Tuberculosis 95 (2015) S35–S41

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

Morphological and biomolecular evidence for tuberculosis in 8th century AD skeletons from Bélmegyer-Csömöki domb, Hungary

Erika Molnár [a, *], Helen D. Donoghue [b], Oona Y.-C. Lee [c], Houdini H.T. Wu [c], Gurdyal S. Besra [c], David E. Minnikin [c], Ian D. Bull [d], Gareth Llewellyn [e], Christopher M. Williams [e], Olga Spekker [a], György Pálfi [a]

[a] Department of Biological Anthropology, University of Szeged, Szeged, Hungary
[b] Centre for Clinical Microbiology and Centre for the History of Medicine, University College London, London, UK
[c] Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK
[d] Organic Geochemistry Unit, School of Chemistry, University of Bristol, Bristol, UK
[e] National Mass Spectrometry Service Centre, School of Medicine, Grove Building, Swansea University, Swansea, UK

Keywords:
Ancient DNA
Lipid biomarkers
Mycobacterium tuberculosis complex
Palaeopathology
PCR

SUMMARY

Macromorphological analysis of skeletons, from 20 selected graves of the 8th century AD Bélmegyer-Csömöki domb, revealed 19 cases of possible skeletal tuberculosis. Biomolecular analyses provided general support for such diagnoses, including the individual without pathology, but the data did not show coherent consistency over the range of biomarkers examined. Amplification of ancient DNA fragments found evidence for the *Mycobacterium tuberculosis* complex DNA only in five graves. In contrast, varying degrees of lipid biomarker presence were recorded in all except two of the skeletons, though most lipid components appeared to be somewhat degraded. Mycobacterial mycolic acid biomarkers were absent in five cases, but the weak, possibly degraded profiles for the remainder were smaller and inconclusive for either tuberculosis or leprosy. The most positive lipid biomarker evidence for tuberculosis was provided by mycolipenic acid, with 13 clear cases, supported by five distinct possible cases. Combinations of mycocerosic acids were present in all but three graves, but in one case a tuberculosis-leprosy co-infection was indicated. In two specimens with pathology, no lipid biomarker evidence was recorded, but one of these specimens provided *M. tuberculosis* complex DNA fragments.

1. Introduction

The macromorphological diagnosis of skeletal tuberculosis (TB) in human remains is based upon the detection of secondary skeletal lesions [1]. The most common representation of skeletal TB is *spondylitis tuberculosa*, which affects the vertebral column. After vertebral involvement, the second most frequent alteration in TB is arthritis of the large, weight-bearing joints [2]. Initially, the diagnosis of TB in osteoarchaeological samples focused only on these classical TB lesions, representing a fairly developed stage of tuberculosis. However, TB may have affected many individuals without classical pathological changes, thus patients died in an earlier stage of tuberculosis long before these symptoms could have developed. Clearly, this early-stage TB is not recognizable on the basis of classical TB alterations, so if we consider only individuals with visible TB-related lesions, it is likely this will significantly underestimate the prevalence of tuberculosis in the examined historical populations [1,3].

Because of the problems of tuberculosis diagnostics, the importance of establishing diagnostic criteria for early-stage TB became recognized in the late 20th century. A number of studies – mainly based on the examination of skeletal collections with known causes of death – have focused on searching for atypical or early-stage lesions in connection with tuberculosis infection. These investigations enabled the recognition of three types of atypical or early-stage TB alterations: rib lesions, superficial vertebral changes including hypervascularisation, and endocranial alterations [3–7].

* Corresponding author. H-6724, Szeged, Közép fasor 52. Tel./fax: +36 62 544 314.
E-mail addresses: balinte@bio.u-szeged.hu (E. Molnár), h.donoghue@ucl.ac.uk (H.D. Donoghue), o.y.lee@bham.ac.uk (O.Y.-C. Lee), h.wu.2@bham.ac.uk (H.H.T. Wu), g.besra@bham.ac.uk (G.S. Besra), d.e.minnikin@bham.ac.uk (D.E. Minnikin), ian.d.bull@bristol.ac.uk (I.D. Bull), g.llewellyn@swansea.ac.uk (G. Llewellyn), christopher.matthew.williams@swansea.ac.uk (C.M. Williams), olga.spekker@gmail.com (O. Spekker), palfigy@bio.u-szeged.hu (G. Pálfi).

http://dx.doi.org/10.1016/j.tube.2015.02.032
1472-9792/

Positive correlations between tuberculosis and stress indicators, such as long bone periostitis, *cribra orbitalia* and *cribra cranii*, were also recognized [7,8]. Since the 1990s, the identification of skeletal tuberculosis in ancient human remains has been facilitated by the confirmation of atypical or early-stage TB lesions by new biomolecular methods based on mycobacterial ancient DNA (aDNA) and lipid biomarker analyses [1,9–12].

In 1990, the first paleopathological analyses of the 8th century AD series Bélmegyer-Csömöki domb were essentially based on macromorphological and radiological examinations, biomolecular methods were used only in a few cases. From a macromorphological point of view, those investigations only considered classical TB alterations [9,13–15]. An advanced-age female skeleton from the grave No. 65 showed severe osteolytic lesions of the anterior portion of the thoracic and lumbar vertebral bodies, causing an unequal collapse, which led to angular kyphosis (Suppl. Fig. S1 a–b) [14]. Mycobacterial DNA targets IS*6110* and the 65-kDa antigen gene, of the *Mycobacterium tuberculosis* complex (MTBC), were found in samples from this specimen [9]. In another case, of a mature male individual (grave No. 90), the pathological remodelling and fusion of the lumbosacral region and the irregular *ante-mortem* erosion on the ventral surface of the sacrum, support the diagnosis of a lumbo-sacral tuberculous involvement with cold abscess. In addition, the severe destruction both of the left hip bone and proximal femur is suggestive of tuberculous arthritis or *coxitis tuberculosa* (Suppl. Fig. S1 c–d) [15]. The diagnosis of skeletal TB was confirmed by biomolecular results, the identification of the DNA-fragment (65-kDa antigen gene) of the MTBC was successful [9]. In a further case, the complete ankylosis of the right knee indicated *gonitis tuberculosa* of an elderly male individual from grave No. 215 [13].

Marcsik and co-workers published two further classical TB cases in 2007 [16]. A young female skeleton from grave No. 38 exhibited signs of probable tuberculous arthritis (*coxitis tuberculosa*) of the right hip joint. Skeletal remains of an adult male individual (grave No. 189) presented complete ankylosis of the 9th and 10th thoracic vertebrae and fusion of the 1st and 2nd and the 3rd and 4th lumbar vertebrae. In addition, new bone formation and osteophytes were found on the ventral surfaces of all lumbar vertebral bodies. These alterations suggest the diagnosis of *spondylitis tuberculosa* [16].

The above mentioned former investigations of the series from the Bélmegyer-Csömöki domb have provided interesting paleopathological cases of skeletal tuberculosis. However, the complete skeletal material has never been analysed systematically for both classical and early-stage TB lesions, and biomolecular analyses had been undertaken only in a few cases. The recent development of diagnostic criteria in the field of paleopathology of TB and biomolecular methods for detection of the MTBC encouraged us to perform a re-examination of the series from 2009. The aim of this study is to summarize the results of this re-examination.

2. Material and methods

2.1. Archaeological background

The skeletal material for this study derives from the archaeological site of the Bélmegyer-Csömöki domb, rising about three kilometres south-east of the village Bélmegyer, in South-Eastern Hungary. During a long-running excavation (1985–1989), skeletal remains of 240 individuals were unearthed. On the basis of the grave goods found in the completely excavated cemetery, it was used for burials between 670 and 800 AD during the late Avar Period [17,18].

Our research strategy was to combine different diagnostic methods in order to get independent verification using different biomarkers. First we conducted the morphological analysis of the skeletal series. Next, bone samples were taken from the skeletal remains of the suspected TB cases. Small pieces from the same rib were selected and sent to separate centres for the aDNA and lipid biomarker analyses.

2.2. Macromorphological analysis

The paleopathological examination of the mostly well-preserved skeletal remains of the 240 individuals (95 males, 72 females, 73 undeterminable) was carried out in the Department of Biological Anthropology, University of Szeged, Hungary. These investigations were performed using macromorphological methods, focussing on previously detailed classical [2] and atypical TB alterations [3–7].

2.3. Mycobacterial aDNA analysis

2.3.1. Mycobacterial DNA extraction

Possible cases of skeletal TB, defined according to skeletal morphological alterations, were examined for the presence of aDNA from the *M. tuberculosis* complex (MTBC). Recommended protocols for aDNA work were followed [19] with separate rooms and equipment for different stages of the process. Well-established methods were employed for aDNA extraction and amplification [20–27] as detailed in Donoghue et al. in this volume [28] and in Supplementary data. The approach used was of a slow but thorough period of sample disruption, one aliquot treated with N-phenacylthiozolium bromide (PTB), to cleave any covalent cross-links thus facilitating DNA strand separation and amplification [21]. Subsequently, samples were treated with guanidium thiocyanate, followed by sample and bacterial cell disruption, using boiling and snap-freezing in liquid nitrogen. All fractions of the sample were used in the extraction. DNA was captured with silica and the pellets washed and dried [28]. Silica supernates from PTB-negative samples were also processed by removal of protein followed by DNA precipitation with isopropanol (−20 °C) [28]. Dried samples were re-hydrated with elution buffer and used immediately or stored at −20 °C. Negative extraction controls were processed in parallel with the test samples.

2.3.2. DNA amplification and detection

Two specific regions of the *M. tuberculosis* complex were targeted – the repetitive elements IS*6110* (1–25 copies/cell) and IS*1081* (6 copies/cell). The IS*6110* primers used for conventional PCR had a target region of 123 bp [22] and the IS*1081* primers produce an amplicon of 113 bp [23]. Later, specific *M. tuberculosis* primers and a fluorescent probe were used [24] to enable shorter DNA fragments to be detected in a real-time PCR reaction (Supplementary data).

2.3.3. The PCR conditions

The PCR mix included 2 mM bovine serum albumin to reduce PCR inhibition [25] and 2.0 mM $MgCl_2$. PCR assays were initially run at an annealing temperature of 58 °C and amplified DNA was examined by agarose gel electrophoresis [26]. Subsequently, amplification was performed in a final volume of 25 μl using a RotorGene© 3000 (Qiagen) real-time platform [27] to enable the detection of DNA using SYBR Green and melt analysis or specific primers with fluorescent probe. Annealing was at 60 °C. A hot-start *Taq* polymerase was used to minimize non-specific primer and template binding. Negative DNA extraction and PCR controls were processed alongside the test samples.