Microbiology Spectrum
American Society for Microbiology Press

# Paleomicrobiology of Human Tuberculosis

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**ABSTRACT** Tuberculosis is a significant global disease today, so understanding its origins and history is important. It is primarily a lung infection and is transmitted by infectious aerosols from person to person, so a high population density encourages its spread. The causative organism is *Mycobacterium tuberculosis*, an obligate pathogen in the *M. tuberculosis* complex that also contains closely related species, such as *Mycobacterium bovis*, that primarily infect animals. Typical bone lesions occur in about 5% of untreated infections. These can be recognized in historical and archaeological material, along with nonspecific paleopathology such as new bone formation (periostitis), especially on ribs. Based on such lesions, tuberculosis has been found in ancient Egypt, pre-Columbian America, and Neolithic Europe. The detection of *M. tuberculosis* ancient DNA (aDNA) by using PCR led to the development of the new field of paleomicrobiology. As a result, a large number of tuberculosis cases were recognized in mummified tissue and bones with nonspecific or no lesions. In parallel with these developments, *M. tuberculosis* cell wall lipid biomarkers have detected tuberculosis suggested by paleopathology and confirmed aDNA findings. In well-preserved cases, molecular typing has identified *M. tuberculosis* lineages and genotypes. The current interest in targeted enrichment, shotgun sequencing, and metagenomic analysis reveals ancient mixed infections with different *M. tuberculosis* strains and other pathogens. Identification of *M. tuberculosis* lineages from samples of known age enables the date of the emergence of strains and lineages to be calculated directly rather than by making assumptions on the rate of evolutionary change.

## THE MODERN DISEASE

Tuberculosis remains one of the world's deadliest communicable diseases. In 2014, tuberculosis developed in an estimated 9.6 million people, and 1.5 million died of the disease (1). The principal causative organism is *Mycobacterium tuberculosis*, an obligate pathogen that is a member of the *M. tuberculosis* complex (MTBC), a group of closely related organisms that primarily infect different animal hosts. 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. Swallowing infected sputum can cause intestinal tuberculosis. Transmission can occur via direct contact in cases of scrofula (skin tuberculosis). In addition, ingestion of milk or food from an infected animal can cause human infection with *Mycobacterium bovis* or other members of the MTBC. However, subsequent transmission of these animal MTBC lineages from person to person is rare. *M. tuberculosis* can survive and grow within macrophages, so that it is able to evade the host immune system. An active cell-mediated immune response is required to contain and kill the tubercle bacilli, so any underlying conditions that reduce its efficiency increase susceptibility to tuberculosis. One-third of the global population is estimated to have latent

**Received:** 9 December 2014, **Accepted:** 12 January 2015, **Published:** 8 July 2016

**Editors:** Michel Drancourt, Aix Marseille Université Faculté de Médecine, Marseille, France, and Didier Raoult, Aix Marseille Université Faculté de Médecine, Marseille, France

**Citation:** Donoghue HD. 2016. Paleomicrobiology of human tuberculosis. *Microbiol Spectrum* 4(4):PoH-0003-2014. doi:10.1128/microbiolspec.PoH-0003-2014.

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tuberculosis infection. These individuals do not have active disease but may develop it in the near or remote future, a process called tuberculosis reactivation. The lifetime risk for reactivation is estimated to be 5% to 10%, with tuberculosis developing in the majority of cases within the first 5 years after initial infection. However, the risk is considerably higher in the presence of predisposing factors (2).

## PALEOPATHOLOGY OF TUBERCULOSIS

### Skeletal Changes Indicative of Tuberculosis

The most characteristic visible skeletal changes in archaeological cases of tuberculosis are those to the spine, such as Pott's disease (Fig. 1A) and cold (chronic) abscess (Fig. 1B). Pott's disease is diagnosed by characteristic changes that result in kyphosis, or gibbus, in which there is loss of function in the lower limbs due to damage to the spinal column. Tuberculosis can affect any part of the skeleton, but bony joints are common sites of involvement. Changes associated with tuberculosis are periosteal reactive lesions on tubular bones, hypertrophic ostearthropathy, and osteomyelitis (3, 4). It is estimated that approximately 40% of cases of skeletal tuberculosis result in tuberculosis of the spine (5). However, as skeletal tuberculosis occurs in only 3% to 5% of untreated cases, the incidence of tuberculosis in the past was undoubtedly far higher than that suggested by the number of bony lesions observed (6). Historical texts contain recognizable descriptions of tuberculosis, in which it is identified as phthisis, scrofula, King's Evil, lupus vulgaris, or consumption, for example (7). Detailed morphological studies enabled diagnostic criteria to be agreed upon, based on more recent historical skeletal collections with contemporaneous records of individual cases, including age, sex, occupation, symptoms, and cause of death (8–10). It was noted that periostitis (surface changes caused by new bone formation) on ribs was significantly associated with individuals in whom clinical tuberculosis had been diagnosed (Fig. 1C). Other conditions linked to recognized tuberculosis changes include hypertrophic ostearthropathy (11, 12) and serpens endocrania symmetrica—a morphological sign of respiratory distress and increased vascularization around the brain (11).

### Archaeological Reports of Tuberculosis around the World

Paleopathology suggestive of tuberculosis has been reported from predynastic Egypt (3500 to 2650 BC) (13, 14), middle Neolithic Italy at the beginning of the fourth millennium BC (15), and an eastern Mediterranean Pre-Pottery Neolithic site (9250 to 8160 years BP) (16). There are fewer reports from eastern and southeastern Asia, but tuberculosis was present in northeastern Thailand at an Iron Age site dated from 2500 to 1700 years BP (17) and in Japan and Korea at least 2,000 years ago (18). Precolonial tuberculosis in the Americas was first identified in humans in a mummified child with bone pathology suggestive of tuberculosis, dated to approximately 700 AD, from the Nazca culture of southern Peru (19, 20). It was also recognized in northwestern Argentina (21) and northern Chile (22), with most morphological evidence found in the period from 500 to 1000 AD, corresponding to fully agropastoral societies. More recently, tuberculosis has been confirmed in Peru from Chiribaya cultures (750 to 1350 AD) associated with the Middle Horizon/Late Intermediate Period (23).

### Relationship of Tuberculosis to Early Human Populations

Because *M. tuberculosis* is an obligate pathogen with no environmental reservoir, its persistence is related to the density of the human population. Therefore, the long hunter–gatherer stage of human evolution, consisting of small populations, would select for commensal organisms or for pathogens that could be transmitted decades after infecting a host, after new susceptible individuals had been introduced into the population via births or migration (24). Typically, commensals are transmitted vertically from parent to child, whereas pathogens are transmitted horizontally. However, tuberculosis is an intermediate case because in a low-density population individuals are more likely to spread infection to family members than to strangers.

The Neolithic transition and development of agriculture were associated with a pronounced increase in tuberculosis prevalence (25, 26). Indirect evidence of this association between urbanization and tuberculosis is the relationship between human natural resistance to the disease and long-term urban settlements (27). Although a majority of individuals have a long or lifetime tuberculosis infection, 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 cause selection pressure on the survival of its human host. Early in life, there is the opportunity for tuberculosis transmission, as infants with an immature cell-mediated immune system can develop active disease with a high mortality rate. Late transmission can occur when adults become susceptible from causes that increase their susceptibility, such


**FIGURE 1** **(A)** Paleopathology diagnostic for skeletal tuberculosis: Pott's disease, angular kyphosis in Th8–L2. Hungary: Zalavár-Vársziget-Kápolna, juvenile, grave No. 17/03. **(B)** Paleopathology highly suggestive of tuberculosis: evidence of infection shown by fusion of vertebrae (Th6–8) with slight gibbus, cavities, and traces of cold abscess (chronic lytic lesion). Hungary: Zalavár-Vársziget-Kápolna, juvenile, grave No. 74/03. **(C)** Paleopathology showing nonspecific changes consistent with a tuberculosis infection; disseminated, small, new bone formations can be observed on the costal groove and on the inner surface of the ribs. Romania: Peteni, grave No. 107. (Courtesy of Tamás Hadju, Department of Biological Anthropology, Eötvös Loránd University, Budapest, Hungary. Fig. 1A, B reprinted from *HOMO - Journal of Comparative Human Biology* [95] with permission of the publisher. Fig. 1C reprinted from *Spine* [96] with permission of the publisher.)

as malnutrition, warfare, and old age. The transition 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 a less varied diet (28, 29).

Agriculture in the Old World is evident from about 10,000 years ago, where five independent areas of cultivation emerged in Mesopotamia, sub-Saharan Africa, southeastern Asia, northern China, and southern China. Initially, it was believed that humans acquired tuberculosis from animals, especially after domestication (30), because this coincided with the observed human paleopathology. As we now know that the human tubercle bacillus is of a more ancestral lineage (31), it is likely that animal domestication was important in sustaining a denser human population, thereby enabling tuberculosis to become endemic (16). However, it is most unlikely that the bovine tuberculosis lineage was derived from the lineage that principally infects humans (32).

## DETECTION AND MOLECULAR DIAGNOSIS OF TUBERCULOSIS

The traditional method of diagnosis, still used in many parts of the world today, is chest radiology plus the microscopic examination of sputum smears following Ziehl-Neelsen staining. This method identifies only 10% to 30% of cases, even when enhanced by fluorescence microscopy, so diagnosis is confirmed by culture in solid or liquid media. Because *M. tuberculosis* can take 4 to 6 weeks to grow, the World Health Organization recommends rapid diagnostic methods based on *M. tuberculosis* genetic markers and PCR, such as the Xpert MTB/RIF rapid TB test, for the diagnosis of pulmonary and extrapulmonary tuberculosis in adults and children (33). It was the early development of *M. tuberculosis* molecular diagnostic markers that led to the discovery of tuberculosis in archaeological material.

### Detection of Archaeological and Historical *Mycobacterium tuberculosis* Ancient DNA

*M. tuberculosis* was first identified in the pre-Columbian Americas by using tissue from a mummified child from the Nazca culture of southern Peru (19, 20). As previously described, this mummy had bone pathology suggestive of tuberculosis and microscopic evidence of acid-alcohol–resistant bacilli. Although these findings are highly suggestive of active tuberculosis, molecular evidence was required to confirm the diagnosis.

### Characteristics of Ancient DNA

Modern DNA sequences will outnumber ancient DNA (aDNA) in any sample, so stringent precautions must be taken, throughout the excavation and sampling process, to reduce extraneous contamination to a minimum. In living cells, DNA is subjected to enzymatic repair processes, but after death DNA is rapidly degraded by enzymes derived from both the host and the macro and microbial flora that form part of the natural decay process (34). As a result of cumulative changes over time (diagenesis), aDNA may develop hydrolytic and oxidative lesions. The breakdown of the N-glycosyl bond between the sugar and the base, in the presence of water, leads to hydrolytic cleavage and DNA fragmentation. Hydrolytic depurination causes a preferential loss of guanine and adenine, whereas the pyrimidines cytosine and thymine are 40-fold more susceptible to hydrolytic deaminization (35). Oxidative damage, especially to pyrimidines, can result in the formation of substances such as hydantoins, which block extension during PCR (36). DNA strands may also become chemically cross-linked as a result of the formation of Maillard products (37) by condensation reactions between sugars and primary amino groups in proteins and nucleic acids (34). Local environmental conditions have a strong impact on the persistence of aDNA, such as the temperature, the pH at the site, the availability of water and oxygen, and the fluctuations of all these factors over time (38). Indeed, these factors outweigh the impact of the chronological age of samples.

Mycobacterial DNA is more robust than the DNA of mammals (39), but its persistence depends not only upon the local environmental conditions but also on the nature of the infection at the time of death of its host. Therefore, *M. tuberculosis* aDNA is often highly localized, and DNA extraction protocols may have to be optimized for specimens from different sites (40). DNA extraction normally involves the disaggregation of samples with ethylenediamine-tetraacetic acid (EDTA) and proteinase K. Covalent cross-links can be reduced by the reagent N-phenacylthiazolium bromide (PTB), which cleaves glucose-derived protein cross-links (37). The final stage is disruption of samples with lysis buffer based on guanidium thiocyanate or hydrochloride, followed by silica capture or isopropanol precipitation of aDNA, washing, and drying.

### Methods of *Mycobacterium tuberculosis* Complex Ancient DNA Analysis

The MTBC was one of the first groups of microorganisms to benefit from the introduction of molecular diagnostics because of their very slow growth rate and clinical significance. Early molecular detection of

*M. tuberculosis* was based on short palindromic repeat sequences. Insertion sequences IS*6110* and IS*1081* were identified as useful specific targets for PCR analysis (41). IS*6110* ranges from 1 to 24 copies per cell but is absent in rare strains from southeastern Asia (42), whereas IS*1081* is present at 1 copy per cell (41, 43) and so can be used for quantitative analysis.

Initially, conventional PCR was used to detect ancient and historical tuberculosis, followed by agarose gel electrophoresis for the detection of amplicons. Because of the tendency of aDNA to fragment, there should be an inverse correlation between the length of the target sequence and amplification efficiency, with claims of long amplicons subject to scrutiny. Results should be repeated in a second extract and verified in an independent laboratory. The use of real-time PCR, based on specific primers and fluorescent probes enables shorter DNA fragments to be examined.

## Verification of *Mycobacterium tuberculosis* Complex Ancient DNA Findings

Initially, there was considerable skepticism among anthropologists when tuberculosis was reported in skeletal material with nonspecific or no paleopathology, although to clinical microbiologists, the findings were unsurprising. Suggested criteria for analysis were based on host aDNA, in which protein preservation was used as a marker to indicate the likelihood of successful detection of aDNA, although this relationship has since been questioned (44). In any event, because of the thick, lipid-rich bacterial cell wall and the DNA high guanine–cytosine (GC) content, mycobacterial aDNA is more persistent than the surrounding host aDNA (39), so such prior screening is unnecessary.

In the early days of aDNA research, there were genuine concerns about the prevention of cross-contamination between samples and amplified DNA. For work on host DNA, stringent containment facilities with one-way access and negative air pressure have been designed to minimize the possibility of contamination with modern DNA or amplicons. Although careful precautions are required, work on the MTBC can be accomplished with the use of good microbiological technique and the strict separation of different stages of DNA extraction, amplification, and subsequent analysis (45, 46). This is because the organisms are pathogens with no known environmental reservoir.

## *Mycobacterium tuberculosis* Complex Genotypes, Strains, and Lineages

PCR-based typing methods have facilitated epidemiological studies of tuberculosis. An early example is spoligotyping, which is based on the direct repeat (DR) region of the MTBC (47). PCR primers are used to amplify 43 unique spacer regions that lie between each DR locus, and amplicons from individual spacers are visualized by dot–blot hybridization on a membrane. Spoligotyping and typing based on other repetitive elements clearly distinguish members of the MTBC and can identify different lineages. *M. tuberculosis* strains commonly show deletions, and because the loss of spacers is unidirectional, the data can indicate evolutionary trends (31, 48). Synonymous single-nucleotide polymorphisms (SNPs) or variants (SNVs) are functionally neutral and so can also be used to distinguish between lineages, aided by the virtual lack of horizontal gene transfer. This has led to the recognition of seven phylogeographical lineages (Fig. 2), each associated with specific human populations (49–52), with the animal lineages sometimes described as lineage 8 (53). High-throughput sequencing of entire genomes, coupled with updates in bioinformatics analysis, is the latest tool used to elucidate the relationships between lineages and strains. Recent genomic analyses suggest that *M. tuberculosis* has evolved from a pool of smooth colony-like mycobacteria (STMs) that gained additional virulence and persistence mechanisms, including loss of gene function, acquisition of new genes via horizontal gene transfer, interstrain recombination of gene clusters, and fixation of SNPs (54). The individual members of the MTBC (excluding the STMs classified as *Mycobacterium canettii*) are 99.95% identical on the basis of nucleotide sequence. This has led to the suggestion that there was an evolutionary bottleneck at the time of speciation. The estimated date of this event (Fig. 3) varies from 3 million years ago if the STMs are included (48) to 40,000 years ago (55), to 70,000 years ago (56), to only 6,000 years ago—based on pre-European contact Peruvian material (23). Clearly, the identification of the Most Recent Common Ancestor (MRCA) is crucial in such calculations (32).

## *Mycobacterium tuberculosis* Cell Wall Lipid Biomarkers

In parallel with *M. tuberculosis* aDNA studies, the use of specific mycobacterial cell wall lipid biomarkers has been developed. *M. tuberculosis* has a cell envelope incorporating a peptidoglycan-linked arabinogalactan esterified by long-chain mycolic acids. A range of "free" lipids is associated with the "bound" mycolic acids, producing an effective envelope outer membrane. The distribution of these lipids varies among mycobacteria, and such lipids can act as specific biomarkers

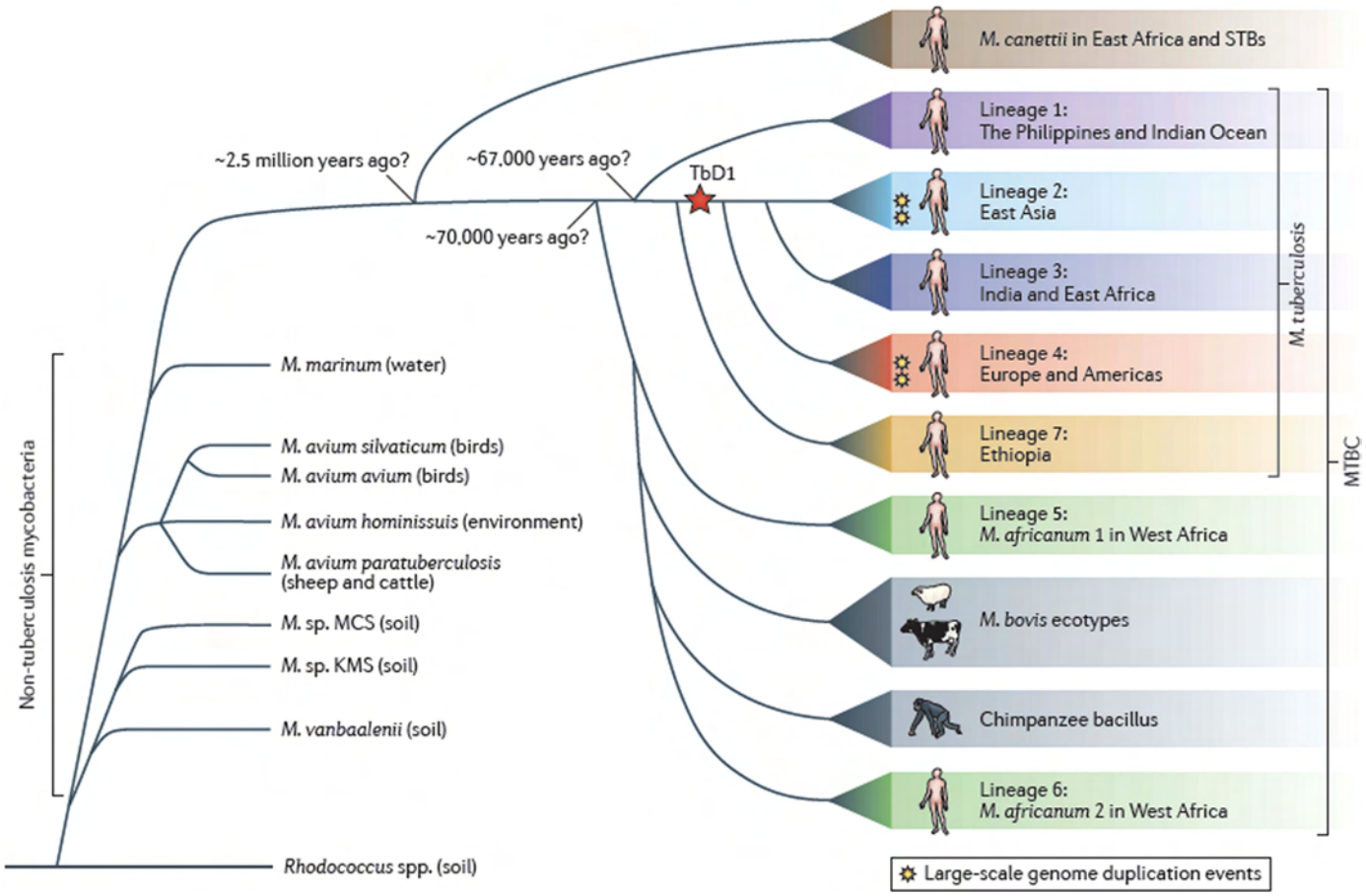


**FIGURE 2** Evolutionary relationship between selected mycobacteria and members of the *Mycobacterium tuberculosis* complex (MTBC). The MTBC was thought to arise as a clonal expansion from a smooth tubercle bacillus (STB) progenitor population. The animal-adapted *Mycobacterium bovis* ecotypes branch from a presumed human-adapted lineage of *Mycobacterium africanum* that is currently restricted to West Africa. Human-adapted *M. tuberculosis* strains are grouped into seven main lineages, each of which is primarily associated with a distinct geographical distribution. The dates of branching events are only crude estimates. (Courtesy of James E. Galaghan, Department of Biomedical Engineering, Bioinformatics Program and National Emerging Infectious Diseases Laboratory, Boston University, Boston, Massachusetts, USA, and Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts, USA. Reprinted from *Nature Reviews Genetics* [97] with permission of the publisher.)

in the identification of *M. tuberculosis* and in tracing its evolution (40, 57). The advantage of lipid biomarkers is that they are detected by extremely sensitive methods, so that there is no amplification of material. Initially, detection of the 70 to 90 carbon mycolic acids was used to complement DNA amplification and paleopathology (58, 59). The biomarker range now includes multimethyl-branched mycocerosic and mycolipenic acids (Fig. 4A, B) (40, 60). Mycolic acids were originally analyzed by fluorescence high performance liquid chromatography (HPLC) of slightly unstable methylanthryl esters (59), so a special robust derivatization protocol, involving pyrenebutyrates of pentafluorobenzyl (PFB) esters, was systematically developed (16, 59, 60). Selected ion monitoring (SIM) negative ion-chemical ionization gas chromatography mass-spectrometry (NICI-GCMS) is an exquisitely sensitive detection method for the mycocerosate and mycolipenate PFB esters (60–63).

With the aim of limiting destructive analyses, it is useful to know that the aqueous residues from DNA extractions can be used for lipid extractions because these use hydrophobic reagents that release different components from samples (64).

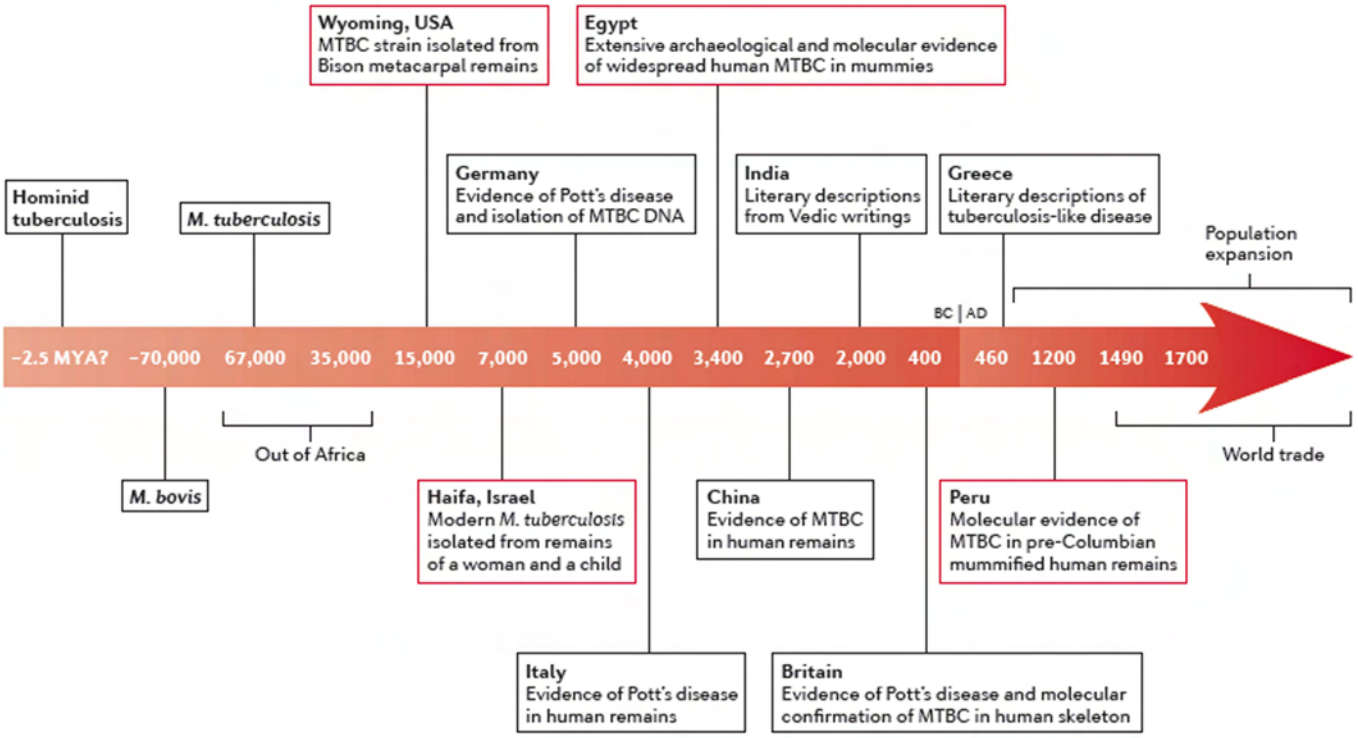


**FIGURE 3** A possible timeline of evolutionary events and archaeological data; the location for archaeological evidence is indicated in each box. Boxes outlined in black indicate morphological evidence only, whereas boxes outlined in red denote both morphological and molecular evidence. (Courtesy of James E. Galaghan, Department of Biomedical Engineering, Bioinformatics Program and National Emerging Infectious Diseases Laboratory, Boston University, Boston, Massachusetts, USA, and Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts, USA. Reprinted from *Nature Reviews Genetics* [97] with permission of the publisher.)

## *MYCOBACTERIUM TUBERCULOSIS* FINDINGS BASED ON MOLECULAR BIOMARKERS

### Overview of *Mycobacterium tuberculosis* Ancient DNA Research

Spigelman and Lemma (65) were the first to demonstrate MTBC DNA in ancient skeletal material. The following year, it was detected in 1,000-year-old human tissue from an Andean mummy and confirmed by sequencing (66). This showed that tuberculosis was definitely present in the Americas before historical European contact. Thereafter, there were several reports of individual cases (6) and cases with no signs of paleopathology, such as those in China from 2,000 years ago (67). Multiple burials enable populations to be studied and the epidemiology of past infections to be investigated. It is especially useful to study infections in the absence of any effective treatment because this has the potential to investigate the host–pathogen interaction at a molecular genetic level. In Thebes-West, ancient Egypt, tuberculosis was quite frequent across a long time period, from the Predynastic Period (c. 3500 to 2650 BC) to the Late Period (c. 1450 to 500 BC). It was suggested that the relatively high incidence of disease might have been related to the dense crowding in the city at a time of prosperity (68). Spoligotyping of the MTBC aDNA demonstrated human *M. tuberculosis* that had experienced the TbD1 deletion, similar to one of the major clades in the world today.

The earliest known published human cases of tuberculosis were from the Pre-Pottery Neolithic site of Atlit Yam in the eastern Mediterranean, dating from 9250 to 8150 BP (16, 60). DNA preservation was excellent because the skeletal remains had been buried in thick clay under the sea. It was possible to demonstrate that two individuals were infected with a strain of *M. tuberculosis* in which the TbD1 deletion had occurred, thus identifying it as the human and not the bovine strain of the MTBC. In northern Europe, *M. tuberculosis* aDNA was detected in eight of 21 early Neolithic samples (5400

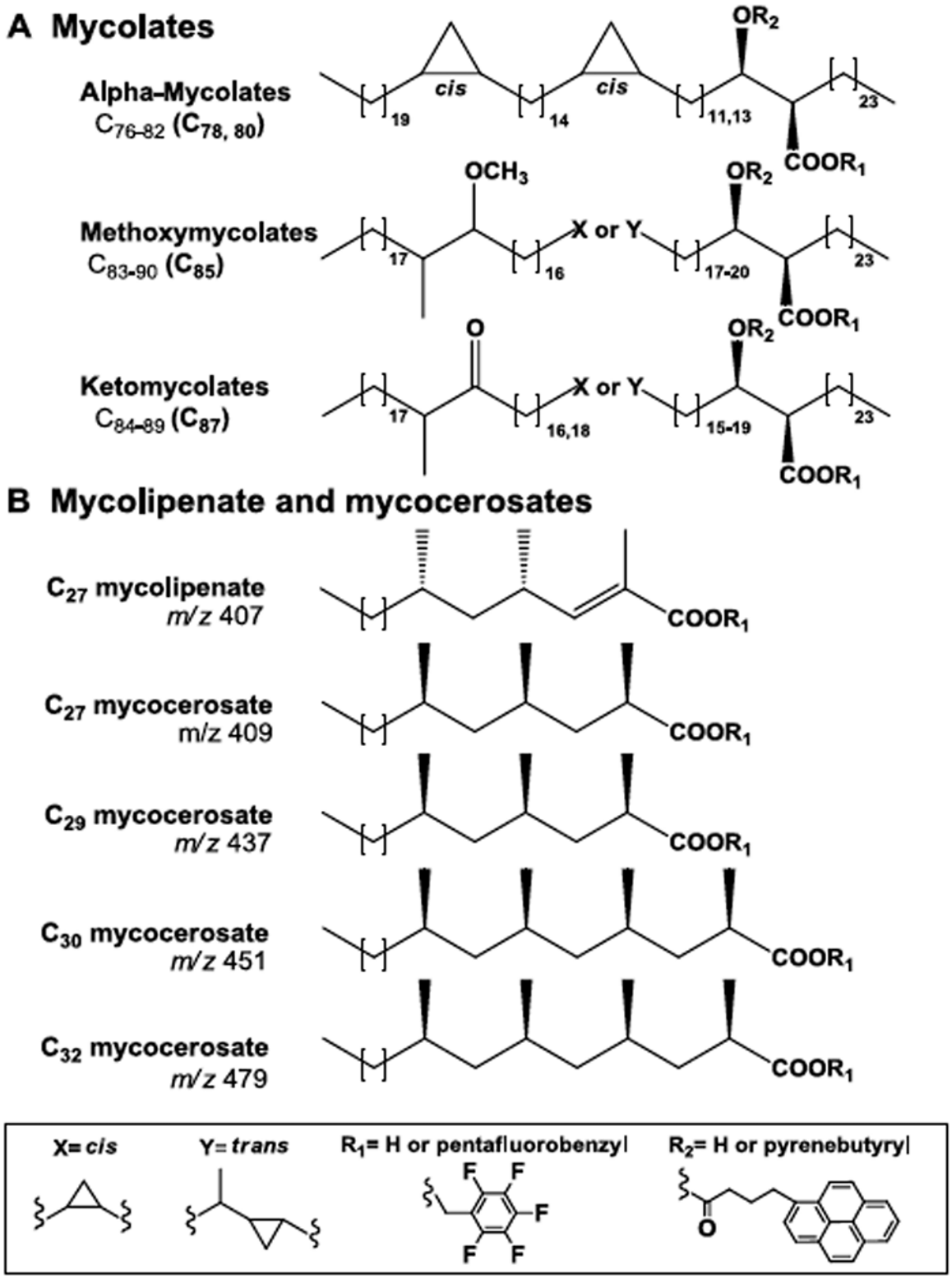


**FIGURE 4** Structures of *Mycobacterium tuberculosis* selected lipid biomarkers. **(A)** The main components of each mycolic acid class are shown; each class comprises a limited range of homologous components with different chain lengths. **(B)** Mycolipenic and mycocerosic acids; for each component, the ions (*m/z*) monitored on negative ion-chemical ionization gas chromatography-mass spectrometry (NICI-GCMS) of pentafluorobenzyl esters of these acids are given. (Courtesy of David E. Minnikin, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK.)

to 4800 BC) from central Germany (69), including three individuals with no visible pathology. Six samples were positive for spoligotyping. A further example of Late Neolithic tuberculosis was reported from central Hungary and dated to 7000 BP (70). In addition to molecular biomarkers, this was a striking case of tuberculosis with characteristic paleopathology—namely, hypertrophic pulmonary osteopathy with rib changes and cavitations in the vertebral bodies.

High-throughput sequencing of entire genomes, coupled with continuing updates in bioinformatics analysis, is now being applied to the examination of historical tuberculosis cases. Bouwman et al. (71) used next-generation sequencing, based on hybridization capture directed at specific polymorphic regions of the *M. tuberculosis* genome, to identify a detailed genotype for a historical *M. tuberculosis* strain from an individual buried in the 19th century in St. George's Crypt, Leeds, West Yorkshire, England. A recent high-profile study (23) examined skeletal material from a large number of pre- and post-contact sites in the New World. Samples were processed via established protocols and screened for MTBC DNA by an in-solution capture assay designed for the *rpoB*, *gyrA*, *gyrB*, *katG*, and *mpt40* genes. Capture products for samples and negative controls were sequenced on an Illumina MiSeq System and mapped to the corresponding regions in the *M. tuberculosis* H37Rv reference genome. There were three positive samples that had been recovered from excavations in Peru and derived from Chiribaya cultures associated with the Middle Horizon/Late Intermediate Period (750 to 1350 AD).

Using a metagenomic approach of shotgun sequencing without prior enrichment, Chan et al. (72) identified two *M. tuberculosis* genomes in one 18th century naturally mummified individual from Vác, Hungary. The Vác mummies are remarkably well preserved because of the local environmental conditions in the sealed crypt where they were found. In addition, there is a contemporaneous archive, so family groups and age at death can be determined (73). The latest findings confirmed an earlier PCR-based study (74) in which each member of a small family group appeared to be infected with a different strain of *M. tuberculosis*. Whole-genome sequencing showed that the mother and her older daughter were both infected with the same two strains of *M. tuberculosis*, but in different proportions (75). In addition, of six other individuals in the same crypt, one was co-infected with three different strains of *M. tuberculosis*, two individuals were co-infected with two strains and the remaining three individuals were each infected with one strain. Six different sub-lineages were detected in this population. PCR-based genotyping has also demonstrated at least one possible mixed infection from British historical samples (76). The presence of mixed infections with more than one strain of *M. tuberculosis* is of particular interest because this phenomenon has been noted in modern tuberculosis infections and described as microevolution, both within a patient and between patients (77). Finding evidence of the same phenomenon in the pre-antibiotic era indicates that this phenomenon is related more to human population density than to antimicrobial therapy.

## Human Infections with Other Members of the *Mycobacterium tuberculosis* Complex

In their spoligotyping study of three different populations in ancient Egypt, Zink et al. (68) showed evidence of human *M. tuberculosis* that had experienced the TbD1 deletion. In addition, there were some strains lacking spacer 39, in samples from a Middle Kingdom tomb in Thebes-West (2050 to 1650 BC). This latter pattern is typical of *Mycobacterium africanum*. *M. bovis* is very rare in the archaeological record. However, it was found in a group of Iron Age Siberian pastoralists (4th century BC to 4th century AD) who wintered in huts with their animals (78). The paleopathogenic lesions (79) were typical of tuberculosis, and the analysis of *M. bovis*–specific genetic markers confirmed the diagnosis. The most recent example of an archaeological human infection with an animal lineage of the MTBC is the study from Peru (23). These ancient strains were most closely related to those adapted to seals and sea lions, known as *Mycobacterium pinnipedii*.

## Past Human Migrations and *Mycobacterium tuberculosis* Epidemiology

There is a striking parallel between human lineage and the corresponding *M. tuberculosis* lineage that is harbored, which apparently persists even if people relocate to other parts of the world. A recent example is shown by a study of a locally dominant *M. tuberculosis* genetic lineage currently circulating among aboriginal populations in Alberta, Saskatchewan, and Ontario, as well as among French Canadians in Quebec, Canada (80). Substantial contact between these human populations was limited to a specific historical era (1710 to 1870 AD), when individuals met to barter furs. Therefore, this study of *M. tuberculosis* provides independent evidence of past contact between distinct peoples.

## Applications of *Mycobacterium tuberculosis* Cell Wall Lipid Markers

Initially, lipid biomarkers were used as an independent method of detecting pathogenic mycobacteria and verifying aDNA data. For example, Redman et al. (61) investigated a group of 49 individuals from the 1837 to 1936 Coimbra Identified Skeletal Collection (Portugal), half of whom had records giving tuberculosis as a cause of death. There was a 72% correlation of the detection of mycocerosate acid biomarkers with individuals who were listed as likely to have died of tuberculosis. Because there is no amplification in lipid analysis, the amount of specific lipid biomarkers can be quantified and used for comparative purposes. Another use is the examination of archaeological samples in which there is poor or no preservation of aDNA. Although samples may show signs of diagenesis, especially samples several thousand years old, the lipid biomarkers are significantly more stable than aDNA and provide independent evidence of infection.

## Use of Other Biomarkers

Carbohydrate or protein antigens are molecules that can induce antibody production, so they can be used to detect infectious organisms. These are often more stable than nucleic acids, but even so, antigenic determinants in ancient tissues may be damaged or destroyed, which limits their use. Antibodies may also be detected in mummified tissues by using a method such as an enzyme-linked immunoelectrotransfer blot. Although the host produces antibodies in response to an infection, their direct detection is difficult because they are generally less stable than antigens. A rare example of a study based on the host response to infection is the work by Corthals et al. (81). These authors reported the first use of shotgun proteomics to detect the protein expression profile of buccal swabs and cloth samples from two 500-year-old Andean mummies. The profile of one of the mummies was consistent with an immune system response to a severe bacterial lung infection at the time of death. One buccal swab contained a probable pathogenic *Mycobacterium* species that was confirmed by DNA amplification, sequencing, and phylogenetic analyses. However, the species was not determined.

An initial proteomic study using shotgun proteomics of mummified lung tissue from Vác, Hungary, revealed a suite of proteins, predominantly derived from the human host. Only one sample demonstrated weak evidence of organisms from the MTBC (82). It appears that most identified proteins were derived from high abundance human extra-cellular matrix proteins, although some immune system and catabolic proteins were identified.

## Host Susceptibility and Ancient Tuberculosis

### Co-infections

It is rare to find visual paleopathological changes that indicate more than one infection. However, in addition to specific aDNA markers, both *M. tuberculosis* and *Mycobacterium leprae* lipids can be identified and distinguished from each other. This led to the discovery that in the past some individuals were co-infected (83). In Europe, the decline of leprosy in the late middle Ages coincided with a rise in tuberculosis. Suggested reasons for this observation include cross-immunity (5) and the increased virulence of tuberculosis (83). Both scenarios are epidemiologically feasible (84).

Evidence of parasitic infections is widespread in human remains. Co-infection with *M. tuberculosis* and parasites is an important public health problem today in areas of the world where both are endemic and is therefore likely to have been so in the past. This has been demonstrated in ancient Lower Egypt dating to c. 800 BC, where four mummies were found with aDNA from both *M. tuberculosis* and *Plasmodium falciparum* (85). Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, was prevalent in pre-Columbian northern Peru, and tuberculosis has also been demonstrated in this population (86). A combination of paleopathology and aDNA analysis demonstrated both diseases in a 12-year-old girl from 910 to 935 BP (87). Leishmaniasis is caused by a protozoan flagellated parasite with a sand fly vector that is associated with acacia trees. Northern Sudan is a region where this disease is endemic today, and *Leishmania* kinetoplast aDNA has been detected from Early Christian Nubia and Middle Kingdom ancient Egypt, where the lack of acacia trees and sand flies led to the assumption that the infection had been spread by trade connections with Nubia (88). Based on aDNA analysis, tuberculosis and leishmaniasis co-infections have been confirmed in Early Christian Nubia (89). It is known that intestinal unicellular parasites and worms are responsible for immunomodulatory effects in their host (90), including the modulation of responses to tuberculosis infections (91). As intestinal parasites are often found in the remains of early human populations, it is highly likely that such modulation of the host response occurred in the past.

### Co-morbidities

Natural resistance to infection is reduced by physical and mental stress, which in turn is caused by invasion,

warfare, displacement, and exclusion from society due to stigma. Also, a pre-existing infection decreases innate host resistance and increases susceptibility to further infections. However, another important consideration is host genetic susceptibility to infectious diseases. Innate immunity is an important arm of the host antimycobacterial defenses that sense various pathogenic microbes by pattern recognition receptors. Toll-like receptors (TLRs) play a crucial role in the recognition of *M. tuberculosis* and other pathogenic mycobacteria (92). Host immune activation occurs only in the presence of functional TLRs. Therefore, any coding changes in TLRs are associated with a substantial drop in susceptibility to these pathogens.

Different types of cancer (neoplasms) have a detrimental effect on host resistance. For example, Langerhans cell histiocytosis, now recognized as a neoplasm, has distinct paleopathology and has been diagnosed in skeletal remains. Langerhans cell histiocytosis is related to immune dysfunction, an increased risk for acquired infections, and early death. A case of archaeological Langerhans cell histiocytosis in an infant, who also had aDNA evidence of tuberculosis infection, has been recognized in one of the Vác Hungarian mummies (93). It is likely that the genetic impairment in the host immune response in such cases increases susceptibility to tuberculosis. In this same 18th century Hungarian population, a 37-year-old woman, with a massive vertebral deformity that would have reduced lung function and therefore increased susceptibility to infection, was found to have tuberculosis (94).

## CONCLUDING REMARKS

The study of ancient tuberculosis based on aDNA, published in 1993, was the first to directly investigate a human infectious disease by using microbial aDNA. Since that date, the field has become recognized around the world, and an increasing range of microbial pathogens is being examined. We now have a clearer understanding of the occurrence of tuberculosis in the past, its epidemiology, and its geographical location. The paleomicrobiology of tuberculosis has verified historical records of past infections and confirmed or refuted the findings of paleopathologists, anthropologists, and archaeologists. Palaeomicrobiology enables the recognition of co-infections, multiple infections, and comorbidities such as tuberculosis and cancer. Links with medical anthropology and biomedical archaeology enable data on human diet, society, location, migrations, stress, and trauma to be considered in relation to past tuberculosis infection and host susceptibility. Collaboration with geneticists and evolutionary biologists has increased our understanding of the origins of the MTBC, *M. tuberculosis*, and the time scale for their emergence.

The newer technologies of high-throughput sequencing, bioinformatics, and metagenomics have made it possible to obtain a complete picture of the host and the microbial contents of samples based on skeletal or mummified remains. An unexpected finding was the discovery of mixed infections with different *M. tuberculosis* lineages. Initially, it was believed that these were linked to scenarios such as the one in modern sub-Saharan Africa, where there are highly dense human populations, many immunocompromised patients, and rising levels of antibiotic resistance. However, we now know that a high incidence of infection, with multiple strains of *M. tuberculosis*, occurred in 18th century Hungary, during a time of peace but also of a rising human population and the start of industrialization. In the present day, there is widespread human mobility around the world, huge changes in lifestyle, and evolutionary changes increasing exponentially in line with the human population. In this scenario, we need to know the origins and development of human microbial pathogens such as *M. tuberculosis* in order to better understand the future. Paleomicrobiology is one of the tools that we can use.

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