Confirmation of the presence of *Mycobacterium tuberculosis* complex-specific DNA in three archaeological specimens.

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ABSTRACT

This journal published the first reported identification of *Mycobacterium tuberculosis* complex (MTB) DNA in ancient human remains but concerns were raised about the article two years after publication. These were based on methodology which, in the field of ancient DNA, was still developing. Here we present a re-examination of the 1993 research conducted on three specimens which exhibited palaeopathologies indicative of tuberculosis. The specimens were: an ulna from pre-European contact Borneo, a spine from Byzantine Turkey, and a lumbar-sacral spine from 17th century Scotland. There was insufficient material to permit re-examination of all of the original samples. The earlier results were confirmed in two independent laboratories using different methodologies. MTB DNA complex-specific DNA amplicons were obtained, and sequenced in both laboratories, in a re-analysis of samples which supported the earlier findings.

Keywords: ancient DNA; *Mycobacterium tuberculosis*; PCR.

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Introduction

In 1993 the Department of Bacteriology (now Medical Microbiology) at University College London published the first paper that reported the finding of pathogenic bacterial DNA in ancient human remains (Spigelman & Lemma, 1993). However, this research received some criticism as technical procedures were not described fully and the results were not confirmed as *Mycobacterium tuberculosis* complex (MTB) DNA by sequencing (Blondiaux & Stanford, 1995).

Subsequent developments in the field of ancient DNA have focused on palaeopathology and its genetic identification in the archaeological record. The identification of MTB-specific DNA and DNA from other pathogens, from a wide variety of specimens from different times and geographical sites, supports our initial findings on the preservation and persistence of ancient microbial DNA in infected biological remains (Arriaza *et al*., 1995; Baron *et al.*, 1996; Donoghue *et al.*, 1998 & 2001; Faerman *et al*. 1997; Haas *et al.*, 2000; Mays *et al*., 2000; Nuorala, 1999; Spigelman & Donoghue, 2001; Taylor *et al*., 1996 & 1999; Zink *et al*., 2001).

The original paper identified *M. tuberculosis* in pre-European-contact human remains from Borneo, Asia (D. Brothwell, personal communication), and this was of particular significance for the history of disease. The initial palaeopathology of this Borneo sample suggested a diagnosis of yaws (D. Brothwell, personal communication), but a lesion in the ulna was due to osteomyelitis but not morphologically characteristic for any specific diagnosis. Due to the significance of the finding of MTB DNA from Borneo, the analysis was repeated using more developed techniques: more efficient high cycle number PCR, additional PCR target sequences, and DNA sequencing. In addition, the experiment was conducted by different researchers in two different laboratories, as suggested at the Second Meeting of the Ancient Biomolecules Initiative (Cambridge, 1996). The Medical Microbiology Department (Laboratory 1) at University College London was the first to conduct this research, and the Kuvin Center for the Study of Tropical and Infectious Diseases, the Hebrew University, Hadassah Medical School, Jerusalem (Laboratory 2) acted as the independent verification laboratory.

Two additional samples from the 1993 research were also re-examined to test the reliability of the original *M.tuberculosis* identification. Laboratory 1 re-examined a sample of lumbar-sacral spine showing evidence of Pott's Disease from 17th Century Scotland, and a child's spine from Byzantine Turkey where at least three of the vertebral bodies had collapsed and fused. Laboratory 2 examined the sample from Byzantine Turkey. Insufficient sample prevented the re-extraction of 17th Century Scotland remains for verification in Laboratory 2, or any re-examination of the fourth sample, a talus from Medieval England (Spigelman & Lemma, 1993). Details of which samples were re-examined and in which laboratory are summarised in Table 1.

Materials and Methods

The sampling of the specimens from Borneo and Turkey was carried out at the Institute of Archaeology, University College London. The Scottish specimen was sampled at the Hunterian Museum of the Royal College of Surgeons, London. Neither institution has a DNA facility or is involved in research on *Mycobacterium tuberculosis*. None of the three specimens have ever physically been removed from their storage place to be taken to our laboratory and for this experiment fresh samples were obtained from each of the specimens. The samples were taken on different days using standard precautions (O'Rourke *et al*., 2000), with protective clothing and sterile instruments. For the Borneo ulna specimen a dental drill was used. Using two separate bits, the

surface layer was removed and a new bit was used to collect the sample. These procedures were identical to those used in the original work, although the precautions against contamination were not then described fully.

All the recommended protocols of ancient DNA (aDNA) work (O'Rourke *et al*., 2000) were followed, with separate rooms for different stages of the process. Laboratory 1 only carries out work with modern MTB DNA in a designated laboratory with negative air pressure and filtered air supply. Laboratory 2 carries out no work with modern MTB DNA. In both laboratories, final crushing of the sample was carried out in a sterilised pestle and mortar. Extraction negative control tubes were always processed in parallel with tubes containing samples.

Laboratory 1 protocols

DNA extraction

A demineralization solution (100 µl of 0.5M EDTA, pH 8.0 and Proteinase K (Finnzymes) 1 mg ml^{-1}) was added to approximately 10-25 mg of sample in a sterile 1.5 ml Eppendorf tube containing 10 glass beads of 1.5-2 mm diameter (Merck), mixed on a mini bead beater, and incubated at 56 ^{o}C overnight or until the sample was dispersed. A positive DNA extraction control was included, which consisted of a sample of calcified pleura from Karkur in ancient Palestine, dating from 600 AD (Donoghue *et al*., 1998). Lysis buffer L6 (Boom *et al*., 1990) was added (250 µl), the tube vortexed and incubated at 37 ^{o}C for 2 h followed by a vortex mix and centrifugation (1 min at 13000 rpm). The supernatant was placed in another sterile tube with 25 µl of silica suspension (12% SiO_2 (Sigma) w/v, in water, adjusted to pH 2.0 with HCl) vortexed and agitated for 1 h at room temperature, to allow cell lysis and binding of DNA to the silica. Samples were re-suspended, centrifuged for 1 min as before and the supernatant discarded. 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 of ultrapure water (Sigma) and incubation at 56 ^{o}C for 30 min followed by centrifugation at 13,000 rpm for 3 min. This was repeated once, the eluates pooled and stored at -20 ^{o}C.

DNA amplification

A two-tube nested PCR was used to detect MTB DNA by targeting a repetitive fragment, the insertion sequence IS6110 (Taylor et al. (1996). This element is normally present in multiple copies in MTB strains and the first pair of primers (P1 and P2) amplify a 123 bp region of IS6110 (Eisenach et al, 1990). The second set of primers, (IS-3 and IS-4) bind internally with some overlap to P1 and P2, and amplify a 92 bp product (Table 2). Nested PCR is exquisitely sensitive, so stringent precautions were necessary to avoid cross-contamination. Clean protective clothing was worn with frequent glove changes. A separate room was used for handling PCR product, with a different set of pipettors and protective clothing. Pipettors were washed with neat household detergent, rinsed with ultrapure water and dried with ethanol before use. Surfaces were cleaned, rinsed and dried as above. All tips and tubes were sterile and plugged tips were used. Reagents were purchased rather than prepared in the laboratory.

Double-strength pre-aliquoted PCR mix from ABgene® (Epsom, UK) was used with a final volume of 50 µl. The final composition of the PCR mixture consisted of: 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 nucleotide (dATP, dCTP, dGTP and dTTP); 0.5 µM of each primer (Oswel Research Products Ltd, Southampton, UK); and 1.25 units *Taq* DNA polymerase. The primer pair (2.0 µl), BSA (at a final concentration of 2.0 mM,

Sigma), and DNA preparation (5.0 µl) were added to each tube. A control tube with water (Sigma, 'water for PCR') in place of template was always included.

Amplification consisted of initial denaturation at 94 °C for 1 min. and 25 cycles of: 40 s at 94°C, 1 min at 68 °C and 20 s at 72 °C, followed by 1 min at 72 °C. For the nested PCR, BSA, 2.0 µl of the IS-3/IS-4 primer pair and 0.5 µl of first stage PCR product were added to each reaction tube and the volume made up to 50 µl. Amplification in this second stage PCR followed the first protocol but with primer annealing at 58 °C.

Detection and sequencing

PCR product (7 µl) was added to 3 µl loading buffer (Sigma) and electrophoresed in a 3.0% (w/v) NuSieve® 3:1 agarose gel (FMC Bioproducts, Flowgen) in TBE buffer (0.09 mM Tris-borate and 0.002 mM EDTA) or TAE buffer (0.04 mM Tris-acetate and 0.001 mM EDTA) at 8.8 volts cm^{-1} for 80 min. Amplified DNA was visualised by ethidium bromide staining exposed under ultraviolet light and was recorded with a Polaroid camera or digital imaging system.

PCR product (8-15 µl) was mixed with 4 µl loading buffer and separated on a gel as above, by electrophoresis with TAE buffer. The selected band (150-250 mg) was removed from the gel with a sterile scalpel blade into a sterile 0.5 ml tube. DNA was extracted from the gel slices using a MERmaid™ Spin Kit for samples 10-200 bp (Anachem, UK), the purified product was then sequenced by MWG-BIOTECH AG (Ebersberg, Germany).

Laboratory 2 protocols

DNA extraction

No demineralization procedure was performed in Laboratory 2. Approximately 50 mg of sample was placed in a 1.5ml tube containing 500 µl of lysis buffer L6 (Boom *et al*., 1990), vortexed for 1 min and incubated at 56 ºC overnight under gentle agitation.

The sample was then heated at 94 ºC for 10 min, centrifuged at 12,000 rpm for 3 min and the supernatant transferred to another sterile 1.5 ml tube. Sodium iodide (6 M) solution (1 ml) and 10 µl of silica suspension (SiO_2 (Sigma) 12% w/v in water) was added to the sample. The tube was mixed for 20 s and placed on ice for 1 hour with agitation every 15 min. The sample was centrifuged at 12,000 rpm for 30 s and the supernatant discarded carefully. Next, 500 µl of washing buffer (2 mM Tris-HCl, pH 7.5; 10mM EDTA, pH 8.0; 10 mM NaCl in a 50% (v/v) water/alcohol mixture (-20 °C) was added, mixed and centrifuged for 30 s at 12000 rpm. The supernatant was discarded. The wash buffer step was repeated if necessary, to clean the pellet. The silica pellet was washed with 200 µl absolute ethanol as above and allowed to air dry. DNA was eluted with 150 µl of water (Mini-Plasco®, Braun Melsungen AG), mixed for 20 s and incubated at 56 ºC for at least 1h. To stabilize the DNA, 5 µl of TE buffer (10 mM Tris, pH 7.5) and 1mM EDTA, pH 7.5-8.0), was added to each extract. The sample was centrifuged at 12000 rpm, for 3 min and the DNA extract stored at 4 ºC.

DNA amplification

Standard precautions to minimise contamination were employed. Face masks, clean laboratory coats and double gloves were used. All equipment and surfaces were washed with 10% bleach and UV-irradiated between each use. Work was carried out under UV hoods and separate rooms were used for preparation of PCR reagents, sample preparation and PCR set up. Two different sets of primers were used for the PCRs carried out in Laboratory 2: the INS-1/INS-2 primers which target a 246 bp sequence (based on van Embden *et al*., 1993), and the S12-F/S12-R primers, which target a region in the ribosomal protein S12 gene (Finken *et al.*,1993), and amplify a 204 bp sequence of MTB

DNA (Table 2). For each sample PCR was replicated four times, with successful amplification each time and each product was sequenced.

The PCR was performed in a final volume of 25 µl in a mixture consisting of: 200 mM Tris-HCl, pH 8.4; 500 mM KCl; 2.0 mM $MgCl_2$; 2.5 mM of each nucleotide (dATP, dCTP, dGTP and dTTP); 0.25 µM of each primer; and 1.25 units of Platinum *Taq* polymerase. The DNA extract (10.0 µl) was added to each tube and both negative and positive controls were always included. The amplification program consisted of a 3 min initial denaturation at 94 ^{o}C, followed by 45 cycles of: 94 ^{o}C for 1 min, 60 ^{o}C for 1 min and 72 ^{o}C for 2 min; followed by 10 min at 72 ^{o}C.

Detection and sequencing

PCR product (10 µl) was added to 1.0 µl loading dye (MBI Fermentas) and electrophoresed in a 2.0% (w/v) NuSieve® (FMC Bioproducts) in TAE at 108 volts for 35 min. Amplified DNA was visualised by ethidium bromide staining exposed to ultraviolet light and recorded with digital imaging equipment (Pharmacia Biotech, ImageMaster).

PCR product was excised from the NuSieve® gel and was amplified on a sequencing reaction of 50 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 30 s and 72 ^{o}C for 1 min. The product was directly sequenced on 6% acrylamide gel (57 g urea; 18 ml 10% TBE; 10-12 ml 40%; acrylamide 19-1, 50 ml dd water, 35 µl TEMED, 900 µl 10% APS) using a radioactive manual sequencing kit (Sequenase Kit, Amersham) and exposed to X-ray film (Kodak) to visualise.

Results

M.tuberculosis complex-specific DNA was identified in all three samples in Laboratory 1 (Figure 1, with the Borneo sample shown in lane 5, the Turkey sample in lane 9 and the Scottish sample in lane 3) although not every DNA extraction yielded positive results (Table 3); negative extraction and PCR controls were satisfactory. The 17th-18th century Scottish sample was sequenced (Figure 2). The positive strand sequence of 76 bp from Laboratory 1 has 100% identity with the MTB repetitive element IS*6110* sequence in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST/). The negative strand sequence of 73 bp also has 100% identity with this sequence, with no gaps, mismatches, or unknown bases, and both sequences include the whole of the target sequence between the inner primers.

MTB DNA was detected in both samples examined in Laboratory 2. The sequence of 246 bp obtained with primers INS-1 and INS-2 from the Borneo and the Byzantine Turkey samples have 100% identity with the MTB repetitive element IS*6110* (Figure 3). Using the S12 primers, the sequences of 204 bp obtained for both samples have 100% identity with MTB (Figure 4). Both sequences display no gaps, mismatches or unknown bases.

Discussion

The original findings of the successful identification of *M.tuberculosis* complex DNA (Spigelman and Lemma 1993) have been confirmed in all three samples re-examined. This re-analysis of the samples employed nested PCR and more efficient high cycle number single-stage PCR than in the original study. Work done on 18th century material has shown that nested PCR, which enables a total of 50 rounds of amplification, gives approximately twice the number of positive results compared with 45 rounds of single-stage PCR carried out on the same DNA extracts with only the outer primers P1/P2 (Fletcher *et al*. 2002). Although 40 amplification rounds were used in the original work by Spigelman and Lemma (1993), the new generation of thermal cyclers enable programmes to

be completed more rapidly and with greater efficiency. As a result, the re-examination of samples proved successful in the identification of MTB DNA.

The DNA extraction protocols differed in the two laboratories. The Laboratory 1 protocol had been optimised for the extraction of mycobacterial DNA based on modern environmental species such as *Mycobacterium chelonae* and *M. vaccae,* which are resistant to commercial protocols. However, the protocol used in Laboratory 2 was optimised for the recovery of mammalian DNA which is notoriously fragile. Therefore, the methodologies may at least partially explain the different results obtained in the two centres. In addition, larger quantities of sample were examined in Laboratory 2. The difficulty in obtaining consistent positive results from very small quantities of material was apparent in Laboratory 1. It may also reflect the very low level of MTB DNA in the samples and its uneven distribution. This is a common observation in aDNA studies of MTB DNA in our experience but has been rarely reported. It would be helpful if future studies on aDNA included an indication of the reproducibility of any data obtained. Laboratory 1 used a commercial DNA sequencer which may partially explain the slight discrepancy (10 bp) between target size and length of sequence obtained, as normal commercial protocols are based on excluding any molecules below 100 bp. However, the low amount of amplicon obtained is undoubtedly another factor.

Although tuberculosis occurred in antiquity (Rothschild *et al*., 2001; Zink *et al*., 2001), it has been postulated that the modern TB epidemic began in Europe in the 1700's then spread to the New World and Africa (Stead *et al*., 1995). The confirmation of MTB DNA in human remains that pre-date European contact in Borneo, suggest that a form of this disease existed in the human population before the subsequent contacts between Europeans and Asians in the Indonesian archipelago. Brothwell's original diagnosis of the lesion sampled was of yaws or osteomyelitis (D. Brothwell, personal communication). The finding of MTB DNA indicates that the individual was suffering from tuberculosis but does not exclude the possibility of another infection in this bone.

The MTB complex consists of several closely related species of mycobacteria that can cause tuberculosis in humans and animals, the most significant of which are *M. tuberculosis* and *M. bovis*. Whereas *M. tuberculosis* is primarily found in humans, *M. bovis* has a broad host range and has natural reservoirs in both wild and domesticated animals. Human infections are believed to be mainly of zoonotic origin (Grange, 2001). It is now possible to distinguish between *M. tuberculosis* and *M. bovis* by PCR (Taylor *et al*., 1999). However, the method is based on a deletion at a single chromosomal site so is much less sensitive than PCR based on a repetitive element such as IS*6110*. Therefore, only well-preserved DNA is likely to yield results and this method was not attempted in the present study. However, it is also possible to identify *M. bovis* by spoligotyping (Kamerbeek *et al*., 1997) based on the DR locus of the MTB complex, where short direct repeats are separated by nonrepetitive spacers and identified by a dot blot hybridisation. This is ideal for aDNA studies of MTB as the spacer regions are each only 34 to 41 bp long. The samples from Borneo and Turkey were examined by spoligotyping in Laboratory 2 and found to contain *M. tuberculosis* (Lev *et al*., 2001). In the few fully published investigations that have sought to distinguish between *M. tuberculosis* and *M. bovis* in archaeological material (Mays *et al*., 2001, Rothschild *et al*., 2001; Taylor *et al*., 2001) only *M. tuberculosis* has been detected. As the epidemiology of infection differs between *M. tuberculosis* and *M. bovis*, it is clearly of interest in future studies to determine which species is present in archaeological specimens, where there is material in sufficient quantity and state of preservation to enable this to be carried out.

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Table 1. Details of re-examination of samples positive for *Mycobacterium tuberculosis* complex DNA (Spigelman & Lemma, 1993)

Sample	Date	Original suspected disease	Examined by Laboratory 1	Examined by Laboratory 2
Ulna, Borneo	Pre-European contact	Yaws	Yes	Yes
L-S spine, Turkey	Byzantine	Tuberculosis	Yes	Yes
L-S spine, Scotland	17th-18th century	Tuberculosis	Yes	No
Talus, England	Medieval	Leprosy	No	No

Table 2. *M.tuberculosis* complex-specific primers used in this study

Name	Target region	Target size	Primer
Laboratory 1			
P1	IS*6110*	123 bp	5'CTCGTCCAGCGCCGCTTCGG 3'
P2	"		5'CCTGCGAGCGTAGGCGTCGG 3'
IS-3	"	92 bp	5' TTCGGACCACCAGCACCTAA 3'
IS-4	"		5' TCGGTGACAAAGGCCACGTA 3'
Laboratory 2			
INS-1	IS*6110*	246 bp	5' CGTGAGGGCATCGAGGTGGC 3'
INS-2	"		5' GACGTAGGCGTCGGTGACAAA 3'
S12-F	Ribosomal protein	204 bp	5' TCGTCGGGACAAGATCAGTAAG 3'
S12-R	S12 gene		5' ATCGAGTGCTCCTGCAGGTTG 3'

Table 3. Summary of results with MTB DNA primer sets

Laboratory and DNA Extract	Samples* and target region					
	Spine Turkey		Ulna Borneo		L-S Spine Scotland	
	IS*6110*	S12	IS*6110*	S12	IS*6110*	S12
Laboratory 1 Extract 1	NT	NT	-	NT	+	NT
Laboratory 1 Extract 2	-	NT	-	NT	NT	NT
Laboratory 1 Extract 3	+[1]	NT	+[1]	NT	-	NT
Laboratory 2 Extracts 1-4	+	+	+	+	NT	NT

* Spigelman & Lemma 1993
NT Not Tested
- Negative PCR
+ Positive PCR
[1] not sequenced

Figure 1. Gel electrophoretic analysis of IS*6110* PCR products (92 bp) from Laboratory 1. Lanes 1 and 11: 123 bp ladder; lane 2: PCR negative control; lane 3: 17th century Scottish specimen, subsequently prepared for sequencing; lane 4: specimen from Karkur (Donoghue *et al*., 1998) - positive extraction control; lane 5: specimen from Borneo; lane 6: PCR negative control; lane 7: negative DNA extraction control; lane 8: PCR negative control; lane 9: specimen from Turkey; lane 10: negative DNA extraction control.

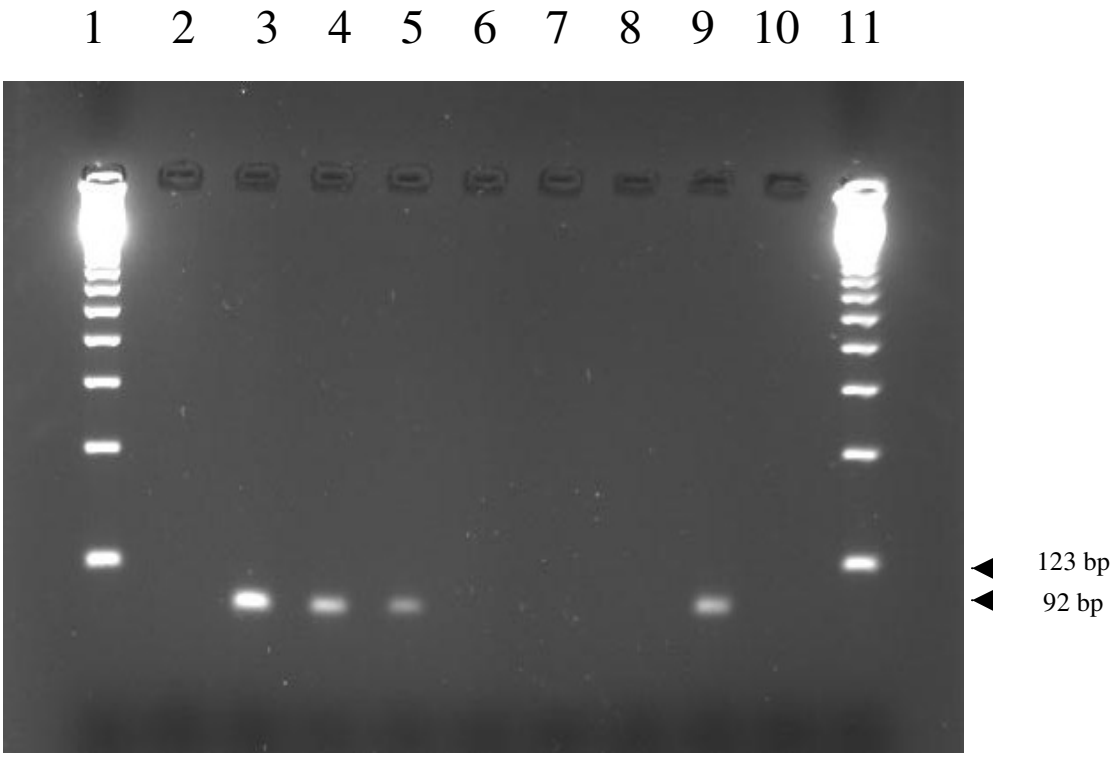

Figure 2. Alignment of genome sequences from X17348.1 *Mycobacterium tuberculosis* IS*6110* IS-like element; Laboratory 1 nested PCR product from Scottish L-S spine sample.

Positive strand primer: IS-3 (5' - 3')

```
M tb:       788   AGCACCTAACCGGCTGTGGGTAGCAGACCTCACCTATGTGTCGACCTGGG 837
Scottish    1     ************************************************** 50

M.tb:       838   CAGGGTTCGCCTACGTGGCCTTTGTC 863
Scottish    51    ************************** 76
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Negative strand primer: IS-4 (3' - 5')

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M tb:       854   CACGTAGGCGAACCCTGCCCAGGTCGACACATAGGTGAGGTCTGCTACCC 805
Scottish    1     ************************************************** 50

M.tb:       804   ACAGCCGGTTAGGTGCTGGTGGT 782
Scottish    51    ********************** 73
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Underline indicates primer sequence
* indicates sequence homology

Figure 3. Alignment of genome sequences from X17348.1 *Mycobacterium tuberculosis* IS*6110* IS-like element; single stage PCR product obtained at Laboratory 2 from the Borneo and Turkey samples and sequences showing some homology.

Positive strand primer: INS-1 (5' - 3')

```
M tb:        633   CGTGAGGGCATCGAGGTGGCCAGATGCACCGTCGAACGGCTGATGACCAA 682
Borneo:      1     ************************************************** 50
Turkey:      1     ************************************************** 50
S.liv[1]:    659   ..............*******C*C***********G*********..... 689
N.men[2]:    10287 ............................**********.....  10270
H.sa[3]:     143892 ............**********.....................  143875


M.tb:        683   ACTCGGCCTGTCCGGGACCACCCGCGGCAAAGCCCGCAGGACCACGATCG 732
Borneo:      51    ************************************************** 100
Turkey:      51    ************************************************** 100
Ps.aer[4]:   6657  ..................................................** 6658
H.sa[5]:     115326 ....**********.....................  115343


M.tb:        733   CTGATCCGGCCACAGCCCGTCCCGCCGATCTCGTCCAGCGCCGCTTCGGA 782
Borneo:      101   ************************************************** 150
Turkey:      101   ************************************************** 150
Ps.aer:      6659  **********.................................. 6676
Am.med[6]:   1679  ..............**********.....................  1658
S.coe[7]:    22100 ..................**********C**********.....  22123
Am.or[8]:    32773 ..............**********.....................  32773
C.cr[9]:     8901  ........................**********.....  8918
R.leg[10]:   3149  ......................*******G*C**********.  3124


M.tb:        783   CCACCAGCACCTAACCGGCTGTGGGTAGCAGACCTCACCTATGTGTCGAC 832
Borneo:      151   ************************************************** 200
Turkey:      151   ************************************************** 200


M.tb:        833   CTGGGCAGGGTTCGCCTACGTGGCCTTTGTCACCGACGCCTACGTC..  880
Borneo:      201   ********************************************..  246
Turkey:      201   ************************.....................  248
G.gall[11]:  1559  ..............**********.....................  1578
A.tum[12]:   528   ..........****T**********T*****.............  501
```

Underline indicates primer sequence

* indicates sequence homology

[1]U50076.1 *Streptomyces lividans* chromosomal terminal inverted repeat containing insertion sequence IS1372
[2]AE002472.1 *Neisseria meningitidis* serogroup B strain MC58 section 114 of 206 of the complete genome
[3]AL121781.38 Human DNA sequence from clone RP5-1164C1 on chromosome 20.
[4]AE004581 *Pseudomonas aeruginosa* PA01, section 142 of 529 of the complete genome
[5]AC005940 *Homo sapiens* chromosome 17, clone hRPK.167_N_20, complete sequence
[6]Y16952.1 *Amycolatopsis mediterranei* 9.9kB DNA fragment from oxyA to oxyE genes
[7]AL132648.1 *Streptomyces coelicolor* cosmid I41
[8]AJ223999.1 *Amycolatopsis orientalis* cosmid PCZA363
[9]AE005680 *Caulobacter crescentus* section 6 of 359 of the complete genome
[10]AF228578.1 *Rhizobium leguminosarum* bv. viciae MazG (mazG) gene, partial cds
[11]U40598.1 *Gallus gallus* NRAMP1 (Bcg) mRNA, complete cds
[12]X55075.1 *A.tumefaciens* DNA for TA region of Ti plasmid

Figure 4. Alignment of genome sequences from X70995.1 *Mycobacterium tuberculosis* ribosomal protein S12 gene PCR product obtained at Laboratory 2 from the Borneo and Turkey samples and sequences showing some homology.

```
M.tb:        33    TCGTCGGGACAAGATCAGTAAGGTCAAGACCGCGGCTCTGAAGGGCAGCC 82
Borneo:      1     ************************************************** 50
Turkey:      1     ************************************************** 50
M.av[1]:     10    **********G**G*C*********************C********** 59
M.int[2]:    60    ..................*****************G**C********** 90
M.sz[3]:     10    ****************G*C*********************T********** 59
M.go[4]:     29    ..................********A**********C********** 59
M.le[5]:     33    ******A********TG*C**********T***************A** 82
M.sm[6]:     29    ..................*****G**********T**C********** 59
C.cr[7]:     4434  ....................................*********T*** 4422

M. tb:       83    CGCAGCGTCGTGGTGTATGCACCCGCGTGTACACCACCACTCCGAAGAAG 132
Borneo:      51    ************************************************** 100
Turkey:      51    ************************************************** 100
M.av:        60    *******C*****C*************************C********* 109
M.int:       91    *******C*****C*********************************** 140
M.sz:        60    *******C*****C*************************C********* 109
M.go:        60    *******C*****C**G*********************************** 109
M.le:        83    *A********C*****T********T********TT****C********* 132
M.sm:        60    **********C**C**G*****G*****T********************** 109
C.cr:        4421  ****A*****CG*C**T**********T********G**T********* 4372

M. tb:       133   CCGAACTCGGCGCTTCGGAAGGTTGCCCGCGTGAAGTTGACGAGTCAGGT 182
Borneo:      101   ************************************************** 150
Turkey:      101   ************************************************** 150
M.av:        110   **********************C************C*******C***** 159
M.int:       141   ********C**T**********C************C*******C***** 190
M.sz:        110   **C*********************C**T**T******C************* 159
M.go:        110   ************************C*****T******C****A**C***** 159
M.le:        133   ****************C*****************C************* 182
M.sm:        110   **************C********C**G*********C****C**C***** 159
C.cr:        4371  ***********T*****T*****..........................  4349

M. tb:       183   CGAGGTCACGGCGTACATTCCCGGCGAGGGCCACAACCTGCAGGAGCACT 232
Borneo:      151   ************************************************** 200
Turkey:      151   ************************************************** 200
M.av:        160   *********C**C*****C*********************************  209
M.int:       191   T*****G*****C*****C**********A******C*******C****  240
M.sz:        160   T********C**C*****C**G**T****C***************A****  209
M.sz:        160   T*****************C**A**T**********T*************  209
M.le:        183   T********A********A**A********T********A*****A****  232
M.sm:        160   T*****G**C**T*****C**G**T**A**********T**********  209

M. tb:       233   CGAT.....................................................  236
Sample:      201   ****.....................................................  204
M. av:       210   *........................................................  210
M.int:       241   ****.....................................................  244
M.sz:        210   ****.....................................................  213
M.go:        210   ****.....................................................  213
M.le:        233   *........................................................  233
M.sm:        210   ****.....................................................  213
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Underline indicates primer sequence

* indicates sequence homology

[1] X80120.1 *Mycobacterium avium rpsL* gene

[2] L08171.1 *Mycobacterium intracellulare* ribosomal protein S12 gene, complete cds, and ribosomal protein S7 gene, 5' end
[3] X80121.1 *Mycobacterium szulgai rpsL* gene
[4] X80123.1 *Mycobacterium gordonae rpsL* gene
[5] X80119.1 *M.leprae rpsL* gene
[6] X80125.1 *M.smegmatis rpsL* gene
[7] AE005984.1 *Caulobacter crescentus* section 310 of 359 of the complete genome