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Review

Insights gained from ancient biomolecules into past and present tuberculosis—a personal perspective

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SUMMARY

Ancient and historical tuberculosis (TB) can be recognized by its typical paleopathology in human remains. Using paleomicrobiology, it is possible to detect many more individuals infected with TB but with no visible lesions. Due to advances in molecular analysis over the past two decades, it is clear that TB was widespread in humans from the Neolithic period and has remained so until the present day. Past human populations were associated with different lineages of the *Mycobacterium tuberculosis* complex, thereby elucidating early human migrations. Using paleomicrobiology, it is possible to detect individuals infected with TB who are also co-infected with other bacteria or parasites. TB is also found in hosts with co-morbidities such as neoplasms, or metabolic disorders such as rickets and scurvy. In well-preserved human skeletal or mummified tissue, whole genome sequencing has detected individuals with multiple infections of different *M. tuberculosis* strains. Such studies put modern findings into context and emphasize the importance of human population density in such circumstances.

1. Recognition of ancient and historical TB by paleopathology

Paleopathologists are readily able to identify the characteristic appearance of Pott's disease that results from tuberculosis (TB) of the spine. There is damage to the spinal column that results in kyphosis, associated with damage to the spinal column and paralysis in the lower limbs.[1,2] Symptoms of TB also include a chronic or 'cold' abscess, periosteal reactive lesions on tubular bones, hypertrophic osteoarthropathy, and osteomyelitis, but these conditions are not specific.[3–6] Brucellosis and chronic inflammation can also cause bony changes, so further tests are required for confirmation of infection. In addition, it is estimated that skeletal TB occurs in only 3–5% of untreated cases, so it is clear that any diagnosis that relies solely on gross morphology will detect only a small fraction of ancient TB infections.[7]

In recent years, additional techniques have been used to detect past pathological conditions, including infectious diseases. Microscopic techniques, such as fluorescent con-focal microscopy, enable the fine detail of pathogenic lesions to be assessed, but may not provide definitive identification of the infecting agent in the absence of macroscopic bone changes.[8] Computed tomography (CT) and micro-CT scanning have largely replaced traditional radiographs. However, although these can identify pathological conditions, the high level of radiation can inactivate ancient DNA (aDNA), so it is important to optimize the protocols and to follow some simple rules to minimize any aDNA damage.[9]

2. Detection and characterization of *Mycobacterium tuberculosis* ancient DNA

The development of molecular diagnostic methods, including DNA amplification via PCR, has enabled anthropologists and paleopathologists to examine historical and archaeological specimens for the presence of pathogen aDNA and other molecular markers. Ancient TB and leprosy were the first human infectious diseases to be confirmed by PCR.[10–12] Initially there was much concern about contamination with modern DNA, coming principally from those working on human and other mammalian material.[13,14] Although it is easier to prevent such contamination when investigating an obligate human pathogen with no known environmental reservoir,[15,16] many studies have incorporated independent verification from other laboratories, including the use of totally different techniques to identify pathogen molecular markers, such as bacterial cell wall lipids.[17,18] It is now clear that TB was very common in the past and that typical paleopathology is the exception rather than the rule.

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The introduction of PCR enabled aDNA from the *Mycobacterium tuberculosis* complex to be detected and also opened the way to the direct characterization of past strains and lineages. The clinical importance of modern TB and the need for rapid diagnosis led to the development of PCR-based diagnostics, and these were soon applied to *M. tuberculosis* aDNA. Specific short repetitive sequences, such as IS*6110* and IS*1081*, have been used as targets for DNA amplification, although care is needed to ensure the primer specificity.[19,20] Some skeletal and mummified tissues were sufficiently well preserved to enable characterization based on synonymous single nucleotide polymorphisms (SNPs).[21] Spoligotyping, based on spacer regions in the repetitive DR locus, also enabled characterization of strains and lineages.[22] Spoligotyping, SNP analysis, and other polymorphisms were used in an early biomolecular study of some well-preserved 18th century naturally mummified human remains from the town of Vác in Hungary that were infected with *M. tuberculosis*.[23,24] As there were contemporaneous church and civic archives, it was possible to draw conclusions about the epidemiology of the disease in this population.[25]

The sequencing of the *M. tuberculosis* genome enabled the recognition of current *M. tuberculosis* lineages, revealing the association of different human populations with different lineages of *M. tuberculosis*.[26] It also led to an understanding of the probable origin of the different *M. tuberculosis* lineages and their geographical distribution.[27,28] It was soon realized that the identification of different lineages of *M. tuberculosis* aDNA could be used as an indicator of past human migrations. An early example of the recognition of different lineages was the study of TB in ancient Egypt. *M. tuberculosis* aDNA was detected in the early dynastic (ca 3500–2650 BC), Middle Kingdom to Second Intermediate Period (ca 2100–1550 BC), the New Kingdom and the Late Period (ca 1450–500 BC).[29] *M. tuberculosis* aDNA was found in many bones that had no pathological lesions. Spoligotyping indicated that both *M. tuberculosis* and *Mycobacterium africanum* were present. The strains of *M. tuberculosis* included those in which the TbD1 deletion had occurred, but also strains that were TbD1-intact. However, there was no evidence of *Mycobacterium bovis*.[30] Indeed, *M. bovis* aDNA has been found only in a community of Siberian pastoralists, who overwintered in huts that also contained their animals.[31,32] However, human remains close to a Peruvian river that flows into the Pacific Ocean were found to contain an *M. tuberculosis* complex strain that was most closely related to *Mycobacterium pinnipedii*, suggesting that sea mammals may have been a source of infection in that community (see Section 6).[33]

3. Detection of ancient tuberculosis via other biomolecules

Several studies have reported the use of matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF MS) for the detection of ancient proteins or peptides.[34] Mycocerosic acids and other specific components of the *M. tuberculosis* cell wall were detected in the ribs of 49 individuals from the 19th and 20th century Coimbra Identified Skeletal Collection, half of those being historically documented with TB infections.[17] Other organic chemical methods, such as fluorescence high performance liquid chromatography (HPLC), or selected ion monitoring (SIM) negative ion chemical ionization (NICI) gas chromatography mass spectrometry (GCMS) have both the sensitivity and specificity to directly detect and quantify ancient mycobacterial cell wall lipid biomarkers, without the need for amplification.[18,35]

The analysis of ancient microbial proteins has been less productive than studies based on *M. tuberculosis* aDNA or cell wall lipids due to their lower specificity, but it was predicted that MALDI-TOF MS should be able to detect host signatures specific to infection.[34] In 2012 the protein expression profile of buccal swabs from two 500-year-old Andean mummies was obtained by the use of shotgun proteomics.[36] Several proteins were detected that are not normally present in blood or saliva, but are consistent with a host immune response to infectious disease. In addition, a probable pathogenic Mycobacterium was detected. It appears that ancient microbial proteins are less informative than other biomarkers, as in a recent study of seven samples from 18th century Hungarian mummified lung, chest, and pleural tissues where TB was prevalent, shotgun proteomics identified only four peptides with unique matches to the *M. tuberculosis* complex.[37]

4. Tuberculosis in relation to early humans

The earliest case of an *M. tuberculosis* complex infection was recognized in a Pleistocene bison that was excavated from the Natural Trap Cave in Wyoming, USA. The environmental conditions enabled excellent preservation of aDNA and other molecular markers. Evidence of TB was obtained from just below the articulating surface of a metacarpal bone.[38] The early findings were queried but a subsequent analysis of mycobacterial cell wall lipid molecular biomarkers confirmed the diagnosis.[39]

Before the development of molecular diagnostic techniques it was assumed that, in the Neolithic period, domesticated animals were the source of TB that subsequently infected humans.[40] However, recent genomic studies have confirmed that *M. tuberculosis* is more ancestral than *M. bovis*,[41] so it is believed now that TB became common in the Neolithic period due to animal domestication supporting larger human populations, thereby facilitating the spread of infection. Using both aDNA and mycobacterial cell wall lipid analysis, *M. tuberculosis* was detected in a submerged 9000-year-old village off the eastern Mediterranean coast,[42,43] but the associated animal bones were negative.[44] Limited molecular evidence of TB was also obtained from an 11 000-year-old pre-domestication site (8800–8300 BCE cal.) and an early domestication site (8200–7600 BCE cal.) in Syria,[45] but yet again, the animal bones provided no evidence of TB. There has been speculation that TB may have occurred even earlier in hominids such as *Homo erectus*,[46] although the paleopathology in this case was disputed.[47] Recently it has been suggested that the *M. tuberculosis* complex may be yet more ancient than has previously been believed[48] and that Neanderthals were possibly infected.[49]

The association of TB with population density is due to the combination of the aerosol route of infection of *M. tuberculosis* and the fact that it is an obligate pathogen. It appears that early lineages of *M. tuberculosis* are less virulent than the predominant strains today and this may have resulted from the necessity for the pathogen to survive in small, scattered human populations until there was an opportunity for transmission. However, more virulent strains of *M. tuberculosis* have emerged in ancient centres of population where they still persist.[50] It is believed that the *M. tuberculosis* complex evolved from smooth colony strains termed 'Mycobacterium prototuberculosis'[51] that subsequently gained additional persistence and virulence mechanisms.[52,53] The modern smooth colony strains in the *M. tuberculosis* complex are classified as *Mycobacterium canettii*, are found in the horn of Africa and may have an environmental reservoir. They are very diverse, experience horizontal gene transfer, yet share a highly conserved core genome with other members of the *M. tuberculosis* complex.[53] Infections are rare in the local human population and there is little evidence of person-to-person spread.[54] In contrast, the other members of the *M. tuberculosis* complex show no evidence of horizontal gene transfer. They appear to have emerged via an