Chapter 6
Palaeomicrobiology of Tuberculosis

Helen D. Donoghue

Abstract The study of tuberculosis palaeomicrobiology has proved to be most rewarding. Due to the characteristic palaeopathological lesions, tuberculosis was recognised in archaeological material and was the first infectious disease to be studied by modern biomolecular methods. The combination of a tough bacterial cell wall and GC-rich DNA has resulted in excellent DNA preservation in some specimens. A wide range of specific molecular diagnostic and typing methods, developed by clinical microbiologists, are available. These have been applied successfully to archaeological material, resulting in the genotyping of the infecting organisms. There has been a fruitful interaction with modern genomic studies, and ancient findings support current views on the evolution of the species in the *Mycobacterium tuberculosis* complex. Questions remain to be answered, including the nature of pre-Columbian tuberculosis in the Americas, and the evolution of tuberculosis in animals. The important topics of interactions with other pathogenic microbes, and the host, are now being explored.

6.1 Introduction

Tuberculosis is a major scourge in the world today and the World Health Organisation estimates that around 2 billion people, about one-third of the world's total population, are infected with tubercle bacilli (WHO 2006). Only about 10% of infected persons will become ill with active disease, and those with weakened immune systems such as the very young and old, people who suffer from malnourishment, other diseases, physical or mental stress, or other immunosuppressive conditions, are more likely to suffer from the disease. The extremely high level of latent infection is an indication of long-term co-existence of human host and bacterial

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pathogen (Hirsh et al. 2004), and the evolution of the causative organism is an active area of research.

6.1.1 Causative Organisms

Tuberculosis is caused by a group of closely related bacterial species termed the *Mycobacterium tuberculosis* (MTB) complex. Other mycobacterial species are widespread in the environment but members of the MTB complex are obligate pathogens. All mycobacteria have DNA that is rich in guanine and cytosine, and all have a lipid-rich cell wall that is hydrophobic and extremely resistant to damage and degradation. Many of the mycobacteria, including the MTB complex, 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.

The principal cause of human tuberculosis is *M. tuberculosis. Mycobacterium bovis* has a wider host range and is the main cause of tuberculosis in other animal species. Humans can become infected by *M. bovis*, usually via milk, milk products or meat from an infected animal. Other members of the MTB complex include the human pathogens *Mycobacterium canettii*, *Mycobacterium africanum*, and several species associated with particular animals such as voles (*Mycobacterium microti*), goats and deer (*Mycobacterium caprae*), and seals (*Mycobacterium pinnipedii*).

6.1.2 Natural Course of Tuberculosis

Tuberculosis (TB) is spread principally by infectious aerosols, which are released from the lungs of an infected person who has pulmonary disease. 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. During the lifetime of the host the organisms may remain dormant but viable for decades and, if the immune response is 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, causing extra pulmonary tuberculosis, e.g. miliary TB or meningitis (Garay 1996).

Occasionally tuberculosis can be acquired by ingestion of infected animal products, causing intestinal tuberculosis. This can also result from swallowing infected sputum. Therefore both urine and faeces may contain tubercle bacilli and act as vehicles of infection. Extra pulmonary tuberculosis can result in infected lymph glands. Cervical lymphadenitis and skin lesions were previously known as scrofula

or lupus vulgaris, and were often associated with *M. bovis* infections (Grange 1995). In developing countries with little or no effective prevention or treatment, it is estimated that around 6% of human tuberculosis cases are caused by *M. bovis*, with a higher proportion in children (Hardie and Watson 1992; O'Reilly and Daborn 1995).

6.1.3 Laboratory Diagnosis of Tuberculosis

The basic method of diagnosis of tuberculosis in patients with lung disease is to microscopically examine stained smears of the sputum and, if facilities are available, to isolate and grow a culture of the infecting organism. Because of the hydrophobic bacterial cell wall, the organisms can be stained only by special procedures, such as heating the slide, that enable the stain to penetrate into the cell. Subsequent decoloration by cold acid and/or alcohol will fail to remove the stain, so this group of bacteria is described as acid-fast. However, this method is not sufficiently sensitive to detect low levels of bacteria, or to diagnose cases of extra pulmonary disease. Culture is still the universally accepted method of confirmation of clinical diagnosis, but requires specialised facilities to protect laboratory staff. In addition, the organisms take several weeks to grow. Therefore, members of the MTB complex were amongst the first microorganisms for which molecular diagnostic methods were devised. The complete genome sequences are available for several mycobacterial species and this has increased the number of methods available (Drobniewski et al. 2003).

6.1.4 Molecular Diagnosis of Tuberculosis

Organisms in the MTB complex have many repetitive sequences in their DNA. These are of no known function and it is probable that the majority are not transcribed. There are several insertion sequences, including IS*6110* and IS*1081*, normally present in multiple copies within the cell (Gordon et al. 1999). Following the development of the polymerase chain reaction (PCR), assays targeted regions of these insertion elements specific to the MTB complex were devised (Eisenach et al. 1990). Care is needed as several environmental species have similar sequences, e.g. in the 16S ribosomal DNA locus, insertion sequences and other widespread alleles such as the 65-kDa heat shock protein gene, *hsp65* (Dziadek et al. 2001; Huggett et al. 2003). It was realised that there were three principal genetic groups within the MTB complex, based upon two functionally neutral single nucleotide polymorphisms (SNPs) in the catalase-peroxidase-encoding gene *katG* and a subunit of the DNA gyrase gene *gyrA* (Sreevatsan et al. 1997; Mathema et al. 2006). Whole genome sequencing has led to a revision of our earlier ideas about the evolution of the *M. tuberculosis* complex (Brosch et al. 2002)

and it appears that this group has accumulated deletions over time that can be used to distinguish individual species (Parsons et al. 2002). It is also now clear that the human pathogen *M. tuberculosis* has evolved from a more ancestral lineage than *M. bovis*.

6.1.5 Molecular Typing Methods

Different strains of *M. tuberculosis* may have up to 26 copies of the IS*6110* element, or rarely, none at all. *M. bovis* has a single copy of IS*6110*. Both species have six copies of IS*1081* (Collins and Stephens 1991). An early method of molecular fingerprinting was to target the IS*6110* locus by restriction enzyme digestion, separate the fragments on a gel and to analyse the restriction fragment length polymorphism (RFLP). However, this expensive and time-consuming method is being replaced by PCR-based techniques (van Soolingen 2001). Spoligotyping is based on the direct repeat (DR) region of the MTB complex organisms (Kamerbeek et al. 1997). PCR primers in the DR region amplify up to 43 unique spacer regions that lie between each DR locus. The presence of these individual spacers is visualised by means of dot-blot hybridisation on a membrane, giving a fingerprint. The more ancestral lineages have a complete spoligotype, but different strains commonly show deletions. The loss of spacers is unidirectional, so the data can be used for evolutionary studies. Spoligotyping clearly distinguishes *M. bovis* from *M. tuberculosis*, and different families of strains are defined by characteristic patterns. An international database is available at www.pasteur-guadeloupe.fr/tb/spoldb4 (Brudey et al. 2006). Further typing is possible 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 (Barnes and Cave 2003). A combination of spoligotyping and MIRU typing has the greatest discriminatory power. Meanwhile, research continues on typing based on genomic deletion analysis and SNPs (Baker et al. 2004; Mathema et al. 2006).

6.2 Tuberculosis in the Past

6.2.1 Palaeopathology of Tuberculosis

The disease has long been recognised by the characteristic changes that occur in the spine, i.e. gibbus formation leading to Pott's disease. In addition, periosteal reactive lesions on tubular bones and osteomyelitis are indications of tuberculosis (Ortner and Putschar 1981). Such palaeopathological changes have been reported in pre-dynastic

(3500–2650 B.C.) Egypt (Zink et al. 2001); middle Neolithic Sweden (3200–2300 B.C.) – culturally associated with the Funnel Beaker Culture, the earliest cattle breeders in Sweden (Nuorala et al. 2004); and middle Neolithic Italy at the beginning of the fourth millennium B.C. (Formicola et al. 1987; Canci et al. 1996). The disease was present in Asia, for example, in northeast Thailand at an Iron Age site dated from 2,500 to 1,700 years BP (Tayles and Buckley 2004). Tuberculosis was also present in China 2,000 years ago (Fusegawa et al. 2003). Erosive lesions suggestive of tuberculosis have been found on fossil fauna from the natural Trap Cave in Wyoming, dated from the 17,000 to 20,000 year level (Rothschild and Martin 2003). Initially, it was believed that humans acquired tuberculosis from animals, especially after domestication (Steinbock 1976; Manchester 1984; Clark et al. 1987), but now we know that human tuberculosis is more ancestral (Armelagos and Harper 2005). Animal domestication is likely to have been important in sustaining a denser human population, enabling *M. tuberculosis* to become endemic (Weiss and McMichael 2004; Armelagos et al. 2005).

6.2.2 History of Tuberculosis

Recognisable descriptions of tuberculosis are found in ancient Egyptian, Greek and Roman texts, and continued throughout history (Haas and Haas 1996; Roberts and Buikstra 2003a, 2003b). Tuberculosis reached epidemic levels in Europe during the Industrial Revolution and was responsible for one in four deaths from the sixteenth to eighteenth centuries (Hutás 1999). In recent years it has become accepted that tuberculosis existed in the Americas before European contact (Daniel 2000; Gómez I Prat and Mendonça de Souza 2003; Mackowiak et al. 2005), although debate continues over which species was responsible (see below). The disease is thought to have reached the Americas via animals (Rothschild and Martin 2006) or early nomads (Daniel 2000) who crossed the Beringa land bridge at least 10,000 years ago. However, the nature of pre-Columbian tuberculosis is still unknown (Wilbur and Buikstra 2006), and the suggestion that more virulent strains of the tubercle bacilli originated in Europe and were spread to the Far East and the Americas during the colonial expansions from the fifteenth century onwards (Clarke et al. 1987), still awaits confirmation.

6.3 Work on *Mycobacterium tuberculosis* Complex Ancient DNA

6.3.1 Recommended Good Laboratory Practice

DNA is an unstable molecule, and modern DNA sequences will always outnumber those of ancient DNA (aDNA) in any sample. Therefore, stringent precautions must be taken to reduce extraneous contamination to a minimum. These should be

applied during the initial removal of samples from the archaeological site (Spigelman and Greenblatt 1998), and throughout all subsequent examinations. Criteria for mammalian aDNA work have been devised for use in the verification of findings (O'Rourke et al. 2000; Hofreiter et al. 2001; Pääbo et al. 2004). 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 MTB complex DNA studies, modified criteria may be more appropriate (Table 6.1).

Table 6.1 Procedures used to determine authenticity of pathogenic mycobacterial ancient DNA (MTB complex aDNA). PCR polymerase chain reaction, SNP single nucleotide polymorphism

Procedure	Comments and examples
The choice of sampling sites should be determined by the natural history of the disease	For pulmonary tuberculosis select inner rib surfaces, or lung tissue; for disseminated tuberculosis sample vascularised tissues
The size and copy number of the MTB aDNA alleles sought should be appropriate for the sampling site	Sites that give protection from the natural decay process are the dental pulp region and the ends of the long bones; abdominal tissue is likely to pose the greatest challenge
Pre- and post-PCR activities must be strictly separated	To prevent cross-contamination
Ensure no modern DNA is used in aDNA laboratory	Modern DNA is non-fragmented and if present will inevitably be the major PCR amplicon
Use multiple negative controls of DNA extraction and PCR	This detects cross-contamination of reagents and by glove-tip
Confirm results by replication within laboratory	Results are often inconsistent due to uneven distribution of pathogen. Replication should be tested with a repeat DNA extract if possible
Independent confirmation of results by external laboratory	Many replicates are advised as discrepant results may be genuine
Results should be consistent with natural history of the infectious disease	TB likely to be disseminated in infants, and pulmonary in adults
An inverse relationship between fragment size and quantity of PCR amplicon should be observed	GC-rich microbial templates such as MTB can yield remarkably large PCR amplicons e.g. ~300 bp if preservation is good
aDNA sequence data should make phylogenetic sense	Direct sequencing is adequate for GC-rich mycobacterial aDNA
Cloning or multiple sequencing	This may be useful when investigating SNPs and MTB genetic variations

6.3.2 DNA Extraction

The first stage is to disaggregate the sample. Published protocols include drilling bone, grinding in a pestle and mortar, successive freezing in liquid nitrogen and thawing, demineralisation with proteinase K and EDTA, and incubation in a lysis buffer based on a guanidium salt. Pre-incubation with the reagent *N*-phenacylthiazolium bromide (PTB) enables DNA to be released from material with glucose-derived protein cross-links that can form over time (Poinar et al. 1998). After release from the specimen, DNA is normally captured onto silica (Boom et al. 1990; Höss and Pääbo 1993), and repeated silica extraction is a simple way to remove inhibitors (Kemp et al. 2006). Alternatively, DNA is precipitated by isopropanol, which also removes inhibitors (Hänni et al. 1995). Finally, DNA is eluted or re-hydrated into solution. DNA extracts are not stable so are often aliquoted into 'no stick' plastic tubes, before storing at −20°C or, preferably, −80°C to avoid unnecessary freezing and thawing.

6.3.3 DNA Amplification

Stringent precautions against cross-contamination must be taken, with physical and temporal separation for different stages of the process, e.g. extraction, PCR set-up and product analysis. Separate sets of pipettes should be used for PCR set-up and product analysis, and cleaned thoroughly before use. Filter tips are routinely used and all surfaces and equipment in contact with sample tubes (centrifuges, rotors, mixers, etc.) cleaned before each assay. Multiple sample blanks should be used for negative controls during the DNA extraction and water blanks included in PCR amplifications to ensure there is no contamination. PCR facilitators, such as bovine serum albumin (BSA) (Forbes and Hicks 1996) and betaine (Abu Al-Soud and Rådström 2000) are often required when amplifying aDNA. Additional *Taq* polymerase can also improve the yield (Sutlovic et al. 2005). Normally 40–45 rounds of amplification are used and further amplification in a nested PCR may be necessary.

6.4 Relationship of MTB complex aDNA to Other Markers

6.4.1 Host Proteins and DNA

In many areas of aDNA research, other markers of molecular diagenesis are used to determine the choice of specimen for examination. For example, the degree of amino acid racemisation in a specimen is taken as a measure of the extent of DNA preservation (Poinar and Stankiewicz 1999). If aDNA was found in samples with

demonstrable amino acid degradation, findings were regarded sceptically. Later work suggests that both hydroxyapatite and collagen yield may be better indicators of biomolecular stability (Götherström et al. 2002).

However, the DNA of *M. tuberculosis* is intrinsically more stable than that of mammalian DNA due to its high percentage GC content. In addition, the bacterial cell wall is both persistent and protective, being lipid-rich and hydrophobic (Spigelman and Donoghue 2003; Donoghue et al. 2004), with large amounts of C60–C90 fatty acids – the so-called mycolic acids (see below) – and many unusual extractable free lipids (Minnikin et al. 2002). These provide protection from environmental extremes and also limit the permeability of the wall (Lambert 2002). This resistant cell wall is believed to be responsible for the persistence and survival of tubercle bacilli throughout the lifetime of a mammalian host and the initial decay process after death (Weed and Bagenstross 1951; Sterling et al. 2000). Therefore, MTB complex aDNA can be found in samples that otherwise appear to be poorly preserved (Donoghue and Spigelman 2006).

6.4.2 Lipid Biomarkers

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 MTB complex from other species (Butler and Guthertz 2001). HPLC has detected cell-wall mycolic acids specific for the *M. tuberculosis* complex from archaeological specimens (Gernaey et al. 1998, 2001), and confirmed findings of *M. tuberculosis* complex DNA (Donoghue et al. 1998). More recently, mass spectroscopy (MS) has been used to detect such molecules (Mark et al. 2006). Unfortunately, the equipment and expertise are not yet widely available, yet this is potentially a method of great promise. The advantage of using HPLC or MS in ancient tuberculosis studies is that these lipid biomarkers are very stable and the methods exquisitely sensitive so the molecules can be detected directly without amplification.

6.5 The Start of Palaeomicrobiology

6.5.1 Early Molecular Studies

Tuberculosis was the first ancient infectious disease to be detected via the DNA of the causative organism. This was due to the combination of clear skeletal markers of the disease, coupled with the availability of specific molecular diagnostic methods based on PCR. The first study, on skeletal samples (Spigelman and Lemma 1993), used MTB complex-specific PCR primers that targeted a short region of

123 bp in the repetitive locus IS*6110* (Eisenach et al. 1990). It is important to use a short target sequence, preferably <130 bp, because aDNA fragments, and damage accrues over time (Pääbo et al. 2004). A repetitive sequence enhances the chance of obtaining a positive result. This first study demonstrated tuberculosis in 4 of 11 specimens that had been morphologically diagnosed with tuberculosis, including 1 from Borneo dated prior to known European contact. These findings were later repeated in independent laboratories for verification, and confirmed by sequencing (Spigelman et al. 2002). Shortly after the initial report, Salo et al. (1994) used the same primers to clone and sequence amplicons from lung tissue that had been sampled from a Peruvian mummy 1,000 years BP. This and subsequent studies (Arriaza et al. 1995; Braun et al. 1998; Konomi et al. 2002) showed that tuberculosis was undoubtedly present in the Americas before Columbus.

Salo et al. (1994) is the earliest molecular study that made use of nested PCR based on IS*6110*, giving a 97 bp amplicon. The technique was also used in the study of Mediaeval remains from a London cemetery, where the burial conditions were not ideal for DNA preservation (Taylor et al. 1996). Here, a smaller nested product of 92 bp was sought, and these primers have often been used since.

Some early molecular studies used primers that were not specific for the MTB complex, or had too large a target to give reliable results. For example, Nerlich et al. (1997) examined ancient Egyptian tissue from the New Kingdom (1,550–1,080 B.C.). PCR was carried out using primers for a 133 bp sequence from the *hsp65* gene and confirmed by sequencing. Although the primers were based on the *M. tuberculosis* sequence, the DNA from many environmental mycobacteria will amplify. The 65 kDa heat shock protein gene was used as an additional target by Crubézy et al. (1998) in their examination of skeletal remains of a pre-dynastic Egyptian (5,400 years BP) with Pott's disease. Haas et al. (2000) also used primers for *hsp65* but concluded that the IS*6110* locus had greater specificity and should be used in preference. Amplification with non-specific primers followed by sequencing of the amplicons is a strategy that has been used where the causative organisms are unknown, such as the case of the Tyrolean Iceman (Cano et al. 2000). However, there is a danger in such studies, using primers for loci such as 16s rRNA, that chimaeric amplicons will be obtained that bear no relation to any original sequence.

The 10-year anniversary of palaeomicrobiology in 2003 resulted in several reviews, which summarise the early studies (Zink et al. 2002; Donoghue et al. 2004; Drancourt and Raoult 2005).

6.5.2 Relationship of Bony Lesions to MTB complex aDNA

It is estimated that around 40% of skeletal tuberculosis cases result in tuberculosis of the spine (Aufderheide and Rodriguez Martin 1998). However, it is important to appreciate that tuberculosis of the bone is comparatively rare, and possibly occurs in only 3–5% of cases allowed to run their natural course (Resnick and Niwayama 1995). Therefore, according to the natural history of tuberculosis, in the great

majority of cases there should be no skeletal lesions, and the incidence of tuberculosis was undoubtedly far higher than that suggested by the level of bony lesions observed by palaeopathologists.

Non-microbiologists, who did not appreciate the comparative rarity of skeletal tuberculosis, initially viewed early reports that bones without lesions were found to be positive for MTB complex DNA with scepticism (Baron et al. 1996; Faerman et al. 1997). As work has continued in this field, however, there are an accumulating number of studies that have reported MTB complex DNA in skeletons without pathological changes (Haas et al. 2000; Zink et al. 2001, 2003a; Mays et al. 2002; Fusegawa et al. 2003), although at a lower frequency than skeletons with lesions. There is continuing interest in the relationship of disease to the presence of bony lesions (Zink et al. 2005a; Raff et al. 2006; Santos and Roberts 2006).

It should be remembered that MTB complex DNA will be unevenly distributed within a host, and that it will always be in the minority, compared with residual human DNA and that of the commensal and saprophytic microflora associated with the skeletal remains. Therefore, a bone with typical tuberculosis pathology provides an excellent marker for a site where MTB complex DNA may be localised. Another excellent site is the pleural surface of the ribs, as these often contain MTB complex DNA due to contact with infected lungs. To detect MTB complex DNA that was present in the bloodstream, the heads of the long bones and centre of all tubular bones should be sampled, as these will contain residues of the bone marrow. The residual material in the dental pulp cavity is another excellent source of microbial DNA in disseminated infections.

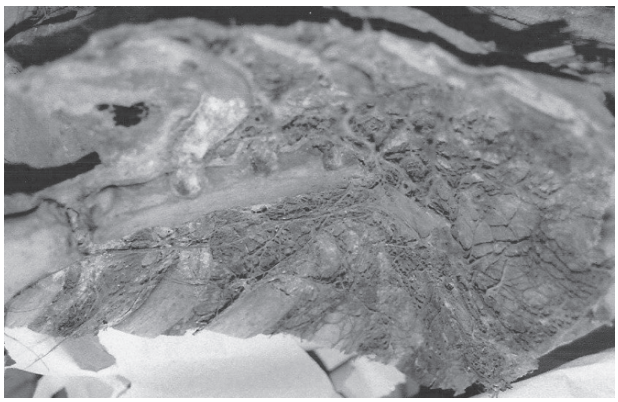
6.5.3 MTB complex DNA in Populations

The examination of tuberculosis within past populations is especially worthwhile, as data are obtained from infections that were allowed to run their natural course, in the absence of effective treatment. This offers the potential to investigate the host–pathogen interaction at a molecular level. The earliest populations studied were those of ancient Egypt (Zink et al. 2001, 2003a), and it is clear 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.). In some cases the age and sex of the individuals could be determined, but the main conclusion drawn was that MTB complex could be detected in bones with typical palaeopathology, non-specific palaeopathology, and without visible palaeopathology. The tombs examined were mainly the ‘Tombs of the Nobles’ in Thebes-West, and the relatively high incidence of disease was related to the dense crowding in the city at a time of prosperity.

An early study of pre-Columbian tuberculosis in Northern Chile examined 483 skeletons, dating from 2,000 B.C. to 1,500 A.D. (Arriaza et al. 1995). Morphological evidence of tuberculosis was found mainly in the period 500–1,000 A.D., which correlated with fully agaropastoral societies, and about 2% showed tuberculosis lesions. However, molecular data were obtained from only one 12-year-old girl with Pott’s disease.

The discovery of a crypt containing 263 wholly or partially naturally mummified bodies in the Hungarian town of Vác, led to an on-going study of tuberculosis at a time when the disease was reaching epidemic levels just before the industrial revolution in that part of Europe (Pap et al. 1999). As there is a contemporaneous archive of the individuals buried in the crypt, it is possible to determine the age, sex, name, family relationship and even the occupation in some cases. In some individuals the preservation of the human remains was remarkable (Fig. 6.1a,b). It was soon discovered that tuberculosis was widespread

a



b

Fig. 6.1a,b Naturally-mummified individuals from eighteenth century Vác, Hungary. **a** Whole body of a 76-year-old man who died in 1796. Tissue from his right chest contained *Mycobacterium tuberculosis* ancient DNA (*M. tuberculosis* aDNA). Other samples were negative. **b** Inner surface of chest cavity of partially mummified 36-year-old man who died suddenly in 1808 vomiting blood, and after long-lasting spitting of blood. Note the well-preserved vascular tissue. His chest and abdomen tissues were strongly positive for *M. tuberculosis* aDNA and a radiograph showed small calcified lesions in his thorax, typical of pulmonary tuberculosis

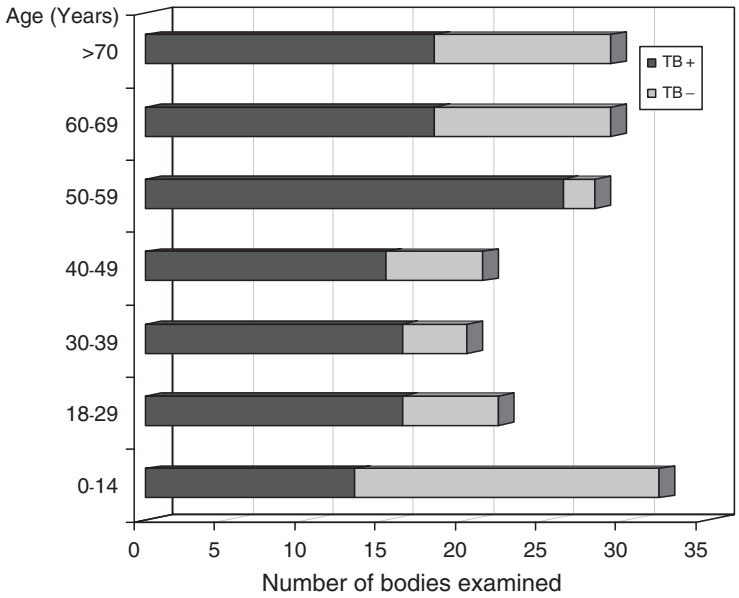


Fig. 6.2 Distribution of *M. tuberculosis* aDNA by age at death in eighteenth century mummies, in Vác, Hungary. There is a very high level of infection in all age groups, particularly in those aged 50–59 years. However, some infected individuals lived to a great age

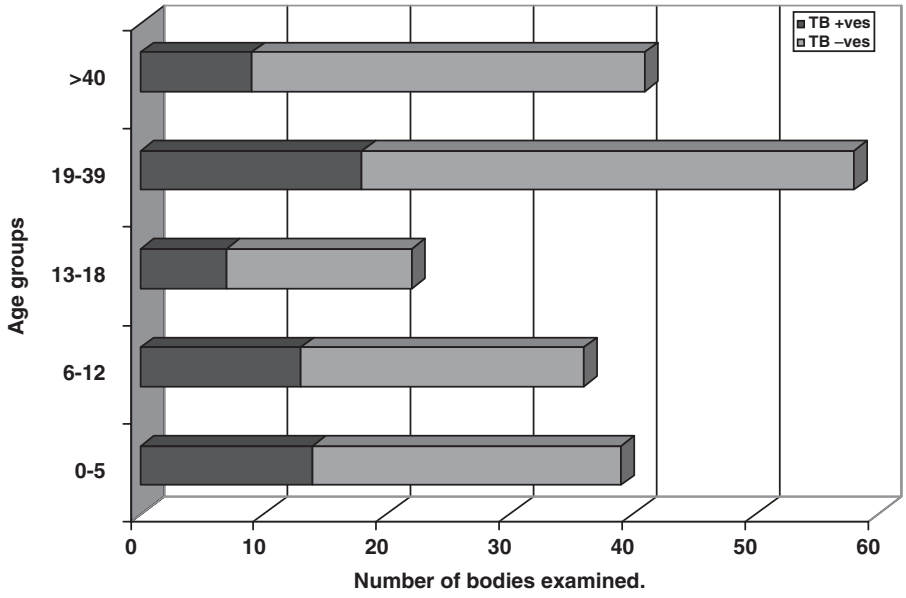


Fig. 6.3 Distribution of MTB complex DNA by age at death in Kulubnarti, early Christian Nubia. Individuals show a lower level of infection compared with eighteenth century Hungary, with highest levels in adults aged 19–39 years, and young children

in the population (Fletcher et al. 2003a), with almost every individual in some age groups infected (Fig. 6.2). Because of the availability of mummified lung tissue, it was possible to distinguish between individuals who probably died from the disease, from those who were infected but lived to a great age. Examination of tuberculosis infection with year of birth indicated that the highest rates of infection occurred during a period of planned expansion of the town with net immigration.

Another large on-going study is based on several hundred early Christian partially mummified remains from two sites in Kulubnarti, Nubia (van Gerven et al. 1981). These have been thoroughly studied by anthropologists, so there is excellent information on their age, sex and nutritional status. Tuberculosis was widespread although at a lower level than in eighteenth century Hungary (Fig. 6.3). Individuals died at a much younger age and there appeared to be more tuberculosis infection in young adults and children under 5 years of age, but the data have not yet been fully published (Spigelman et al. 2005).

6.6 Molecular Characterisation of Ancient MTB complex aDNA

6.6.1 Detection of Deletions and Spoligotypes

In an effort to characterise the strains of MTB detected by PCR, many genetic loci have been examined in addition to IS*6110*, although none are as sensitive. It is inevitable that single copy genetic loci will give less consistent results, as they will be more prone to the impact of poor DNA preservation. However, Taylor et al. (1999) attempted to determine whether *M. tuberculosis* or *M. bovis* was present in three mediaeval skeletons from the London Royal Mint site. They used primers for mtp40, a region found in most *M. tuberculosis* isolates, and obtained positive results. In addition, a locus in the *oxyR* pseudogene was amplified and sequenced, revealing a guanine residue at position 285, typical of *M. tuberculosis* but not of *M. bovis* (Sreevatsan et al. 1996). Finally, spoligotyping was performed, and showed a pattern consistent with *M. tuberculosis*. Subsequent work on material from a deserted Mediaeval village site in Yorkshire (UK) included PCR based on regions known to be deleted (DR regions) in different members of the MTB complex. In addition, genetic loci related to virulence, such as *rpoB* and *pncA*, associated with susceptibility or resistance to rifampicin and pyrazinamide, respectively, were explored for their use in characterising MTB aDNA (Mays et al. 2001; Taylor et al. 2001). This biomolecular approach, expanded by the inclusion of flanking and internal primers for the TbD1 deletion (Brosch et al. 2002), successfully identified the organisms isolated by Robert Koch and stored in a museum display case, to be of a ‘modern’ evolutionary lineage of *M. tuberculosis* (Taylor et al. 2003).

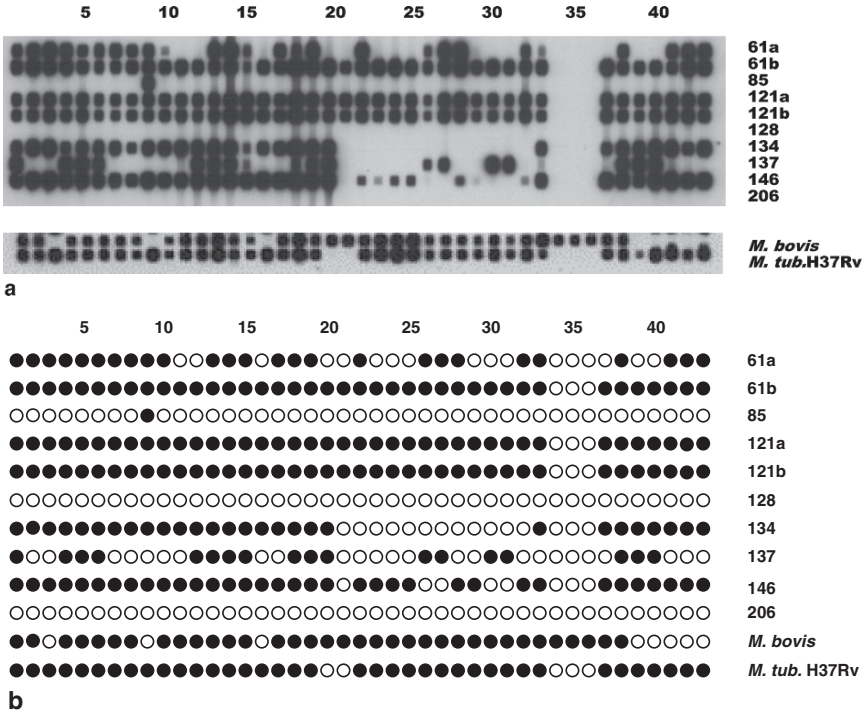


Fig. 6.4 Spoligotypes of MTB complex aDNA from different individuals in Vác, Hungary, in comparison with *M. tuberculosis* H37Rv and *Mycobacterium bovis*. The data are consistent with all individuals being infected with *M. tuberculosis* of 'modern' lineage

This biomolecular approach was adopted by Fletcher et al. (2003a, 2003b) in their study of the Vác mummies (Fig. 6.4). Both genotyping and spoligotyping were carried out, and the infecting organisms were shown to be 'modern' strains of *M. tuberculosis,* of principal genomic groups 2 and 3. Interestingly, three members of a family group apparently were infected with different strains. This study also clearly demonstrated the inverse relationship between the number of amplified copies per microlitre and increasing PCR target size, caused by fragmentation of aDNA. The nature of the tuberculosis in 85 Egyptian mummies was also investigated by the use of additional PCR target regions and spoligotyping (Zink et al. 2003b). Spoligotyping gave the most clear-cut results, and in 12 cases indicated *M. tuberculosis* or *M. africanum* patterns, but clearly not *M. bovis*.

The use of spoligotypes in MTB complex aDNA studies has initiated a discussion on whether it is appropriate to use 'consensus' spoligotypes, where positive results from repeated blots are amalgamated. As the individual spacer regions are single-copy oligonucleotides, palaeomicrobiologists tend to use consensus patterns, although this is viewed with suspicion by those responsible for maintaining the international spoligotyping database.

6.6.2 *The Search for* M. bovis *DNA in Archaeological Material*

M. bovis infects many host species, so both wild and domesticated animals can act as reservoirs of infection. In one of the very few studies on animal material, IS*6110* PCR and spoligotyping were used to examine a sample from a Pleistocene bison with an erosive lesion from the Wyoming Natural Trap Cave (Rothschild et al. 2001). However, the spoligotypes failed to match any in the database. Statistical analysis of the data indicated that the patterns had the closest match to *M. africanum*, although more recent analysis suggests ancestral *M. tuberculosis* (Huard et al. 2006). There was no indication of *M. bovis*. This is consistent with our current understanding of the evolution of the MTB complex, as we now know that *M. tuberculosis* is the more ancestral lineage (Brosch et al. 2002).

PCR based on IS*1081* is a better means of identifying *M. bovis*, as there are six copies per cell. Although earlier attempts to detect *M. bovis* in British Iron Age material (2,200 years BP) by this means were unsuccessful (Taylor et al. 2005), it has been found recently in a group of Siberian Iron Age semi-nomadic pastoralists (Taylor et al. 2007). However, its scarcity reflects the low levels of *M. bovis* infection reported in the absence of effective control measures today, of around 6% of cases (Grange 1995), and suggests that a long-term close association with an infected herd is the most likely scenario in which to detect this organism.

6.6.3 *Genotypes and Genetic Lineages*

It is clear that 'modern strains' of *M. tuberculosis*, defined as lineages with the TbD1 deletion, occurred in ancient Egypt, alongside more ancestral strains (Zink et al. 2003, 2005b). So far, mainly European and Near-Eastern archaeological samples have been subjected to further molecular analysis, and it would be of great interest to determine the nature of the indigenous tuberculosis found in archaeological material from pre-Colombian America, Africa, the Indian subcontinent and the Far East.

Further whole genome sequencing of modern clinical isolates has resulted in large international databases of the molecular characteristics of MTB strains, based on numbers of repetitive sequences, spoligotypes and SNPs. Recent meta-analyses of these databases has led to distinct lineages of *M. tuberculosis* strains being recognised, which are associated with different geographical regions and human populations (Baker et al. 2004; Hirsh et al. 2004; Gagneax et al. 2006), possibly contemporaneous with early hominids in Africa (Gutierrez et al. 2005).

6.6.4 *Tuberculosis and Ancestral Sequence Inference*

Our knowledge of the stability of molecular typing methods and their rate of change makes it possible to estimate the rate of evolutionary change under different

scenarios. Meta-analyses of modern molecular data support the hypothesis that the other MTB complex species are clonally derived from an "*M. canettii*"-like organism, so this may offer a good genomic reference point to investigate how genes have evolved to greater virulence in *M. tuberculosis*. Analysis of one *M. tuberculosis* genotype (Beijing) has led to the hypothesis that it may have originated in central Asia in humans migrating from the Middle East during a second out-of-Africa migration in the Upper Palaeolithic 45,000–30,000 years ago (Mokrousov et al. 2005). In the future it may be possible to obtain direct evidence of MTB aDNA dating from these remote times, in order to explore the co-evolution of host and pathogen.

6.7 Interactions of *Mycobacterium tuberculosis*

6.7.1 Co-infections

It is important to appreciate that the relationship between human hosts and their microbial pathogens is dynamic and although in the case of tuberculosis the disease may remain latent for most of a lifetime, perturbations in the host cell-mediated immune response can lead to a re-activation of disease. Intestinal parasites, such as worms, have a profound effect upon host immunity, which can result in atopy rather than a cell-mediated protective response (Elston 2006). Other co-infections can also bring this about, and there is limited evidence in the archaeological record of individuals infected with both MTB and *Mycobacterium leprae* (Donoghue et al 2005), or *Leishmania* (Spigelman et al. 2005: Zink et al. 2006). The liver from a Korean 'wet' mummy (Kim et al. 2006) has been diagnosed with DNA from both MTB and hepatitis B virus (Donoghue et al. 2007).

6.7.2 Lowered Host Resistance or Increased Susceptibility

Host-related factors that can exacerbate the impact of MTB infection are extremes of age, nutritional stress, and neoplasms. Both the Vác and Nubian studies have shown a pronounced effect of age in relation to disease. The population at Kulubnarti in early Christian Nubia had a remarkably high incidence of the stress indicator *criba orbitaria*, which may be related to the high rates of infection and early deaths in the older settlement. One cause of *criba orbitaria* is iron deficiency anaemia, and in severe cases this enhances the virulence of MTB infection (Ratledge 2004). It has been suggested that the iron status within pre-Columbian populations in the Americas may have had a profound impact on the clinical presentation of the disease (Wilbur and Buikstra 2006). Work is just starting on the impact of neoplastic disease, but tuberculosis infection has been detected in an

infant with Langerhans cell histiocytosis from the Vác mummy study group (Spigelman et al. 2006).

It is now becoming increasingly recognised that the genetic variability of *M. tuberculosis* strains has an impact on the clinical presentation of disease (Malik and Godfrey-Fausset 2005). A small proportion of strains currently cause a disproportionate number of cases of tuberculosis. Similarly, there is substantial evidence for the role of genetic factors in the susceptibility of humans to mycobacterial disease (Fernando and Britton 2006).

6.8 Conclusions

The palaeomicrobiology of tuberculosis has been illuminating to archaeologists, palaeopathologists, molecular epidemiologists and experts in microbial genomics alike. For the future, palaeomicrobiology offers us an exciting prospect of exploring the relationship between the microbial pathogen *Mycobacterium tuberculosis*, and its human host. This may enable us to examine directly different MTB strains and human genotypes, from a time before the selection pressure created by the global epidemic associated with the Industrial Revolution in the Western World.

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