



## 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.

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## 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.<sup>1,2</sup> 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.<sup>3–6</sup> 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.<sup>7</sup>

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.<sup>8</sup> 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.<sup>9</sup>

## 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.<sup>10–12</sup> Initially there was much concern about contamination with modern DNA, coming principally from those working on human and other mammalian material.<sup>13,14</sup> Although it is easier to prevent such contamination when investigating an obligate human pathogen with no known environmental reservoir,<sup>15,16</sup> 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.<sup>17,18</sup> 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 IS6110 and IS1081, have been used as targets for DNA amplification, although care is needed to ensure the primer specificity.<sup>19,20</sup> Some skeletal and mummified tissues were sufficiently well preserved to enable characterization based on synonymous single nucleotide polymorphisms (SNPs).<sup>21</sup> Spoligotyping, based on spacer regions in the repetitive DR locus, also enabled characterization of strains and lineages.<sup>22</sup> Spoligotyping, SNP analysis, and other polymorphisms were used in an early biomolecular study of some well-preserved 18<sup>th</sup> century naturally mummified human remains from the town of Vác in Hungary that were infected with *M. tuberculosis*.<sup>23,24</sup> As there were contemporaneous church and civic archives, it was possible to draw conclusions about the epidemiology of the disease in this population.<sup>25</sup>

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*.<sup>26</sup> It also led to an understanding of the probable origin of the different *M. tuberculosis* lineages and their geographical distribution.<sup>27,28</sup> 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).<sup>29</sup> *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*.<sup>30</sup> Indeed, *M. bovis* aDNA has been found only in a community of Siberian pastoralists, who overwintered in huts that also contained their animals.<sup>31,32</sup> 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).<sup>33</sup>

### 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.<sup>34</sup> Mycocerosic acids and other specific components of the *M. tuberculosis* cell wall were detected in the ribs of 49 individuals from the 19<sup>th</sup> and 20<sup>th</sup> century Coimbra Identified Skeletal Collection, half of those being historically documented with TB infections.<sup>17</sup> 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.<sup>18,35</sup>

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.<sup>34</sup> In 2012 the protein expression profile of buccal swabs from two 500-year-old Andean mummies was obtained by the use of shotgun proteomics.<sup>36</sup> 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 18<sup>th</sup> 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.<sup>37</sup>

### 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.<sup>38</sup> The early findings were queried but a subsequent analysis of mycobacterial cell wall lipid molecular biomarkers confirmed the diagnosis.<sup>39</sup>

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.<sup>40</sup> However, recent genomic studies have confirmed that *M. tuberculosis* is more ancestral than *M. bovis*,<sup>41</sup> 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,<sup>42,43</sup> but the associated animal bones were negative.<sup>44</sup> 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,<sup>45</sup> 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*,<sup>46</sup> although the paleopathology in this case was disputed.<sup>47</sup> Recently it has been suggested that the *M. tuberculosis* complex may be yet more ancient than has previously been believed<sup>48</sup> and that Neanderthals were possibly infected.<sup>49</sup>

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.<sup>50</sup> It is believed that the *M. tuberculosis* complex evolved from smooth colony strains termed 'Mycobacterium prototuberculosis'<sup>51</sup> that subsequently gained additional persistence and virulence mechanisms.<sup>52,53</sup> 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.<sup>53</sup> Infections are rare in the local human population and there is little evidence of person-to-person spread.<sup>54</sup> In contrast, the other members of the *M. tuberculosis* complex show no evidence of horizontal gene transfer. They appear to have emerged via an

evolutionary bottleneck followed by clonal expansion,<sup>27,41,55</sup> and modern *M. tuberculosis* lineages are associated with different human populations.<sup>56</sup>

## 5. Past co-infections and co-morbidities

Pulmonary TB was widespread in the past, so it is unsurprising that *M. tuberculosis* aDNA can be found in association with other infections and non-infectious conditions. However, it is difficult to distinguish between active and latent disease. Injury or other infections lower host resistance, thereby allowing latent TB to re-activate. Similarly, the very young or old are more susceptible to infection. In contrast, worms and parasites were common in past human societies and these are known to have a developmental and regulatory role in the host immune response.<sup>57</sup> There are examples of human skeletal remains that demonstrate paleopathology that indicates co-infections, for example of TB and leprosy. In central Europe, *M. tuberculosis* and *Mycobacterium leprae* aDNA have been detected in the same specimens.<sup>58,59</sup> This led to the suggestion that leprosy declined as urbanization and thereby the level of TB increased.<sup>59</sup> The population data suggest that this is a feasible theory.<sup>60</sup> TB has also been associated with malaria in Ancient Egypt<sup>61</sup> and with Chagas disease in 10<sup>th</sup> century northern Peru.<sup>62</sup> *M. tuberculosis* and *Leishmania spp* have been detected as co-infections in Early Christian Nubia and Middle Kingdom ancient Egypt.<sup>63</sup>

Co-morbidities include cancer and metabolic disorders such as rickets and scurvy. These can be detected by paleopathology of skeletal material, including cribra orbitalia (pitting of the eye sockets), which is interpreted as indicating nutritional or metabolic stress. Vitamin deficiency and malnutrition will decrease host resistance to infection and may result in re-activation of latent infection.<sup>64,65</sup> An example is shown by a study of the Cross Bones burial site in Southwark, London, UK, dating from the mid-19<sup>th</sup> century that contained a majority of adult women and infants. The site was notorious for prostitution and the burial ground was for those who were destitute. Many of the human remains show clear signs of infectious and metabolic diseases, but none were identified by paleopathology as having TB. However, a biomolecular analysis showed that 27% of individuals in this population were positive for *M. tuberculosis* aDNA.<sup>66</sup> Other conditions in this population identified by paleopathology have included syphilis, Paget's disease, prostate cancer, healed rickets, scoliosis, and osteoarthritis. Another cancer-like condition that has been found in association with active TB is Langerhans cell histiocytosis. Such a case was discovered in one of the Vác 18<sup>th</sup> century mummies – a 4-year-old boy who was infected with *M. tuberculosis*.<sup>67</sup> This demonstrates the complexities in past populations where many factors contribute to the final outcome.

## 6. Insights from whole genome sequencing (WGS)

Advances in sequencing and instrumentation have enabled whole genomes of historical strains of *M. tuberculosis* to be determined. However, most historical and archaeological specimens will include high levels of environmental microbial DNA. Therefore, various enrichment strategies have been devised, such as capture of targets by probes or microarrays, or selective capture of damaged DNA molecules using probe-based, solid phase or whole genome target enrichment.<sup>68,69</sup> The use of WGS provides additional confirmation of the authenticity of aDNA by the analysis of DNA damage patterns.<sup>68,69</sup> A next generation sequencing (NGS) approach that included hybridization capture of specific regions of interest enabled a detailed genotype to be identified from an individual buried in a 19<sup>th</sup> century church crypt in Leeds, UK.<sup>70</sup> In

the Peruvian study described in Section 2, a selection of skeletal samples from pre- and post-contact New World sites were examined, initially using screening based on a capture assay designed for five *M. tuberculosis* genes. Three samples showed convincing evidence of *M. tuberculosis* DNA and were subsequently examined by whole genome analysis. DNA libraries were treated with uracil DNA glycosylase to remove and repair damaged nucleotides, and were subsequently used for full genome hybridization capture.<sup>33</sup> In a different strategy, Chan et al. used a metagenomic approach, with open-ended sequencing of aDNA, without target-specific amplification or enrichment.<sup>71</sup> This was possible due to the excellent preservation of the 18<sup>th</sup> century naturally mummified tissue from Vác, Hungary. The source of the tissue, a young woman aged 28 years at the time of death, was infected with two different strains of *M. tuberculosis*, both similar to the Haarlem and Erdman lineages. In a further study of eight Hungarian 18<sup>th</sup>-century mummies, a majority were infected with two *M. tuberculosis* genotypes and one individual contained three genotypes.<sup>72</sup> Overall, 14 genotypes were obtained, all belonging to *M. tuberculosis* lineage 4.

The phenomenon of mixed strain *M. tuberculosis* infection has recently been highlighted as an increasing problem today, as it may affect the outcomes of treatment for infected individuals and influence the impact of population-level interventions against TB.<sup>73,74</sup> Modern studies have emphasized the impact of HIV co-infection, antimicrobial therapy, and drug resistance on this phenomenon, but the data from the pre-antibiotic era highlight the importance of human population density in this interaction between host and pathogen.

## 7. Recent findings from epigenetics and transcriptomics

An exciting new area of study is emerging in the field of physical anthropology—that of epigenetics and transcriptomics. The epigenome is viewed as a collection of regulatory layers that control when, where, and how genes are turned on or off.<sup>75</sup> Epigenetic changes, such as methylation patterns, have been detected in ancient humans and other mammals.<sup>76</sup> This opens the way to the analysis of regulatory changes underlying adaptation and species divergence. This approach was used in the analysis of methylation data from the hair of a 4000-year-old paleo-Eskimo, where the extent of methylation indicated that this individual probably died in his 50s.<sup>77</sup> It is possible to selectively enrich microbial DNA from a background of vertebrate host DNA,<sup>78</sup> although this is less of a problem when examining historical or ancient material. Recent studies using a methylated binding domains (MBD)-based enrichment method<sup>79</sup> have shown that when aDNA is preserved under favourable conditions, methylation can survive over 45 000 years, whereas in less favourable conditions such as warmer regions, its half-life can be as short as approximately 1500 years.<sup>75</sup> However, MBD enrichment appears to be appropriate only for the analysis of very well preserved samples where DNA fragmentation and deamination are limited.<sup>79</sup>

*M. tuberculosis* transcriptomics is currently an active field of study, but the techniques have not yet been applied to historical or ancient human remains.<sup>80,81</sup> This is clearly an area where important insights should be obtained, provided suitable well-preserved specimens containing sufficient *M. tuberculosis* aDNA are available for analysis, such as historical remains from the permafrost or possibly the 18<sup>th</sup> century Hungarian Vác mummies. The combination of epigenetics, proteomics, and transcriptomics should throw light on the differences in gene expression and virulence between *M. tuberculosis* strains and lineages.<sup>82,83</sup>

## 8. Summary and conclusions

The detection of *M. tuberculosis* aDNA more than 20 years ago led to the new field of paleomicrobiology. It was initially viewed as a useful method to confirm the presence of TB in human remains with typical paleopathology. However, it rapidly became clear that visible paleopathology was the exception and that TB was far more prevalent than had been assumed previously. Also, additional paleopathological indicators of *M. tuberculosis* infection were thereby authenticated. It became possible to identify different *M. tuberculosis* genetic lineages and to associate these with early human populations, thus providing data on migrations. The use of *M. tuberculosis* aDNA and cell wall lipids has allowed past *M. tuberculosis* infection to be studied alongside other markers of migration and diet, such as stable isotope analysis. *M. tuberculosis* ancient biomolecules have also provided insights into the interpretation of modern findings, such as infections involving mixed lineages of *M. tuberculosis*. Perhaps the greatest contribution of *M. tuberculosis* aDNA research is that it enables genetic changes to be studied in real time and the rate of evolutionary change to be directly calibrated. This demonstrates the value of 'blue skies' research, as we now appreciate the extent to which paleomicrobiology can enhance our understanding of both ancient and modern infection.

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