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New skeletal tuberculosis cases in past populations from Western Hungary (Transdanubia)

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ABSTRACT

The distribution, antiquity and epidemiology of tuberculosis (TB) have previously been studied in osteoarchaeological material in the eastern part of Hungary, mainly on the Great Plain. The purpose of this study is to map the occurrence of skeletal TB in different centuries in the western part of Hungary, Transdanubia, and to present new cases we have found. Palaeopathological analysis was carried out using macroscopic observation supported by radiographic and molecular methods. A large human osteoarchaeological sample ($n = 5684$) from Transdanubian archaeological sites ranging from the 2nd to the 18th centuries served as a source of material. Spinal TB was observed in seven individuals (in three specimens with Pott's disease two of which also had cold abscess) and hip TB was assumed in one case. The results of DNA for *Mycobacterium tuberculosis* were positive in seven of the eight cases identified by paleopathology, and negative in the assumed case of hip TB. However, the molecular results are consistent with highly fragmented DNA, which limited further analysis. Based on the present study and previously published cases, osteotuberculosis was found in Transdanubia mainly during the 9th–13th centuries. However, there are

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no signs of TB in many other 9th–13th century sites, even in those that lie geographically close to those where osteotuberculous cases were found. This may be due to a true absence of TB caused by the different living conditions, way of life, or origin of these populations. An alternative explanation is that TB was present in some individuals with no typical paleopathology, but that death occurred before skeletal morphological features could develop.

Introduction

Tuberculosis (TB) and other infectious diseases have been a major natural selective factor in human evolution and have deeply influenced population history and civilization (Marcsik et al., 2006; Ubelaker, 1996). TB affects all ages and ranges in severity from latent to hyperacute (Kelley and El-Najjar, 1980). It is responsible for more deaths than any other bacterial disease (Kelley and El-Najjar, 1980; Vincent and Gutierrez, 1999). It is caused by a group of closely related bacterial species termed the *Mycobacterium tuberculosis* complex, which are obligate pathogens. The principal cause of human TB is *Mycobacterium tuberculosis*. *Mycobacterium bovis* is the main cause of TB in other animal species. Human TB is principally transmitted via infectious aerosols released from the lungs of an infected person who has pulmonary disease. *M. bovis* can be acquired from animals by direct contact, or the ingestion of infected meat or dairy products (Aufderheide and Rodríguez-Martín, 1998; O'Reilly and Daborn, 1995; Vincent and Gutierrez, 1999).

With rare exceptions, skeletal TB is a secondary infection from either the lungs or the lymph nodes, the results of limited hematogenous dissemination (Panuel et al., 1999). The great majority of statistical analyses indicate that the three major sites of skeletal TB are the vertebral column (spinal TB), the hip, and the knee (Panuel et al., 1999). In general, the joints of the lower extremities are affected much more often than are the joints of the upper extremities (Steinbock, 1976).

Fig. 1. Sites of the examined skeletal material in the Carpathian Basin – hydrographic map in the 13th century (Bak, 2003): (1) Esztergályhorváti, (2) Zalavár (two sites), (3) Vörs (two sites), (4) Kaposvár, (5) Fonyód, (6) Kereki, (7) Dombóvár, (8) Szekszárd, (9) Daruszentmiklós, (10) Bölcske, (11) Zsámbék, (12) Budapest (four sites).

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Table 1
Some basic information about the examined skeletal materials from Transdanubian sites from the 2nd to the 18th centuries.

Site	Century	$N_{0–20\ years}$	$N_{adult\ ♂}$	$N_{adult\ ♀}$	$N_{adult\ ?}$	N_{total}	Authors
Budapest. II. ker. Sajka u. 4–6	2nd–3rd	2	3	3	1	9	Hajdu (in preparation)
Budapest II. ker. Lajos utca-Cserfa utca	2nd–3rd	2	2	1	0	5	Hajdu (in preparation)
Budapest III. ker. Graphisoft park	2nd–4th	149	220	220	16	605	Bernert (in preparation)
Szekszárd-Tószigeti dűlő	5th–8th	519	414	421	30	1384	Bernert (in preparation)
Dombóvár-Tesco	3rd–4th, 16th–17th	8	11	14	0	33	Pap-Hajdu (in preparation)
Daruszentmiklós (lh. F-05)	7–8th	8	6	8	0	22	Hajdu (in preparation)
Kaposvár (lh. 26)	8th	98	73	73	10	254	Évinger and Bernert (2005)
Kereki-Homokbánya	8th	36	64	49	4	153	Bernert (2003)
Esztergályhorváti-Alsóbáránd puszta	9th–10th	179	65	75	0	319	Marcsik et al. (2004)
Vörs-Papkert B	8th–11th	292	206	202	6	706	Bernert (in preparation)
Vörs-Majori dűlő	10th	145	122	116	5	388	Bernert (in preparation)
Zalavár-Vársziget-Hadrianus templom	9th	154	124	102	1	381	Évinger (in preparation)
Zalavár-Vársziget-Kápolna	11th–12th	55	108	94	6	263	Wolff et al. (2009)
Bölcske-Református templom	13th–16th	31	12	7	5	55	Pap-Hajdu (in preparation)
Zsámbék-Premontrei templom	12th–18th	135	165	87	7	394	Hajdu (2006)
Budapest II. ker Kapás utca	14th–16th	58	126	64	2	250	Bernert-Évinger-Hajdu (in preparation)
Fonyód-Bézsenypuszta	16th–17th	205	168	87	3	463	Bernert-Évinger (in preparation)
Total		2076	1889	1623	96	5684	

Skeletal TB is found mostly in the lower thoracic and upper lumbar vertebrae involving two to four vertebrae (Brothwell, 1963; Ortner, 2003). The central and anterior portions of the vertebral bodies are the most common sites (Aufderheide and Rodríguez-Martín, 1998; Ortner, 2003). The infection rarely involves the transverse processes, pedicles, lamina, or spinous processes of the vertebrae (Aufderheide and Rodríguez-Martín, 1998). As a result of the extensive trabecular and cortical destruction (without new bone formation), collapse of the involved vertebral bodies occurs (Panuel et al., 1999). The unequal collapse results in an angular posterior deformity or kyphosis (Aufderheide and Rodríguez-Martín, 1998; Ortner, 2003). A common complication of vertebral tuberculosis is the formation of unilateral or bilateral paravertebral abscess that can be accompanied by an associated fistula (Aufderheide and Rodríguez-Martín, 1998; Ortner, 2003).

Complicating the differential diagnosis, many conditions closely resemble spinal TB: e.g. compression fractures, pyogenic osteomyelitis, blastomycosis, brucellosis, actinomycosis, Paget's disease, neoplasms, metastatic carcinoma, etc. (Aufderheide and Rodríguez-Martín, 1998; Ortner, 2003).

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Table 2
PCR probe and primer sequences used in the biomolecular investigation.

PCR locus	Primer name	Primer sequence (5′–3′)	Amplicon size (bp)
IS6110	P1	CTCGTCCAGCGCCGCTTCGG	
IS6110	P2	CCTGCGAGCGTAGGCGTCGG	123
IS6110	IS-3	TTCGGACCACCAGCACCTAA	
IS6110	IS-4	TCGGTGACAAAGGCCACGTA	92
IS6110	FAM6110Probe	5′-FAM-ACCTCACCTATGTGTCGACCTG-BHQ1-3′	
IS6110	6110F	CACCTAACCGGCTGTGG	
IS6110	6110R	TGACAAAGGCCACGTAGG	75
IS1081	1081F2	CTGCTCTCGACGTTCATCGCCG	
IS1081	1081R3	TGGCGGTAGCCGTTGCGC	113
IS1081	FAM1081Probe	5′-FAM-GGGCTACCGCGAACGCA-BHQ1-3′	
IS1081	NF	TGATTGGACCGCTCATCG	
IS1081	NR	CTTGATGGGGGCTGAAGC	72

Palaeopathological research of skeletal TB in Hungary

Paleopathological studies of the distribution, antiquity and epidemiology of TB have previously been based on osteoarchaeological material from the eastern part of Hungary, mainly the Great Plain (Donoghue et al., 2005; Haas et al., 1999; Marcsik et al., 2002, 2006, 2007; Pálfi and Marcsik, 1999). In addition, there is one description of a severe case of skeletal TB (spinal TB and spina ventosa), which originated on the Great Plain (Bačka-Topola) at a site now in Serbia (Farkas et al., 1976).

The Dominican Church of Vác, a town near Budapest, is very important, because of the large number of spontaneously mummified persons that were buried there mainly in the 18th century. In total, 265 individuals in coffins and another remains of approximately 46 individuals from an ossuary were found, and among them, morphological and radiological studies revealed cases of spinal TB (Pott's disease and other vertebral destructions). Based on molecular analysis, the prevalence of TB was very high (Donoghue et al., 2011; Fletcher et al., 2003a,b; Pálfi et al., 2004; Pap et al., 1999, 2002, 2008; Spigelman et al., 2006).

Fig. 2. Probable hip tuberculosis (on the left side); Szekszárd-Tószegi dűlő, male, Object 1074.

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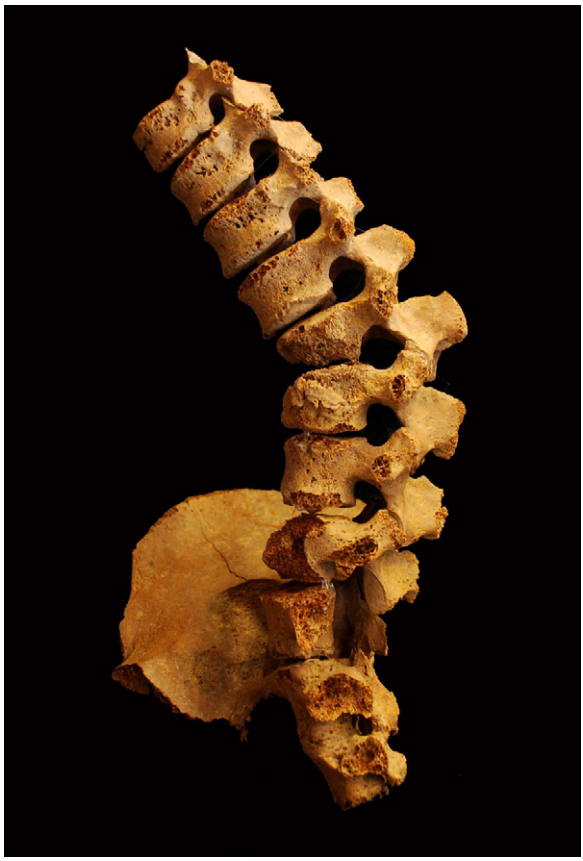

Fig. 3. Spinal tuberculosis in L2–3; Zalavár-Városziget-Hadrianus templom, juvenile, Grave No.: 135/01.

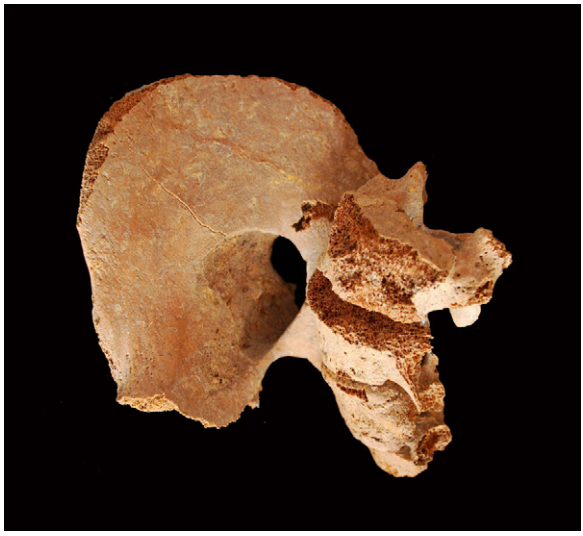

Fig. 4. The sign of cold abscess in the hip; Zalavár-Városziget-Hadrianus templom, juvenile, Grave No.: 135/01.

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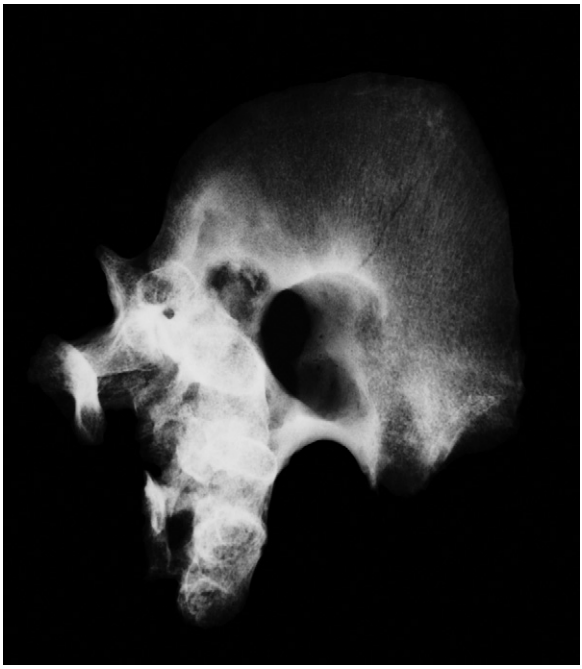

Fig. 5. Radiograph shows cold abscess and other lytic destruction, and the fusion in sacroiliac joint; Zalavár-Vársziget-Hadrianus templom, juvenile, Grave No.: 135/01.

In Western Hungary (Transdanubia), only isolated examples have been reported. An adult spinal TB case was described by Merczi (2001), and another adult case suggestive of spinal TB, dated to the 16th century, was reported by Éry (1982). The Székesfehérvár burial-ground includes skeletal material dated to various centuries; there, spinal TB was diagnosed in four cases (Donoghue and Spigelman, 2008; Éry et al., 2008a,b). Another case of spinal TB was described in material from Zalavár-Vár (Acsádi et al., 1962).

Fig. 6. Erosive changes in lumbar vertebra; Zalavár-Vársziget-Hadrianus templom, male, Grave No.: 50/01.

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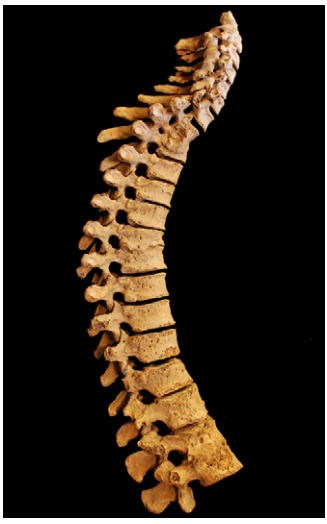

Fig. 7. Lateral view of the spine (right side) with lesions at three sites (Th1–3, Th7–8, L1–3); Zalavár-Vársziget-Hadrianus templom, female, Grave No.: 39/02.

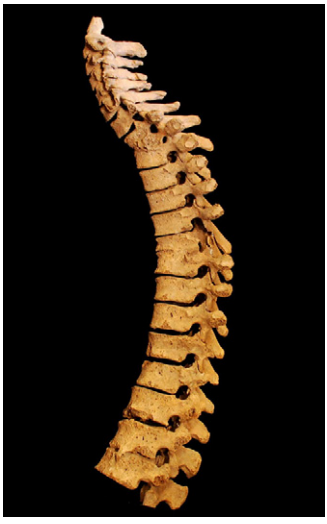

Fig. 8. Lateral view of the spine (left side) with lesions at three sites (Th1–3, Th7–8, L1–3); Zalavár-Vársziget-Hadrianus templom, female, Grave No.: 39/02.

The purpose of the current study was to map the occurrence of skeletal TB in various centuries in Western Hungary, Transdanubia, and to present new cases we have found.

Materials and methods

A large skeletal sample ($n = 5684$) served as a source of material for the palaeopathological and general anthropological investigations (Table 1 and Fig. 1).

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The skeletal materials are housed at the Department of Anthropology, Hungarian Natural History Museum, Budapest, and at the Balaton Museum, Keszthely, Hungary.

Palaeopathological analysis was based on macroscopic observation. For possible TB cases identified that way, further radiographic and molecular methods were used.

Due to the variation in the preservation of skeletons, it was not possible to apply statistical methods to characterise the distribution of skeletal tuberculous lesions. These lesions can be localised in different parts of the bones, but soil acidity and other forms of postmortem destruction can damage osteoarchaeological material, and obscure any possible evidence of disease. Furthermore, the bones of infants are often missing, therefore the exact prevalence of the disease in different populations and time periods cannot be presented.

Molecular analysis of DNA for the *M. tuberculosis* complex

Possible cases of skeletal TB, defined according to skeletal morphological changes, were examined for the presence of DNA from the *M. tuberculosis* complex. Recommended protocols for ancient DNA (aDNA) work were followed (O'Rourke et al., 2000; Taylor et al., 2010), with separate rooms and equipment for different stages of the process. A small quantity (50–100 mg) of each sample was crushed by a sterile pestle in a mortar and added to 400 μl of Proteinase K/EDTA. The slurry was incubated at 56 °C (Donoghue et al., 2005), and mixed on a bead beater daily. When the sample was solubilised, it was divided and one aliquot treated with 40 μl of 0.1 mol^{-1} of N-phenacylthiozolium bromide (PTB), to cleave any covalent cross-links thus enabling DNA strand separation and amplification (Poinar et al., 1998). Sample tube contents were transferred into separate 9 ml tubes of NucliSens® (bioMérieux) lysis buffer containing 5 mol^{-1} guanidium thiocyanate and incubated for 1–3 days at 56 °C. To complete the disruption of bone and any mycobacterial remnants, samples were boiled, then snap-frozen in liquid nitrogen and thawed in a 65 °C waterbath. This procedure was repeated twice. Sample tubes were centrifuged at 5000 × *g* for 15 min at 5 °C and the supernatants carefully removed into clean, sterile tubes. DNA was captured by adding 40 μl silica suspension (NucliSens®) and mixing on a rotator wheel for 1 h. Tube contents were centrifuged and silica pellets washed once with wash buffer (NucliSens®), twice with 70% (v/v) ethanol (−20 °C) and once with acetone (−20 °C). After drying in a heating block, DNA was eluted using 60 μl elution buffer (NucliSens®), aliquoted and used immediately or stored at −20°C. Silica supernates (500 μl) from PTB-negative samples were also collected from the 9 ml tubes of lysis buffer, and the 2.0 ml screw-capped Eppendorf tubes used to wash the silica. After chilling at 5 °C, supernates were mixed vigorously for 20 s with 200 μl of Protein Precipitation Solution (SLS Ltd., UK) and centrifuged for 3 min at 10,000 × *g*. Any pellet was discarded and 600 μl isopropanol (−20°C) added to 550 μl of each supernate. Tubes were mixed by inversion 50 times and spun 3 min. Supernates were discarded and tubes washed once with 500 μl 70% ethanol (−20°C). After draining, tubes were dried in a heating block. Any precipitated DNA was re-hydrated with 60 μl elution buffer (NucliSens®), aliquoted and used immediately or stored at −20 °C. Negative extraction controls were processed in parallel with the test samples.

DNA amplification and detection

Two specific regions of the *M. tuberculosis* complex were targeted in the repetitive elements IS6110 and IS1081. Initially primers were used which give PCR products of 129 bp (Eisenach et al., 1990) and 113 bp (Taylor et al., 2003), respectively. Subsequently, specific *M. tuberculosis* complex primers and fluorescent probes were designed, to enable shorter DNA fragments to be detected (Table 2). The PCR mix included 2 mM BSA to reduce PCR inhibition (Abu Al-Soud and Rådström, 2000; Forbes and Hicks, 1996), 2.0 mM $MgCl_2$ and annealing was at 60 °C. A hot-start Taq polymerase was used to minimise non-specific primer and template binding. Negative DNA extraction and PCR controls were processed alongside the test sample. Amplification was performed in a final volume of 25 μl using conventional PCR, on the Corbett Research RotorGene 3000 real-time platform (Taylor et al., 2007). PCR product from conventional PCR was detected by gel electrophoresis using 3.0% (w/v) NuSieve 3:1 agarose gel (FMC Bioproducts, Flowgen) in TBE buffer (0.09 mol^{-1} Tris-borate and 0.002 mol^{-1} EDTA) at 8.8 volts cm^1 for 80 min. Amplified DNA was visualized by ethidium bromide staining exposed under ultraviolet light

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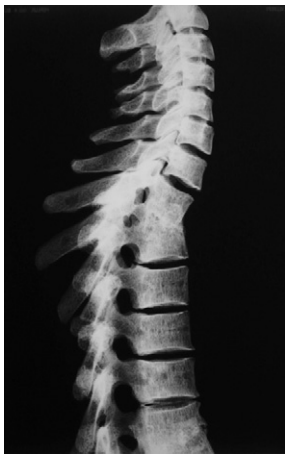

Fig. 9. Radiograph shows the fusion of Th1–3; Zalavár-Városziget-Hadrianus templom, female, Grave No.: 39/02.

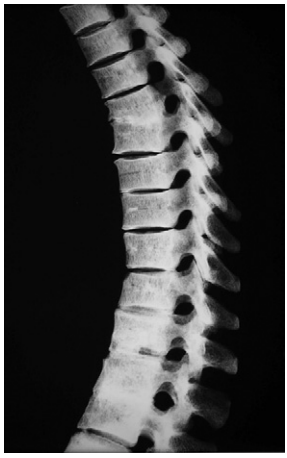

Fig. 10. Radiograph shows the fusion of Th7–8 and L1–3; Zalavár-Városziget-Hadrianus templom, female, Grave No.: 39/02.

and was recorded with a Polaroid camera. SYBR Green was initially included in the real-time PCR mix to directly visualize double-stranded DNA via fluorescence and the T_m determined by a subsequent melt analysis (Taylor et al., 2007). The specific probes enabled direct observation of specific amplicons and the determination of cycle threshold (C_t) indicated relative concentration of template. When sequencing was undertaken, PCR amplicons were separated on low melting point 2% agarose gels in TAE buffer. Bands of the anticipated molecular weight were excised under ultraviolet light with single-use sterile scalpel blades, prepared and sequenced, using a commercial DNA purification kit (Anachem Ltd. GeneClean® II) and sequencing service (Cogenics Ltd., Takely, Essex, UK).

Results

Based on skeletal morphological changes, eight cases of skeletal TB were identified in the sample. These new cases are described in detail below.

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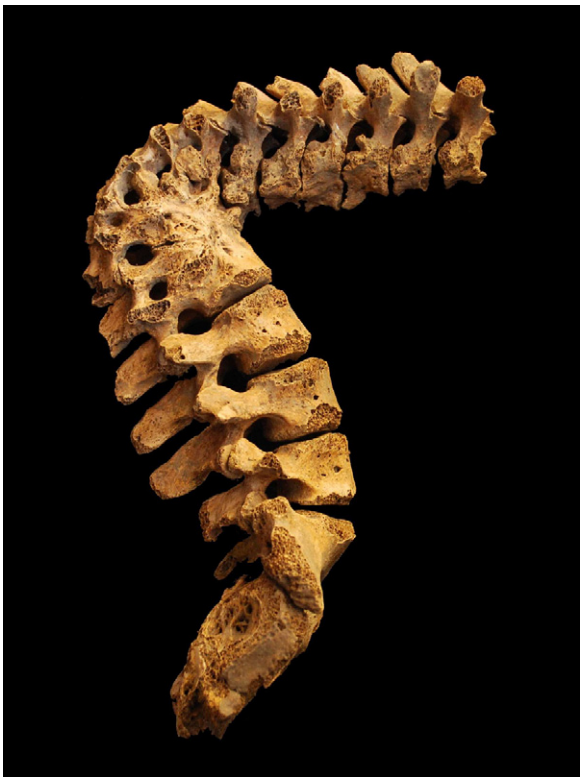

Fig. 11. Pott's disease, angular kyphosis in Th8–L2; Zalavár-Városliget-Kápolna, juvenile, Grave No.: 17/03.

Case reports

Site: **SZEKSZÁRD-Tószegi dűlő (6th–7th centuries)**
Grave no.: 1074, object (6th–7th centuries)
Inventory no.: 2008.7.132.
Male, 25–30 years

On the left side, the acetabulum and the head of femur have partly disappeared due to a disease process; subluxation and osteoporosis of the femur and the ilium are visible, and evidence of severe inflammation is present on the body of the femur and on the pelvis between the acetabulum and the greater sciatic notch. The acetabulum shows several defects and its articular surface is granular. The margin of the acetabulum is characterised by new bone formation probably representing a healing phase of this tuberculous infection. The lateral surface of the ilium is eroded (Fig. 2).

Diagnosis: probable hip tuberculosis, diagnostic options include other infectious processes.

DNA for *M. tuberculosis* complex: negative.

Site: **ZALAVÁR-Városliget-Hadrianus templom (9th–13th centuries)**
Grave no.: 135/01 (9th century)
Inventory no.: 2007.4.104.
Juvenile, 15–17 years

There are multiple destructive foci in the body of the second and third lumbar vertebrae; the body of L2 is partially missing (Fig. 3). The right auricular surfaces of the sacrum and the ilium are fused. Next to the fusion, there is a large perforation with a diameter of ~33 mm on the ala of ilium. Its surface is very rough and shows signs of inflammation. Similar severe destructions can be seen on the medial and lateral surfaces of the ala of ilium due to inflammatory processes (Fig. 4). On the radiograph

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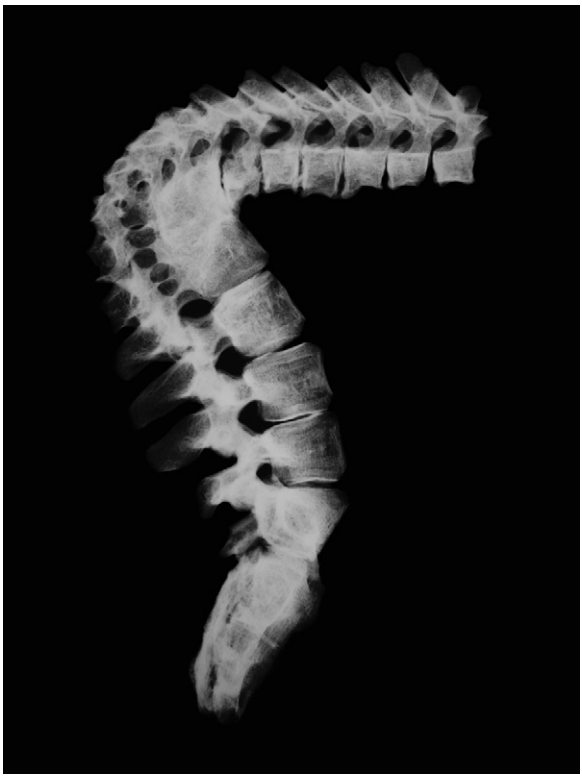

Fig. 12. Radiograph shows Pott's disease; angular kyphosis with osteosclerosis in Th8–L2; Zalavár-Városziget-Kápolna, juvenile, Grave 17/03.

showing the hip area, the large perforation can be demonstrated, and osteosclerosis is present at the area of the fusion (Fig. 5).

Diagnosis: spinal tuberculosis in the lumbar vertebrae, with prevertebral cold abscess.

DNA for *M. tuberculosis* complex: positive for IS6110 (75 bp).

Site: **ZALAVÁR-Városziget-Hadrianus templom (9th–13th centuries)**
Grave No.: 50/01 (9th century)
Inventory no.: 2007.4.37.
Male, 22–24 years

There are multiple small and larger confluent cavities with severe destruction on the anterior parts of the third and fourth lumbar vertebral bodies. Much of their spongious substance has disappeared mostly in the dorsal direction. These changes are localised only in L3–4 (Fig. 6).

Diagnosis: probably spinal tuberculosis.

DNA for *M. tuberculosis* complex: positive for IS1081 (113 bp).

Site: **ZALAVÁR-Városziget-Hadrianus templom (9th–13th centuries)**
Grave No.: 39/02 (9th century)
Inventory no.: 2008.4.118.
Female, 30–40 years

There are multiple destructive loci in the spine. Th1–3 are fused probably as a consequence of a fracture on Th2. Fused vertebra Th7–8, with the body height of Th8 being normal, but with the body

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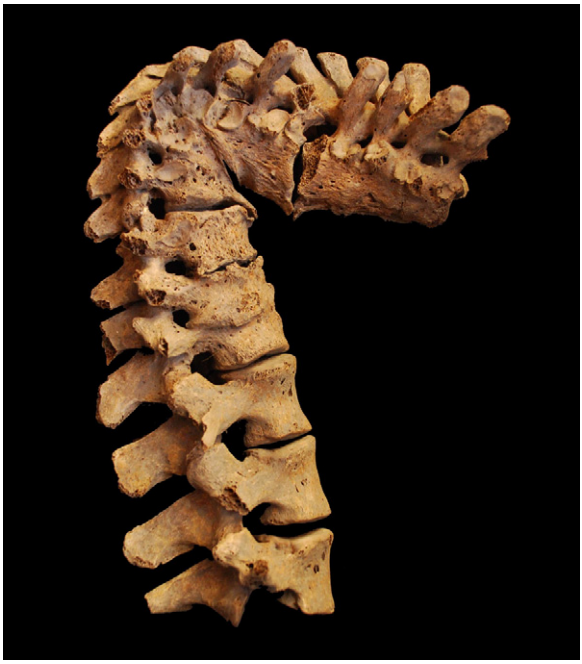

Fig. 13. Pott's disease; angular kyphosis in Th8–10, and three fused vertebral „blocks" (Th1–4, Th5–11, Th12–L2); Zalavár-Vársziget-Kápolna, juvenile, Grave No.: 32/02.

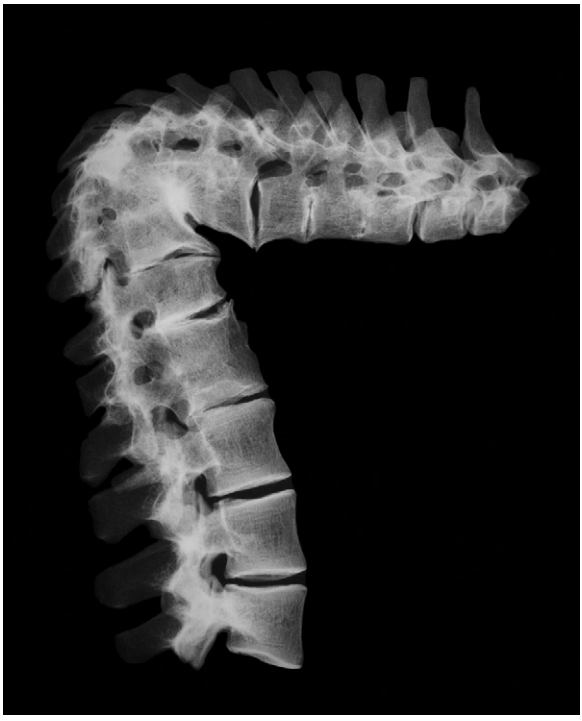

Fig. 14. Radiograph shows Pott's disease, angular kyphosis in the thoracic region; Zalavár-Vársziget-Kápolna, juvenile, Grave No.: 32/02.

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height of Th7 being decreased. The inferior surface of the body of Th8 and the superior surface of Th9 have multiple larger and smaller cavities. Lytic foci can be seen in the vertebral body of L1, and the L2 and L3 vertebrae have fused (Figs. 7–10).

Diagnosis: spinal tuberculosis (it is probable that the destructive process involved two sites: Th7–8 and L1–3).

DNA for *M. tuberculosis* complex: positive for IS6110 (75 bp) and IS1081 (113 bp).

Site: **ZALAVÁR-Városziget-Kápolna (11th–13th centuries)**
Grave No.: 17/03 (11th–13th centuries)
Inventory no.: 2008.9.17.
Juvenile, 15–16 years

The anterior portion of the vertebral bodies is seriously affected. An angular kyphosis (Pott's disease) is produced by the unequal collapse of Th10–11. There is a fusion from Th8 to L2. Pott's deformity is stabilized by new bone formation due to healing (Figs. 11 and 12).

Diagnosis: spinal tuberculosis (advanced Pott's disease).

DNA for *M. tuberculosis* complex: positive for IS6110 (75 bp).

Site: **ZALAVÁR-Városziget-Kápolna (11th–13th centuries)**
Grave No.: 32/02 (11th–13th centuries)
Inventory no.: 2008.9.101.
Male, 35–40 years

Several vertebrae show tuberculous destruction and collapse. There are three fused vertebral "blocks" in the spine (Th1–4, Th5–11, Th12–L2). A severe angular kyphosis is produced by the tuberculous collapse of Th8–10 (Fig. 13). On a lateral radiograph advanced osteosclerosis is shown at the angulation of the spine (Fig. 14).

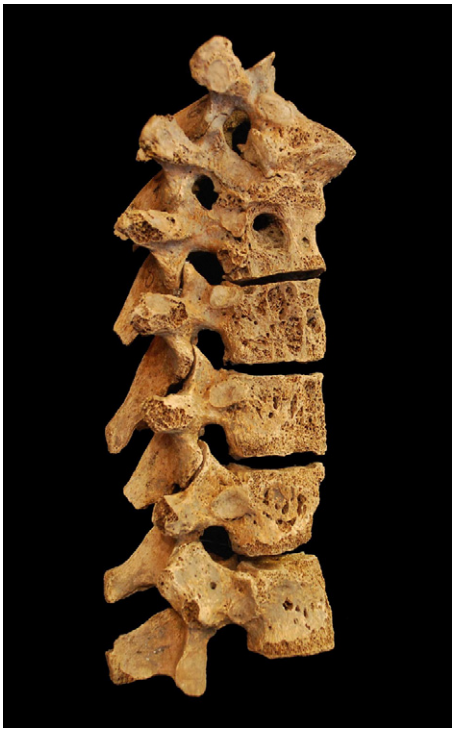

Fig. 15. Spinal tuberculosis, lateral view, fusion of vertebrae (Th6–8) with slight gibbus, cavities and traces of cold abscess; Zalavár-Városziget-Kápolna, juvenile, Grave No.: 74/03.

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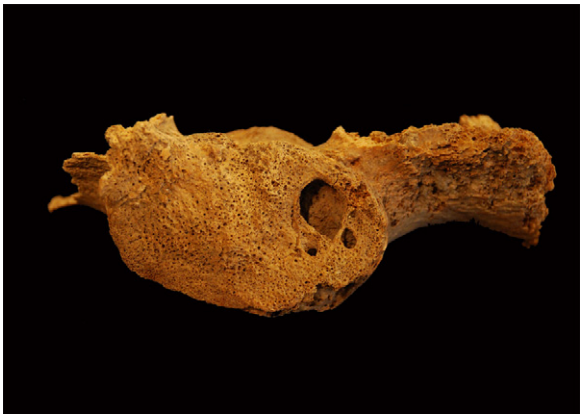

Fig. 16. Lumbosacral tuberculosis with erosion and cavities; Zsámbék, premontrei templom, female, Grave No.: 161.

Diagnosis: spinal tuberculosis (advanced Pott's disease).
DNA for *M. tuberculosis* complex: positive for IS6110 (75 bp).

Site: **ZALAVÁR-Városziget-Kápolna (11th–13th centuries)**
Grave No.: 74/03 (11th–13th centuries)
Inventory no.: 2008.9.69.
Male, 20–40 years

Fusion of vertebrae can be seen at Th6–8, with the body of Th7 having disappeared. The collapse has resulted in a slight gibbus in the infected vertebra (Pott's disease). The gibbus is wedge-shaped and cavities can be seen from anterior and lateral views. Effects of prevertebral cold abscesses can be found on the thoracic vertebrae 8–10 (Fig. 15).

Diagnosis: spinal tuberculosis with the sign of cold abscess.
DNA for *M. tuberculosis* complex: positive for IS6110 (75 bp), weak positive for IS1081 (113 bp).

Site: **ZSÁMBÉK – Premontrei templom (11th–18th centuries)**
Grave No.: 161 (18th century)
Inventory no.: 2002.2.82.
Female, 50–60 years

The fifth lumbar vertebra is fused with the sacrum. There is a resorption of the L5, and smaller and larger cavities with severe destruction, and resorptive changes can be viewed on the superior surface of the bodies L3–5. There are reactive bone formations on the anterior side of the sacrum (Figs. 16 and 17). (The lumbar 1–2, cervical and thoracic vertebrae are missing postmortem).

Diagnosis: spinal (lumbosacral) tuberculosis.
DNA for *M. tuberculosis* complex: positive for IS6110 (75 bp).

In summary, based on morphology of eight skeletal TB cases, spinal TB was observed in seven individuals, two of them with advanced gibbus and one with a slight gibbus. In two specimens the signs of cold abscesses were present. There was one case of probable hip TB from the Szekszárd-Tószegi dűlő site, although the morphological changes could be due to an infectious process other than TB.

Neither rib, nor endocranial lesions were observed in these cases.

Results of molecular examination

A positive result for the *M. tuberculosis* complex was obtained from two samples, with freshly eluted DNA, using the IS1081 primers that gave a PCR product of the expected size (113 bp) and T_m

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Fig. 17. Lumbosacral tuberculosis with erosion and cavities in the L4; Zsámbék, premontrei templom, female, Grave No.: 161.

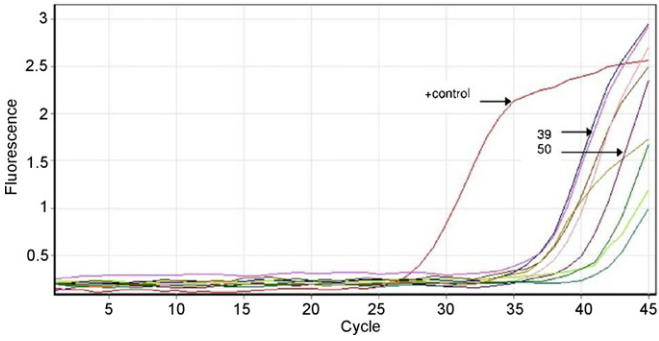

Fig. 18. Real-time PCR of archaeological samples using IS1081 primers.

(Figs. 18 and 19). Although the 113 bp PCR product from sample 50/01 was clearly demonstrable on the gel (Fig. 20), only a partial sequence was obtained (Figs. 21 and 22). However, no positive results were obtained with other primers for IS1081, nor with the IS6110 primers with target sequences of 123 bp. Five samples gave positive results for the shorter IS6110 locus of 75 bp. An example of a real-time PCR experiment is shown in Fig. 23.

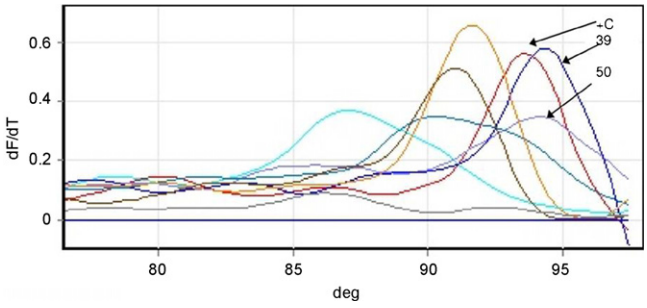

Fig. 19. Melt analysis of real-time PCR products.

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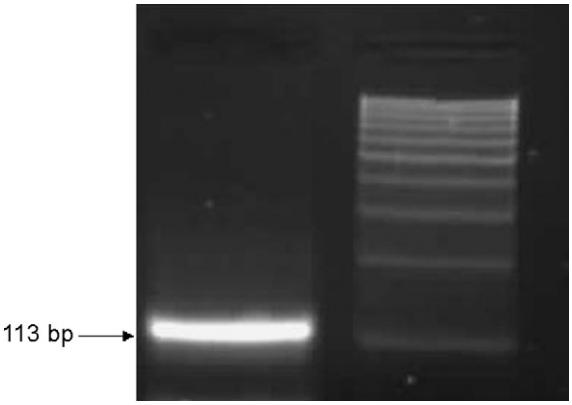

Fig. 20. Gel of purified IS1081 PCR product from sample 50/01 with molecular markers (bright line on the left indicates the 113 bp PCR product).

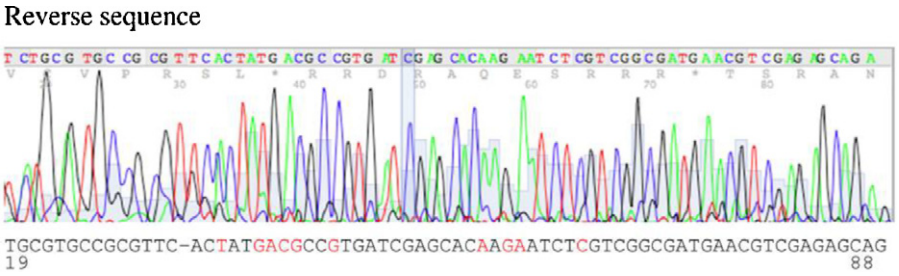

Fig. 21. Partial DNA sequence of IS1081 PCR product from sample 39/02.

Discussion

The biomolecular investigations were carried out following rigorous protocols for studies of ancient microbial DNA and with plentiful negative extraction and PCR controls. Each extraction was examined in quadruplicate, and extractions were repeated at least once in every case. The specific loci targeted on the insertion elements have a high percentage of guanine and cytosine bases (IS6110 60–65% GC and IS1081 68% GC), which confers enhanced stability due to the additional hydrogen bond in the DNA molecule. DNA specific for the *M. tuberculosis* complex was detected on one or more occasions in seven of the eight cases identified by palaeopathological macroscopic examination, and negative in one case. Five of the seven positive samples only yielded amplified DNA with specific probe and

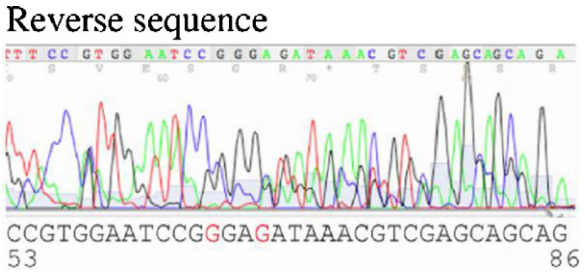

Fig. 22. Partial DNA sequence of IS1081 PCR product from sample 50/01.

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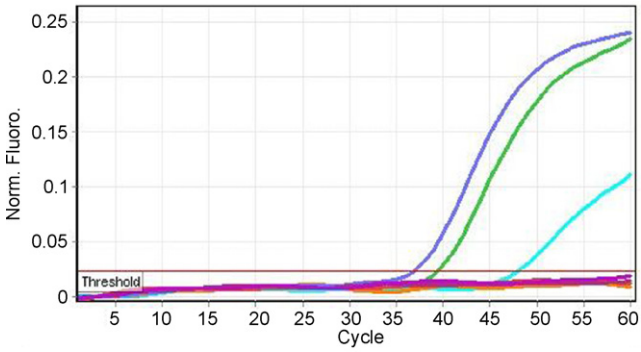

No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
5		32	Unknown	39.46			
7		39	Unknown	48.09			
9		wb1	NTC				

(Continued on next page)...

No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
14		74	Unknown	37.02			
18		wb2	NTC				
27		wb3	NTC				
31		EC	Negative Control				

Fig. 23. Real time PCR with IS6110 probe and primers showing cycling and cycle thresholds from samples 74/03, 39/02, 32/02 with negative extraction and PCR control.

primers for a 75 bp length of the IS6110 insertion element that can have up to 25 copies per bacterial cell in *M. tuberculosis*, which increases the sensitivity of the assay. These data are consistent with the DNA being in a highly fragmented state, presumably caused by taphonomic changes over the course of time. The IS1081 region is present at six copies per cell in all members of the *M. tuberculosis* complex. It is not possible to conclude whether the disease was caused by the human pathogen *M. tuberculosis*, or by another member of the complex such as *M. bovis*. However, where it has been possible to determine which member of the *M. tuberculosis* complex was responsible for past cases of archaeological tuberculosis, the overwhelming majority of cases have been caused by the human tubercle bacillus (Donoghue, 2008).

Throughout history, skeletal tuberculosis appears to occur sporadically. In Transdanubia, on the basis of the published and presented cases, more osteotuberculosis was observed during the 9th–13th centuries (e.g. Zalavár-Vársziget, Székesfehérvár). However, there are no signs of osteotuberculosis in geographically closer regions of Zalavár and Szekszárd in Transdanubia from the same centuries (Kaposvár, Kereki, Esztergályhorváti, two sites at Vörs, Fonyód; Table 1 and Fig. 1). Thus we can reach no clear conclusions from such a small number of positive TB cases. It is theoretically possible that these other contemporaneous populations experienced different conditions (e.g. lived in smaller communities than the once densely populated Zalavár, that was a main regional centre at that time), had adopted an alternative lifestyle and/or were of a different origin that reduced their susceptibility to the disease. Alternatively, TB may have been present but without the development of skeletal lesions, as it is estimated that such lesions occur only in 3–5% of TB cases from the pre-antibiotic era (Resnick and Niwayama, 1995). Further investigations should include specimens with no visible lesions to examine this possibility. However, the differential preservation of skeletal remains and the relatively limited number of excavated sites and examinable specimens pose problems in drawing broader conclusions on the palaeoepidemiology of historical tuberculosis in this region.

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According to Roberts and Buikstra (2003) skeletal TB appeared in Hungary at least 1300 years ago. However, an adult spinal tuberculosis case was published from the Roman Period by Merczi (2001) and a case of hypertrophic osteoarthropathy, consistent with TB, has been described (Masson et al., 2008) in a Neolithic population from the Great Plain of Southern Hungary from the Late Neolithic tell settlement (Tisza Culture) of Hódmezővásárhely-Gorzsa (4790–4594 BCE). Thus, the appearance of osteotuberculosis dates back to even earlier centuries. Based on previous studies, skeletal TB seems to be the most widespread during the 7th–8th centuries, less so during the 10th–11th centuries, and more during the 14th–17th centuries in Hungary (Pálfi and Marcsik, 1999). The present study expands and contributes to the general knowledge about the occurrence of skeletal TB in past populations in Hungary, and confirms earlier conclusions, that severe lesions such as the signs of cold abscess and Pott's disease (advanced angular kyphosis) are good indicators of TB.

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