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STUDIES ON THE PRESERVATION OF THE INTESTINAL MICROBIOTA'S DNA IN HUMAN MUMMIES FROM COLD ENVIRONMENTS

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SUMMARY

Analysis of ancient microorganism DNA represents one of the newest and most promising branches of molecular archaeology. In particular, microbial DNA associated with human remains can provide direct evidence of the occurrence and frequency of infectious diseases in historic times. Human mummies represent very interesting subjects for palaeomicrobiological investigations as they retain soft tissues. Recently reports on the identification of ancient bacterial pathogens in human mummies by DNA analysis are steadily becoming more numerous. However, despite this favourable trend, the analysis of ancient microbial DNA is still a contentious issue. As a model system, we studied the preservation of the intestinal microbiota's DNA in two naturally freeze-dried human mummies found on the Alps. This kind of mummy is an ideal subject for ancient DNA investigations. The first is a male body historically dated 1918 A.D. while the second is the famous Tyrolean Iceman (3.350-3.100 BC).

Introduction
Human populations constantly share the ecosystems with a wide

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variety of microorganisms (bacteria, viruses and protozoa) establishing with them a delicate equilibrium; changes in the environment, transitions in human ecology or mutations and adaptations in microorganisms often affect this delicate interaction.

Most microorganisms are benevolent, and some are symbiontic, because their presence is beneficial to both host and microbe, whereas some adversely affect the host's biology[1]. In the latter case we have an "infectious disease".

Before the advent of molecular techniques, the diagnosis of a disease in antiquity was fundamentally based on three sources of information: artistic representations of pathological conditions, ancient descriptive tests, and direct observations of human remains (skeletal or mummified material) by radiologic or histological examinations[2]. The first two methods may be criticized, but the last source is without any doubt more reliable. In the early nineties, Wood and colleagues[3] discussed "the osteological paradox" based on the evidence that in most cases the bones of the deceased do not show signs of disease, simply because in many acute conditions the illness results in death before a skeletal response occurs. The manifestations of diseases in bones are, generally, expressions of chronic conditions. For this reason, skeletons without signs of disease may belong either to healthy individuals or to individuals which have died as a result of acute illness.

Hence to reconstruct the general condition of health in the past, the information available in bones should be offset with analyses of the soft tissues. Mummies (human or animal bodies that have been preserved through artificial or accidental means) are ideal for this purpose.

The analysis of the microbial DNA associated with human remains provides direct evidence of the occurrence and frequency of infectious diseases and bears witness to the delicate relationship among humans, environment, and microbes in historic times. Moreover, the analysis of bacterial DNA in ancient tissue samples offers an interesting approach for the study of the evolution of microorganisms, their interaction with hosts, the origin and spatial distribution of a disease, and aetiologies of epidemics.

In recent times, various old infectious diseases, such as tuberculosis,

malaria and cholera, that seemed to be eliminated, have been on the increase. Data from studies of past infectious diseases may offer insights into the present management of public health, as well as an understanding of the emergence and reemergence of infectious disease.

This paper reviews the polymerase chain reaction (PCR) based molecular studies which have aimed to detect and identify bacteria associated with archaeological human mummies.

Long-term persistence of bacterial DNA

The persistence of DNA throughout the course of time remains a contentious issue. DNA molecules are relatively unstable compared with other cellular components and will degrade with time if not repaired. DNA undergoes a spontaneous depurination process that rapidly leads to strand breakage. The speed of this process can vary as a function of temperature and pH of the medium. Irrespective of the speed, however, the final result will always be the same, i.e. the degradation of all DNA in the sample[4]. Past reports[5] indicate that while samples coming from Arctic and Antarctic regions have relatively high success rates, this is not the case for those coming from warm regions. On the basis of the extent of racemization of aspartic acid, Poinar *et al*[6] stated that *"the survival of DNA is limited to a few thousand years in warm regions such as Egypt and to roughly 10[5] years in cold regions."*

Marota *et al*.[7] studied the DNA decay rate in papyri and human remains from Egyptian archaeological sites. They established that the DNA half-life in papyri is about 19-24 years which corresponds to a maximum survival time of about 530-670 years.

Theoretical calculations of DNA survival, based on the Arrhenius equation and depurination kinetics[8], suggest that a bacterial genome of 3.0×10^6 bp (alternating purines and pyrimidines in a ratio of 1:1, and assuming constant activation energy) will be broken into fragments of *ca*. ~100 bp in length within 500 years at 15 °C, 81,000 years at -10 °C and 1.7 million years at -20 °C[9].

Recently, Willerslev *et al*.[10], published a study of DNA durability and degradation of a broad variety of bacteria preserved under

optimal freezing conditions. They investigated twelve permafrost samples dated between 0 and 8.1 million of years. DNA was extracted from the samples and PCR amplified, using a variety of primer pairs designed to bind to different fragments of the 16S ribosomal RNA gene. Amplifications products of 120 bp and 600 bp were obtained from samples 400,000-600,000 years old. Moreover, DNA concentrations and taxonomic diversity were found to decrease with age. The results suggest that non-spore-forming grampositive Actinobacteria are the most durable, outsurviving endospore formers such as Bacillaceae and Clostridiaceae (Fig. 1).
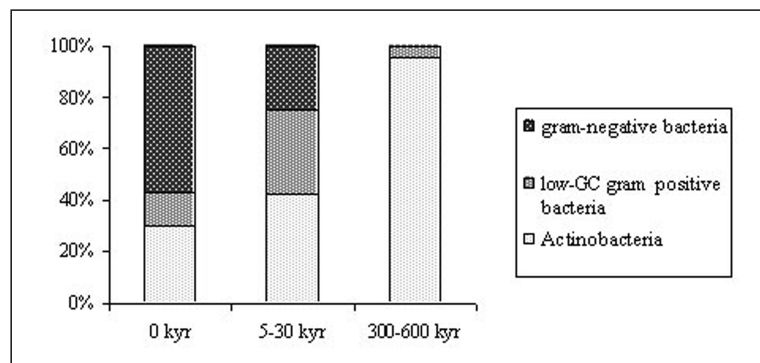

Fig. 1 - Long term persistence of bacterial DNA in permafrost. Relative frequency of gram-positive *Actinobacteria*, low-GC gram-positive bacteria, and gram-negative bacteria as derived from the screening of 16S rRNA gene libraries (Willerslev *et al*., 2004b, modified).

Identifying ancient bacterial DNA

A fundamental issue in all palaeomolecular studies is how to discriminate between ancient and modern DNA. This problem is particularly relevant in the case of ancient microbes.

The following two criteria have been proposed.

1. Use of PCR systems designed on the basis of DNA sequences which are specific for the pathogenic forms of a certain bacterium, but absent in free-living isolates. Examples of this are the quest for *Mycobacterium tuberculosis*[11] and *Yersinia pestis*[12] using, respectively, the insertion sequence IS6110 (*M. Tuberculosis*) and the RNA polymerase beta-subunit-encoding gene and the virulence-associated plasminogen activator encoding gene (*Y. pestis*).

2. Application of the so-called criterion of "palaeoecological consistency"[13]. According to this criterion, the remains are first tested for their diagenetic state by analyzing the preservation of the original human mitochondrial DNA and the level of aspartic acid racemization[14]. If the response is favorable, DNA preparations obtained from different bioptic samples are amplified using "universal" PCR systems designed on the basis of the 16S ribosomal RNA (16S rRNA) gene sequence. Following cloning and sequencing of the amplified DNA, the ancient bacteria are putatively identified by database search. The results are finally evaluated for the consistency of the (putative) bacterial type and distribution with that of the living person.

The two criteria are by no means conflicting. The first is appropriate when the search concentrates on a single pathogen, normally on the basis of the indications given by previous osteological or histological investigations. The second, on the other hand, overcomes the limitations imposed by the first when no previous indication is available. Practically, the second criterion can help us to determine which microbes, either symbionts, opportunists or pathogens, were associated with a certain historical individual during his last days of life.

Authentication of the molecular data in palaeomicrobiology

Despite the growing number of papers addressing this topic, the analysis of ancient microbial DNA is still a contentious issue. Two cases, in particular, have been the subject of debate. The first is the above cited detection of *Y. pestis*, the aetiological agent of plague, in human teeth from Middle Ages and Renaissance victims found in European archaeological sites. While reports from one laboratory[15] claimed a very high percentage of successful identifications, not a single positive result could be obtained in two others[16].

The second case is the identification of *M. tuberculosis* DNA in mummified human remains from Egyptian archaeological sites[17]. These and the associated results on the preservation of human nuclear DNA[18] were challenged on the basis of empirical and theoretical calculations suggesting the unlikeness of long-term

survival of ancient DNA in the Egyptian environment[19].

Among the difficulties in the ancient microbial DNA investigations, sample contamination due to aerosol in laboratories where the DNA of modern microbes is manipulated[20] and the ubiquitous dispersal of microorganisms[21] seem to play some of the most prominent roles.

In addition to general criteria for identifying ancient DNA[22], more specific protocols have been proposed[23]. It may however be worth remarking that the criterion proposed by Drancourt and Raoult[24] for the authentication of ancient pathogens focuses on the identification of the pathogen and pays little or no attention at all to the consistency of the find with the diagenetic level of the specimen (Table 1). In the past, we have suggested that molecular data in

Figure legends

Tab. 1 - Criteria for the authentication of molecular data in palaeomicrobiology (Drancourt and Raoult, 2005)

Absence of a positive control
-   The positive control should be removed from the laboratory in which ancient specimens are processed.

Negativity of negative controls
-   Several negative controls should be analyzed in parallel with the specimens being processed.
-   Negative controls should be as similar as possible to the ancient specimens.
-   Negative controls should remain free of amplicons.

Sequencing of PCR amplicons
-   PCR alone does not ensure the specificity of the diagnosis, and amplicons have to be sequenced to identify ancient microorganisms.

Targeting a new sequence in the laboratory
-   PCR should target a specific sequence that has not previously been amplified in the laboratory.

Amplification and sequencing of a second target
-   A positive result must be confirmed by amplification and sequencing of a second specific molecular target.

-   Originality of the ancient sequence

-   The acquisition of an original sequence that differs from modern homologues in mutation or deletion excludes contamination.

palaeomicrobiology should provide an exhaustive description of the microbial ecology of the specimen. In particular, they should take into account the issue of the specimen's recolonization by the environmental flora and the erasing action of the latter over the remains of the oldest genetic material[25].

Gilbert *et al.*[26] recently proposed further authentication criteria in the form of a series of key questions to be asked about ancient DNA (Table 2).

Tab. 2 - Key questions to ask about ancient DNA (Gilbert *et al.*, 2005)

- Do the age, environmental history and preservation of the sample suggest DNA survival?

- Do the biomolecular and/or macromolecular preservation of the sample, the amplified molecular target, the innate nature of the sample and its handling history suggest that contamination is a risk?

- Do the data suggest that the sequence is authentic, rather than the result of damage, jumping PCR, and contamination? Would patterns in the data indicate other artefacts, such as phantom mutations? Do the Authors offer sufficient additional proof that the sequences are authentic?

- Do the results make sense, and are there enough data to make the study useful and/or support the conclusions?

The digestive tract of frozen mummies as a model system for palaeomicrobiological investigations

Given the close correlation between long-term preservation of DNA and environmental conditions, mummified human remains kept at temperatures below 0 °C are ideal subjects for palaeomolecular studies. This kind of mummy is found in the Arctic region, in Siberia and at high altitudes in the Andes and the Alps[27]. In general, the bodies have all undergone a natural freeze-desiccation (lyophilization) process either due to water phase separation (permafrost) or sublimation (high altitude).

To date, most of the scientifically valuable information regarding frozen mummies derives from histopathological, immunological and electron microscopic observations. Relatively few molecular data are available.

The human digestive tract harbors a highly complex and abundant community of microorganisms[28]. The composition and activity of the intestinal bacterial community (microbiota) has a significant impact on the health of the host because of its influence on nutrition, bowel habit, the physiology of the mucin barrier, and the ontogeny of the mucosal immune system[29]. In normal conditions, the upper part of the small intestine (duodenum, jejunum) is considered to have a low microbial content, while its lower portion (ileum) is characterized by higher cell counts. The highest microbial content is found in the large intestine[30].

In the past, the composition of the intestinal microbiota was studied using traditional microbiological techniques. In the last 10 years, however, several 16S rRNA gene libraries have been prepared and screened[31] providing an important element of reference for the palaeomicrobiological investigators.

As stressed above, the authentication of the data referring to single bacterial species in archaeological specimens may be problematic, due to the ease of laboratory contamination and the ubiquity of microorganisms. These issues are markedly reduced if the object of the investigation is not a single bacterial species but a community of bacteria, such as that inhabiting the digestive tract.

An early study on the preservation of the intestinal microbiota's DNA in a frozen-desiccated mummy was published in 1998[32]. The mummy, at the present kept at the National Museum of Anthropology in Florence, comes from the Peruvian Andes and has been radiocarbon dated to the years 980-1170 A.D.. The analysis of 16S ribosomal RNA gene fragments in bioptic samples from the small and large bowels of the mummy showed that the concentration of the fragments was considerably higher in the latter, a situation reminiscent of that currently found in the living. In a further step[33], 16S rRNA gene fragments from fecal residues were used to construct a library. Screening of the library by sequencing and comparison with known bacterial sequences from Genbank, demonstrated that traces of the original microbiota were still present. In particular, it was possible to putatively

identify the DNA of *Haemophylus parainfluenzae*. As suggested by the name, meaning "blood lover" in Greek, the whole genus *Haemophylus* consists of species that are associated with warm-blooded animals. In the past, the unfavorable growth characteristics of this organism and the poor specificity of traditional methods for species identification were responsible for inaccuracies in the diagnosis of infection caused by *H. parainfluenzae* and related organisms, leading to a substantial underestimation of their pathogenic role. More recently, thanks to the application of molecular techniques, *H. parainfluenzae* was recognized as a cause of serious invasive diseases such as endocarditis and community-acquired pneumonia.

Currently, the most famous frozen mummy is the so-called "Tyrolean Iceman", or "Similaun Man", or Ötzi. This mummy was discovered on 19th September 1991 at 3270 m above sea level in an Alpine glacier near the Austro-Italian border. The most relevant feature of the discovery, radiocarbon dated to 3350–3100 B.C.[34], corresponding to the Late Neolithic or Early Copper Age, is the excellent state of preservation of the body and the pieces of clothing and equipment found with it[35].

Since his discovery, the Iceman has been the object of molecular investigations aimed at determining the mitochondrial haplogroup[36], the composition of the last meals[37], the species of grass utilized to manufacture some pieces of the equipment[38] and the types of microorganisms associated with the clothes[39].

A preliminary study of the intestinal microbiota's DNA of the Iceman was performed in the late nineties by Cano and coworkers[40]. The poor quality of the specimens available at that time, however, made it possible to obtain only a very small number (17) of bacterial sequences. They were putatively attributed to the species *Clostridium perfringens*, *C. ghonii*, *C. sordellii*, *Eubacterium tenue*, and *Bacteroides sp*. Fortunately, new investigations could be carried out following the first complete defrosting of the body which took place on 25th September 2000 at the South Tyrol Archaeological Museum in Bolzano, Italy.

Last year, in addition, chance made it possible to study another Alpine mummy of relatively recent origin. On 22nd August 2004, three human bodies were recovered at 3596 m a.s.l. in the Forni glacier (Alta Val di Pejo, northern Italy). Remains of clothes and equipment made it possible to identify the bodies as belonging to three Austrian soldiers of the 1st Regiment Tyroler Kaiserschützen. They were killed by a burst of some shells on 3rd September 1918 during a counterattack on the Italian lines. Because of the impediments posed by the battle, the corpses were forsaken on the glacier (either thrown into a crevasse or covered with snow) rather than being transported down the valley. Before being transferred to the Merano war cemetery, to be buried with military honors, the corpses were examined by Dr. Eduard Egarter-Vigl of the Regional Hospital of Bolzano and found to be perfectly mummified. As the natural mummification process (freeze-drying) closely resembled that of the Iceman, one of the bodies was subjected to necroscopic examination, and bioptic samples of the internal organs were collected and stored for DNA analysis.

DNA was isolated from the intestinal (colon) content of the 1918 mummy and the Iceman, and PCR amplified using different sets of primer pairs designed to bind to different fragments of the 16S rRNA gene. Amplified DNA was then cloned. Sequence analysis of the resulting two libraries (49 sequences for the 1918 mummy, 119 sequences for the Iceman) showed that the characteristic composition of the intestinal microbiota of man (mainly members of the classes Alpha-, Beta- and Gammaprotebacteria, Bacteroides and Clostridia) could still be traced in the recent mummy while, in the Iceman, more than 90% of the flora was represented by Clostridia (Fig. 2). Comparison of these data with recent reports describing the screening of 16S rRNA gene libraries from other parts of the Iceman's body, such as muscle and skin[41] and environmental samples such as specimens of present-day and ancient Siberian permafrost[42] suggests that the change of bacterial DNA composition inside the mummies may be attributed partly to the proliferation of the cadaveric flora and in
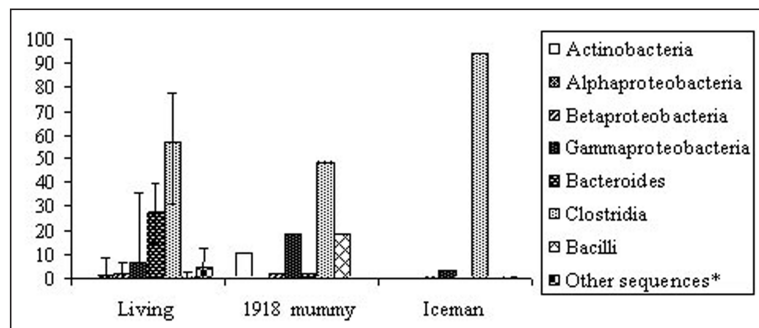
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Fig. 2 - Composition of the intestinal (colon) microflora in the living and in two mummies as derived from the screening of 16S rRNA gene libraries (*other sequences: *Flavobacteria*, *Fusobacteria*, *Mollicutes*, *Verrucomicrobiae*).

part to the lower stability of the DNA of gram negative bacteria compared to that of endospore-former low-GC gram positive bacteria.

Conclusions

The study of bacterial DNA in human mummies is an emerging field. While its potential for palaeomicrobiological, palaeopathological and anthropological studies is undisputed, it is limited a lack of standard protocols. It seems clear that the development of the field will benefit from advanced understanding of our knowledge of the mechanisms responsible for DNA preservation. In particular, the emerging issue of the differential preservation of bacterial DNA in archaeological materials is likely to play a major role in future palaeomicrobiological research.

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