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ARTICLE

Ancient human tooth samples used for TB paleomicrobial research

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ABSTRACT Questions about the evolution of tuberculosis and its pathogens are of primary importance in today's research. We need a thorough understanding, both of the origins and history of the disease and of the evolutionary potential of its pathogens, in order to make valid prognoses for the future. Paleomicrobial analysis of ancient microbial DNA (aDNA) helps to identify human pathogens in human remains. During the last twenty years the application of modern biomolecular techniques, such as PCR, spoligotyping and DNA sequencing, in possible cases of skeletal tuberculosis has provided additional evidence for the differential diagnosis of ancient tuberculosis (e.g. Spigelman and Lemma 1993; Nerlich et al. 1997; Zink et al. 2001; Donoghue 2009). The presence of *Mycobacterium tuberculosis* (MTB) DNA in ancient human samples has confirmed morphological and radiological evidence of tuberculosis in archaeological human remains and furnished key material to understand the evolutionary history of the pathogen. The large majority of MTB aDNA studies used bone samples (mainly compact bone or vertebrae) or mummified tissues. Although, since the late nineties tooth samples have already been used successfully for the isolation of aDNA remains of other pathogens, e.g. for *Yersinia pestis* (Drancourt et al. 1998), they were only sporadically utilized in TB research. In a recent research project we tried to compare the preservation of mycobacterial aDNA in different bone elements and found a very heterogenous picture, indicating an important 'chance factor' in these researches (Pósa et al. 2012). In the newest phase of this project, tooth samples have also been used. We tested bone and tooth samples from two osteoarchaeological series from Hungary: the anthropological remains of the Bácsalmás-Óalmás and the Vésztő-Mágor sites. Our preliminary results prove the preservation of MTB aDNA in both series indicating the necessity to use parallel bone and tooth samples in further paleomicrobial analyses. Our future aims include a larger scale comparative investigation in order to precisely determine which anatomical elements of a human skeleton give the best MTB aDNA preservation.
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Tuberculosis (TB), with millions of victims each year, is still the infectious disease that is responsible for the most deaths in the world today. The 'success' of the infection can be only partly explained by the growing susceptibility of human populations, due to AIDS, poverty, other factors weakening the immune systems of populations in developing countries and the socially marginalised groups in developed countries. Another key to the advancement of the disease lies in the exceptional evolutionary abilities of *Mycobacteria*. The 'traditional' human pathogen *Mycobacterium* develops a variety of new, multi-resistant strains; at the same time, atypical *Mycobacteria* formerly harmless for humans, grow pathogenic (Bloom 1994; Cotran et al. 1994; WHO 2010).

As a consequence, questions about the evolution of the disease and its pathogen are of primary importance in today's research (Supply et al. 2013). We need a thorough understanding, both of the origins and history of tuberculosis and of the evolutionary potential of its pathogens, in order to make valid prognoses for the future. To date, it has become essential to systematise our knowledge about the past of TB. Besides written sources, paleopathological evidence is also of significance, because it provides primary evidence of biological material for the study of microbial evolution (e.g. Roberts and Manchester 1995; Ortner 2003).

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In the last twenty years, biomolecular methods were efficiently used in the paleopathology of mycobacterial infections and opened new perspectives for the study of the evolution of these diseases (e.g. Spigelman and Lemma 1993; Nerlich et al. 1997; Donoghue et al. 1998, 1999, 2005; Gernaey et al. 1999; Pap et al. 1999; Haas et al. 2000; Rothschild et al. 2001; Minnikin et al. 2011; Donoghue 2008).

Human osteoarchaeological remains used as samples for MTB aDNA research

Different elements of ancient human remains were used as samples in MTB aDNA research during the last two decades. We collected a series of references in order to study the main tendencies in sample selection. In our paper, we present only some of them: a simplified, non-exhaustive list of more than 40 publications concerning MTB aDNA research is presented in Table 1.

Bone remains were used by Spigelman and Lemma in their first paleomicrobiological study (Spigelman and Lemma 1993). Ancient mummies were also subjects of the earliest paleomicrobial works: Salo and collaborators took samples of mummified tissue for MTB aDNA extraction (Salo et al. 1994). Baron and his colleagues worked with bones in their early study (Baron et al. 1996) and Nerlich and coworkers identified MTB aDNA from lung remains of an Egyptian mummy (Nerlich et al. 1997). In the second half of the nineties, Faerman and colleagues detected the presence of *M. tuberculosis* aDNA in skeletal remains from Lithuania. They used compact bone samples from long bones. However, they also successfully used tooth samples for aDNA extraction (Faerman et al. 1997). Two years later, the same team published a completed version of this paleomicrobial study on Lithuanian material – this second publication also mentioned the successful aDNA extraction from teeth (Faerman et al. 1999). Even though our literature review is not exhaustive, it is surprising that we had to wait a decade for the next reference of this type. However, a lot of TB paleomicrobiology works were published between the end of the nineties and the beginning of the 2010' years. In the late nineties several researchers used vertebrae (e.g. Braun et al. 1998), ribs (e.g. Crubézy et al. 1998), or calcified material (Donoghue et al. 1998). Several studies were published in 1999: Haas and his team, Horáčková and collaborators and the team of Dutour used bones (Haas et al. 1999; Horáčková et al. 1999; Dutour et al. 1999). In this year Donoghue and her team, or Taylor and coworkers worked with ribs (Donoghue et al. 1999; Taylor et al. 1999). Some researchers used calcified fragments (Pálfi et al. 1999; Spigelman and Donoghue 1999) or mummified soft tissues (Pap et al. 1999; Zink et al. 1999). In 2000, Haas and his collegues performed the aDNA investigation on bones (mainly vertebrae and ribs) and in one case on calcified pleura (Haas et al. 2000). In 2001 Zink and his team continued to use bones (Zink et al. 2001). Maczel and collaborators (working together with Nerlich and Zink) also used vertebrae, long bones or skull fragments for the identification of MTB infection in Hungarian and French osteoarchaeological materials (Maczel et al. 2001; Maczel 2003). Fletcher and her colleagues used bones and soft tissues in 2003: these samples came from different organs of the mummies from Vác (Fletcher et al. 2003). In 2004 Bathurs and his group worked with carpals (Bathurs et al. 2004). Taylor, in different studies, worked with rib and/or long bone samples (Taylor et al. 2005, 2007). In the same

Table 1. References indicating samples used in the last 20 years for MTB aDNA studies. (The references are in chronological order. The list of the references is not exhaustive.).

Some references about ancient TB DNA studies	Samples from ancient human remains used from TB DNA analyis
Spigelman and Lemma 1993	bone ~~samples~~
Salo et al. 1994	mummified soft tissues
Baron et al. 1996	Bones
Nerlich et al. 1996	mummified soft tissues (lung)
Faerman et al. 1997	teeth, compact bone
Braun et al. 1998	Vertebra
Crubézy et al. 1998	rib, vertebra
Donoghue et al. 1998	calcified pleura
Donoghue et al. 1999	Rib
Dutour et al. 1999	bones (vertebral remains)
Faerman et al. 1999	teeth, long bone
Haas et al. 1999	bones, calcified pleura
Horáčková et al. 1999	Bones
Pálfi et al, 1999	calcified fragments (pleura)
Pap et al. 1999	mummified tissues
Spigelman and Donoghue 1999	calcified fragments
Taylor et al. 1999	Vertebra
Zink et al. 1999	mummified tissues
Haas et al. 2000	bones (vertebra, rib), calcified pleura
Maczel et al. 2001	bone remains (vertebra, rib, skull)
Rothschild et al. 2001	ancient animal bone
Zink et al. 2001	Bones
Konomi et al. 2002	mummy, dried tissue samples
Fletcher et al. 2003	mummies: bone and soft tissue remains
Maczel 2003	bone remains (vertebra, rib, skull)
Zink et al. 2003	bones, soft tissue
Bathurst et al. 2004	carpal bones
Donoghue et al. 2005	Bones
Taylor et al. 2005	ribs, long bone, vertebra, skull fragment
Zink et al. 2005	Bones
Zink et al., 2007	Bones
Taylor et al. 2007	vertebra, long bone, ribs
Hershkovitz et al. 2008	long bones
Donoghue et al. 2009a	mummified lung tissues
Matheson et al. 2009	teeth, long bone
Nerlich et al. 2010	Bones
Lemma et al. 2010	Bones
Neparáczki et al. 2011	rib, vertebra
Lee et al. 2012	Bones
Masson et al. 2012	Bones
Nicklisch et al. 2012	long bones
Pósa et al. 2012	ribs, long bone, vertebra, skull fragment

period Donoghue and her team and Zink and collaborators used bone samples for skeletons and mummified soft tissues for mummies (e.g. Zink et al. 2003, 2005, 2007; Donoghue et al. 2005, 2009a). Compact bone samples were used for the detection and molecular characterization of a 9000-year-old *Mycobacterium tuberculosis* infection from a Neolithic settlement in the Eastern Mediterranean (Hershkovitz et al. 2008). Several results were published in 2010, using bones also (e.g. Nerlich et al. 2010; Lemma et al. 2010). In the most recent publications, a large variety of bone types were used – long bones, ribs, vertebrae, skull fragments (e.g. Neparáczki et al. 2011; Masson et al. 2012; Lee et al. 2012; Nicklisch et al. 2012; Pósa et al. 2012).

Although our list of references is not complete at all, it is quite evident that the great majority of the MTB aDNA analyses are carried out on different bone samples for skeletons and dried soft tissues for mummies. Since the early work of Faerman and collaborators we had to wait ten years till 2009 when the team of Matheson and collaborators published their results on the detection of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in the remains from the Tomb of the Shroud in Israel. Teeth and bone samples were selected: a phalange and the pulp of a molar tooth were positive for M. tuberculosis (Matheson et al. 2009).

In our previous study, we tried to compare the preservation of mycobacterial aDNA in different bone elements (long bones, vertebrae, ribs, skull remains) and we found a very heterogenous level of preservation, indicating an important 'chance factor' (Pósa et al. 2012). This is the reason why we decided to add one more item to the list of our samples *i.e.* the use teeth as well in our analysis.

Human tooth samples in paleomicrobiology

Because of the special anatomical characteristics of teeth, several researches have started in the nineties to use this organ as a sample in paleomicrobial studies. The advantage of using dental pulp for aDNA studies lies in its potential better preservation in a closed cavity. In the pulp chamber, soft tissues are preserved from environmental contamination and degrading agents. In an ideal case, the external enamel layer is able to protect the DNA remains in the dental pulp (Keyser-Tacqui and Ludes 2007; Nguyen Hieu et al. 2011). Authors argued that the aDNA yield of teeth is necessarily better than that of bones and the quality is also necessarily better, since soil bacteria can not change its characteristics and the contamination risks are lower during handling and washing (e.g. Gilbert et al. 2004, 2005; Drancourt and Raoult 2005). Experimental animal models infected with *Coxiella burnetii* and *Bartonella* ssp. had also confirmed the possibility of finding bacterial DNA in dental pulp long time after bacteremia (Aboudharam et al. 2000, 2005).

In paleomicrobial research the first tooth sample-based examination was carried out by the group of Drancourt in the mid-nineties. They could detect *Yersinia pestis* aDNA from 400 year-old human dental pulp remains. In the second half of the nineties, some other studies were conducted by the same team on different plague mass grave materials from France (e.g. Drancourt et al. 1998). Dental remains were used later for aDNA studies of many other pathogens, such as *Anelloviridae, Bartonella henselae, Bartonella quintana, Mycobacterium leprae, Mycobacterium tuberculosis, Rickettsia prowazekii, Samonella enterica serovar Typhi* and *Yersinia pestis* (Drancourt et al. 1998; Drancourt et al. 2004; Raoult et al. 2000; Papagrigorakis et al. 2006; Bianucci et al. 2008; Drancourt et al. 2007; Bianucci et al. 2009; Haensch et al. 2010; Wiechmann et al. 2005; Tran et al. 2011; Nguyen-Hieu et al. 2010; Raoult et al. 2006; Mathenson et al. 2009).

Although MTB aDNA remains were successfully extracted from ancient human teeth by the team of Faerman and these results were published relatively early (Faerman et al. 1997) before the publication of the *Yersinia pestis* identification (Drancourt et al. 1998), the use of tooth samples is not common in ancient TB research. Teeth were used especially in case of diseases without skeletal involvement, but for mycobacterial infections their use was very sporadic, as mentioned previously. However, the success of the work by the group of Matheson for the identification of a coinfection by *Mycobacterium leprae* and *M. tuberculosis* (Matheson et al. 2009) proves the potential usefulness of tooth samples in ancient TB research.

In our project concerning MTB aDNA preservation in Hungarian osteoarchaeological series, the heterogenous DNA yield of the different bone samples in our preliminary studies (Pósa et al. 2012) suggested the necessity to complete the list of our samples with teeth for the second step of our studies.

Material – the osteoarchaeological series and samples

In the present investigation, we tested bone and tooth samples from two osteoarchaeological series from Hungary, housed in the collections of the Department of Biological Anthropology of the University of Szeged: the anthropological remains of the Bácsalmás-Óalmás and the Vésztő-Mágor sites.

The 16-17th century AD skeletal series of Bácsalmás-Óalmás (Hungary) has already been the subject of several paleopathological studies. Initial macromorphological analysis suggested tuberculosis infection in this population (*e.g.* Molnár and Pálfi 1994). Infection by the *M. tuberculosis* complex was confirmed in several cases (*e.g.* Haas et al. 2000). In a recent pilot project paleomicrobiological analysis was used to study the presence of MTB aDNA in morphologically positive and negative cases. Our preliminary results indicated a better preservation of mycobacterial DNA in the compact layer of long bones than in vertebrae or ribs. However, the results of this pilot study suggested an important role of the 'chance factor' in the sampling. Altogether 19 samples were collected

from those 6 individuals that later gave positive results, but only 7 of these samples were actually PCR positive (Pósa et al. 2012). In our current research 6 new cases were selected, and besides usual aDNA sampling sites in the skeleton tooth samples were also collected in each case.

The second series of our study is the Vésztő-Mágor anthropological material from the Neolithic Age (Tisza culture). A more detailed description of this approximately 5000 year-old cemetery is given by Spekker and co-workers in the present volume of Acta Biologica Szegediensis (Spekker et al. 2012). The authors identified 4 cases of potential skeletal tuberculosis in this series of 30 skeletons. Tooth samples were extracted from these 4 cases and from one morphologically negative case for the paleomicrobial analysis. In one of the 5 cases, humerus sample was used as there was no tooth available for the study.

Paleomicrobial analysis, results and discussion

The paleomicrobiological analysis was carried out in the EURAC Institute for Mummies and the Iceman in Bolzano, Italy. Initially, a PCR-based assay targeting the *Mycobacteria* multicopy IS6110 region has been conducted to a subset of the samples.

All sample preparations and DNA extractions were performed in a dedicated pre-PCR area following strict procedures required for studies of aDNA: 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 by using a Dremel speed rotary tool. Cleaned samples were pulverized using a Retsch mixer mill. Teeth were washed in sodium hypochlorite and washed-out ultra-pure water.

DNA extraction was performed with 250 mg bone powder, which was added to 5 ml EDTA and 20 µl 20mg/ml Proteinase K by pipette and was mixed then incubated overnight at 40°C. After this the samples' supernatants were added to 2.5 ml binding buffer and 100 µl silica suspension and incubated for 3 hours. The slightly modified extraction protocol of Nadine Rohland (Rohland et al. 2009) was followed subsequently. Samples were examined for a specific, 123 bp locus in the insertion sequence IS6110 of *Mycobacterium tuberculosis* complex (MTBC) (Donoghue 2009). The IS6110 region is a multicopy repetitive element usually found with up to 24 copies in each cell (Baron et al. 1996; Salo et al. 1994) and was amplified with primer pairs IS6110F and IS6110R. We used hot-start PCR with purchased kit (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA). The PCR reaction mix contained 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.875 mM $MgCl_2$, 200 µM of each deoxynucleotide trisphosphate, 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 4 µl of extracted DNA to a final volume of 50 µl. TB positive cases were further analyzed by spoligotyping. The method was performed with the Spoligotyping Kit (Ocimum Biosolutions) as described by Kamerbeek et al. (1997) with minor modifications.

Table 2. PCR results of the preliminary paleomicrobial investigations, indicting the samples, the subjects an the osteoarchaelogicalsere.

Bácsalmás-Óalmás	Register Nr.	Localisation/sample	PCR result
Grave Nr. 175	1359	temporal bone	--
	1360	vertebra	--
	1361	radius	--
	1362	rib	--
	1363	tooth	--
Grave Nr. 208	1377	radius	--
	1378	vertebra	--
	1379	rib	--
	1380	tooth	--
Grave Nr. 326	1369	radius	--
	1370	vertebra	--
	1371	rib	--
	1372	tooth	PCR+
Grave Nr. 409	1381	radius	--
	1382	vertebra	--
	1383	rib	--
	1384	tooth	--
Grave Nr. 410	1373	radius	--
	1374	vertebra	--
	1375	rib	--
	1376	tooth	--
Grave Nr. 423	1364	temporal bone	--
	1365	tibia	--
	1366	vertebra	--
	1367	rib	--
	1368	tooth	--
Vésztő-Mágor			
Grave Nr. 6.	1355	tooth	--
Grave Nr. 13.	1358	tooth	PCR+
Grave Nr. 33.	1356	humerus	--
Grave Nr. 39.	1357	tooth	--
Grave Nr. 5.	1354	tooth	--

As for the PCR results, one TB infection is detected on the 6 samples coming from the Bácsalmás-Óalmás series, TB infection of one individual was proven by the biomolecular analysis. The ratio is less spectacular if we consider that there was only one positive sample for MTB aDNA out of 26 samples analysed (Table 2). However, we have to mention that even for this particular skeleton (an adult male skeleton from Grave Nr 326) the bone samples (radius, vertebra and rib) did not give positive paleomicrobial result: only the tooth sample contained evaluable MTB aDNA remains. This skeleton was one of the 'morphologically negative' controls, which means that the adult man was infected by *Mycobacterium tuberculosis* (or by a member of the *M. tuberculosis* complex), but he died before the disease could produce observable osteological symptoms.

Figure 1. The positive tooth sample for MTB aDNA; Vésztő-Mágor Grave Nr. 13.

Figure 2. Periosteal remodelling of probable infectious origin on a long bone surface; Vésztő-Mágor Grave Nr. 13.

We got a very strong signal in the agarose gel picture from a tooth sample from the Vésztő-Mágor Neolithic material. The tooth sample (Fig. 1) of the adult male skeleton from Grave Nr. 13 furnished evidence for the presence of MTB aDNA in the dental pulp. Based on the morphological analysis presented in detail in the present volume of this journal (Spekker et al. 2012), this case was one of the potential TB cases. Typical signs of early stage TB infection were observable on the skeleton, such as the association of vertebral hypervascularisation and resorptive lesions, bilateral periostitis (Fig. 2) on tibiae and slight rib periostitis. The complementary morphological and biomolecular proofs of TB infection together strengthen the preliminary diagnosis of this case – and the presence of tuberculosis in this population 5 thousand years ago.

These two positive biomolecular results for MTB aDNA from teeth prove the usefulness of tooth samples in paleomicrobial study of tuberculosis, especially if we consider the negative results received from the study of the 3 bone samples of the young male skeleton from Bácsalmás. Our preliminary results indicate the necessity to use parallel bone and tooth samples in further paleomicrobial analyses. The future aims of our ancient TB research program include a larger scale comparative investigation in order to precisely determine which anatomical elements of a human skeleton give the best MTB aDNA preservation.

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