ANCIENT DNA RESEARCH METHODS

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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 application 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 remains was almost an educated divertissement (like the public "unwrapping" of the mummies), but scientists later began to use scientific methods based on macroscopic observation and, in case of mummified remains, on microscopic evaluation borrowed from classical pathological anatomy. Thus, paleopathology could soon benefit from new imaging methods such as CT almost in concomitance with their use in routine medicine.

The aim of this abstracts to provide initial insights into paleogenetics and ancient DNA study and to illustrate the limits, risks and potentiality of the research on the genetic material of ancient specimens, whose results have a strong impact on the present and future medicine.

Materials and methods. 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 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; 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.

Research results. 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. 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. 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. 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.

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. 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. 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”, which is specific of the so-called “M. tuberculosis complex” 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.

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 diseases.

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 1918-1919 Spanish influenza genome. 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. 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.

BIOCHEMICAL ASPECTS OF SYMBIOSIS RESEARCH

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Introduction. In symbioses, the irreducible complexity of an organism is compounded by persistent symbiotic interactions with one, two, or many phylogenetically different organisms, each of which is adapted to function in the context of its partner or partners. Until recently, the molecular basis of symbiosis could be studied only on the basis of one or a few genes and their products at a time. For example, in their research on interactions between a Legionella-like bacterium and its host Amoeba proteus, Jeon and colleagues were able to correlate the reduced expression of a single host gene product, S-adenosyl methionine synthetase (SAMS), with the rapid evolution from a pathogenic to a mutualistic relationship. It is very likely that this dramatic evolutionary transition involved multiple coevolved changes in the metabolic and regulatory networks of the two organisms, but a systematic analysis of these putative changes accompanying the change in host SAMS expression was technically unrealistic at that time. Today, just 9 years later, the association in A. proteus and other fascinating symbioses can be interrogated by a range of high-throughput methods that reveal the total (or near-total) complement of a particular class of biological molecules: genes, transcripts, proteins, lipids, metabolites, etc.

The “omics” revolution of the last decade has transformed our capacity to understand symbioses at the molecular level. It is now possible, for example, to construct an inventory of the genes coded by each partner, to quantify patterns of transcription under different environmental conditions, to establish the relationship between transcript and protein abundance for every protein-coding gene, and to determine the metabolite set that makes up the metabolic pool of the interacting symbiotic partners.

The minimal output of omics is lists of genes, proteins, metabolites, etc., that are a partial or near-complete molecular catalog of an organism or symbiosis. To use omics methods to answer biological questions requires great care in experimental design and interpretation. In the second part