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Characterization of *Mycobacterium tuberculosis* Complex DNAs from Egyptian Mummies by Spoligotyping

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Bone and soft tissue samples from 85 ancient Egyptian mummies were analyzed for the presence of ancient *Mycobacterium tuberculosis* complex DNA (aDNA) and further characterized by spoligotyping. The specimens were obtained from individuals from different tomb complexes in Thebes West, Upper Egypt, which were used for upper social class burials between the Middle Kingdom (since ca. 2050 BC) and the Late Period (until ca. 500 BC). A total of 25 samples provided a specific positive signal for the amplification of a 123-bp fragment of the repetitive element IS*6110*, indicating the presence of *M. tuberculosis* DNA. Further PCR-based tests for the identification of subspecies failed due to lack of specific amplification products in the historic tissue samples. Of these 25 positive specimens, 12 could be successfully characterized by spoligotyping. The spoligotyping signatures were compared to those in an international database. They all show either an *M. tuberculosis* or an *M. africanum* pattern, but none revealed an *M. bovis*-specific pattern. The results from a Middle Kingdom tomb (used exclusively between ca. 2050 and 1650 BC) suggest that these samples bear an *M. africanum*-type specific spoligotyping signature. The samples from later periods provided patterns typical for *M. tuberculosis*. This study clearly demonstrates that spoligotyping can be applied to historic tissue samples. In addition, our results do not support the theory that *M. tuberculosis* originated from the *M. bovis* type but, rather, suggest that human *M. tuberculosis* may have originated from a precursor complex probably related to *M. africanum*.

Within the last few years a considerable number of molecular studies have provided evidence for the presence of *Mycobacterium tuberculosis* complex DNA in ancient skeletal and mummified material (1, 7, 9, 18, 27, 28, 34, 38, 41, 47). Besides the mere evidence of *M. tuberculosis* complex DNA, initial information suggested a high frequency of tuberculosis in ancient populations (13), and we have recently provided evidence that this also holds true for pharaonic Egypt (47). Moreover, the first studies on the differentiation of subtypes of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canettii*) have successfully been performed on ancient tissue samples (33, 42; H. Fletcher, H. D. Donoghue, J. Holton, M. Thomas, I. Pap, and M. Spigelman, Abstr. 5th Int. aDNA Conf., Ancient Biomolecules **3:**294, 2001; G. Lev, H. Bercovier, D. Brittain, and C. Greenblatt, Abstr. 5th Int. aDNA Conf., Ancient Biomolecules **3:**306, 2001). This work was carried out mainly by the spoligotyping technique (20), which is based on the variation of the direct-repeat (DR) region in *M. tuberculosis* complex members. Using this technique, differentiation up to a subspecies level is possible. Spoligotyping is widely used and accepted in medical microbiology for the initial genotyping of the *M. tuberculosis* complex at the population level. In addition, spoligotyping seems to be the most suitable method for analyzing ancient material, since usually only minute amounts of a significantly fragmented mycobacterial ancient DNA (aDNA) remains in the samples under investigation. Likewise, other recent methods, such as IS*6110* restriction fragment length polymorphism (43), ligation-mediated PCR (31), and variable number of tandem repeat typing (24), require cell culture conditions or at least high-molecular-weight bacterial DNA and are therefore not applicable to ancient tissue material.

In addition, spoligotyping seems to be suitable for investigating evolutionary aspects of human tuberculosis (36) and may clarify the origin and transmission of the disease in humans of various historical periods and populations (35). When its results are combined with other data, they can be used to construct phylogenetic trees reaching back to the beginning of the pathogenesis and spread of the disease in humans and animals (37).

In this regard, there is still an open debate about the origins of tuberculosis in human and animal species. One previous hypothesis (5) suggests that *M. bovis* is the probable ancestor which was transmitted from cattle to humans during domestication. Other theories assume that an *M. tuberculosis* complex precursor evolved from *M. africanum* and that the present-day *M. tuberculosis* and *M. bovis* developed in parallel (39). This theory is supported by nucleotide sequence analyses of current *M. tuberculosis* isolates, revealing an absence of allelic variation. The evolutionary origin of *M. tuberculosis* was therefore suggested to be 15,000 to 20,000 years ago (39).

In a recent paper, a new evolutionary scenario was presented based on the occurrence of several deletions in the genomes of the tubercle bacilli (4). These findings allow a differentiation of *M. tuberculosis* strains into modern and ancestral strains depending on the presence or absence of an *M. tuberculosis*-

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TABLE 1. Detailed information on the tombs analyzed

Tomb complex[a]	Period of construction[c]	Period of use (approx)	No. of individuals identified[d]	No. of individuals subjected to molecular investigation
TT 196	Middle Kingdom	2050–1650BC	211	37
DAN 95.1	Middle Kingdom	2050–500BC	92	10
DAN 93.11	New Kingdom	1550–500BC	70	10
TT 84	New Kingdom	1550–500BC	20	2
TT 85	New Kingdom	1550–500BC	147	6
TT 95	New Kingdom	1450–500BC	73	10
TT 453[b]	New Kingdom	1450–500BC	33	4
TT 183	New Kingdom	1250–500BC	92	6

[a] Numbers of TT tombs are those used by Porter and Moss (30).
[b] Modified TT number as used by Kampp (21).
[c] The dates for tomb construction were obtained by archaeological observations.
[d] Total number of individuals identified in the respective tomb complex.

specific deletion (TbD1). Moreover, successive loss of DNA reflected by subsequent deletions demonstrates an evolutionary lineage represented by *M. africanum*, *M. microti*, and *M. bovis* that diverged from the progenitor of *M. tuberculosis*. These results also contradict the classical hypothesis that *M. bovis* is the probable ancestor.

Evolutionary models are usually developed on data obtained from recent strains by comparing their frequencies, the occurrence of certain polymorphisms, and their genetic differences. Since such statistical models are restricted to present-day data and cannot take into account particular events of molecular evolution (such as bottlenecks and environmental disasters), only the investigation of ancient biomaterial can provide the real story of molecular evolution and the development and spread of mycobacterial disease.

In this study, we successfully applied spoligotyping to ancient Egyptian material dating back to approximately 4,000 years. Prior to this, human material had been successfully investigated only as far back as the medieval period (42). There is one study using 17,000-year-old material from fossilized animal remains that produced results (33). The spoligotyping patterns are compared to a recent database containing most of the known common mycobacterial types. The results provide interesting insights into the presence of particular subtypes of the *M. tuberculosis* complex in ancient Egypt. These data can potentially be of great use in evaluating evolutionary models and can help investigators get closer to the origin and the spread of a disease which has plagued humans for such a long time.

MATERIALS AND METHODS

Materials. All investigated material comes from recent excavations, which were performed in the necropolis of the Tombs of the Nobles in Thebes West, Upper Egypt. These anthropological and paleopathological investigations were performed under the auspices of the German Institute of Archaeology, Cairo, Egypt (headed by D. Polz), and the Institute of Egyptology and Coptology, University Münster, Münster, Germany (headed by E. Graefe). The tombs investigated were built during either the Middle Kingdom (approximately 2050 to 1650 BC) or the New Kingdom (approximately 1550 to 1080 BC) and had been used until the Late Period (until 500 BC). The tomb TT196 was used exclusively during the Middle Kingdom as evidenced by extensive archaeological investigations (E. Graefe et al., unpublished data). These studies provided evidence that this tomb was not used later than the late 17th dynasty (approximately 1600 BC). Detailed data on the tombs are given in Table 1. During the examination of the human remains, 738 individuals were identified. In spite of the extensive postmortem damage due to grave robberies and disruptions of the mummified individuals, the accessible bone and soft tissue material was in excellent condition. This can be ascribed to the mummification procedure and the favorable climatic conditions in Egypt. The remains were stored within burial shafts in the tombs, where a dry environment with stable temperature protected the tissue from advanced decomposition. From the archaeological sites, we analyzed bone and soft tissue samples from 85 individuals (Table 1). As described in our previous study of Egyptian material (47), we selected three groups of samples: (i) with morphological evidence for tuberculosis ($n = 12$ [Fig. 1A]), (ii) with nonspecific alterations ($n = 25$ [Fig. 1B]), and (iii) without any evidence of pathological alterations ($n = 48$). For a more detailed description of the samples with the morphological evidence, refer to a further study on the epidemiology of tuberculosis in ancient Egypt (A. Zink, W. Grabner, U. Reischl, H. Wolf, and A. G. Nerlich, submitted for publication).

Sample preparation and DNA extraction. To eliminate contamination, bone tissue was cleaned with a 0.5% sodium hypochlorite solution and then the outer surface was removed mechanically with appropriate sterile tools. Samples were taken exclusively from the inner portion of the bones and pulverized with a mixer mill (MM200; Retsch, Haan, Germany). A 1-g portion of the pulverized material was then incubated with 2 ml of 0.5 M EDTA solution containing proteinase K (0.25 mg/ml) at room temperature for 2 days on a rotary mixer (3). Soft tissue samples from inner organs (mostly lung tissue) were obtained at autopsy of otherwise intact internal body cavities, such as the pleural cavity, where residues of lung tissue could be identified (28). Strict precautions were applied to ensure sterile material. This material was also subjected to proteinase K digestion as indicated above. Following centrifugation for 15 min at 3,000 × *g*, 0.5 ml of the supernatant was removed and 1 ml of guanidine isothiocyanate solution and diatomaceous earth (12) was added. After incubation on a rotary mixer for another 2 h, the diatomaceous earth was pelleted by centrifugation and washed twice with 70% ethanol and once with acetone. The DNA was eluted with 80 μl of sterile water. Finally, another washing and concentration step was performed with Microcon-30 filters (Millipore, Bedford, Mass.), and the final DNA solution was diluted to 20 μl with sterile water.

General precautions to avoiding contamination. Besides the above-indicated procedures, several more precautions were taken to avoid contamination during the extraction procedure and in the PCR amplifications. The extraction and the PCR and post-PCR analyses were all conducted in separated rooms of the building, where no studies of modern mycobacterial or human DNA have ever been performed. The analyses of modern mycobacterial DNA (as described below) were performed in separate rooms of the building. All reagents were purchased as DNase- and RNase-free molecular biology grade chemicals or autoclaved when appropriate. No positive PCR controls were used. Disposable gloves were worn during all procedures and changed frequently. Sterile filter tips were used exclusively to avoid cross-contamination. Two extraction blanks were always performed in the same procedure, and additionally a PCR blank, containing no DNA template, was included in each PCR amplification. Finally, the specimens analyzed were divided between two different institutions (Munich and Regensburg), using the same analysis procedure in parallel.

Amplification of human DNA. To test whether amplifiable DNA was present in the samples and to ascertain that the PCR was not inhibited, a 202-bp segment of the human β-actin gene was amplified in parallel (16). The PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each de-

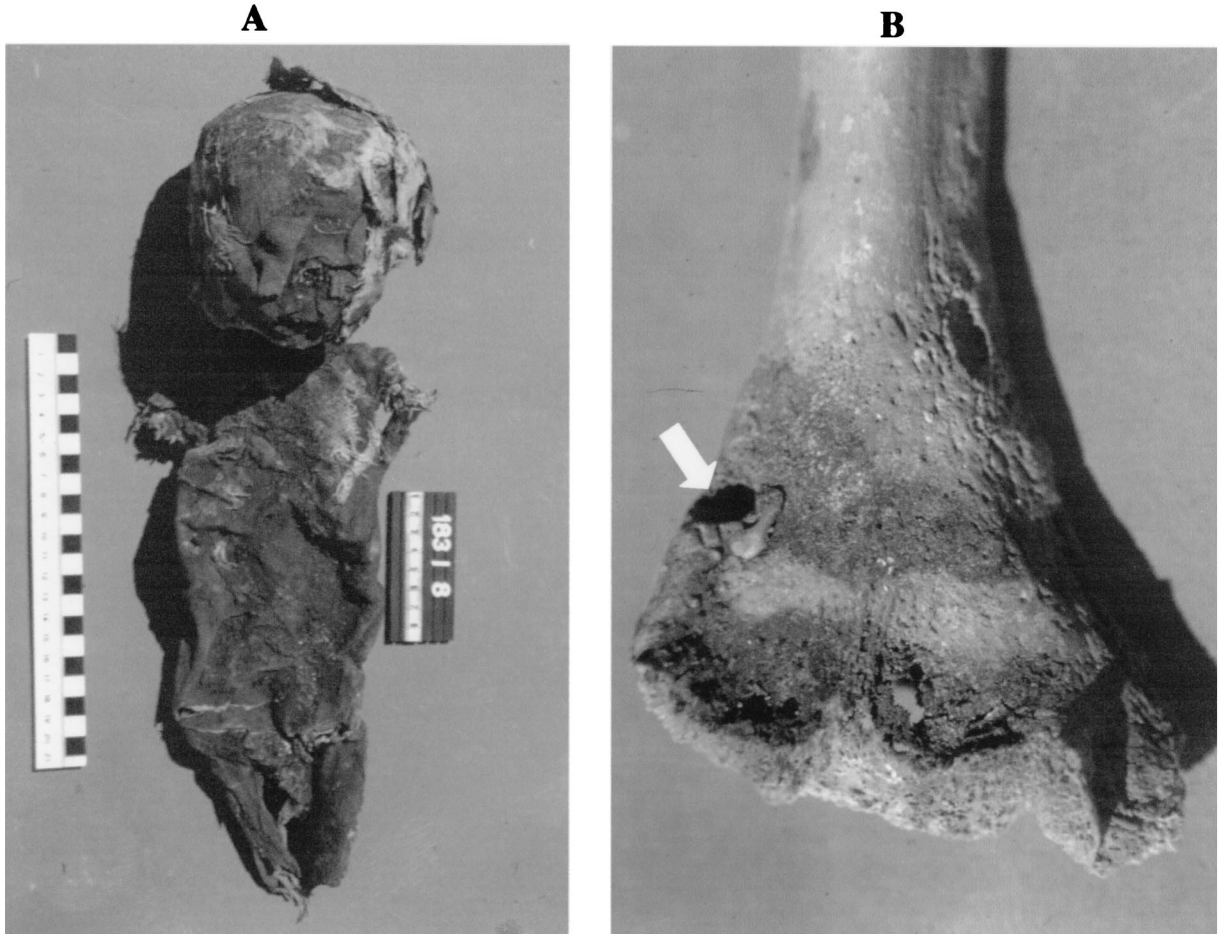

FIG. 1. (A) Case TT183-8. The mummy of a newborn male child without any sign of evisceration. After careful opening of the chest wall, fine pleural adhesions of the lung indicated a tuberculose infection. (B) Case TT95-PC169. The right humerus shows morphological alterations, probably due to a nonspecific inflammation. At the distal end, reactive new-bone formation and fistular defects (arrow) are visible.

oxynucleoside trisphosphate (Amersham Pharmacia, Uppsala, Sweden), 1 μM each primer, 0.025 U of AmpliTaq Gold (PE Biosystems, Foster City, Calif.) per μl, and 0.5 μl of extracted DNA to a final volume of 20 μl. The following amplification protocol was used: 10 min at 95°C, followed by 45 cycles of 94°C for 1 min, 60°C for 3 min, and 72°C for 3 min, followed by a final extension at 72°C for 8 min.

Detection of PCR and digestion products. All PCR products were electrophoresed on a 4% agarose gel and visualized on a UV screen after being stained with ethidium bromide.

Amplification of mycobacterial IS*6110* DNA and analysis of product specificity. For the specific amplification of mycobacterial DNA, we used a primer pair targeting a 123-bp segment of the repetitive sequence IS*6110* of *M. tuberculosis* complex, which covers *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, and *M. canetti* (10, 45). The PCR template was prepared as described above. PCR conditions were as follows: 10 min at 95°C followed by 45 cycles of 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min. After the final cycle, another 8 min at 72°C was added.

In several cases, the 123-bp PCR product of IS*6110* was digested with *Hae*III (2). For the digestion, 8 μl of the PCR product was incubated with 10 U of *Hae*III (Roche Diagnostics, Mannheim, Germany) for 2 h at 37°C. This resulted in 94- and 29-bp fragments. The nucleotide sequences of the PCR products in several cases were determined by direct sequencing: after electrophoresis on a 4% low-melting-point agarose gel, the respective fragment of the PCR product was eluted with a purification kit (Freeze'n Squeeze; Bio-Rad, Hercules, Calif.). With the eluted DNA, cycle sequencing was performed with a dye terminator cycle sequencing kit (PE Biosystems). Automatic sequencing was performed on an ABI PRISM 310 Genetic Analyzer (PE Biosystems).

Analysis of various PCR products for the differentiation of *M. tuberculosis* and *M. bovis*. We tested different PCR amplification products, which have previously been described to differentiate between *M. bovis* and *M. tuberculosis*. To evaluate

TABLE 2. Tested genes for the distinction between *M. tuberculosis* and *M. bovis*

Primer	Target	Product size (bp)	Reacting species	Reference
Mbov-1/2	*M. bovis*-specific region	122	*M. bovis*	
mtp40-1/2	Phospholipase C gene *mtp-40*	150	*M. tuberculosis*	42
mtp40-3/4	Phospholipase C gene *mtp-40*	152	*M. tuberculosis*	
oxyR-1/2	*oyxR* pseudogene	150	*M. bovis* vs. *M. tuberculosis*	42

TABLE 3. Results of PCR-based distinction between *M. tuberculosis* and *M. bovis*

PCR product	Result[b] for:					
	M. bovis	mtp40-1/2	mtp40-3/4	*oxyR*	IS*6110*	Spoligotyping
Modern control DNA						
M. tuberculosis H37Rv (10 ng/μl)	+	++	++	+	+	+(*M. tuberculosis*)
M. bovis BCG (10 ng/μl)	++	+	++	+	+	+(*M. bovis*)
M. tuberculosis H37Rv (0.01 ng/μl)	−	+	+	+	+	+(*M. tuberculosis*)
M. bovis BCG (0.01 ng/μl)	+	−	+	+	+	+(*M. bovis*)
Recent autopsy cases						
Paraffin sample *M. bovis*	+	−	−	−	+	+(*M. bovis*)
Paraffin sample *M. tuberculosis*	−	−	−	−	+	+(*M. tuberculosis*)
Historic tissue samples[a]						
TT453-9	+	−	−	−	+	+(*M. tuberculosis*)
TT453-14	+	−	−	−	+	+(*M. tuberculosis*)
TT95-122	+	−	−	+	+	+(*M. tuberculosis*)
TT95-40	−	−	−	+	+	+(*M. tuberculosis*)
TT95-169	−	−	−	−	+	+(*M. tuberculosis*)
DAN93.11	+	+	−	−	+	+(*M. tuberculosis*)
DAN95.1-1	+	−	+	+	+	+(*M. tuberculosis*)
TT196-M5	−	−	−	−	+	+(*M. africanum*)

[a] Indicated are the number of the tomb and of the respective specimen.
[b] −, no amplification product; +, amplification product; ++, abundant amplification product.

the specificity of these amplifications, we used the following set of samples. (i) As positive controls for *M. tuberculosis* and *M. bovis*, DNAs of the recent *M. tuberculosis* strain H37Rv and the *M. bovis* strain BCG P3 were obtained from a commercial source (Isogen, Maarsen, The Netherlands). These were used at two different concentrations to evaluate any concentration-dependent difference in the reaction patterns (10 and 0.1 ng/μl). (ii) We analyzed two contemporary tissue samples obtained from our autopsy files, one with (microbiologically) confirmed tuberculosis caused by *M. tuberculosis* and the other with a microbiologically proven infection by *M. bovis*. We had tested the spoligotyping patterns of both samples, which clearly confirmed the infections to be *M. tuberculosis* and *M. bovis*, respectively. From both cases, lung tissue specimens had been fixed in formaldehyde, routinely embedded into paraffin wax, and used for DNA analysis as described previously (2). (iii) Eight cases were tested from our ancient Egyptian samples (see above) which had tested positive for the presence of IS*6110* and for which a spoligotyping pattern had been obtained in parallel.

We chose primer sets amplifying DNA fragments smaller than 200 bp, since aDNA is known to be significantly degraded so that the amplification of targets of more than 200 bp is very difficult (29).

For PCR analysis, we selected four different amplification products from the Mbov, *mtp-40*, and the *oxyR* regions (Table 2). (i) The Mbov sequence was chosen, since it has been published as a specific product of *M. bovis* (31). Therefore, the following primers were designed: 5′ GAAATTCGCCTCTGTA GTGCCAC 3′ (forward) and 5′ GCTGCGTTGACTGAGAAAATGTATG 3′ (reverse). The cycling parameters were 45 cycles of 94°C for 1 min and 60°C for 1 min with a final cycle of 72°C at 1 min. (ii) Two primer sets for the *mtp-40* gene were used that have previously been successfully tested on medieval bone material (41). The *mtp-40* sequence (a region including a phospholipase C gene) is present in *M. tuberculosis* and thereby allows a distinction between *M. tuberculosis* and most *M. bovis* isolates (7). The primers and reaction conditions were essentially the same as those described by Taylor et al. (42). (iii) We designed two new primer sets for the *mtp-40* gene on the basis of published *M. tuberculosis*- and *M. bovis*-specific gene regions (7). These produce a 152-bp product, and the primers are mtp40-3 (5′ AGTCGCAAAGTTGAACGCTGAGGTC 3′) (forward) and mtp40-4 (5′ GTTCCCTGCATTGTCATTTCGCC 3′) (reverse). The cycling parameters were 45 cycles of 94°C for 1 min and 62°C for 1 min with a final cycle of 72°C at 1 min. (iv) Finally, we examined the pseudogene *oxyR* (oxidative response regulator), which contains a sequence polymorphism between *M. tuberculosis* and *M. bovis* (39). The primer sequence and the cycling conditions were used as described in the literature (42).

Spoligotyping. Spoligotyping was used for further analysis of the samples with a positive signal for the IS*6110* region. The method was performed as described by Kamerbeek et al. (20), except that the cycle number was increased to 45 cycles. For this analysis, we used a commercial spoligotyping kit (Isogen, Maarsen, The Netherlands). The extracted DNA was amplified with primers DRa and DRb, which enable the amplification of the whole DR region, so that the spacers between the DR targets can be evaluated. A biotin-labeled reverse primer, Dra, was used to obtain biotin labeled PCR products after amplification. Subsequently, the PCR products were perpendicularly hybridized to lines of immobilized spacer oligonucleotides that represent spacers of known sequence. The oligonucleotides were covalently linked to an activated membrane in parallel lines. Following the hybridization, the membrane was incubated with streptavidin peroxidase. For the detection of the hybridization signals, an enhanced chemiluminescence (ECL) system was used (Amersham Pharmacia). In this system, peroxidase catalyzed a reaction, resulting in the emission of light, which can be visualized by autoradiography (Hyperfilm ECL; Amersham Pharmacia).

Database comparison of spoligotyping results. The resulting spoligotyping patterns were compared to an international database that contained the following: (i) a total of 813 spoligotyping patterns reported at least twice, representative of 11,708 clinical isolates from more than 90 countries, and (ii) 1,300 unique spoligotyping patterns (I. Filliol et al., unpublished data).

RESULTS

Identification of *M. tuberculosis* complex DNA (IS*6110*). Of the 85 samples tested for the presence of mycobacterial aDNA, 48 (56.5%) yielded amplifiable aDNA as shown by the amplification of the human β-actin gene. From these 48 cases with amplifiable aDNA, 25 individuals provided a positive result for the IS*6110* sequence, indicating the presence of *M. tuberculosis* complex DNA. The highest incidence was found in the cases with typical morphological alterations, where 9 of 12 samples (75%) were positive for the presence of mycobacterial DNA. The specimens with nonspecific alterations (32%) and unremarkable morphology (17%) revealed significantly fewer positive cases. To confirm the specificity of the results, the positive PCR products were subjected to digestion with the restriction enzyme *Hae*III and/or direct sequencing of the IS*6110* insertion sequence. In all cases, the PCR results were unambiguously confirmed by these subsequent analyses.

PCR-based distinction between *M. tuberculosis* and *M. bovis* in recent and historic samples. The PCR methods used to differentiate between *M. tuberculosis* and *M. bovis* provided no clear results that would allow a distinction on the basis of the

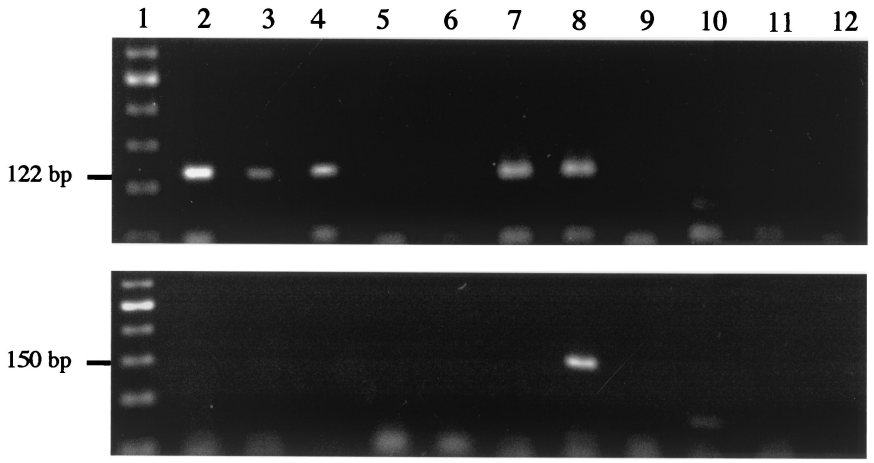

FIG. 2. Amplification of the *M. bovis*-specific sequence (Mbov, above) and the *mtp-40* region (mtp40-3/4, below) in some samples of the ancient mummified material. Lanes: 1, 50-bp standard ladder; 2 to 9, historic tissue samples as listed in Table 3; 10 to 12, blank controls.

historic material. The commercially available control DNAs showed positive amplification products for all sequences tested (Table 3).

Commercial controls. At high concentration, both commercial controls showed amplification products with all sets of primers, thus providing evidence for cross-reactions. The only difference was in the signal intensity. The *M. bovis*-specific primers (Mbov) revealed a stronger signal with the *M. bovis* control DNA than with *M. tuberculosis* DNA, while the *M. tuberculosis* primers showed a more pronounced signal with *M. tuberculosis* DNA than with *M. bovis* DNA (mtp40-1 plus mtp40-2 and mtp40-3 plus mtp40-4 primers). The two primer sets used for the *mtp-40* gene did not provide different patterns.

When control DNA samples that had been diluted 1,000-fold were used, more specific results were evident. Thus, the *M. bovis*-specific primers exclusively amplified the *M. bovis* control. The mtp40-1 plus mtp40-2 primer-targeted gene was amplified only in the *M. tuberculosis* control; however, the mtp40-3 plus mtp40-4 primer-targeted gene reacted with both *M. tuberculosis* and *M. bovis*.

Recent autopsy material. In the recent autopsy material (formalin fixed and paraffin embedded), only the Mbov primers produced a specific amplicon. Here, neither *mtp-40* primer pair reacted. The paraffin sample with known *M. tuberculosis* infection gave no reaction with either *M. bovis* or *M. tuberculosis* primers. Nevertheless, this material was positive for IS*6110* (and the β-actin gene), so that the mere absence of amplifiable DNA can be ruled out.

Ancient tissue samples. In the series of ancient tissue samples, an inconsistent, "patchy" reaction pattern was found to provide no secure distinction between *M. bovis* and *M. tuberculosis* strains. Five of the eight samples showed a positive signal with the *M. bovis*-specific primers, but only two samples were successfully amplified with the *mtp-40* primers (Fig. 2). Interestingly, these two samples also reacted positively for the *M. bovis*-specific amplicon, strongly suggesting cross-reaction. The *oxyR* gene was detected in three samples. Besides these problems in specificity, the sensitivity of the tested primers is clearly lower than that of the IS*6110* PCR approach. The results are summed up in Table 3.

Spoligotyping observations. Following the detection of mycobacterial aDNA (IS*6110*), spoligotyping was performed on all positive samples. To reduce the risk of the occasional absence of single spacers—due to the degradation of the DNA—all blots were performed in triplicate. From the results, a consensus pattern was established. Allowing any differences between the repeated spoligotypings of one individual to be kept to a minimum, reproducible spoligotyping patterns were obtained for 12 individuals. The detailed patterns are listed in Table 4.

Spoligotyping database comparison. The spoligotyping patterns were compared to those found in a third updated version of an international spoligotyping database representative of around 13,000 clinical isolates, split into 813 shared types and 1,300 unique types. Three isolates of the ancient samples matched some isolates from the database. The three profiles TT183-8, DAN 93.11, and TT95-PC40 match types 393, 291, and 53 of the database, respectively. These isolates are all close or identical to the ubiquitous *M. tuberculosis* type 53, which is one of the most common profiles and is, according to a hypothesis of evolution by loss of DRs (44), close to the origin of development of mycobacterial diversity.

The four samples from tomb TT196 and the sample TT453-PC14 showed a pattern close to that of *M. africanum*. Indeed, an attempt to define an *M. africanum*-specific spoligotyping signature (deletion of spacer 39 and, to a lesser extent, spacers 7, 8, and 9) was suggested by Viana-Niero et al. (46). Consequently, TT196-M5 fulfills, stricto sensu, all the conditions (with regard to the spoligotyping signatures) for classification as *M. africanum*. The other profiles that lacked spacer 39 may also be *M. africanum*.

The remaining patterns were not found in the database, but they are all clearly related to the *M. tuberculosis* type, as indicated by the absence of spacers 33 to 36 and the presence of several spacers between positions 39 and 45. In none of the samples analyzed were any *M. bovis*-specific patterns detect-

TABLE 4. Spoligotyping results

Sample	Sequence[a]
TT196-44	xxxxxxxx.xxxxx..xxxxxxxxxxxxxxxx.xxxx.xx..
TT196-M5	.xxxxx...xxxxxxxx.xx.xxxx..xx.xxx.x.xx.xxxx
TT196-MW7	x.xxxxxxxxxx...xxxx.xxxxxxxx.xx....xx.xxxx
TT196-MW18	x.xx.xx.x.xxx..xx.x..xxxxxxxxxx....xx.xxxx
TT453-PC14	xxxxxxxxxxxxxxxxx.xxxxxxxxx.x....xx.xxxx
TT453-PC9	xxxxxxxxxxxxx.xxxx.....xxxxxxx....xxxxxx.
TT183-8	xxxxxxxxxxxx.xxxxxxxxxxxxxxxx....xxxxxxx
TT95-PC40	xxxxxxxxxxxxxxxxxxxxxxxxxxxx....xxxxxxx
TT95-PC122	xxxxxxxxxxxxxxxxxxx.xxxxx..x.....xxxxx.
TT95-PC169	xxxxxxxxxxxxxxxxxxxxxxxxxxxx....xxxx..x
DAN 93.11	xxxxxxxxxxxxxxxxx.xxxxxxxxx....xxxxxxx
DAN 95.1-1	xxxxxxxxxxx..xxxxx..xxxxxxxxx....xxxxxxx
M. tuberculosis H37Rv	xxxxxxxxxxxxxxxxx..xxxxxxxxx....xxxxxxx
M. bovis BCG P3	xx.xxxxx.xxxxxx.xxxxxxxxxxxxxxxxxxxxx.....

[a] Sequence data are for positions 1 to 43.

able, which would have been characterized by the absence of spacers 39 to 45.

DISCUSSION

The molecular identification of *M. tuberculosis* complex DNA in ancient skeletal and mummified material is based mainly on the multicopy insertion element IS*6110*, where the PCR-based amplification of a 123-bp fragment provides a highly sensitive technique to detect DNA of the *M. tuberculosis* complex. The specificity of this marker has previously been questioned (22), but several extensive reevaluations of this issue confirm the specificity of IS*6110* as a marker for human-pathogenic mycobacteria (19).

In current microbiological diagnosis, the differentiation of any of the subtypes of the *M. tuberculosis* complex is achieved by the analysis of certain metabolic differences between these strains, e.g., the accumulation of niacin, susceptibility to pyrazinamide, pyrazinamidase activity, nitrate reduction, and thiophene carboxylic acid hydrazide susceptibility (6). All these phenomena, however, can be investigated only in living mycobacteria (e.g., under cell culture conditions) and are not applicable to the investigation of mycobacteria from historic samples.

Several attempts have been undertaken to differentiate the *M. tuberculosis* complex by use of molecular markers. This has led to the design of PCR-based methods to specifically differentiate certain strains. The *M. tuberculosis*-specific *mtp-40* gene (8), a 500-bp fragment specific for *M. bovis* (31), and an *M. bovis* specific *mpb-64* flanking sequence (14) have been described. Subsequent studies provided evidence that the presumed differences were not specific enough to securely differentiate among the assumed mycobacterial species (25, 26). This notion is strongly supported by our tests of several primer sets, since we showed that *M. tuberculosis* DNA could also be amplified with "*M. bovis*-specific" primers and vice versa depending on the DNA concentration used for PCR. The amplification of *M. bovis* control DNA with both *mtp-40* primers demonstrates a lack of specificity of this molecular approach. This may be due to a high homology of the respective gene sequences to comparable counterparts in the other mycobacterial species.

On the basis of these insecure amplification reactions, the finding that several of our ancient samples provided a positive amplification pattern suggesting the presence of *M. bovis* must be interpreted with great caution. Subsequent spoligotyping patterns clearly ruled out these diagnoses and highlighted the obvious problems with the "species-specific" PCR amplification patterns. In addition, the two paraffin samples from defined mycobacterial infections did not provide a reliable pattern, since part of the material failed to provide a specific amplification at all. Therefore, we conclude that the application of PCR-based markers previously assumed to specifically react with defined mycobacterial species is of only limited value for the investigation of historic tissue samples. Furthermore, other PCR-based methods like the single-sequence variation found in the *oxyR* gene seem to be useful only to a limited extent for applications involving ancient or otherwise degraded material. Only three out of the eight ancient samples provided an amplification product at all.

One major limitation of ancient samples is that the available DNA is highly degraded and therefore only small fragments of DNA can be amplified. Usually only small amounts of aDNA can be expected. Therefore, a high sensitivity of the primers used is required to obtain amplification products in a reasonable number. This can be expected for the 123-bp fragment of IS*6110*, which is a multicopy gene. It should be noted that the IS*6110* copy number in "ancestral" and many African-Indian strains can be very low, and therefore amplification of IS*6110* may have missed those samples, although the high rate of positive cases in this study tends to contradict such an assumption. The high rate of amplifiable material in the Egyptian mummies is probably due to the excellent preservation of biomaterial resulting from both artificial and natural mummification processes and the constantly arid climatic conditions in Egypt.

Traditional genotyping methods do not allow further phylogenetic study of ancient material due to the low structural gene variation present in *M. tuberculosis* complex strains. Therefore, repetitive DNA markers such as insertion sequences (IS*1081* or IS*6110*), variable numbers of tandem DNA repeat loci, mycobacterial interspersed repetitive units (40), and the DR locus are much more suitable for investigating evolutionary

and phylogenetic aspects of the *M. tuberculosis* complex due to the extensive polymorphism of these markers (15, 39).

On the basis of these methods, spoligotyping seems to be the preferable method for ancient material, due to the amplification of several small fragments. The probability of obtaining reliable spoligotyping patterns from ancient samples is significantly higher and has already been proven in a few previous studies on individual findings or small series (42).

In our study, a reliable pattern from 12 individuals was obtained when the analysis was repeated for three times and a consensus pattern was established for each sample. Minor changes in the pattern obtained between the individuals analyzed and the known present-day isolates are good arguments for the authenticity of aDNA.

One of the key observations from our study was that there is no evidence for the presence of any *M. bovis* isolate in the ancient samples. The *M. bovis* isolates are usually characterized (for all known isolates) by the lack of the five 3′-end spacers (spacers 39 to 43) of the spoligotyping pattern, while *M. tuberculosis* isolates typically show an absence of spacers 33 to 36 (20). The classification of *M. africanum* is somewhat more difficult, since there are only limited data on this species. In a recent publication, Viana-Niero et al. (46) clearly demonstrated that the absence of spacer 39 is the most striking spoligotyping signature of all *M. africanum* isolates tested. In their extensive investigations of 81 *M. africanum* isolates, they described the absence of spacer 39 in all isolates and the absence of spacers 8 and 9 in most of the isolates. Accordingly, they proposed that an isolate lacking spacers 8, 9, and 39 while containing spacers 33 to 36 and 40 to 43 may be tentatively classified as *M. africanum*.

In our samples, these requirements are almost completely fulfilled for the sample TT196-M5 and partly for the sample TT196-44. Moreover, our data suggest that the other two samples from tomb TT196 and the sample TT456-PC14 may also be *M. africanum*. Most interestingly, all these samples—except for TT453—were from the oldest tomb included in our study. In this tomb complex, we did not find any other types of mycobacteria (although minor differences between the spoligotyping patterns strongly argue against any general "contamination" within that tomb complex). The samples obtained from tomb TT196 originate exclusively from the Middle Kingdom (2050 to 1650 BC), since there is archaeological evidence that this tomb complex was not used in subsequent periods such as the New Kingdom (1500 to 1000 BC) or even later. Further archaeological data indicate that tomb DAN95.1 was also built in the Middle Kingdom but that it was used until the Late Period (until ca. 500 BC) and so attributing the sample investigated to any specific period is not possible. All the other tomb complexes were constructed in the New Kingdom (i.e., since ca. 1500 BC) and were used until the Late Period (i.e., until ca. 500 BC). Although our material was restricted to a few samples from only one necropolis (Thebes West) of one country (Egypt) and date back not more than ca. 4,000 years, we show no data supporting the previous hypothesis that *M. tuberculosis* and *M. bovis* closely coexisted or even that *M. bovis* may have been a precursor of *M. tuberculosis* (4). In contrast, our results are in favor of the theory that the members of the *M. tuberculosis* complex may have originated from a precursor complex, which may have split into branches. Since we detected only *M. africanum* isolates (in the oldest tomb complex investigated) and *M. tuberculosis* strains (in the "younger" material), these two mycobacteria may have been more closely related to each other than to *M. bovis*. This would support the notion that *M. bovis* split off either earlier or significantly later. Brosch et al. (4) have shown that a particular DNA region (RD9) is missing in *M. africanum*, *M. microti*, and *M. bovis* but is present in *M. tuberculosis*. This suggests that *M. africanum* belongs to a different evolutionary lineage from *M. tuberculosis* and is more closely related to *M. microti* and *M. bovis*. This agrees with our finding that *M. bovis* does not resemble the precursor of *M. tuberculosis* but is in contrast to our assumption of a close relationship of *M. africanum and M. tuberculosis*. In a further study, the presence or absence of the RD9 region in the ancient Egyptian samples will be evaluated for a better characterization of the supposed *M. africanum* findings.

Since molecular biology-based estimates from recent strains backdate the origin of *M. tuberculosis* 10,000 to 20,000 years before the present (22, 39), the final development of present-day *M. tuberculosis* may indeed have happened only in the last few 4,000 years.

The comparison of the spoligotyping patterns with the shared-types database revealed that three samples (TT183-8, DAN 93.11, and TT95-PC40) match some isolates of the database. Interestingly, the isolates are close to the ubiquitous type 53, representing one of the most common profiles. According to the theory of evolution by loss of DRs (44), this profile is assumed to be close to the original pattern. This parallels our assumption that the spoligotyping signatures obtained from our Egyptian tissue sample series probably represent an "ancient pattern" which may come close to any presumed precursor forms. However, it has been shown that the absence of spacers 33 to 36 in the spoligotyping signatures of *M. tuberculosis* is characteristic of strains that carry a particular *katG* codon 463 mutation (CTG to CGG) and showing the specific *M. tuberculosis* deletion (TbD1) in the *mmpL6* gene (4). Based on the absence of spacers 33 to 36 (Table 4), most of the *M. tuberculosis* spoligotypes obtained from the Egyptian mummies would be classified as characteristic of "modern" strains, belonging to phylogenetic group 2 or 3 (39). This would suggest that the TbD1 deletion and the *katG463* mutation, as well as the loss of spacers 33 to 36, occurred at least 2,500 years ago. Moreover, the delineation of group 2 strains from group 1 (e.g., Beijing type) strains prevalent in Asia could have happened before that time. Further studies are required to test the ancient Egyptian samples for the presence or absence of the TbD1 deletion and the *katG463* locus and for further classification into the group 2 or 3 *gyrA95* sequence polymorphism. The investigation of these genetic markers in even older material from Egyptian cemeteries may eventually shed more light on the evolutionary process of mycobacterial development.

It is difficult to rank *M. bovis*, since we have no idea when *M. bovis* actually appeared first in human history. It is conceivable that *M. bovis* had already existed for several thousand years without affecting humans. In this regard, it is of interest that the as yet oldest successful spoligotyping pattern has been obtained from a fossilized bison sample. However, this material, dating back to ca. 17,000 years before present, did provide a spoligotyping pattern, but this pattern does not match with

any known spoligotyping signature (33). In any case, the resulting pattern was different from known *M. bovis* patterns. It is obvious that the risk of becoming infected by contact with cattle increased with the steady rise of agriculture and cattle breeding. The close contact between humans and animals may have promoted the transmission of *M. bovis* from cattle to humans. This theory, however, is not supported by the few spoligotyping results obtained with ancient material from different periods. In all available studies, no case matched with the *M. bovis* type. Taylor et al. (42) identified the infective strain in three medieval bone samples to be similar to those of present-day *M. tuberculosis* isolates. Recently, Mays et al. reported controversial spoligotyping results arguing in favor of the presence of tubercle bacilli of the East-African-Indian clade (EAI) (37) in a medieval village in England (27).

When we consider the small size of our study group, it is too early to discount *M. bovis* as the agent of early human tuberculosis, but at least our results from ancient Egypt argue against the classical theory that human tuberculosis has arisen from bovine tuberculosis (5). Other theories, like transmission from domesticatied goats rather than cattle as the origin of human tuberculosis (11, 17), fail because there is no evidence of *M. bovis* caprine strains in historic tissue samples. Further studies are required to obtain more information about the role of *M. bovis* in the evolution of the disease. Ancient mycobacterial isolates can probably be included in phylogenetic trees with modern isolates to enhance the plausibility of such attempts.

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