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ORIGINAL ARTICLE

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# Inter-laboratory validation of PCR-based detection of *Mycobacterium tuberculosis* in formalin-fixed, paraffin-embedded tissues

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**Abstract** The present study is based on the initiative for quality assurance in pathology of the German Society of Pathology and the Professional Association of German Pathologists. Four panel laboratories with experience and expertise in polymerase chain reaction (PCR) detection of *Mycobacterium tuberculosis* were selected to establish the prerequisites for continuous external laboratory trials, in particular, by providing pre-tested specimens and evaluation criteria for participating institutes. In the first step, the four panel laboratories performed an internal trial to test their own reliability and reproducibility. Paraffin sections and DNA preparations from 34 tissues (25 clinical specimens and 9 controls) totalling to 66 samples were evaluated by each panel institute according to their own protocols. The methodologies differed and are described in detail. Despite these differences, a high degree of inter-laboratory reliability was achieved. In this report, we summarise our results including the correlation with the histology and provide recommendations for applying PCR-based methodology for the detection of mycobacterial DNA in surgical specimens. Supplementary data are available online at http://www.charite.de/ch/patho (rubric "Forschung"). Pre-tested specimens are now available for the external trial and can be ordered from the steering institute via Oligene (http://www.oligene.com/). All molecular pathology laboratories are invited to participate in this quality assurance initiative.

**Keywords** PCR · Tuberculosis · Multi-centre study · Quality assurance · Reference standards

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## Introduction

Polymerase chain reaction (PCR)-based analytical technologies have developed to a standard methodology in nearly all disciplines of biosciences. Among others, this approach provides the option to look at targeted gene sequences and to amplify them, thus, enormously increasing sensitivity for detection. Detection and identification of disease-related genes in surgical pathology specimens, i.e. in biopsies, cytology samples, surgical resections and specimens from autopsies, have gained increasing importance in recent years and have created the discipline of molecular pathology [19]. The use of formalin fixation and paraffin embedding (FFPE) in pathology, however, implies several methodological obstacles, which need to be accounted for and cleared appropriately [13, 40, 48]. The commonly used fixation agent formalin is known to cause damage to DNA via fragmentation and/or cross-linkage. For this reason, the primer sets should be selected in such a way that specific and small, preferably multi-copy or repetitive, sequences are amplified. Additionally, reaction periods of the PCR cycles need to be sufficiently long.

In the literature, a large number of successfully applied set-ups are recommended for this purpose [2, 8, 17, 20, 26, 30, 39]. However, their reliability and reproducibility have only rarely been validated by comparative inter-laboratory trials [3, 28].

PCR-based investigation for mycobacterial DNA is an important application of molecular pathology, because granulomatous tissue responses are frequently, although not exclusively, attributed to mycobacterial infections. Detection of mycobacterial DNA is mandatory in tissues affected by granulomatous disorders, because the Ziehl–Neelsen (ZN) staining for acid-fast bacteria often fails to yield conclusive results [18, 24, 43].

Similarly, other approaches such as auramine staining or immunohistochemical identification using polyclonal antibodies against mycobacteria proved to be not sensitive enough for this purpose [29, 41]. Moreover, none of these classical techniques enables the distinction among *Mycobacterium tuberculosis*, atypical mycobacteria [also called mycobacteria other than tuberculosis complex (MOTT), or non-tuberculous mycobacteria (NTM)] and their subtyping [9, 35]. With FFPE, this task can only be solved by PCR-based methodology to date.

In addition to FFPE-related problems, mycobacteria carry a further methodological obstacle because of their thick mycolate-rich outer cell wall, which requires special measures of cell disintegration without causing further damage to DNA [14]. Thus, detection of mycobacteria in pathology samples is particularly critical and needs validation.

As part of a joint initiative of the German Society of Pathology (Deutsche Gesellschaft für Pathologie, http://www.mh-hannover.de/institute/pathologie/dgp/) and the Professional Association of German Pathologists (Berufsverband Deutscher Pathologen, http://www.bv-pathologie.de/) for quality control in pathology (QuIP), we performed an internal comparative inter-laboratory trial in the field of PCR-based detection of *M. tuberculosis*. Four institutes were selected by both societies because of their expertise and experience in this area [13, 16, 40, 47]. Our aim was to compare and to validate methodological variants employed by the four panel laboratories in order to draw general conclusions how to achieve high-level standards for PCR-based diagnostics of mycobacteria, but also to provide recommendations for nucleic acid amplification technology of surgical pathology specimens in general. This study followed the general guidelines in molecular pathology [19] and is similar to another multi-centre trial on Ki-67 labelling index [27]. General minimal demands for employing PCR and its terminology have been defined by the German Industrial Standard [7].

Despite a number of methodological variations, PCR-based diagnostics of mycobacteria proved to be reliable and reproducible. The specimens tested constitute the stock of a sample collection that is available for external molecular pathology laboratories in order to test their methodology and to increase quality assurance.

## Materials and methods

### Design of study

Each of the four panel groups (A–D) supplied eight cases for examination of *M. tuberculosis* by PCR including *M. tuberculosis*-positive and *M. tuberculosis*-negative samples (see below). Both DNA stock solution and the corresponding paraffin block were provided.

These materials were collected by the steering institute in Berlin, encoded and sent to the single panel laboratories. Each laboratory received a set of 66 samples consisting of 30 cases from clinical material and two experimentally infected human lung tissues for which paraffin sections and DNA stock solutions were provided as well as two paraffin samples from *M. tuberculosis*-infected mice.

The samples were provided in an anonymous fashion so that the respective investigators of the laboratories were blinded concerning the expected results.

### Tissue samples

Of the 32 human samples, 25 were clinical cases that were previously investigated by one of the panel laboratories for *M. tuberculosis* because of clinical or histopathological suspicion for infection.

Negative controls consisted of three foetal lung tissues as well as two adult lung samples, one with lipoid pneumonia around a squamous cell carcinoma and one with little unspecific inflammatory reaction. Two cases of freshly operated human lung samples that were infected in tissue culture with *M. tuberculosis* BCG similarly as recently described for *Chlamydia pneumoniae* [37] acted as positive controls. Furthermore, lungs of *M. tuberculosis*-infected mice adjusted to defined colony forming units (0.7–0.8 CFU/lung) served as positive controls. These specimens originated from the Max Planck Institute for Infection Biology, Berlin [41]. Two positive and one negative control was submitted to HOPE (Hepes Glutamic Acid Buffer Mediated Organic Solvent Protection Effect) fixation instead of formalin followed by paraffin embedding [42].

For the 32 human tissues, one tube with 5 to 10 paraffin slices of 3-μm thickness as well as one tube containing 30–50 μl of DNA stock solution were assessed. For the two experimental mouse specimens, tubes with five paraffin slices of 3-μm thickness were sent to the panel laboratories. A survey of the tested specimens is provided in Table 5.

### Histological evaluation

The paraffin blocks used for PCR analysis were histologically reassessed. Tissue sections were stained by hematoxylin and eosin (H&E), ZN, auramine–rhodamine and immunohistochemistry (DAKO, rabbit anti-*Mycobacteri-*

*um bovis* BCG), and semi-quantitatively evaluated in the following way:

- Necrosis. Extent: 0, negative; 1, minimal/punctuate; 2, low; 3, medium/confluent; 4, strong/greater than 50%. Type (predominant): 0, negative; 1, mainly caseous; 2, cellular; 3, fibrinoid
- Perinecrotic reaction. Intensity of inflammation: 0, negative; 1, little; 2, medium; 3, strong. Fibrosis: 0, negative; 1, little/focal granulation; 2, medium; 3, strong/circular sclerosis
- Granulomas. Each of the three granuloma types (caseous, pseudotuberculoid and sarcoidosis-like) were assessed as 0, absent; 1, present; 2, abundant
- Giant cells. Samples were searched for Langhans type or other (generally foreign body) types of giant cells and scored as 0, absent; 1, present; 2, abundant
- General inflammation intensity: 0, none; 1, little; 2, medium; 3, strong
- ZN, auramine and the immunohistochemical stainings were each scored as 0, negative; 1, weakly; 2, medium; 3, strongly positive

The histological parameters were statistically correlated with the PCR results using the SPSS software package (version 11.5). The detailed histological evaluation and representative microscopic images of each case are available as supplementary data at the abovementioned Charité web site.

## Preparation of DNA from paraffin-embedded tissue

A survey on the experimental strategy is shown in Fig. 1. The methods for tissue preparation and DNA extraction differed slightly and are specified in Table 1.

In general, sections were cut from each tissue block and collected in 1.5 ml of Eppendorf vials. After each block, the microtome was cleaned and the knife changed. Paraffin was removed by extraction with xylene and centrifugation. This procedure was carried out either once, twice or three times; the pellet was then washed one to three times with ethanol (see Table 1). The supernatant was removed and the pellet air-dried. In parallel, blank extraction controls (dummies) containing no mycobacterial material were processed. These controls consisted of extractions containing all of the reagents without tissue and extractions of tissues ascertained to be negative for mycobacterial DNA.

All panel laboratories applied proteinase K digestion to disrupt the tissue, but the procedures to obtain the mycobacterial DNA as well as extraction and enrichment methods differed. Details of the respective protocols of the four laboratories are specified in the following.

### *Laboratory A*

Air-dried tissue pellets were processed using the QIAamp DNA Mini tissue kit (http://www.qiagen.com/). Briefly, the pellets were suspended in lysis buffer and heated for 15–20 min at 95°C. After cooling to room temperature, the following steps were carried out according to the manufacturer's instructions with the exception that elution of DNA was performed with 50–80 µl of buffer.

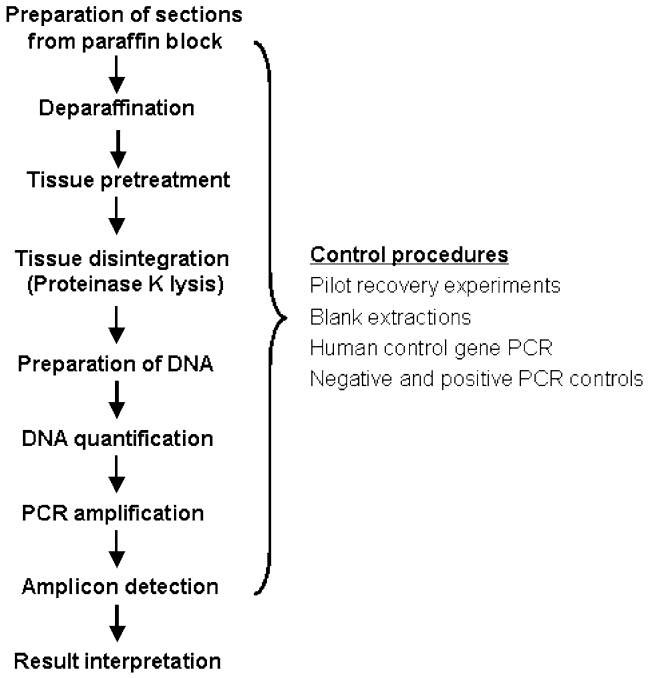


**Fig. 1** Experimental steps in PCR-based detection of *M. tuberculosis* from paraffin-embedded tissue

### *Laboratory B*

DNA preparation was performed according to the protocol by Ghossein et al. [12] and Popper et al. [31]. For digestion, the pellet was suspended in 100 µl of TEN-buffer (0.04 M Tris–HCl, 0.2 M NaCl, pH 8.0, 1 mM EDTA), 5 µl of proteinase K (Merck, Darmstadt, Germany, 20 mg/ml) and 5.25 µl of sodium dodecyl sulphate [final concentration 1% (w/w)] at 55°C for 3–4 h. To inactivate proteinase K, the solution was boiled for 10 min and centrifuged, and the DNA was precipitated with 96% ethanol (two volumes) in the presence of NaCl (final concentration 0.2 M) at room temperature for 30 min, recentrifuged at 13,000 rpm for 30 min and dissolved in 30–50 µl of ultrapure water (HPLC grade, Merck).

### *Laboratory C*

DNA was extracted by the use of the Gen-ial First-DNA Kit (Fa. Gen-ial, Troisdorf, Germany, http://www.gen-ial.de/). Briefly, after removal of xylene, 500 µl of lysis buffer 1, 50 µl of lysis buffer 2 and 10 µl of proteinase K were added and incubated at 37°C overnight. To get access to mycobacterial DNA, the solution was heated to 90°C for 4 min and then shock-frozen in liquid nitrogen five times. After this step, 375 µl of lysis buffer 3 were added, vortexed and

**Table 1** Methodological variants for tissue preparation and DNA extraction

| Laboratory[a] | Sections | Deparaffinization | Tissue pre-treatment | Proteinase K lysis | Preparation of DNA | DNA amount[b] (µl, ~µg/PCR) | PCR volume (µl) |
|---|---|---|---|---|---|---|---|
| A | 5–10 (3 µm) | 1×xylene, 1×ethanol, air drying | Heating in lysis buffer for 15–20 min at 95°C | 2–3 h at 55°C | QIAamp DNA Mini Kit | 1–5 µl, 0.2–0.5 µg | 25 |
| B | 5 (10 µm) | 3×xylene, 3×ethanol, air drying | Nothing | 3–4 h at 55°C | Precipitation with 96% ethanol | 1–10 µl | 50 |
| C | 2–5 (5 µm) | 1×xylene | 5×heating to 90°C followed by shock freezing with liquid nitrogen after proteolysis | at 37°C over-night | Gen-ial First-DNA Kit, precipitation with 96% ethanol | 1–3 µl according to intensity after agarose (2%) gel electrophoresis | 20 |
| D | 5 (2 µm) | 2×xylene, 2×ethanol, air drying | Freeze-thawing before and after proteolysis | 16 h at 55°C | QIAamp DNA Micro Kit | 1–10 µl, 0.4 µg | 50 |

[a]Laboratories: A, Berlin; B, Dresden; C, Munich; D, Borstel
[b]Extraction volume/DNA mass per reaction

cooled down for 5 min at −20°C. The samples were centrifuged at 13,000 rpm for 20 min, and the supernatant was transferred into new sterile tubes. At last, the DNA was precipitated with ethanol, and the pellet was diluted with sterile $H_2O$ to a final volume of 50 µl.

*Laboratory D*

Extraction of DNA was performed by the use of QIAamp DNA Micro Kit (QIAGEN) with the following modifications: Two temperature treatments were included before and after the proteinase K incubation step (16 h at 55°C). Each of these treatments consisted of three cycles of 2-min incubation of the samples at 37°C, 30-s vortexing and 2-min incubation at −80°C in a dry ice/ethanol bath.

## DNA quantification

To standardise the conditions and to estimate the amount of extracted DNA, the concentration of DNA was measured spectrophotometrically (laboratories A, B and D). In addition, the OD ratio (260 nm/280 nm) was measured to obtain information about the purity of the template DNA (laboratories B and D). The integrity and the quantity of the DNA was assessed semi-quantitatively via gel electrophoresis by laboratory C. DNA amounts and PCR reaction volumes of each laboratory are specified in Table 1. To examine whether amplifiable DNA was present in the samples, all panel laboratories assessed the quality of the prepared DNA by different control PCR to amplify an appropriate segment of a human gene (Table 2).

## PCR amplification and amplicon detection

PCR of mycobacterial DNA sequences was performed employing different primer sequences for the amplification of distinct target genes of *M. tuberculosis* (Table 3). The genus-specific 16*S* rDNA and 65-kDa antigen genes harbour consensus sequences of mycobacterial organisms and thus cover the broadest detection range. The IS*6110* gene from the *M. tuberculosis* complex is suitable to detect

**Table 2** Human control genes

| Laboratory | Gene | Direction | Sequence 5′→3′ | Amplicon (bp) | Reference |
|---|---|---|---|---|---|
| A | β-Globin | sense | GAA GAG CCA AGG ACA GGT AC | 268 | [15] |
| | | antisense | CAA CTT CAT CCA CGT TCA CC | | [5] |
| B | GAPDH | sense | CGGAGTCAACGGATTTGGTCGTAT | 306 | [46] |
| | | antisense | AGCCTTCTCCATGGTGGTGAAGAC | | |
| C | β-Actin | sense | CCT TCC TGG GCA TGG AGT CCT G | 202 | [12] |
| | | antisense | GGA GCA ATG ATC TTG ATC TTC | | |
| D | β-Actin | sense | AGC GGG AAA TCG TGC GTG | 303 | [33, 34] |
| | | antisense | CAG GGT ACA TGG TGG TGC | | |

**Table 3** Set of primers to detect mycobacterial DNA sequences

| Laboratory | Target (Method No.)[a] | Direction (primer names) | Sequence 5′→3′ | Amplicon (bp) | Gene | Detected *Mycobacterium* subtypes | Reference |
|---|---|---|---|---|---|---|---|
| B, D | *M. tub* complex (1) | sense (A)<br>antisense (tb) | AGA GTT TGA TCC TGG CTC AG<br>ACC ACA AGA CAT GCA TCC CG | 193 | 16S rDNA | *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti*, *M. africanum*) | [34] |
| A–C, A | *M. tub* complex (2) | sense<br>antisense<br>probe | CCT GCG AGC GTA GGC GTC GG<br>CTC GTC CAG CGC CGC TTC GG<br>Biotin-CAT AGG TGA GGT CTG CTA CC′ | 123 | IS*6110* | *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. bovis BCG*, *M. microti*, *M. africanum*) | [8, 45] |
| D | *M.* genus | sense (A)<br>antisense (247)<br>primer (9) | AGA GTT TGA TCC TGG CTC AG<br>TTT CAC GAA CAA CGC GAC AA<br>CGT GCT TAA CAC ATG CAA GTC | 590 | 16S rDNA | Variety of bacteria, conserved region within the mycobacterial species, sequencing of atypical mycobacteria | [1, 34, 33] |
| A | *M.* genus (3) | sense<br>antisense | CTA GGT CGG GAC GGT GAG GCC AGG<br>CAT TGC GAA GTG ATT CCT CCG GAT | 125–165 | 65 kDa Ag | Variety of mycobacteria, conserved region of mycobacteria | [30] |
| A | *M.* genus (nested PCR) (4) | T1U1, T1U2, T1D<br><br>T2U<br>T2D | First round: AAG GAG ATC GAG CTG GAG GA, AGG CGT TGG TTC GCG AGG G TGA, TGA CGC CCT CGT TGC C<br><br>Second round with internal primers: GTC TCA AAC GCG GCA TCG, GTC ACC GAT GGA CTG GTC | 310, 231<br><br>133 | 65 kDa Ag | Variety of mycobacteria, conserved region of mycobacteria | [4] |

[a]Method numbering corresponds to that of Tables 5 and 6

*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti*.

The PCR protocols, i.e. reaction conditions, cycle programmes and detection method also differed and are compiled in Tables 1 and 4. Further details are as follows.

### *Laboratory A*

For detection of *M. tuberculosis*, the oligonucleotide primers described by Eisenach et al. [8] were used to amplify a 123-bp fragment of the IS*6110* insertion sequence. The PCR reagents were commercially available (Reddy Mix, *AB* gene, http://www.abgene.com/). The final reaction volume of 25 µl contained 75 mM Tris–HCl (pH 8.8 at 25°C), 20 mM ammonium sulphate, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide trisphosphate, 0.01% (v/v) Tween 20, 1.25 units Thermoprime DNA polymerase, as well as loading buffer and dyes for electrophoresis. The twofold concentrated kit reagents were complemented by a PCR DIG labelling mix (DIG detection, Roche, http://www.roche.com/), with a final concentration of 120 µM to render possible PCR-ELISA detection. The primer concentration was adjusted to 0.1 µM. The cycle conditions are specified in Table 4. A 10-µl aliquot of the reaction mix was subjected to 3.3% agarose gel electrophoresis and ethidium bromide staining. A second 2.5-µl aliquot containing the digoxigenin-labelled PCR products was hybridised with the biotinylated probe (Table 3), immobilised onto a streptavidin-coated microtiter plate and detected as described in the instruction manual (Roche, see above).

The amplification of the consensus 65-kDa antigen gene was similarly performed by the use of Reddy Mix (*AB* gene) without labelling using a 0.125-µM primer concentration.

The nested PCR was performed according to Cook et al. [4] by the use of PCR Master Mix (Promega, http://www.promega.com/) without restriction enzyme analysis of PCR product. One microliter of the first round product was amplified in the second PCR. Again, the PCR products were detected by 3.3% agarose gel electrophoresis.

**Table 4** PCR conditions and amplicon detection

| Laboratory | PCR reaction mixtures | Final Concentrations | Thermocycler profile | Detection procedure |
|---|---|---|---|---|
| A | Reddy Mix: Thermoprime DNA polymerase (*AB* gene) PCR, Dig-labelling mix (Roche) | 0.65 units enzyme, 1.5 mM $MgCl_2$, 0.1 µM primer, 0.2 mM dNTPs, 120 µM dNTPs and 6 µM DIG-dUTP | I*6110*: 94°C 5 min, 40× (94°C 1 min, 68°C 2 min, 72°C 1.5 min), 72°C 7 min | *DIG PCR/ELISA*: 2,5 µl digoxigenin labelled PCR products were hybridised with biotinylated probe, immobilised and detected on microtiter plates (Roche) |
| | Reddy Mix: Thermoprime DNA polymerase (*AB* gene) | 0.65 units enzyme, 1.5 mM $MgCl_2$, 0.125 µM primer, 0.2 mM dNTPs | 65 kDa Ag: 94°C 5 min, 40× (94°C 1 min, 60°C 1 min, 72°C 1 min), 72°C 5 min | Electrophoresis: 3.3% agarose gel, ethidium bromide staining and UV visualisation |
| | Promega PCR Master Mix (2×) | 0.65 units enzyme, 1.5 mM $MgCl_2$, 0.3 µM primer, 0.2 mM dNTPs | Nested PCR 65 kDa Ag: 94°C 5 min, 40× [94°C 1 min, 57°C 1 min (first round), 52°C (second round), 72°C 1 min], 72°C 5 min | |
| B | Ampli Taq Gold (Perkin Elmer Applied Biosystems) | 1 unit enzyme, 1.5 mM $MgCl_2$, 0.1 µM primer, 0.2 mM dNTPs | IS*6110*: 94°C 9 min, 35× (94°C 1 min, 68°C 1 min, 72°C 1 min), 72°C 7 min | Electrophoresis: 6% polyacrylamide gel |
| | Ampli Taq Gold (Perkin Elmer Applied Biosystems) | 1 unit enzyme, 1.5 mM $MgCl_2$, 0.1 µM primer, 0.2 mM dNTPs | M. tub. 16S rDNA: 94°C 9 min, 40× (94°C 1 min, 57°C 1 min, 72°C 1.5 min), 72°C 15 min | |
| C | Ampli Taq Gold (Perkin Elmer Applied Biosystems) Nucleotides (Amersham Pharmacia) | 0.025 units enzyme, 1.5 mM $MgCl_2$, 1 µM primer, 0.2 mM dNTPs | IS*6110*: 95 °C 10 min, 45× (94 °C 1 min, 66°C 1 min, 72°C 1 min) 72°C 8 min. | Electrophoresis: 4% polyacrylamide gel |
| D | Invitrogen | 5 units enzyme, 5 mM $MgCl_2$, 0.8 µM primer, 0.2 mM dNTPs | M. tub. 16S rDNA: 94°C 5 min, 40× (94°C 1 min, 57°C 1 min, 72°C 1,5 min) 72°C 15 min | Electrophoresis: 2% agarose-gels stained with ethidium-bromide and UV visualisation. Sequencing: according (Richter 1995) |

*Laboratory B*

For detection of *M. tuberculosis*, the IS*6110* and the 16*S* rDNA genes were amplified. All reactions were throughout performed in a volume of 50 µl using Ampli Taq Gold DNA polymerase (1U PE Applied Biosystems, http://www.appliedbiosystems.com/) and reagents according to the manufacturer's instructions. Further details are given in Tables 3 and 4. PCR products were run on 6% polyacrylamide gel and visualised by staining with SYBR Green I (Biozym FMC) and UV transillumination.

*Laboratory C*

Similar to laboratories A and B, the detection of *M. tuberculosis* complex DNA was performed by amplification of the IS*6110* sequence. The cycler profile is given in Table 4. The detection was performed by gel electrophoresis after cleaving the PCR product by a specific restriction endonuclease digestion with *Hae*III (Roche Diagnostics, Mannheim, Germany, http://www.roche.com/) [2]. The PCR products were separated on a 4% agarose gel and visualised on a UV screen after staining with ethidium bromide. All samples with positive amplification of IS*6110* were subjected to further analysis by spoligotyping. The method was performed as described previously [22, 32, 44]. We used a commercial Spoligotyping Kit (Isogen, Maarsen, The Netherlands, http://www.isogen.nl/). Due to differences in the presence of non-repetitive DNA spacers, the hybridisation patterns vary among *M. tuberculosis* complex strains. Due to the absence of spacers 39 to 43 in all known *M. bovis* isolates, this approach allows a clear differentiation between *M. tuberculosis* and *M. bovis* strains, as well as the identification of sub-species or defined genetically related families.

*Laboratory D*

Amplifications were performed by the use of oligonucleotide primer sets for a 590-bp genus-specific fragment of mycobacterial 16*S* rDNA and a 193-bp fragment of the 16*S* rDNA of *M. tuberculosis* complex, respectively (Table 3), as described previously [34]. For amplification, 400 ng DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM

$MgCl_2$, 200 μM of each deoxynucleotide trisphosphate (Invitrogen), 5 units Taq-Polymerase (Invitrogen) and 0.8 μM of each primer were added to a final volume of 50 μl. PCR was performed in a Biometra Uno-II thermocycler with cycle conditions as specified in Table 4. The amplicons were detected by 2% agarose gel electrophoresis.

When the outcome of the 193 bp PCR was negative and the 590 bp PCR was positive, the long PCR products were subjected to sequencing by the use of primer 9 as previously described by Richter et al. [34]. Sequence analysis was carried out using an automated fluorescent DNA-sequencer (AbiPrism 377, Applied Biosystems) to determine the respective (atypical) mycobacterial species. Database comparison was done with the aid of GenBank at the NCBI applying the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). Because this analysis was performed only in single selected cases, it was not included in the sensitivity and specificity analysis.

### Control procedures

Two different types of negative controls were included in the study to rule out contaminations: In the first one, sterilised water was used instead of the sample to check the PCR. In the second one, material from 'blank extractions' (dummies, see above) was used in order to exclude any contamination in the extraction system [14]. To examine the reliability of both reaction mixtures and reaction conditions, each analysis was combined with a positive control of mycobacterial DNA obtained from highly diluted suspensions of *M. tuberculosis* DNA or *M. szulgai* (genus PCR, laboratory D).

## Results

Of the 34 cases, 25 were clinical samples that were previously investigated by one of the panel laboratories for tuberculosis because of clinical or histopathological suspicion for infection. In addition, five negative human control samples and four positive, two human and two mice, tissues were included (Table 5).

### Amplification of control genes

DNA stock solutions ($n$=32) and paraffin sections ($n$=34) were sent to the four panel laboratories for PCR, thus, totalling to 264 control reactions. Only a small share of them did not supply the expected results. Thirteen were negative and six displayed only a weakly positive outcome. A higher share of negativity occurred when large target sequences were amplified (16/19, laboratories B, D) compared to small target sequences (3/19, laboratories A, C). A somewhat higher abundance of negative outcomes was observed with the DNA stock solutions (8× negative, 3× weakly positive) compared to DNA that was extracted from the paraffin sections by each laboratory (5× negative, 3× weakly positive).

### PCR detection of *M. tuberculosis*

The results for PCR detection of *M. tuberculosis* genes are shown in detail in Table 5. It represents the analysis of each case using DNA solutions and DNA from paraffin sections by all four laboratories using five different detection methods. Each laboratory provided a definite final assignment (positive or negative) of the 34 specimens that was based on the investigation of the different DNA preparations and PCR approaches for detection of mycobacterial DNA (column 'laboratory score', 0 = negative, 1 = positive). These were added for the calculation of a total score ranging from 0 to 4.

For 29 out of 34 sample sets, all four laboratories achieved identical assessments. The specimens from mouse tissues with defined CFU (samples 33 and 34) were evaluated consistently positive. From these samples, it could be calculated that 5–10 copies of the bacterium per set-up were reliably detected.

In five cases, conflicting data were obtained by one of the four laboratories, i.e. three aberrant positive results (samples 7, 21, 31) and two aberrant negative ones (samples 12 and 28). Case 7 was positive with the paraffin sample, but not with the DNA solution, which was interpreted as final positive result by the panel laboratory C. Both sample types also gave a positive result in the nested PCR of laboratory A; however, it was finally interpreted as negative because the IS*6110 M. tub*. complex PCR was negative. Similarly, case 21 gave positive results in the IS*6110* complex PCR and spoligotyping analysis in both sample types. This case was consistently negative in all analyses of the other laboratories. The third case (31) was positive in the DNA preparation from the paraffin sections, but not in the conveyed solution in laboratory D. In the same case, laboratory B detected mycobacterial DNA in the IS*6110* region, which, however, was not confirmed by the 16*S* rDNA analysis (A/tb primers) leading to a final negative evaluation. In one of two cases that were aberrantly evaluated as negative (case 28), amplification of the 193 bp sequence of 16SrDNA yielded no amplicon; however, the PCR for IS*6110* was positive. In the second case (sample 12), an amplicon of wrong size was seen with the A/tb primers thus leading to the negative final interpretation.

Three aberrant results, two aberrant positive and one aberrant negative, were obtained in the two laboratories that used freeze-thawing for DNA extraction as compared to one aberrant negative result in the two laboratories that did not employ this technique.

The results of the majority of the panel laboratories were defined as PCR consensus and served for the comparison of the applied methodologies regarding sensitivity and specificity (Table 6). The highest sensitivity was achieved for the amplification of the IS*6110* gene followed by the

**Table 5** Results for PCR detection of *M. tuberculosis* genes

| Sample | | | Laboratory | A | | | | | | B | | | | | C | | | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Method No. | (2) | | (4) | | (3) | Laboratory score | (2) | | (1) | | Laboratory score | (2) | | Spoligotyping (5) | | Laboratory score | (1) | | Laboratory score |
| Code | Localization | Type | Total score | DNA | P | DNA | P | P | | DNA | P | DNA | P | | DNA | P | DNA | P | | DNA | P | |
| 1 | Lung | Clinical | 4 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 2 | Aorta | Clinical | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| 3 | Aorta/Lung | Clinical | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 4 | Liver | Clinical | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | n.a. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Lymph node | Clinical | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | Lymph node | Clinical | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 7 | Lymph node | Clinical | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| 8 | Foetal lung | n.c. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | Lung | Clinical | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 10 | Lung/Lymph node | Clinical | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 11 | Foetal lung | n.c. | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 12 | Lymph node | Clinical | 3 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| 13 | Lung | Clinical | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 14 | Lung/Lymph node | Clinical | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 15 | Foetal lung | n.c. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Lung | Clinical | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | Lung | p.c. | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 18 | Lung | p.c. | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 19 | Prostate | Clinical | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | Lung | n.c. | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 21 | Lung | Clinical | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| 22 | Lung | n.c. | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | Pleura/diaphragma | Clinical | 4 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| 24 | Diaphragma | Clinical | 4 | 1 | 1 | n.a. | n.a. | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| 25 | Vertebra | Clinical | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 26 | Soft tissue/serous cyst | Clinical | 4 | 1 | 1 | n.a. | n.a. | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| 27 | Lung | Clinical | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 28 | Epididymis | Clinical | 3 | 1 | 1 | n.a. | n.a. | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| 29 | Prostate | Clinical | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 5** (continued)

| Sample | | | Laboratory | A | | | | | | B | | | | C | | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Method No. | (2) | | (4) | | | (3) Laboratory score | (2) | | (1) | | Laboratory score | (2) | | (5) | | (1) | | Laboratory score |
| Code | Localization | Type | Total score | DNA | P | DNA | P | P | | DNA | P | DNA | P | | DNA | P | DNA | P | DNA | P | |
| 30 | Pleura | Clinical | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | n.a. | n.a. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31 | Lung | Clinical | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 32 | Epididymis | Clinical | 4 | 1 | 1 | n.a. | n.a. | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 |
| 33[a] | Mouse lung | p.c. | 4 | n.a.[a] | 1 | n.a.[a] | n.a. | 1 | 1 | n.a.[a] | 1 | n.a.[a] | 1 | 1 | n.a.[a] | 1 | n.a.[a] | | n.a.[a] | 1 | 1 |
| 34[a] | Mouse lung | p.c. | 4 | n.a.[a] | 1 | n.a.[a] | n.a. | 1 | 1 | n.a.[a] | 1 | n.a.[a] | 1 | 1 | n.a.[a] | 1 | n.a.[a] | | n.a.[a] | 1 | 1 |

Method numbering (see also Table 3 and Table 6): (1), 16SrDNA, A/tb primer; (2), IS6110/M. tub. complex; (3), 65 kDa Ag/*M.* genus; (4), 65 kDa Ag/nested PCR
*p.c.* Positive control, *n.c.* negative control, *DNA* results for DNA solutions, *P* results for paraffin sections, *n.a.* not analysed
[a]Only paraffin slices were shipped

16*S* rDNA of *M. tuberculosis* complex, whereas regarding specificity, the latter was superior to the IS*6110* detection.

### Correlation with microscopic findings

During the course of this study, all samples were microscopically reassessed and semi-quantitatively scored for the parameters necrosis, granuloma, giant cells, inflammation/fibrosis and acid-fast bacilli. The data were statistically correlated with the PCR consensus results. The extent of necrosis was highly significantly associated with the positive PCR result ($P$=0.006 by Fisher's exact test). Furthermore, there was also a positive correlation with immunohistochemical detection of acid-fast bacilli ($P$=0.027). For the other parameters, there were no statistically significant correlations. Details of the histological evaluation together with representative histological images of each case are available as supplementary data at http://www.charite.de/ch/patho under the rubric "Forschung".

## Discussion

Detection of pathogen-specific DNA or RNA by using methodology of molecular pathology is implicated in diagnostics and characterisation of infections. This task includes the choice of the suitable methodological approach, its correct handling by an expert and experienced staff as well as critical interpretation of results and their impact in the final diagnosis.

PCR is a highly sensitive procedure, which is extremely susceptible to interference by contamination [23, 36]. Moreover, particular requirements are needed as to disintegration of sample material and quality of nucleic acid preparation. Therefore, a number of preconditions should be fulfilled to prevent both false-positive and false-negative results. Some essential general preconditions for PCR analysis are provided as supplementary data to the manuscript (http://www.charite.de/ch/patho, rubric Forschung).

The present inter-laboratory trial indicated a high degree of inter-laboratory reliability of the PCR-based detection of *M. tuberculosis* genes. Importantly, all positive and negative controls were evaluated consistently. Positive controls originated from experimentally *M. tuberculosis*-infected mice and human lung tissue cultured in the presence of *M. bovis* BCG. Negative samples were from foetal and adult human lung that had no clinical or histological evidence of infection and were previously PCR negative. The majority of the samples consisted of clinical material that harboured characteristic morphological signs suggestive for mycobacterial infection, i.e. caseous necrosis, granulomas and/or giant cells. Interestingly, the extent of necrosis carried the most significant association with the detection of *M. tuberculosis* DNA. In contrast, neither the presence and type of giant cells, granuloma type, nor the staining results for ZN and auramine showed a significant correlation. The insufficient association with the conventional stainings is probably due to

**Table 6** Sensitivity and specificity of the *M. tuberculosis* detection methods

| No. | Method | Sensitivity[a] | | | Specificity[b] | | |
|---|---|---|---|---|---|---|---|
| | | All | *P* | DNA | All | *P* | DNA |
| 1 | *M. tub.* complex (16S rDNA) | 0.87, *n*=76 | 0.91, *n*=40 | 0.84, *n*=36 | 0.95, *n*=39 | 0.95, *n*=20 | 0.95, *n*=19 |
| 2 | *M. tub.* complex (IS*6110*) | 0.95, *n*=114 | 0.94, *n*=60 | 0.96, *n*=54 | 0.88, *n*=84 | 0.89, *n*=42 | 0.86, *n*=42 |
| 3 | *M.* genus | | 0.83, *n*=20 | | | 1.0, *n*=14 | |
| 4 | *M.* genus nested | 0.92, *n*=12 | | | 0.84, *n*=26 | | |
| 5 | Spoligotyping | 0.88, *n*=38 | 0.83, *n*=20 | 0.95, *n*=18 | 0.82, *n*=28 | 0.88, *n*=14 | 0.78, *n*=14 |

Samples with score 0 or 1 are defined as true negative, samples with score 3 and 4 are defined as true positive. This agreement is important for the estimation of sensitivity and specificity

[1–4]Method numbering corresponds to that of Table 3

[a]Sensitivity [true positive results/(true positive plus false negative results)]

[b]Specificity [true negative results/(true negative plus false positive results)]

the preselection of cases, because generally, only the ZN-negative cases are further analysed by PCR. Auramine revealed more positive cases that, however, by PCR, turned out to be related to unspecific staining. Only immunohistochemical detection correlated significantly with the PCR results and may thus increase the armamentarium of morphology-based methods.

Except for the positive controls, no microbiological data were available. Although this may be a limitation of our study, it is representative for the inherent problem of tuberculosis detection in pathology specimens. On the one hand, clinicians and surgeons do not always pay attention to the possibility of an infection, and therefore, do not send adequate material to the microbiology department. On the other hand, the suspicion of such an infection is frequently raised only by histomorphological analysis. Thus, the available specimen is already formalin-fixed and cannot be further analysed microbiologically.

The molecular pathological analysis needs to be considered in the context of the histopathological and clinical setting. On the one hand, it may strongly enhance the sensitivity and considerably shorten the time period for achieving the specific diagnosis of an infection. On the other hand, the PCR methodology itself with all the pre-amplification, amplification and detection procedures implicates multiple obstacles that may hinder the detection of the pathogen unless appropriate conditions are maintained.

## Sectioning of paraffin blocks

Paraffin blocks, usually generated after formalin fixation of the tissue, are the fundamental working material in pathological institutes. These blocks need to be cut to gain paraffin sections that are suitable for DNA extraction. To our knowledge, there are so far no general rules for tissue sectioning in molecular pathology analysis.

The panel laboratories took the following measures for paraffin block sectioning:

- Cleaning of the microtome after each tissue block from unused paraffin material by aspirator
- Cleaning of knives after each block to remove paraffin and tissue remnants
- Change of knives between each block (in particular if the same microorganism is detected)
- Precautions for contaminations during transfer of tissue sections into the tubes

Within the panel laboratories, the section thickness varied between 2 and 10 μm, the number of sections was between 2 and 10, thus giving a range of tissue thickness between 10 and 50 μm. This variation is mainly due to the area of tissue within the paraffin block and its cellular composition. The optimal amount depends largely on the experience of each laboratory and should be tested. Interestingly, one report recently described a higher sensitivity for the detection of mycobacterial DNA using thin tissue sections [25].

For this investigation, the sections were provided by one laboratory for the in-house preparations by each panel member (P). The additionally distributed DNA preparation (DNA) originated from all four laboratories that contributed DNA solutions and paraffin blocks.

## DNA extraction

The use of formalin-fixed and paraffin-embedded tissue is hampered by a high degree of cross-linking, which renders its disintegration difficult. This difficulty is reinforced by the presence of a thick mycolate-rich outer cell wall in mycobacteria. Therefore, additional and even drastic methods of cell rupture were applied by the laboratories. The aim is to keep a balance between DNA recovery from the bacteria and tissue and DNA integrity for later amplification. Although the influence of the DNA extraction method was not systematically tested, our experience suggests that tissue pretreatment enhances the amount of recovered DNA whereas higher number of cycles in tissue pretreatment does not necessarily increase the sensitivity of detection. It was observed in the present study that heating prior to proteinase K digestion promotes tissue disintegration and may thus increase DNA recovery. Additional

cycles of freeze thawing were applied by two laboratories and resulted in two additional (aberrant) positive results. Whether this reflects contamination or an increase in sensitivity needs to be clarified by future studies. The optimal conditions should be examined in pilot recovery experiments by each laboratory independently.

## DNA quantification

The variation of amount and composition of a tissue will greatly influence DNA recovery and purity. Differences in the preparation procedures like extraction volume, addition of proteinase K, duration of digestion, procedures for DNA concentration and others may also contribute to this variation. In daily practice, this variability is firstly accounted for by the tissue amount entering the extraction procedure and secondly by measurement and standardisation of the DNA amount for PCR. In addition to quantification, photometric measurement of the OD ratio at 260/280 nm may also provide an indication for sufficient DNA purity and is thus highly recommended.

Each laboratory should find out the optimal DNA concentration for its PCR set-up. The few discrepancies of the PCR results between the P and DNA preparations within the panel laboratories may be attributed to differences in DNA preparation and partitioning for amplification. In our experience, amounts of 100 ng for the control gene and up to 400 ng for the detection of mycobacteria, respectively, yielded best results.

## PCR amplification

Formalin fixation results in cross-linking and degradation of the DNA, thus, limiting its use as template for amplification. The impact of the fragment size for the detection of the pathogen and the control gene was clearly evident from our inter-laboratory trial. Small target sequences should be applied in order to minimise the risk of false-negative results. We observed the highest frequency of positive control gene amplification for β-globin (268 bp fragment, 1 dropout) followed by β-actin (202 bp fragment, 2 dropouts).

Similarly, the 123-bp fragment of the IS*6110* gene of *M. tuberculosis* complex showed the highest sensitivity, which is additionally owed to the fact that it constitutes a repetitive genomic sequence of the bacterium. To achieve high yields of PCR products, cycle numbers between 35 and 45 as well as prolonged reaction times between 60 and 90 s were applied.

Nested PCR being additionally performed by one laboratory for amplification of the 65-kDa antigen harbours the potential of an even higher sensitivity. However, in our experience, this method should only be applied in the context of further tests due to the relatively low specificity (Table 6).

Recently, it has been shown that application of the novel *HOPE* fixation boosts the sensitivity of PCR to detect mycobacteria in paraffin-embedded samples at least 100-fold along with a well-preserved morphology [42]. The HOPE-fixed samples (code no. 17, 18, 20), including two positive and one negative control, gave highly reproducible results. The morphology was similar to the conventionally fixed specimens and can be checked on the supplementary data web site. Formalin, however, is and probably will remain for some time the standard fixative in most hospitals and pathological institutes, thus requiring accordingly optimised procedures in molecular pathology.

## Amplicon detection

The most common and convenient way to confirm a successful amplification is agarose or polyacrylamide gel electrophoresis. However, an amplicon of the correct size does not necessarily represent the specific target. Therefore, further confirmative analysis is recommended. This is one reason why all panel laboratories applied different primer sets for PCR to detect mycobacterial DNA. For additional confirmation, restriction enzyme digestion, hybridisation to a specific internal probe followed by ELISA detection, DNA sequencing and spoligotyping were performed. Hybridisation techniques and restriction enzyme digestion are easier and faster than DNA sequencing followed by database comparison. Therefore, the latter approach is often restricted to the detection and subtyping of atypical mycobacteria.

Extremely high sensitivity can be achieved by the spoligotyping technique [22]. In the present study, as compared to conventional PCR, the typing was successful, providing additional information on distinct strains of the *M. tuberculosis* complex. Most of the samples exhibited a *M. tuberculosis*-specific signature. In two lung samples, *M. bovis* BCG strains were detected. A number of the *M. tuberculosis* strains revealed spoligotyping patterns, which are listed in an international database [11] and resemble common and well-characterised signatures. However, two samples provided aberrant positive spoligotyping results, which were found out by PCR to be negative as to the presence of *M. tuberculosis* DNA. Interestingly, these cases yielded only incomplete, patchy spoligotyping patterns. Further studies are needed to clarify whether these findings are due to non-specific amplification or hybridisation products or whether they indicate an extremely low level of *M. tuberculosis* DNA, whose clinical relevance has to be established. At present, this approach is not mandatory and generally recommended for diagnostic purposes, but may be used to address alternative issues, such as evolution and spread of the *M. tuberculosis* complex.

## Interpretation of PCR results

The final plus/minus assignment of the PCR results for *M. tuberculosis* genes (Table 5, laboratory score) stands for a summing up of all analyses that were performed by each laboratory. Similar to the amplification of the control genes,

there were also discrepancies in single cases between the multiple approaches for *M. tuberculosis* detection (DNA solution vs new extractions from paraffin section, different PCR methods). The specifications in Table 5 may thus reflect complex considerations. The criteria that were applied by the laboratories will be discussed in the following:

Generally, a case was scored positive when the PCR and the following detection yielded a definite outcome in one of the preparations. As an exception of this rule, laboratory B required two positive parameters, i.e. either positive outcome in the PCR for IS*6110* and A/tb or positive outcome in both DNA preparations (D+P) for a definitive assessment. The backgrounds for these more stringent criteria are reports from the literature indicating that the use of the primer set for IS*6110* by Eisenach et al. [8] may identify *M. tuberculosis* in cases of sarcoidosis [6, 10, 21, 38]. Thus, the positive detection of the A/tb was scored with a higher priority than the IS*6110* fragments. From the experience of the other panel laboratories, positive outcome for IS*6110* in cases of sarcoidosis is not or only occasionally observed. However, this phenomenon was not systematically investigated in this inter-laboratory trial and merits further investigation. In general, this issue highlights the need to apply multiple approaches for the detection of mycobacterial DNA, in particular, in case of suspicion of sarcoidosis.

Similarly, the nested PCR performed by laboratory A was scored only when both preparations showed equal results in repetitive analyses, thus, considering the high frequency of false-positive results.

Discrepancies within and between the laboratories can also be due to low-copy infection and unequal distribution of the mycobacteria in the tissue. Considering the multiple difficulties in the *M. tuberculosis* detection as mentioned here, we achieved a high rate of consistent results.

## Conclusions

The present inter-laboratory trial has clearly demonstrated reliability and reproducibility of PCR-based detection of *M. tuberculosis*, provided that appropriate conditions are kept. Moreover, the prerequisites are now established for a subsequent external inter-laboratory trial in which other molecular pathology laboratories are invited to participate.

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