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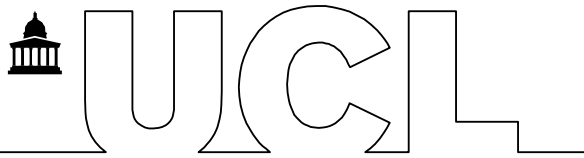

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ARTICLE

Insights gained from palaeomicrobiology into ancient and modern tuberculosis

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Abstract
The direct detection of ancient *Mycobacterium tuberculosis* molecular biomarkers has profoundly changed our understanding of the disease in ancient and historical times. Initially diagnosis was based on visual changes to skeletal human remains, supplemented by radiological examination. The introduction of biomolecular methods has enabled the specific identification of tuberculosis in human tissues and has expanded our knowledge of the palaeopathological changes associated with the disease. We now realise that the incidence of past tuberculosis was greater than previously estimated as *M. tuberculosis* biomarkers can be found in calcified and non-calcified tissues with non-specific or no visible pathological changes. Modern concepts on the origin and evolution of tuberculosis are informed by the detection of lineages of known location and date.

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

Early morphological studies

'You have to know the past to understand the present' Dr Carl Sagan (1934–1996)

Tuberculosis is a disease that was recognised in ancient times, primarily by the characteristic changes to the spine (kyphosis or gibbus) that result in Pott's Disease. The eminent Greek physician Hippocrates (460–370 BC) gives a clear description of kyphosis, stating that it can be the result of disease, can occur above or below the diaphragm, is associated with hard tubercles in the lungs, and abscesses in the lumbar region [1]. He also noted that patients had a poor prognosis if the spinal curvature was above the diaphragm and if the patient was a child or young adult. Many other ancient and historical texts contain recognisable descriptions of tuberculosis, where the disease is identified as phthisis, scrofula, King's Evil, lupus vulgaris, consumption, etc [2]. As palaeopathology emerged from physical anthropology and forensic science as a distinct discipline, diagnostic criteria based on skeletal changes were agreed [3,4].

As part of an on-going process, detailed morphological studies were performed on more recent historical skeletal collections with contemporaneous records of the individual cases, including age, sex, occupation, symptoms and cause of death [5–7]. This led to the realisation that some bony changes, such as periostitis (surface changes due to new bone

formation) on ribs, were significantly associated with individuals who had been diagnosed as having clinical tuberculosis. Other conditions linked to recognised tuberculosis changes include hypertrophic osteoarthropathy (HOA) [8,9] and *serpens endocrania symmetrica* (SES) – a morphological sign of respiratory distress and increased vascularisation around the brain [8].

The use of palaeohistology [10] also aids the recognition of more subtle changes associated with tuberculosis in calcified and non–calcified tissues. Mummified remains can demonstrate signs of tuberculosis such as granulomas in lung and other organs. Indeed, Allison et al [11] used histological methods to re-hydrate such tissue from a pre-Columbian Andean mummy (200–800 AD) and demonstrated bacteria within granulomas that were acid-fast by the Ziehl-Neelson stain, with the typical microscopic appearance of mycobacteria. Radiological imaging gives additional data, and the use of computerised tomography (CT) facilitates the three-dimensional examination of morphological features. Micro-CT is becoming used more frequently, although there are concerns at the increased radiation load that may impact on the recovery of biomolecular markers such as ancient DNA (*vide infra*).

Early studies, based on visual appearance, radiology, and a limited number of microscopic and histological investigations, led to the conclusion that tuberculosis definitely occurred in ancient populations but that it was comparatively rare. The earliest cases recognised were from Neolithic times [12,13], so the hypothesis that tuberculosis was associated with animal domestication and that human TB originated from *Mycobacterium bovis* [3] became widely accepted. The significance of the finding of acid-fast bacilli within granulomas in Pre-Columbian mummified tissue [11] was not generally appreciated, and several subsequent publications included the suggestion that Columbus and subsequent European contacts introduced tuberculosis to the Americas.

Early biomolecular studies

The use of cloning to detect DNA from the extinct quagga in 1984 [14] opened up the prospect of direct genetic studies of archaeological material. However, this only became practicable after the development of PCR [15], which enables rapid amplification of specific genetic loci defined by oligonucleotide primers. There was an explosion of studies based on extinct animals, plants and other remains, several of which are now suspect due to the lack of appropriate measures to prevent contamination with modern DNA. The early development of this field in archaeology and palaeontology had a strong influence on the initial criteria that emerged, on the necessary precautions required for the production of verifiable data based on ancient DNA (aDNA) [16,17]. These have since been modified [18] to take account of our increased understanding of the sources of contamination [19] and the stability of MTBC aDNA [20] compared with other biomolecules [21,22].

Tuberculosis was the first infectious disease to be successfully detected by PCR in bones [23] and mummified tissue [24], due to a fortuitous combination of circumstances. There were agreed morphological diagnostic criteria, appropriate DNA extraction procedures had been developed and clinical microbiologists had designed specific PCR primers. The driving force for molecular diagnostics of tuberculosis was the extremely slow growth rate of the causative organism, *Mycobacterium tuberculosis* and the other closely related members of the *M. tuberculosis* complex (MTBC). Clinical treatment is the same for all members of the MTBC so no attempt was made at the time to distinguish between its members. The first primers used were based on an MTBC-specific locus in the insertion sequence IS*6110* and because it is usually present in multiple copies within the bacterial cell, it was the preferred locus for detection of tubercle bacilli. These early studies confirmed tuberculosis in Byzantine Turkey, pre-European contact Borneo, and a pre-Columbian Andean mummy (1000 BP). They were quickly followed by reports of MTBC aDNA in ancient Egypt, China, America and Europe, summarised in a series of reviews [18, 25–29].

It soon became apparent that MTBC aDNA could be detected in material with non- specific pathology, such as ribs with periostitis [30] and calcified pleura [31]. Specific MTBC mycolic acids, found in the bacterial cell wall, can provide independent confirmation of tuberculosis [31,32], which is of particular significance as the method is based on high performance liquid chromatography and there is no amplification of the target molecules. Recently another category of cell wall lipid biomarkers, the phthiocerol dimycocerosate waxes, have enabled the detection of tuberculosis in archaeological material by the use of negative ion chemical ionisation gas chromatography mass spectrometry of their diagnostic mycocerosic acid components. Redman et al [33] investigated a group of 49 individuals from the 1837–1936 Coimbra Identified Skeletal Collection (Portugal), half of whom had records giving tuberculosis as a cause of death. There was a 72% correlation between detection of mycocerosate acid biomarkers with individuals who were listed as likely to have died from tuberculosis.

Re-evaluation of tuberculosis palaeopathology as elucidated by biomolecular techniques

It had been observed by Baron et al [34] that bone samples from sites with obvious pathology, and from the same bone but of apparently normal appearance, were positive for MTBC aDNA. Their specimens were from a historical collection of individuals given a diagnosis of tuberculosis at autopsy and they concluded that the tubercle bacilli had been carried into the bone with the bloodstream. They speculated further that this raised the possibility that other infectious agents retrievable in blood should be detectable by PCR in the absence of lesions – a supposition now known to be correct [28].

A small study of skeletal remains from 15th–17th century Lithuania may have been the first to deliberately include bones from individuals with no bone pathology. Faerman et al [35] examined pathological lesions versus healthy tissue from the same skeleton, using four individuals. Three additional skeletal remains, chosen at random from the same site, were also examined, plus soil samples to test the specificity of the PCR primers. Compact bone and teeth were examined. All seven individuals contained MTBC aDNA. One soil sample was also positive but further analysis showed that only a sub-specimen that contained small pieces of skin and cartilage was positive. The authors concluded that they had obtained direct evidence for haematogenous spread of tubercle bacilli. They observed that many clinical forms of tuberculosis leave no specific traces on the skeleton, and suggested the disease may be detectable even in individuals represented by only a single bone or tooth.

Zink et al [30] examined the relationship between morphological bony changes and tuberculosis in ancient Egypt. They examined 41 bones: 37 from the necropolis of Thebes-West (2120–500 BC) and four bones from the necropolis of Abydos (3000 BC). Only three subjects had pathological changes typical of tuberculosis, whilst 17 showed non-specific pathology but were likely to be due to tuberculosis. The remaining 20 specimens were viewed as controls and had no visible pathology. Of 30 specimens that demonstrated human DNA, nine samples were positive for MTBC DNA: two of the three with typical pathology, 5/13 non-specific but probable cases of tuberculosis (including two from 3000 BC), and 2/14 with no pathological changes.

Mays et al [36] noted that tuberculous lesions in the ribs arise by extension from spinal lesions, from haematogenous spread from some remote soft-tissue focus, or by direct spread from disease in the lungs, pleura, or chest-wall lymphatic system. In the clinical literature lytic lesions, caused by dissemination of disease from remote soft-tissue foci via the bloodstream, are believed to be the most frequent form of rib involvement in tuberculosis and in such cases bony changes would not be anticipated. Mays et al examined 14 ribs from a deserted mediaeval village in northeast England (10th–16th century AD), seven with pathological surface pathology and seven forming an age and sex-matched control group, but with no visual or radiological sign of pathology. MTBC aDNA was detected in one rib with

pathology and two control ribs, indicating that the absence of visible pathology does not preclude detection of tuberculous biomarkers. The failure to detect MTBC aDNA in bones with pathology is most likely due to poor DNA preservation and/or localised distribution within the bone.

A further example of the detection of tuberculosis in the absence of visible markers is given by a study of skin tissues from 12 Pre-Columbian (140–1240 AD) Andean mummies, where Konomi et al [37] demonstrated MTBC DNA in two individuals. In addition, the study by Redman et al [33] reported that there were no visible signs of tuberculosis in 11 of the 49 skeletons that were positive for mycocerosates. Therefore both MTBC aDNA and cell wall lipid biomarkers have demonstrated the molecular traces of tuberculosis in the absence of specific palaeopathology.

When MTBC aDNA was detected in bones from individuals with no obvious palaeopathology at all [30,35,36], this initially attracted criticism and the assumption of laboratory contamination, until anthropologists realised the low proportion of skeletal tuberculosis in clinical infections from the recent pre-antibiotic era. It is estimated that bone tuberculosis occurs in only 3–5% of infections [38], and that the spine is involved in around 40% of these. Therefore, the earlier views of the incidence of tuberculosis based solely on bone morphology were necessarily severe under-estimates. The detailed studies on relationship of rib lesions with tuberculosis [5–7] had indicated that this might be the case, but until the use of specific aDNA and lipid biomarkers, this could not be proved (Panel 1).

Identification of strains and lineages

The parallel development of molecular diagnostics, and increased understanding of the MTBC genome, encouraged efforts to identify individual members of the MTBC in ancient material and to distinguish between strains and lineages. Methods are based mainly on copy number of repetitive sequences, deletion analysis and single nucleotide polymorphisms (SNPs). Sreevatsan et al [39] described three SNP types based on *katG*^{463} CTG to CGG and subsequent *gyrA*^{95} ACC to AGC mutations, thereby proposing an evolutionary pathway of the tubercle bacilli.

Spoligotyping is based on the direct repeat (DR) region of the MTBC [40]. PCR primers in the DR region amplify up to 43 unique spacer regions that lie between each DR locus. The spacers are visualised by dot-blot hybridisation on a membrane giving a fingerprint and patterns are stored on an international database. Thus, Taylor et al [41] were able to demonstrate, using spoligotyping and SNPs, that bones from Mediaeval London (1350–1538 AD) were infected with *M. tuberculosis* rather than *Mycobacterium bovis*. Both human and cattle bones with periostitis from an earlier mediaeval site in northern England were examined for *M. tuberculosis* and *M. bovis* [42], but only *M. tuberculosis* was detected and only in human remains. Indeed, to date there is only one site where human archaeological cases of *M. bovis* have been identified, and this of Iron Age pastoralists (360 BC–230 AD) from southern Siberia [43,44].

Animal bones have proved a difficult subject for the study of past tuberculosis. There is one report of an Iroquoian dog from the 16th century, with the tuberculosis-associated but non-specific palaeopathological condition HOA [45], in which MTBC aDNA was found. A histological study of the HOA pathological lesions in these remains [46] indicated that the dog had experienced a long and most likely arduous period of illness. The animal was buried with purpose and lived within a longhouse environment [45], thus providing the perfect opportunity for the transmission of an interspecies (and intraspecies) tuberculosis infection.

However, the rarity of animals in the pathological record is, no doubt, because most collections of animal bones are from domesticated animals used for food. These rarely live a sufficient time to develop recognisable lesions or to die a natural death. Once killed,

carcases will be butchered, cooked and eaten. Bones will be used if possible, and if not, discarded. An exception to this scenario was discovered in the Natural Trap Cave in Wyoming, USA. This cave is 30 m deep and presents an unavoidable hazard on a game trail, thereby providing an unbiased sample over >100,000 years of the many passing species. Due to the depth of the cave, falls are invariably fatal, and the temperature remains constant at 4–5 ^{o}C, conditions known to be conducive for aDNA persistence. Palaeopathological changes were occasionally noted in bovids and a metatarsal from an extinct long-horned bison (17,870 ± 230 BP) was found to contain MTBC aDNA [47]. Further characterisation was attempted and consensus spoligotyping indicated an ancestral version of the MTBC DR region, rather than the typing patterns associated with *M. bovis* or most extant strains of *M. tuberculosis*.

The first indication of human tuberculosis infections caused by another member of the MTBC was in a population study from Thebes West in ancient Egypt [48]. Spoligotyping indicated *M. tuberculosis* was present in most of the infected individuals, but there appeared to be one case where *M. africanum* was implicated. A follow-up study of bone and soft tissue samples from 118 individuals compared the incidence of tuberculosis over three time periods. This revealed several samples with an *M. africanum* spoligotype pattern from a Middle Kingdom tomb (2050 –1650 BC), whilst samples from later periods were all typical of *M. tuberculosis* [49]. There was no indication of *M. bovis*. The authors concluded that these findings did not support the theory that *M. tuberculosis* had evolved from *M. bovis*.

Palaeoepidemiology of tuberculosis

To estimate disease prevalence over a long time span, the occurrence of tuberculosis in vertebral bones only was examined at Thebes-West in Ancient Egypt. Specimens were from three time periods: the pre-dynastic to early dynastic period (*c.* 3500–2650 BC), the Middle Kingdom to Second Intermediate Period (*c.* 2100–1550 BC) and the New Kingdom to the Late Period (*c*. 1450–500 BC) [50]. MTBC aDNA was found in 18 of the 50 individuals with detectable human aDNA throughout these time periods, indicating the long-term association of host and bacterial pathogen. There were more positives in individuals with typical tuberculosis pathology, but 7/50 positive cases had bones of normal appearance.

A population study from a more recent, but exceptionally well-preserved collection of naturally mummified or partially mummified remains, was based on a collection of 263 individuals buried during the 18th century, in a church crypt that was sealed and only re-discovered in the early 1990's [51,52]. Contemporaneous civic and church records were available, so it was possible to determine tuberculosis prevalence according to age, sex, occupation and family group. Tuberculosis was assessed initially by visual and radiographic evidence of palaeopathology and contemporary records. Independent research groups determined the presence of MTBC aDNA and found over 60% of individuals to be infected [18]. Molecular typing showed that three members of a family group were infected with separate strains of *M. tuberculosis*. A mother was infected with a different SNP genotype [39] from her two daughters [52], thus suggesting that in this high-incidence population, infection was acquired from the community, not within the family.

An intriguing aspect of palaeomicrobiology is the ability to detect co-infections by the use of specific biomarkers. Donoghue et al [53] detected both MTBC and *Mycobacterium leprae* aDNA in skeletal material from individuals with obvious palaeopathology for lepromatous leprosy. This had been prompted by reports in the clinical literature of leprosy patients dying of tuberculosis, as *M. tuberculosis* is the more virulent pathogen in such patients. Other past populations with molecular evidence of two or more infectious diseases include the Early Christian Nubian population of Kulubnarti, where both tuberculosis and leishmaniasis were identified, and 15th–16th century 'wet' Korean mummies in which many intestinal parasites, tuberculosis, and hepatitis B virus have been detected [18].

Setting the molecular clock – direct determination of past MTBC lineages

Brosch et al [54] demonstrated that the SNP types identified by Sreevatsan et al [39] occurred in a lineage of *M. tuberculosis* strains that had already lost TbD1 (Fig.1), and re-defined them as markers of three Principal Genetic Groups (PGGs). The results by Fletcher et al [51] therefore demonstrated that *M. tuberculosis* aDNA amplified from naturally mummified Hungarians from the 18th and 19th century belonged to *katG*^{463}/*gyrA*^{95} PGG2 and PGG3, and that the TbD1 deletion occurred in the lineage of *M. tuberculosis* before the 18th century. At the time of publication this was of interest, as it suggested the dramatic increase of tuberculosis cases later in the 18th century in Europe involved the TbD1-deleted *M. tuberculosis* strains that are now prevalent in the developed world. It also provided a further demonstration that tuberculosis was caused by *M. tuberculosis* and not by *M. bovis*.

The time-scale for the emergence of distinct human lineages was dramatically extended by the findings of Hershkovitz et al [55]. Studies from the Pre-Pottery Neolithic site of Atlit Yam in the Eastern Mediterranean, dating from 9,250-8,150 BP demonstrated that a woman and infant were infected with *M. tuberculosis*, based on morphological changes to bones, the detection of MTBC-specific cell wall lipid biomarkers, and of aDNA. Bones were well preserved as the skeletal remains had been buried in thick clay under the sea, and DNA preservation was also good so some molecular characterisation was possible. Several different target loci were detected, including that of the TbD1 region. This demonstrated that these two individuals were infected with *M. tuberculosis* from a TbD1–deleted lineage.

Phylogenomics of MTBC strains

The individual members of the MTBC (excluding the strains with smooth colonies classified as *Mycobacterium canettii*) are 99.95% identical, based on nucleotide sequence. This gave rise to the suggestion that there had been an evolutionary bottleneck at the time of speciation [39], estimated by ancestral sequence inference to have occurred 15,000–20,000 years ago. However, there are clear differences in phenotype, host tropism and pathogenicity, so comparative genomic studies were undertaken. Brosch et al [54] analysed 20 variable regions situated around the genome of a representative and diverse set of 100 MTBC strains, obtained from different hosts and with a broad range of geographical origins. The major finding was that MTBC strains have undergone reductive evolution by the unidirectional loss of chromosomal sequences. Once a non-repetitive region has been deleted it cannot be replaced and the deletion is a biomarker of a clone derived from a single cell and all its descendants [56]. The application of deletion analysis thus demonstrates unambiguously that *M. bovis* has undergone numerous deletions relative to *M. tuberculosis* and therefore *M. tuberculosis* is a more ancestral lineage. The *M. tuberculosis* specific deletion 1 (TbD1) locus is absent from the majority of strains now prevalent in Europe and the Americas, but occurs in strains of different geographic origin and in MTBC lineages associated with animal hosts. Therefore, Brosch et al [54] and subsequent authors identified the TbD1 deletion as a marker of importance in our understanding of the evolution of the MTBC. This is supported by total genome sequencing of *M. tuberculosis* [57] and *M. bovis* [58], which show that the *M. bovis* genome is slightly smaller in size (98.5%) and has undergone 11 deletions in comparison with *M. tuberculosis*.

In the search for the source of the MTBC Gutierrrez et al [59] re-examined *M. canettii*, which Brosch et al [54] had concluded was a distinct outlier group with the most ancestral characteristics. Indeed, it became clear that different strains of *M. canettii* displayed more variation than is found in all the other species or ecotypes [56] in the MTBC. This led the authors to the hypothesis that the MTBC had emerged, via an evolutionary bottleneck, from a postulated species with the expected range of internal clonal variation, termed "Mycobacterium prototuberculosis". Using synonymous sequence diversity to estimate the minimal age of the last common ancestor, they concluded that the bacteria responsible for tuberculosis have co-evolved with early humans since at least the time of early hominids between 2.6 and 2.8 million years ago. There is continuing debate about this suggested

scenario, but meanwhile a possible case of tuberculosis in *Homo erectus* dated to 490–510 ± 0.05 Ka was described in a partial skeleton discovered in Western Turkey [60]. The diagnosis was based on endocranial palaeopathology distinct from SES, termed *Leptomeningitis tuberculosa*, believed to be associated with tuberculous meningitis, but critics believe that this diagnosis is premature and based on inadequate evidence [61]. This remains an intriguing possibility that awaits the application of next-generation molecular methods for clarification.

Relationship of tubercle bacilli to different human societies

The use of standardised methods of reporting skeletal collections, and the study of past populations in the context of their diet, living conditions, work patterns and type of society, has facilitated the study of health and disease over time [62]. Evidence of habitations, population density, animal domestication, tools and agriculture is supplied by traditional archaeological assessments. Carbon and nitrogen stable isotope analysis can give information on the type of diet eaten, e.g. terrestrial, marine, freshwater, meat, or dairy. Coprolite analysis can also provide data on nutrition and calorie intake, based on identification of bones, seeds, pollen etc in preserved faeces. Strontium and oxygen isotopes can give an indication of location and mobility during a lifetime. There may also be evidence of major climatic events, such as earthquakes and tsunamis changing sea levels, or the height of the annual flooding of the Nile [63].

Tuberculosis is a disease that can exist as a latent infection throughout the lifetime of a host, but which may be re-activated or re-acquired when host resistance is impaired. In addition to the extremes of life, individuals become susceptible to active disease when suffering from poor nutrition, other serious medical conditions, or severe mental or physical stress. Today it is estimated by the World Health Organisation that one third of the global population is infected with tuberculosis but that only 10% of individuals will develop disease in their lifetime (Fig. 2). The implication of the high proportion of latent infections is that there has been a prolonged period of co-evolution of host and pathogen. This is also indicated by the detection of a TbD1-deleted lineage in the early Neolithic site at Atlit Yam.

Evolutionary biologists have postulated that in the long period of human evolution that pre-dated the Neolithic, tubercle bacilli occupied an unusual ecological niche in human biology. While populations were primarily small, mobile and often family groups, the organisms could survive only by surviving throughout the lifetime of their host, with transmission occurring from adults with re-activated disease due to an age-related failing immune system, or to other severe disease or stress. Infants are more susceptible to acquiring disease, and due to their immature immune system, may have a high mortality with primary disseminated tuberculosis, thereby providing an opportunity for transmission soon after infection. Therefore tubercle bacilli adopt a quasi-commensal relationship with their host in the majority of cases [64].

This scenario explains the development of the clonal relationship of the MTBC complex members to their host, where particular bacterial lineages are associated with human lineages, even in the modern world and in second or third generation inhabitants of ethnically diverse cities [65] (Fig. 3). However, when the human population density rises significantly there is a greater opportunity for transmission to susceptible hosts, so bacterial virulence is likely to increase due to natural selection. This has now been observed by the development of evolutionary modern lineages of *M. tuberculosis*, which induce a lower early inflammatory response in the host and demonstrate faster progression to active disease [66]. Therefore, reflecting the co-evolution of host and pathogen, it is unsurprising that length of urbanisation is a predictor of host genetic resistance to tuberculosis [67].

Concluding remarks

At this time of rapid population growth and the emergence of new virulent *M. tuberculosis* lineages, it is especially important that we understand the history of tuberculosis and evolution of the host/pathogen relationship. This chronological review also allows us to follow the sequence of observations, emergence of concepts and the changes in our perception of this relationship (Panel 2). Palaeomicrobiology offers a unique opportunity to detect past lineages that may no longer be extant. It also enables us to calibrate the molecular clock when calculating time to Most Recent Common Ancestor by ancestral sequence inference. By providing direct evidence of specific biomolecular markers, palaeopathologists are able to make better assessments of past prevalence of disease and to identify tuberculosis-associated skeletal changes with more confidence. In this regard, it is important to extend palaeomicrobiological studies around the world to obtain as comprehensive a picture as possible. The development of next-generation technology and additional biomarkers, with greater stability than DNA, should enable us to explore even further back into the past.

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Panel 1. Objectives of studying tuberculosis palaeomicrobiology

Confirmation of palaeopathological changes associated with tuberculosis

Gain a better understanding of the majority of past tuberculosis infections that leave no visible signs of disease

To examine ancient tuberculosis in relation to diet, health and social structures

Geographic range in the past in relation to human and pathogen lineages

Comparison of microbial pathogens from the past with those of today

Provision of real-time markers of genetic changes

Enable monitoring of changes in virulence over a longer time-scale

Panel 2. **Insights gained from palaeomicrobiological studies of tuberculosis**

Confirmation of diagnosis from palaeopathology

Additional palaeopathological indicators of tuberculosis authenticated

Evidence of haematogenous and direct spread of tubercle bacilli within the body

Understanding that *M tuberculosis* DNA is in bones with no pathology

Appreciation that former estimates of tuberculosis infection were far too low

Confirmation of geographical distribution in ancient times e.g. tuberculosis existed in Borneo before European contact, in Pre-Columbian America, and was widespread in ancient Egypt and Rome

M tuberculosis complex DNA detected in North American bison from Pleistocene (17 500 years ago)

Confirmation of palaeopathological lesions on bovids indicates that *M tuberculosis* in North America was spread from Asia by ungulates that crossed the Bering land bridge

Direct evidence that humans were infected with a TbD1-deleted lineage of *M. tuberculosis* 9000 years ago

M africanum and *M tuberculosis* both shown to exist in ancient Egypt over 4000 years ago

M bovis only clearly identified in human remains from Iron Age pastoralists in Southern Siberia

Co-infections identified in some human remains

Figure legends

Fig. 1 Working model of the evolutionary scheme of tubercle bacilli [68], which was based on earlier versions [54,59]. There is a unidirectional successive loss of DNA and the scheme is based on the presence or absence of conserved regions of difference RDs (TbD1) and sequence polymorphisms in five selected genes. It is schematic and the distances between certain branches do not necessarily correspond to calculated phylogenetic differences.

Fig. 2 The global burden of tuberculosis estimated by the World Health Organisation. Each section of the pyramid drawn is roughly to scale and the bar to the right represents the spectrum of different lesion types seen in latent infections [69].

Fig. 3 A single clone or small subset of a population of a free-living bacterium (left) invades a new pathogenic niche, founding a population of low diversity. The pathogen becomes reproductively isolated from the ancestral population (right), and the pathogenic lifestyle precludes genetic exchange among members of the clone as it expands into its new niche. The accumulation of mutation in different branches generates a fingerprint of polymorphisms characteristic of each branch, enabling the phylogeny to be precisely reconstructed from whole-genome sequence data [70].