Yearbook of Mummy Studies, Vol. 1, pp. 51–56, 4 figs., 1 tab., June 2011
 – ISBN 978-3-89937-137-6

Detection and Characterization of *Mycobacterium tuberculosis* DNA in 18th Century Hungarians with Pulmonary and Extra-pulmonary Tuberculosis

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Abstract

Skeletal and naturally mummified tissues from a previously archived group of 18th century Hungarian remains were examined for the presence of *Mycobacterium tuberculosis* complex (MTB) DNA, using specific nested PCR for the IS*6110* locus. Paleopathological changes in bones and from radiographs were noted in a minority of cases. Overall, specimens from 157/232 (67.7 %) of individuals proved positive, ranging from 20/43 (46.5 %) in children, 26/29 in middle-age (89.7 %) and 32/46 individuals aged 65-95 years (69.6 %). Single samples gave a positive result in 67/120 of cases (55.8 %). Most were ribs where the surface adjacent to lungs and pleura was sampled. When multiple sites were examined, 73/93 (78.5 %) individuals were positive; most of these had MTB only in the pulmonary region but 26 had disseminated disease (35.6 %) and 12 (16.4 %) had extra-pulmonary disease only. To distinguish *M. tuberculosis* from *Mycobacterium bovis*, well-preserved positive samples were examined for several additional genetic loci including the TbD1 deletion – characteristic of modern European strains of *M. tuberculosis*, and spoligotyped. No evidence other than of human *M. tuberculosis* was found, but different strains were detected. Tuberculosis was widespread in this community and whilst some individuals succumbed early in life, the majority co-existed with the infection. Therefore, this study may lead to the identification of host alleles and MTB strains associated with active and latent disease.

Introduction

During reconstruction work in the Dominican Church of Vác, Hungary (Pap et al. 1999), sealed crypts were discovered, which contained many human remains. The great majority were in pine coffins which were stacked up to the ceiling of the crypts. Many of the coffins also had pine shavings around the body. There was a contemporaneous civic archive and church records, which showed that the crypts were used continuously for burials of several middle class families and clerics, from 1731–1838. The crypts were cold with an ambient temperature of about 10 °C. They were also dry, with very poor but continuous ventilation through two small air vents at either end of the crypts. Under these conditions it appears that the normal decay process was delayed and 70 % of the bodies were totally or partially naturally mummified.

There were several gross pathological changes, visible by naked eye or radiographic examination, which suggested a tuberculous infection. Tuberculosis was recognized in ancient and historical times and is still a major global cause of death and disease. Several specific molecular methods are available to detect the DNA from the group of very closely-related bacteria responsible for the disease, known

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collectively aa the *Mycobacterium tuberculosis* (MTB) complex. A pilot study was carried out on samples from Vác using the polymerase chain reaction (PCR) for the specific amplification of MTB complex DNA (Pap et al. 1999). Positive results were obtained and further investigation established that *M. tuberculosis* infection was widespread in this community (Fletcher et al. 2003a). In several cases the DNA preservation was sufficiently good to enable molecular fingerprinting of the infecting *M. tuberculosis* strain (Fletcher et al. 2003b). Due to these encouraging results mainly from adult remains, specimens were obtained from infants and children. The aims of this study were to determine the incidence and type of tuberculosis, and the nature of the infection, in relation to the age at death in these adult and child remains. This part of Hungary was then pre-industrial, so the material provides a means of examining the local endemic strains of *M. tuberculosis* that were prevalent before the epidemic of tuberculosis associated with the Industrial Revolution in Europe.

Materials and Methods

The recommended protocols of ancient DNA (aDNA) work (O'Rourke et al. 2000, Donoghue & Spigelman 2006, Taylor et al. 2010) were followed. In brief, samples were processed in laboratories where no work with modern *M. tuberculosis* has ever been performed. There was strict separation of work areas between DNA extraction, PCR set-up, nested and post-PCR procedures using different laboratories, demarcated by labels, color of laboratory coats, and backed up by staff training. Within laboratories strict guidelines were followed regarding cleaning and non-sharing of equipment. Dedicated sets of pipettes were used for PCR set-up and were stripped and cleaned in detergent and ethanol before each experiment. Fresh sterile filter tips were used routinely. Surfaces and equipment in contact with sample tubes (centrifuges, rotors, mixers, etc.) were cleaned before each assay and use of sterile and pre-purchased reagents minimised contamination risk.

DNA extraction procedures have been described previously (Donoghue et al. 2005). In brief, approximately 25 mg of sample was pre-incubated in Proteinase K/EDTA at 56 °C for 24–72 hours. all samples were lysed in guanidium thiocyanate solution at 56 °C (Boom et al. 1990) and vigorously mixed with small glass beads to release any mycobacterial DNA. DNA was captured onto silica by using spin columns (DNeasy® tissue kit, Qiagen Ltd). After washing and drying, DNA was eluted from the silica, aliquoted and used immediately or stored at −20°C. Negative extraction controls were always included. Members of the MTB complex have many clustered regularly interspaced short palindromic repeat sequences in their DNA, which are known as insertion sequences (IS). Their function is not known but they are normally present as multiple copies within the cell. The *M. tuberculosis* complex was detected by targeting a specific region of the repetitive element IS*6110* (Eisenach et al. 1990, Hellyer et al. 1996). A two-tube nested PCR was used which yielded an outer product of 123 bp and a nested PCR product of 92 bp (Spigelman & Lemma 1993, Taylor et al. 1996). The PCR mix included 10 mM bovine serum albumin to reduce PCR inhibition (Forbes & Hicks 1996, Abu Al-Soud & Rådström 2000). A 50 µl reaction mix was used with 5 µl of DNA extract. A hot-start *Taq* polymerase was used to minimize non-specific primer and template binding. Negative controls were always included. PCR product was detected by gel electrophoresis, DNA staining with ethidium bromide and visualized under ultraviolet light.

Following initial screening for the presence of MTB DNA further characterization was attempted on better-preserved specimens, using genomic deletion analysis and single nucleotide polymorphisms (SNPs) (Brosch et al. 2002). The strategy followed is shown in Figure 1. Functionally neutral SNPs in the catalase-peroxidae-encoding gene *katG* and a subunit of the DNA gyrase gene *gyrA* enable three principal genetic groups (PGGs) to be recognized within the MTB complex and this method has been applied to specimens from Vác. Spoligotyping is based on the direct repeat (DR) region of the MTB complex (Kamerbeek et al. 1997). DR-based PCR primers amplify up to 43 unique spacer regions that lie between each DR locus. Amplicons from individual spacers are visualized by dot-blot hybridization on a membrane, giving a fingerprint. Spoligotyping clearly distinguishes *M. bovis* from *M. tuberculosis*, and different families of strains are defined by characteristic patterns. Table 1 lists the genetic loci and primers used.

Data were analyzed according to the age and sex of the individuals, body site examined, and any relevant physical characteristics. Statistical significance was determined using the Chi-square test. An attempt was made to distinguish between individuals with active or latent disease, based on the following criteria: Active disease was assumed where there were multiple MTB-positive sites in the body,

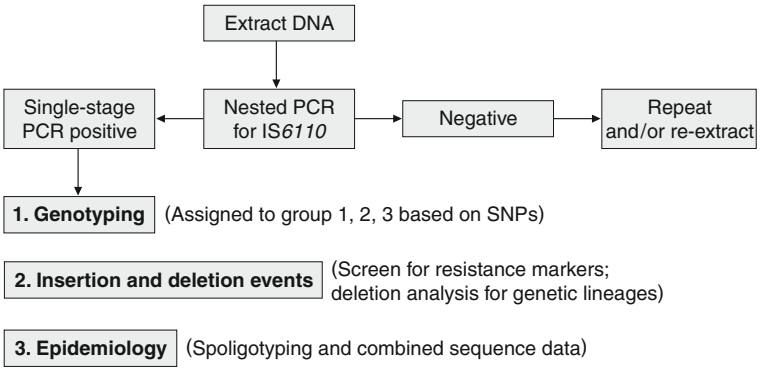

Fig. 1. Analytical approach for MTB work.

and/or physical signs of disease e.g. wasting, calcified lesions visible by CT scans or radiographs, or bony lesions. In addition, active disease was assumed in any neonates or children aged <5 years. Latent disease was assumed when only a single site was found MTB-positive even though several sites were examined, and the individual had no physical signs of disease.

Results

Overall, specimens from 157/232 (67.7 %) of individuals proved positive. Examining several samples from different sites in the body significantly increased the detection rate. Only 67/120 of cases (55.8 %) of individuals were positive where only a single sample was examined – normally a rib, where the surface adjacent to lungs and pleura was sampled. In comparison, 73/93 (78.5 %) of individuals were positive

Table 1. Primer sequences and PCR details[1].

Locus	Primers (5'–3')		$MgCl_2$ [mM]	Annealing temp. [°C]	Product [bp]
IS*6110* Outer	P1: P2:	CTCGTCCAGCGCCGCTTCGG CCTGCGAGCGTAGGCGTCGG	1.5	68	123
IS*6110* Nested	IS3: IS4:	TTCGGACCACCAGCACCTAA TCGGTGACAAAGGCCACGTA	1.5	58	92
gyrA 95 L27512	Gyr1: Gyr2:	CGATTCCGGCTTCCGCCCGG CCGGTGGGTCATTGCCTGGCG	1.5	68	194
katG 486 X68081	KatGa: KatGb:	GGCCGCTGGTCCCCAAGCAG GGCTGCAGGCGGATGCGACC	1.5	68	220
TbD1 Flanking outer	TbD1a: TbD1b:	CTAACGGGTGCAGGGGATTTC CCAAGGTTACGGTCACGCTGGC	1.5	60	128
TbD1 Flanking inner	TbD1c: TbD1d:	GCAGGGGATTTCAGTGACTG GCTGGCCAGCTGCTCGCCG	1.5	58	103
DR	DRa: DRb:	GGTTTTGGGTGTGACGAC CCGAGAGGGGACGGAAAC[2]	3.0	55	not applicable

1 An initial denaturation step (95 °C for 15 min – hot start PCR, or 94 °C for 1 min); DNA amplification (initially 40 cycles, with 25 cycles in nested reactions) of strand separation at 94 °C for 40 sec, 1 min of primer annealing, followed by strand extension at 72 °C for 20 sec plus 1 sec/cycle; and a final extension step at 72 °C, were used for all PCR amplifications.

2 The DRb primer was biotinylated at the 5' end to enable subsequent detection of amplified DNA by reverse hybridization.

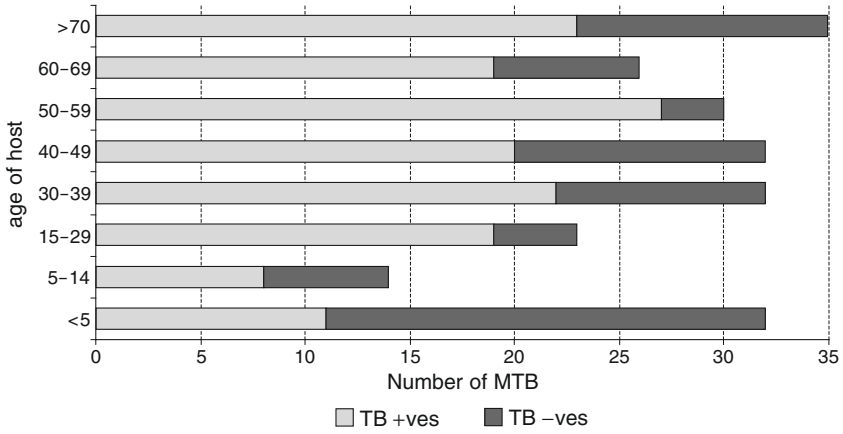

Fig. 2. Results of screening for MTB DNA by age of host.

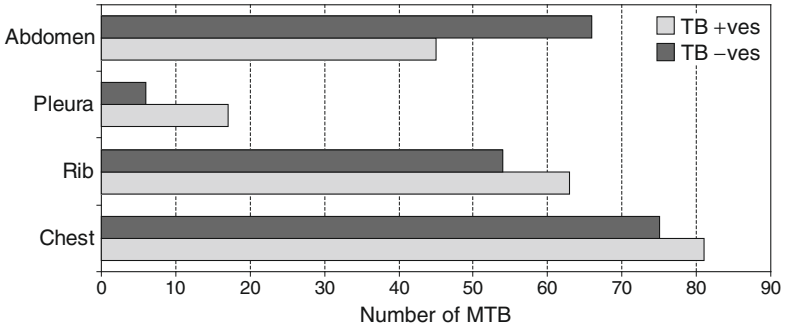

Fig. 3. MTB DNA in relation to sample site.

where multiple sites were sampled ($p < 0.001$). Most of these individuals had MTB only in the pulmonary region but 26 had disseminated disease (35.6%) and 12 (16.4 %) had extra-pulmonary disease only. The results of screening for MTB DNA by the age of the host are shown in Figure 2. There is evidence of tuberculosis in neonates and young children. Fewer older children were positive, but overall figures showed 20/43 (46.5 %) were positive for MTB DNA. High levels of infection were noted in young adults and specially the middle-aged., where 26/29 (89.7 %) were positive. A high level of positivity was also seen in the oldest group of individuals with 32/46 (69.6 %) aged 65-95 years positive for MTB DNA. There was no significant difference in MTB-positivity between males and females. When the effect of sampling site was examined, a greater proportion of chest tissue and ribs were MTB positive compared with abdominal tissue (Fig. 3), and the highest proportion of positive samples was found in specimens identified as calcified pleura ($p < 0.01$).

Strains from PGG2 and PGG3 have been identified, and where the DNA has sufficient preservation, the MTB appears to be from a TbD1-deleted lineage. Examples of spoligotyping data are shown in Figure 4. The observed patterns result from a combination of true spacer deletions, and poor preservation of some DNA samples.

Discussion and conclusions

The effect of host age on the detection of MTB DNA can be explained by the natural history of the infection. Tuberculosis is transmitted via infectious aerosols released from the lungs of an infected person. In the alveolus of the lung, inhaled tubercle bacilli are ingested by macrophages and the host immune response

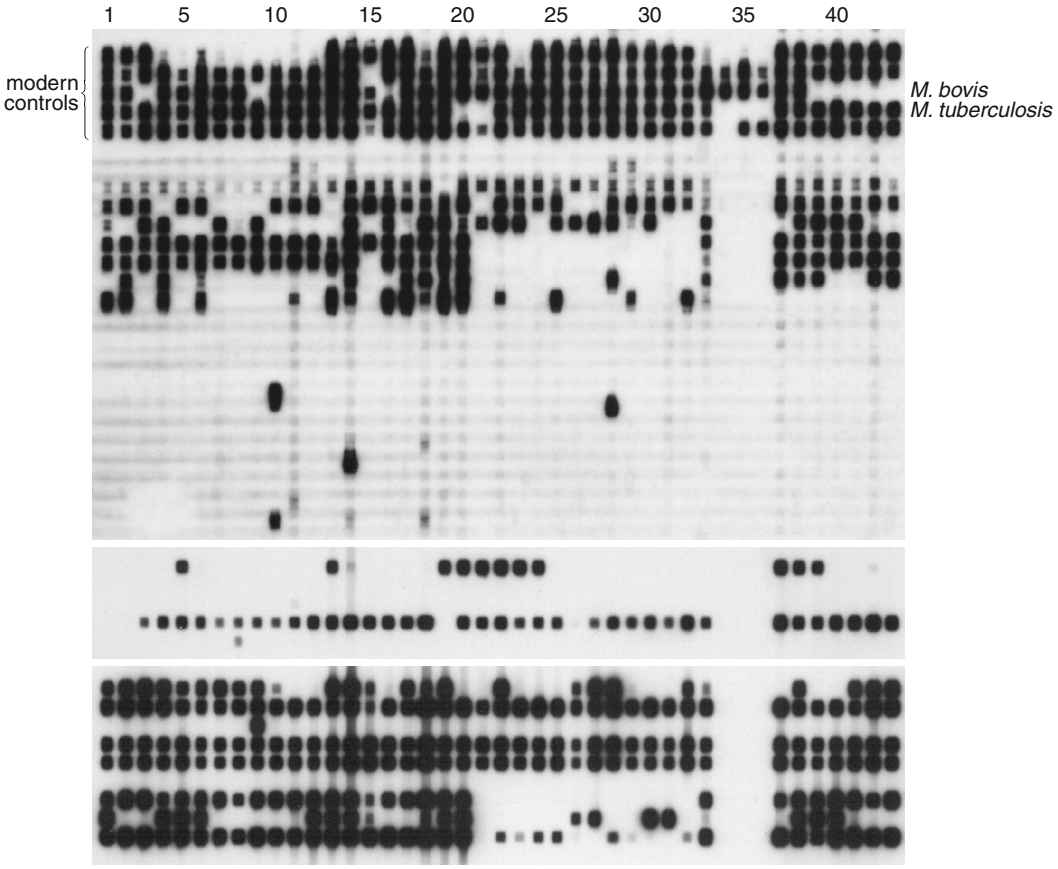

Fig. 4. Spoligotyping of MTB DNA from Vác.

leads to granuloma formation and eventually to calcified lesions. The bacilli may remain dormant for decades, but if the host immune response becomes less effective, for example by old age, poor nutrition, or other stresses, the bacteria may escape into the lungs causing re-activated pulmonary tuberculosis. The very young can develop primary disseminated tuberculosis due to their immature immune response. When bacteria spread to other host tissues via the lymphatic system and blood, and disseminate throughout the body, this results in miliary or extra-pulmonary tuberculosis. Gastro-intestinal tuberculosis can result from swallowing infected sputum or by ingestion of infected meat or milk.

Therefore, the finding that MTB DNA was widespread in this population does not mean that all had active tuberculosis at the time of death. Using the criteria described above, individuals could be identified who were likely to have had active or latent disease. Infection was disseminated in 35 % of individuals which indicates an active infection that would have been fatal in the absence of any effective treatment. However, in others it was highly localized so these may be individuals with latent disease. These are of especial interest as there may be host susceptibility or bacterial virulence factors that can be elucidated by a comparison between these two groups of individuals. A future aim of the study is to determine the genetic characteristics of both host and pathogen in these two groups. The initial genetic characterization of the MTB DNA from the Vác mummified individuals has been reported previously and demonstrated that members of the same family group were infected with distinct strains of MTB (Fletcher et al. 2003b). This observation suggests community spread of infection – typical in a society with a high TB incidence.

Acknowledgements

The authors appreciate the contribution made by the many collaborating laboratories, researchers and students on this project – also the authorities which made this work possible. Early funding was from the Wenkart Foundation (Australia). More recently, the Hungarian Scientific Research Fund (OTKA) Grant No. 61155 part-funded this work.

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