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ARTICLE

Preliminary results from the paleomicrobiological studies of *Mycobacterium tuberculosis* infection in the Bácsalmás-Óalmás anthropological series

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ABSTRACT The aim of the actual study of the 16-17th centuries AD series of Bácsalmás-Óalmás (Hungary) is to combine the osteological and complete paleomicrobiological studies of a large series of approximately half a thousand skeletons. This material, which is stored in the collections of the Department of Biological Anthropology, University of Szeged, was chosen because of the good state of preservation and of some previous aDNA results. Some cases of TB infections have already been confirmed during the analysis of the first third of the series. In the actual state of the Bácsalmás ancient TB project, a general morpho-pathological study of the complete series is in progress in the frame of a PhD thesis at the University of Szeged. Molecular paleomicrobiological analysis to diagnose bacteria of the *Mycobacteria tuberculosis* complex has been initiated in the ancient DNA Laboratory of the Institute for Mummies and the Iceman, EURAC Research, Bolzano, Italy. A PCR-based assay targeting the multicopy IS6110 region has been conducted to a subset of the samples. First results already indicate the presence of *Mycobacteria* in some of the rib samples. These positive cases will be further subtyped via Spoligotyping.

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KEY WORDS

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The background of the molecular paleomicrobial analysis

Nowadays many ancient diseases such as tuberculosis, leprosy, treponematosis or cholera are re-emerging. Despite significant efforts to reduce the spread of those diseases, they are still threatening large populations in various countries. For this reason, it seems to be of increasing interest to understand host-pathogen interaction and the mechanisms responsible for epidemic spread. Knowledge, however, about the history and evolution of the pathogens, their hosts and their interactions is still scarce. Paleomicrobial research with ancient DNA helps to identify human pathogens in ancient human remains and thus provides considerable information on the onset and development of infectious diseases. Furthermore ancient DNA analysis can help to answer questions about the origin and virulence of pandemic strains.

The paleopathology and paleomicrobiology of tuberculosis

Human tuberculosis has a very rich paleopathology: a high number of typical osteoarcheological manifestations of the disease was described during the last century (*e.g.* Steinbock 1976; Ortner 1999, 2003; Pálfi et al. 1999). Since the 1990's, the identification of TB in ancient human material was facilitated by the introduction of new biomolecular methods (*e.g.* Donoghue 2008; Donoghue et al. 1998, 1999, 2005; Haas et al. 2000; Nerlich et al. 1997; Pap et al. 1999; Spigelman and Lemma 1993), as well as by the study of its early stage or atypical skeletal manifestations of tuberculosis (*e.g.* Roberts et al. 1994; Pálfi 2002; Maczel 2003). The earliest evidence of the disease goes back as far as at least 9000 years ago (Hershkovitz et al. 2008). However, large paleoepidemiological studies are rare and are based mainly on morphological or on biomolecular aspects (*e.g.* Dutour et al. 1998; Pálfi and Marcsik 1999; Fletcher et al. 2003; Maczel 2003).

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Materials and Methods

The Bácsalmás skeletal series and its paleopathological context

The village of Bácsalmás is located in the southern part of the Danube-Tisza interfluve in Hungary. Near the village there is a sand-mine in an area called Óalmás, where a late medieval graveyard containing the remains of 481 individuals was excavated. The population of the site emigrated from the southern

Figure 1. Potential early stage skeletal TB: periosteal appositions and remodelling on the visceral surface of a rib (Bácsalmás-Óalmás, Grave No 61, Juvenile Male; ref: Molnár and Pálfi 1994).

Figure 2. Severe destruction of a cervical vertebral body due to probable TB spondylitis (Bácsalmás-Óalmás, Grave No 39, 50-60 year old Male; ref: Molnár and Pálfi 1994).

part of Serbia or Montenegro during the Turkish occupation. As the soil of the excavation site is mostly sandy, the majority of the skeletons are in a very good state of preservation.

This material, which is stored in the collections of the Department of Biological Anthropology, University of Szeged, was chosen because of the good state of preservation and of some previous aDNA results. Some cases of TB infections have already been confirmed by morphological and/or molecular biological analysis of the first third of the series (*e.g.* Haas et al. 1999, 2000; Molnár and Pálfi 1994; Pálfi et al. 1997; Pálfi and Ardagna 2002; Zink et al. 2007) (Fig. 1-2). In the actual state of the Bácsalmás ancient TB project, a general morpho-pathological study of the complete series is in progress in the frame of a PhD thesis at the University of Szeged (*e.g.* Lovász et al. 2008; Bereczki et al. 2009).

Molecular paleomicrobial analysis and the experimental procedures

The aim of the actual molecular paleomicrobiological analysis is to diagnose bacteria of the *Mycobacteria tuberculosis* complex in the Bácsalmás sample. Quite recently this study has been initiated in the ancient DNA Laboratory of the Institute for Mummies and the Iceman, EURAC Research, Bolzano, Italy. The studies are carried out in cooperation with researchers of the University of Szeged, Hungary. 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 ancient DNA: use of protective clothing, UV-light exposure of the equipment and bleach sterilization of surfaces, use of PCR workstations and filtered pipette tips.

Within a designated sample preparation room the surface of the rib samples was mechanically removed by using a dremel. So-cleaned samples were pulverized using a Retsch mixer mill.

DNA extraction was performed with approximately 150 mg bone powder by using two different extraction methods, a Phenol/Chloroform-based DNA extraction according to Hawass and colleagues (Hawass et al. 2010) and a Silica-based DNA extraction described by Rohland and collaborators (Rohland et al. 2009). Both methods were used with minor modifications.

There were two sets of primers used for molecular analysis of the samples, one pair targeting a short region of the

Table 1. Primer sequences and polymerase chain reaction conditions used in this study.

Target region	Direction	Primer sequence	Product size	Parameters
mtDNA hypervariable region 1	F	CACTAGGATACCAACAAACC	162 bp	95°C, 5 min; 95°C, 55°C, and 72°C, 45 sec each for 45 cycles; 72°C, 4 min
	R	GCGGGATATTGATTTCACGG		
IS6110 repeat element	F	CTCGTCCAGCGCCGCTTCGG	123 bp	95°C, 5 min; 95°C, 66°C, and 72°C, 45 sec each for 45 cycles; 72°C, 4 min
	R	CCTGCGAGCGTAGGCGTCGG		

Table 2. Details of the cases studied and the summary of the Polymerase chain reaction (PCR) results for both genetic targets. DNA extraction was performed using two methods.

ID number EURAC	Grave number Bácsalmás -Óalmás	Age (year)	Sex	Potential TB related lesion	Phenol/Chloroform-based DNA extraction		Silica-based DNA extraction	
					mtDNA*	IS6110**	mtDNA*	IS6110**
383	39	50-60	male	Spondylitis (Fig.2)	-	-	+	-
457	115	17-18	male	spondylitis	-	-	+	-
379	35	18-20	?	early-stage vertebral lesions	-	-	-	-
387	44	2-3	?	endocranial lesions	-	-	+	-
365	21	60-70	male	---	-	-	-	-
443	101	40-50	female	---	-	-	+	-
486	143	1-2	?	endocranial lesions (Fig.3,4)	+	+	-	+
507	165	40-50	female	early-stage rib lesions	-	+	-	-

* mtDNA Hypervariable region 1 PCR (162 bp fragment)
** IS6110 repeat element PCR (123 bp)

mtDNA hypervariable region 1 and another primer pair for amplifying the *Mycobacteria* multicopy IS6110 element (Table 1). 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 2µl of extracted DNA to a final volume of 20µl. Polymerase chain reactions were carried out according to the parameters in Table 1.

Results and Discussion

A subset of the Bácsalmás samples was examined and analyzed for the presence of *Mycobacteria tuberculosis* DNA (Table 2).

One individual (ID.EURAC 486, grave Nr 143) had a positive PCR result for the insertion sequence IS6110 in both DNA extraction methods. The skeletal remains show typical lesions of early-stage skeletal TB (endocranial lesions due to TB meningitis, Fig. 3-4).

Another *Mycobacteria* positive case (ID.EURAC 507, grave Nr 165) was only diagnosed in a Phenol/Chloroform-

Figure 3. Superficial inflammatory changes on the endocranial surface due to TB meningitis (Bácsalmás-Óalmás, Grave No 143, 1-2 year old Child). The presence of *M. tuberculosis* DNA is confirmed by two methods.

Figure 4. Endocranial superficial remodelling due to TB meningitis (Bácsalmás-Óalmás, Grave No 143, 1-2 year old Child). The presence of *M. tuberculosis* DNA is confirmed by two methods.

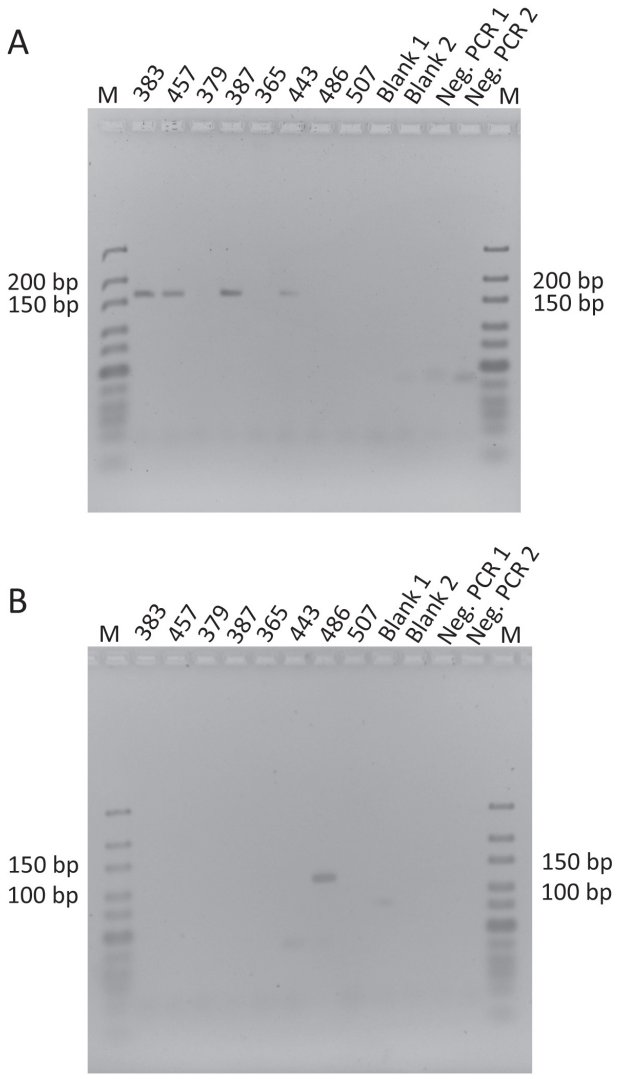

Figure 5. Analysis of the Polymerase chain reaction (PCR) amplification products using agarose gel electrophoresis. All displayed results were retrived with DNA of the Silica-based DNA-extraction method. (A) PCR amplification of a 162 bp fragment of the mtDNA Hypervariable region 1. (B) PCR amplification of a 123 bp fragment of the IS6110 repeat element.

based DNA extract. However, including also the results of the mtDNA hypervariable region 1 PCR the Silica-based DNA extraction clearly outperformed the Phenol-Chloroform extraction (Fig. 5).

A reason for this could be the higher extraction efficiency of the Silica method and/or the fact that less PCR inhibitory substances are co-extracted. To confirm this observation further comparative tests are required.

This preliminary molecular study already indicates the necessity in paleomicrobiology to adapt and test the planned molecular analysis on a subset of the samples before starting the analysis of a whole series. Apart from adapting to the samples the DNA-extraction methods (not so far rigorously tested for DNA-yield and DNA-purity in the aDNA field), we also recommend and plan testing the influence of the sampling site on the molecular diagnosis of a blood-borne pathogen in an individual. However, aforementioned pre-analyses can only be done if there is enough starting material available, which is not often the case in paleomicrobiology.

In the next steps of our project (2011-12), in addition to the methodological studies mentioned above, the paleomicrobiological analysis of the complete series and comparative studies of aDNA, as well as morphological analyses will be undertaken, in order to have a more precise picture of the prevalence of *M. tuberculosis* infection in this 16-17th century Hungarian population.

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