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

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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 IS*6110*, *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.

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INTRODUCTION

Building works carried out in 1994 at the Dominican church in Vác, Hungary, led to the chance discovery of two crypts, which had been sealed since 1838. One of these was found to contain a total of 263 human bodies within wooden coffins; about two-thirds of the bodies were naturally mummified. The human remains proved to be those of prominent local families and clerics accorded interment between the years 1731 and 1838. Information from the coffin plates and local archival records, combined with the appearances of some of the bodies, led to the suggestion that many of the individuals were suffering from tuberculosis at the time of death. Certainly, tuberculosis was one of the most prevalent infections in Europe during this period, accounting for one in four deaths from the 16th to 18th centuries (Dubos & Dubos, 1952). The presence of tuberculosis in the Vác samples was confirmed by computerized tomographic (CT) examination of one of the bodies at the Natural History Museum in Budapest, histopathology of lung tissue, and a pilot study using PCR to amplify the IS*6110* element specific to the *Mycobacterium tuberculosis* (MTB) complex (Thierry *et al.*, 1990; Páp *et al.*, 1999). Preliminary testing on the Vác remains suggested that the preservation of DNA was sufficiently robust to permit in-depth study of the mycobacterial DNA from different strains persisting in the bodies.

Contemporary written records were available for many of the individuals found in the crypt, including date of death, age, sex, family name, relatives and, in some cases, a brief description of the cause of death. These records enabled several bodies to be placed into family groups. Of particular interest in this respect was a small family group consisting of a mother and two daughters, all of whom had died over a four year period between 1793 and 1797. Body 28 was that of a 55-year-old woman who died on 16 December 1793, the mother of the two other individuals reported here. Her height was noted as 1·45 m. Soft tissue was taken from the tracheal region and abdomen of body 28, using Stortz endoscopes and an aseptic technique as precautions against

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Abbreviation: MTB, *Mycobacterium tuberculosis*.

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 cachectic 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 cachectic 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 mummified 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.

Table 1. Samples collected from the family group studied here

Body no.	Relationship	Year of birth/death	Age at death (years)	Sample type
28	Mother	1738/1793	55	Abdomen Trachea
68	Daughter 1	1769/1797	28	Chest
72	Daughter 2	1780/1795	14	Abdomen Chest Pleura

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 8·0 (Promega), 0·4 mg proteinase 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 IS*6110* (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 site for IS*6110* 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 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.

Locus (accession no.)	Primers (5′→3′)	Product size (bp)	$MgCl_2$ (mM)	Parameters	Cycles
IS*6110**†	P1, CTCGTCCAGCGCCGCTTCGG P2, CCTGCGAGCGTAGGCGTCGG	123	1·5	94 °C, 40 s 68 °C, 1 min 72 °C, 20 s	45 or 25 if nested
IS*6110**†	IS3, TTCGGACCACCAGCACCTAA IS4, TCGGTGACAAAGGCCACGTA	92	2·5 or 1·5	94 °C, 40 s 58 °C, 1 min 72 °C, 20 s	25
19 kDa antigen gene*	NB3, TCTTTCCGGATGTTCAAGCA NB5, GTGACGTTCTGGTCCTTACC	131	1·5	95 °C, 40 s 68 °C, 1 min 72 °C, 20 s	40
gyrA 95* (L27512)	GyrU1, CGATTCCGGCTTCCGCCCGG GyrU2, CCGGTGGGTCATTGCCTGGCG	194	1·5	95 °C, 40 s 68 °C, 1 min 72 °C, 20 s	40
gyrA 95†	F, CGAGACCATGGGCAACTACCA R, CATTGCCTGGCGAGCCGAA	131	1·5	94 °C, 10 s 65 °C, 30 s 72 °C, 10 s	43
katG 486* (X68081)	KatGa, GGCCGCTGGTCCCCAAGCAG KatGb, GGCTGCAGGCGGATGCGACC	220	1·5	95 °C, 40 s 68 °C, 1 min 72 °C, 20 s	40
katG 486†	F, TCAGCCACGACCTCGTCGG R, AGGCGGATGCGACCACCGTT	163	1·5	94 °C, 10 s 65 °C, 30 s 72 °C, 10 s	43
katG 203†	F, TTCGGCCGGGTCGACCAGT R, GGTTCACGTAGATCAGCCCATC	142	1·5	94 °C, 10 s 62 °C, 30 s 72 °C, 10 s	43
*dnaA–dnaN**	Pr1, GTCACAGAGATTGGCTGTGAGTGT Pr2, AGGTCGACGTCGGTCGGAGTTGT Pr3, GCCTACTACGCTCAACGCCAGAG	Pr1/Pr2 =159 or ~1500 Pr2/Pr3 =230 or 160–200	1·5	95 °C, 40 s 48 °C, 1 min 72 °C, 20 s	40
mtp40† (M57952)	F, CTGGTCGAATTCGGTGGAGT R, ATGGTCTCCGACACGTTCGAC	152	1·5	94 °C, 10 s 62 °C, 30 s 72 °C, 15 s	43
MT1801–*plcD* region†	M3, AGAATTACTTTCAGGCTCTGGA M4, CCATCCCATAGCCACGAAT	177	1·5	94 °C, 10 s 60 °C, 30 s 72 °C, 15 s	43
plcD region*	PD1, AGGCTCTGGATTACCGCATC PD2, GACCCCACCGATGGCGACG	123	1·5	95 °C, 40 s 60 °C, 1 min 72 °C, 20 s	43 or 25 if nested
	PD3, ACCGCATCCTTCCGGCGGA PD4, CGGTGGGGTCGAGGATGTCGC	102	1·5	As for PD1/2	25
oxyR† (U16243)	F, CGCGCTGTCAGAGCTGACTTT R, TCTGCGGAATCAGTGTCACC	150	2·0	94 °C, 10 s 62 °C, 30 s 72 °C, 15 s	43
RD7†	F, ACTTCAGTGCTGGTTCGTGG R, ATCTTGCGGCCCAATGAATC	211	2·0	94 °C, 20 s 66 °C, 30 s 72 °C, 20 s	43
DR region†‡	DRa, GGTTTTGGGTCTGACGAC DRb, CCGAGAGGGGACGGAAAC	Various	3·0	96 °C, 20 s 57 °C, 40 s 72 °C, 30 s	40

*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.

Gene codon	Genotypic group			
	1A	1B	2	3
katG 463	Leu, CTG	Leu, CTG	Arg, CGG	Arg, CGG
katG 203*	Thr, ACT	Thr, ACC	Thr, ACC	Thr, ACC
gyrA 95	Thr, ACC	Thr, ACC	Thr, ACC	Ser, AGC

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, IS*6110* 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 (10^6 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 IS*6110*. 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 IS*6110* and negative for an IS*6110* fragment in the *dnaA–dnaN* region, indicating that they were members of the MTB complex but not of the group 1 subtype. An IS*6110* 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.

Sample analysed (body no.)	Tests that differentiate *M. bovis* and MTB*				
	mtp40 fragment	MT1801–*plcD*	*oxyR* nucleotide 285	Genotypic group	Spoligotype
Abdomen (28)	+	+	G	2	53
Chest (68)	+	+	G	3	50
Chest (72)	+	− (177 bp) + (123 bp)	G	3	50

*+, Positive; −, negative; G, guanine, denotes MTB.

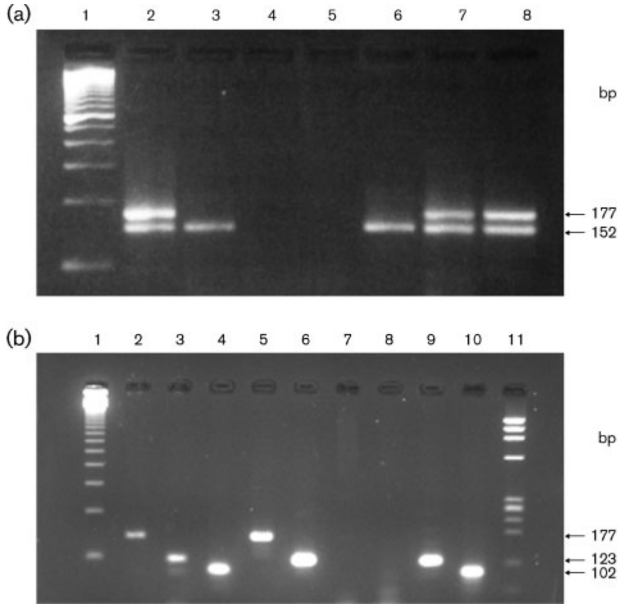

Fig. 1. (a) Multiplex PCR to screen for a deletion in *plcD*. Lanes: 1, 100 bp DNA size marker; 2, strain CDC 1551; 3, strain H37Rv; 4, extraction control; 5, PCR control; 6, body 72; 7, body 68; 8, body 28. (b) Examination of effect of PCR target size in *plcD*. Lanes: 1, 123 bp DNA ladder; 2, body 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, ϕX 174/*Hae*III 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 IS*6110* in the *dnaA–dnaN* region

A 159 bp product was amplified from the Vác 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 777777777730771 and 777777777760771, 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 densiometric 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 IS*6110* 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: IS*6110* (92 bp) $y=-3{\cdot}8124$; 19 kDa antigen gene (131 bp) $y=-3{\cdot}68439$; *dnaA–dnaN* region (159 bp) $y=-3{\cdot}5276$; MPB70 antigen gene (372 bp) $y=-3{\cdot}9832$.

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.*, 1994). 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

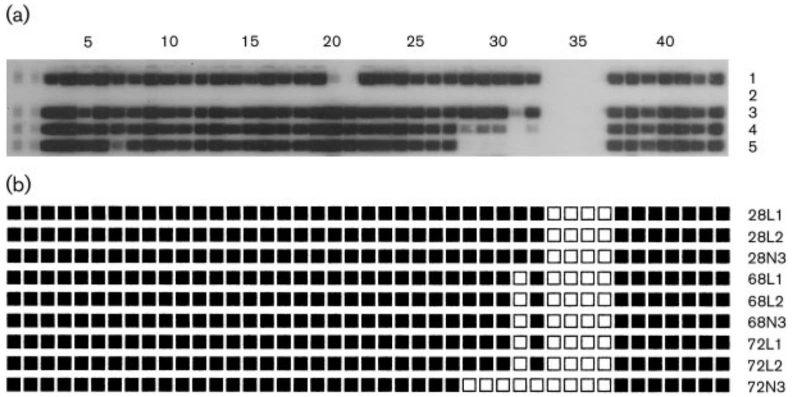

Fig. 2. (a) Spoligotyping of DNA from bodies 28, 68 and 72. Rows: 1, strain H37Rv; 2, PCR control; 3, body 28; 4, body 68; 5, body 72 (spacers 28, 29, 30 and 32 are present but faint). (b) Schematic representation of spoligotyping results from London and The Netherlands. Suffixes: L1, extract 1 typed in London; L2, extract 2 typed in London; N3, extract 3 typed in The Netherlands.

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

Data are from one experiment.

Sample (body no.)	PCR target sequence (bp)			
	IS*6110* (92 bp)	19 kDa antigen (131 bp)	*dnaA–dnaN* (159 bp)	MPB70 antigen (372 bp)
M. bovis AN-5, reference strain	$8{\cdot}6\times10^{10}$	$7{\cdot}9\times10^{10}$	$8{\cdot}8\times10^{10}$	$2{\cdot}4\times10^{11}$
Abdomen (28)	$1{\cdot}8\times10^{11}$	$1{\cdot}1\times10^{11}$	$9{\cdot}8\times10^{10}$	$1{\cdot}0\times10^{9}$
Trachea (28)	$2{\cdot}1\times10^{11}$	$1{\cdot}1\times10^{11}$	$8{\cdot}6\times10^{10}$	ND
Chest (68)	$4{\cdot}9\times10^{11}$	$4{\cdot}2\times10^{11}$	$5{\cdot}6\times10^{11}$	$2{\cdot}2\times10^{11}$
Abdomen (72)	$1{\cdot}1\times10^{11}$	$1{\cdot}9\times10^{10}$	ND	ND
Chest (72)	$3{\cdot}3\times10^{11}$	$1{\cdot}4\times10^{11}$	$8{\cdot}2\times10^{10}$	$4{\cdot}3\times10^{9}$
Pleura (72)	$1{\cdot}3\times10^{11}$	$3{\cdot}6\times10^{9}$	$3{\cdot}4\times10^{10}$	$3{\cdot}2\times10^{9}$

ND, Not detected.

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 IS*6110* insertion in this target region.

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-discriminatory. 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 IS*6110* 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 IS*6110* element in spacer 31 as opposed to a true type 50 (Legrand *et al.*, 2001). Screening for the presence of IS*6110* 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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