APMIS 113: 586–93, 2005
Printed in Denmark . All rights reserved

APMIS
ISSN 0903-4641

Improved diagnosis of mycobacterial infections in formalin-fixed and paraffin-embedded sections with nested polymerase chain reaction

ANDREY G. AZOV,[1,2] JØRN KOCH[1] and STEPHEN J. HAMILTON-DUTOIT[1]

[1]Institute of Pathology, Aarhus University Hospital, Aarhus, Denmark and
[2]Pathology Department, I.M. Sechenov Moscow Medical Academy, Moscow, Russia

Azov AG, Koch J, Hamilton-Dutoit SJ. Improved diagnosis of mycobacterial infections in formalin-fixed and paraffin-embedded sections with nested polymerase chain reaction. APMIS 2005;113:586–93.

Traditional histological diagnosis of mycobacterial infection in formalin-fixed and paraffin-embedded (FFPE) tissues is insensitive and poorly specific. To improve this, we developed nested polymerase chain reaction (PCR) protocols for detecting a *Mycobacterium* genus-specific 65-kDa heat shock protein (HSP65) sequence and the *M. tuberculosis* complex-specific insertion sequence IS6110 in FFPE sections. Protocols were optimized on tissues from 20 patients with a final clinical diagnosis of mycobacterial infection. Amplicons were controlled by sequencing and restriction endonuclease digestion. PCR could detect as few as three mycobacterial genomes per reaction. Assays showed 100% sensitivity and specificity for both *M. tuberculosis* complex and *M. avium* complex infection. Paraffin blocks from a second group of 26 patients with histological evidence of necrotizing granulomas of unknown etiology were then analyzed as a surrogate group to test the assay under conditions similar to those applying during routine diagnosis. Twenty-three of these blocks contained amplifiable DNA; nine were positive for *M. tuberculosis* complex DNA and four for other types of mycobacterial DNA. Furthermore, digestion of HSP65 amplicons with NarI could distinguish *M. tuberculosis* from *M. avium* complex. In conclusion, our nested PCR assays can be used as reliable tools for the detection of mycobacterial infections in FFPE tissues. The assays are simple and rapid to perform and show improved sensitivity and specificity compared to previously reported protocols.

Key words: Atypical mycobacteria; tuberculosis; nested PCR; paraffin sections.

Andrey G. Azov, Institute of Pathology, Aarhus University Hospital, Noerrebrogade 44, DK-8000 Aarhus C, Denmark. e-mail: andrey.azov@ki.au.dk

The incidence of tuberculosis is rising worldwide. According to World Health Organization estimates, the number of new cases had increased globally from 8.0 million in 1997 to 8.7 million by the year 2000 (1, 2). Some 2 million deaths annually are attributable to tuberculosis, making it, after human immunodeficiency virus infection, the world's second commonest cause of death from infectious diseases (3). Approximately 1.7 billion people – nearly one third of the world's population – are thought to be infected with *Mycobacterium tuberculosis* (4). Although this pandemic is most severe in the developing countries, industrialized nations are by no means protected from it.

Symptoms of tuberculosis are protean and non-specific. The disease should be suspected in any patient with persistent febrile illness, particularly if it is accompanied by chest X-ray shadowing or lymph node enlargement. Appropriate samples can then be collected and sent for

Received 18 March 2005.
Accepted 22 June 2005.

culture to confirm the disease. A problem may arise, however, if tuberculosis is not initially suspected, and tissue specimens are fixed before being submitted for routine histopathological examination, as this precludes microbiological culture. The lack of culture may confound the correct diagnosis, particularly since the morphological features of mycobacterial infections are shared by other diseases. The correct diagnosis will then often be dependent on demonstrating acid-fast bacilli in the lesions by Ziehl-Neelsen staining, a method with low sensitivity. If Ziehl-Neelsen staining is negative, specific anti-mycobacterial treatment may be delayed or may have to be started on clinical grounds alone, without a definitive diagnosis having been made. Thus, there is a clear need for a sensitive and reliable method to detect mycobacteria in paraffin sections that can be easily introduced into routine pathological practice. In this study, we have modified polymerase chain reaction (PCR) protocols in order to develop a highly specific and sensitive molecular technique suitable for routine detection of mycobacteria in formalin-fixed and paraffin-embedded (FFPE) tissues.

MATERIALS AND METHODS

Patients

We included two groups of patients in our study. The first group, which was used to optimize PCR protocols and to test the sensitivity and specificity of the reactions, consisted of 20 patients with a final clinical diagnosis of tuberculosis or atypical mycobacterial infection made on surgically removed tissues. Acid-fast bacilli were detected by Ziehl-Neelsen staining in all cases. The diagnosis was confirmed in most cases by culture. The second group was chosen to reflect a common diagnostic problem in routine histopathology. This group consisted of 26 patients with a histological diagnosis of necrotizing granulomatous inflammation of unknown origin in which Ziehl-Neelsen staining did not show any acid-fast bacilli. Tissues from these patients were used to test the ability of our PCR-based assays to provide additional specific diagnostic information regarding the presence of different mycobacteria.

Tissue samples

Three 15-μm-thick tissue sections were cut from each paraffin block and placed in PCR microtubes. Routine precautions were taken to prevent cross-contamination between cases, including changing microtome blades for every new case. A dummy paraffin block containing no tissue was cut before each test sample; these sections were processed in parallel with the study samples and analyzed for possible contaminating DNA with PCR. They were consistently negative.

Tissue processing

The sections were deparaffinized in UltraClear (an iso-paraffin-based clearing agent; J.T. Baker, Deventer, The Netherlands) at 60 °C for 2×20 min, followed by incubation with 99% ethanol for 2×20 min, also at 60 °C. Two drops of acetone were then added to each tube, and the samples were air-dried. Dry samples were then resuspended in 300 μL of digestion buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% sodium dodecyl sulfate, 50 mM NaCl and 300 μg/mL proteinase K, and left at 56 °C with shaking for at least 24 h, until the tissue was completely digested. Proteinase K was then inactivated by incubating the samples at 95 °C for 20 min.

DNA extraction

Sample DNA was purified by phenol extraction followed by ethanol precipitation. Three hundred microliters of phenol/chloroform/isoamyl alcohol (25:24:1; v/v) was added to each tube, and these were vortexed for 10–15 s and centrifuged at 14,000 *g* for 5 min. The aqueous supernatant was transferred to new tubes and mixed with a 1/10 volume of 3 M sodium acetate and 2.5 volumes of 99% ice-cold ethanol. The mixtures were incubated at −20 °C for 30 min and centrifuged at 14,000 *g* for 5 min, after which the supernatant was discarded. The pellets were washed with 1 mL of 70% ethanol, centrifuged again at 14,000 *g* for 5 min, the alcohol was removed, and the pellets were air-dried. Dry pellets were resuspended in water, after which the resultant DNA solution was ready for analysis.

Positive controls for polymerase chain reaction

We used DNA extracts from *M. tuberculosis* strain H37RV (420 μg/mL; kindly provided by Statens Serum Institut, Copenhagen, Denmark) as positive controls for polymerase chain reactions. The sensitivity of PCR for detection of mycobacteria under optimal conditions was tested by analyzing serial 10-fold dilutions of purified control mycobacterial DNA.

Polymerase chain reaction for human beta-globin

The β-globin gene was amplified to control for the adequacy of extracted DNA. Only samples with detectable DNA were analyzed further for mycobacterial genes. The reaction mixture, 50 μL in volume, contained 250 μM of each dNTP, 1.5 mM of $MgCl_2$, 1 U of Taq polymerase (AmpliTaq DNA Polymerase, Applied Biosystems, Foster City, CA, USA), 5 μL of 10×PCR buffer (supplied with the enzyme), and 0.2 μM of each primer. Amplification was performed with an initial 5-min denaturation at

TABLE 1. *Primer sequences*

HSP-65	Outer	5′-AGGCGTTGGTTCGCGAGGG-3′
		5′-TGATGACGCCCTCGTTGCC-3′
	Inner	5′-CCAACCCGCTCGGTCTCAA-3′
		5′-CCGATGGACTGGTCACCC-3′
IS6110	Outer	5′-CGGGACCACCCGCGGCAAAGCCCGCAGGAC-3′
		5′-CATCGTGGAAGCGACCCGCCAGCCCAGGAT-3′
	Inner	5′-CCTGCGAGCGTAGGCGTCGG-3′
		5′-CTCGTCCAGCGCCGCTTCGG-3′

94°C, followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 59°C, and elongation for 1 min at 72°C, with a final extension for 10 min at 72°C.

Polymerase chain reaction for mycobacterial DNA

PCRs for mycobacterial DNA were performed as nested procedures consisting of two consecutive reactions where the second reaction amplified a DNA sequence within the first amplicon. The reactions were modified from protocols previously described by Marchetti et al. (5).

1) *PCR for the gene encoding a 65-kD heat shock protein (HSP65)*

This reaction targets a short segment of the highly conserved HSP65 gene common to most known species of mycobacteria. The outer primers (Table 1) flank a 234-bp fragment of the gene, and the inner primers amplify a 142-bp segment within it. The reaction mixture, 50 μL in volume, contained 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 2.5 U of Taq polymerase (AmpliTaq DNA Polymerase, Applied Biosystems), 5 μL of 10×PCR buffer and 0.4 μM of each primer. The first PCR was performed with an initial 4-min denaturation step at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 57°C, and elongation for 2 min at 72°C, with a final extension for 7 min at 72°C. Five microliters of the first reaction product was used for the second-round PCR. The reaction was performed under the same conditions as described for the first reaction, except for a lower annealing temperature of 55°C.

2) *PCR for the repetitive insertion sequence IS6110*

This reaction targets the repetitive insertion sequence IS6110, which is found in a limited number of mycobacterial species, namely in *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, and *M. simiae.* The outer primers (see Table 1) recognize a 220-bp fragment, within which the inner primers amplify a 123-bp sequence. The reaction mix for the first-round PCR (50 μL in volume) contained 250 μM of each dNTP, 1 mM of $MgCl_2$, 1.25 U of Taq polymerase (AmpliTaq DNA Polymerase, Applied Biosystems), 5 μL of the PCR buffer, 0.1 μM of each primer, and 20 μL of DNA sample. Two and a half microliters of the first reaction product was transferred to the second PCR mixture, which differed from the first in the concentration of dNTPs (125 μM each), $MgCl_2$ (1.5 mM), and primers (0.3 μM each). Thermal cycling was carried out with an initial 4-min denaturation step at 94°C, followed by 20 (first reaction) or 40 (second reaction) cycles of denaturation for 1.5 min at 94°C, annealing for 1.5 min at 63°C, and elongation for 1.5 min at 72°C, with a final extension for 7 min at 72°C.

Purification of DNA products and DNA sequencing

Amplification products from the positive control DNA were purified by anion-exchange chromatography (with MicroSpin™ S-400 HR columns, Amersh-

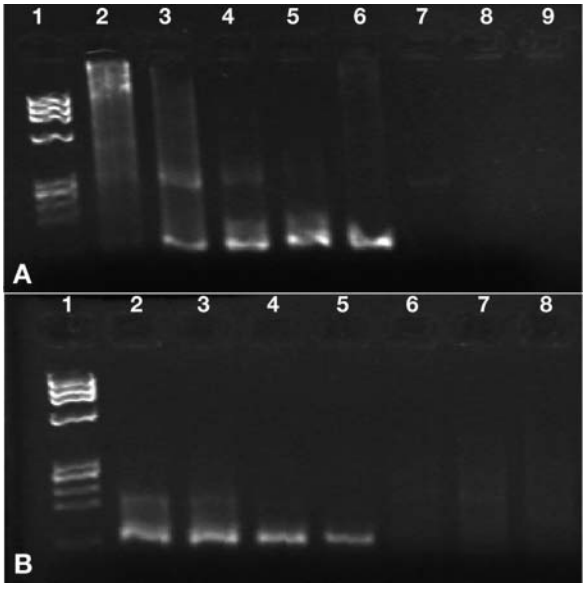

Fig. 1. Sensitivity of PCR analysis for HSP65 and IS6110. A. HSP65 PCR. Lane 1: molecular weight marker; lanes 2–8: PCRs for multiple DNA dilutions from 4.2 ng/ml to 4.2 fg/ml; the minimal detectable DNA concentration (lane 6) is 420 fg/ml. B. PCR for IS6110. Lane 1: molecular weight marker; lanes 2–8: PCRs for multiple DNA dilutions from 4.2 ng/ml to 4.2 fg/ml; the minimal detectable DNA concentration (lane 5) is 4.2 pg/ml. (Agarose gel; staining with ethidium bromide).

TABLE 2. *Correlation of PCR results with clinical and microbiological data from 17 control cases with amplifiable DNA*

Clinical \ PCR	IS6110$^+$/ HSP65$^+$	IS6110$^-$/ HSP65$^+$	IS6110$^+$/ HSP65$^-$	IS6110$^-$/ HSP65$^-$
TB (culture positive) – 9 cases	8	0	1	0
TB (clinically suspected) – 3 cases	1	0	1	1[a)]
MAC (culture positive) – 1 case	0	1	0	0
MAC (clinically suspected) – 4 cases	0	3	0	1[b)]

[a)] A culture-negative case.
[b)] The culture yielded a rare atypical mycobacterium – *M. branderi.*
Abbreviations: MAC, *M. avium* complex; TB, *M. tuberculosis.*

am Biosciences, Little Chalfont UK) and sequenced (performed by DNA technology A/S, Aarhus, Denmark).

Digestion of DNA with restriction endonucleases

To confirm amplification specificity, the amplified products were digested with the following restriction endonucleases: IS6110 fragments with SalI, and HSP65 fragments with NarI. Each digestion mixture (25 μL in volume) contained 10 μL of DNA sample and either 5 U (for SalI) or 10 U (for NarI) of restriction enzyme. The digestion mixtures were incubated overnight at 37°C.

RESULTS

PCR sensitivity under optimal conditions

PCR analysis of serial 10-fold dilutions of purified mycobacterial control DNA (*M. tuberculosis*, strain H37RV) could detect HSP65 sequences down to a DNA concentration of 4.2×10^{-13}g/ml, which corresponds to three mycobacterial genomes per PCR tube. The PCR for IS6110 was slightly less sensitive, but could detect mycobacterial DNA down to a concentration of 4.2×10^{-12} g/ml (4.2 pg/ml), or about 30 microorganisms per tube (Fig. 1).

PCR analysis of the control cases

Archive FFPE tissues from 20 patients with a final diagnosis of tuberculosis or atypical mycobacterial infection were studied. In 17 cases (85%), PCR-grade DNA could be extracted, as assessed by successful amplification of the β-globin control gene. Of the 17, 9 cases were positive for both IS6110 and HSP65 sequences, 4 were positive for HSP65 but negative for IS6110, and 2 were positive for IS6110 but negative for HSP65. Two cases were negative for both mycobacterial genes.

Comparison with the clinical data (Table 2) showed that of nine culture-positive tuberculosis cases, eight were IS6110^{pos}/HSP65^{pos}, whilst one was IS6110^{pos}/HSP65^{neg}. Two tuberculosis cases, diagnosed clinically but not confirmed microbiologically, were positive for mycobacterial genes, one being IS6110^{pos}/HSP65^{pos}, the other IS6110^{pos}/HSP65$^{neg.}$ Four cases of atypical mycobacterial infection in HIV-positive patients (one confirmed as *M. avium* complex by culture, three diagnosed on clinical grounds alone) showed IS6110^{neg}/HSP65^{pos} by PCR. Finally, one case clinically diagnosed as tuberculosis but not confirmed by culture, and one case that was culture positive for a rare mycobacterium (*M. branderi*) were negative for both genes.

PCR analysis of cases with necrotizing granulomas of unknown origin

Twenty-three of twenty-six study cases with necrotizing granulomas of unknown origin (88.5%) were positive for the control β-globin gene and were thus suitable for PCR analysis. Of the 23 cases, 8 were IS6110^{pos}/HSP65^{pos} (consistent with the presence of mycobacteria of the tuberculosis complex), 4 were IS6110^{neg}/HSP65^{pos} (consistent with the presence of atypical mycobacteria), one was IS6110^{pos}/HSP65^{neg} (interpreted as being consistent with infection with *M. tuberculosis* complex), and 10 were negative for both genes. Thus, of 23 informative cases in which the etiology of granulomatous inflammation could not be established by routine morphological methods, 13 (57%) turned out to be associated with mycobacteria. In contrast, 10 cases with adequate DNA were negative for mycobacteria, suggesting an alternative cause for the disease.

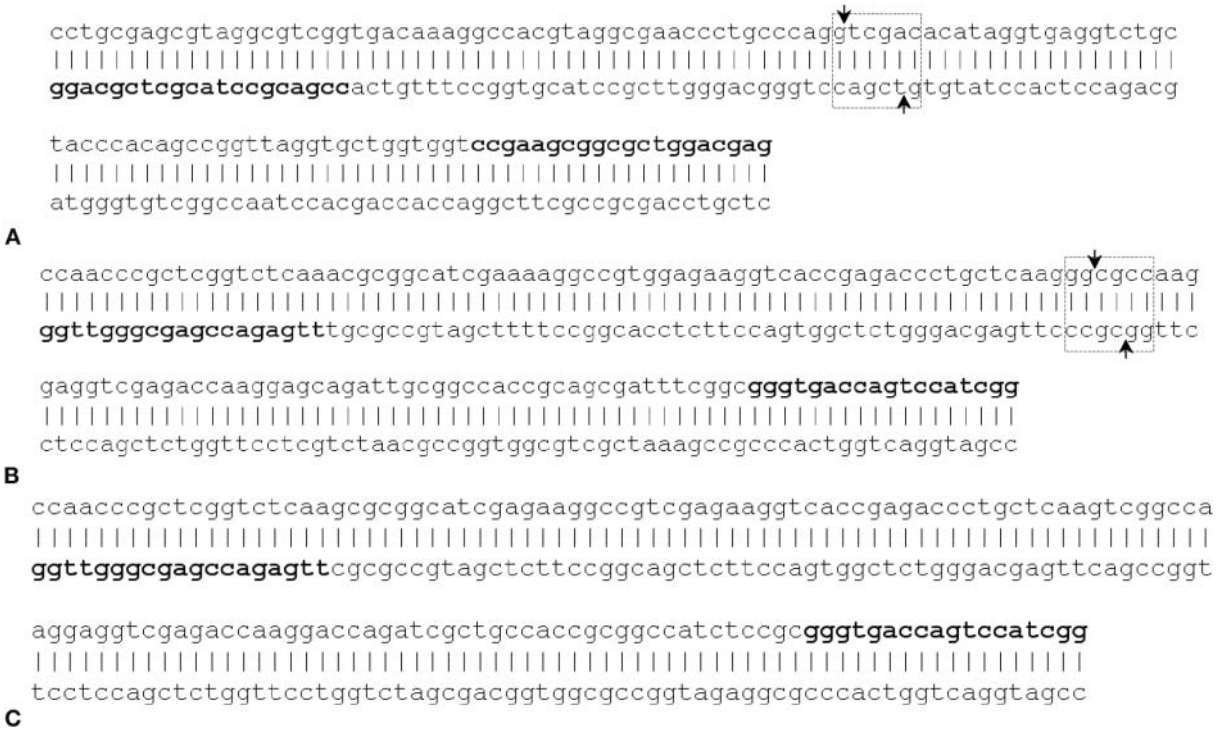

Fig. 2. Sequences of the amplified fragments (priming sites marked in boldface). A. IS6110 amplicon (source: purified *M. tuberculosis* DNA strain H37RV). B. HSP65 amplicon (source: purified *M. tuberculosis* DNA strain H37RV). C. HSP65 amplicon (source: a case of *M. avium* complex infection). Boxes: recognition sites for restriction enzymes (A: SalI; B: NarI); arrows: cleavage sites. Note that in *M. avium* HSP65 fragment lacks NarI restriction site.

DNA sequencing

Sequences of both IS6110 and HSP65 are shown in Figs. 2A & 2B.

Enzymatic digestion of the amplified fragments

Digestion of the amplified region of IS6110 with SalI yielded two fragments, 53 bp and 70 bp in length (Fig. 3). NarI cut the HSP65 segment of *M. tuberculosis* into two fragments of similar size (70 bp and 72 bp, respectively), which could not be resolved separately on gel electrophoresis. Importantly, the HSP65 segment of *M. avium* complex was not digested by NarI, and sequencing confirmed that this lacked a NarI restriction site (see Fig. 2C).

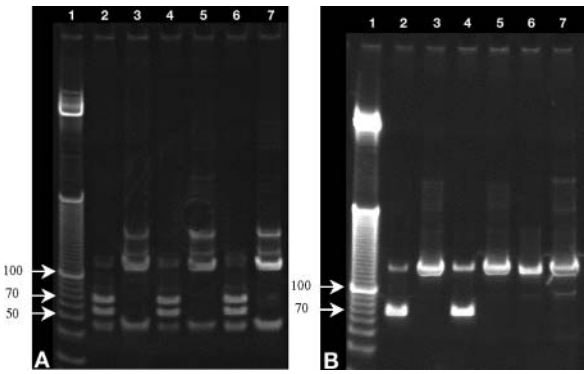

Fig. 3. Restriction enzyme digestion of the amplicons. A. Digestion of the IS6110 amplicon with SalI. Lane 1: molecular weight marker (10-bp DNA ladder); lanes 2, 4 & 6: Sall added; lanes 3, 5 & 7: Sall not added (control). B. Digestion of the HSP65 amplicon with NarI. Lane 1: molecular weight marker (10-bp DNA ladder); lanes 2–5: *M. tuberculosis* DNA (lanes 2 & 4, NarI added; lanes 3 & 5, NarI not added); lanes 6 & 7: *M. avium* DNA (lane 6, NarI added; lane 7, NarI not added). Note that NarI does not digest the DNA in lane 6. (Polyacrylamide gel; staining with SyBR® Green)

DISCUSSION

The differential diagnosis of necrotizing granulomatous inflammation is a common problem in routine histopathological practice. In the absence of microbiological culture, correct diag-

nosis of the cause will often depend on the demonstration of acid-fast bacilli, a staining technique that is notoriously insensitive in tissue sections. Even if material is sent for culture, this may take up to 6 weeks to perform, often leading to a delay in the start of appropriate treatment. PCR-based molecular diagnosis of tuberculosis and other mycobacterial infections is used increasingly in microbiology(6), and has also been reported for use in routine FFPE tissues (7–10). This has, however, been slow to catch on as a routine assay in histopathology. Although there may be several explanations for this, an important contributing factor is the widespread misconception that the method is technically challenging, expensive to perform, and insensitive in paraffin-embedded tissues. We have used previously reported primers in modified nested PCR protocols, and shown that this allows easy, rapid, specific and sensitive amplification of mycobacterial DNA from FFPE tissues, well suited to routine histopathological practice.

The sequences amplified in our PCR techniques have been well established as suitable targets for detection of mycobacteria in clinical specimens. The use of the IS6110 fragment as a PCR marker of *M. tuberculosis* complex was first reported in 1989 (11), whilst amplification of the HSP65 gene was described soon after (12). In spite of initial scepticism, numerous studies during the last decade have shown that it is possible to extract PCR-quality DNA from archive FFPE tissues.

Approaches to mycobacterial DNA amplification from FFPE tissues differ. Some authors (7, 13) recommend traditional PCR protocols, which in our experience may not be sensitive enough. There may be several reasons for this lack of sensitivity. First, mycobacteria possess a thick waxy coat, which resists simple disruption procedures and hinders the release of bacterial nucleic acids (14). Secondly, formalin fixation reduces the quality of tissue DNA and impairs PCR efficiency. The precise nature of the chemical modifications induced in DNA during formalin fixation is only partially understood. However, fixation is known to lead to both reversible changes in nucleic acids (such as the formation of nucleotide monomethylol adducts (15)), and more permanent alterations (reviewed in (16)). Furthermore, several reports have shown that formalin-induced DNA damage may reduce not only the efficiency, but also the fidelity of PCR, resulting in non-reproducible sequence variations in amplified products (17, 18). Finally, there is some evidence that tissue preparation, including paraffin embedding, may introduce molecules that act as PCR inhibitors (19, 20). The cumulative effect of these fixation-related phenomena is to markedly reduce the efficiency of PCR amplification of DNA extracted from FFPE tissues. These adverse effects can to a certain extent be compensated for by using nested PCR, which improves sensitivity approximately 100-fold compared with traditional PCR methods (21). An additional advantage of the technique is a 10–20-fold dilution of any potential PCR inhibitors, which occurs when products of the first PCR round are included in the second PCR mix.

Our assay is based on the parallel use of more than one target for nested PCR, as this increases the discriminating power of the reaction. Thus, detection of targets within both genus-specific (HSP65, common for all mycobacteria) and species-specific (IS6110, restricted to mycobacteria of the tuberculosis complex) DNA sequences allows for differentiation between tuberculosis and other mycobacterial infections.

Our reaction proved to be highly sensitive for detecting low-copy-number mycobacterial genomes in the test material. When compared with the clinical data, our assay showed a sensitivity of at least 82% for *M. tuberculosis* (9 $IS6110^{pos}/HSP65^{pos}$ cases out of 11) and of 100% for *M. avium* (4 $IS6110^{neg}/HSP65^{pos}$ cases out of 4). The results become even more striking (100% concordance with the clinical and microbiological data) if the diagnostic criteria for tuberculosis are expanded to include $IS6110^{pos}/HSP65^{neg}$ cases. This combination was reproducible, suggesting that it was not an artifact. Rather, it is likely to reflect differences in the efficiency of the two reactions or the effects of DNA damage induced during fixation and processing of FFPE tissues. We therefore consider the combination $IS6110^{pos}/HSP65^{neg}$ to be consistent with tuberculosis.

In diagnostic practice, it is important to control for possible false negativity caused by inaccessible or suboptimal DNA in FFPE tissue. We did this by testing all cases for am-

plifiable human β-globin gene. As expected, not all samples had adequate DNA. For example, the frequency of satisfactory DNA extraction from archive cases stored for up to 7 years was below 90%. However, since developing our protocol and introducing it in our laboratory routine, we have encountered no cases with unsatisfactory DNA (unpublished data), which underlines the influence of age of the FFPE tissue blocks on the chances of generating false-negative results.

To test the specificity of the reactions, we sequenced the amplified regions of purified mycobacterial DNA and compared them with published sequences. In addition, we selected restriction endonucleases with appropriate 6-bp recognition sites, and used these to digest the amplified fragments. These enzymes successfully cleaved the amplified DNA of the test cases. Interestingly, the HSP65 segment of *M. avium* differs in sequence from that of *M. tuberculosis*, and is, therefore, not cut by one of our chosen enzymes (NarI). This provides a cheaper, if somewhat more time-consuming, method of analysis as an alternative to the amplification of both genes.

In conclusion, nested PCR is an effective method of amplification that can be used for detecting small amounts of poor-quality target DNA. Using this technique, it is possible to identify mycobacterial DNA and to differentiate tuberculosis from atypical mycobacterial infections in routine FFPE tissues, with a sensitivity approaching single mycobacterial genomes per PCR tube. We use a diagnostic protocol consisting of three PCRs: one for a human gene to control for DNA quality, a second to detect the common mycobacterial gene HSP65, and a third to detect the tuberculosis-specific insertion sequence IS6110. Of the possible results, $IS6110^{pos}$/$HSP65^{pos}$ and $IS6110^{pos}$/$HSP65^{neg}$ cases are consistent with tuberculosis, $IS6110^{neg}$/$HSP65^{pos}$ cases are consistent with mycobacterial infections other than tuberculosis, and $IS6110^{neg}$/$HSP65^{neg}$ cases are likely to be free of mycobacteria. Digestion of the HSP65 segment with endonuclease NarI, which recognizes and cleaves only the HSP65 amplicon of *M. tuberculosis*, but not that of *M. avium* complex, provides a control of the specificity of the PCRs, and may contribute to the differentiation of these mycobacteria.

We wish to thank Statens Serum Institut (Copenhagen, Denmark) for the *M. tuberculosis* strain H37RV reference DNA. AGA was sponsored by the Scholarship for Study Abroad awarded by the President of the Russian Federation.

REFERENCES

1. World Health Organization. Global tuberculosis control: surveillance, planning, financing 2001; WHO report 1999.
2. World Health Organization. Global tuberculosis control: surveillance, planning, financing 2002; WHO Report 2000.
3. Dye C, Scheele S, Dolin P, Pathama V, Raviglione MC. Global burden of tuberculosis: estimated incidence, prevalence and mortality by country: WHO Global Surveillance and Monitoring Project. JAMA 1999;282:677–86.
4. Dolin PJ, Raviglione MC, Kochi A. Global tuberculosis incidence and mortality during 1990–2000. Bull World Health Organ 1994;72:213–20.
5. Marchetti G, Gori A, Catozzi L, et al. Evaluation of PCR in detection of Mycobacterium tuberculosis form formalin-fixed, paraffin-embedded tissues: comparison of four amplification assays. J Clin Microbiol 1998;36:1512–7.
6. Shamputa IC, Rigouts L, Portaels F. Molecular genetic methods for diagnosis and antibiotic resistance detection of mycobacteria from clinical specimens. APMIS 2004;112:728–52.
7. Frevel T, Schafer KL, Totsch M, Bocker W, Dockhorn-Dworniczak B. PCR based detection of mycobacteria in paraffin wax embedded material routinely processed for morphological examination. J Clin Pathol Mol Pathol 1999;52: 283–8.
8. Hsiao PF, Tzen CY, Chen HC, Su HY. Polymerase chain reaction based detection of Mycobacterium tuberculosis in tissues showing granulomatous inflammation without demonstrable acid-fast bacilli. Int J Dermatol 2003;42:281–6.
9. Salian NV, Rish JA, Eisenach KD, Cave MD, Bates JH. Polymerase chain reaction to detect Mycobacterium tuberculosis in histologic specimens. Am J of Resp Crit Care Med 1998;158: 1150–5.
10. Ghossein RA, Ross DG, Salomon RN, Rabson AR. Rapid detection and species identification of mycobacteria in paraffin-embedded tissues by polymerase chain reaction. Diagn Mol Pathol 1992;1:185–91.
11. Brisson-Noel A, Gicquel B, Lecossier D, Levy-Frebault V, Nassif X, Hance AJ. Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. Lancet 1989;4:1069–71.
12. Hance AJ, Grandchamp B, Levy-Frebault V, Le-

cossier D, Rauzier J, Bocart D, Gicquel B. Detection and identification of mycobacteria by amplification of mycobacterial. DNA Mol Microbiol 1989;3:843–9.
13. Totsch M, Bocker W, Brommelkamp E, Fille M, Kreczy A, Ofner D, Schmid KW, Dockhorn-Dworniczak B. Diagnostic value of different PCR assays for the detection of mycobacterial DNA in granulomatous lymphadenopathy. J Pathol 1996;178:221–6.
14. Reischl U. Application of molecular biology-based methods to the diagnosis of infectious diseases. Frontiers Biosci 1996;1:72–7.
15. Feldman MY. Reactions of nucleic acids and nucleoproteins with formaldehyde. Prog Nucleic Acid Res Mol Biol 1973;13:1–49.
16. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002;161: 1961–71.
17. Quach N, Goodman MF, Shibata D. In vitro mutation artifacts after formalin fixation and error prone translesion synthesis during PCR. BMC Clin Pathol 2004;4:1.
18. Williams C, Ponten F, Moberg C, Soderkvist P, Uhlen M, Ponten J, Sitbon G, Lundeberg J. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 1999;155:1467–71.
19. An SF, Fleming KA. Removal of inhibitor(s) of the polymerase chain reaction from formalin fixed, paraffin wax embedded tissues. J Clin Pathol 1991;44:924–7.
20. Bascunana CR, Belak K. Detection and identification of mycobacteria in formalin-fixed, paraffin-embedded tissues by nested PCR and restriction enzyme analysis. J Clin Microbiol 1996;34: 2351–5.
21. Kim J, Chae C. Optimized protocols for the detection of porcine circovirus 2 DNA from formalin-fixed paraffin-embedded tissues using nested polymerase chain reaction and comparison of nested PCR with in situ hybridization. J Virol Methods 2001;92:105–11.