ORIGINAL ARTICLE

Molecular strain identification of the *Mycobacterium tuberculosis* complex in archival tissue samples

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Aims: To investigate the use of different molecular analyses that can identify distinct strains of human pathogenic mycobacteria in formalin fixed and paraffin wax embedded archival tissue samples to see whether it is possible to differentiate between the members of the *Mycobacterium tuberculosis* complex (*M tuberculosis*, *M bovis*, *M africanum*, *M microti*, or *M canettii*) and/or substrains in a high number of samples. This would be of interest for identifying individual infection traits and superinfection by different mycobacterial strains.

Methods: Forty nine archival tissue samples with clinically and/or histologically suspected tuberculosis infection were subjected to molecular DNA analysis.

Results: The molecular analysis revealed the presence of *M tuberculosis* complex DNA in 20 samples, whereas acid fast bacilli could be detected by Ziehl-Neelsen staining in only eight samples. All IS6110 positive samples were further characterised by spoligotyping and seven cases provided *M tuberculosis* specific signatures, whereas *M bovis* specific signatures were obtained in four cases. The analysis of mtp40, oxyR, and pncA partial gene sequences confirmed the presence of *M tuberculosis* in six cases and *M bovis* in one case. The amplification and sequencing of four further genetic regions (katG, gyrA, TbD1, RD9) characterised six "modern" *M tuberculosis* strains belonging to genetic groups 2 or 3.

Conclusion: This study provides clear evidence that archival paraffin wax embedded material can be used for further studies on the strain identification of *M tuberculosis* complex strains and can therefore unequivocally be used for the study of the epidemiology and evolution of tuberculosis pathogens.

I n general, the sensitivity of routinely applied histochemical staining for acid fast bacilli in paraffin wax embedded tissues is low and allows the detection of human pathogenic members of the *Mycobacterium tuberculosis* complex in only a proportion of affected cases. Frequently, even when lesions appear morphologically to be typical for an active tuberculosis (TB) infection only a few cases are stained positive. Therefore, polymerase chain reaction (PCR) based amplification of mycobacterial DNA in paraffin wax embedded material is increasingly used to confirm suspected mycobacterial infections. Several previous studies provide data on this issue and suggest that this approach has considerably higher sensitivity.[1–5]

Despite this diagnostically important improvement, there have been few reports on molecular techniques for the identification of different mycobacterial strains in archival tissue material. This is becoming increasingly important as the tracing of re-emerging disease, the identification of de novo or superinfection, and/or the identification of "new" substrains with higher resistance against multidrug treatment potentially become clinically relevant.[6]

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To the best of our knowledge, until now only two studies have used emerging molecular techniques such as spoligotyping on paraffin wax embedded tissue samples to identify *M tuberculosis* complex strains.[7][8] These studies showed that formalin fixed and paraffin wax embedded tissue samples from patients with positive TB culture and/or clinically unambiguously identified tuberculosis can be used for the detection and strain differentiation of members of the

M tuberculosis complex. However, no data are as yet available on the rate of success of those techniques in archival material or on the rate of positive strain identification in unclear or clinically suspected, but uncertain, cases.

Our present study applied the spoligotyping technique and a panel of distinct primer pairs to identify (sub)strains of the *M tuberculosis* complex. We describe here the technical details, and provide clear evidence that these approaches can successfully be applied to archival paraffin wax embedded tissue samples in approximately two thirds of cases with molecularly confirmed *M tuberculosis* complex infection.

MATERIAL AND METHODS
Samples and study population

Our study was conducted on routinely formalin fixed and paraffin wax embedded biopsy tissue samples from 48 patients where the presence of tuberculosis had been suspected either by clinical assessment and/or histomorphological investigation. All samples were consecutively selected from the files of the institute of pathology of the Academic Hospital Munich-Bogenhausen, Germany between June 2001 and June 2003. Cases without histomorphological evidence for granulomatous or necrotising inflammation were excluded from our study, even when the clinical differential diagnosis had included TB. Table 1 presents detailed information on all cases, including clinical diagnosis, localisation of the biopsy, and major histopathological features. Furthermore, we listed the nationality of the affected individuals so that we could potentially trace back the TB strains on a geographical basis. Accordingly, all samples except two (46) showed typical epitheloid granulomas. In 27 cases, giant cells of the Langhans type were

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Abbreviations: PCR, polymerase chain reaction; PZA, pyrazinamide; RD9, region of difference 9; TB, tuberculosis

present and 32 cases revealed necrosis, which showed in most instances the typical features of caseous necrosis. Therefore, in each of the 48 cases either epitheloid granulomas or necrosis was present. Using Ziehl-Neelsen staining, only eight samples contained typical acid fast bacilli, and this histochemical staining method provided no diagnostic information in the remaining samples (table 1).

Sample preparation and molecular identification of *M tuberculosis* complex DNA

As a first step, the presence of *M tuberculosis* complex DNA was analysed in all 48 samples. Two to five 5 µm thick sections were prepared from each appropriate paraffin wax block and placed into a sterile tube. All procedures were performed under strictly controlled precautions—such as the use of new cutting blades for each case—to avoid cross-contamination between the samples. Subsequently, the samples were dewaxed in xylene and the DNA was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to standard protocols.

All samples were tested for the presence of *M tuberculosis* complex DNA by the amplification of a 123 bp fragment of the insertion sequence IS6110 (table 2).[9][10] In parallel, a 202 bp fragment of the human β actin gene was amplified to test for the presence of amplifiable DNA and to assure that the PCR reaction was not inhibited.

Confirmation of amplification specificity

The specificity of the 123 bp PCR product of IS6110 was confirmed either by restriction enzyme digestion or direct sequencing. For the digestion, 8 µl of the PCR product was incubated with 10 U Hae III (Roche Diagnostics, Mannheim, Germany) for two hours at 37°C. This resulted in a 94 bp and a 29 bp fragment. In several cases, the nucleotide sequences of the PCR products were determined. After electrophoresis on a 4% low melting point agarose gel, the respective fragment of the PCR reaction was eluted with a purification kit (Freeze'n Squeeze; Bio-Rad, Hercules, California, USA). Using the eluted DNA, cycle sequencing was performed with the big dye terminator cycle sequencing kit (Applied

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Table 1 Summary of the clinical and histomorphological data from our study population

Case	Age	Sex	Country (of origin)	Clinical diagnosis	Localisation	Epitheloid granuloma	Giant cells (LH)	Necrosis	ZN staining
1	61	F	Germany	TB	Distal tibia	+	+	+	−
2	50	F	Germany	Uro TB	Kidney	+	+	+	−
3	64	F	Germany	Tumour	Liver	+	+	−	−
4	79	F	Germany	Abscess	Vulva	+	−	+	−
5	67	F	Germany	TB	Paravertebra	+	−	−	−
6	42	M	Arabia	Lymphoma	Lymph node	+	+	+	−
7	74	M	Poland	Inflammation	Epididymis	+	+	+	−
8	79	M	Germany	Tumour	Prostate	+	−	−	−
9	40	F	Germany	TB	Lymph node	+	+	+	+
10	65	M	Germany	Spondylitis	Vertebra	+	−	+	−
11	23	M	Vietnam	Lymphoma	Lymph node	+	+	+	−
12	70	F	Germany	Granuloma	Finger	+	−	+	−
13	43	M	Germany	Granuloma	Thigh	+	−	+	−
14	54	M	Germany	Spondylitis	Vertebra	+	−	−	−
15	63	M	Germany	BCGitis	Urinary bladder	+	+	+	−
16	77	M	Germany	Chronic inflammation	Chest wall	+	−	+	−
17	49	M	Turkey	Granuloma	Larynx	+	−	+	−
18	61	M	Germany	BCGitis	Prostate	+	−	−	−
19	55	M	Germany	Tumour	Lung	+	−	−	−
20	58	M	Germany	Spondylitis	Vertebra	+	−	+	−
21	50	M	Germany	BCGitis	Prostate	+	+	+	−
22	64	M	Germany	TB	Lung	+	−	−	−
23	83	M	Germany	Spondylitis	Vertebra	−	−	+	−
24	61	M	Germany	BCGitis	Prostate	+	−	−	−
25	23	F	Germany	TB	Lung	+	+	−	−
26	45	M	Turkey	TB	Skin	+	+	−	−
27	35	F	Africa	Tumour	Tonsilla	+	+	−	−
28	45	F	Germany	Tumour	Lung	+	−	−	−
29	67	M	Germany	Tumour	Prostate	+	−	+	−
30	58	F	Germany	TB	Lung	+	+	−	−
31	34	M	Arabia	TB	Lung	+	−	−	−
32	58	M	Germany	Tumour	Lymph node	+	+	−	−
33	61	M	Germany	TB	Lung	+	+	+	−
34	75	F	Germany	TB	Lung	+	+	+	−
35	54	M	Germany	Tumour	Prostate	+	−	+	−
36	76	M	Germany	Tumour	Lung	+	−	+	−
37	59	M	Germany	Tumour	Lung	+	+	+	+
38	50	F	Germany	Epidural tumour	Spinal	+	+	−	−
39	87	F	Croatia	Tumour	Lung	+	+	+	+
40	24	F	Germany	Tumour	Pelvis	+	+	+	+
41	71	F	Germany	TB	Lung	+	+	+	+
42	30	M	Russia	Tumour	Lymph node	+	+	+	−
43	19	M	Arabia	Spondylitis	Vertebra	+	+	+	−
44	55	F	Germany	TB	Lung	+	+	+	+
45	30	M	Pakistan	Spondylitis	Vertebra	+	+	+	−
46	31	F	Pakistan	Tumour	Pelvis	+	+	+	−
47	87	F	Germany	TB	Lung	−	−	+	+
48	77	F	Germany	Spondylitis	Vertebra	+	+	+	+

Age is given in years.
+, positive; −, negative; LH, Langhans type; TB, tuberculosis; ZN, Ziehl-Neelsen stain (for acid fast bacilli).

Table 2 Primer sequences and polymerase chain reaction conditions used in our study

Locus	Direction	Primers	Product size	Parameters	Cycles	Ref
IS6110	F	CTCGTCCAGCGCCGCTTCGG	123 bp	94°C, 68°C, and 72°C	45	10
	R	CCTGCGAGCGTAGGCGTCGG		1 min each		
katG	F	TCAGCCACGACCTCGTCGG	163 bp	94°C, 65°C, and 72°C	43	18
	R	AGGCGGATGCGACCACCGTT		1 min each		
gyrA	F	CGAGACCATGGGCAACTACCA	131 bp	94°C, 65°C, and 72°C	43	18
	R	CATTGCCTGGCGAGCCGAA		1 min each		
TbD1 int	F	AAGGACGAAAGGATCGTCAA	135 bp	94°C, 63°C, and 72°C	40	–
	R	AAAACAGCAAGATCGGCAAC		1 min each		
TbD1 fla	F	CGGTTATCGAAAGGCTAACG	96 bp	94°C, 62°C, and 72°C	40	–
	R	TACCGTCGATCGTGTCAAAG		1 min each		
RD9 int	F	GTGGTCATCGAGGTCCAGAT	204 bp	94°C, 60°C, and 72°C	40	–
	R	GCCTGGGTGCATTACCTACT		1 min each		
RD9 fla	F	GTGACGGTATCGTCGAGCAG	178 bp	94°C, 55°C, and 72°C	40	–
	R	TACCTGGAGGACCAGCTGAA		1 min each		
mtp40	F	CTGGTCGAATTCGGTGGAGT	152 bp	94°C, 64°C, and 72°C	45	19
	R	ATGGTCTCCGACACGTTCGAC		1 min each		
oxyR	F	CGCGCTGTCAGAGCTGACTTT	150 bp	94°C, 60°C, and 72°C	45	19
	R	TCTGCGGAATCAGTGTCACC		1 min each		
pncA	F	ATCAGCGACTACCTGGCCGA	180 bp	94°C, 66°C, and 72°C	45	19
	R	GATTGCCGACGTGTCCAGAC		1 min each		

F, forward; R, reverse.

Biosystems, Weiterstadt, Germany). Automatic sequencing was performed on an ABI PRISM 310 genetic analyser (Applied Biosystems).

Spoligotyping

All samples with positive amplification of IS6110 were subjected to further analysis by spoligotyping. The method was performed as described previously.[11][12] Briefly, this analysis permits the evaluation of variable sequences located in the direct repeat region of the genome of *M tuberculosis* complex members by hybridisation of biotin labelled PCR products to a set of immobilised spacer oligos that represent spacers of known sequence. Hybridisation patterns vary between *M tuberculosis* complex strains because of differences in the presence of the non-repetitive DNA spacers. This allows a clear differentiation between *M tuberculosis* and *M bovis* strains, as a result of the absence of spacers 39 to 43 in all known *M bovis* isolates, and the identification of subspecies or certain genetically related families.[12]

Application of strain specific/typical primer pairs

In a further step, we tested the specimens for the absence or presence of an *M tuberculosis* specific deletion (TbD1) and the loss of region of difference 9 (RD9), which is characteristic of *M africanum, M microti*, and *M bovis* strains.[13] In addition, partial gene sequences of oxyR, pncA, mtp40, katG, and gyrA, which have been shown to vary between different strains of tuberculosis bacilli, were analysed to characterise the detected *M tuberculosis* strains further.[13] The mtp40 region, located in the phospholipase A gene, is present in about 90% of *M tuberculosis* strains and absent in most of the *M bovis* isolates.[14] The pseudogene oxyR presents a polymorphism at nucleotide 285 (oxyR285) and the pncA (pyrazinamidase) gene differs between *M tuberculosis* and *M bovis* by a mutation at codon 57 (pncA57).[15][16] *Mycobacterium tuberculosis* strains were further divided into three different genetic types based on sequence polymorphism of katG codon 463 (katG463) and gyrA codon 95 (gyrA95), as defined by Sreevatsan *et al*.[17]

Group 1 is characterised by katG463 CTG (Leu), gyrA95 ACC (Thr); group2 shows katG463 CGG (Arg), gyrA95 ACC (Thr); and group3 exhibits katG463 CGG (Arg), gyrA95 AGC (Ser).

For all amplifications, the PCR reaction mix contained 10mM Tris/HCl (pH 8.3), 50mM KCl, 1.5mM MgCl$_2$, 200 µM of each deoxynucleotide trisphosphate (Amersham Pharmacia, Uppsala, Sweden), 1 µM of each primer, 0.025 U/µl AmpliTaq Gold (PE Biosystems, Foster City, California, USA), and 0.5 µl extracted DNA to a final volume of 20 µl. Primers targeting partial gene sequences of katG and gyrA,[18] oxyR, pncA, and mtp40[19] were used as described in the literature. New primer sets were designed for the amplification of RD9 and TbD1. Table 2 lists the details of the primer sequences and PCR conditions used.

Identification of amplification products

All PCR products were identified by gel electrophoresis in a 2% agarose gel and were visualised by ethidium bromide staining plus ultraviolet light. For sequencing, the products were purified and subjected to sequencing as described above.

RESULTS
Rate of molecular identification of *M tuberculosis* complex DNA

Nineteen of the 48 biopsy samples showed a specific positive amplification product in the IS6110 PCR reaction (fig 1). The specificity of all positive amplification reactions was confirmed by typical cleavage in restriction enzyme digestion and in several cases by direct sequencing showing the expected sequence known from database source (BLASTN).[20] Therefore, in 19 cases the presence of human pathogenic mycobacterial DNA was molecularly confirmed. Furthermore, all eight samples with histochemically positive acid fast bacilli staining were confirmed by the molecular analysis. In addition, a specific molecular diagnosis was seen in 11 cases without histological evidence of tubercle bacilli (table 3).
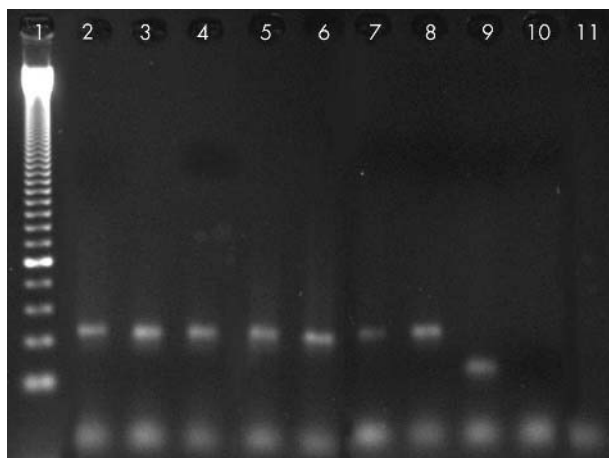
Figure 1 Polymerase chain reaction (PCR) amplification of a 123 bp fragment of IS6110. Lane 1, 50 bp ladder; lanes 2–8, paraffin wax embedded samples, lanes 9–10, extraction controls; lane 11, PCR control.

Further characterisation of mycobacterial DNA by spoligotyping

The 19 IS6110 positive cases were then subjected to further characterisation by spoligotyping. Typical spoligotyping signatures were obtained for 11 samples, whereas five cases produced an incomplete pattern with only few and "patchy" hybridisation signals (fig 2). No spoligotyping signature could be obtained from the remaining three cases. Of the 11 successfully obtained spoligotyping signatures seven revealed a typical *M tuberculosis* pattern. This is characterised by the presence of spacers 37 to 43. Furthermore, in six cases spacers 33 to 36 were absent, which is also characteristic of *M tuberculosis*.[12] Two cases (47 and 48) showed spoligotyping patterns that are listed in an international database and resemble two of the most common signatures worldwide (shared types numbers 50 and 53).[21] One further case (7) has previously been described in Houston, Texas,[22] and resembles a further shared type (280) of the database. The signatures of three further *M tuberculosis* cases were distinct from all of the as yet classified and identified substrains.

In four samples, spacers were detected at positions 33 to 36, whereas no hybridisation signal was detected for spacers 39 to 43, a pattern that is characteristic of *M bovis* strains.[14]

One case (37) showed a characteristic signature for *M bovis* subtype *caprae*,[23] whereas the remaining three cases could not be attributed to the signatures identified to date.

In eight cases, only incomplete or no spoligotyping signatures were obtained. Therefore, strain identification or differentiation between the members of the *M tuberculosis* complex could not be performed in these samples.

Mycobacterial strain and substrain identification by specific gene analysis

PCR amplification of the variable regions TbD1 and RD9 and the partial gene sequences oxyR, pncA, mtp40, katG, and gyrA (figs 3 and 4) provided fewer positive cases when compared with the IS6110 amplification data. This is probably the result of differing amounts and states of preservation of mycobacterial DNA between the various samples, and it is not surprising that the multicopy gene IS6110 was more informative than the single copy genes.

The deletion analysis revealed that all seven *M tuberculosis* cases (as demonstrated by the spoligotyping pattern) lacked TbD1, and in six of these samples RD9 was present. This confirms the presence of *M tuberculosis* DNA. In addition, in five *M tuberculosis* cases a katG(463) CTG → CGG and/or gyrA(95) ACC → AGC mutation was detected. According to Sreevatsan *et al*,[17] these samples belong to *M tuberculosis* genetic groups 2 or 3 and resemble "modern" *M tuberculosis* strains. In the two remaining cases, no katG or gyrA specific sequences could be retrieved.

In two of the *M bovis* cases TbD1 was present and in one RD9 was deleted. This is again very typical of *M bovis*. In addition, no mutations were found in the katG or gyrA sequences, which again confirms the presence of *M bovis*.

In all seven *M tuberculosis* samples, the partial gene sequence of oxyR showed the typical nucleotide G at position 285, whereas in one *M bovis* case a characteristic mutation was detected at this position (G → A; fig 5). No mutations were found in the pncA gene sequence at position 57, specific for *M tuberculosis* and pyrazinamide (PZA) susceptible *M bovis* isolates. A fragment of the mtp40 gene, typically present in *M tuberculosis* strains, was amplified in six of the *M tuberculosis* and one of the not specified cases.

Table 3 summarises the results of the further characterisation of the *M tuberculosis* complex positive samples.

DISCUSSION

We successfully applied the spoligotyping technique for the identification of strains of human pathogenic mycobacteria

Table 3 Molecular results of IS6110 positive cases

| Case	β actin	IS6110	Spoligotyping	oxyR	pncA	mtp40	katG	gyrA	TbD1 int	TbD1 fla	RD9 int	RD9 fla	Genetic group
1	+	+	*M bovis*	−	−	−	−	+	+	−	−	−	?
2	+	+	Incomplete	G	+	−	−	C	+	−	−	−	1,2
6	+	+	−	−	−	−	−	C	−	−	+	−	1,2
7	+	+	*M tuberculosis*	G	C	+	−	G	−	+	+	−	3
9	+	+	−	−	−	−	−	+	+	−	−	−	?
11	+	+	*M tuberculosis*	−	C	+	−	+	−	+	+	−	?
15	+	+	*M bovis*	−	−	−	−	+	−	−	−	−	?
20	+	+	Incomplete	−	−	−	−	−	−	+	+	−	?
21	+	+	*M bovis*	−	−	−	−	C	−	−	−	−	1,2
25	+	+	Incomplete	−	−	−	−	−	−	−	−	−	?
33	+	+	−	−	−	−	−	+	−	−	+	−	?
36	+	+	Incomplete	−	−	−	−	C	−	−	−	−	1,2
37	+	+	*M bovis*	A	C	−	T	C	+	−	−	+	1
39	+	+	*M tuberculosis*	G	C	−	G	C	−	+	−	−	2
40	+	+	*M tuberculosis*	G	C	+	−	C	−	+	+	−	2,3
41	+	+	Incomplete	G	C	+	−	C	−	+	+	−	2,3
44	+	+	*M tuberculosis*	G	−	+	−	−	−	+	+	−	?
47	+	+	*M tuberculosis*	G	C	+	G	+	−	+	+	−	2,3
48	+	+	*M tuberculosis*	G	−	+	G	C	−	+	+	−	2

+, positive; −, negative.
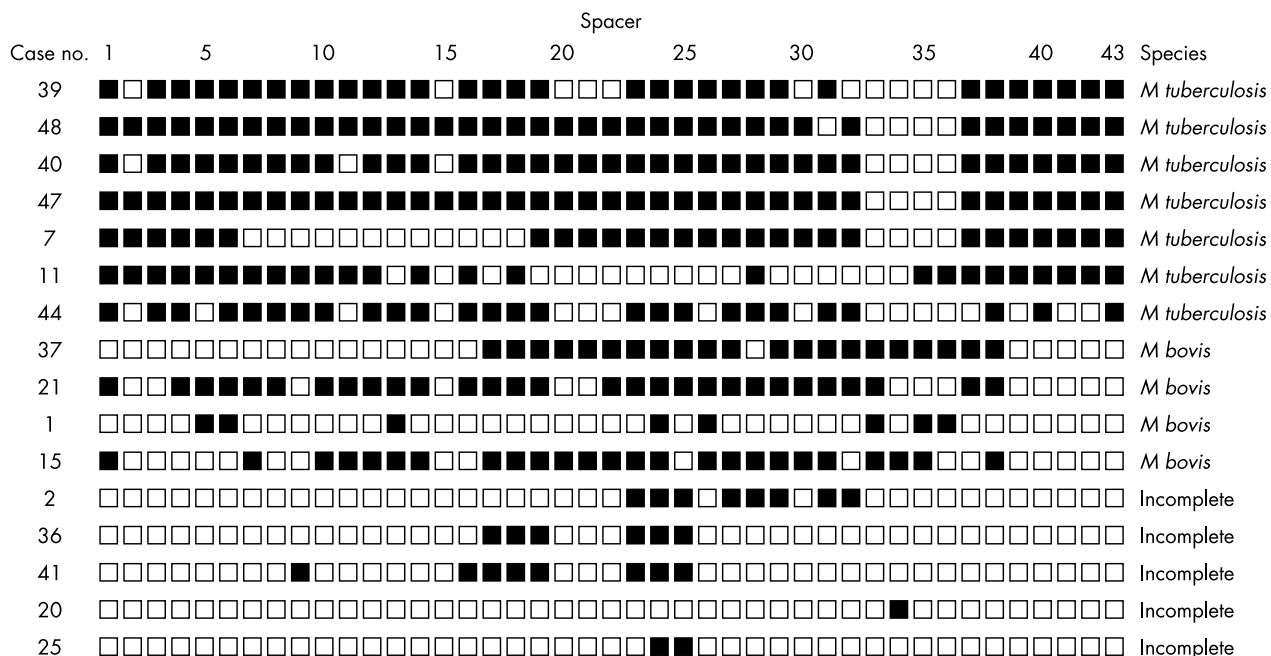
Figure 2 Spoligotyping results of the paraffin wax embedded samples.

in approximately two thirds of our archival tissue samples. In contrast to former studies on paraffin wax embedded samples,[7][8] cases with unclear histomorphology were also included and tested for the presence of mycobacterial DNA. Using this emerging technique for the identification and characterisation of the direct repeat locus of the mycobacterial genome, we provide clear evidence that such an analysis can be performed successfully in a high proportion of

samples. Therefore, this approach not only extends and confirms the molecular diagnosis in those cases identified by the frequently used IS6110 sequence of the mycobacterial DNA complex, but it also provides information on distinct strains of human pathogenic mycobacteria. Using this approach, the identification of mycobacterial strains and substrains may show changes in re-emerging mycobacterial infection or may trace double infections by various
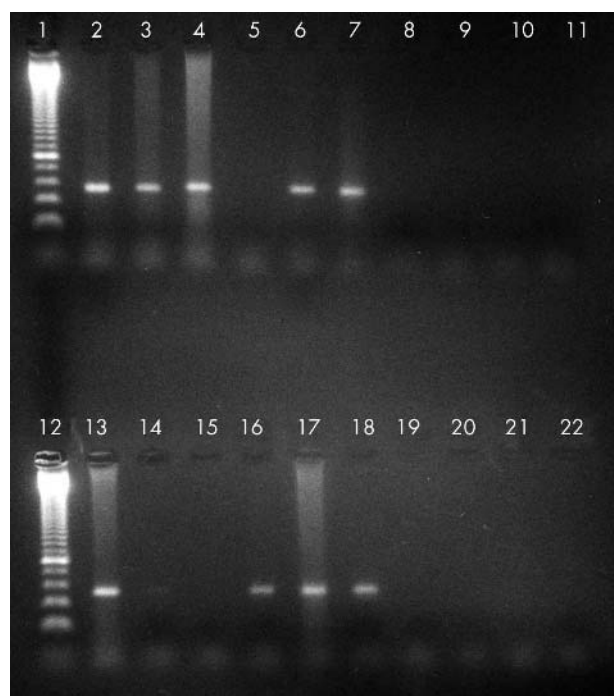
Figure 3 Polymerase chain reaction (PCR) amplification of a 131 bp fragment of the gyrA gene. Lanes 1 and 12, 50 bp ladder, lanes 2–11 and 13–14, paraffin wax embedded samples; lanes 15–21, extraction controls, lane 22, PCR control.
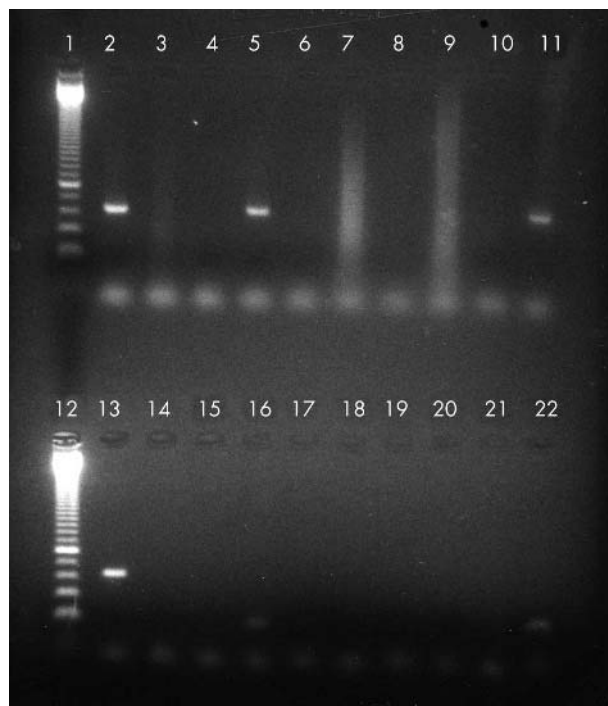

Figure 4 Polymerase chain reaction (PCR) amplification of a 163 bp fragment of the katG gene. Lanes 1 and 12, 50 bp ladder; lanes 2–7 and 13–18, paraffin wax embedded samples; lanes 8–11 and 19–21, extraction controls; lane 22, PCR control.

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No. 37 : 1        cgcgctgtcagagctgactttgttgtgactgcatgaggggcactgcctacgcgaccagac
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
M. tb  : 301054   cgcgctgtcagagctgactttgttgtgactgcatgaggggcactgcctacgcgaccagac


No. 37 : 61       gctcgatgctgcccaacacccgggcggtgtagctggccaccgtcgtgcagtgcgtgaccg
                  ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
M. tb  : 300994   gctcgatgctgcccaacacccgggcggtgtgggctggccaccgtcgtgcagtgcgtgaccg


No. 37 : 121      gcgggctgggggtgacactgattccgcaga  150
                  ||||||||||||||||||||||||||||||
M. tb  : 300934   gcgggctgggggtgacactgattccgcaga  300905
```

Figure 5 Partial sequence of the oxyR pseudogene. Compared with the *Mycobacterium tuberculosis* reference strain sequence data, case number 37 showed a mutation (G to A, grey marking) in the oxyR pseudogene characteristic of *M bovis*.

mycobacterial strains. It may also facilitate the recording of the epidemiology and spread of mycobacteria even in archival material. Furthermore, our analysis provides clear evidence that spoligotyping is the technique of choice in archival material, because it was more informative than single gene analysis. This is not surprising, because fixation and chemical processing of the archival material induces alterations of the DNA, potentially resulting in complete or partial loss of the DNA. However, we also provide circumstantial evidence that both approaches (spoligotyping and single gene analysis) produce almost identical results.

As a first step, we made use of the frequently used segment IS6110, which is specifically seen in human pathogenic bacteria of the *M tuberculosis* complex. Recent discussions on eventual "crossreactions" with other (non-pathogenic) mycobacteria provided clear evidence that the approach used here excludes such false results.[24 25] Using this approach, we were able not only to confirm the presence of mycobacteria in the eight cases where histochemical Ziehl-Neelsen staining had already demonstrated mycobacterial infection, but also found that 11 of the 41 cases with histologically unclear or suspected mycobacterial infection were molecularly positive. This rate is well within the limits of several other previous studies where formalin fixed, paraffin wax embedded tissue samples from patients with clinically suspected TB had been included.[3 26] The amplification of mycobacterial DNA in the acid fast bacilli positive specimens clearly underlines the high specificity of the IS6110 based TB PCR. Therefore, we confirm the results of former studies that had focused on the specificity and sensitivity of this molecular approach.[3 26–29]

Moreover, the extracted DNA can be used for further differentiation of the species of the *M tuberculosis* complex by spoligotyping. Comparison of the signatures with an international database revealed conformity with patterns obtained from other studies from different geographical regions. Two spoligotyping signatures have been identified as being common worldwide, and another has been described in Houston, Texas, USA.[22] Three further patterns are also typical of *M tuberculosis*, but have not been found on the database. At present, we can only speculate whether or not these three signatures represent "new", hitherto unreported substrains, or whether they were incomplete and should therefore not be further evaluated. However, we have to be aware that the absence of single spacers can also be caused by isolated dropouts, potentially leading to misinterpretation. One *M tuberculosis* specific signature appears not to be fully complete, but the presence of spacers 33 to 43 leads to the assumption that this resembles an "ancient" *M tuberculosis* strain.[13] Interestingly, at least four *M bovis* specific signatures were also unambiguously detected by spoligotyping. One of

these came from a patient with clinical evidence of pulmonary tuberculosis. This case was clearly identified as a typical pattern of *M bovis* subtype *caprae*,[30] which to date has been isolated mainly from sheep and goats.[31] Recently, Kubica *et al* showed that *M bovis* subtype *caprae* was responsible for one third of all human tuberculosis cases caused by the bovine tubercle bacillus in Germany between 1999 and 2001.[32] The three remaining patterns all showed the absence of spacers 3 and 9, and in two cases spacer 16 was also absent. These spacers are usually absent in *M bovis* BCG strains.[12] In this context it is very interesting that two of these patients were treated with BCG for urothelial carcinoma of the bladder and, therefore, most probably had developed a local BCG infection. Five further cases revealed an incomplete, patchy pattern, where no further strain differentiation was possible. This can be attributed to poor DNA conservation as a result of fixation and the embedding procedure. It can be assumed that this chemical modification of the tissue samples during processing for histology enhances DNA degradation processes and reduces the amount of detectable bacilli.[33 34] Nevertheless, in a recent study we successfully applied the spoligotyping method to mummified human remains from ancient Egypt.[35] This revealed the presence of different *M tuberculosis* and *M africanum* strains 4000 years ago and demonstrates the high sensitivity of this approach.

> "Our analysis provides clear evidence that spoligotyping is the technique of choice in archival material, because it was more informative than single gene analysis"

The analysis of partial gene sequences of oxyR, pncA, mtp40, katG, and gyrA and testing for the presence or absence of variable gene regions are useful for the further characterisation of the identified *M tuberculosis* complex strains. The mtp40 sequence has been shown to be specific for *M tuberculosis* and *M africanum*.[14] Single point mutations of the pncA gene and the oxyR locus have been used to differentiate between *M bovis* and *M tuberculosis* isolates.[15 16] The sequence polymorphism of the katG and gyrA gene allows the division of the *M tuberculosis* strains into three genetic groups.[17] In our study, all *M tuberculosis* strains identified by spoligotyping could be confirmed by either the presence of the mtp40 sequence or by showing the typical oxyR and pncA wild-type sequences. Two further cases with incomplete spoligotyping patterns also contained the *M tuberculosis* specific oxyR sequence and are therefore most probably *M tuberculosis* strains. One *M bovis* case showed the typical mutation of the oxyR locus at nucleotide 285 (G to A), but had a wild-type pncA gene. In this case, the spoligotyping

Take home messages

- Archival paraffin wax embedded material can be used for molecular analysis to identify different *Mycobacterium tuberculosis* complex strains and can therefore unequivocally be used for the study of the epidemiology and evolution of tuberculosis pathogens
- We successfully applied the spoligotyping technique for the identification of strains of human pathogenic mycobacteria in approximately two thirds of our archival tissue samples
- This technique could potentially provide data on superinfection by various tuberculosis strains and clinically relevant data on multidrug resistant strains

signature showed a pattern specific for the *M bovis* subtype *caprae*, which is unusually susceptible to PZA. The PZA resistance, similar to that seen in the *M bovis* subtype *bovis* strains, is based on a single point mutation in the pncA gene (see above). Thus, the absence of the pncA mutation in this case confirmed the spoligotyping result.

Based on the results of the katG and gyrA sequence analysis, five of the *M tuberculosis* samples could be classified as group 2 or 3 according to the scheme proposed by Sreevatsan *et al*.[17] As expected, no mutations of the katG or gyrA gene were found in the *M bovis* strains. These findings are substantiated by the investigation of the *M tuberculosis* specific deletion TbD1 and RD9, which is absent in the *M africanum*, *M microti*, and *M bovis* strains.[13] In all seven *M tuberculosis* cases, TbD1 was deleted and a further six cases showed the presence of RD9, whereas in one *M bovis* sample RD9 was deleted. Therefore, all *M tuberculosis* cases identified in our study could be classified as ''modern'' *M tuberculosis* strains.

In summary, our study clearly shows that archival paraffin wax embedded material can be used for epidemiological and evolutionary studies. We present circumstantial evidence that the epidemiology of *M tuberculosis* complex strains and substrains can be evaluated on archival material that predates major population movements in recent times. Our results suggest that this information might be uncovered with considerable success. Furthermore, this technique could potentially provide data on superinfection by various TB strains and clinically relevant data on multidrug resistant strains.

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