Historical Pathologica

Ancient DNA and paleogenetics: risks and potentiality

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Summary

Paleopathology, the science that studies the diseases of the past, has always been
addressed to the future in the use of new diagnostic methods. One of its relatively recent
branches is paleogenetics, which is the study of genetic material from the past. Nuclear
and mitochondrial DNA recovered from archaeological and paleontological specimens is
called ancient DNA (aDNA), which can be extracted from a large variety of biological
materials, of different origin, state of preservation and age, such as bones, teeth, copro-
lites, mummified tissues and hairs. There are many applications for ancient DNA research
in the field of archaeology and paleopathology: population demography, genealogy, dis-
ease studies, archaeological reconstruction of plant vegetation, calibration of the molecu-
lar clock, phylogenetic relationship between different mammals and interpretation of the
paleoclimate. However, the study of ancient genetic material is extremely difficult due to its
poor quality and quantity, as well possible contamination with modern DNA. New advanced
methods will allow extracting DNA from a greater variety of materials, and improvements in
sequencing techniques will unveil data that are currently concealed.
The aim of this paper is to provide initial insights into paleogenetics and ancient DNA
study and to illustrate the limits, risks and potentiality of the research on the genetic mate-
rial of ancient specimens, whose results have a strong impact on the present and future
medicine.

Key words: ancient DNA, paleogenetics, paleopathology, mummies, metagenomics

Introduction

Paleopathology, the science that studies the diseases of the past through
skeletal or mummified remains, has always been addressed to the future
in the use of new diagnostic methods and at the forefront in the applica-
tion of new tools gradually discovered and used in the medical routine.
At the end of the 19th century, the approach to the study of human re-
mains was almost an educated *divertissement* (like the public "unwrap-
ping" of the mummies), but scientists later began to use scientific meth-
ods based on macroscopic observation and, in case of mummified re-
mains, on microscopic evaluation borrowed from classical pathological
anatomy [1]. Thus, paleopathology could soon benefit from new imaging
methods such as CT [2] almost in concomitance with their use in routine
medicine. Therefore, it is no coincidence that genetics entered the study
of ancient human remains some decades ago [3], even if, in the light of the
latest findings, the first papers are no longer reliable due to contaminat-
ing modern DNA [4].
Published articles on paleogenetics have increased exponentially in re-
cent years and the advent of sophisticated new methodologies such as
genomics and proteomics are providing unimaginable results only a few
years ago.

DNA, mtDNA, ancient DNA and the problem of degradation

DNA is a complex two-stranded molecule that holds the instructions for building the proteins and that contains all the information necessary to build and maintain an organism. DNA is present in the nucleus of each cell of an organism (therefore called "nuclear") and represents the genetic patrimony that is transmitted to descendants. However, each child receives an equal contribution from each parent, so that nuclear DNA will be a mixture of parental DNAs. In the cytoplasm of all animal cells (matrix) with aerobic metabolism and plant eukaryotic cells there are generally elongated organelles called "mitochondria". Mitochondria possess their own genetic inheritance, mitochondrial DNA (mtDNA), partially autonomous compared to that contained in the nucleus. This genetic inheritance is composed of circular molecules of double-helix DNA. Each molecule is about 16,500 pairs of bases. The transmission of nuclear and mitochondrial DNA from parents to children follows different mechanisms since mitochondrial DNA derives exclusively from the mother (maternal inheritance). The fact that mtDNA is transmitted through the maternal line means that it will not undergo segregation or recombination. In fact, mtDNA is transmitted unchanged through generations and changes can only intervene through mutations. Two individuals who have an ancestor in common will present a more divergent mtDNA the greater the time separating their from the ancestor. It is therefore particularly suited to study the history of the human populations that have been accomplished over a few million years [5]. Mitochondrial DNA analysis in the paleoanthropological field offers multiple perspectives, in particular, the possibility to check whether an individual in antiquity contributed to the genetic variability of modern populations over the same territory. The most important example is offered by studies on the mitochondrial DNA of the Neanderthals [6]. The nuclear and mitochondrial DNA recovered from archaeological and paleontological remains is called ancient DNA (aDNA). aDNA can be extracted from a large variety of biological materials, of different origin, state of preservation and age, such as bones, teeth [7], coprolites [8], mummified tissues [9] and hairs [10]. However, ancient remains always contain only small fragments of aDNA, generally in a poor state of preservation. Postmortem instability of nucleic acids is responsible for the degradation of DNA. Marota and Rollo used an incisive example in order to understand how much the thanatological processes are damaging for the integrity of the double-helix molecule: "aDNA is like an approximate thirty-kilometre-long ribbon reduced to fragments of 10 ÷ 20 cm" [11].

DNA is subject to various types of damage, mainly caused by two phenomena: hydrolysis and oxidation. In the living cell, a series of enzymatic processes intervene to repair the damaged DNA. However, at the death of the cell, the repair processes are interrupted. From this moment on, DNA starts to accumulate different types of alterations that become more and more numerous. The degradation of DNA depends mainly on the state of preservation of the sample rather than its antiquity. The most typical type of damage is the fragmentation of the double helix. In general, the degradative processes that alter the structure can be attributed to multiple causes, which are summarized in direct modification (the action of enzymes released by microorganisms) and cellular nucleasic activity by exo-endo nucleasic enzymes. In this respect, the types of damage the aDNA can encounter are generally classified into two categories: 1) modifications of the nucleotide bases; 2) fragmentation of single-strand break (SSB) helix or double-strand break (DSB) helix due to hydrolysis. Among the four nitrogenous bases (adenine, guanine, thymine and cytosine), the first two in particular (purine bases) tend to detach by hydrolysis. These reactions are influenced both by temperature and pH. In the site without the base, a reaction of detachment of the deoxyribose results in an interruption of the filament. When these interruptions accumulate and coincide on the two filaments, the double helix breaks. In addition to hydrolysis, the bases may undergo oxidative modifications, either directly as a result of interaction with ionizing radiation or indirectly as a result of the action of free radicals [(superoxide anion radical (O_2^-), singlet oxygen $(^1O_2)$, hydroxyl radical (OH-) and perhydroxyl radical (HO_2-)].

Environmental factors such as temperature and climate events have a direct influence on the long- term survival of aDNA molecules. The link between temperature and spontaneous chemical decay is described by the Arrhenius equation: $k = A \exp(-Ea/RT)$, where k is the constant or the coefficient of reaction velocity, A is a factor that depends on the reaction itself, Ea the activation energy, R the gas constant (8.31 KJ mol-1 a 1 atm) and T the temperature in Kelvin. According to this formula, theoretically, each decrease in temperature corresponds to an exponential reduction in the reaction speed. As a concrete example, a bacterial chromosome consisting of 3×10^6 base pairs (bp) will fragment into pieces with a length of about 100 bp in "just" 500 years at a temperature of 15°C. The same process will require 81,000 years if the temperature is -10°C [11]. If we turn these considerations into paleopathology, we easily understand that the chances of being able to isolate DNA from ancient human remains

are very poor and require advanced laboratory techniques capable of extracting and analyzing minimal genetic fragments.

Materials, methods and pitfalls

Ancient DNA can be extracted from a wide variety of biological samples, but due to its extreme fragmentation, the analysis can be very challenging. Endogenous DNA has been successfully obtained from human teeth (including dentine, crushed cementum of the roots and dental calculus, used for the exploration of the oral microbiome), compact bone (femur and tibia) and, more recently, from the petrous portion of the temporal bone [12] as the osteocytic *lacunae* behave like "protective niches" for the nucleic acids. It is precisely from these "natural protective shells" that it is possible to extract the ancient genetic heritage. In particular, the high quality of the endogenous genetic material extracted from the petrous bone may be due to the high density of the bone that reduces the bacteria-mediated and other post-mortem DNA decay.

The application of several technologies, from polymerase chain reaction (PCR) and traditional Sanger sequencing to High-Throughput DNA Sequencing (HTS) methods like Next Generation Sequencing (NGS), dramatically changed the study of ancient DNA. In PCR, a segment of DNA defined by two synthetic oligonucleotide primers is amplified through repeated cycles of denaturation of the DNA, annealing of the oligonucleotide primers, and synthesis of new DNA strands. Early extraction protocols for aDNA were not very different from the protocols used to extract DNA from contemporary sources. A new revolution in ancient DNA research started with the introduction of HTS and Next-Generation Sequencing (NGS) techniques. These technologies generate large quantities of highly accurate DNA sequences at lower costs than it was possible by using first-generation sequencing technologies. While traditional PCR methods could only amplify a small number of specific DNA targets, HTS combines amplification and sequencing of up to several billions of individual DNA library templates at a time [13]; moreover, HTS can sequence shorter DNA fragments. NGS technologies have been integral to the study of paleogenomes, which is the entire genetic material extracted from an organism of the past. It consists of DNA and includes both the genes (the coding regions) and the noncoding DNA, as well as the genetic material of the mitochondria and, eventually, chloroplasts. The first sequenced human paleogenome dates to 2010 and was isolated from a well-preserved Saqqaq paleoeskimo hair sample [14].

The most serious criticism in the paleogenetics field is the potential contamination of samples by contemporary DNA [15]. Since modern DNA consists of intact template molecules, it will be copied with much higher efficiency during the PCR process than the fragmented and damaged aDNA templates. It is thus fundamental to conduct aDNA research in a laboratory devoted exclusively to ancient studies, where no investigations with modern DNA are performed. Moreover, specimen collection and sampling must be performed by taking severe precautions to avoid contamination. For example, HTS data from a specimen of a Neanderthal sampled without precautions in the 1980s showed that 10.2% of sequences belonged to modern human contaminating DNA [16].

For a correct paleogenetics study, several precautions must be taken from the archaeological excavation (or the first handling in general) up to the analysis in the laboratory [17].

During sampling, paleopathologists and archaeologists must:
- wear sterile surgical coats, sterile latex gloves, sterile masks, headdresses and overshoes;
- clean tools (e.g. trowels, picks, brushes etc.);
- not wash specimens with water since it may be contaminated by bacterial DNA;
- immediately store and seal the specimens in sterile containers.

Many essential precautions must be taken in the aDNA laboratory:
- to remove DNA contamination, the outer surface of the bones should be removed and the resulting core irradiated [18]. Similarly, the external surface of the teeth must be decontaminated by bleach treatment before dentine removal;
- physical separation of pre- and post-PCR procedures;
- use of UV-irradiated hoods with sterile equipment;
- strict protocols to prevent the introduction of modern DNA in the aDNA samples;
- use of negative controls;
- replication of DNA studies of all specimens to confirm the authenticity of the results;
- assessment of sequence data to confirm the results;
- observation of an inverse relationship between fragment size and PCR efficiency;
- study the DNA profile of every researcher participating in the work. Comparing their DNA profiles with those obtained from ancient samples, we could rule out the possible contamination of modern DNA.

Applications

There are several applications for ancient DNA research in the field of archaeology and paleopathology. It is, in fact, possible to study the history of humans and their ancestors, their lifestyle and physical characteristics. But it is also feasible to understand the interaction of individuals from the past with the environment around them. The direct genetic analysis of genomes from an environmental sample is called metagenomics. The advent and development of metagenomics is one of the most important events in the field of microbial ecology in the last decade [19]. In particular, the study of the intestinal microbiome plays an extremely important role in the study of ancient individuals. The intestinal (or gut) microbiome is the whole genetic inheritance possessed by the microbiota, which is the totality of the microorganisms – bacteria, fungi and protozoa – and of the viruses that live and colonize the intestine. These microbes have a heavy impact on physiology, both in health and in disease. They contribute to metabolic functions, protect against pathogens, regulate the immune system, and affect most of our physiologic functions directly or indirectly.

Even if little is still known about the microbiome resulting from the process of mummification of the human gut, it was possible to reconstruct the intestinal population of some pre-Inca mummies from Peru, to identify the *Trypanosoma cruzi, Leishmania donovani* and Human Papilloma Virus and to reconstruct their phylogenetic evolution [20]. Moreover, South American mummies and Italian Renaissance mummies showed several antibiotic resistance genes in concentrations not dissimilar to those present today. Surprisingly, the presence of antibiotic-resistance genes indicates that these genes pre-date the therapeutic use of these compounds and that they are not necessarily associated with a selective pressure of antibiotic use [21]. Studies on the ancient microbiome represent an opportunity to better understand microbe-host interactions, membership and ecology of microbes, the evolution of commensal and pathogenic microorganisms and their impact on health and disease.

In summary, the various applications of paleogenetics can be listed as follows:

1 Population demography [22].
2 Evaluation of the actual number of individuals found within multiple burials, in order to identify with certainty the skeletal segments belonging to each individual in case of dubious attribution by anthropologists. Alternatively, allow for sex determination in the case of bony remains belonging to sub-adults or infants, for which the development of sexual traits is incomplete and poorly evident.
3 Genealogy. As described previously, the mitochondrial genome could establish family relationships. For example, the royal mummies of the late 18th dynasty in Egypt were studied to obtain a correct identification and to establish genealogical relationships (King Tutankhamun Family Project). In particular, genetic fingerprinting has made it possible to construct a pedigree of 5 generations of the Tutankhamun lineage [23].
4 Understanding the admixture between different hominin species (e.g. genetic relationship between Neanderthals and Denisovans) [24].
5 Disease studies. This is the paleopathology application *stricto sensu*. The study of ancient pathological conditions is crucial since it may have a direct impact on the understanding of modern diseases and their treatment. At the beginning of the 1990s, with the detection of Mycobacterium tuberculosis complex DNA, PCR-based amplification methods started to be used to diagnose diseases in skeletal and mummified remains [25]. Roberts and Ingham specified that aDNA is also used to identify diseases that may not cause visible changes in the skeleton (e.g. malaria, plague, *E. coli*), and to understand the exact aetiology of non-specific pathological lesions [4]. Among several pathogens, the most studied is the *Mycobacterium tuberculosis*. The principle of paleomolecular diagnosis of the mycobacterium is based on PCR amplification of a short tract of the bacterial chromosome called "insertion sequence (IS) 6110" [26], which is specific of the so-called *"M. tuberculosis* complex" (MTB) that includes *M. bovis* and *M. africanum*, as well as *M. tuberculosis*. It is thus possible to discriminate between the mycobacterial DNA of the soil, which is abundant in the excavation materials, and that of ancient pathogens.
6 Archaeological reconstruction of vegetation and understanding of the process of plant domestication [27].
7 Calibration of the molecular clock in phylogenetic evolutionary studies [28].
8 Interpretation of the palaeoclimate by the use of palaeoenvironmental DNA [29].
9 Phylogenetic relationship among different mammals [30].
10 The study of ancient pathogens genomes to inform the future management of emerging and re-emerging diseases.
11 Unmasking fraud and refutation of fake news. Even if it seems a hot topic only in the contemporary world, fake news in science has existed for centuries. For example, at the beginning of the 20th century, the famous Piltdown Man hoax, consid-

ered the greatest anthropological scam in history, happened. The finding consisted of fragments of skull and mandibular bone, declared by the discoverers as collected in the Piltdown area of East Sussex. The unknown hominid was given the scientific name of *Eoanthropus dawsoni*, after the name of the discoverer Charles Dawson. The discovery of the new species was the subject of controversy, which was only resolved in 1953, when the fake was definitively unmasked since it was supposed that the remains were probably obtained by combining the mandibular bone of an orang-utan with fragments of the skull of a modern man. Recent re-evaluation of the Piltdown fossils using the latest scientific methods (DNA analyses, high-precision measurements, spectroscopy and virtual anthropology) showed that it is highly likely that a single orang-utan specimen and at least two human specimens were used to create the fake fossils [31]. Then, numerous mummies, thanks for example to imaging techniques, turned out to be false or composed by material different from the supposed one. A particular case is the Atacama mummy which, according to some pseudoscientists, was thought to be even of alien origin. Whole-genome sequencing demonstrated the human nature of the fetus which was probably the carrier of a genetic mutation linked to dysplasia, dwarfism and scoliosis [32].

Conclusions

The study of genetic material extracted from ancient samples requires careful and scrupulous management in all steps, from archaeological excavation to laboratory procedures. Close collaboration between archaeologists, paleopathologists and geneticists is therefore necessary. Paleogenetics, from an expensive and rare technique, has become accessible and almost routine in paleopathological studies since it is fundamental to obtain results that would otherwise be hidden forever. The study of ancient molecular paleopathology in human and animal remains is a matter of fundamental importance because of the implications for understanding human evolution, predicting emerging and re-emerging diseases and possibly their future management.

For example, the study of the full genome from teeth and bones of victims of the 1348-1350 Black Death in England revealed that the perceived increased virulence of the disease during the Black Death may not have been due to bacterial characteristics, but due to a combination of climate, vector dynamics, social conditions and synergistic interactions with concurrent dis-

eases. But above all, the medieval plague "was probably responsible for its introduction and widespread distribution in human populations. This indicates that the pathogen implicated in the Black Death has close relatives in the twenty-first century that are both endemic and emerging" [33].

Analyzing another more recent pandemic, the study from an archived formalin-fixed lung autopsy specimen and from the frozen lung tissues of an Alaskan influenza victim buried in permafrost in November 1918 allowed reconstructing the 1918-19 Spanish influenza genome [34]. This discovery provided insight into the nature and origin of this pathogen, which caused millions of victims worldwide. Comparison of the 1918 pandemic virus with contemporary human influenza H1N1 viruses indicates that the 1918 pandemic virus was able to replicate in the absence of trypsin, and this might be one of the reasons why the virus had a uniquely high-virulence phenotype [35]. This information may be useful to design management programs aimed at preventing other influenza epidemics and at developing future vaccines.

Science, and genetics in particular, is constantly evolving, so new methodologies will allow extracting DNA from a greater variety of materials, and improvements in sequencing technologies will provide data that will allow us to thoroughly investigate the past to better face the future.

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