Human tuberculosis – an ancient disease, as elucidated by ancient microbial biomolecules

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Abstract

Tuberculosis is a major cause of death but infected people with effective immunity may remain healthy for years, suggesting long-term co-existence of host and pathogen. Direct detection and characterisation of ancient microbial DNA and lipid biomarkers confirms palaeopathological diagnoses. Archaeological *Mycobacterium tuberculosis* resembles extant lineages indicating the timescale for evolutionary changes is considerably longer than originally believed.

Keywords: Ancient DNA; lipid biomarkers; *Mycobacterium tuberculosis*; palaeogenetics; palaeomicrobiology

1. Introduction

Tuberculosis was recognised in ancient and historical times and is still a major global cause of death and disease. It is believed that around 2 billion people, about one third of the world's total population, are infected with tubercle bacilli and in 2007 1.77 million people died from the disease, according to the World Health Organisation: http://www.who.int/tb. Only about 10% of infected persons will become ill with active disease – generally those with a less effective immune system such as the very young and old, or who suffer from malnourishment, other diseases, physical or mental stress. This high level of latent infection indicates a long-term co-existence of human host and bacterial pathogen [1], and the evolution of this host/pathogen relationship is an active area of research.

Tuberculosis is caused by a group of closely related bacterial species termed the *Mycobacterium tuberculosis* complex (MTBC). Other mycobacterial species are widespread in the environment but members of the MTBC are obligate pathogens. Many of the

mycobacteria, including the MTBC, are very slow growing. The pathogenic species are able to survive and grow within macrophages, which enables them to evade the host immune system. An active cell-mediated immune response is required to contain and kill the tubercle bacilli. Today the principal cause of human tuberculosis is *Mycobacterium tuberculosis*. *Mycobacterium bovis* has a wider host range and is the main cause of tuberculosis in other animal species. Unpasteurised milk and milk products are regarded as the main route of transmission of zoonotic TB caused by *M. bovis* in countries where there are no effective eradication programmes. Other members of the MTB complex occasionally cause human infections, such as *Mycobacterium canettii*, *Mycobacterium africanum*, and species associated with particular animals such as voles (*Mycobacterium microti*), or goats (*Mycobacterium caprae*).

Tuberculosis may involve every organ in the body but the most common clinical presentation is pulmonary disease, in which transmission is via infectious aerosols released from the lungs of an infected person. In the alveolus of the lung, inhaled tubercle bacilli are ingested by macrophages and are normally contained by the host immune response. This leads to granuloma formation and eventually to calcified lesions. Spread of the bacteria within a year of initial infection results in primary disease. However, the organisms may remain dormant but viable for decades. If the immune response is subsequently compromised, the bacteria may escape into the lungs causing re-activated pulmonary tuberculosis. In a minority of cases the bacteria spread to other host tissues via the lymphatic system and blood, thereby becoming disseminated throughout the body, resulting in miliary or extra-pulmonary tuberculosis (EPTB). In immunocompetent adults it is estimated that primary EPTB disease occurs in 15-20% of all cases. Infection of the lymph nodes results in swollen glands and is the most common clinical presentation of EPTB. Cervical lymphadenitis and skin lesions were previously known as scrofula or lupus vulgaris. Pleural effusions, genito-urinary tract

tuberculosis, meningitis, skeletal, ocular and abdominal tuberculosis are additional clinical presentations of the disease, especially in communities where no effective chemotherapy is available. Gastro-intestinal tuberculosis can result from swallowing infected sputum or by ingestion of infected animal products, resulting in further transmission of infection via faeces and urine. In the past, and today in the absence of effective eradication programmes, it is estimated that *M. bovis* is responsible for about 6% of human deaths from tuberculosis [2].

2. Molecular detection and epidemiology of the MTB complex

The traditional criteria for diagnosing tuberculosis are: chest radiology, detection of acid-fast bacilli by Ziehl-Neelsen stain and culture. Microscopy is the most rapid diagnostic tool. In ideal settings it can produce same day results, but it is very insensitive, yielding only 10-30% of culture-positive samples. Culture is sensitive, but may take four weeks to obtain conclusive results even with enhanced culture systems. Therefore, molecular diagnostic markers have been developed, facilitated by the complete genome sequencing of *M. tuberculosis* and *M. bovis*. This has made it possible to directly detect DNA from *M. tuberculosis* and *M. bovis* in archaeological material and to examine their molecular characteristics.

Members of the MTBC have many clustered regularly interspaced short palindromic repeat sequences in their DNA. These are of no known function and it is probable that the majority are not transcribed. Insertion sequences, including IS*6110* and IS*1081*, are normally present in multiple copies within the cell. Specific regions of IS*6110* and IS*1081* are used as target loci of widely used polymerase chain reaction (PCR) assays [3–5]. Epidemiological studies of tuberculosis have been facilitated by PCR-based typing methods. Spoligotyping is based on the direct repeat (DR) region of the MTB complex [6]. DR-based PCR primers

amplify up to 43 unique spacer regions that lie between each DR locus. Amplicons from individual spacers are visualised by dot-blot hybridisation on a membrane, giving a fingerprint. Different strains commonly show deletions and as the loss of spacers is unidirectional, the data can indicate evolutionary trends. Spoligotyping clearly distinguishes *M. bovis* from *M. tuberculosis*, and different families of strains are defined by characteristic patterns. An international database is available at http://www.pasteur-guadeloupe.fr/tb/spoldb4. Further typing is based upon variable number tandem repeat (VNTR) loci, and mycobacterial interspersed repetitive units (MIRU), which are tandem repeats of 40-100 bp located in microsatellite regions around the chromosome. A standardized protocol for MIRU-VNTR typing with an international database is now available for easy strain identification. Typing is also possible based on genomic deletion analysis and single nucleotide polymorphisms (SNPs) [7].

Functionally neutral SNPs in the catalase-peroxidae-encoding gene *katG* and a subunit of the DNA gyrase gene *gyrA* enable three principal genetic groups (PPGs) to be recognised within the MTBC [7]. Whole genome sequencing led to the recognition of deletions that can distinguish between members of the MTBC and to the development of ideas about its evolution [8]. SNPs act as phylogenetically informative mutations because the low DNA sequence variation in *M tuberculosis* makes independent recurrent mutation very unlikely. In addition, the virtual lack of horizontal gene exchange reduces the likelihood of independent recurrent SNPs, and, in the case of large sequence polymorphisms (LSPs) such as deletions, precludes *M tuberculosis* strains from reacquiring genomic regions that have been lost. Thus, SNPs and LSPs are ideal markers for inferring deep phylogenies.

Using such methods the global population structure of *M. tuberculosis* can be defined by six phylogeographic lineages, each associated with specific human populations. In an urban cosmopolitan environment (San Francisco, USA) with a high rate of tuberculosis

transmission, mycobacterial lineages were much more likely to spread in sympatric than in allopatric patient populations. Tuberculosis cases in allopatric hosts disproportionately involved high-risk individuals with impaired host resistance. These observations suggest that mycobacterial lineages are adapted to particular human populations [1, 9]. Very similar results were obtained from Montreal, Canada, a city with low transmission of tuberculosis and a very different human population structure [10]. A similar association of host and *M. tuberculosis* genotypes is found in the different ethnic populations in Taiwan, whose migratory activities occurred between 55 and 500 years ago [11]. This is a compatible timescale to that calculated by Hirsh et al [1] as the Time to Most Recent Common Ancestor of the *M. tuberculosis* carried by East Asian and Philippine human populations. They found that the *M. tuberculosis* belongs to distinct lineages that have been separated for 240–1,000 years, and concluded that the strong association between host place of birth and parasite genotype is one of the evolutionary consequences of the longstanding human–pathogen association, suggestive of an interaction between host and bacterial genotypes.

3. Genotyping and ancestral sequence inference

Variations in geographical distribution, host preference, virulence, and relative human infectivity differentiate tubercle bacilli. A detailed study of genetic polymorphisms supports the hypothesis that the other MTBC species are actually clonally derived from an "M. canettii"-like organism [8, 12], which may have formed part of a larger group of genotypically divergent MTBC organisms, described as “Mycobacterium prototuberculosis”. Sequence data suggest that a severe evolutionary bottleneck occurred in the MTBC 20,000–35,000 years ago. In support of this hypothesis, the extant representatives of this ancient group are localised to East Africa and are also human pathogens, suggesting that the MTBC

may have coevolved with the human lineage since at least the time of early hominids between 2.6 and 2.8 million years ago [13]. Hershberg et al [14] reached similar conclusions after surveying sequence diversity within a global collection of strains belonging to the MTBC using seven megabase pairs of DNA sequence data. They showed that the species that infect humans are more genetically diverse than generally assumed, and can be linked to the ancient human migrations out of Africa, as well as to movements of human populations in Europe, India, and China during the past few hundred years. Further analyses based on globally neutral markers with mutation rates estimated from human *M. tuberculosis* infection cases, a descent-sampling scheme and multiple, convergent population genetic estimators, led to more precise conclusions on the timescale of MTBC evolution. Wirth et al [15] present genetic evidence indicating that the most common ancestor of the bacterial complex emerged some 40,000 years ago from its progenitor in East Africa. This is the region from where modern human populations disseminated around the same period. This was followed 10,000 to 20,000 years later by the radiation of two major lineages, one of which spread from human to animals. In more recent years, coinciding with the human population explosion and the industrial revolution, the human-associated pathogen lineages have strongly expanded. These results thus reveal the strikingly parallel demographic evolution between humans and one of their primary pathogens.

The genotype of *M. tuberculosis* has important clinical consequences since it influences the presenting features of pulmonary and extra-pulmonary tuberculosis. The East Asian/Beijing lineage, predominantly found in Asia, is associated with greater dissemination and a higher incidence of drug-resistance. It alters disease presentation by influencing the intracerebral inflammatory response, resulting in more meningeal disease [16]. The outcome of exposure to *M. tuberculosis* depends on both human and bacterial genotypes. For example, a particular mutation in the Toll-like receptor, TLR2 T597C, is more commonly found in

patients infected with East-Asian/Beijing strains of *M. tuberculosis* [17]. It is highly likely that more examples of such interactions will come to light and these may explain the observations that mycobacterial lineages appear to have adapted to particular human populations.

4. Human society and the origins of tuberculosis

It is believed that the emergence of human infectious diseases is linked to population density. Therefore, the long hunter-gatherer stage of human evolution would be a bottleneck for highly virulent human pathogens. Small population sizes would select for symbionts or for pathogens that could be transmitted decades after infecting a host, after new susceptible individuals had been introduced into the population via births [18]. Typically, indigenous (commensal) organisms are transmitted vertically from mother to child, whereas pathogens are transmitted horizontally. However, tuberculosis is an intermediate case as an individual is more likely to cough on family members than on strangers, and the microbes transmitted from mother to offspring may be affected by her environmental exposures. In tuberculosis, a majority of hosts have a long or lifetime infection, but disease may be latent, or have phases of activity, which then subside. Pathogen and host can co-exist, which provides a reservoir of infection for the pathogen, and may have caused selection pressure on the survival of its human host. There is the opportunity for early transmission after infection as infants may develop active disease with a high mortality. Late transmission may occur when adults become susceptible from a variety of causes, such as malnutrition, lowered host resistance due to old age or stresses caused by warfare.

The historical change from foraging to settled farming communities in the Neolithic period coincided with the appearance of diseases associated with larger, denser populations, a

sedentary lifestyle, widespread domestication of animals, social stratification, and possibly a less varied diet [19]. Agriculture in the Old World is evident from about 10,000 years ago, where five independent areas of cultivation are found in Mesopotamia, Sub-Saharan Africa, Southeast Asia, northern China and southern China. Independent sites in Mesoamerica and South America were additional independent centres of domestication. Additional factors associated with agriculture, which increase the risk of infectious diseases, are ecological disruption from cultivation and the rise of social and economic inequality. Another factor to be considered is that of local climatic changes, such as prolonged droughts fluctuating with cool, rainy conditions. Nerlich and Lösch [20] argue that such changes in the palaeoclimate of ancient Egypt can be identified and that it is feasible to investigate the interaction of climate conditions and pathogens in historic populations.

5. Palaeopathology of tuberculosis

Tuberculosis may be recognised in human skeletal remains by characteristic vertebral lesions, such as the gibbus formation leading to Pott's disease. It can affect any part of the skeleton, but bony joints are common sites of involvement, and ribs may show lesions. Changes associated with tuberculosis are periosteal reactive lesions on tubular bones, hypertrophic osteoarthropathy, and osteomyelitis [21, 22]. It is estimated that around 40% of skeletal tuberculosis cases result in tuberculosis of the spine [23]. However, skeletal tuberculosis is comparatively rare, occurring in only 3–5% of cases allowed to run their natural course, so in the great majority of cases there should be no skeletal lesions. Therefore, the incidence of tuberculosis in the past was undoubtedly far higher than that suggested by the level of bony lesions observed by palaeopathologists.

Palaeopathology suggestive of tuberculosis has been observed in pre-dynastic Egypt (3,500-2,650 B.C.) [24], middle Neolithic Italy at the beginning of the fourth millennium BC [25] and an Eastern Mediterranean pre-pottery Neolithic site (9,250-8,160 years B.P.) [22]. There are fewer reports from East and Southeast Asia but tuberculosis was present in northeast Thailand at an Iron Age site dated from 2,500 to 1,700 years B.P. [26], and in China, Japan and Korea at least 2,000 years ago [27]. Pre-Columbian tuberculosis was noted in northern Chile, with most morphological evidence found in the period 500 to 1000 A.D., corresponding to fully agraropastoral societies [28]. Few animal studies have been undertaken, but erosive lesions suggestive of tuberculosis have been found on bovids from the natural Trap Cave in Wyoming, dated from the 17,000 to 20,000 year level [29]. Initially, it was believed that humans acquired tuberculosis from animals, especially after domestication [30], because this coincided with the observed human palaeopathology. As we now know that the human tubercle bacillus is of a more ancestral lineage, it is likely that animal domestication was important in sustaining a denser human population, thereby enabling tuberculosis to become endemic [22]. However, it is unlikely that the foundling strain of bovine TB was derived directly from a strain of human TB [31].

6. Historical tuberculosis

Tuberculosis was described by Hippocrates (400 B.C.) in “Of the Epidemics” and was documented by Claudius Galen during the Roman Empire. Recognisable descriptions of tuberculosis are found in other ancient texts, and continued throughout history, identified as consumption, King’s Evil, lupus vulgaris, phthisis, etc [7, 32]. During the Industrial Revolution tuberculosis reached epidemic levels in Europe and was responsible for one in four deaths from the sixteenth to eighteenth centuries. It is now accepted that tuberculosis

existed in the Americas before European contact [28, 32, 33] although it has not yet been determined which species or genotype of the MTBC was responsible. It is hypothesised that the disease reached the Americas via animals [29] or early nomads [32] who crossed the Beringa land bridge at least 10,000 years ago. The suggestion that more virulent strains of the tubercle bacilli originated in Europe and spread to the Americas during the colonial expansions from the fifteenth century onwards [30] has not yet been verified nor disproved.

7. Early molecular studies of archaeological tuberculosis

The combination of clear skeletal markers of tuberculosis, coupled with the availability of specific PCR-based molecular diagnostic methods, led to the detection of MTBC ancient DNA (aDNA) – the first direct detection of microbial pathogenic DNA in archaeological material – in 1993 [34]. MTB aDNA was demonstrated in four of eleven specimens that had been morphologically diagnosed with tuberculosis, including one from Borneo dated prior to known European contact. Subsequently, these findings were verified in independent laboratories, and confirmed by sequencing. Shortly afterwards Salo et al [35] used the same PCR method to examine a sample of lung tissue from a Peruvian mummy 1,000 years B.P., then cloned and sequenced the amplicons. This and subsequent investigation [28] showed that tuberculosis was undoubtedly present in the Americas before Columbus.

The first study, and the majority of those which followed, used the MTBC-specific PCR primers that targeted a short region of 123 bp in the repetitive locus IS*6110* [3]. The use of a multi-copy sequence increases the chances of its detection, and the choice of a short target locus minimises the effect of the DNA fragmentation found in aDNA [36]. The PCR reaction can be made more sensitive by using nested PCR. First used for archaeological samples by Salo et al [35], it was subsequently applied to the study of Mediaeval remains in a

London cemetery where DNA preservation was not good so a smaller nested product of 92 bp was sought [4]. These primers [3, 4] have since been used in many other MTBC aDNA studies. Other PCR primers were used by some early investigators, but were not specific for the MTBC, or had too large a target to give reliable results. The alternative strategy of amplifying aDNA with non-specific primers followed by sequencing of the amplicons poses a risk of amplifying chimaeric sequences that bear little relation to the original. This is a particular problem with aDNA, which is likely to have accumulated hydrolytic and oxidative damage over time, which leads to fragmentation during the PCR reaction [36].

8. Verification of MTB aDNA

DNA is an unstable molecule, and its preservation depends upon the environmental conditions to which it has been exposed rather than the chronological age [36]. Residual DNA from the host and the commensal and saprophytic microflora associated with the remains and the immediate environment will normally predominate. Therefore, stringent precautions must be taken to reduce extraneous contamination to a minimum, during the initial removal of samples from the archaeological site and throughout all subsequent examinations. Criteria for mammalian aDNA work have been devised for use in the verification of findings [36, 37]. Due to the tendency of aDNA to fragment, there should be an inverse correlation between length of target sequence and amplification efficiency, with claims of long amplicons scrutinised. Results should be repeated in a second extract, and verified in an independent laboratory. It is also recommended that the number of amplifiable DNA molecules be quantified, and that PCR products be cloned and sequenced. For palaeomicrobiological MTBC aDNA studies, where the persistence of human DNA and assessment of the state of preservation are less relevant for verification of authenticity, modified criteria have been

devised [38]. If there is clear evidence of excellent preservation of pathogenic microbial DNA, cloning is unnecessary, although it has been used to verify direct sequences.

Early studies of archaeological tuberculosis soon reported positive findings in samples of bones with no palaeopathological signs of disease In addition, positive MTBC aDNA was found in samples where no host DNA could be amplified [38, 39]. Scientists working on human or animal aDNA viewed these findings with scepticism and interpreted such data as contamination and laboratory artefacts, especially as in ancient Egypt the decay rate of DNA from the host or associated materials (papyri) from such an environment was believed to be very rapid, and the amino acid preservation in the specimens apparently poor [40]. However, such results are consistent with our knowledge of the organism and the natural history of tuberculosis [41]. MTBC DNA is rich in guanidine and cytosine so is intrinsically more stable than that of mammalian DNA. In addition, the bacterial DNA is contained within a lipid-rich cell wall that is hydrophobic and extremely resistant to damage and degradation. The natural history of tuberculosis also aids our interpretation of negative findings. MTBC aDNA is more likely to be localised within a host, and will always be a minor component compared with residual DNA from the host and the commensal and saprophytic microflora associated with the remains. The distribution of the microbe within the host is not uniform and will vary according to the stage and intensity of infection. Bones with typical palaeopathology indicate sites where MTBC aDNA may be localised, but its persistence will depend upon the environmental conditions, which the remains have experienced over the years. Unlike host DNA, therefore, it is essential that both positive and negative findings be subjected to independent verification, as different samples from the specimen and minor differences in laboratory procedures can result in success or failure. For example, removal of the outer surface of a bone, such as a rib adjacent to the lungs and pleura, may inadvertently remove superficial MTBC aDNA. The relationship of microbes to lesions is not consistent so

samples should be taken from sites appropriate to what is known of the natural history of the infection [42].

Independent, robust confirmation of the presence of tuberculosis is provided by direct biomarker analysis, without any amplification step. All mycobacteria, including *M. tuberculosis*, have characteristic long-chain fatty acids and other cell wall components. These can be detected by high performance liquid chromatography (HPLC) and present techniques can distinguish the MTBC from other species. HPLC has detected cell-wall mycolic acids specific for the MTBC from archaeological specimens and confirmed findings of MTBC DNA [22, 43]. The presence of Neolithic *M. tuberculosis* was demonstrated by converting long chain fatty acids to pyrenebutyric acid-pentafluorobenzyl mycolates, and demonstrating profiles similar to standard *M. tuberculosis* by reverse phase HPLC [22]. Further normal and reverse phase HPLC gave detailed profiles for each sample and were used to determine the percentage ratios and absolute amounts of mycolic acids extracted from the bone samples. These data verified the *M. tuberculosis* aDNA findings from the same bones. Another class of mycobacterial lipids, the mycocerosic acids, have also recently been shown to be suitable biomarkers for TB diagnosis in archaeological human remains [44]. In mycobacteria these are found mainly as phthiocerol dimycocerosate (PDIM) waxes or phenolic glycolipids. Both these groups of molecules are thought to be involved in virulence [45].

9. Population studies of MTB aDNA

The study of MTBC aDNA within populations is especially useful, as infections occurred in the absence of any effective treatment, thus potentially enabling the investigation of the host--pathogen interaction at a molecular level. The earliest known human cases were from the Pre-Pottery Neolithic site of Atlit Yam in the Eastern Mediterranean, dating from

9,250-8,150 B.P. [22]. The skeletal remains had been buried in thick clay under the sea, and DNA preservation was good. Some molecular characterisation was possible, which demonstrated that two individuals were infected with an *M. tuberculosis* lineage in which the TbD1 deletion had occurred.

It is clear from ancient Egyptian studies [39] that tuberculosis infections were relatively frequent, from predynastic (ca. 3,500-2,650 B.C.) to the Late Period (ca. 1,450-500 B.C.). Most of the tombs examined were in Thebes-West, and the relatively high incidence of disease may be related to the dense crowding in the city at a time of prosperity. MTBC aDNA was detected, and spoligotyping demonstrated both human *M. tuberculosis* that had experienced the TbD1 deletion, and some strains lacking spacer 39 – suggesting possible infection with *M. africanum* [39, 41].

A large on-going study also from the Old World, is based on several hundred early Christian partially mummified remains from two sites in Kulubnarti, Nubia [38, 46]. There is excellent information on their age, sex and nutritional status. Tuberculosis was widespread, with some individuals dying at a young age. There appears to be more tuberculosis infection in young adults and children under 5 years of age, and there are palaeopathological features indicating that these groups suffered from nutritional stress that would exacerbate any underlying tuberculosis infection.

A remarkable collection of naturally mummified 18th century bodies was discovered in a sealed crypt in the Dominican church in the town of Vác, Hungary. The crypt contained 263 wholly or partially naturally mummified bodies, in a good state of preservation. This has led to an on-going study of tuberculosis from a time when the disease was reaching epidemic levels just before the industrial revolution in that part of Europe. Details of the buried individuals are available from church records and the town archives so there is excellent demographical information. Tuberculosis was widespread [47], with almost every individual

in some age groups infected. Radiography and molecular studies enabled individuals who probably died from the disease to be distinguished from those who were infected but lived to a great age. The infecting *M. tuberculosis* strains could be spoligotyped and the *M. tuberculosis* PGG2 and PGG3 could be distinguished in some samples because of their excellent preservation [48]. Different strains of *M. tuberculosis* were demonstrated in a small family group, suggesting that transmission occurred in the community rather than the home.

The study by Redman *et al.* [44] was based on 49 adults (> 15 years) selected as representative from the 505 individuals in the Coimbra Identified Skeletal Collection (Portugal), dating from 1837–1936. The 49 adults died between 1910 and 1936, with documentary data indicating tuberculosis as a cause of death in 48%. A 72% correlation with the burial records supported tuberculosis as a major cause of death, based on *M. tuberculosis* cell wall mycocerosic acids.

M. bovis was not detected in archaeological material until reported in semi-nomadic pastoralists from the Siberian Iron Age, dating from approximately the 4th century BC to the 4th century AD [49]. The preservation of some specimens was sufficient to enable further molecular characterisation, possibly due to the cold continental climate of the region. Examination of regions of difference (RDs) in the MTBC demonstrated that the TbD1 and RD17 regions were intact, but RD12, RD13 and RD4 were deleted. Combined with characteristic SNPs in the *oxyR* and *pncA* loci, it was possible to determine that the infecting organism was classical *M. bovis* yet not of the latest lineage in which RD17 has been lost. Therefore, the RD4 deletion had occurred 2000 years B.P.. *M. bovis* infects many host species, so both wild and domesticated animals can act as reservoirs of infection. It appears that a long-term close association with an infected herd, such as that experienced by the Siberian semi-nomadic pastoralists, is the most likely scenario for the detection of this organism. Interestingly, the earliest report of MTBC aDNA was in a Pleistocene bison dated

to 17,500 years B.P. [29]. It was possible to demonstrate partial spoligotypes that suggested an ancestral pattern of *M. tuberculosis* rather than *M. bovis*. However, consensus spoligotypes were constructed due to the inconsistent results obtained using the techniques then available, so firm conclusions cannot be drawn.

10. Ancient MTB DNA and phylogenetic studies

It is interesting to examine whether the estimated Time to Most Recent Common Ancestor for the MTBC is consistent with the data obtained by the direct detection of MTBC aDNA and other biomolecules. As mentioned above, Wirth et al [15] concluded that the MTBC emerged 41,500 years ago (CI 29,100-60,000) from its progenitor in East Africa. The clade characterised by the loss of the TbD1 region, which evolved into the lineages pathogenic for humans, followed 21,3000 years later (CI 14,300-31,600). Therefore, the demonstration of TbD1–deleted *M. tuberculosis* DNA from 9,000 years B.P. [22] is consistent with this estimated age. Smith *et al*. [31] point out that the Most Recent Common Ancestor of any species can change over time as lineages die out and the population evolves, but suggest that molecular analysis of *M. tuberculosis* aDNA may enable strains that pre-date the current Most Recent Common Ancestor to be characterised. The observation of non-specific lesions consistent with tuberculosis found in a 500,000 year-old skeleton of *Homo erectus* [50] is an intriguing indication that the evolutionary timescale suggested by Gutierrez et al [13] for the emergence of “Mycobacterium prototuberculosis” is worthy of serious consideration.

11. Concluding remarks

The new field of palaeomicrobiology has been of particular value in elucidating the ancient origins of *M. tuberculosis* and the other members of the MTBC. The direct detection and characterisation of aDNA enables accurate dating of changes that have been inferred by phylogenetic studies. Data from historical material contribute to our anthropological understanding of human migrations, settlements and interactions. An increased appreciation of the factors that influence this host/pathogen relationship is of great importance today [45], when *M. tuberculosis* is undergoing rapid changes in response to antimicrobial agents, an increasing human population in denser settlements, and rapid global travel. It is hoped that additional archaeological material will become available for biomolecular studies from the indigenous tuberculosis found in archaeological material from pre-Columbian America, Africa, the Indian subcontinent and the Far East, thus enabling a broader understanding of the nature of ancient tuberculosis to be obtained.

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