Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians

Helen A. Fletcher, Helen D. Donoghue, G. Michael Taylor, Adri G. M. van der Zanden and Mark Spigelman

The naturally mummified remains of a mother and two daughters found in an 18th century Hungarian crypt were analysed, using multiple molecular genetic techniques to examine the epidemiology and evolution of tuberculosis. DNA was amplified from a number of targets on the *Mycobacterium tuberculosis* genome, including DNA from IS6110, gyrA, katG codon 463, oxyR, dnaA–dnaN, mtp40, plcD and the direct repeat (DR) region. The strains present in the mummified remains were identified as *M. tuberculosis* and not *Mycobacterium bovis*, from katG and gyrA genotyping, PCR from the oxyR and mtp40 loci, and spoligotyping. Spoligotyping divided the samples into two strain types, and screening for a deletion in the MT1801–plcD region initially divided the strains into three types. Further investigation showed, however, that an apparent deletion was due to poor DNA preservation. By comparing the effect of PCR target size on the yield of amplicon, a clear difference was shown between 18th century and modern *M. tuberculosis* DNA. A two-centre system was used to confirm the findings of this study, which clearly demonstrate the value of using molecular genetic techniques to study historical cases of tuberculosis and the care required in drawing conclusions. The genotyping and spoligotyping results are consistent with the most recent theory of the evolution and spread of the modern tuberculosis epidemic.
cross-contamination. Body 68 was the daughter of the above, and she died on 25 December 1797 aged 28 years. No abnormalities were detected in a chest radiograph of body 68 but she was noticeably small for her age and of cachetic appearance. A tissue sample from the chest was examined. Body 72 was the younger daughter of body 28 and sister to body 68, and she died on 2 March 1795. Although the archive stated that body 72 was aged 14 years at death, when examined she was initially believed to be aged only eight or nine years due to her very small size and cachetic appearance. No abnormalities were seen in a chest radiograph. Samples of body 72 were taken from the abdomen, chest and from possible calcified pleura.

Several studies have examined skeletal and mumified remains for the presence of mycobacterial DNA (Spigelman & Lemma, 1993; Salo et al., 1994; Baron et al., 1996; Faerman et al., 1997; Nerlich et al., 1997; Braun et al., 1998; Crubézy et al., 1998; Donoghue et al., 1998; Taylor et al., 1996, 1999, 2001; Haas et al., 2000; Mays et al., 2001; Zink et al., 2001). However, the state of surviving DNA has only seldomly permitted detailed molecular examination of samples (Mays et al., 2001). Consequently, few population-based studies have been reported for ancient tuberculosis (Zink et al., 2001). However, the completion of the sequencing of the MTB genome (Cole et al., 1998) and comparative genomic studies of members of the MTB complex have revealed an increasing number of targets for the study of historical cases of tuberculosis. In the present study a number of PCR-based methods were applied to the MTB complex strains from the family group described above. The aim was to evaluate the use of these markers for the analysis of archaeological specimens and to assess the extent to which such studies may contribute to increasing our understanding of the evolution and molecular epidemiology of tuberculosis.

**METHODS**

**Samples.** Taking precautions against cross-contamination, samples of dehydrated soft tissue were collected from the selected family group, bodies 28, 68 and 72, respectively (Table 1). Samples were stored in sterile universal bottles at 4 °C until analysis.

**Precautions against contamination.** Stringent precautions were taken against laboratory cross-contamination: clean protective clothing was worn; gloves were changed frequently; sterile tubes and aerosol-resistant tips were used. A two-laboratory, three-workstation strategy was used for DNA extraction and PCR amplification, which also served to verify the results. Pipettes, other equipment and surfaces were cleaned with concentrated household detergent or bleach, rinsed with ultrapure water and dried with ethanol. Molecular biology grade reagents (sterile where possible) were purchased and used only for the ancient DNA studies. Pre- aliquoted PCR master mix was used in laboratory one (Abgene). In laboratory two, the UNG-ready Excite core kit with hot-start Taq polymerase (BioGene) was used in later experiments for confirmation of genotyping experiments.

**Reproducibility.** Data were verified by repeating PCRs on the same and on fresh extracts of samples. In addition, in several cases duplicate reactions were carried out independently in one of the other collaborating laboratories.

**DNA extraction.** DNA extraction in laboratory one was performed using an adaptation of the Boom method (Boom et al., 1990) as described previously (Donoghue et al., 1998). Repeat extracts were performed using the Qiagen DNeasy Tissue Kit with the following adaptations: 25 mg of sample was added to a 1·5 ml tube containing 1·5–2 mm glass beads and incubated overnight at 56 °C with 200 μl digestion buffer [0·5 M EDTA, pH 8·9 (Promega), 0·4 mg protease K ml-1 (Finnzymes)]. Each sample was homogenized for 50 s at medium speed with a Mini Bead Beater (Stratech Scientific) and transferred to a column where it was further processed according to the manufacturer's instructions. In laboratory two DNA was extracted using a silica-based method as described previously (Taylor et al., 1999).

**Screening methods.** Primer sequences, amplification conditions and details of where the work was carried out are given in Table 2. Samples were screened for an MTB-complex-specific region of the insertion sequence IS6110 (Eisenach et al., 1990) using a two-tube nested PCR as described previously (Taylor et al., 1996; Donoghue et al., 1998). Thereafter, target sequences in the 19 kDa antigen gene (Mustafa et al., 1995) and the dnaA–dnaN region were examined.

**Genotyping.** To place the strains into genotypic groups (Sreevatsan et al., 1997; Frothingham et al., 1999), polymorphisms at codons 203 and 463 of katG and codon 95 of gyrA were determined (Table 3). This was performed independently in laboratories one and two.

**Distinction of MTB from Mycobacterium bovis.** Samples from the three family members were screened for the presence of the mtp40 element, located in the phospholipase A gene (plcA) (Del Portillo et al., 1991). This element is retained in around 90% of MTB isolates but lost as part of a deletion event (RD5) in the majority of M. bovis strains (Del Portillo et al., 1996; Gordon et al., 1999). PCR for mtp40 was multiplexed with a second method, designed to amplify a region in the plcD-cutinase region of strain CDC 1551 known as MT1801. This region, deleted from the H37Rv laboratory strain of MTB (RvD2), is a hot-spot for IS6110 insertion and is generally subject to deletion events. PCR for the MT1801 oxidoreductase DNA has been used as an indicator of deletion events in this region (Ho et al., 2000). The multiplex PCR method therefore yields information as to the presence of all four phospholipase gene copies (plCA, plcB, plcC and plcD). Amplification of these target sites (mtp40 and the plcD loci) was performed in laboratory two using a hot-start method as described previously (Taylor et al., 1999), as was determination of the polymorphism at nucleotide 285 in the oxyR pseudogene. In laboratory one, PCR of the plcD region was carried out using a shorter target sequence of 123 bp, which can be included in a nested reaction.

**RD7 PCR.** As a further screening test to refute the presence of M. bovis DNA in the samples, flanking primers were used in a PCR

### Table 1. Samples collected from the family group studied here

<table>
<thead>
<tr>
<th>Body no.</th>
<th>Relationship</th>
<th>Year of birth/death (years)</th>
<th>Sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>Mother</td>
<td>1738/1793</td>
<td>Abdomen</td>
</tr>
<tr>
<td>68</td>
<td>Daughter 1</td>
<td>1769/1797</td>
<td>Trachea</td>
</tr>
<tr>
<td>72</td>
<td>Daughter 2</td>
<td>1780/1795</td>
<td>Abdomen</td>
</tr>
</tbody>
</table>

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Table 2. Primer sequences and PCR conditions used in laboratories one, two and three

An initial denaturation step at 94 °C and a final extension step at 72 °C were used for all PCR amplifications.

<table>
<thead>
<tr>
<th>Locus (accession no.)</th>
<th>Primers (5’→3’)</th>
<th>Product size (bp)</th>
<th>MgCl₂ (mM)</th>
<th>Parameters</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS6110†</td>
<td>P1, CTCGTCCAGCGCCCGCTCGG P2, CCTGCGAGCGGTAAGGCGGTCGG</td>
<td>123</td>
<td>1-5</td>
<td>94 °C, 40 s, 68 °C, 1 min 72 °C, 20 s</td>
<td>45 or 25 if nested</td>
</tr>
<tr>
<td>IS6110†</td>
<td>IS3, TTCGGGCCACCCGACCGACCTA A IS4, TCGGTGACAAAGGCGACGTA</td>
<td>92</td>
<td>2-5 or 1-5</td>
<td>94 °C, 40 s, 58 °C, 1 min 72 °C, 20 s</td>
<td>25</td>
</tr>
<tr>
<td>19 kDa antigen gene*</td>
<td>NB3, TCTTTCCGAGATGTCAAGCA NB5, GTGACGTTCTGGTCCTTACC</td>
<td>131</td>
<td>1-5</td>
<td>95 °C, 40 s, 68 °C, 1 min 72 °C, 20 s</td>
<td>40</td>
</tr>
<tr>
<td>gyrA 95* (L27512)</td>
<td>GyrU1, CGATTTCCGCTTCGCCCGG GyrU2, CCGTTGCGTTGCAATCTCGGTGGC</td>
<td>194</td>
<td>1-5</td>
<td>95 °C, 40 s, 68 °C, 1 min 72 °C, 20 s</td>
<td>40</td>
</tr>
<tr>
<td>katG 486* (X68081)</td>
<td>KatGa, GGCGCTCTGGTCCCCAAGCAG KatGb, GGCTGCAGGCGGATGCGACC</td>
<td>220</td>
<td>1-5</td>
<td>95 °C, 40 s, 68 °C, 1 min 72 °C, 20 s</td>
<td>40</td>
</tr>
<tr>
<td>katG 203†</td>
<td>F, TTCGGCCGGGTGACGACAT R, GTTTTCAGTAGATCGCCCATC</td>
<td>142</td>
<td>1-5</td>
<td>94 °C, 10 s, 62 °C, 30 s 72 °C, 10 s</td>
<td>43</td>
</tr>
<tr>
<td>dnaA–dnaN*</td>
<td>Pr1, GTCACAGAGATTGGCTGAGTG Pr1/Pr2 =159 or ~1500 Pr2, AGGTCGACGTCGATGGTGGT Pr3, GCCTACTACGCTCAACGCCAG Pr2/Pr3 =230 or 160–200</td>
<td>159 or 1500</td>
<td>1-5</td>
<td>95 °C, 40 s, 48 °C, 1 min 72 °C, 20 s</td>
<td>40</td>
</tr>
<tr>
<td>mtp40† (M57952)</td>
<td>F, CTGTCGAGAGTTGCTGGAGG R, ATGTTCTCGACACGTTGCAC</td>
<td>152</td>
<td>1-5</td>
<td>94 °C, 10 s, 62 °C, 30 s 72 °C, 15 s</td>
<td>43</td>
</tr>
<tr>
<td>MT1801–plcD region†</td>
<td>M3, AGAATTACTTTCAGGGCTCGTGGA M4, CACATCCCATAGCGCACAG</td>
<td>177</td>
<td>1-5</td>
<td>94 °C, 10 s, 60 °C, 30 s 72 °C, 15 s</td>
<td>43</td>
</tr>
<tr>
<td>plcD region*</td>
<td>PD1, AGGCTTCGGATACTACGGCATC PD2, CACGCCACGGATGGCGACG</td>
<td>123</td>
<td>1-5</td>
<td>95 °C, 40 s, 60 °C, 1 min 72 °C, 20 s</td>
<td>43 or 25 if nested</td>
</tr>
<tr>
<td>oxyR† (U16243)</td>
<td>F, CGCGCTGTGCAGCGCTGACTTT R, TCTGCGGAATCAGTGGTTCAC</td>
<td>150</td>
<td>2-0</td>
<td>94 °C, 10 s, 62 °C, 30 s 72 °C, 15 s</td>
<td>43</td>
</tr>
<tr>
<td>RD7†</td>
<td>F, ACTTCACTGTGGCTCTGTCGG R, ATCTTGGCGCCCAATGAAATC</td>
<td>211</td>
<td>2-0</td>
<td>94 °C, 20 s, 66 °C, 30 s 72 °C, 20 s</td>
<td>43</td>
</tr>
<tr>
<td>DR region††</td>
<td>DRa, GGTTTTGGTCTGGACGAC DRb, CGGAGAGGGACGGAAC</td>
<td>Various</td>
<td>3-0</td>
<td>96 °C, 20 s, 57 °C, 40 s 72 °C, 30 s</td>
<td>40</td>
</tr>
</tbody>
</table>

*PCR performed in laboratory one (University College London).
†PCR performed in laboratory two (Imperial College London).
‡PCR performed in laboratory three (Apeldoorn, The Netherlands).
Table 3. Mutations in the katG and gyrA codons enabling division of MTB complex strains into genotypic groups 1A, 1B, 2 and 3

Data are from Sreevatsan et al. (1997) and Frothingham et al. (1999)*. Mutated nucleotides are underlined.

<table>
<thead>
<tr>
<th>Gene codon</th>
<th>Genotypic group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1A</td>
</tr>
<tr>
<td>katG 463</td>
<td>Leu, CTG</td>
</tr>
<tr>
<td>katG 203*</td>
<td>Thr, ACT</td>
</tr>
<tr>
<td>gyrA 95</td>
<td>Thr, ACC</td>
</tr>
</tbody>
</table>

to detect deletion region 7 (RD7). This deletion, of around 12-7 kb, extends from base pair 2 208 003 to base pair 2 220 721 in the MTB genome (Zumárraga et al., 1999; Gordon et al., 1999). A product of 211 bp is amplified from M. bovis isolates but under the conditions used no product is expected from MTB, particularly in fragmented archaeological material (Mays et al., 2001).

Molecular fingerprinting. Spoligotyping (Kamerbeek et al., 1997) was carried out in laboratories two (Taylor et al., 1999) and three (van der Zanden et al., 1998).

Detection and analysis of amplified DNA. In laboratories one and two, PCR products were electrophoresed in a 3-0 % (w/v) NuSieve (Flowgen) or agarose gel, respectively. DNA was visualized by ethidium bromide staining plus UV light. Images were recorded with a digital image capture system. Products for sequencing were run subsequently on 0-8 % low-melting-point agarose gels. DNA was purified and sequenced using in-house sequencing services or MWG-Biotech.

Quantification and amplification efficiency. In laboratory one, the amount of amplified product obtained from the 18th century samples was determined in a single experiment by gel densiometric analysis using a DNA mass marker (Gibco) and LABWORKS software (UltraViolet Products). This was compared with DNA from an M. bovis culture, strain AN-5, which was prepared and set up in an isolated, totally separate laboratory, which was under negative air pressure. Four target sequences were used for this comparison, IS6110 using the inner primers (92 bp), the 19 kDa antigen gene (131 bp), the dnaA–dnaN region (159 bp) and the MPB70 antigen gene (372 bp). The quantity of amplicon was converted to the number of copies per microlitre using Avogadro’s constant. Modern DNA was used to ascertain whether differences in yield were due to variations in amplification efficiency between the PCRs. A dilution curve (10⁶ to 10⁻¹) of each product was subjected to real-time PCR amplification (ABI 7000 SDS; Applied Biosystems) using Qiagen Quantitect master mix.

RESULTS

Evidence of MTB infection

All samples were strongly positive with both nested and single-stage PCR for IS6110. Positive results were also obtained consistently from PCRs based on the 19 kDa antigen and the dnaA–dnaN target sequence, and from spoligotyping.

Genotypic grouping

Fragments of DNA spanning the regions of polymorphisms in the katG and gyrA genes (Table 3) were amplified and sequenced in laboratories one and two. Body 28 classified as group 2 with katG codon 463 CGG, katG 203 ACC and gyrA 95 ACC. Bodies 68 and 72 classified as group 3 with katG 463 CGG, katG 203 ACC and gyrA 95 AGC (Table 4).

oxyR pseudogene

Sequencing of both strands of the PCR products from bodies 28, 68 and 72 revealed a guanine residue at position 285 characteristic of MTB, as opposed to an adenine residue characteristic of M. bovis (Sreevatsan et al., 1996).

mtp40 and plcD-cutinase PCR

Samples from all three individuals were positive for the mtp40 fragment, which strongly indicated that the DNA was from MTB rather than M. bovis (Table 4). Samples from bodies 28 (abdomen and trachea) and 68 (chest) were consistently positive for plcD but body 72 yielded a negative result with single-stage PCR based on the larger target sequence (177 bp; Fig. 1a). However, in laboratory one,....

Table 4. Summary of results obtained for bodies 28, 68 and 72

All three samples were positive for the presence of IS6110 and negative for an IS6110 fragment in the dnaA–dnaN region, indicating that they were members of the MTB complex but not of the group 1 subtype. An IS6110 fragment in the dnaA–dnaN region is typical of members of the multi-drug-resistant Beijing family of strains. Also, the M. bovis-specific deletion region 7 (RD7) was not amplified from any of the samples.

<table>
<thead>
<tr>
<th>Sample analysed (body no.)</th>
<th>Tests that differentiate M. bovis and MTB*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mtp40 fragment</td>
</tr>
<tr>
<td>Abdomen (28)</td>
<td>+</td>
</tr>
<tr>
<td>Chest (68)</td>
<td>+</td>
</tr>
<tr>
<td>Chest (72)</td>
<td>+</td>
</tr>
</tbody>
</table>

*+, Positive; –, negative; G, guanine, denotes MTB.
Two spoligotype patterns were observed for the three bodies 28, 68 and 72 were remarkably complete (Fig. 2a). The spoligotyping patterns obtained from the extracts from Spoligotyping family of multi-drug-resistant MTB (Kurepina insertion sequence in this region specific for the Beijing primers Pr2 and Pr3 (Table 4), indicating that there was no primers Pr1 and Pr2 and no product was obtained with A 159 bp product was amplified from the Va´c samples with Hae

Fig. 1. (a) Multiplex PCR to screen for a deletion in plcD. Lanes: 1, 100 bp DNA size marker; 2, strain CDC 1551; 3, strain H37Rv; 4, extraction control; 5, PCR control; 6, body 72; 7, body 68; 8, body 28. (b) Examination of effect of PCR target size in plcD. Lanes: 1, 123 bp DNA ladder; 2, body 28 (177 bp); 3, body 28 (123 bp); 4, body 28 (nested PCR, 102 bp); 5, body 68 (177 bp); 6, body 68 (123 bp); 7, body 72 (177 bp); 8, body 72 (123 bp); 9, body 72 (nested PCR, 123 bp); 10, body 72 (nested PCR, 102 bp); 11, φ X 174/ HaeII molecular markers.

with single-stage PCR of the 123 bp target region, some positive results were obtained from body 72, although samples from the chest, pleura and abdomen differed in the quantity of amplifiable MTB DNA, and repeated DNA extracts also yielded differing amounts. Nested PCR of apparently negative PCR products of either the 177 or 123 bp target regions was required to give clear positive results from body 72 (Fig. 1b).

**RD7 PCR**

No product was obtained from any of the samples, which was the result expected from MTB DNA.

**Screening for IS6110 in the dnaA–dnaN region**

A 159 bp product was amplified from the Va´c samples with primers Pr1 and Pr2 and no product was obtained with primers Pr2 and Pr3 (Table 4), indicating that there was no insertion sequence in this region specific for the Beijing family of multi-drug-resistant MTB (Kurepina et al., 1998).

**Spoligotyping**

The spoligotyping patterns obtained from the extracts from bodies 28, 68 and 72 were remarkably complete (Fig. 2a). Two spoligotype patterns were observed for the three bodies, patterns 50 and 53 (Sola et al., 1999), which can also be described by an octal code as 7777777730771 and 7777777760771, respectively (Dale et al., 2001). Body 28 (mother) had pattern 53 and bodies 68 and 72 (daughters) had pattern 50. To confirm these results, spoligotyping was repeated in laboratory two using separate extracts from the bodies. In addition, a third set of extracts from the same bodies was taken to laboratory three for spoligotyping. Fig. 2(b) is a schematic representation of the results obtained from the two laboratories. The extracts from body 72 prepared in laboratory one and amplified in laboratory two gave extremely faint hybridization in spacers 29, 30 and 32, although these spacers were negative when the DNA was amplified according to the normal protocol used in laboratory three.

**Quantification and amplification efficiency**

From densitometric analysis the quantity of amplicon from modern M. bovis DNA was similar for each PCR and was not related to the size of target sequence (Table 5). The 18th century Hungarian samples gave a comparable yield for the IS6110 PCR (92 bp). Differences between samples were apparent with the other PCRs and, overall, there was a decline in copy number of amplicon per microlitre with the size of target sequence. Only one sample (body 68 chest) gave a comparable yield to modern DNA with the MPB70 PCR (372 bp), and two samples (body 28 trachea and body 72 abdomen) gave no detectable amplicon. Real-time amplification of dilution curves demonstrated that there was no relationship between amplification efficiency and PCR product size. In a plot of threshold cycle (detection level) versus log of the starting copy number, the greater the $y$ value the greater the efficiency of amplification, yet values were as follows: IS6110 (92 bp) $y = -3.8124$; 19 kDa antigen gene (131 bp) $y = -3.68439$; dnaA–dnaN region (159 bp) $y = -3.5276$; MPB70 antigen gene (372 bp) $y = -3.9832$.

**DISCUSSION**

This study of MTB DNA from a family of 18th century Hungarians has revealed several points of interest. It is apparent that the MTB DNA was remarkably well preserved. DNA fragments as it degrades, and the rate of degradation is dependent upon the environmental conditions in which the specimen is stored (Hoss et al., 1996). Light, moisture and temperature fluctuations are known to be particularly detrimental to DNA. Studies on ancient human DNA have shown that bones and teeth are the specimens most likely to yield amplifiable DNA and mummified tissue has been shown to be an extremely poor source (Lassen et al., 1999). However, MTB DNA was isolated consistently from the mummified tissues examined here. We have suggested elsewhere (Fletcher et al., 2003) that the properties of the MTB bacillus, which enable the organism to persist latently in the human lung, afford protection of the bacterial DNA post-mortem. This study demonstrates that naturally mummified tissue is likely to be a valuable resource for
the palaeomicrobiologist. The reduction in the rate of PCR positives in relation to product size obtained from the mummified tissues was shown by real-time PCR to be due to DNA preservation and not to variation in amplification efficiency. The inverse relationship between PCR yield and target sequence size is a characteristic of ancient DNA (O’Rourke et al., 2000) and confirms that no contamination with modern MTB has occurred.

Bodies 68 and 72 were physically small in comparison to the majority of the remains found in the crypt, some of which were of individuals who lived to a great age and who showed no signs of malnourishment or wasting (Fletcher et al., 2003). Indeed, it is unlikely that the physical appearance of bodies 68 and 72 was due to malnutrition as it is known that only wealthy, middle class families were buried in the crypt. Therefore, the cachectic appearance of these bodies, in association with the age of death and ease of detection of MTB DNA, strongly suggests that death was as a result of active tuberculosis infection. Therefore, it can be concluded that the MTB characterized here was virulent.

Due to the close association of humans with domesticated animals, it was believed that many cases of tuberculosis in antiquity were due to M. bovis (Dankner et al., 1993). In addition, it has been suggested that as M. bovis can be transmitted via the consumption of dairy products it may be found infecting the abdomen. The abdomen of body 28 was found to be strongly positive for MTB; therefore, five different tests were performed that can differentiate between the DNA of MTB and M. bovis. All the results indicate that the DNA was from MTB and not M. bovis. M. bovis is a member of the MTB complex genotype 1, yet the strains we have analysed classify as genotypes 2 and 3. Finally, the presence of spacers 37–43 on the spoligotype confirm that these genotype 2 and 3 strains are MTB and not M. bovis.

Initially, the failure to detect evidence of M. bovis infection, also reported by others (Mays et al., 2001), was surprising. However, M. bovis infections are zoonotic and person-to-person spread is thought to occur only in exceptional circumstances (Grange, 2001). Therefore, in a population with endemic MTB infection, M. bovis infections are always likely to comprise a small proportion of the total number of cases.

The findings from the dnaA–dnaN spacer region are consistent with both the genotyping and spoligotyping data. Beijing strains are of genotype 1 and have a unique spoligotype. In addition, the multi–drug-resistant strain W has a characteristic IS6110 insertion in this target region.

Table 5. Quantity of amplified DNA (copies per microlitre) after 40 rounds of amplification

Data are from one experiment.

<table>
<thead>
<tr>
<th>Sample (body no.)</th>
<th>PCR target sequence (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IS6110 (92 bp)</td>
</tr>
<tr>
<td>M. bovis AN-5, reference strain</td>
<td>8·6×10^{10}</td>
</tr>
<tr>
<td>Abdomen (28)</td>
<td>1·8×10^{11}</td>
</tr>
<tr>
<td>Trachea (28)</td>
<td>2·1×10^{11}</td>
</tr>
<tr>
<td>Chest (68)</td>
<td>4·9×10^{11}</td>
</tr>
<tr>
<td>Abdomen (72)</td>
<td>1·1×10^{11}</td>
</tr>
<tr>
<td>Chest (72)</td>
<td>3·3×10^{11}</td>
</tr>
<tr>
<td>Pleura (72)</td>
<td>1·3×10^{11}</td>
</tr>
</tbody>
</table>

ND, Not detected.

![Fig. 2.](image-url)
The Beijing/W group of strains is believed to be of recent origin (Glynn et al., 2002), which is supported by the failure to detect any members of this group in the mummified material from Vác examined so far (H. D. Donoghue, unpublished observations).

The spoligotypes obtained from the Hungarian MTB DNA samples were remarkably reproducible. Patterns 50 and 53 are the patterns most commonly found across the globe today and are non-disruptive. Sola et al. (1999) have proposed a phylogenetic tree, the root of which is pattern 53. Although tuberculosis occurred in antiquity (Sola et al., 1999), it is thought that the modern tuberculosis epidemic began in Europe in the 1700s, then moved to the New World and Africa (Stead et al., 1995). Consistent with the above theories, we have demonstrated that patterns 50 and 53 were present in Europe in the 18th century. Spoligotypes 50 and 53 differ by the absence of one spacer (spacer 31). Recent studies have demonstrated that in some cases the absence of spacer 31 in spoligotype 50 may be due to the presence of IS6110 in this region (Filliol et al., 2000; Legrand et al., 2001). In an extensive genotyping study it has been shown that strains with spoligotype 53 are groups 2 or 3 and that strains with spoligotype 50 are group 2 (Soini et al., 2000).

However, our spoligotype pattern 50 strains are group 3 rather than group 2. It is possible that the strains we are observing are indeed spoligotype 53 with an IS6110 element in spacer 31 as opposed to a true type 50 (Legrand et al., 2001). Screening for the presence of IS6110 in spacer 31 of the direct repeat (DR) region should determine if this is the case, although so far we have been unsuccessful in obtaining sequence data from this region.

The present study, based on multiple targets in the MTB genome, has confirmed that the amplified DNA from the Hungarian samples was that of MTB. The two-centre system used to analyse the Hungarian samples confirms the inter- and intra-laboratory reproducibility of the results. Using MTB-specific primers followed by sequencing or probing, DNA has been amplified from eight loci on the MTB genome, including both single-copy and multiple sites. The detection of small-scale genomic deletions has been shown to be a useful technique for exploring the molecular epidemiology, microbial evolution and pathogenesis of tuberculosis (Ho et al., 2000; Kato-Maeda et al., 2001). Using a novel multiplex PCR technique we have demonstrated the use of screening historical cases of tuberculosis for such deletions. Spoligotyping revealed a difference between the strain infecting the mother and the strain infecting her two daughters; the mother typing as type 53 and the daughters as type 50. Using multiplex PCR of the mtp40 and plcD loci we initially concluded that there was a further difference between the strains infecting the two daughters, the isolate from the younger daughter having undergone a deletion in the plcD-oxidoreductase region that was detected using the larger of the two target sequences used (Fig. 1a, lane 6). About one-third of clinical isolates of MTB studied appear to have undergone a deletion in this region (Ho et al., 2000).

However, the use of a shorter target sequence and nested PCR demonstrated (Fig. 1b) that this initial assumption was incorrect and that the material from the younger daughter was less well preserved, shown by the difficulty in obtaining positive results when larger target sequences were used. The spoligotyping data also suggest poor preservation of part of the DR spacer regions from MTB in this individual. As determination of deletion subsets in this field is likely to be increasingly attempted for phylogenetic purposes, it will be important, as a check on DNA preservation, to multiplex PCRs such as plcD with primers to another target region that produce a slightly longer product.

In conclusion, it appears that the three members of the family group studied here were infected with two distinct strains of MTB. This is the first demonstration of spoligotyping combined with screening of small-scale deletions for determining the molecular epidemiology of MTB from archaeological material. In comparison with the strain infecting the mother, there was one point mutation and one deletion event, the loss of spacer 31, detected in the MTB from the two daughters. This supports the theory that MTB undergoes more deletion events as it evolves (Ho et al., 2000; Kato-Maeda et al., 2001; Brosch et al., 2002). However, screening of the entire genomes of these strains would be needed to confirm this. The findings are also consistent with the major genotypic grouping described by Sreevatsan et al. (1997), which is thought to broadly represent an evolutionary scenario for the MTB complex. Genotypes 2 and 3 are believed to be younger in evolutionary terms than genotype 1 organisms, so their demonstration in 18th-century Europe supports the latest hypothesis of MTB evolution based on deletion analysis of the genomes of MTB and M. bovis (Brosch et al., 2002). Future studies adopting the methods described here should contribute further to our knowledge of the evolution and epidemiology of tuberculosis.

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