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Relevance of dental medicine in paleomicrobiology: narrative review

Universidade Fernando Pessoa

Faculdade de Ciências da Saúde

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Relevância da medicina dentaria na paleomicrobiologia: revisão narrativa

Atesto a originalidade do trabalho

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Bensaadat Badr Eddin

Trabalho apresentado à Universidade Fernando Pessoa

como parte dos requisitos para obtenção do grau de

Mestre em Medicina dentaria

Resumo:

A paleomicrobiologia é um ramo especial da microbiologia relacionado com o estudo de fósseis bacterianos. Esta revisão dá uma visão geral da história da investigação do ADN antigo, e as implicações da paleomicrobiologia oral. O estudo deste material genético antigo abre uma nova porta para o mundo antigo, através da compreensão da viagem dos microorganismos antigos através do tempo e do espaço, particularmente durante os eventos pandémicos.

A polpa dentária e também o cálculo dentário foram identificados como fontes ricas de DNA microbiano antigo. Nesta revisão, concentrámo-nos na relevância da medicina dentária, especialmente nas técnicas de acesso a amostras de DNA de boa qualidade da cavidade oral, mas também na alteração do maxilar devido a doença inflamatória infecciosa.

Palavras chaves: Paleomicrobiologia oral, polpa dentária antiga, cálculo dentario, extracção de ADN, paleomicrobiologia, pandemias.

Abstract

Paleomicrobiology is a particular field of study of microbiology where bacterial fossils are studied. It opens a new door to the ancient world, by the understanding of ancient microorganisms' travel through time and space, particularly during the pandemic events

This review gives an overview of history of ancient DNA research, and the implications of the oral paleomicrobiology. This work intents to give a general understanding of the role a dentist can play in this field of study.

Dental pulp and also dental calculus have been identified as rich sources of ancient microbial DNA. We have focused on the relevance of dental medicine, especially on the techniques to access good quality DNA samples from oral cavity but also about the alteration of the jaw due to inflammatory infection disease.

Keywords: Oral paleomicrobiology, *ancient dental pulp*, *calculus*, DNA extraction, paleomicrobiology, pandemics.

Dedicatória

Dedico este trabalho a meu pai e a minha mãe.

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Índice

Figure index

Index of tabels

Abbreviation index

PCR	Polimerase chain reaction
DNA	Desoxyribonucleic acid
MRI	Magnetic resolution imaging
CT	Computed tomography
GY	Gray
UV	Ultraviolet

I. Introduction

Paleomicrobiology is a branch of microbiology that allows the identification of ancient infectious diseases thanks to pathogenic microorganisms found in human or environmental samples. This recent discipline has been developed in parallel with the progress of genome sequencing techniques. (Dagli *et al.*, 2015; M. Drancourt e Raoult, 2016)

The dental pulp, as well as the tartar, have thus been shown to be interesting sources of ancient microorganisms; the latter colonize the pulp by the hematogenous route. The tooth, being the most resistant organ of the human body, preserves the pulp and its pathogens over hundreds, even thousands of years. (Dagli *et al.*, 2015; M. Drancourt e Raoult, 2016)

The oral ecosystem is full of diversity and harbors second most diverse human microbiome after gut. As described by (Warinner *et al.*, 2015), the oral cavity is an entire world unto itself, populated by fusobacteria, streptococci and treponemes. (Dagli et al., 2015)

Dental paleomicrobiology has thus allowed the study of many diseases over time, including the bubonic plague of *Y. pestis*. For 5,000 years, numerous epidemics have taken place throughout history and have considerably reduced the European population.(Derbise *et al.*, 2012)

The current technique for retrieving dental pulp from the cavity is based on the longitudinal opening of the tooth, which involves significant exposure of the material to the laboratory environment, resulting in potential contamination of the dental pulp.(Drancourt *et al.*, 1998; Calvo *et al.*, 2001; Gilbert *et al.*, 2004)

It is also possible to remove the dental pulp from the dental pulp cavity after drilling a small hole to expose the dental pulp cavity.(Woodward et al., 1994)

Both techniques require dentistry expertise in the fine gestures to manipulate the teeth. Due to these limitations, some laboratories have been unable to develop a universal protocol for obtaining DNA from the pulp for molecular detection of hosts and bacteria in the pulp.

A protocol has also been proposed in which the entire tooth is embedded in silicone(fig4). (Gilbert et al., 2003).

Therefore, studying the ancient oral microbiota gives us important clues about life in the ancient world.(Dagli et al., 2015)

Our main objective it to understand the relevance of paleomicrobiology, the technique involved in this area of study, particularly the acquisition of samples and the relevance of the medical dentist capacities to improve that acquisition.

A literature search in PubMed/Medline, Google Scholar, Academia and Elsevier databases was performed for this narrative review using the following keywords in multiple combinations: "Oral paleomicrobiology"; "dental pulp"; "Ancient dental pulp"; "DNA extraction", Paleomicrobiology " Forensic odontology and DNA extraction".

Articles published in the last 28 years (1994 - 2022) in English and French were included. The search encompassed narrative and systematic review articles, randomized clinical trials, and clinical cases. Articles that did not meet the objectives of the study were excluded. The reason why the selection of articles that old is because it’s when most of the studies were realised. In all, 31 articles were selected that proved to be most relevant to the development of the theme.

II. Development

1. Historic

A pandemic is a large-scale outbreak of infectious disease that can significantly increase morbidity and mortality over large geographic areas and cause serious economic, social and political turmoil. There is evidence that increased travel and global integration, urbanization, changing land use, and increased use of the natural environment have increased the likelihood of a pandemic over the past century (Jones and others 2008; Morse 1995). These trends are expected to intensify. Key policy attention focuses on the need to identify and contain new epidemics that may lead to pandemics, and to increase and maintain investment in health preparedness and health literacy.(Madhav *et al.*, 2017)

Many pandemics have occurred in human history. Plague, smallpox, cholera, and the Spanish flu were the longest-lasting, most recurrent, and most life-threatening. It is estimated that at least 20 million people died of the Spanish flu in 1918 in the early 20th century.(Akin e Gözel, 2020)

In April 2009, a new pandemic occurred. The cause was the H1N1pdm09 virus that occurred in Mexico. Within a few weeks, the disease spread to many countries.(Akin e Gözel, 2020)

After three influenza pandemics in 20th century, and the first one of the 21st century in 2009, Humankind encounter a novel pandemic, but this time of a corona virus. (Akin e Gözel, 2020)

Pandemics, in history have spread around the world due to people traveling from one country to another within, two or three months. In the past, pandemics spread predictably along military passages or major trade routes, but globalization has multiplied and obscured the dominant routes. Given their numbers and the speed with which human and animal vectors of transmission can move, there is no longer a single dominant route for the movement and geographical spread of infectious diseases. Pandemics are inherently uncertain. Based on our experience and preparations for past pandemics, it is appropriate to assess the epidemiological characteristics of the pandemic.(Akin e Gözel, 2020)

The development of new multidisciplinary approaches is necessary to prevent new pandemic episodes.(Hammer-Dedet e Licznar-Fajardo, 2020)

2. • The interest of paleomicrobiology

Paleomicrobiology is a recent discipline that has been developed in parallel with advances in biomolecular sequencing techniques. (Drancourt e Raoult, 2005a)

Paleomicrobiology is a specialty of microfossil science related to the study of microfossils. The term "microfossils" means bacterial fossils.(Dagli *et al.*, 2015)

As the name implies, this branch is related to the study of ancient human microbiota, but its scope is much broader as it includes studies of human evolution, human migration, and bacterial evolution... (Dagli *et al.*, 2015)

The era of paleomicrobiology began only decades ago when ancient DNA was first extracted from the dry muscles of extinct zebra-like species (*Equus quagga*). Shortly after this discovery, the first human DNA was found in a muscle extract from a pre-Egyptian mummy. The next important event was the successful DNA sequencing of Neanderthal specimens. Mitochondrial genome of mammoths were targeted for extraction and study. (Dagli *et al.*, 2015)

The next major event was the restoration of the first 4,000-year-old Paleo-Eskimo genome sequence and the draft sequence of the Neanderthal genome from a tuft of hair from a museum specimen.(Dagli *et al.*, 2015)

2.1. Objectives of paleomicrobiology

Its goals are the diagnosis of past infectious diseases, hundreds of years later, through the identification of specific molecular sequences in ancient remains.(Drancourt e Raoult, 2005a)

The diagnosis of an infectious disease is based on the identification of the pathogenic microorganism in the tissue of the infected individual, and the diagnosis of ancient infectious diseases should not escape this approach. Thus, paleomicrobiology offers the researcher in anthropology the possibility of confirming in a demonstrative way, a suspected diagnosis in front of the confrontation of anthropological data and historical data (Zink et al. 2002)

Elucidation of the epidemiology of past infectious diseases by reconstructing the temporal and geographical distribution of infected individuals, reservoirs and vectors.(Drancourt e Raoult, 2005a)

Tracing of the genetic evolution of the microorganisms themselves through genotyping (Drancourt e Raoult, 2005a).

Historical and anthropological contributions, by confirming or refuting historical hypotheses.(Drancourt e Raoult, 2005a)

2.2. From dental forensic medicine to paleomicrobiology

Forensic odontology is a domain of forensic medicine based on the study of jaws and teeth. It has 3 fields of use.(Jeddy *et al.*, 2017)

The first step is to evaluate and inspect jaw, oral tissue, and tooth damage from a variety of causes. Second, examine the possible identification of the suspect as a predator or any signs of subsequent exclusion, and finally examine the complete tooth fragment or debris (including all types of restorations) to make possible identification.(Balachander *et al.*, 2015)

Conventional methods used for this purpose include good dental record keeping (comparative analysis), bite analysis, imaging techniques, DNA analysis, cheiloscopy and rugoscopy (study of palatal papillae). (Jeddy *et al.*, 2017)

Forensic dentistry often requires an interdisciplinary approach to dentistry to properly diagnose a case. For example, an orthodontist can be very helpful if a forensic dentist needs to verify the identity of an individual. Teeth can record lifelong and long-lasting information about their anatomical / physiological changes and treatments such as orthodontic treatments, restorations and prostheses.(Reddy *et al.*, 2016)

Teeth are the most indestructible part of the human body. They survive not only after death but remain unchanged for many thousands of years. A well-known example is the teeth found in the mandible of Tabun man, aged about 35,000 years old. (Balachander *et al.*, 2015)

Dental identification is becoming more important because tooth tissue is often preserved when the deceased is skeletalized, disassembled, burned, or dismantled.(Jeddy et al., 2017) The tooth is an excellent source of DNA; pulp, dentin, cementum and periodontal ligament can be used in these investigations and can extend the time for genetic identification of decomposed human remains. The genomic DNA is then compared to ante-mortem samples to identify the deceased.(Jeddy et al., 2017; Adserias-Garriga et al., 2018)

These pulp-based DNA techniques have been extended to paleomicrobiology in the search for ancient microorganisms. (Raoult *et al.*, 2000; Jeddy *et al.*, 2017)

The pulp can be infected in a variety of ways, i.e. exposed dentinal direct pulp exposure, or through lateral or apical foramen, and of blood origin. (La *et al.*, 2008)

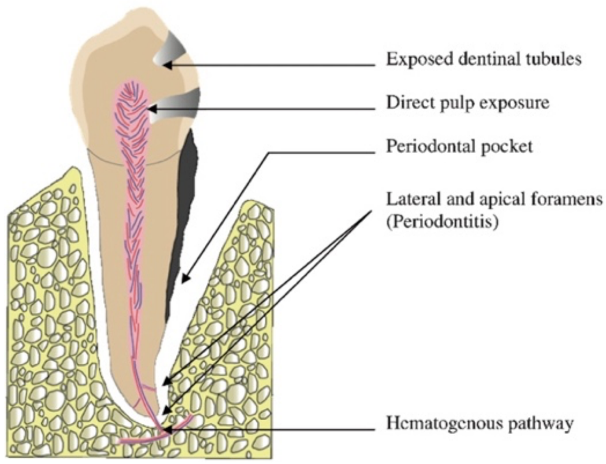

Fig 1 Pathways of pulpal infection (La *et al.*, 2008)

3. **Different sources of samples used**

Traces of pathogens can be found in various tissues

3.1. Bone tissue:

For bone specimens, this probably identifies potentially infectious anthropological relics using the resulting pathology, where certain microorganisms can produce visible lesions during chronic infection.(Drancourt et al., 1998)

COVID-19 is currently causing pandemics around the world. As the number of patients increased, the incidence of various complications, such as facial and jaw infections and inflammatory diseases, increased slightly in both early and late-stage infections.

(Boymuradov *et al.*, 2021)

However, and unlike COVID-19, tuberculosis and others, not all infectious diseases cause bone damage (Gilbert *et al.*, 2004; Drancourt e Raoult, 2016; Boymuradov *et al.*, 2021). There is also a problem of environmental contamination. (Drancourt e Raoult, 2005a)

3.2. Dental pulp:

It is well known that the pulp is a vascular tissue. The vascular density of the pulp is comparable to the vascular density of the human brain. Therefore, when a person is infected, the causative microorganisms are very likely to be found in the pulp. (Dagli *et al.*, 2015)

The pulp, when properly prepared, is a good sample for detecting past infections through the identification of DNA and proteins. In addition, it helps to understand the factors that cause historical illnesses and epidemics.(Mai *et al.*, 2020)

3.3. Dental tartar:

Tartar has been identified as a potential substrate for studying the genomic analysis of ancient oral microflora and its hosts. Many microorganisms in the oral cavity are trapped in tartar. Therefore, it’s a rich source of genetic material in the oral microflora. (Dagli et al., 2015)

The importance of archaeological calculus as a source of oral health and nutritional information is increasingly recognized. Early studies of archaeological calculus can be traced back almost a century.(Leigh, 1925). It was not until the 1970s that tartare was taken seriously by archaeologists, dental anthropologists and dentists who explained the outbreaks in human and animal populations and determined their mineral composition.

(Warinner *et al.*, 2015).

In the 1990s and 2000s, tartstone starch analysis made a fundamental contribution to the reconstruction of the starch components (roots, tubers, seeds, etc.) of the human and archaic hominine diet. (Warinner *et al.*, 2015).

Traces of host mitochondrial DNA were found in ancient tartar (Warinner *et al.*, 2015)

3.4. Dentin

Dentin is not widely used in archaeological studies to recover ancient DNA, probably because the amount of DNA recovered from it is so small. However, recent studies have shown that there is a strong binding affinity between DNA and dentin. In a study by Malin Brundin et al. When DNA extracted from *Fusobacterium nucleatum* was incubated with dentin, DNA bound to dentin was found to be recoverable by PCR, but no DNA was detected in the control (i.e., without dentin). This is because dentin is rich in hydroxyapatite. The stabilizing and protective effects of hydroxyapatite on DNA are involved. (Dagli *et al.*, 2015)

4. Interest of the dental pulp

The oral microbial flora is the second largest human microbial community after the intestine. Pulp and tartare have been identified as potential reservoirs of the oral microflora.(Dagli *et al.*, 2015)

It is well known that the pulp is a vascular tissue. The density of blood vessels in the pulp is comparable to the density of the human brain. Therefore, when the body is infected, it is very likely that the causative organism will be found in the pulp. DNA residues of systemic pathogens that cause bacterialemia were previously detected in old pulp. The genome can be detected in dead bacteria even years after cell death. *Bartonella quintana* was found in the pulp of a 4000 years old human sample (by Drancourt e Raoult, 2005).

Surrounded by dentin and enamel, it naturally protects the pulp from environmental pollution and has as much high vascular distribution as the brain and and has a long archaeological life in samples, which makes the pulp suitable for the study of retrospective diagnosis of infections. In conditions such as bacterialemia, the old dental pulp contains a sufficient amount of the causative agent's genetic material for testing. (Drancourt e Raoult, 2005a; Dagli et al., 2015)

Although the use of pulp is supported by several studies showing that the pulp is contaminated by microorganisms that have invaded through the apical foramen.(Dagli *et al.*, 2015)

Some studies have shown that a greater amount of amplifiable DNA was found in dental pulp (37%) than in bone tissue (5.7%). (Drancourt e Raoult, 2011)

5. **Different techniques used on the identification of microorganism pathogens from dental pulp. (Fig 2)**

Depending on the tissue studied, several methods are used to detect microorganisms in old tissue:

-Microbial isolation and culture: very complex to demonstrate on old samples in terms of bacterial survival. (Drancourt e Raoult, 2005a)

- Microscopic observations; for example, detection of *Mycobacterium tuberculosis* in Andean mummies (Drancourt e Raoult, 2005a)

- Study of proteins: immunodetection and serology (Drancourt e Raoult, 2005a; Barbieri *et al.*, 2017)

- DNA analysis based on PCR and sequencing. (Dagli *et al.*, 2015)

Based mainly on the amplification and sequencing of human DNA, as well as on the study of proteins, the evidence of *Y.pestis* in the dental pulp of subjects buried in French tombs dating from the seventeenth century could be realized for the first time in 1998. (Drancourt e Raoult, 2005a; Mai *et al.*, 2020)

5.1. Microscopy

The first study of pulp bacterial colonization in intact teeth was carried out by Tunnicliff and Hammond in 1937. After disinfecting the intact teeth and confirming their outer surface sterility, pulp samples were taken with sterile finely curved forceps and sterile split scissors. Smear specimens were stained with Gentian violet and Giemsa. The pulp was fixed with 1% formalin and the sections were stained with Gram-Weigert solution, hematoxylin-eosin and carbonthionin. Histology showed no leukocyte infiltration and *cocci* and *bacilli* found in different parts of the flesh. This was the first study of bacterial colonization of the pulp without rinsing the teeth (usually by preparing the cervical cavity of the tooth)

In the second study, cavities were prepared on the teeth of dogs that were intravenously injected with *E. coli* and *Streptococcus*. After tooth extraction, the teeth were fixed with 10% formalin or cold acetone, decalcified with 5% formic acid and embedded in paraffin. Bacteria are observed under a microscope in the pulp removed at various points after infection. (La et al., 2008)

5.2. Immunodetection

The only immunological detection study to date on the formation of bacterial colonies in the pulp was conducted by Gier and colleagues in 1968, with 88 teeth of four dogs producing deep class V cavities and E. coli was inoculated intravenously. A small piece of absorbent paper soaked in croton oil was sealed with half of the cavity under a concentrated mixture of zinc oxide and eugenol, leaving the other half open for oral fluid. Bacteria were found in tissue sections of 48 unexposed pulps out of 67 teeth of both caries types prepared and confirmed by culture. However, bacteria were also found in some blood vessels, but not in one pulp tissue of the control tooth (unprepared) that was removed 30 minutes after inoculation. The authors state that the pulp inflammatory response was proportional to the degree of damage and that bacteria were not found in the 23 undamaged control teeth. (La et al., 2008)

5.3. Culture

Aboudharam used guinea pigs to identifiy the presence of *Streptococcus* in the dental pulp. A deep cervical cavity was made and was injected intravenously with the bacteria in study. Of the 15 unexposed dental pulps, 11 produced group A *Streptococcus* and *E. coli* in culture. Similarly, pulp culture revealed *C. burnetii* in 2 of 12 specimens after experimental bacteraemia in guinea pigs. Cavities were not prepared, and the cultures were positive 7 and 21days after infection. These results suggested that the pulp was comparable to a small blood sample for the recovery of pathogenic agents. Viable *C. burnetii* was found in the pulp of guinea pigs after a week of bacteraemia. The authors suggested that the pulp could be a paradise for *C. burnetii* but the relationship between this finding and the patient is unknown. Guinea pigs have a constantly developed dentition with open apexes, which is very different to the situation in humans. (La *et al.*, 2008)

5.4. Nucleic-Acid-Based Detection

Nucleic acid-based detection is the most commonly used method in this type of biomedical research. Nucleic acid extracted from the pulp can be used for genome amplification. In most cases, PCR is followed by sequencing and identification. This method was used to make a retrospective diagnosis of infection by detecting a specific microbial sequence in the pulp. In these studies, teeth were decontaminated, pulp was removed using a variety of methods, and PCR was done to search for unique fragments of the microbial genome. An experiment like this should be controlled carefully at every step. The reasons are that this method is very sensitive and it has a high risk of contamination, by so leading to a false positive result. This approach is very useful when examining ancient specimens where DNA has been shown to be better preserved in the pulp than in bone. (La *et al.*, 2008)

Numerous protocols have been established to reduce at the maximum the external contamination and to conduct experiments in laboratories where the bacteria of interest have never been introduced or treated yet. (Raoult *et al.*, 2000)

6. From dental tissue selection to Detection of Microorganisms in Dental Pulp

6.1. The choose of dental tissue

- Prefer intact teeth with a closed apex, such as untraumatized and healthy teeth, to limit contamination (without cracks, fractures, excessive abrasion or carious lesions...) It is important to analyze several teeth in the same individual to maximize the chances of detection. (Drancourt e Raoult, 2005b; Mai *et al.*, 2020)

If possible, give preference to teeth surrounded by bone tissue such as erupting teeth, impacted teeth, wisdom teeth... (Sterile and bone protected).

- Selection according to the volume of the pulp chamber. (Mai *et al.*, 2020).The pulp volume is proportional to the volume of DNA recovered. Monoradicular teeth are to be preferred because their handling is easier and they can present a larger pulp chamber (incisors, premolars and especially canines) (Mai *et al.*, 2020)

- Consider the estimated age of the subject: the teeth of mature subjects are more resistant over time; the DNA is less degraded. This is due to a more mineralized enamel, a less porous and thicker dentin and cementum. (Mai *et al.*, 2020)

6.2. Sample Preparation

It's important to not separate the tooth from the socket at the sampling site to avoid contamination. This must be done in the laboratory in a sterile manner. (Tran *et al.*, 2011; Mai *et al.*, 2020)The preservation of DNA in old teeth is highly influenced by storage temperatures; in samples buried in soil a 1/2 reduction in DNA half-life was detected just with an increase of 2°C. In the laboratory, proper storage of teeth should at -20°C. (Mai *et al.*, 2020)

Jaws and teeth are photographed in all planes of space, for comparative (for further studies) and statistical purposes (Mai *et al.*, 2020)

Preoperative radiographs are also necessary to select the most appropriate teeth. This allows visualization of the pulp morphology and anticipates the presence of calcification or fissures that could contaminate the specimens. Retroalveolar radiographs, MRI and CT scans are the most used. There is no risk of DNA damage below 200Gy. (Mai *et al.*, 2020)

6.3. Removal of Dental Pulp (tab 2)

Pulp can be removed in a variety of ways, depending on the condition and purpose of the test. Generally, before removing the pulp, the outer surface of the tooth is wiped with bleach and exposed to ultraviolet (UV) light to extract and decontaminate the tooth. (La *et al.*, 2008)

6.4. Extraction of Nucleic Acids

Any extraction protocol can be used to extract nucleic acids directly without the demineralization step, because the pulp is soft tissue. Based on experience with older samples, the phenol-chloroform protocol for total nucleic acid extraction is the best one (fig 5). Controls should be performed to avoid the risk of contamination (Barnett e Larson, 2012)

6.5. Prevention of Contamination (Tab1)

Depending on the nature of the sample a variety of external decontamination methods can be used. For example, washing the tooth with distilled water, soaking it in absolute or 70% ethanol, and exposing it to UV light. In terms of protocols, the best one is to completely cover the tooth with sterile resin and then by centrifugation, recovering the pulp through the apex (fig 4). However, this technique is not applicable for molars with small and thin root canals. It’s more useful for monoradicular teeth. Each step of the experiment should be performed in a separate room with disposable equipment and freshly prepared reagents. All PCR-based experiments should be performed in designated one-way PCR sets with appropriate ventilation. By introducing numerous contaminations and negative controls into any amplification reaction can help determine the source of any contamination, and by so helping to avoid the contamination in the next experiment. (La *et al.*, 2008)

III. Discussion

Paleomicrobiology is a recent discipline at the crossroads of medicine, archaeology and microbiology, dental paleomicrobiology has, in about ten years, strongly contributed to the understanding of the history and evolution of diseases and ancient micro ecosystems. In this respect, it also represents a means to fight against the re-emergence of infectious diseases. (Fornaciari, 2017; Bos et al., 2019)

By studying the pulp, we can diagnose previous infections and identify the causative organism to elucidate epidemiologies from the past.

Thanks to its durability the use of the dental pulp to detect microorganisms could help us determine when the microbes infect a the host. Dental pulp can also be used to provide information about emerging infectious diseases, helping to establish models of emerging infections and by doing it, it's contribute to the advancement and development of appropriate preventative measures.(Drancourt e Raoult, 2005b)

However, the underlying problem is the need for careful measures to protect the material from external contamination. Therefore, a more universally applicable protocol is needed to facilitate the use of dental pulp in paleomicrobiology. This is one of the reasons why it's important to have a dentist or a dental specialist present during these steps. In the step of choosing the tooth (for example) to be used as the source of information, the dentist it's the best choice regarding the knowledge of the teeth anatomy. The knowledge of extractions protocols of tissues or organ(s) from the oral cavity, the process of decontamination of the source needs to be carried out by a dentist or a specialist in dentistry, experienced. The quality and accuracy of the final results, as well as the correctness of the final diagnosis, depends on it.

The dentist and the dental medicine have a major role on the paleomicrobiology, the knowledge and the experience of a dental specialist are essential for this field of study to make a step forward.

IV. Conclusion

Paleomicrobiology is important for the diagnosis of past infectious diseases through the identification of specific molecular sequences in ancient remains, also the elucidation of the epidemiology of past infectious diseases by reconstructing the temporal and geographical distribution of infected individuals, reservoirs and vectors; by so it helps to improve management of new pandemic episodes. Paleomicrobiology uses many different sources of samples, in this review we've focused on the oral cavity, where the dentist might be a relevance.

We have two types of information. Firstly, the anatomical one which includes the structure of the oral jawbone and the maxilla. As we saw briefly, inflammatory infection diseases can affect the structure of the oral bone, as it we can observe in the face and jaw of COVID-19 patients during early infection stages but also later on the disease evolution.

The second type of information is the DNA information which includes, in particularly the dental pulp, dental calculus and the dentin. We saw from the processes of choosing the tooth until the DNA information, by talking about the preparation of the sample, the different techniques used to extract dental pulp and the prevention of decontamination of the sample. We've verified the importance of the dentist and the dental medicine in this field of study; dentists and dental specialists are the best choice regarding oral paleomicrobiology because of their knowledge about oral tissues and expertise in the application of the tooth manipulation protocols. The correctness of the past diseases diagnosis', by getting better results, depends on it.

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Anexos

Fig. 1:Some of the Cultural time periods and epidemics or pandemics of human history(Spyrou *et al.*, 2019)

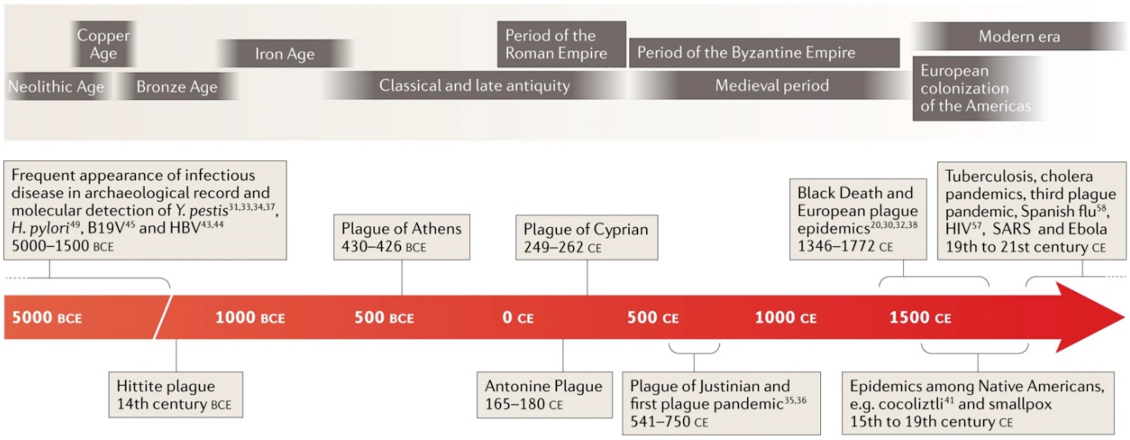


Fig 2 Methods used for the detection of microorganisms in dental pulp (La *et al.*, 2008)

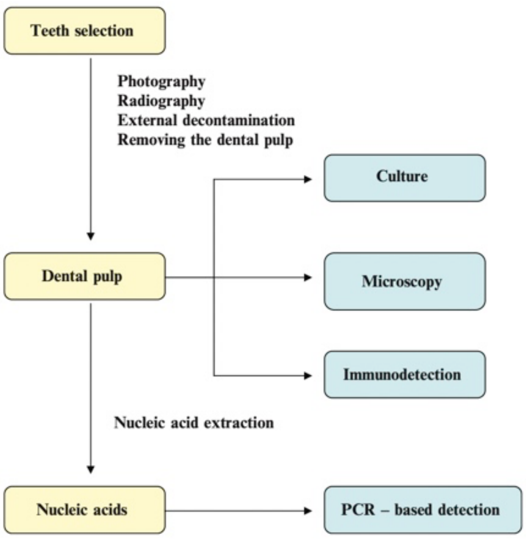


Fig 3 Complete opening of the pulp chamber by longitudinal section of the tooth. (La et al., 2008)

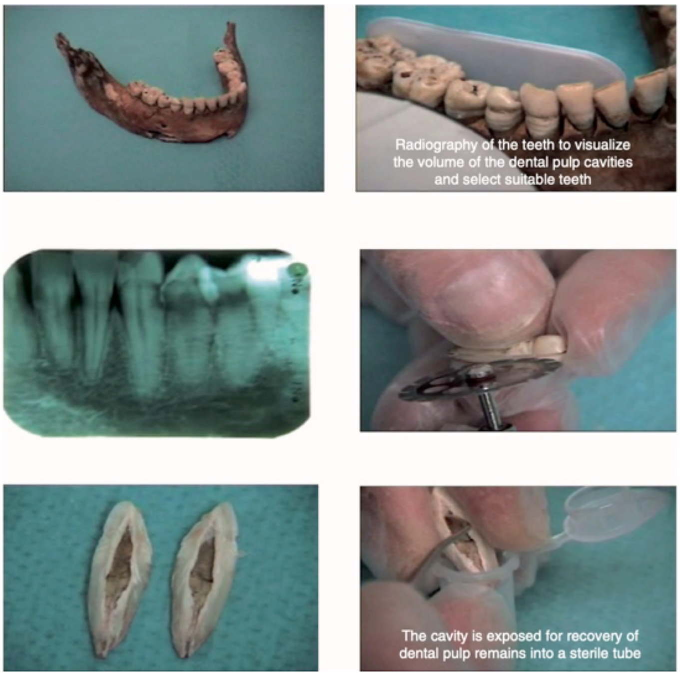
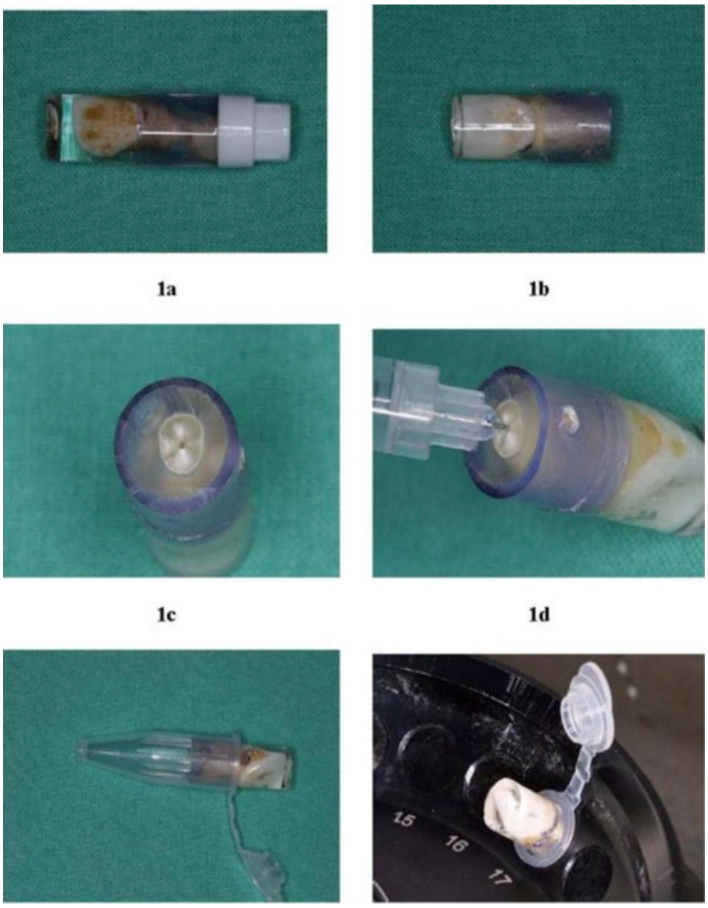


Fig 4 protocol for recovery of dental pulp through the apex.(Tran-Hung *et al.*, 2007)



Tab 1:Prevention of contamination in paleomicrobiology (Woodward et al. 1994; Drancourt et al. 1998; Drancourt et Raoult 2005; Mai et al. 2020)

procedure	Sample Preparation	DNA extraction	PCR
risk	-Contamination by environmental flora -Contamination by manuported	-Contamination by environmental flora Positive control -Contamination with previous PCR amplicons	Cross-contamination (by other pathogens previously studied in the laboratory)
Preventive mesures	-Be equipped with personal protection personal protection when handling samples; gloves, gowns, masks gowns... -Removal of the external tissues of the tooth by scraping, sandblasting or by using a burr mounted on a surgical handpiece. -Rinsing with distilled water. -Washing with ethanol, hydrogen peroxide or sodium hypochlorite - Drying with compressed air. -Drying by exposure to ultraviolet rays (254nm) Coating the teeth in a sterile resin -Handling and study of samples should be done in a controlled room that has never been studied, grown and amplified	- The extraction and amplification of DNA must take place in a controlled and ventilated room where it has never been grown, studied and amplified -Be equipped with personal protective equipment when handling samples; gloves, gowns, masks, gowns...	- There must be no positive controls -have several negative controls - Sequence all DNA fragments obtained by PCR -The handling and study of the samples must take place in a controlled room that has never been studied, cultivated and amplified - The primers used must be single-use -The PCR must be unidirectional. -Be equipped with personal protective equipment when handling samples; gloves, gowns, masks.

Tab 2 The different techniques for pulp DNA recovery

technique	Principle	advantages	incovenients
fully ground tooth	The dental tissue is analyzed in its entirety	Dental tissue is analyzed in its totality	- Subject to contamination - No advantage over bone tissue - Loss of dental morphology
pulp removal through the apex	Embedding of the tooth in sterile resin or silicone; Sectioning of the apex to provide access to the pulp complex. Addition of solutions for extraction and purification of DNA directly in the directly into the canal; the tooth is used as a natural test tube. natural test tube. The digestion products are recovered by centrifugation. The orifice can also be made at the coronal level	- Respect of the dental morphology - Limits very strongly the external contamination of the dental pulp by the apex	- Technique requiring the intervention of a dental surgeon
Complete opening of the pulp chamber by longitudinal section of the tooth.	Creation of an incision path opposite the pulp, without reaching it, thanks to a diamond or carborundum disk mounted on a handpiece. It allows to anticipate the fracture line. This incision path varies according to the type of tooth: Monoradicular teeth and maxillary premolars: this line is made on the vestibular is made on the vestibular and lingual surfaces. Maxillary molars: a path is made in the palatal area, as well as in the vestibular area (between the 2 roots) and in the vestibular area of the root. vestibular surface of the palatal root. Mandibular molars: a path is made distally over the entire tooth height and mesially between the two roots and a reminder on the mesial side of the distal root. The pulp chamber is opened by a clean fracture using a syndesmotome inserted into the incision line and accompanied by a dislocation movement. Recovery of the pulp sample (as well as some traces of juxta-pulpal dentin) is recovered by scraping. Approximately 7.5 mg of powder is recovered per tooth.	-The two parts of the tooth can be glued back together for a later morphological study - Allows to recover a higher concentration of DNA - Limits contamination	-Technique requiring the intervention of a dental surgeon -It is a deleterious analysis technique for the tooth, but if it is well done, the tooth can still be reconstructed by bonding for possible later morphological analyses.

(Drancourt e Raoult, 2005a, 2011; Tran-Hung *et al.*, 2007; Mai *et al.*, 2020)

Fig 5 Phenol chloroform extraction (Barnett e Larson, 2012)