chowdhury et al. (E4) focused on sex determination by amplification of the amelogenin gene from dental pulp tissue using PCR. This in vitro study involved 130 premolars. DNA was extracted using the phenol/chloroform method. Teeth were subjected to various treatments: immersion in salt water, desiccation at room temperature, burial in soil (all for durations of 30 to 90 days), and incineration at 150°C, 205°C and 350°C. The results showed complete sensitivity and specificity in sex determination. However, at extreme temperatures (350°C), no DNA could be recovered.

Alvarez García et al., in 1996, (E5) examined the effect of environmental factors on PCR analysis of DNA extracted from dental pulp. This in vitro research involved 570 teeth, including 6 teeth extracted between 10 and 30 years ago and 4 teeth from forensic cases. All teeth were premolars and molars, and DNA was extracted using a specialized kit. The teeth were also subjected to various treatments: immersion in salt water and river water (from 15 days to 6 months), storage at temperatures of 4°C, 20°C and 40°C (from 2 weeks to 36 months), burial in soil or sand (from 2 weeks to 6 months), outdoor exposure (from 2 weeks to 6 months), and incineration for 1 to 2 minutes at temperatures of 75°C, 100°C, 200°C, 300°C, 400°C and 500°C. The results showed that sex determination was possible for all samples stored at different temperatures, buried or left outdoors. Similarly, sex determination was successful for all samples immersed in water for 3 and 6 months, as well as for all samples incinerated, except those exposed to 500°C for 2 minutes. Sex determination was also 100% successful for old samples (10 to 30 years old) and for forensic samples.

Urbani et al. (1999) (E6) describe the effect of temperature on sex determination using PCR analysis of DNA extracted from dental pulp. This in vitro study included 88 impacted third molars, with and without carious lesions, as well as 6 teeth in cadaver bone sockets. DNA was extracted using the phenol/chloroform method. Teeth were incinerated for 15 or 30 minutes at temperatures of 100°C, 200°C and 300°C, and cadaver teeth in bone sockets surrounded by soft tissue were incinerated for 15 minutes at temperatures of 150°C, 250°C and 350°C. The results showed that sex determination was possible with 100% accuracy for samples subjected to 100°C for 15 minutes, but above 100°C or for longer durations, the accuracy of sex determination decreased. Nevertheless, it remained possible to determine sex for teeth embedded in bone up to a temperature of 250°C.

Finally, the study conducted by Musse et al. in 2008 (E11) examined the influence of fresh and salt water on human identification by DNA analysis, as part of an epidemiological and laboratory study. This in vitro research involved 40 tooth samples as well as saliva samples used as controls. DNA was extracted using the phenol/chloroform method. Samples were subjected to immersion treatments in fresh and salt water for 1 month, and results showed successful DNA isolation from 37.5% of tooth samples, while all saliva samples achieved successful DNA isolation. Sex determination was successful in 83.3% of tooth samples and 100% of saliva samples. Water was found to have a direct impact on DNA preservation, affecting the ability to extract DNA from tooth samples.

Concerning the publications without treatment, the 2013 study by Zapico et al. (E7) examined sex determination from dentin and pulp in a forensic context. Carried out in vitro, this research involved 2 incisors and 12 molars with periodontal disease. DNA was extracted using a specialized kit with no special processing conditions applied to the teeth, and the results showed that it was possible to determine gender using pulp or dentin samples. Similar amounts of DNA were obtained using different extraction methods however, the efficiency of DNA isolation from dentin depended on the type of tooth used.

Moreover, Praveen Kumar et al. in 2016 (E8) explored DNA isolation from teeth using organic extraction and gender determination by analyzing the *AMEL* genetic marker using PCR. This in vitro research involved 40 teeth. DNA was extracted using the phenol/chloroform method, without further sample processing, and the results showed that sufficient good-quality DNA could be isolated from the teeth. Sex determination was successful for all samples, and the PCR method proved sensitive and effective for sex determination.

Komuro et al. (1998) (E9) investigated sex determination from dental pulp using capillary gel electrophoresis of the amelogenin locus. This in vitro investigation involved 20 teeth, with DNA extraction carried out using the phenol/chloroform method, without further sample processing, and the results showed that sex determination was successful for all samples.

Work of Srivastava et al. in 2017 (E10) examined sex determination from mesiodens in Indian children using the amelogenin gene. This in vitro research involved 8 mesiodens, with DNA extraction performed using the phenol/chloroform method, without further sample processing, and the results showed successful DNA isolation and sex determination for 75% of the samples studied.

Research conducted by Sivagami et al. in 2000 (E12) developed a simple, cost-effective method for preparing DNA from hard tooth tissue, and used it in the PCR amplification of a segment of the amelogenin gene for sex determination in an Indian population. This in vitro research was carried out on 10 teeth, with the DNA isolated by ultrasonication and phenol/chloroform extraction, without further sample processing, and the results showed that sex determination was possible for all the samples studied.

Finally, Nogami et al. in 2008 (E13) developed a rapid and simple method for sex determination from dental pulp using LAMP. This in vitro investigation involved 32 teeth

and was carried out without DNA isolation or further sample processing, and the results showed that sex determination was possible for all samples analyzed using the LAMP method. The essential data on the methodologies and results of these thirteen articles are summarized in Table 4.

Table 4. Essential data taken from the selected publications.

	Author (year)	Type of study	Title	Methodology	Results
E1	Nayar et al. (2017)	In vitro	Determination of age, sex, and blood group from a single tooth	-60 teeth -With treatment: normal environmental conditions and under saline water (for 2 days and 6 weeks each) -PCR performed directly in pulp tissue -PCR followed by gel electrophoresis (PAGE)	-High specificity in sex determination using PCR in all samples. -No significant effect of time or environmental conditions on sex determination.
E2	Williams et al. (2004)	In vitro	Sex determination by PCR analysis of DNA extracted from incinerated, deciduous teeth	-84 deciduous primary molars and 2 mandibular decidual teeth of a man who died in a fire -With treatment: incineration for 15 minutes at temperatures ranging from 100°C to 500°C -DNA extracted from pulp tissue with kit -PCR followed by gel electrophoresis (agarose)	-Successful sex determination in control teeth and teeth incinerated to temperatures of 100-200ºC -Successful sex identification in teeth from fire victim -Sex determination not possible in teeth incinerated at temperatures above 200ºC
E3	Dutta et al. (2017)	In vitro	Amelogenin Gene - The Pioneer in Gender Determination from Forensic Dental Samples	-50 teeth (premolars and molars) with and without carious lesions -With treatment: salt water; desiccation at RT; buried in soil; incineration at 500-1050ºC -DNA extracted from pulp tissue with phenol/chloroform method -PCR followed by gel electrophoresis (agarose)	-Possible sex determination in most samples -Samples submitted to sea water or incinerated above 800ºC required increase in PCR cycles for adequate amplification

E4	Chowdhury et al. (2018)	In vitro	Sex Determination by Amplification of Amelogenin Gene from Dental Pulp Tissue by Polymerase Chain Reaction	-130 premolars -With treatment: salt water (30 to 90 days); desiccation at RT (30 to 90 days); buried in soil (30 to 90 days); incineration at 150ºC, 250ºC and 350ºC -DNA extracted from pulp tissue with phenol/chloroform method -PCR followed by gel electrophoresis (agarose)	-Sex determination demonstrates complete sensitivity and specificity -at extreme temperatures (350°C) no DNA could be recovered
E5	Alvarez García et al. (1996)	In vitro	Effect of environmental factors on PCR-DNA analysis from dental pulp	-570 teeth; 6 teeth extracted 10-30 years before; 4 teeth from forensic casework. All teeth were premolars and molars -With treatment: salt and river water (15 days to 6 months); kept at 4ºC, 20ºC and 40ºC (2 weeks to 36 months); buried in soil or in sand (2 weeks to 6 months); outdoors (2 weeks to 6 months); incineration for 1-2 minutes at 75ºC, 100ºC, 200ºC, 300ºC, 400ºC and 500ºC -DNA extracted from pulp tissue with kit -PCR followed by gel electrophoresis (PAGE)	-Sex determination in all samples kept at different temperatures, buried or left outdoors -Sex determination in all samples kept in water for 3 and 6 months -Poorest DNA isolation in water immersed samples -Sex determination in all incinerated samples. Exception: no sex determination when exposed to 500ºC for 2 min -100% sex determination in old samples (10 to 30 years old) -100% sex determination in forensic casework samples

E6	Urbani et al. (1999)	In vitro	The effect of temperature on sex determination using DNA-PCR analysis of dental pulp	-88 impacted 3rd molars, with and without carious lesions; 6 teeth in bone sockets from a cadaver -With treatment: incineration for 15 or 30 minutes at 100ºC, 200ºC and 300ºC; cadaver teeth embedded in bone and surrounded by soft tissue incinerated for 15 minutes at 150ºC, 250ºC and 350ºC -DNA extracted from pulp tissue with phenol/chloroform method -PCR followed by gel electrophoresis (agarose)	-Sex determination with 100% accuracy in samples submitted to 100°C for 15 minutes -Above 100ºC or longer time, the accuracy on sex determination decreased -Remained possible at 250°C for teeth embedded in bones
E7	Zapico et al. (2013)	In vitro	Sex determination from dentin and pulp in a medicolegal context	-2 incisors and 12 molars with periodontal disease -Without treatments -DNA extracted from pulp and dentin tissues with kit -PCR followed by gel electrophoresis (agarose)	-Possible to determine sex using pulp or dentin samples -Similar amounts of DNA are obtained using different extraction methods -Efficiency of DNA isolation from dentin depends on the type of tooth used
E8	Praveen Kumar et al. (2016)	In vitro	DNA isolation from teeth by organic extraction and identification of sex of the individual by analyzing the AMEL gene marker using PCR	-40 teeth -Without treatments -DNA extracted from hard tooth tissues with phenol/chloroform method -PCR followed by gel electrophoresis (agarose)	-Enough good quality DNA isolated from teeth -Sex determination in all samples -PCR was sensitive and effective for sex determination
E9	Komuro et al. (1998)	In vitro	Gender determination from dental pulp by using capillary gel electrophoresis of amelogenin locus	-20 teeth -Without treatments -DNA extracted from pulp tissue with phenol/chloroform method -PCR followed by PAGE or CGE	-Sex determination in all samples

E10	Srivastava et al. (2017)	In vitro	Sex determination from mesiodens of Indian children by amelogenin gene	-8 mesiodens -Without treatments -DNA extracted from hard tooth tissues with phenol/chloroform method -PCR followed by gel electrophoresis (agarose)	-Successful DNA isolation and sex determination from 75% of samples
E11	Musse et al. (2008)	In vitro	Freshwater and salt-water influence in human identification by analysis of DNA: an epidemiologic and laboratory study	-40 teeth and saliva samples as control -With treatments: immersion in fresh and salt-water for 1 month -DNA extracted from hard tooth tissues with phenol/chloroform method -PCR followed by gel electrophoresis (agarose)	-Successful DNA isolation from 37.5% of teeth samples; successful DNA isolation from all saliva samples -Sex determination in 83.3% of teeth samples and in 100% of saliva samples -Water interfered directly in DNA preservation
E12	Sivagami et al. (2000)	In vitro	A simple and cost-effective method for preparing DNA from the hard tooth tissue, and its use in polymerase chain reaction amplification of amelogenin gene segment for sex determination in an Indian population	-10 teeth -Without treatments -DNA isolated from hard tooth tissues by ultrasonication and phenol/chloroform extraction -PCR followed by gel electrophoresis (agarose)	-Sex determination in all samples
E13	Nogami et al. (2008)	In vitro	Rapid and simple sex determination method from dental pulp by loop-mediated isothermal amplification	-32 teeth -Without treatments -LAMP method on pulp tissue (without DNA isolation) -PCR followed by PAGE	-Sex determination in all samples

2.4 Discussion

From the scientific literature consulted for this scoping review and the analysis of the thirteen articles meeting the eligibility criteria, it has been demonstrated the applicability of biological methods for sex determination based on identification and analysis of sex-specific genetic markers, including the *AMEL* gene.

These techniques offer several advantages over traditional methods, particularly in terms of speed and precision. To determine the sex of an unidentified person, several techniques have been employed, targeting the gene coding for amelogenin, a protein involved in tooth development, specifically in the formation of tooth enamel.

Among the various methods used for molecular analyses of sex determination, PCR stands out for its high precision, sensitivity, and specificity. PCR is widely adopted in forensic science and paleogenetics due to its speed, accuracy, and efficiency, even with low-quality or degraded samples. This method amplifies specific DNA sequences, making it possible to detect the presence of the *AMELX* and *AMELY* genes and thus determine the sex of the individual from whom the sample was taken. The advantage of PCR in sex determination lies in its ability to produce results from minute quantities of DNA, which is particularly beneficial in forensic cases where sample amounts can be extremely limited. Additionally, PCR's robustness allows for the successful amplification of genetic material from degraded or ancient samples, making it invaluable in both modern forensic investigations and archaeological studies.

The thirteen selected publications were classified into two categories: the ones “without treatments” and those “with treatments”, meaning that samples were submitted to conditions that mimic forensic scenarios. The treatments described in the various publications included the immersion in fresh, salt or river waters, incineration at different temperatures, exposure to the outdoor environmental conditions, buried in sand or soil, desiccation at room temperature or even cadaver teeth packed in bone and surrounded by soft tissue. Thus, articles E1, E2, E3, E4, E5, E6 and E11 are listed as “with treatment”, while articles E7, E8, E9, E10, E12 and E13 are classified “without treatments”.

In the thirteen selected papers, different methodologies were used to extract DNA from dental tissues, such as using a commercial kit, with phenol/chloroform, or by PCR or

LAMP directly in pulp tissue cells. In this way, papers were grouped mentioning the method used for DNA extraction.

2.4.1. Comparison of the DNA extraction methods and samples

In the selected studies, the most frequently used DNA isolation method was the phenol/chloroform extraction (E9 - Komuro et al., 1998; E6 - Urbani et al., 1999; E12 - Sivagami et al., 2000; E11 - Musse et al., 2008; E8 - Praveen Kumar et al., 2016; E3 - Dutta et al., 2017; E4 - Chowdhury et al., 2018; E10 - Srivastava et al., 2017). Exceptions were the works of Alvarez García et al. (1996) (E5), Williams et al. (2004) (E2) and Zapico et al. (2013) (E7), who used commercial kits, Nayar et al. (2017) (E1), who employed PCR directly in the dental pulp tissue and Nogami et al. (2008) (E13) who used the LAMP method without previous DNA isolation.

Moreover, samples used for DNA isolation were also not similar. Most studies used dental pulp tissue for DNA extraction (E1 - Nayar et al., 2017; E2 - Williams et al., 2004; E3 - Dutta et al., 2017; E4 - Chowdhury et al., 2018; E5 - Alvarez García et al., 1996; E6 - Urbani et al., 1999; E7 - Zapico et al., 2013; E9 - Komuro et al., 1998; E13 - Nogami et al., 2008), while others used dental hard tissues (E8 - Praveen Kumar et al., 2016; E10 - Srivastava et al., 2017; E11 - Musse et al., 2008; E12 - Sivagami et al., 2000).

The study by Zapico et al. (2013) highlights the differences between tooth types (e.g. incisors) and dental tissue types (pulp and dentin) for DNA extraction and amplification. DNA extraction was performed with a commercial kit from the pulp and failed in only one tooth. The authors showed that DNA yield varies according to tooth type, being lowest in small teeth such as incisors. Most samples allowed similar quantities of extracted DNA from both tissues (24.2 to 57 µg/mL). However, lower amounts were obtained from dentin. Despite these differences in performance, researchers were able to identify sex in all samples, opening the possibility of identifying other DNA sequences, such as short tandem repeats. PCR correctly identified sex independently of tissue type (pulp or dentin) via two regions of the *AMEL* gene. The amplification pattern of β-actin gene, used as control, was comparable to that of *AMEL* gene, showing that differences in amplification efficiency between dentin and pulp were mainly due to the initial DNA concentration.

With a similar purpose, Srivastava et al. (2017) focused on the isolation and analysis of genomic and mitochondrial DNA from mesiodens (supernumerary teeth between

incisors) to determine the sex of individuals by PCR. In this study, DNA was extracted using the phenol/chloroform method. In a control group, DNA was obtained from both mesiodens and sex identification was accurate using PCR analysis. Genomic and mitochondrial DNAs were successfully isolated in six out of eight samples with subsequent sex identification, a success rate of 75%. In one sample, although DNA was not visible on the agarose gel, PCR amplification enabled sex determination. In this study, the amount of isolated DNA ranged from 4 to 14 µg/mL, contrasting with concentrations of 24.2-57 µg/mL in the study of Zapico et al. (2013). In conclusion, the methodology used by Zapico et al. (2013) allowed a higher DNA yield when compared to Srivastava et al. (2017). However, these studies are not directly comparable since they used different teeth tissues as samples.

Similarly to Srivastava et al (2017), the study by Praveen Kumar et al. (2016) focused on the use of PCR after DNA extraction with phenol/chloroform, and both exploit teeth for their resistance to degradation and their ability to provide good quality DNA. However, the latter mainly highlights the advantages and challenges of this method for determining gender and dental hard tissues (enamel and dentin), as an important source of DNA. Similarly, Zapico et al. (2013) focused on the efficiency of DNA extraction from different dental tissues (pulp and dentin). Praveen Kumar et al. (2016) affirms that dental tissues, such as enamel, dentin, pulp and cementum, have the advantage of resisting physical and environmental degradation, being, for that reason, a reliable source of genomic DNA. Dutta et al. (2017) emphasized the importance of analyzing the effects of environmental conditions on human tissues, by examining the dentition as it is crucial in determining human identity, since other tissues often degrade under extreme forensic conditions.

Dutta et al. (2017) attested significant recovery of DNA in all samples. In fact, in this work, genomic DNA was successfully extracted from all tooth samples, with a recovery rate of 100%. However, since these researchers did not state the yield of isolated DNA, it is not possible to compare the efficiency of the extraction method with other studies. Moreover, in this work, samples were submitted to extreme conditions and, it was observed that DNA isolation from these teeth was problematic. In fact, in samples submitted to several treatments, in order to be able to detect small amounts of DNA the number of PCR cycles had to be increased.

Praveen Kumar et al. (2016) were able to isolate 55 to 86 µg of DNA per tooth and of a good purity to use as a forensic evidence material. This result cannot be compared with

DNA concentrations obtained by Zapico et al. (2013) or Srivastava et al. (2017), since amounts are not expressed in the same way.

Moreover, Sivagami et al (2000) extracted DNA by the phenol/chloroform method after ultrasonication of hard tooth tissues. As asserted by Praveen Kumar et al. (2016), teeth and bones, due to their mineralized structure, preserve DNA well, making them ideal for post-mortem investigations. However, extracting DNA from these hard tissues is often difficult due to the resistance of the cells, so ultrasound (US) has been used to disintegrate hard tissues, facilitating DNA extraction. Since these researchers did not state the total amount of DNA extracted from each sample, it is not possible to compare and conclude if, in fact, the use of ultrasonication facilitates DNA extraction. PCR-amplified segments of the *AMEL* gene showed comparable results between DNA extracted from teeth and the control sample, confirming the efficacy of this method.

In the study of Praveen Kumar et al. (2016), sex could be determined with total accuracy, validated by statistical analysis. Nevertheless, this study revealed certain limitations to the use of PCR: first, the reagents and equipment required for PCR are expensive; second, possible contamination by foreign DNA can lead to false positives; and third, magnesium ion concentration (Mg^{2+}) is crucial, as inadequate concentration can reduce or alter results. These technical limitations of PCR are also addressed in the study by Srivastava et al. (2017). These studies demonstrate the effectiveness of using teeth for DNA extraction and sex determination via PCR. Srivastava et al. (2017) and Praveen Kumar et al. (2016) highlight the advantages of teeth as a resilient source of DNA while highlighting the common technical challenges associated with PCR. The differences between studies show the possible variability in tooth types and protocols used, but the overall positive results reinforce the idea that teeth are a valuable resource for forensic analysis.

The evaluation of several methods of DNA isolation for sex determination reveals significant advances in the field of forensic genetics. The following studies belonging to the “without treatment” category highlighted innovative approaches, each with its own strengths and specific applications. Komuro et al (1998) relied on the emerging capillary gel electrophoresis (CGE) as a sophisticated technique for accurate separation of mixture components based on charge and size. In the context of sex determination, CGE accurately identifies X and Y loci after a PCR reaction. Female dental pulp DNA shows a single peak as the X locus and male dental pulp DNA shows two peaks with the first

corresponding to the X locus and the second to the Y locus. In terms of advantage, CGE offers precise identification of X and Y loci thanks to the measurement of migration times, facilitating rapid and accurate sex identification. Additionally, unlike plate gel electrophoresis, CGE does not require simultaneous electrophoresis cycles with ladder markers, simplifying the process.

An innovative alternative approach is presented by Nogami et al. (2008) with the use of the LAMP technique. This method, based on isothermal DNA amplification, enables rapid and accurate sex determination by observing post-amplification turbidity. To improve the efficiency and speed of the process, loop primers are added to the initial primers, reducing reaction times while maintaining amplification specificity. Results are validated by two complementary methods: visual observation of post-amplification turbidity and confirmation by PAGE. These methods ensure the reliability and accuracy of the results obtained by the LAMP technique.

Ultimately, although these studies examine different methods for DNA-based sex determination and highlight various aspects of accuracy, speed and applicability, they all share a common goal of improving forensic and medical analysis by providing reliable and effective tools: CGE has a fine discrimination capability between X and Y loci. The results of this method are reliable and rapid, enabling precise identification even with minimal sequence differences, making it particularly useful in the forensic science context. Similarly, the LAMP technique offers a fast, accurate and reliable approach to DNA-based sex determination. Its use has great potential in various fields of research and medical application, offering an effective alternative to traditional DNA amplification methods. Finally, the use of the *AMEL* gene-based method and ultrasound for DNA extraction from teeth is also described as offering reliable tools for sex determination, which could increase the accuracy and efficiency of forensic investigations, although speed is not specifically mentioned. In summary, these studies highlight the constant evolution of DNA-based sex determination methods, offering solutions that are accurate, reliable and suitable for different application contexts, from forensics to biomedical research.

2.4.2. Effect of different treatments on DNA isolation and sex determination

Regarding the "with treatment" classification, Nayar et al. (2017) examined the effectiveness of the PCR technique for sex determination using dental pulp under different environmental conditions, including exposure to salt water. Dental pulp samples were stored under normal environmental conditions, without exposure to external agents like salt water and the PCR technique showed high specificity and accuracy in sex determination under these conditions. After immersion of the dental pulp samples in salt water for a period of 2 days, the specificity and precision of the PCR technique remained high. Similarly, after prolonged exposure to salt water for a period of 6 weeks, the PCR technique maintained high specificity and accuracy in sex determination. This study (Nayar et al., 2017) highlights the importance and reliability of dental pulp as a source of DNA for forensic applications and the effectiveness of the PCR technique in various environmental contexts, providing a robust method for sex determination. Dental pulp, protected within the tooth structure, is less susceptible to external damage compared to other sources of DNA, making it particularly valuable for forensic analysis where samples may be compromised. by severe environmental conditions. The results of Nayar et al. (2017) have significant implications for forensic practice. They suggest that even in scenarios where biological samples are limited or damaged, dental pulp remains a viable source of DNA, and PCR an essential tool for analysis. Thus, this study reinforces the importance of adequate preservation of dental samples and the systematic application of advanced molecular techniques to ensure the integrity of genetic evidence and the reliability of forensic findings.

In the same way, Alvarez García et al. (1996) aimed to evaluate the reliability of DNA analysis techniques under several environmental conditions, to establish robust methods for forensic applications. However, unlike the study by Nayar et al. (2017) which focused on the effectiveness of PCR for sex determination from dental pulp, these authors used various methods to prepare the tooth sample: whole teeth crushed/cut transverse / endodontic access, and DNA extraction was performed using a commercial kit. Alvarez García et al. (1996) analyzed several genetic markers (HLA DQA1, D1S80, HUMTH01, HUMFES/FPS, and the homologous XY *AMEL* gene). Samples were exposed to various conditions. Samples were exposed to different temperatures (4°C, 20°C and 40°C) during periods of 3, 6 or 36 months. No significant differences were observed between the tested

temperatures and periods of time. Teeth were also submitted to immersion in fresh and salt water (discussed below) or exposed to environmental conditions or buried in sand or soil. Authors stated that better results were observed for samples kept outdoors when compared with the buried ones. Nevertheless, it was possible to determine sex in all outdoors/buried samples and no significant differences were observed between the sand buried and soil buried samples. These authors were also able to determine sex by the identification of the *AMEL* gene from 100% of old samples (teeth aged 10 to 30 years). Samples incinerated at temperatures from 100°C to 500°C, for different periods of time (1 to 10 minutes) also allowed sex determination using the *AMEL* gene. However, negative results were observed for STR analysis in samples submitted to 500°C. But as expected, best results were observed at lower temperatures (100°C for 10 minutes). Finally, cremated bodies allowed positive sex determination for all markers, due to the protection given by muscular and skeletal structures and dental enamel. These experiments affirm that the STR markers and the amelogenin XY gene are very robust, providing positive results under most tested conditions. Thus, *AMELX/Y* genes are extremely robust, even under harsh conditions like extreme temperatures or prolonged immersion.

Dutta et al. (2017) also used several different treatments: immersion in salt water; desiccation at room temperature; buried in soil; and incineration at temperatures from 500 to 1050ºC. These authors stated that sex determination was possible in most samples. Teeth kept in sea water or incinerated at temperatures above 800ºC did not give positive results unless the number of PCR cycles was increased to allow adequate amplification of DNA. Under normal circumstances, PCR uses between 30 and 35 amplification cycles to copy enough DNA for analysis. However, for these specific samples, authors increased the number of cycles to 45, as extreme conditions can damage DNA. Thus, Dutta et al. (2017) recommended the use of the LAMP method as a rapid alternative to PCR, providing faster results (in around half an hour) and operating at isothermal temperatures. Despite extreme environmental conditions, including incineration up to 1050°C, the band width of DNA fragments from the amelogenin gene did not change, showing that analysis of this gene for sex determination is effective even under extreme conditions, but for more accurate individual identification, this method should be combined with age estimation techniques.

Chowdhury et al. (2018) also applied different treatments to teeth samples, before DNA phenol/chloroform extraction: immersion in salt water for 30 to 90 days; desiccation at room temperature for 30 to 90 days; buried in soil for 30 to 90 days; and incineration at 150ºC, 250ºC and 350ºC. This work concluded that sex identification was positive for most samples. In this study, only teeth subjected to extreme temperatures (350ºC) did not permit the isolation of DNA. This is not in accordance with the work of Alvarez García et al. (1996) that extended the range of temperatures giving positive DNA isolation and sex identification to 500°C, the critical limit for most markers except for the *AMEL* gene. Therefore, in accordance with Chowdhury et al. (2018), DNA can be extracted and analyzed accurately from teeth, except for samples exposed to high temperatures (350°C). DNA amplification and sex determination are reliable.

Two studies (Williams et al., 2004; Urbani et al., 1999) focused exclusively on the effect of incineration temperature in the effectiveness of sex determination from teeth samples. Both studies concentrate on sex determination from incinerated teeth, but they use different DNA isolation methods and incineration conditions. Williams et al. (2004) performed DNA isolation from baby teeth using a commercial kit. These authors stated successful sex identification through PCR on the *AMEL* gene, in control teeth (non-incinerated teeth) and samples incinerated to temperature ranges from 100ºC to 200ºC. Positive results could not be obtained for teeth incinerated at temperatures above 200ºC. In fact, at high temperatures, identification became more difficult in cases where soft tissue protection was not present. At 100°C for 15 minutes, all isolated teeth allowed correct sex identification. However, at 300°C for 15 minutes, sex determination was only possible in 14.3% of samples, indicating significant DNA degradation in these cases. Moreover, at 250°C, teeth protected by soft tissue and embedded in bone showed improved sexing ability, with all teeth allowing positive sex identification. Nevertheless, at 350°C, none of the protected teeth could be properly sexed, highlighting the limits of the protection offered by soft tissue and bone at high temperatures. These authors demonstrated effective identification of human biological sex in samples incinerated at temperatures up to 200°C.

Concerning the work of Urbani et al. (1999), DNA was extracted using the phenol/ chloroform method and probably from permanent teeth. These authors examined the effect of different temperatures and times of exposure, as well as protection by soft tissue and bone, on the ability to determine sex by amplification of the *AMEL* gene through

PCR. These authors were able to obtain accurate results at lower temperatures than Williams et al. (2004). The ability to determine sex with the methodology used by these authors was 100% efficient when using 100ºC for teeth treatment, however diminished when using incineration temperatures above 100°C or longer exposure to high temperatures than 15 minutes. Nevertheless, DNA isolation and consequent sex identification was still possible at 250°C for teeth protected by soft tissue and bone.

Both studies show that sex identification from incinerated teeth is possible, but results vary according to the methods and conditions used. Williams et al. (2004) demonstrated improved sensitivity with laser-induced fluorescence, enabling identification of PCR products up to 400°C. In contrast, Urbani et al. (1999) showed that 100% of isolated teeth exposed to 100°C for 15 minutes could be accurately sexed, with the ability to determine diminishing beyond this temperature and duration, although this was still possible at 250°C for bone-encrusted teeth. These variations illustrate the importance of experimental conditions and analysis methods in determining sex from cremated teeth.

Some of the selected studies where treatments were applied to tooth samples, also focused on the impact of immersing teeth in different types of water on the quality of the recovered DNA. For example, Alvarez García et al. (1996) examined teeth immersion in salt and river water for 30 to 90 days. Dutta et al. (2017) analyzed teeth in salt water over periods of 15 days to 6 months, while Chowdhury et al. (2018) and Nayar et al. (2017) also studied salt water, with immersion periods up to six weeks in the first study, and from two days to six weeks in the second one. Musse et al. (2008) studied immersion in fresh and salt water for one month, focusing on drowning deaths, drowning conditions, victim characteristics and body identification methods. In this study, DNA was recovered from 37.5% of samples, mainly from teeth immersed in fresh water (45% of samples coming from teeth immersed in fresh water versus 30% from those in salt water). All saliva samples used as controls showed positive DNA amplification. Sex determination was possible in 83.3% of samples. The study by Musse et al. (2008) highlights the challenges and successes of drowning victim identification, with a predominance of saltwater drownings, mainly affecting young men. Other studies confirmed the results obtained by Musse et al. (2008). For instance, Nayar et al. (2017) acknowledged PCR as a highly specific and effective method for determining sex under adverse environmental conditions, such as prolonged immersion in salt water, although it is often necessary to complement with other techniques. These authors did not observe significant effect of

time of exposure or environmental conditions on DNA isolation and subsequent sex identification through the detection of the *AMEL* gene. A slight decrease in the percentage of accuracy was observed with increased treatment exposure time (normal environmental conditions or salt water for 2 days or 6 months). Results of Alvarez García et al. (1996) showed no significant differences between fresh and saltwater immersion, but authors stated that this treatment gives the poorest DNA isolation when compared to other treatments. Even so, in this work it was obtained complete success in sex identification for all samples (immersed in salt or fresh water for 3 or 6 months) when using detection of the *AMEL* gene. However, sex determination using other markers was only possible for 50% of samples.

In conclusion, the exposure of teeth to water plays a critical role in determining the quality of isolated DNA. Our findings highlight that the type of water whether saltwater or freshwater significantly impacts DNA preservation. Saltwater, due to its high salinity, accelerates the degradation of DNA. This process makes subsequent DNA extraction and analysis more challenging, often resulting in lower yields and poorer quality genetic material. The harsh environment created by saltwater can break down cellular structures and nucleic acids more rapidly, compromising the integrity of the samples. On the other hand, teeth exposed to freshwater exhibit a much better preservation of DNA. Freshwater, with its relatively neutral pH, provides a more favorable environment for maintaining the structural integrity of DNA over time. This better preservation facilitates more efficient extraction processes and yields higher quality DNA suitable for detailed genetic analysis.

These insights are particularly relevant for forensic where the condition of DNA can be a limiting factor. Understanding the effects of different water environments on dental DNA can inform best practices for sample collection and preservation, ultimately enhancing the reliability of genetic analysis in various scientific fields. Therefore, careful consideration of the environmental conditions to which dental samples have been exposed is crucial for obtaining optimal results in DNA-based studies.

3. CONCLUSION

The research presented in these 13 articles highlights the importance and reliability of using teeth for forensic identification, particularly for sex determination. Studies show that even under extreme environmental conditions, such as high temperatures or exposure to salt water, teeth remain a reliable source of DNA for PCR analyses.

The *AMEL* gene is commonly used for sex determination in forensic science. This gene, located on the X and Y sex chromosomes, codes for a protein essential in the development of tooth enamel. By amplifying the specific sequences of this gene via PCR, it is possible to distinguish the sexual chromosomes according to the length of the amplified products, allowing rapid and reliable identification of the sex.

Although the *AMEL* gene is an effective marker, polymorphisms in primer binding sites can lead to errors, such as dropouts of X alleles in males and deletions in the Y chromosome. These variations can compromise the accuracy of the sex determination. To overcome these limitations, it is recommended to use additional tests such as Y-STR and the *SRY* gene.

PCR is a crucial technique in forensic medicine for the amplification of DNA extracted from teeth. It allows enough DNA to be rapidly produced from small initial quantities, even when samples are degraded or exposed to adverse environmental conditions. Several studies have demonstrated that this method, used with primers based on the *AMEL* gene, showed high sensitivity and specificity. For example, studies have reported 100% DNA recovery and accurate sex determination with optimal sensitivity of the technique.

Some authors recommend the use of ultrasonication of dental hard tissue samples which improves the quality and quantity of extracted DNA, increasing the efficiency of PCR analyses.

Teeth exposed to temperatures up to 250°C allowed accurate sex identification, although the ability to determine sex diminished beyond this temperature. On the other hand, at 350°C, no DNA could be extracted.

DNA extracted from teeth, including pulp and dentin, is often of good quality and enough for PCR analyses, even after several years of storage or under harsh environmental conditions. Various studies show that dental pulp is a valuable tissue for DNA typing, allowing accurate identification even after prolonged storage periods.

Thus, although teeth provide a valuable source of DNA for forensic identification, further research as well as the integration of various techniques, such as: PCR, PAGE, CGE and LAMP, as well as complementary markers, is essential to ensure accurate and reliable identification.

Overall, the integration of biological methods, particularly those utilizing genetic markers such as the *AMEL* gene and the PCR technique, represents a significant advancement in the field of sex determination. These methods not only enhance the accuracy and reliability of sex identification but also streamline the process, offering rapid results crucial in various scientific and forensic applications.

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