Widespread Occurrence of *Mycobacterium tuberculosis*-DNA from 18th-19th Century Hungarians

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ABSTRACT A large number (265) of burials from 1731-1838 were discovered were discovered in sealed crypts of the Dominican Church, Vác, Hungary in 1994. Many bodies were naturally mummified so that both soft tissues and bones were available. Contemporary archives enabled the determination of age at death, and the identification of family groups. In some cases, symptoms before death were described and, occasionally, occupation. Initial radiological examination of a small number of individuals had indicated calcified lung lesions and demonstrable acid-fast bacteria suggestive of tuberculosis infection. Tuberculosis was endemic in 18th–19th century Europe, so human remains should contain detectable *Mycobacterium tuberculosis* complex (MTB) DNA, enabling comparisons with modern isolates. Therefore, a comprehensive examination of 168 individuals for the presence of MTB DNA was undertaken. Specific amplification methods for MTB showed that 55% of individuals were positive and that the incidence varied according to age at death and sampling site in the body. Radiographs were obtained from 27 individuals and revealed an association between gross pathology and the presence of MTB DNA. There was an inverse relationship between PCR positivity and MTB target sequence size. In some cases, the preservation of MTB DNA was excellent, and several target gene sequences could be detected from the same sample. This information, combined with MTB DNA sequencing data and molecular typing techniques, will enable us to study the past epidemiology of TB infection, and extends the timeframe for studying changes in molecular fingerprints.

A large number of well-documented, naturally-mummified individuals were discovered in sealed crypts during reconstruction work in the Dominican Church of Vác, Hungary (Pap et al., 1999). The crypts were used continuously for burials of several middle class families and clerics, from 1731-1838. The crypts were cold but dry. The ambient temperature was about 10°C and there was very poor but continuous ventilation through two small air vents at either end of the crypts. In addition, the remains were protected against humidity by coffins, many of which were filled with pine shavings, and which were stacked up to the ceiling of the crypts. These were ideal conditions for natural preservation, and 70% of the bodies were totally or partially naturally mummified. Examination by computed

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tomography (CT) full-body scans of a number of bodies indicated three individuals who showed evidence of tuberculosis. This was confirmed by sampling one of these individuals, i.e., a 56 year-old man who died in 1783. Histological examination of a sample from his right lung showed acid-fast intracellular bacteria, and polymerase chain reaction (PCR) amplification detected *Mycobacterium tuberculosis* complex (MTB)-specific DNA (Pap et al., 1999).

Following this, a comprehensive study was undertaken to determine the extent of TB infection, its distribution in the town community, and to examine the feasibility of molecular fingerprinting of MTB DNA. Tuberculosis was endemic in Europe during the 18th–19th centuries, but only reached its peak during industrialisation (Hutás, 1999). The Vác material predates this, so it should be possible to examine the types of MTB strains in circulation before the main epidemic expansion of the disease in Hungary.

MATERIALS AND METHODS

Analysis of the human population buried in the crypt

Contemporary written records are available for many individuals and include date of death, age, sex, family name, relatives and sometimes a brief description of cause of death. These records enabled several bodies to be placed into family groups. Where records were not available, the remains were examined to determine the sex and provisional age of the individual by visual examination of bones, joints and teeth. Well-preserved remains from 27 individuals were subjected to radiographic examination.

Samples

Over 350 samples from 168 individuals were collected, including lung, pleura, abdomen, ribs, hair, teeth and clothing, taking precautions against contamination. Protective clothing was worn and new gloves used for each individual. Where body cavities were intact, samples were taken using a no-touch technique with an endoscope that was decontaminated between samples with detergent and 70% ethanol. Samples were placed into sterile containers and stored at 4 ^{o}C. Where remains were skeletalised, a lower rib was taken as the sample from that individual.

Precautions against contamination

Stringent precautions were taken against cross-contamination in the laboratory. No work with MTB cultures or tuberculosis clinical specimens has ever been carried out in any of the laboratories used. Clean protective clothing was worn with frequent glove changes. Different workstations were used for DNA extraction and PCR set-up. Separate rooms were used for PCR set-up, preparing nested PCR reactions and the electrophoresis of PCR product, with different sets of pipettors and protective clothing. Pipettors and surfaces were cleaned with neat household liquid detergent, rinsed with ultrapure water and dried with ethanol before use. Sterile tubes and plugged tips were used. Wherever possible,

sterile reagents were purchased (not prepared in the laboratory), and pre-aliquoted PCR mix was purchased for DNA amplification (ABGene®, Advanced Biotechnologies Ltd, Epsom, UK).

DNA extraction

A demineralization solution consisting of 100 µl 0.5 M EDTA at pH 8.0 plus proteinase K (1 mg/ml) was dispensed into sterile 1.5 ml tubes, each containing 10 glass beads (1-2 mm). Approximately 25 mg of sample was added, tubes were mixed on a mini bead beater (Stratech Scientific Ltd, Luton, Bedfordshire, UK) and incubated at 56 ^{o}C overnight. Extraction negative control tubes were always processed in parallel with tubes containing samples. Lysis buffer L6 (250 µl) was added (Boom et al., 1990), and tubes were mixed briefly and incubated at 37 ^{o}C for 2 h . After remixing, silica suspension (SiO_2 12% (w/v), pH 2.0) was added (25 µl), and tubes were mixed and gently shaken for 1–2 h at room temperature to allow cell lysis and adsorption of DNA to the silica. Tubes were remixed and centrifuged for 1 min at 13,000*g*, and the were supernates collected into new sterile tubes. The silica was washed with 100 µl washing buffer L2 (Boom et al., 1990), washed twice with 70% (v/v) ethanol at –20 ^{o}C, and once with acetone at –20 ^{o}C. Tubes were drained on clean, absorbent paper and dried in a 56 ^{o}C heating block. DNA was eluted with 50 µl ultrapure water (Sigma) and incubation proceeded at 56 ^{o}C for 30 min. This was repeated once; the eluates were pooled and stored at -20 ^{o}C.

Where silica eluates gave negative results, the silica supernates were processed. After remixing, the suspension was recentrifuged for 3 min and the supernate collected, chilled, and processed by a modified protocol for the Puregene™ DNA extraction kit (Flowgen Instruments Ltd, Lichfield, UK). Protein precipitation solution (200 µl) was added to each tube, which was vortex-mixed 20 sec and centrifuged for 3 min at 13,000g. The supernate was added to 600 µl isopropanol at –20 ^{o}C to precipitate any DNA. The tube was inverted 50 times, chilled, and spun 3 min, and the supernate was discarded. Ethanol (70% v/v), pre-cooled to –20 ^{o}C was added (600 µl), and the contents were mixed by inversion and centrifuged for 2 min at 13,000*g*. The tube was drained on clean, absorbent paper and dried in a 56 ^{o}C heating block. Samples were re-hydrated with100 µl of water and incubated at 56 ^{o}C. The sample was stored at –20 ^{o}C until use (Donoghue et al., 1998).

Some samples were extracted using the Qiagen® DNeasy Tissue Kit (Qiagen Ltd, Crawley, UK), which was used according to the manufacturer's instructions with the following adaptations. Briefly, 25 mg of sample was added to a 1.5-ml sterile screw-capped Eppendorf tube which contained 0.5 mm glass beads to one third of the volume and 200 µl demineralization solution (see above). The sample was incubated overnight at 56 ^{o}C, and homogenized on a minibead beater for 50 sec at 200 rpm. The homogenized sample was transferred to a column and processed according to the instructions from Qiagen Ltd.

DNA amplification to detect *M. tuberculosis* complex-specific DNA

All samples were screened for the presence of MTB DNA by PCR (Table 1). Primers P1 and P2 target a 123-bp region of the insertion sequence IS*6110* for DNA amplification (Eisenach et al., 1990). This element is normally present in multiple copies in

M. tuberculosis isolates, which increases the likelihood of detection of MTB DNA. Negative samples were screened by nested PCR (Taylor et al., 1996), where a second set of primers, IS3 and IS4, which bind internally with some overlap to the outer primers, amplify a 92-bp product.

Prealiquoted double-strength PCR mix was purchased (ABGene®). The final composition of the PCR mixture (50 µl) was: 75 mM Tris-HCl (pH 8.8); 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 0.01% (v/v) Tween-20; 200 µM (each) dATP, dCTP, dGTP and dTTP; primer, 0.5 µM (each); and 1.25 units *Taq* DNA polymerase. Bovine serum albumin (BSA) was added to give a final concentration of 10 mM, as this has been shown to improve the yield (Forbes and Hicks, 1996; Abu Al-Soud and Rådström, 2000). The primer pair and DNA preparation (5.0 µl) were added to each prealiquoted tube plus sufficient water to bring the volume to 50µl. Tubes with water in place of template were always included as negative controls.

Amplification with primers P1/P2 consisted of initial denaturation at 94 ^{o}C for 4 min, followed by 45 cycles of strand separation at 94 ^{o}C for 40 sec, primer annealing at 68 ^{o}C for 1 min, and strand extension at 72 ^{o}C for 20 sec; this was followed by 1 min of strand extension at 72 ^{o}C. In the nested PCR, only 25 cycles of amplification were used, with 0.5 µl of P1/P2 product, and primers IS-3 and IS-4. This second stage PCR was identical to the first, with the exception that primer annealing was at 58 ^{o}C.

In cases where the DNA had been extracted with the Qiagen DNeasy kit, the Qiagen® HotStar Taq® Master Mix Kit with 10% (v/v) Q solution was used. *Taq* polymerase was activated by heating for 15 min at 95 ^{o}C, which was followed by 40 cycles of strand separation at 94 ^{o}C for 1 min, primer annealing at 68 ^{o}C for 1 min, and strand extension at 72 ^{o}C for 1 min. This was followed by final strand extension at 72 ^{o}C for 10 min.

Samples yielding a 123-bp product were examined for other target gene sequences, to determine the extent of DNA preservation and to explore the characteristics of tuberculosis strains from the 18th century. Samples were examined for the presence of silent point mutations in codon 463 of *kat*G and codon 95 of *gyr*A (Sreevatsan et al., 1997), as this enables genotyping of TB strains (Table 2). In addition, PCR was performed using a target sequence in the 19-kDa antigen gene (131 bp; Mustafa et al., 1995). Better-preserved specimens were examined for the MPB70 antigen gene (372 bp; Cousins et al., 1992). In addition, a series of PCRs were performed to detect the presence of an IS*6110* insertion in the *dnaA-dnaN* region specific for the multi-drug-resistant Beijing family of MTB (Kurepina et al., 1998). Strains without this specific insertion yield a PCR product of 151 bp. Details of all reactions are listed in Table 1.

Verification of data

Twenty-seven samples from 26 individuals were examined independently by between 1–4 separate laboratories based in London, The Netherlands, Israel and Australia, using a variety of MTB target sequences. Two of these were in Departments of Medical Microbiology, and two were in nonmedical departments which do not work with MTB.

Detection and analysis of amplified DNA

PCR product (7µl) was added to 4µl loading buffer (Sigma) and electrophoresed in a 3.0% (w/v) NuSieve 3:1 agarose gel (Flowgen) in TBE buffer (0.09 mol Tris-borate and 0.002 mol EDTA) at 8.8 volts/cm for 80 min. Amplified DNA was visualized by ethidium bromide staining plus ultraviolet light, and was recorded with a Polaroid camera or a digital image capture system. Quantification of amplified DNA was performed by intensity densitometry of DNA bands on gels, in comparison with a DNA mass marker (Gibco BRL).

Where appropriate, purified amplified DNA was sequenced by MWG-BIOTECH GmbH, Qiagen Ltd., or an in-house sequencing service in the Department of Biology, University College London.

Statistical analysis

The χ^2 test was used to determinine the significance of any association between MTB positivity and other factors. A probability level ≤ 0.05 was deemed significant. The sign test was used to examine any association of MTB positivity with family group.

RESULTS

Detection of MTB DNA by PCR

Nested PCR based on IS*6110* with a product of 92 bp was the most sensitive method. Single-stage IS*6110* PCR detected only 51% of samples positive by nested PCR. Other target sequences detected by PCR included sites in the 19-kDa antigen gene; the *dnaA-dnaN* spacer region; and the MPB70 antigen gene. Preliminary experiments in a separate laboratory confirmed that single-stage PCRs based on target sites ranging from 92–372 bp gave comparable yields when modern DNA from MTB or *M. bovis* was used as the template and the same number of amplification rounds were used. Only 4/20 of samples positive by nested IS*6110* PCR were also positive for the MPB70 PCR, with a target sequence of 372 bp (Fig. 1), which suggests both that amplicon size is a crucial factor, and that some specimens contained extremely well-preserved MTB DNA. In addition, quantification of amplicons from seven well-preserved samples that were each subjected to the same four PCRs, demonstrated that whilst similar yields of 92-bp product were obtained from each, the number of amplified copies/µl fell with increasing target size or became undetectable.

DNA extraction with the Qiagen® Dneasy Tissue Kit gave excellent results when used in conjunction with the Qiagen® HotStar Taq PCR, and was used when PCR products were to be sequenced.

Results from independent laboratories confirmed the presence of MTB in 23/27 samples. In one sample, a doubtful result was obtained. The three remaining samples were positive by nested IS*6110* PCR, but negative when examined by single-stage PCR. The one independent laboratory that examined these samples found them negative by single-stage PCR for IS*6110*. Therefore, all results were compatible with those obtained in our laboratory.

Presence of MTB DNA in relation to age of individuals

To date, 168 bodies have been examined by specific PCR for MTB. Results were analyzed by age of the individual, sex, sampling site, and type of PCR used (Donoghue et al., 2000). Age at death peaked between 51–60 years, but a small number of individuals survived >80 years. Overall, 93/168 (55%) of the individuals were MTB positive (Fig. 2), but this varied according to age, and 79% of those aged 51-60 years were MTB-positive. Of 6 teenagers, 4 were MTB-positive. Comparing age groups from 0–20 years, 21–40 years, successive 10-year groups from 41–70 years, and >70 years, there was a significant difference in MTB positivity with age ($P<0.05$). There were greater proportions of MTB-positive individuals aged 21–40 years and 51–60 years. Examining the 69 individuals aged 41–50 years, 51–60 years, and 61–70 years, there was a greater proportion of individuals aged 51–60 years positive for MTB DNA ($P<0.01$).

As infection with MTB is thought to occur early in life, results were examined in relation to year of birth. The proportion of MTB-positive bodies was approximately 48% for individuals born before 1740. Thereafter, the proportion rose and reached a peak between 1760–1774, when all 15 individuals born during this period were MTB-positive.

MTB-positivity in relation to other host factors

There were 95 males, 70 females and 3 individuals of unknown sex examined. It appeared that a higher proportion of males (59%) were MTB-positive, compared with females (44%). However, this was not statistically significant.

There was no statistically significant association between MTB infection and family group (Fig. 3). Numbers were too low to indicate any association between occupation and MTB infection, but the three surgeons identified in the group were MTB-positive. There were nine clerics, identified by their clothes and/or from the archive, including priests and more senior churchmen. Six were MTB-positive, but the same proportion was found in nonclerical men of similar age.

Effect of sampling site

Of 253 samples examined, they were equally likely to be MTB-positive from the abdomen (46%), chest (47%) or ribs from totally skeletalised remains (43%). A higher proportion of pleural samples were positive (68%), but this was not statistically significant due to the small number of samples (22). In some instances, chest samples were taken specifically from the left and/or right side of the chest. When these were analyzed together with the rib and pleura samples (112 samples in total) for the proportion that was MTB-positive, a significant difference was found ($P<0.05$), with a greater proportion of MTB-positive samples from the left chest (14/22) and from pleura (15/22). Only 7/22 of samples from the right chest were MTB-positive.

In 112 individuals, samples were examined from a single site, and 53 were MTB-positive (47%). However, when multiple samples were examined, the proportion of MTB-positive individuals was significantly greater (69%; $P<0.01$). Of these 56 individuals, 19

were positive only at a single site; 6 were positive at different but similar sites, such as the left and right chest; and15 were positive at two or more different sites, such as the chest and abdomen. The remaining 15 individuals were negative for all samples.

Gross morphology and MTB-positivity

Of those individuals who were radiographed, 14/27 showed signs of possible lesions, and of those with chest samples examined for MTB DNA, 11/14 were positive, with good correlation between putative lesions and the site of MTB-positive samples. One of these individuals, who apparently had multiple foci in his lungs, was markedly cahectic (Fig. 4). Six individuals with apparently normal radiographs were MTB-positive. These included one girl aged 9 years; another aged 14 years who was extremely small for her age; her 28-year-old sister, who was also extremely small for her age; a woman of 26 years whose pleura and hair were found to be MTB-positive and who died 2 weeks after childbirth; a 37-year-old woman who died of a "painful wound"; and a 36-year-old man who was found during sampling to have a calcified nodule in the chest and who was described in the archive as having died suddenly with blood from the mouth, after long-term spitting of blood. Comparison of all radiographed individuals showed a significant difference ($P < 0.05$) between those who demonstrated gross pathological signs (radiographs, stunted growth or reported major haemoptysis) and those who had no such abnormalities. Two of the radiographs showed gross spinal deformities (e.g. Fig. 5), suggestive of tuberculosis, and MTB DNA was detected in each. In addition, there were two further individuals who had visible deformities of the spine, another with deformed ribs, and one individual who was noticeably cahectic. All were MTB-positive.

In contrast, the oldest individual known to be buried in the crypt, a well-nourished 95-year-old woman (Fig. 6), had a small calcified lesion visible on radiograph in the left hilar region of the lung (Fig. 7). This was the only site to give a positive result for MTB DNA, as her right lung and abdomen samples were negative. It is suggested, therefore, that this individual and the 18 others who were MTB-positive at only one of multiple sites examined, had latent rather than active MTB infection**.**

Characteristics of MTB DNA

None of the specimens which have been examined to date for the presence of an IS*6110* insertion in the *dnaA-dnaN* region specific for the multidrug-resistant Beijing family of TB had this specific insertion. Sequencing of 11 samples for the presence of silent point mutations in codon 463 of *katG* and codon 95 of *gyrA* has placed these strains into genotypic group 2 or 3 (Table 3).

DISCUSSION

The remains were not subjected to contact or contamination from soil, and *M. tuberculosis* has no natural habitat other than an infected host. The PCR primers devised by Eisenach et al. (1990) are specific for MTB DNA, and the detection of other MTB-specific target DNA sequences in well-preserved samples confirms the original findings. In

addition, in many cases there was clear correlation between pathological sites and the detection of MTB. Therefore, it was assumed that MTB-positive individuals were infected with *M. tuberculosis* and that the results were not due to external contamination from the environment or the investigators. The initial reports of the detection of MTB DNA in archaeological remains (Spigelman and Lemma, 1993; Salo et al., 1994) have been supported by a growing body of work which makes it clear that MTB DNA can be detected in a wide variety of specimens which range in age and geographical location from ancient Egypt (Zink et al., 2001); pre-Colombian America (Arriaza et al., 1995; Braun et al., 1998); the Middle East (Donoghue et al., 1998) and Europe (Baron et al., 1996; Taylor et al., 1996, 1999; Faerman et al., 1997; Haas et al., 2000; Mays et al., 2001).

The inverse relationship between PCR positivity and target size was noted previously (Hagelberg and Sykes, 1989; Pääbo, 1993; Höss et al., 1996) and is believed to be due to the fragmentation of DNA over time. Indeed, this is typical of ancient DNA, and has been recommended as one of the criteria to be used in verifying ancient DNA work (Cooper and Poinar, 2000; O'Rourke et al., 2000). Even in positive samples it was possible to demonstrate an inverse relationship between copy number of the amplified sequence and target size, which does not occur in modern DNA.

The higher proportion of pleural samples that were positive (68%) confirms earlier findings that these can be a reservoir of MTB DNA (Donoghue et al., 1998). A slightly higher proportion of primary tubercular lesions in the right chest compared to the left were reported by clinicians in the early 20th century (reviewed by Garay, 1996). It was believed to be partly due to the local differences in anatomy of the left and right bronchi. However, it appears that there is a different distribution of lesions in chronic pulmonary tuberculosis. In a study of rib lesions from the remains of individuals who were known to have had clinical tuberculosis (Kelley and Micozzi, 1984) twice as many lesions were found in ribs from the left chest, compared to the right. Similar data were reported from the early 19th century, as reviewed by Kelley et al. (1994). Further, in a review of rib lesions in archaeological material, Roberts et al. (1998) noted that new bone formation on rib surfaces tended to be more common on the left rather than the right side of the ribcage, and that it was highly probable that such lesions were the result of pulmonary tuberculosis. Our initial findings support this conclusion, although additional samples should be examined.

The comparable recovery of MTB DNA from bone and mummified tissue is in contrast with findings from studies of mammalian DNA (Lassen et al., 1994). A preliminary report of human DNA in these Hungarian remains (Matheson et al., 2000 and personal communication) confirmed that it is much more difficult to detect human DNA than mycobacterial DNA in the same samples of mummified tissue.

Mycobacteria produce cell walls of unusually low permeability, which contribute to their resistance to environmental stresses. Their cell walls contain large amounts of C60–C90 fatty acids, mycolic acids, and many extractable lipids. Most of the hydrocarbon chains of these lipids assemble to produce an asymmetric bilayer of exceptional thickness (Brennan and Nikaido 1995; Daffe and Draper 1998). This resistant cell wall is believed to be responsible for the persistence and survival of tubercle bacilli throughout the lifetime of a mammalian host and the initial decay process after death (Weed et al., 1951; Sterling et al., 2000). In addition, DNA survival is further enhanced in prokaryotes by biocrystallization (Wolf et al., 1999). The persistence of acid-fast bacilli in a calcified lung

lesion from a Vác sample was reported earlier (Pap et al., 1999). Therefore, it is not surprising that some tissue samples from the Vác material contained MTB DNA that was sufficiently well-preserved to enable amplification of PCR targets from several different single-copy genes, including some >200bp in length.

The incidence of infection with *M. tuberculosis*, indicated by the presence of MTB DNA, may appear extremely high, but it must be remembered that this was a population where the disease was endemic. It is very probable that the number of individuals with active tuberculosis was considerably lower, as the large majority of individuals infected with *M. tuberculosis* suffer no recognizable ill-effects (Kochi, 1991). Analysis of the individuals where only one of several different sites were positive indicates that, as a conservative estimate, 39% had highly localised, possibly latent tuberculosis. It has been estimated that perhaps between one quarter and one third of patients with untreated pulmonary tuberculosis heal spontaneously, although the average survival rate of the others is only 14 months (Springett, 1971). It is likely that the majority of cases with MTB present in both chest and abdomen, suffered from open pulmonary tuberculosis, with secondary infection of the abdomen caused by swallowing sputum. In some instances the contemporary archive described symptoms consistent with open pulmonary tuberculosis. For example, the Director of the Institute for the Deaf and Mute died in 1808 at age 36 years. The archive states that he had suffered from long-lasting spitting of blood and died suddenly, vomiting blood. Both his abdominal contents and calcified lung lesions were MTB positive. However, the initial index case, who had both a positive chest and abdomen sample, showed no sign of wasting and the histopathology suggests that he died at age 56 years from heart failure.

The human population under study was from a prosperous neighbourhood. According to the contemporary archive, many of the families were professionals or skilled artisans. It was noted that individuals buried in the church had German or Hungarian names. However, the ethnic origin of the individuals has not yet been confirmed by molecular methods, so these putative data were not included in the analysis. Tuberculosis is known to be spread by aerosols, and the pattern of spread differs in high- and low-prevalence areas. In areas of low prevalence spread occurs frequently within the family. In contrast, in high-prevalence areas there is spread within the community at large (Classen et al., 1999), so it is unsurprising that there was no significant association between MTB infection and family group (Fig. 3). The rise in proportion of individuals with MTB infection who were born after 1740, with a peak between 1760-1774, coincides with an expansion of the population due to major civic building works and the active encouragement given to agricultural workers to settle in and around the town. It was noted in the very early days of medical microbiology that persons from undeveloped rural areas were very susceptible to tuberculosis if they move to a more developed area (Cummins, 1908), and it is suggested that this may account for the increase in tuberculosis infection at this time.

The MTB DNA from these individuals pre-dates the earliest bacterial cultures and in a few cases may date from the late 17th century, if it is assumed that infection was acquired early in life. Some of the DNA is remarkably well-preserved and is capable of being typed by molecular methods such as spoligotyping (Kamerbeek et al., 1997; Fletcher et al., 2000; Lev et al., 2000), which may throw light on the molecular

epidemiology of infection within this community, and the rate of change of MTB strains in comparison with those in the world today. Initial indications are that there are no molecular changes that would suggest any relationship with the Beijing MTB family, whose evolution is still unknown and which includes several multi-drug resistant strains of current clinical interest. According to the genotyping scheme devised by Sreevatsan et al. (1997) MTB from 11 samples can be placed into genotypic groups 2 or 3, which are suggested to be more recent in evolutionary terms than group 1. As Beijing strains are all members of group 1, this is consistent with apparent absence of Beijing strains in the Hungarian population under study, and indicates that the change from group 1 occurred far longer ago than the last 400 years.

Despite advances in modern medicine, one third of the world's population is infected with tuberculosis. With 3 million deaths a year, tuberculosis is currently the greatest cause of death from any single infectious agent worldwide. In Europe between the 17th–19th centuries, tuberculosis caused one in four deaths. The remains from Vác offer a unique opportunity to characterize the MTB strains that were prevalent in Central Europe just prior to this major outbreak. This gives us the exciting prospect of understanding the changes that have occurred in the tubercle bacillus from the pre-antibiotic era, possibly 200 years before its first cultivation in the laboratory.

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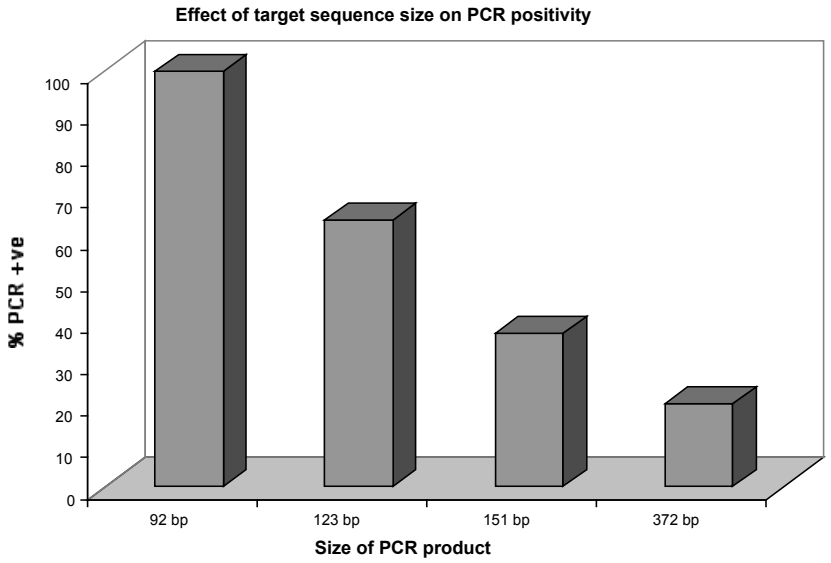

Fig. 1. Proportion of samples positive by nested PCR for IS*6110* that were positive for target sequences of larger size.

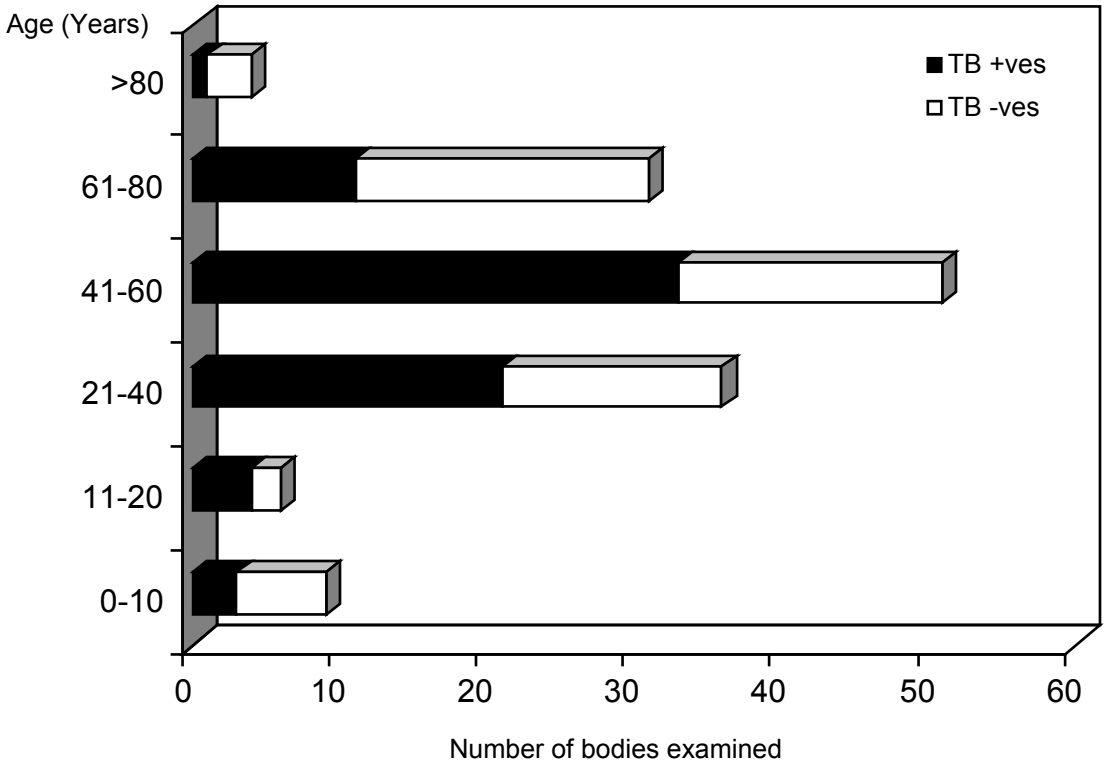

Fig. 2. Distribution of individuals according to age at death and MTB positivity.

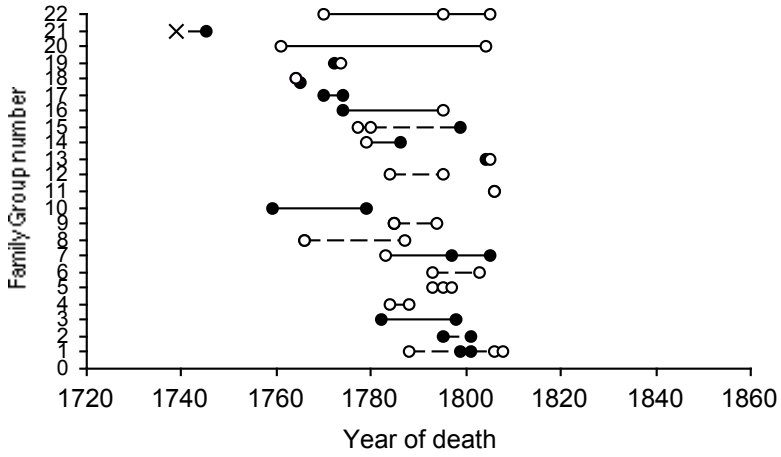

Fig. 3. Distribution of family group members according to year of death and MTB positivity. Solid circles, MTB DNA-negative; open circles, MTB DNA-positive; x, not examined.

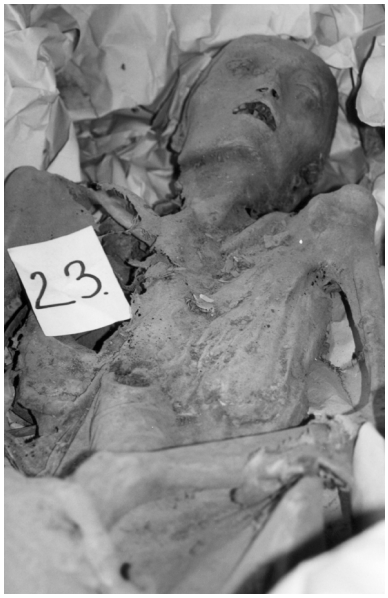

Fig. 4. Unknown young man with wasted appearance; radiograph showed multiple foci in chest. Chest contents were MTB-positive.

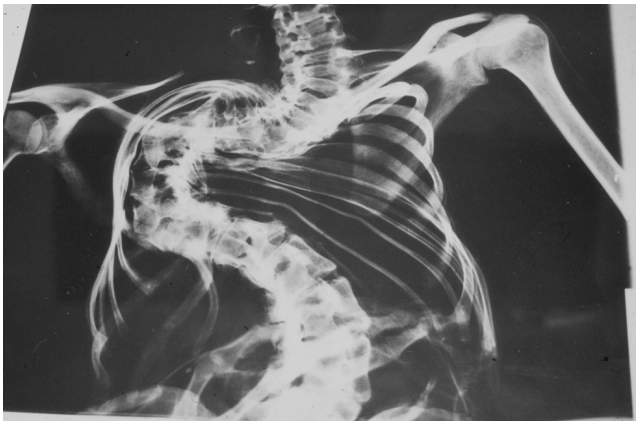

Fig. 5. Radiograph of adult with gross spinal deformity. Abdomen sample was MTB DNA-positive.

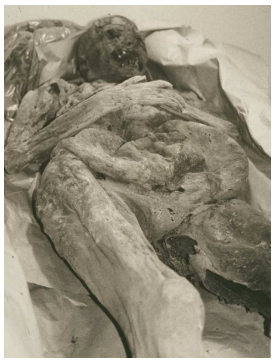

Fig. 6. Ninety-five-year-old woman. Her left lung was MTB-positive, while other sites were negative.

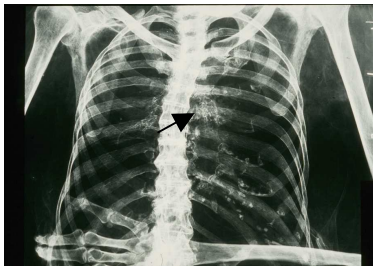

Fig. 7. Chest radiograph of 95-year-old woman, showing calcified lesion in left lung (arrow), which was MTB-positive

TABLE 1. Primer sequences and PCR program used

Locus / accession	Primers (5' - 3')	Product Size	Parameters[1]	Cycles
IS*6110*	P1: CTCGTCCAGCGCCGCTTCGG P2: CCTGCGAGCGTAGGCGTCGG	123 bp	94°C, 40sec 68°C, 1min 72°C, 20sec	25 nested or 45
IS*6110*	IS3: TTCGGACCACCAGCACCTAA IS4: TCGGTGACAAAGGCCACGTA	92 bp	94°C, 40sec 58°C, 1min 72°C, 20sec	25
gyrA 95 L27512	Gyr1: CGATTCCGGCTTCCGCCCGG Gyr2: CCGGTGGGTCATTGCCTGGCG	194bp	94°C, 1min 68°C, 1min 72°C, 1min	40
katG 486 X68081	KatGa: GGCCGCTGGTCCCCAAGCAG KatGb: GGCTGCAGGCGGATGCGACC	220bp	94°C, 1min 68°C, 1min 72°C, 1min	40
dnaA-dnaN	Pr1: GTCACAGAGATTGGCTGTCAGTGT Pr2: AGGTCGACGTCGGTCGGAGTTGT Pr3: GCCTACTACGCTCAACGCCAGAG	Pr1+Pr2 = 151bp or ~1500bp Pr3+Pr2 = 230bp or 160 - 200bp	95°C, 1min 48°C, 1min 72°C, 1min	40

TABLE 1 (continued)

Locus / accession	Primers (5' - 3')	Product Size	Parameters[1]	Cycles
19kD Ag	NB-3: TCTTTCCGGATGTTCAAGCA NB-5: GTGACGTTCTGGTCCTTACC	131 bp	Touchdown PCR from 70°C Then 94°C, 40 sec, 58°C, 1 min, 72°C, 20 sec	12 40
MPB70 Ag	TB-1A: GAACAATCCGGAGTTGACAA TB-1B: AGCACGCTGTCAATCATGTA	372 bp	95°C, 1min 48°C, 1min 72°C, 1min	40

[1] An initial denaturation step (95°C, 15 mins for HotStar PCR; or 94°C, 1 min) and a final extension step at 72 °C was used for all PCR amplifications.

TABLE 2 Mutations in katG *and* gyrA *codons enabling division of* Mycobacterium tuberculosis *strains into genotypic groups 1, 2, and 3 (Sreevatsan et al., 1997)*

Locus	Group 1	Group 2	Group 3
katG codon 463	Leu - CTG	Arg - CGG	Arg - CGG
gyrA codon 95	Thr - ACC	Thr - ACC	Ser - AGC

TABLE 3 Genotypic grouping of MTB DNA from 18th century Hungarian remains

Body no.	Sample site	*gyrA* 95	*katG* 463	Group
13	Chest	-	CGG	2 or 3
25	Left chest	-	CGG	2 or 3
28	Abdomen	ACC	CGG	2
68	Left chest	AGC	CGG	3
72	Pleura	AGC	CGG	3
78	Rib	-	CGG	2 or 3
79	Pelvis	-	CGG	2 or 3
80	Chest	ACC	CGG	2
92	Left chest	AGC	CGG	3
121	Pleura	AGC	CGG	3
134	Sacral region	AGC	CGG	3