Modern Pathology (2005) 18, 274–282

www.modernpathology.org

Species identification of mycobacteria in paraffin-embedded tissues: frequent detection of nontuberculous mycobacteria

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Diagnosis of infections caused by mycobacteria, especially nontuberculous mycobacteria still represents a difficult task both in microbiology and pathology. The aim of this study was to determine the frequency of mycobacterial DNA detectable by PCR in formalin-fixed paraffin-embedded tissues showing suspicious granulomatous lesions. A total of 190 archival specimens were analyzed, using a nested PCR protocol, which amplifies a fragment of the mycobacterial 65-kDa heat-shock protein gene. Restriction fragment-length polymorphisms and sequencing were utilized to further analyze the obtained PCR products. Corresponding microbiological culture results were available for 41 cases. We detected mycobacterial DNA in 119 cases (63%), of which 71 (60%) were positive for *Mycobacterium tuberculosis* complex DNA and 41 (34%) for DNA of nontuberculous mycobacteria. Seven cases (6%) could not be subtyped for technical reasons. The largest group of nontuberculous mycobacteria comprised 29 cases (25% of the 119 positive cases), which were assigned to *Mycobacterium fortuitum* complex. *Mycobacterium avium–intracellulare* complex was detected in eight (7%) cases, *Mycobacterium gordonae* in three (2.5%) and *Mycobacterium rhodesiae* in a single case (0.8%). All cases of *Mycobacterium tuberculosis* were unequivocally identified by restriction fragment-length polymorphism analysis. In contrast, sequencing provided a gain of information over restriction fragment-length polymorphism analysis in 37% of the nontuberculous mycobacteria cases (15 of 41). Alignment studies on DNA of nontuberculous mycobacteria showed frequent sequence variations, supporting the existence of sequevars. Comparison of molecular data to available results of microbiological culture assays showed a good concordance of 83%. In conclusion, amplification and sequencing of the mycobacterial 65-kDa heat-shock protein gene is an excellent tool for species identification of mycobacteria, especially nontuberculous mycobacteria, in formalin-fixed paraffin-embedded tissues.
Modern Pathology (2005) **18,** 274–282, advance online publication, 8 October 2004; doi:10.1038/modpathol.3800289

Keywords: nontuberculous mycobacteria; NTM; 65-kDa heat-shock protein; formalin-fixed paraffin-embedded tissue; FFPE; molecular pathology; granulomatous reactions

The diagnosis of mycobacterial infections in archival pathological specimens still represents a challenge. This is especially true for infections caused by nontuberculous mycobacteria, which frequently show nonspecific clinical symptoms.[1,2] Furthermore, microbiological culture assays can have a fairly low sensitivity in nontuberculous mycobacteria especially in the setting of cervical lymphadenitis, where detection rates are between 50 and 60%.[3] Morphologically, caseating and noncaseating granulomatous reactions can occur, which often do not allow a differentiation between various infectious etiologies and hypersensitivity reactions. Stains for acid-fast bacilli remain frequently negative.[4]

In order to improve the diagnostic sensitivity, many different PCR protocols have been developed

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Received 3 September 2004; revised 13 July 2004; accepted 15 July 2004; published online 8 October 2004

for amplification of mycobacterial DNA. Only some of them are suitable for routine application on formalin-fixed paraffin-embedded material.[5–11] and only few PCR protocols are able to amplify hypervariable gene regions, necessary for identification of nontuberculous mycobacteria. One of the protocols that fulfills these requirements was published by Cook *et al*.[5] This method amplifies a short fragment (133 bp) of the hypervariable region, that belongs to the 65-kDa heat-shock protein gene (*hsp65*).[12,13]

The aim of this study was to test the suitability of this protocol for the detection of mycobacterial DNA, especially of nontuberculous mycobacteria in archival pathological specimens with suspicious granulomatous reactions and to analyze the distribution of mycobacterial species. Furthermore, we compared the specificity of restriction fragment-length polymorphism analysis (RLFP) with sequencing for nontuberculous mycobacteria speciation.

Materials and methods

Materials

Our collection consisted of formalin-fixed and paraffin-embedded material of 190 cases diagnosed histologically as granulomatous reactions suspicious for possible mycobacterial infection, which had been collected over a period of 3 years (1998–2000). The samples originated from a variety of anatomical sites such as respiratory tract, lymph nodes, gastrointestinal tract, genitourinary tract, bone, skin and other miscellaneous localizations (Table 1). Histologically, 61 cases (32%) showed well-defined granulomas with caseation necrosis, 66 cases (35%) well-defined granulomas without caseation necrosis and 63 cases (33%) ill-defined granulomas, including 26 pyogranulomas, and three spindle cell pseudotumors. Majority of specimens were submitted by outside pathologists for consultation. Cases with a history or clinical diagnosis of sarcoidosis, necrotizing sarcoidal granulomatosis, Crohn's disease, Wegener's disease and other variants of arteritis were excluded from this study ($n = 9$). Analysis of stains for acid-fast bacilli as well as standard morphological analysis were performed independently by two pathologists.

Organism controls consisted of DNA extracts of the following cultured mycobacterial strains obtained from the American-type culture collection (ATCC): *Mycobacterium tuberculosis, Mycobacterium gordonae, Mycobacterium fortuitum, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium chelonae*

DNA Extraction

Deparaffinization was performed with xylene and ethanol on serial sections of 40–60 μm in total, depending on the amount of tissue. To avoid contamination of the samples during the preparation process, new disposable microtome blades were used for each sample, and the microtome was cleaned meticulously after each block. After lyophilization the probes were resuspended in 50 μl of 50 mM Tris 8.3 with 200 μg/ml proteinase K and incubated overnight at 37°C.[5,6] A modified temperature step model was used before and after proteinase K digestion three times each (2 min at 37°C, 30 s vortexing, 2 min at −80°C) to disrupt mycobacterial cell walls.[14]

Nested PCR and RFLP Analysis

After pelleting of insoluble debris by centrifugation, DNA integrity was tested by amplification of a 269 bp fragment of the *β-globin* gene (as house-

Table 1 Molecular examinations for mycobacterial DNA on granulomatous reactions

Tissue type (n)	*M. tbc*	*NTM*	*n.s.*	*Negative*
Respiratory tract (46)	19 (41%)	8 (17%)	3 (7%)	16 (35%)
Cervical LN (38)	13 (34%)	11 (29%)	2 (5%)	12 (32%)
Other peripheral LN (15)	5 (33%)	4 (27%)	1 (7%)	5 (33%)
Miscellaneous locations (46)	16 (35%)	9 (20%)	1 (2%)	20 (43%)
Gastrointestinal tract (22)	7 (32%)	6 (27%)	0	9 (41%)
Genitourinary tract (7)	4 (57%)	1 (14%)	0	2 (29%)
Bone (9)	3 (33%)	1 (11%)	0	5 (56%)
Skin (5)	3 (60%)	1 (20%)	0	1 (80%)
Liver (2)	1 (50%)	0	0	1 (50%)
190 Molecular analyses[a]	71 (60%)[b]	41 (34%)[b] 63% positive (119)	7 (6%)[b]	71 37% negative (71)

M. tbc., Mycobacterium tuberculosis; NTM, nontuberculous mycobacteria; n.s., not specifiable, respiratory tract: lungs, trachea, larynx, pulmonary and paratracheal LN; miscellaneous locations: connective tissue, fatty tissue, mammary glands, salivary glands, among others; gastrointestinal tract: complete intestinum and related LN stations; genitourinary tract: kidneys, urinary bladder, prostate, testes, ovaries; Percentages in parentheses in general are related to the total number of samples (*n*) from each type of tissue.

[a]*hsp65* amplification, combined with RFLP and sequencing analysis.

[b]Percentages are related to all positive cases (119).

keeping gene) in a single step PCR (35 cycles).[15] All cases, which showed negative or weak results in *β-globin* amplifications due to poor DNA quality, were excluded from further analysis ($n=7$). PCR for the detection of mycobacterial DNA was performed using the primer combinations described by Cook *et al*,[5] which amplify a fragment of the mycobacterial 65-kDa heat shock protein gene (*hsp65*). A measure of 5 and 0.5 μl of the DNA extract (soluble phase) was put as template in the first-round PCR reaction mix (50 μl total volume, containing 200 μM of each dNTP, 2.5 ng/μl of each primer, 1 U *Taq* polymerase and 1 × *Taq* polymerase buffer). The PCR protocol consisted of an initial denaturation (7 min, 94°C), 35 cycles (1 min at 94°C, 2 min at 57°C, 2 min at 72°C) and a final extension step of 7 min (72°C). For nested reamplification, 5 and 0.5 μl of first-round PCR product were transferred and subjected to the same PCR conditions (35 cycles). Cases positive for *M. tuberculosis* DNA in the *hsp65* PCR but negative in microbiological cultures were confirmed with an additional single step PCR, specific for *M. tuberculosis* complex. It amplifies a sequence of 123 bp of the insertion sequence IS6110 by using a single-step approach (40 cycles).[8] As positive control, we used DNA of *M. tuberculosis* (H37Rv). The negative controls consisted of template-free reaction mix with deionized water. Furthermore, we used normal formalin-fixed paraffin-embedded material tissues from lung (2 ×), lymph nodes (2 ×), soft tissue (2 ×), kidney (2 ×), liver (1 ×) and bone (1 ×) as negative tissue controls. PCR products were visualized by agarose gel electrophoresis. RFLP analysis of the *hsp65* PCR products for identification of mycobacterial species was performed using three different endonucleases (*Hha*I, *BstU*I, *Mbo*I), according to the published protocol.[5] Fragment sizes were estimated by comparison with the DNA size marker (Hae3 digest of Phi × 174).[5]

Sequencing Analysis and Homology Matches

All PCR products with restriction patterns other than *M. tuberculosis* were purified (QiaQuick Gel extraction kit, Qiagen, Hilden, Germany) and directly sequenced, using the fluorescence-based cycle sequencing procedure.[16] Sequence analysis was performed on an automated sequencer (ABI prism 377™, Perkin-Elmer). For homology matches, we used the BLAST search program and the NCBI database. Multiple alignment studies and restriction site analysis were performed using the DNASIS™ software (Hitachi Softw. Eng.) and *hsp65* sequences of the following reference strains (gi and gb accession numbers from BLAST search program, other designations are shown in parentheses): *M. chelonae* gi 4093110 (IP 140420003^T, ATCC 35752^T), *M. fortuitum* gi 4093116 (IP 140410001^T, ATCC 6841^T), *Mycobacterium mucogenicum* gi 4093120 (IP 140430001^T, ATCC 49650^T), *Mycobacterium peregrinum* gi 4093122 (IP 14041002^T, ATCC 14467^T), *M. avium* gi 2108161 (gb U 85641), *M. intracellulare* gi 19070717 (gb/AF 354277, strain 950A-9), *M. gordonae* gi 4093118 (gb/AF 071134, IP 14021001^T), *Mycobacterium rhodesiae* gi 13560193 (emb/AJ 30764.1, MRH 307647).

Detection of *Mycobacteria* in Clinical Samples Via Culture

Clinical samples were incubated for 25 min in decontamination solution (sodium citrate solution 1.55% (w/v) containing *N*-acetyl-L-cysteine 0.005% (w/v) and NaOH 2% (v/v)) to remove any contaminating bacteria. Subsequently, the specimens were cultured on two different solid media (BBL™ Löwenstein–Jensen Medium + PACT (cat # 220502, Becton Dickinson, Loveton Circle, USA), BBL™ Stonebrink TB Medium + PACT (cat # 220505, Becton Dickinson)) as well as one liquid medium (BACTEC™ 12B MYCOBACTERIA CULTURE VIALS Middlebrook 7H12 (cat # 442004, Becton Dickinson). Samples were incubated until the culture yielded growing mycobacteria or were discarded as negative after 6 weeks.

Results

A total of 190 cases were analyzed. Corresponding microbiological culture data were available for 41 (21.6%) cases. Therefore, samples with available culture results (group 1) and without microbiological data (group 2) were analyzed separately. Overall, a total of 119 (63%) cases were positive for mycobacterial DNA, including 25/41 (61%) cases of group 1 and 94/149 (63%) of group 2. Concordant molecular and microbiological results were found in 83% (34/41 cases) of group 1, including 16 cases positive for *M. tuberculosis*, two positive for *M. avium–intracellulare* and 16 negative cases (Table 2). Discrepant results occurred in 17% (7/41 cases), including three positive PCR results for *M. tuberculosis* complex DNA and four positive PCR results for *M. fortuitum* complex DNA, whereas corresponding

Table 2 Correlation of PCR results with microbiological culture assays (group 1)

Culture	*PCR amplification*			
	M. tbc	*MAI*	*M. fort.*	*Neg*
M.tbc	16 (7)			
MAI		2 (2)		
M. fort.				
Neg	3		4	16

M. tbc., *M. tuberculosis complex*; MAI, M. *avium–intracellulare* complex; M. fort., *M. fortuitum* complex; Neg, negative.
In parenthesis (*n*) number of samples, which were positive in Ziehl–Neelsen stainings.

microbiological culture assays remained negative. All three cases of *M. tuberculosis* with negative cultures were validated independently by positive results obtained in a separate PCR assay for the *M. tuberculosis* complex-specific insertion sequence (IS 6110).[8]

All 119 positive PCR products were subjected to RFLP analysis. In all, 71 cases (60% of the positive cases) showed the typical restriction pattern for *M. tuberculosis* complex (Table 1).[5] This was confirmed by sequencing of the PCR product in 15 cases. All of them showed 100% homology to *M. tuberculosis* (data not shown). Eight patients positive for *M. tuberculosis* had a history of immunosuppression, including five with HIV infection. Two patients had received intravesical BCG therapy for urothelial carcinoma, and a positive PCR result was obtained from bladder biopsies. Seven patients had a confirmed history of tuberculosis, and in the majority of the remaining cases, there was a high clinical suspicion of tuberculosis.

DNA of nontuberculous mycobacteria was found in 41 cases (34% of 119 positive cases, Table 1). Sequencing and BLAST homology search assigned the nontuberculous mycobacteria cases to *M. fortuitum* complex in 25% (30/119), including *M. chelonae*, *M. fortuitum*, *M. mucogenicum* and *M. peregrinum*.[6] The second largest group of nontuberculous mycobacteria belonged to *M. avium–intracellulare* complex (6.7%). DNA of *M. gordonae* was found in three cases (2.5%) and of *M. rhodesiae* in a single case (0.8%, Table 3).

In contrast to *M. tuberculosis,* species identification was not possible by RFLP analysis in 15 (36.5%) of nontuberculous mycobacteria cases. The range of different restriction patterns for members of the *M. fortuitum* complex was fairly wide. We observed three different patterns for *M. chelonae*, two of which had been reported previously (data not shown).[2,5] Accordingly, sequences from different *M. chelonae* isolates varied at 1–5 nucleotides (Table 4).

In addition, a new conserved restriction pattern specific for *M. mucogenicum* was identified in eight cases (Figure 1a, b). This subspecies had not been considered in the work of Cook *et al*.[5] Homology matches of the sequenced PCR products (NCBI database) demonstrated a concordance of 93–100% with the reference sequence of *M. mucogenicum* (Table 4). Multiple alignments demonstrated sequence variants (sequevars) showing a divergence in up to 7 bp of the eight *M. mucogenicum* cases. Despite the existence of sequevars, species identification by RFLP was possible in all of the *M. mucogenicum* cases, since restriction sites were conserved.

Five of eight cases with DNA of *M. avium–intracellulare* complex showed the previously reported restriction patterns.[5] The remaining three cases, which could not be clearly distinguished by RFLP, showed a stronger intraspecies divergence from to the *M. avium* reference sequence (Tables 4 and 5). Two of the eight *M. avium–intracellulare* infections had developed in the setting of HIV infection. The advantage of sequencing analysis over RFLP was most obvious for *M. peregrinum* and *M. rhodesiae*, which could only be specified via sequencing (Tables 4 and 5). Despite repeated DNA extraction, seven of the 119 cases (6%) showed only weak, although reproducible PCR products unsuitable for subtype identification by sequencing or RFLP (Table 1). Negative controls as well as negative tissue controls remained completely and reproducibly negative in the *hsp65* PCR.

Correlation of histomorphology with molecular results showed that only the characteristic finding of spindle cell pseudotumors in HIV infection (two cases) allowed to confidently predict an infection with *M. avium–intracellulare*. Furthermore, a fairly good correlation of 79% (48 of 61 cases) existed between the presence of well-defined caseating granulomas and the detection of *M. tuberculosis* complex DNA. Nevertheless, three of these well-defined granulomas turned out to be positive for DNA of nontuberculous mycobacteria ($2\times$ *M. fortuitum* complex, $1\times$ *M. avium–intracellulare* complex), whereas nine cases were completely negative for mycobacterial DNA.

As expected, staining for acid-fast bacilli was of limited value. Only four out of 41 nontuberculous mycobacteria cases were positive, all of them caused

Table 3 Identification of nontuberculous mycobacteria[a]

MAI	*M. fortuitum Complex*				*M. gord.*	*M. rhod.*
	M. chel.	*M. fort.*	*M. muc.*	*M. per.*		
8 (6.7%)	13 (10.9%)	7 (5.9%)	8 (6.7%)	1 (0.8%)	3 (2.5%)	1 (0.8%)
			29 (25.2%)			

[a]Percentages are related to the total number of 119 positive cases after *hsp65* amplification (Table 1), combination of RFLP and sequencing results. MAI, *M. avium–intracellulare; M. chel., M. chelonae; M. fort., M. fortuitum; M. muc., M. mucogenicum; M. per., M. peregrinum; M. gord., M. gordonae; M. rhod, M. rhodesiae.*
Percentages are related to all positive cases (119, Table 1).

Table 4 Multiple alignments for DNA of nontuberculous mycobacteria (*hsp65*-sequences)[a]

	10	*20*	*30*	*40*	*50*	*60*	*70*
M. chelonae	CTGCTGGACT	CCGCCAAGGA	GATTGACACC	AAGGAGCAGA	TCGCGGCCAC	CGCGGGCATC	TCCGCGGGTG
C1 (2x)	----------	----------	----------	----------	----------	----------	----------
C2 (4x)	----------	----------	---C------	----------	----------	----------	----------
C3 (2x)	-------C--	----------	---C------	----------	----------	----------	----------
C4 (2x)	--------CT-	----------	----------	----------	----------	----------	-----T----
C5	--------CT-	----------	---C------	----------	----------	----------	-----T----
C6	-------C--	----------	-G-C--G---	----------	----------	----------	----------
C7	---------T-	----------	---C------	----------	----T-----	----------	-----T----
M. fortuitum	CTGCTGAAGA	GCGCCAAGGA	GGTGGAGACC	AAGGAGCAGA	TCGCTGCCAC	CGCCGGTATC	TCCGCCGGTG
F1 (3x)	----------	----------	----------	----------	----------	----------	----------
F2 (2x)	C-------TC-	----------	----------	----------	----------	----------	----------
F3 (2x)	C-------TC-	----------	----------	----------	----------	-G--G-----	----------
M. mucogenicum	CTGCTGGCTT	CCGCCAAGGA	GGTCGAGACC	AAGGAGCAGA	TCGCTGCCAC	CGCCGGGATC	TCGGCCGGTG
M1 (3x)	----------	----------	----------	----------	----------	----------	----------
M2 (2x)	----------	----------	----------	-----T----	----------	----------	----------
M3	---------C-	----------	----------	----------	----------	----------	----------
M4	----------	----------GA-	----------	----------	----------	----------	----------
M5	----------	----------	CA--------	----------	----------	-----C----	--C--T----
M. peregrinum	CTCCTGAAGT	CCGCCAAGGA	GGTGGAGACC	AAGGAGCAGA	TCGCTGCCAC	CGCCGGTATC	TCCGCCGGAG
P1	----------	----------	----------	----------	----------	----------	----------
M. avium	CTGCTGAAGG	AGGCCAAGGA	GGTCGAAACC	AAGGAGCAGA	TCGCTGCCAC	CGCCGCGATC	TCCGCGGGCG
A1 (3x)	----------	----------	----------	----------	----------	----------	----------
A2	---------T	----------	------T---	----------	------A---	--------C---	----------
A3	-----CT---	----------	-------G---	-----C----	----------	---G-----T	--G-------
A4	-----GC--	-C--------	*C*-----*G*---	C----------	---G--G---	CG---GC---	----------
A5	-----C---T	C----------	-------G---	-----C----	----G--G--T	CG--G---CG--	A--AGT---AT
M. intracellulare	CTGCTCAAGT	CGGCCAAGGA	GGTCGAGACC	AAGGACCAGA	TCGCTGCCAC	CGCGGCGATT	TCGGCGGGCG
I1	----------	----------	----------	----------	----------	----------	----------
M. gordonae	CTGCTCAGCT	CGGCCAAGGA	CGTCGAGACC	AAGGAGCAGA	TCGCGGCCAC	CGCGGGCATC	TCCGCGGGTG
G1	----------	----------	----------	----------	----------	----------	----------
G2	---------T-	----------	----------	----------	----T-----	----------	----------
G3	-----G----	----------C	-A--------	----------	----T-----	---C------	-----T----
M. rhodesiae	CTCCTCAAGA	GCGCCAAGGA	GGTCGAGACC	AAGGATCAGA	TCGCAGCCAC	CGCCGGGATC	TCCGCGGGCG
R1	----------	----------	----------	-----C----	----G-----	----------	----------

[a]Selective demonstration of 70 bp from the 134 bp *hsp65* PCR fragment.
The first nucleotide shown in each line corresponds to position 416 of the published sequence from *M. tbc.*[8]
The number of identical biovariants are put in parentheses; if not further indicated the sequence was only detected once.
Restriction sites are underlined. (*Hha*I: GCG/C, *Mbo*I: /GATC, *BstU*I: CG/CG); – : correspondence of probe with reference sequence.

Table 5 Comparison of RFLP with sequencing results

	MAI	*M. chel.*	*M. fort.*	*M. muc.*	*M. per.*	*M. gord.*	*M. rhod*	*total*
Specifiable by RFLP	5	8	4	8	0	1	0	26
Specifiable by sequencing	8	13	7	8	1	3	1	41
Gain of identification by sequencing	38% (3)	39% (5)	43% (3)	0% (8)	100% (1)	67% (2)	100% (1)	36.5% (15)

MAI, *M. avium–intracellulare*; *M. chel.*, *M. chelonae*; *M. fort.*, *M. fortuitum*; *M. muc.*, *M. mucogenicum*; *M. per.*, *M. peregrinum*; *M. gord.*, *M. gordonae*; *M. rhod*, *M. rhodesiae*. (*n*): cases, which were identifiable only by sequencing.

by *M. avium–intracellulare* complex, either showing the morphology of spindle cell pseudotumors (three cases) or ill-defined granulomas with microabcess formation (one case). Acid-fast bacteria were found in 7/16 cases positive for *M. tuberculosis* by both PCR and microbiological culture assays.

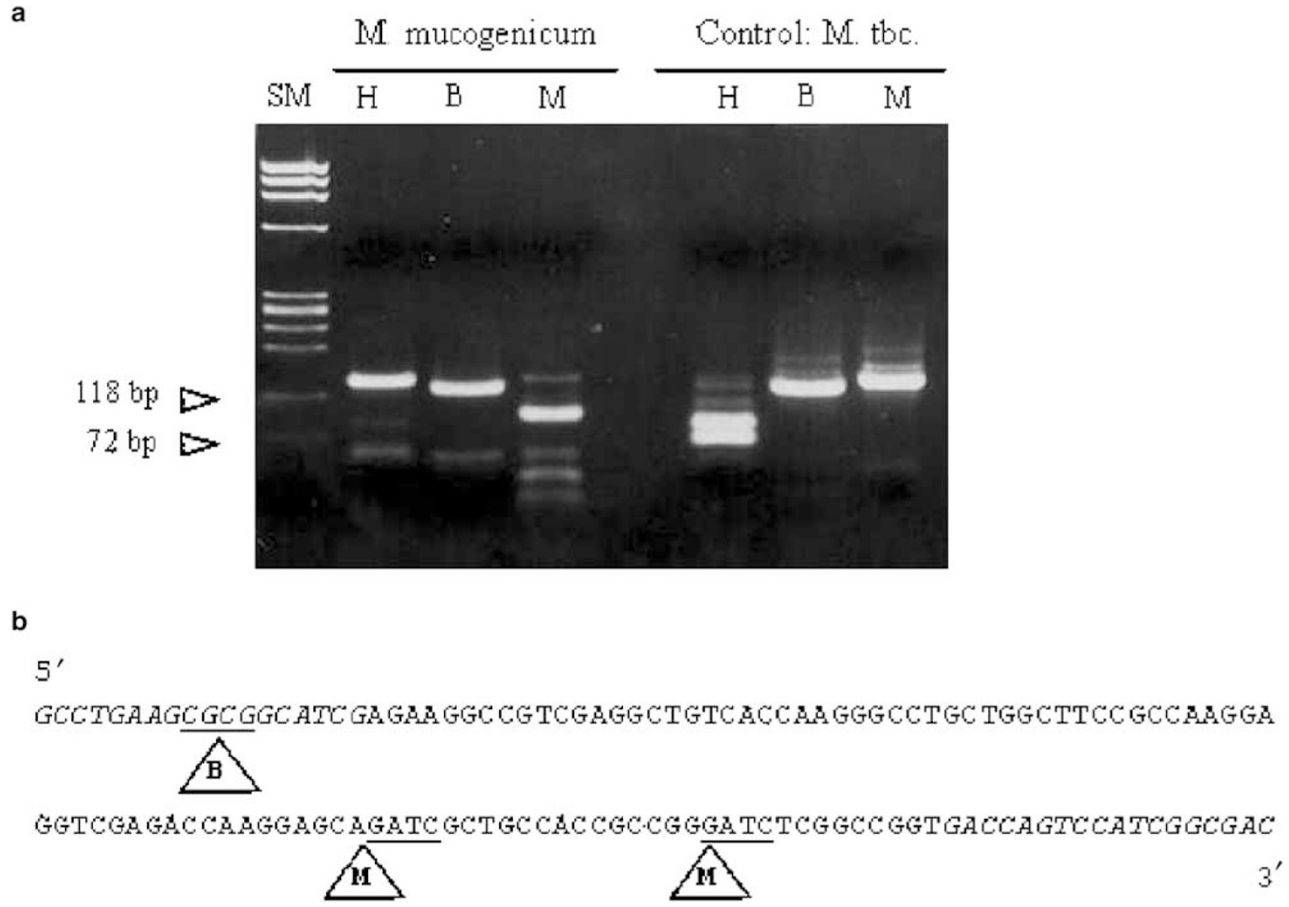

Figure 1 (**a**) A new restriction pattern for *M. mucogenicum* was observed by using RFLP on PCR products (*hsp65* amplification) in eight cases. The agarose gel shows a comparison of the specific restriction pattern found for DNA of *M. mucogenicum* to the restriction pattern of control DNA derived from *M. tuberculosis* complex. Sizes of restriction fragments of *M. mucogenicum*: H: 133 bp (uncut), b: 124 + 9 bp, M: 85 + 31 + 18 bp. SM, size marker (*Hae*3 digest of Phi × 174). Restriction enzymes: H, *Hha*I; B, *Bstu*I; M, *Mbo*I. (**b**) Conserved DNA sequence of the amplified *hsp65* fragment of *M. mucogenicum*, which was found in three of eight cases (Table 4). Restriction sites are indicated in triangles, primer binding sites are shown in italics.

Discussion

The diagnosis of mycobacterial infections still presents a frequently difficult task in clinical and pathological practice. This is especially true for nontuberculous mycobacteria infections, which usually lack both specific clinical symptoms and defining morphological features. The pathological changes, frequently ill-defined granulomas or pyogranulomas with a lack of caseation, few or no giant cells and a negativity for acid-fast bacteria in staining procedures may lead to a suspicion of nontuberculous mycobacteria infection, but usually are nonspecific.[3,4] Nowadays, molecular techniques allow the species identification of mycobacteria even in samples where culture methods and other conventional detection methods have either not been employed or remained negative.[3,4] The application of PCR techniques has demonstrated that the frequency of infections by nontuberculous mycobacteria has probably been underestimated in the past and that the clinical and morphological spectrum of these infections is broader than previously thought.[17–22] This is confirmed by our study, which identified nontuberculous mycobacteria species in a third of the cases of suspicious granulomatous tissue reactions positive for mycobacterial DNA, using a sensitive nested PCR technique amplifying a hypervariable fragment of the *hsp65 gene*.[5]

The only specific histological pattern in our study, which allowed confident identification of the causative organism on morphological grounds alone corresponded to the well-recognized spindle cell pseudotumor in the setting of HIV infection. The two cases showed abundant intracellular acid-fast bacilli and were positive for DNA of *M. avium–intracellulare* complex. The remaining 38 nontuberculous mycobacteria cases (93%) could not be linked to a specific histomorphologic pattern.

PCR-based detection of mycobacterial DNA offers significant diagnostic support in these cases. In our laboratory, the protocol of Cook *et al*[5] proved to be a highly sensitive method for detecting DNA of

nontuberculous mycobacteria in routinely fixed, paraffin-embedded specimens.[2] In addition to identifying nontuberculous mycobacteria infections, this protocol is also a convenient way of testing for *M. tuberculosis*. In contrast to nontuberculous mycobacteria species, *M. tuberculosis* showed a 100% conservation of its characteristic RFLP pattern and complete sequence homology to the reference strain in all analyzed cases, confirming that our protocol in conjunction with RFLP is of equal specificity as PCR protocols amplifying only *M.tuberculosis*-specific sequences. We observed DNA of *M. tuberculosis* complex in 60% and DNA of nontuberculous mycobacteria in 34% of the cases, which showed positive PCR signals. This ratio of 2:1 for *M. tuberculosis* complex to nontuberculous mycobacteria DNA correlates well with other studies and supports the observation of an increasing rate of nontuberculous mycobacteria infections.[23,24]

Our series was split into two groups, on the basis of presence (group 1) or absence (group 2) of corresponding microbiological culture assays. The latter is still considered the goldstandard by many microbiologists, although PCR protocols get increasingly implemented in microbiology laboratories.

Group 1 of the present series showed a concordance of 83% between microbiological culture assays and PCR results. Of note, culture assays remained negative in four cases with positive PCR results for *M. fortuitum* complex, whereas both *M. avium–intracellulare* cases in which cultures had been obtained were positive by both techniques. In additon to the four specimens with *M. fortuitum* complex mentioned above, the other discrepant cases consisted of three samples positive for *M. tuberculosis* by PCR, but with negative culture assays. An independent confirmation of the presence of mycobacterial DNA was achieved by a separate PCR assay specific for *M. tuberculosis* in these three cases.[8]

Although RFLP analysis is a fast and helpful method to discern *M. tuberculosis* from nontuberculous mycobacteria, it is limited in identifying nontuberculous mycobacteria species. Incomplete digestion can result in ambiguous patterns, and small restriction products (<30 bp) cannot be differentiated sufficiently on conventional agarose gels. Another factor of great importance for the identification of nontuberculous mycobacteria is the high frequency of sequevars, resulting in diverse restriction patterns for members of the same subtype, like for *M. chelonae*.[25] In these circumstances, sequence analysis provided a means to overcome the limitations of RFLP. In cases with atypical restriction patterns, sequencing analysis and homology matching identified various species of nontuberculous mycobacteria but never *M. tuberculosis*, confirming that RFLP is probably sufficient to make the clinically important distinction between *M. tuberculosis* and nontuberculous mycobacteria. A problematic, although small group of cases are specimens, which render reproducible signals too weak for RFLP or sequencing. These results should be interpreted with great caution. In our experience, some weak bands which are not cut by any of the restriction enzymes used may actually contain nonmycobacterial sequences which probably represent ubiquitous bacterial DNA amplified at very low efficiency.[5]

Homology matching with large databases such as the NCBI-database are helpful instruments for analysis of mycobacterial DNA sequences. However, identification and differentiation of mycobacterial subspecies on a genetic basis is still evolving.[25–30] Although amplification of a hypervariable region of the *hsp65 gene* followed by sequencing allows identification of most species,[13,25,26,31] a minority cannot be differentiated because of only minor interspecies divergence in these small PCR fragments. This applies for instance to *M. fortuitum* and *Mycobacterium senegalense*, which only differ in a single bp of the analyzed *hsp65* fragment.[25] Whether some of the sequence variants for members of the *M. fortuitum complex* described above represent true biovariants or misidentification of nontuberculous mycobacteria species due to the short sequence available for homology matching remains to be determined. Unfortunately, amplification of the 16S rRNA gene does not represent a real alternative for practical purposes, because the usual PCR fragment sizes are too large (more than 500 bp) for the highly fragmented DNA obtained after formalin fixation (suitable fragment sizes usually below 400 bp). However, therapy regimens usually do not differ for species of the same mycobacterial complex, as long as there are no contradictory results of susceptibility testing.[23,32,33] We therefore believe that reporting the complex, the identified mycobacterial species belongs to, is sufficient for most practical purposes.[2,34–36]

An interesting finding of our study was the frequent identification of *M. mucogenicum*, a subtype of the *M. fortuitum* complex, suggesting that it might be occasionally involved in human infections.[26,37,38] All eight *M. mucogenicum* cases in the present study showed an identical, previously unreported restriction pattern, despite a sequence divergence in up to 7 bp.

The predominance of species from the *M. fortuitum* complex in our collection, consisting of *M. fortuitum*, *M. chelonae*, *M. mucogenicum* and *M. peregrinum*, differs somehow from previously reported series.[39–42] Although this might in part reflect a sampling bias due to the inclusion of specimens submitted in consultation, the biological relevance of the detection of DNA of the abovementioned species needs to be critically evaluated. Some of the nontuberculous mycobacteria species frequently identified in our series such as *M. mucogenicum* are regarded as typical apathogenic water contaminants,[43] and their presence in surgical biopsies may result from tissue processing or other

environmental sources. Although this possibility cannot be excluded entirely for our cases, some facts suggest that a potential pathogenic role for these nontuberculous mycobacteria should be taken into consideration. Besides the fact that our standard negative controls including identically processed control tissues remained negative, the high sequence variability found in our isolates support diverse origins. In addition, well-documented series of infections in nonimmunocompromised patients, such as an outbreak of otitis media caused by patient-to-patient transmission of *M. chelonae* through contaminated medical devices, underlines the facultative pathogenicity of these species.[17] Furthermore, a recent report also demonstrated the facultative pathogenicity of *M. mucogenicum* by using a microbiological culture approach.[38] Nevertheless, one has to keep in mind, that the identification of mycobacterial DNA by PCR does not allow any statements concerning viability or pathogenicity, and treatment decisions have to be based on the integration of clinical, microbiological, histological and molecular findings. Any positive molecular result has to be correlated with morphology and should be interpreted with utmost caution in the absence of adequate histological alterations. Furthermore, we emphasize the importance of periodical quality assessments in diagnostic molecular pathology of infectious disease, in order to advance rules for standardization and interpretation of the different amplification methods.[44,45]

In conclusion, the applied *hsp65 gene* amplification protocol has proven to be a suitable method for detecting mycobacterial DNA in routinely processed pathological specimens.[2,5] RFLP analysis is very helpful for the discrimination of *M. tuberculosis* complex from nontuberculous mycobacteria; however, is limited for species identification of nontuberculous mycobacteria due to the high frequency of sequevars. In cases with ambiguous or novel restriction patterns, sequencing can confirm the presence of mycobacterial DNA and allows species identification.

Acknowledgements

We thank Mrs B Geist and Mrs E Hübner for excellent technical assistance, Dr L Binder for helpful discussions and provision of interesting cases and all the pathologists from other institutes for sending the formalin-fixed paraffin-embedded material of granulomatous reactions.

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