



Tuberculosis infection in a late-medieval Hungarian population



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The AD 16–17th century skeletal series from Bácsalmás-Óalmás (southern Hungary) has already been the subject of previous paleopathological studies concerning TB-related bone lesions. Due to recent development of macroscopic and molecular diagnostic methods in paleopathology and paleomicrobiology, a five-year international research program was recently started in order to re-evaluate the TB-related lesions in the complete series, comprising 481 skeletons.

The skeletal material of these individuals was examined using macromorphological methods focusing on both classical/advanced stage skeletal TB alterations and atypical/early-stage TB lesions. Paleomicrobial analysis was used to study the presence of *Mycobacterium tuberculosis* complex (MTBC) DNA both in morphologically positive and negative cases. Samples were tested for the repetitive element IS6110 and further characterized by spoligotyping.

In the whole series, 283 possible cases of TB infections were identified based on morphological alterations. Skeletal samples of eighteen individuals, morphologically positive as well as negative cases, were selected for further biomolecular examinations. Among them, seven individuals were PCR positive for the repetitive IS6110 sequence of the MTBC genome.

Compared to the few cases of TB from the Bácsalmás-Óalmás series previously described, a much higher prevalence of MTBC infected skeletons was revealed in this study. The atypical/early stage skeletal lesions occurred significantly more frequently than the so-called classical alterations. Paleomicrobial analysis confirmed a prevalence of MTBC infection nearing 40% among the selected sample. Preliminary results also indicated better preservation of bacterial DNA in the compact layer of long bones and teeth, while spoligotyping suggested infection by different MTBC pathogens.

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1. Introduction

Tuberculosis causes about 1.5 million deaths every year, and one-third of the world's current total population may be infected with *Mycobacterium tuberculosis* [1]. These facts and the millions of

new TB cases reported by WHO every year have brought renewed attention to the research of this disease and its causative *Mycobacterium*-species.

Paleopathology provides a vast amount of data on TB and its various osteoarticular occurrence in human skeletal remains found in archeological sites [2,3]. The last decade of the 20th century brought major breakthroughs in the paleopathological diagnostics of TB. The first DNA evidence-based molecular (paleomicrobial) study of *M. tuberculosis* infection in old skeletal remains came out in 1993 [4] and was thereafter followed by many [5–7].

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Paleomicrobial research helps to identify pathogens in ancient human remains, thus providing crucial information on the onset and development of infectious diseases.

The AD 16–17th centuries skeletal series of Bácsalmás-Óalmás (southern Hungary) has been the subject of a wide range of studies including paleopathological investigation. The initial macro-morphological research had already suggested the presence of TB infection in this population [8]. Later studies estimated a relatively high frequency of TB but there was no systematic analysis done to evaluate all types of TB lesions. In the first phase of analysis, only classical chronic forms were taken into consideration. A few years later some cases of early or atypical TB infection were identified, and the first ancient DNA (aDNA) results were able to prove that these bones were infected by MTBC, thus confirming that paleo-microbial diagnostic tools could provide evidence for the reliability of early stage or atypical lesions [9].

Following recent developments of macroscopic and molecular diagnostic methods in paleopathology and paleomicrobiology, a five-year international research program was recently started in order to re-evaluate the TB-related lesions in the complete Bácsalmás-Óalmás series. The aim of the project presented here was, for the first time in the history of ancient TB projects in the Bácsalmás series, to study the potential occurrence of TB in the complete series, carrying out a systematic morphological analysis of both early stage/atypical and advanced stage/typical skeletal TB lesions as well as establishing the presence of MTBC aDNA in the skeletons using paleomicrobial techniques.

2. Material and methods

2.1. Archaeological background

The skeletal material came from the Bácsalmás-Óalmás archeological site (AD 16–17th centuries, southern Hungary), excavated in three phases between 1992 and 2003 [10]. This study was carried out on the series of 481 skeletons housed in the collection of the Department of Biological Anthropology at the University of Szeged. The skeletons' state of preservation was exceptionally good, the sandy soil of Bácsalmás (located in the southern part of the Danube-Tisza interfluvium) having provided favorable conditions for conservation.

On the basis of the archaeological and historical findings, it was known that this population immigrated from the southern part of Serbia or Montenegro during the Turkish occupation in the AD 16–17th centuries [10]. They did not seem to intermingle with other populations, probably for religious reasons, and their community became closely endogamous [10].

2.2. Skeletal material and earlier osteological studies

This study was based on an initial macroscopic morphological examination, complemented with medical imaging techniques in some cases. The paleodemographic analysis revealed 126 males, 113 females and 242 individuals of unknown sex [11].

The skeletal series was very rich in paleopathological cases and previous investigations had already provided a high amount of paleopathological data with some important discoveries [11–16]. Results of these investigations showed that pathological alterations occurred with a high frequency. The abundance of minor skeletal developmental defects could be linked to endogamy, which may indicate that this immigrated population had probably only very limited contact with the autochthonous Hungarian population [10,14–16]. Following the first case studies on rheumatological [12] and infectious [8] conditions, several important cases were described, including two cases of ankylosing spondylitis with a

possible familial context [14] and the recent discovery of the occurrence of skeletal scurvy in this population [11].

As for TB, presence of this disease was first indicated by morphological data and several cases have been described previously [8,15,16]. However, these studies did not consider the entire series and/or focused only on certain types of TB related skeletal lesions. In the course of the investigation presented in this article, lesions possibly associated with MTBC infection were separated into two main groups. In the 'early stage/atypical' group, endocranial-, rib-, vertebral- and long-bone changes probably related to TB were considered [15–17], while the more apparent chronic osseous TB lesions were included in the second 'typical/classical/advance stage' group [2,3].

2.3. Selected material for paleomicrobiological analysis

Eighteen individuals from the complete series of 481 individuals were selected for the molecular investigation, including both morphologically positive (from the aforementioned 283 potentially infected cases) and morphologically negative cases.

In order to compare the preservation of pathogen DNA in different skeletal areas, a total of three samples from each individual were taken from the vertebrae, ribs and long bones. In two cases showing signs of possible TB meningitis (grave no. 264 and grave no. 483) an additional sample was also taken from the cranial bone. Altogether, a total of 56 samples (eighteen individuals with three samples each plus two cranial samples) were utilized in the molecular analysis.

In the second phase of the paleomicrobial investigation, tooth samples were added to the study. This methodological decision was made in order to provide a better estimate of the potential for MTB aDNA preservation in this material. Compared to the abundance of bone samples used in MTB aDNA studies in the literature, there are to date only few references on tooth samples [18–21].

2.4. Biomolecular analysis

All aspects of the molecular analysis were conducted at the ancient DNA Laboratory of the EURAC Institute for Mummies and the Iceman, Bolzano, Italy. The sample preparation and DNA extraction were performed in a dedicated pre-PCR area corresponding to the requirements of aDNA studies in order to strictly avoid any contaminations. This includes the use of protective clothing, UV-light exposure of the equipment and bleach sterilization of surfaces, use of PCR workstations and filtered pipette tips. In a designated sample preparation room the outer surface of the bone samples was mechanically removed using a speed rotary tool (Dremel, Konijnenberg, The Netherlands). Cleaned samples were pulverized using a mixer mill (Retsch, Haan, Germany).

DNA extraction was performed with approximately 250 mg of bone powder using the Silica-based DNA extraction method described by Rohland et al. [22]. The PCR mix contained 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.875 mM MgCl₂, 200 μM of each deoxynucleotide triphosphate, 0.5 μM of each primer, 0.1 mg/ml Bovine serum albumin, 0.05 U/μl AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) and 2 μl of extracted DNA to a final volume of 20 μl. Polymerase chain reaction was carried out according to the parameters described by Nicklisch and colleagues [23]. The presence of TB DNA was assessed based on a PCR-based assay targeting the MTBC multicopy IS6110 region [24]. For further diagnosis and to possibly subtype the MTBC bacteria, spoligotyping was used on selected specimens performed after Kamerbeek et al. [25] with minor modifications.

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