MYCOBACTERIUM TUBERCULOSIS COMPLEX ANCIENT DNA DEMONSTRATION FROM HUMAN BONE REMAINS

SUMMARY OF PH. D THESIS

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Introduction

Tuberculosis (TB) is one of the oldest diseases and still has a high prevalence nowadays (Ortner, 2003; Gutierrez *et al*, 2005; Hershkovitz *et al*, 2008; Donoghue, 2009; Baker *et al*, 2015; Donoghue *et al*, 2015; Pálfi *et al*, 2015). The disease is pandemic, according to the estimation of the WHO 30% of the total population of the World may be infected latently by *Mycobacterium tuberculosis*; this is why the research of TB is so important for mankind.

For a long time it was thought that *Mycobacterium tuberculosis* was developing from *Mycobacterium bovis* during domestication and had infected the human population (e.g. Cockburn, 1963; Ortner, 1999). This hypothesis was accepted until the beginning of the 2000s, thereafter more studies revealed that *Mycobacterium tuberculosis* is a more ancient variant than the *M. bovis* (e.g. Brosch, 2002; Filliol *et al*, 2006; Hershberg *et al*, 2008). It is probable that *Mycobacterium tuberculosis* complex (MTBC) has a general ancestor, which is the *Mycobacterium prototuberculosis* (Brosch *et al*, 2002) which might have developed together with mankind (Camus *et al*, 2002; Gutierrez *et al*, 2005; Brisse *et al*, 2006; Wirth *et al*, 2008; Weber doctoral dissertation, 2013; Comas *et al*, 2013; Bos *et al*, 2014; Boritsch *et al*, 2014, Donoghue *et al*, 2017).

Human tuberculosis has a very rich paleopathology: a high number of typical osteoarcheological manifestations of the disease were described during the last century (e.g. Bartels, 1907; Steinbock, 1976; Ortner, 1999, 2003; Pálfi *et al*, 1999). 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 paleomicrobial diagnostic tools could provide evidence for the reliability of early stage or atypical lesions and thus confirming that paleomicrobial diagnostic tools could provide solid evidence for the early stage or atypical lesions. The first DNA evidence-based molecular (paleomicrobial) study of *M. tuberculosis* infection (demonstrated on old skeletal remains) was issued in 1993 (Spiegelman & Lemma, 1993) and was thereafter followed by many (Nerlich *et al*, 1997; Zink *et al*, 2001; Donoghue *et al*, 2009, 2017). Since the last decades some ‘early stage’ or ‘atypical’ form of TB infection were identified. The positive results of aDNA-examination justified that, the paleomicrobial diagnostic is dependably usable to identify similar cases (Haas *et al*, 2000).

Different elements of ancient human remains were used as samples in MTB aDNA research during the last two decades. New researches justify that, the advantage of using dental pulp – beside the compact tissue of long bones – for aDNA studies holds in its potential better preservation in a closed cavity. In an ideal case, the external enamel layer is able to protect the DNA remains in the dental pulp (Nguyen Hieu *et al*, 2011).

Aims of study

The archeogenetical research has significant antecedents in Hungary; however, the detection of bacterial DNA in human bone remains was not ordinary amongst the Hungarian methods until the late 2000s. Until now this was available only with the support of foreign laboratories and experts.

Since Hungary has plentiful morphologically identified TB cases (Pálfi *et al*, 1999; Pálfi, 2002; Pálfi és Ardagna, 2002), nowadays it is a current issue that these data definitely need supportive molecular biological research.

The aims of my thesis are as follows:

– to adopt the molecular biological identification of mycobacterial infection in Hungary, to confirm in our country us the modern methods which were learned from abroad, in order to use it routinely on samples from Hungary.

– to define the best anatomical location in terms of the bacterial aDNA extraction using long bones and teeth of human bone remains.

– to prove the presence of mycobacterial infection with molecular biological results in case of some different, historical ages – especially with regard to paleo epidemic and demographic impact of TB –, to confirm the morphological results as auxiliary research, in order to assist the biological-historical reconstruction of the Carpathian Basin's ancient population.

– preparation of molecular background identification of archaic mycobacterial infections in Hungary, in order to validate the abroad acquired state of the art testing methods at home, and use them routinely on samples from Hungary.

Materials & methods

In my examination I researched six different historical periods for TB infection, the earliest period is Neolithic, which may give important confirmation to the evolution of *Mycobacterium tuberculosis*. Human remains were studied from the following archaeological sites:

- Alsónyék-Bátaszék is a village situated in Southern Hungary. The excavation of this prehistoric settlement and cemetery took place between 2006 and 2009, during which 2,359 burial places of the Late Neolithic-Early Copper Age were found (first half of the 5th millennium BC). This is therefore to date one of the largest late Neolithic sites excavated in Central Europe. The first TB cases came out from the northern, so-called Kanizsa-dűlő part (or 010/B) of the site, from where the skeletal remains of 862 graves of Lengyel culture were excavated (Zalai-Gaál, 2008; Zalai-Gaál & Osztás, 2009). The other Neolithic cemetery came from the Tisza culture – Vésztő - Mágor – from the same time period. The preliminary morphological research indicated possible TB occurrences from both archaeological sites (Spekker *et al*, 2012; Köhler *et al*, 2013).
- Tápé-Széntéglaégető archeological site is a late Bronze Age cemetery, human remains were found from this place that came from the 14-13th centuries B.C. The archaeological excavations were conducted between 1960 and 1966. In the exploration 686 graves were found. The results of anthropological examinations were published by Gyula L. Farkas and Pál Lipták in 1971, and the anthropological studies have included 579 graves (Farkas & Lipták, 1971). In the recent past paleopathological research was started on this series in the Department of Biological Anthropology – University of Szeged, and seven individuals were detected for TB related atypical lesions. These cases gave samples for my biomolecular research.
- Colleagues of the Department of Biological Anthropology – University of Szeged found atypical TB related lesions on the Avar Age samples (Szeged-Kiskundorozsma Kettőshatár II. (Grave No. 263.) and Csárdaszállás-Hanzélytanya). My paleomicrobiological doctoral research gave opportunity to analyse both samples from the Avar Age.

- The cemetery from the age of the Árpádian Dynasty was excavated in Győr Pósdomb between 1970 and 1973. In the case of Pott's disease – from grave no. 187 – we noticed severe morphological lesions during the molecular biological analysis of the skeleton.
- It was important for my research that the skeletal material came from the Bácsalmás-Óalmás archeological site (AD 16-17th centuries, southern Hungary), excavated in three phases between 1992 and 2003 (Wicker, 2006). This ethnic group originated in the Balkans and intermingled with other populations, probably for religious reasons, and their community became closely endogamous (Wicker *et al*, 1999). This study was carried out on the series of 481 skeletons whose state of preservation was exceptional (Lovász, 2005a, 2009, 2013). In the series there was high prevalence of TB-referring morphological lesions suitable for paleomicrobiological sampling.
- My research has had only one subject from the modern age. The naturally mummified human remains which are unique in Europe, were found in 1994 when the restoration of the Dominican church of Vác took place: partially or completely mummified bodies were found in the 265 ornamented coffins (Pap *et al,* 1997). Significant TB infections were indicated by Former analysis of the series (Fletcher *et al*, 2003). In our work a molecular biological examination was performed on the body of Terézia Sándor for the detection of *Mycobacterium tuberculosis* aDNA.

To find the most efficient way to detect the TB presence was among my objectives, therefore I was using such methods – consideration of the relevant literature – where the DNA preservation and the success of the identification may be the maximum. At the beginning we had used phenol-chloroform DNA-extraction (Hochmeister *et al*, 1991), but we would like to reach a higher concentration of DNA, therefore we started to use the more effective silica-based DNA-extraction (Rohland *et al*, 2009).

Beside the former widely used spongy bones we used long bones and teeth in our research, in order to justify the better DNA extraction from this anatomical location. These sample types also help to avoid the hazard of contamination.

After the DNA extraction we have conducted PCR reaction, where the IS*6110* region of *Mycobacterium tuberculosis* was amplifying by primers (IS6110R, IS6110F &

IS6110intR, IS6110intF), so as to control the extraction, and the aDNA availability (Eisenach *et al*, 1990; Taylor *et al*, 1996). We were using spoligotyping in several cases (Kamerbeek *et al*, 1997; Zink *et al*, 2003).

Results & discuss

In parity with my goals, during my studies I had been sampling from potential TB infected human remains of domestic anthropological series, and I was carrying out with those MTBC and aDNA demonstration in the aDNA laboratory of EURAC Institute for Mummies and Iceman (Bolzano, Italy). The first part of my research was performed in Italy, while, so as to establish the paleomicrobial research in Hungary, in the second phase of my work, certain tasks were carried out in the Archeogenetics Laboratory of the Institute of Archeology, HAS RCH Budapest, Hungary.

I have to emphasize among my methodology results that I successfully establish the sampling of teeth for proving archaic TB, beside generally used vertebrae or ribs. My research has revealed that the preservation of mycobacterial DNA is much more likely within the closed cave of dental pulp (Pósa *et al*, 2012), just like in the case of MTBC. My analyses were also productive on long bone samples.

In the case of the neolith series from Alsónyék, all of the human skeletal remains of a medium size grave group were included in the molecular biological analysis. Five individuals showed positive molecular results for bacterial DNA. The molecular detection was successful in samples from long bones and/or teeth (Pósa *et al*, 2015a, Pósa *et al*, 2016a). The results were in accordance with my molecular biological research and the parallel publicised morphological data (Köhler doctoral dissertation, 2012; Köhler *et al*, 2013) indicate that, the *Mycobacterium tuberculosis* was present in those human remains, which were from the part of the Alsónyék-Bátaszék Neolithic cemetery investigated by us (Pósa *et al*, 2015a). My results corroborate the Neolithic occurrence of tuberculosis from South-eastern Hungary already published by Masson and colleagues (Masson *et al*, 2013, 2015), confirming the presence of TB in the Carpathian Basin 7000 years ago with several new cases. Regarding the Vésztő - Mágor Neolithic cemetery (originated from the Tisza culture) we confirmed only on one occasion the presence of TB's aDNA in human remains, to prove the presence of the Mycobacterial infection in this individual.

Tápé-Széntéglaégető archeological site came from the late Bronze Age. On the location there was discovered TB associated with atypical symptoms in seven individuals in anthropological studies, nevertheless the presence of the pathogen was not confirmed by molecular biological methods.

We could not confirm the infection in the case of the Avar Age samples (Kiskundorozsma Kettőshatár és Csárdaszállás-Hanzélytanya), it may conclude that aDNA was greatly degraded. This experience pointed to that in the future it will be required to use next-generation sequencing and wider sampling for this kind of researches.

The cemetery from the age of the Árpád Dynasty (Grave No. 187) shows pathological lesion (ribs, *femur*, vertebrae and Pott's disease), the presence of Mycobacterial DNA can be justified in the case of the *humerus* and *femur* (Pósa *et al*, 2016b, in press).

In the individuals of Bácsalmás-Óalmás burial ground among the examined 481 skeletons, 283 possible cases of TB infection were recorded based on macro morphological data by anthropologists, also including the typical and atypical alterations, and the itemized paleopathological results were presented in the doctoral dissertation of Gabriella Kajdocsi Lovász (Kajdocsi Lovász Gabriella doctoral dissertation, 2015). Seven out of the eighteen individuals analyzed were positive for the region of IS*6110* of *M. tuberculosis* (Pósa *et al*, 2013; Pósa *et al*, 2015b). Unfortunately, the biomolecular examination of this complete series would be very time-consuming and would require a large amount of funds in order to be completed, however, the high TB-infectious rate of the population was proved. Our new results confirm data of some earlier studies on high TB prevalence in this population (Pálfi and Ardagna, 2002; Zink *et al*, 2007). The spoligotyping pictures different MTBC pathogens, although the precise *Mycobacterium* (*M. bovis, M. tuberculosis*) appearing in these samples could not be identified.

The previously published results (Fletcher *et al,* 2003) were confirmed in the case of Terézia Sándor's mummy, which originated from modern age. She was the individual who was buried in the crypt in the Dominican church of Vác, and who was infected with tuberculosis: we isolated *Mycobacterium tuberculosis* aDNA from her remains.

Evaluation of results

During our research program we successfully adopted long standing paleomicrobiological techniques on the indigenous samples, and the TB infection was confirmed in four different ages.

My research has revealed that the preservation of mycobacterial DNA is much more extensive in the case of long bones and teeth, than in the case of vertebrae or ribs. At the same time, data gained indicate the importance of the selection of the sample, as well as parallel sampling individually from long bones and teeth.

The significance of the Alsónyék-Kanizsa dűlő excavation is that you can observe the fast growth and sudden collapse of the population – according to the radiocarbon results, remarkable population used to live there for relative by short time –, so you cannot disregard the responsibility of TB for the extinction.

The confirmation of the presence of bacterial DNA justifies the huge amount of TB suggestive morphological deformation of the osteological series in the case of Bácsalmás-Óalmás archeological site. The archeologically and paleopathologically presumed endogamy of the population may cause the high prevalence of TB (Wicker *et al*, 1999; Lovász, 2015).

The next-generation sequencing will give us more accurate result in comparison with both past examined series and future ones. With this method the hazard of contamination will be limited or excluded. The more precise isolation between the soil Mycobacteria and the human pathogenic Mycobacteria strains can materialize, and beyond the confirmation of MTBC DNA presence there will be opportunity for the characterization of the ancient strains and answering the questions concerning their evolution.

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2. Published publication in referred journal

2.1. Other publications related to the topic of the dissertation

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3. Other professional publications

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