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Insights into ancient leprosy and tuberculosis using metagenomics

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Leprosy and tuberculosis were widespread in the past and remain significant diseases today. Comparison of ancient and modern genomes of *Mycobacterium leprae* and *Mycobacterium tuberculosis* gives insight into their evolution and a calibration of the timescale for observed changes. Recently, whole genome sequencing has revealed genotypes and mixed-strain infections.

Leprosy is caused by *Mycobacterium leprae*, an obligate pathogen that cannot grow unless it is in a living host. It is the greatest cause of infection-related disability, with around 180 000 new cases reported annually by the World Health Organisation, mainly in tropical and subtropical parts of the globe. It can be recognised in human skeletal remains by typical palaeopathological changes found mainly in the naso-maxillary region of the face, the long bones of the arms and legs, and the small bones in hands and feet. These can be recognised from a 2000 BC site in India, the Roman Empire and especially Western and Central Mediaeval Europe.

Most human tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, and occasionally by other members of the closely related *M. tuberculosis* complex. *M. tuberculosis* is also an obligate pathogen, usually spread by infectious aerosols that result in lung infections. However, the tubercle bacilli can spread via the bloodstream to all parts of the body so can cause typical bone lesions in the vertebrae and elsewhere. Vertebral TB can result in vertebral fusion and collapse, which enabled the disease to be recognised in ancient Egypt and early Neolithic communities [1].

The development of the polymerase chain reaction (PCR) to amplify DNA resulted in ancient DNA (aDNA) research of prehistoric human, animal and plant remains. Soon after, the technique was adapted to seek evidence of ancient TB and leprosy, thereby establishing the new field of palaeomicrobiology. *M. tuberculosis* and *M. leprae* were excellent candidates for this early exploration of ancient human infections, as potential infected human remains could be visually recognised. Also, they were early candidates in the development of molecular clinical diagnostics due to the extremely slow growth rate of *M. tuberculosis* and the impossibility of *M. leprae* culture.

Initially studies sought confirmation of diagnoses based on skeletal pathology, but it soon became apparent that bones and tissue could be infected but without any visual signs of disease. Many useful studies were carried out, including molecular typing, epidemiological investigations of populations [2] and the discovery of mixed infections [3]. However, PCR has the intrinsic problem of requiring DNA amplification, so stringent precautions are needed to prevent external and cross-contamination. Different molecular biomarkers, such as mycobacterial cell wall lipids, provide independent confirmation of *M. leprae* and *M. tuberculosis* without any amplification [4], although without direct evidence of genetic changes.

The development of high throughput sequencing and associated software for data analysis has enabled direct analysis of entire genomes. To overcome the problem of low aDNA yield, next generation sequencing (NGS) enriches the target aDNA by using hybridization capture, using modern sequences as bait on magnetic beads or an array. After size gating, DNA fragments are tagged by oligonucleotide ligation, sequenced and aligned to construct the genome using bioinformatics software. Some recent studies are summarized in Table 1.

The genomic analysis of human and microbial DNA from the 5300 year-old Tyrolean Iceman [5] included a significant proportion of the genome of *Borrelia burgdorferi*, suggesting that he suffered from Lyme disease. In this study, the DNA was well preserved in the frozen body. However, at ambient temperatures DNA degrades unless the combination of temperature, oxygen level and hydration preserve its integrity. Even so, NGS has been used successfully to determine the genome of *Yersinia pestis* from the 1348–1349 outbreak of bubonic plague in London, known as the Black Death [6]. This was achieved by taking samples from the dental pulp cavity in teeth, as this is a sequestered site where aDNA can persist. NGS was used in a study of a 19th century skeleton found in a church crypt in Leeds, UK. It was based on one sample from a rib displaying surface

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Table 1. Insights from whole genome sequencing of microbial aDNA

Bacterial pathogen	Sample site and age	Main findings	Observations	Refs
Borrelia burgdorferi (causative agent of Lyme Disease)	Left ileum, Copper Age (5200 years BP)[a]	Analysis of the human aDNA showed the Ice Man was blood group O, had brown eyes and was probably lactose intolerant. There were signs of atherosclerosis.	The preservation of the spirochaete and human aDNA was due to the cold environment. There was ~60% coverage of the genome, so detailed comparison of ancient and modern DNA was not possible. Lyme disease is associated with vascular calcifications.	[5]
Yersinia pestis	Teeth, 1348–49 AD	Strains from the Black Death were ancestral to modern strains, but no genetic characteristics were found to explain the difference in virulence.	The high virulence reported during the Black Death could be related to the insect vector, and/or changes in host susceptibility.	[6]
Mycobacterium tuberculosis	Rib, 19th century AD	Over 87% of the genome was identified. SNP data identified the strain as similar to H37Rv, in the Europe/Americas clade, but with some discrepancies.	Conventional PCR was used to confirm the detailed SNP analysis.	[7]
Mycobacterium leprae	Bones and teeth, 644–1023 AD	Two *M. leprae* genotypes were found: 2F and 3I. These are associated with Europe and the Middle East in modern strains. No genetic factors were found to explain the high virulence reported in the 14th century.	The reasons suggested for the decline in virulence are the impact of other infections, such as plague and tuberculosis, decreased host susceptibility and improved social conditions.	[8]
Mycobacterium tuberculosis	Mummified lung, 1797 AD	The *M. tuberculosis* DNA was 8% of the total, but human DNA was 0.3%. There were two lineages of *M. tuberculosis* that were ancestral to the Hamburg outbreak clone from 1998–2010.	This illustrates the greater robustness of mycobacterial aDNA compared with that of humans. This is likely to be due to the hydrophobic bacterial cell wall but the high %GC in its DNA may also aid persistence.	[9]

[a]Abbreviation: BP, before present.

bone formation consistent with a case of pulmonary TB [7]. Deletion regions, single nucleotide polymorphisms (SNPs) and repeat sequences were identified.

A recent NGS study of leprosy was based on bone and teeth samples [8] where genomes from five mediaeval leprosy cases were compared with eleven modern strains from around the globe. A potential problem is that the capture technique may fail to detect sequences present in ancient strains but absent today. However, one mediaeval *M. leprae* DNA extract from a tooth could be analysed directly, without enrichment, a procedure known as metagenomics. This is believed to be the first time the entire genome of an ancient pathogen has been obtained from skeletal material exposed to normal ambient temperatures. Historical and modern *M. leprae* sequences showed considerable genomic conservation over the past 1000 years, and enabled genotypes to be determined at different geographical sites.

A subsequent metagenomic study based on naturally mummified lung tissue [9] enabled genetic analysis of *M. tuberculosis* in one of a family group from 18th century Vác, Hungary. The Vác mummies were in a good state of preservation in a sealed church crypt and there are contemporaneous archives indicating family relationships, date and age at death, sometimes with descriptions of occupation or symptoms. Earlier studies [10] showed that a family group of a mother and two daughters each appeared to harbour a different *M. tuberculosis* strain. However, metagenomic analysis of the older daughter showed that she was infected with two strains of *M. tuberculosis*, which can occur when TB is endemic. Both were in the European Haarlem lineage and resembled the modern Hamburg clone that appears to spread more rapidly than other strains.

Leprosy and TB appear to have co-evolved with their human hosts and different pathogen lineages are linked to human lineages around the globe. Ancient DNA studies expand our knowledge of the spread of infectious diseases and have shed light on human migrations based on the pathogens they harbour. Metagenomic analysis of aDNA enables us to calibrate the rate of evolutionary change before the acceleration caused by modern antibiotics. It also facilitates identification of mixed infections, of the same or with different species, and it is clear that these are more frequent than previously realised.

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