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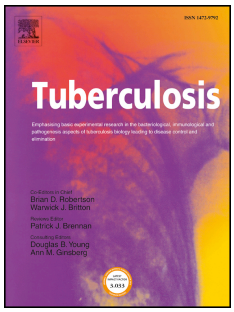

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Ancient DNA analysis – an established technique in charting the evolution of tuberculosis and leprosy

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Summary

Many tuberculosis and leprosy infections are latent or paucibacillary, suggesting a long time-scale for host and pathogen co-existence. Palaeopathology enables recognition of archaeological cases and PCR detects pathogen ancient DNA (aDNA). *Mycobacterium tuberculosis* and *Mycobacterium leprae* cell wall lipids are more stable than aDNA and restrict permeability, thereby possibly aiding long-term persistence of pathogen aDNA. Amplification of aDNA, using specific PCR primers designed for short fragments and linked to fluorescent probes, gives good results, especially when designed to target multi-copy loci. Such studies have confirmed tuberculosis and leprosy, including co-infections. Many tuberculosis cases have non-specific or no visible skeletal pathology, consistent with the natural history of this disease. *M. tuberculosis* and *M. leprae* are obligate parasites, closely associated with their human host following recent clonal distribution. Therefore genotyping based on single nucleotide polymorphisms (SNPs) can indicate their origins, spread and phylogeny. Knowledge of extant genetic lineages at particular times in past human populations can be obtained from well-preserved specimens where molecular typing is possible, using deletion analysis, microsatellite analysis and whole genome sequencing. Such studies have identified non-bovine tuberculosis from a Pleistocene bison from 17,500 years BP, human tuberculosis from 9000 years ago and leprosy from over 2000 years ago.

Key words: Ancient DNA; evolution; *Mycobacterium leprae*; *Mycobacterium tuberculosis*; molecular typing

1. Introduction

According to the World Health Organisation,[1] one third of the global human population is infected with tuberculosis (TB) but most are latent infections. In people with no underlying risk factors, approximately 10% will develop an active infection during their lifetime.[1] However, underlying deficiencies in immunity caused by co-infections such as HIV, or co-morbidities such as cancer, greatly increase the chance of active infection. This high level of latent infection suggests a period of close co-evolution of *Mycobacterium tuberculosis* and its human host.[2] Phylogenetics indicate that the *Mycobacterium tuberculosis* complex (MTBC) emerged via an evolutionary bottleneck and that existing lineages have emerged after a succession of unidirectional deletion events.[3] *M. tuberculosis* is an obligate pathogen and has no environmental reservoir. There appears to be an association between *M. tuberculosis* lineages with different human populations around the globe and this persists within modern cities with a population of diverse origins.[4] An association has been found between population density and the emergence of human infectious diseases.[5] This association is apparent in the early Neolithic period of human development and in the case of TB appears to be continuing with the emergence of highly transmissible and virulent strains of *M. tuberculosis* in major cities that have a long record of continuous habitation.[6]

Leprosy is a chronic human infection caused by *Mycobacterium leprae*. This has declined in recent years but caused approximately 219,000 new cases in 2011,[7] mainly in South East Asia, Africa and South America. It is a major cause of preventable disability and of social exclusion due to stigma. *M. leprae* is extremely slow growing and requires to be in an intracellular environment within a host, primarily human. *M. leprae* also appears to have experienced an evolutionary bottleneck and subsequent clonal expansion between pathogen and host.[8] Different strains of *M. leprae* can be distinguished by variable number tandem repeat (VNTR) and short tandem repeat typing. These can indicate short-term transmission via microsatellite analysis but are unstable due to poor DNA repair by *M. leprae*.[9] Stable long-term changes can be monitored by synonymous single nucleotide polymorphisms (SNPs) and these have identified lineages that are also associated with different human populations.[10]

Much can be inferred by the study of modern isolates of both *M. tuberculosis* and *M. leprae*. However, the direct study of ancient specific biomarkers for these pathogens, such as ancient DNA (aDNA) and cell wall lipid biomarkers, has distinct advantages.[11] These biomarkers enable confirmation of infection in skeletal or soft tissue remains with non-specific or no palaeopathology, as only about 5% of TB cases are believed to result in bony changes. Ancient biomarkers may also answer

historical questions, such as the nature of pre-Columbian TB or the role played by the slave trade across the Atlantic in the dispersal of TB and leprosy to the Americas. Mixed infections can also be detected. Analysis of aDNA may permit the determination of genetic lineages, genotypes or sub-genotypes in specimens of known age, thus providing real time calibration of the date of their emergence. The association of ancient pathogen genotypes with different host populations may also pinpoint and date human migrations.[10,12]

A useful approach in palaeomicrobiology is to obtain independent verification of findings by seeking different specific biomarkers in individual specimens. Our group has concentrated on initial examination for aDNA and subsequent independent analysis of mycobacterial specific cell wall lipids.[13] Until recently, our aDNA data have been obtained by DNA amplification using PCR, first conventional single-stage or nested PCR, and more recently using real-time PCR with specific fluorescent probes and primers for selected target regions. As aDNA is often highly fragmented, the use of specific probes has been very productive as they enable specific detection of selected target regions of as little as 60–80 base pairs (bp). The development of Next Generation Sequencing (NGS) and sophisticated bioinformatic analysis has enabled sequencing and analysis of non-amplified DNA using targeted enrichment approaches.[14,15] Rarely, in exceptionally well-preserved material, it is possible to perform shot-gun sequencing without target enrichment and to obtain an analysis of the entire DNA within a sample. This has been achieved for *M. leprae* found in mediaeval dental pulp[15] and in extremely well preserved lung tissue from a naturally mummified individual from 18th century Vác, Hungary.[16]

2. Ancient DNA methodology

2.1 Extraction of M. tuberculosis *and* M. leprae *aDNA*

The following protocol gives sufficient time for samples to be disaggregated, but includes vigorous bead beading and snap freezing in dry ice to release aDNA from association with any residual lipids from the lipid-rich mycobacterial cell wall. Small samples (bone scrapings 20–80 mg; mummified tissue10– 40 mg) are taken from human remains, according to recommended protocols for aDNA with separate rooms and equipment for different stages of the process.[17] Skeletal material is crushed in a sterile pestle and mortar and samples are added to 400 µl of Proteinase K/EDTA. The slurry is incubated at 56^{o}C and mixed on a bead beater daily until solubilised. An aliquot is treated with 40 µl of 0.1 mol^{-1} of *N*-phenacylthiozolium bromide (PTB), to cleave any covalent cross-links thus enabling

DNA strand separation and amplification.[17] As PTB is inhibitory in the PCR reaction, an aliquot without PTB is processed in parallel, so that short DNA fragments can be precipitated from PTB-free silica supernatants (see below). Sample tube contents are transferred into lysis buffer containing 5 mol^{-1} guanidium thiocyanate and incubated for 1–3 days at 56^{o}C. To complete the disruption of bone and any mycobacterial remnants, samples are boiled, then snap-frozen in liquid nitrogen and thawed in a 65^{o}C water bath. This procedure is repeated twice. Sample tubes are centrifuged at 5000g for 15 mins at 5^{o}C and the supernates carefully removed into clean, sterile tubes. DNA is captured with silica suspension (NucliSens®) and mixed on a rotator wheel for 1 hour. Tube contents are centrifuged and silica pellets washed once with wash buffer (NucliSens®), twice with 70% (v/v) ethanol (-20 ^{o}C) and once with acetone (-20 ^{o}C). After drying in a heating block, DNA is eluted using 60µl elution buffer (NucliSens®), aliquoted and used immediately or stored at -20^{o}C. Silica supernates (500 µl) from PTB-negative samples are also taken from the lysis buffer, and 2.0 ml screw-capped Eppendorf tubes used to wash the silica. After chilling at 5^{o}C, supernates are mixed vigorously for 20 sec with 200 µl of Protein Precipitation Solution (SLS Ltd, UK) and centrifuged for 3 min at 10,000 g. Any pellet is discarded and 600 µl isopropanol (-20^{o}C) added to 550 µl of each supernate. Tubes are mixed by inversion 50 times and spun 3 min. Supernates are discarded and tubes washed once with 500 µl 70% ethanol (-20^{o}C). After draining, tubes are dried in a heating block. Any precipitated DNA is re-hydrated with 60µl elution buffer (NucliSens®), aliquoted and used immediately or stored at -20^{o}C. Negative extraction controls are processed in parallel with the test samples.

2.2 DNA amplification and detection

In the current protocol, two specific regions of each organism are targeted, using repetitive elements to increase the likelihood of detection of pathogen aDNA. For the *M. tuberculosis* complex, IS*6110* (1–25 copies/cell) and IS*1081* (6 copies/cell) are used.[18] For *M. leprae*, RLEP (37 copies/cell) and REPLEP (15 copies/cell) are used.[19] Initially, conventional PCR was used, with primers targeting DNA regions of around 90-bp to 123-bp. PCR was performed in two stages, with 45 rounds of amplification followed, if necessary, by a nested reaction using internal primers, with a further 25 cycles of amplification. PCR products were detected by agarose gel amplification, gel slices were removed, the PCR products purified and sequenced. As aDNA is highly fragmented specific primers and fluorescent probes have since been designed to enable shorter DNA fragments to be specifically detected (Table 1). The Qiagen QuantiTect® Probe reaction mix is used with additional 2 mM BSA to reduce PCR inhibition and additional 2.0 mM $MgCl_2$ to facilitate primer binding. A hot-start *Taq* polymerase is used to minimise non-specific primer and template binding. Negative DNA extraction and PCR

controls are processed alongside the test sample. Amplification is performed in a final volume of 25 µl using the Qiagen RotorGene® real-time platform. After enzyme activation for 15 min at 95°C, amplification consists of 50-55 cycles of strand separation at 94°C for 10 sec, primer binding at 60°C for 20 sec and strand extension at 72°C for 10 sec. The probes enable direct observation of specific amplicons and the determination of cycle threshold (Ct) indicates relative concentration of template. Findings may be confirmed by sequencing. Analysis of cell wall lipid biomarkers, based on the direct detection of cell wall components without any amplification of the signal, enables independent verification of the presence of the target pathogen in the sample.[13,20]

3. Significant questions answered by aDNA studies

3.1 The M. tuberculosis *lineages prevalent in early human history*

TB is spread by infectious aerosols from an infected person, which results in lung infections, although bacteria may spread via the bloodstream to all other parts of the body. Infection of the vertebrae results in pathology typical of TB, such as Pott's disease, that was recognized in ancient Egypt and early Neolithic communities. However, in the majority of cases there is non-specific palaeopathology or none at all, so the extent of past TB infections was greatly underestimated.[11] However, it was noted that there was an increase in TB in the Neolithic compared with hunter-gatherers and this appeared to be associated with animal domestication. This led initially to an assumption that human TB was derived from animals and that *Mycobacterium bovis*, the principal cause of TB in domesticated animals, was the ancestor of *M. tuberculosis*. This was disproved once the phylogenomics of the MTBC was determined,[2] that demonstrated *M. tuberculosis* was more ancestral than *M. bovis*. The earliest demonstration of the MTBC, in Pleistocene bison, suggested that the pathogen resembled *M. tuberculosis* or *M. africanum*,[21] but at that time the significance of the smooth colony "*Mycobacterium canettii"* group was not realized. This very diverse group is believed to be most similar to the original ancestor of the MTBC and demonstrates greater variability than any other member of the MTBC.[2,22]

M. tuberculosis aDNA from ancient Egypt that had not experienced the TbD1 deletion has been reported from Ancient Egypt.[23] Such strains are still extant in the Far East around the Pacific Rim and are believed to be the oldest *M. tuberculosis* lineage. However, the majority of modern *M. tuberculosis* strains are TbD1-deleted and this lineage was demonstrated 9000 years ago in the pre-pottery Neolithic site of Atlit-Yam, in the Eastern Mediterranean.[24] *M. africanum* was found in Middle Kingdom ancient

Egypt[23] but *M. bovis* is very rare in the archaeological record. It was found in a group of Iron Age Siberian pastoralists (4th century BC – 4th century AD) who over-wintered in huts with their animals.[25]

The interest in *M. tuberculosis* lineages has been driven partly because of the realization that the clinical presentation of TB results from a combination of factors related to host susceptibility and *M. tuberculosis* virulence.[2] This can be studied directly in past populations, allowing the effects of industrialization, population density and large population movements to be examined.[3,11]

3.2 The nature of past M. leprae *infections*

Leprosy was recognized in antiquity by the characteristic clinical symptoms. *M. leprae* targets nerves and the bacteria invade the Schwann cells that are essential for the transmission of nerve impulses. The clinical presentation depends upon the host immune reactions. A predominant humeral response leads to multibacillary, or lepromatous leprosy, where there is a strong antibody response that is useless. This is the form of leprosy that results in gross changes to the nasopharyngeal region, the hands and feet, with destruction of tissue and gross deformity. However, in the presence of an effective cell-mediate host response, symptoms are minor although late autoimmune reactions can lead to destruction of nerve function and disability. The disease has been described in ancient China, Egypt and India[7] although it is sometimes difficult to distinguish between leprosy and other diseases with similar external symptoms. In archaeological cases, it is lepromatous leprosy that is recognized. Leprosy occurred during the Roman empire[10] and was spread by traders and invading armies.

A phylogenetic study of global *M. leprae*, that included both modern and aDNA, demonstrated a clear link between global populations and the *M. leprae* genotype and subgenotypes, as determined by SNPs.[10,19] It appears that SNP type 2 strains are associated with early strains that migrated from the Middle East to South East Asia but recent work shows they also spread westwards to northern and western Europe.[12] SNP 3 strains are found in North Africa and the Eastern Mediterranean and were very common in Mediaeval Europe. Different migratory routes were suggested for the spread of *M. leprae* from the proposed source near the Horn of Africa – a land route from the Mediterranean east to central China, and sea routes via India and South East Asia. The genotypes found today in these regions support this theory of dispersal.[26]

3.3 Past co-infections

The ability of aDNA studies to detect and characterize mixed infections had not been an original aim and it was by chance that co-infections of *M. tuberculosis* and *M. leprae* were detected.[27] Using conventional PCR, a decision has to be made on which organisms to target in a sample and choice of

primers is made accordingly. Mixed infections were discovered when mediaeval leprosy samples were examined for evidence of *M. tuberculosis* after contemporary co-infections were reported. This has led to an on-going debate on the possible sequence of events in the decline of leprosy in late Mediaeval Europe and whether TB had any role in bringing this about.

Thereafter, other examples of co-infections were sought. Another disease known in antiquity was malaria, in particular that caused by *Plasmodium falciparum*. Co-infection of *M. tuberculosis* and parasites is an important public health problem in co-endemic areas of the world today, 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 *P. falciparum*.[28] Intestinal and systemic parasites were widespread in the past, also was the carriage of ectoparasites. In addition, it is highly likely that future whole genome studies will identify multiple bacterial and viral infections within individual human archaeological remains, in addition to associations of infection with co-morbidities such as host immune or genetic disorders and cancer. Such complex scenarios form the backdrop to the emergence of modern pathogens and we should endeavour to increase our understanding of the factors involved.

Whole genome sequencing makes it feasible to detect different strains of the same pathogen within a host. This scenario was demonstrated in 18th century naturally mummified lung, where two *M. tuberculosis* strains were detected, apparently one more ancestral than the other and both resembling a modern outbreak strain, which is closely related to modern Haarlem and Erdman strains.[16] This may be relevant as a recent study of current mixed strain infections[29] found that the Haarlem and Beijing genotypes are more likely to occur in a mixed infection than any of the other genotypes tested suggesting pathogen-pathogen compatibility. There is evidence for intra-strain gene flux in *M. tuberculosis*[22] and this is likely to be significant in the emergence of modern *M. tuberculosis* strains that are rapidly diversifying, due to mutation, recombination and natural selection.[30]

4. Conclusions

During the past twenty years, since study of the palaeomicrobiology of human infectious diseases became feasible, the nature of the research questions addressed has broadened in scope and become far more sophisticated. Early palaeopathologists wished for validation of their diagnoses that were based on skeletal morphological changes. However, it soon became clear that the scale of past TB infections was far greater than previously envisaged, as many infections do not have skeletal involvement.

Palaeomicrobiology has been used to answer historical questions, such as whether the European colonialists brought TB to South America. Indeed they did, but pre-Columbian TB existed and its nature is still the subject of study. Population studies of past TB and leprosy enable epidemiological studies from the pre-antibiotic era. Information on living and burial conditions can highlight whether there was social stigma or whether infected individuals were integrated into their society.

As *M. tuberculosis* and *M. leprae* are obligate pathogens, their geographical distribution illustrates past human migrations or dispersal around the globe. Recent developments in genomics have increased our understanding of modern strains of *M. tuberculosis* and *M. leprae*, thereby providing comparators for pathogen aDNA. It is now appreciated that palaeomicrobiology enables direct comparison of ancient and modern lineages. One of the greatest benefits is that palaeomicrobiology enables direct calibration of the timescale over which changes have occurred, in the absence of modern evolutionary pressures caused by antimicrobial therapy and mass transport around the globe.

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Ethical approval

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Author contributions

HD and MS conceived the original aDNA studies and DM and GB the lipid work. MS, IP and IS collected or supplied specimens. HD performed experiments and analyzed aDNA data. JO'G designed the PCR probes and primers. OL and HW performed lipid experiments. DM, GB, OL and HW analyzed data. HD wrote the manuscript and all authors approved the final version.

Competing interests

None declared.

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367 **Table 1**

368 PCR primers and probes to detect *M. tuberculosis* (IS*6110*, IS*1081*) and *M. leprae* (RLEP, RepLep)

PCR locus	Primer name	Primer sequence (5'–3')	Amplicon size (bp)
IS*6110*	6110 Probe	5'-FAM-ACCTCACCTATGTGTCGACCTG-BHQ1-3'	
IS*6110*	6110F	CACCTAACCGGCTGTGG	
IS*6110*	6110R	TGACAAAGGCCACGTAGG	75
IS*1081*	1081 Probe	5'-FAM-GGGCTACCGCGAACGCA-BHQ1-3'	
IS*1081*	NF	TGATTGGACCGCTCATCG	
IS*1081*	NR	CTTGATGGGGGCTGAAGC	72
RLEP	RLEP Probe	FAM-5'- CTCAGCCAGCAAGCAGGCAT-3'-BHQ2	
RLEP	RLEPF	CGCTGGTATCGGTGTCG	
RLEP	RLEPR	ACACGATACTGCTGCACC	80
REPLEP	REPLEP Probe	5'-FAM-CATGTCTATCTCCGTACGCAGCTG-BHQ1-3'	
REPLEP	REPLEPF	GACTGTACTTCTTGGCCAGC	
REPLEP	REPLEPR	GCAAGGTGAGCGTTGTGG	66

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