Sensitive Differential Detection of Genetically Related Mycobacterial Pathogens in Archival Material

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Abstract

A polymerase chain reaction (PCR) assay targeted to the immunogenic protein MPB64 gene was used to detect members of the Mycobacterium tuberculosis complex, and an outward-primed PCR (OPPCR) designed on the IS6110 element allowed differentiation between Mycobacterium bovis and Mycobacterium tuberculosis. Additionally, the amplification of IS1110 and 16S ribosomal RNA sequences combined with a dot blotting assay were able to differentially detect Mycobacterium avium, Mycobacterium intracellulare, and Mycobacterium paratuberculosis. The validity of the experimental procedure was tested on reference material and formalin-fixed paraffin-embedded samples from patients with tuberculosis, sarcoidosis, or Crohn disease. We demonstrated mycobacterial DNA in 59 of 75 cases with histologic lesions typical of tuberculosis; we detected M tuberculosis and M paratuberculosis in 6 of 25 sarcoidosis cases and in 7 of 20 Crohn disease specimens, respectively. The proposed diagnostic procedure is directly applicable to archival material and allows differentiation of genetically related mycobacterial pathogens in more detail than other molecular methods. It provides a tool for the diagnostic study of tuberculosis, sarcoidosis, and Crohn disease.

During the last decade, tuberculosis has reemerged as a serious public health problem in many regions worldwide.1,2 However, apart from tuberculosis, the cause of which has been well substantiated, there is currently increasing evidence that certain mycobacterial species are also implicated in 2 diseases with elusive causes, namely sarcoidosis and Crohn disease.3-5 Although molecular research has proposed many alternatives, the detection of mycobacteria continues to rely on the application of specific staining techniques and cultivation. The latter has been recognized as the “gold standard” method, with sensitivity that reaches 100 bacilli per milliliter of sample.6 Although a number of modifications have shortened the time required for the isolation of mycobacteria, it remains a rather time-consuming method.6-8 Furthermore, the multiple steps involved in processing cultures and the fact that many samples carry very small numbers of mycobacteria make culturing a process inherently prone to errors.9 On the other hand, the Ziehl-Neelsen stain is by far less adequate since its sensitivity is low,4,10 and it does not allow the distinction of Mycobacterium tuberculosis from other mycobacterial species.11 The disadvantages of the methods previously described have dictated the need for more effective diagnostic tools. A plethora of diagnostic procedures for the detection of mycobacteria in a variety of clinical samples have been published.4-7,10,12,13 Several methods are available in the market, and, in most cases, they rely on DNA amplification12,14 or on hybridization with oligonucleotide probes.13,15 Some of the aforementioned diagnostic procedures are recommended for use only in combination with culture, mainly due to their low sensitivity, while the cost of others justifies their use only by highly specialized laboratories. Moreover, all the aforementioned methods have a limited diagnostic spectrum, since they are able to detect the...
presence of either *M. tuberculosis* complex or *Mycobacterium avium-intracellulare* complex, providing no means for further diagnostic investigation.

The time required by the traditional microbiologic method for a definite diagnosis is an important drawback, especially for patients in critical condition. Therefore, the initial laboratory assessment of patients with possible mycobacterial infection usually involves the histologic examination of the appropriate biopsy specimen, followed by Ziehl-Neelsen staining. However, despite the considerable significance of a positive Ziehl-Neelsen result, the method cannot lead to the determination of the infectious agent.

Hence, the need for developing a rapid, specific, and sensitive procedure that would ensure the differential diagnosis of mycobacterial infections becomes evident, particularly when the examined material consists of a routinely processed (formalin-fixed paraffin-embedded [FFPE]) biopsy specimen. This study focuses on the design and validation of a molecular assay allowing the distinction of genetically related mycobacterial pathogens directly on FFPE samples. For this purpose, we designed 3 pairs of primers that can be used separately or in a multiplex polymerase chain reaction (PCR) assay. A combination of 2 amplification reactions followed by a dot blotting assay readily distinguishes *Mycobacterium avium* from *Mycobacterium intracellulare* and *Mycobacterium paratuberculosis*, while an outward-primed PCR (OPPCR) enables the differentiation of *M. tuberculosis* from *Mycobacterium bovis*.

### Materials and Methods

#### Samples

The proposed molecular method was evaluated initially on reference material consisting of various mycobacterial species grown on pure culture and mycobacterial DNA preparations [Table 1](#). This material was collected and identified by hospitals in Athens, Greece (Sotiria Chest Hospital and 401 Army General Hospital), and the Veterinary Investigation Center, Carmarthen, Wales. Genus and species identification was established as previously described.16,17

FFPE tissue samples from patients with tuberculosis, sarcoidosis, or Crohn disease also were tested. Characteristic lesions were recorded initially by histologic examination of the samples that were examined further by the Ziehl-Neelsen stain, by the traditional microbiologic method, and by the molecular methods currently proposed. The Ziehl-Neelsen stain was performed by the staff of the University of Athens School of Medicine on the same samples used for PCR, while corresponding clinical material was used for culture. Unfortunately, in some cases [Table 2](#), the shortage of material did not allow the complete set of tests to be carried out.

Our material consisted of 4 sets of specimens from various sources. Groups 1 (75 cases) and 2 (25 cases) comprised lung and pulmonary lymph node specimens with histologic evidence suggestive of tuberculosis and sarcoidosis, respectively. Group 3 included 20 FFPE intestinal biopsy samples with histologic findings suggestive of Crohn disease. Samples for group 4 were collected from patients who were hospitalized during the present study and showed strong clinical and/or histologic evidence of mycobacterial infection. Thus, group 4 consisted of 50 FFPE samples: lung, 30; pulmonary lymph nodes, 10; spinal cord, 4; and colon, 6 (derived from the same patient).

#### Fixation Procedure

Fresh biopsy samples were initially placed in buffered formalin. They subsequently were incubated for 60 minutes

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**Table 1**

<table>
<thead>
<tr>
<th>Mycobacterium Species</th>
<th>No. of Strains Tested</th>
<th>PCR Reaction</th>
<th>PRAVMPAR Illumination</th>
<th>Outward-Primed PCR FINGA-B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pr1-2 (243 bp)</td>
<td>Pr3-4 (91 bp)</td>
<td>M16SU-D (504 bp)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>50</td>
<td>50</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. avium-intracellulare</em></td>
<td>25</td>
<td>–</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td><em>M. africanum</em></td>
<td>2</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. microti</em></td>
<td>4</td>
<td>4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. marinum</em></td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. gordonae</em></td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>12</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>13</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>15</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. paratuberculosis</em></td>
<td>15</td>
<td>–</td>
<td>8</td>
<td>15</td>
</tr>
</tbody>
</table>

bp, base pair; –, negative result.

* PRAVMPAR is a probe; Pr1-2, Pr3-4, M16SU-D, and FINGA-B are primers. Data for the outward-primed PCR are the number of strains recognized.
in 70%, 80%, 96%, and 100% ethanol; the last 2 steps were repeated twice. The material then was submerged in xylene for 60 minutes and, finally, in paraffin at 50°C for 4 hours. The FFPE samples used had been stored for no more than 18 months before PCR examination.

**DNA Extraction**

Two 15-µm-thick sections from each FFPE sample were used for DNA extraction, which was performed as previously described. In brief, the material was dewaxed by repeated incubations in xylene and ethanol and subsequently was digested with protease K (Sigma, Athens, Greece) in a sodium-dodecyl-sulfate buffer. The resulting nucleic acid solutions were purified by a phenol–chloroform-isooamyl alcohol procedure precipitated in a 70% ethanol solution and eluted in high-performance liquid chromatography (HPLC)-quality water.

The quality of the extracted DNA was determined by optical density (OD) measurements, electrophoresis, and a control PCR assay targeted to the human genome. For the latter, and to avoid false-negative results due to low quality and/or fragmented DNA, we amplified a 523-base-pair (bp) fragment of the p53 gene, which exceeds the PCR products amplified by the proposed assays. Samples that failed to produce a positive result with this PCR reaction were discarded, and the extraction procedure was repeated until the quality of the DNA product was satisfactory. Specimens that comprised the reference material were decontaminated according to Centers for Disease Control and Prevention guidelines for mycobacteriology.

**Table 2**

Comparison of Results Obtained for *Mycobacterium* Species With the PCR, OPPCR, Ziehl-Neelsen, and Culture*

<table>
<thead>
<tr>
<th>Group (No. of Samples)</th>
<th>MTBC</th>
<th>MAC</th>
<th>MI</th>
<th>MP</th>
<th>MTB(^1)</th>
<th>MAC-INT(^3)</th>
<th>MP</th>
<th>Acid Fastness</th>
<th>MTB</th>
<th>MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (75)</td>
<td>38 (51)</td>
<td>7 (9)</td>
<td>14 (19)</td>
<td>0 (0)</td>
<td>38 (51)</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>35 (47)(^1)</td>
<td>29 (39)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>2 (25)</td>
<td>6 (24)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3 (20)</td>
<td>0 (0)</td>
<td>2 (10)</td>
<td>0 (0)</td>
<td>7 (35)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4 (50)</td>
<td>29 (58)</td>
<td>4 (8)</td>
<td>0 (0)</td>
<td>4 (8)</td>
<td>28 (56)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>22 (44)</td>
<td>22 (44)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lung (30)</td>
<td>22 (73)</td>
<td>4 (13)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>22 (73)(^4)</td>
<td>0 (0)(^4)</td>
<td>0 (0)(^4)</td>
<td>16 (53)</td>
<td>16 (53)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lymph node (10)</td>
<td>6 (60)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (60)(^*)</td>
<td>0 (0)(^*)</td>
<td>0 (0)(^*)</td>
<td>6 (60)</td>
<td>6 (60)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Intestine (6)</td>
<td>1 (25)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spinal cord (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (67)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>


* Data are given as number (percentage). Group 1, histologic evidence of caseous granulomatous necrosis; group 2, histologic evidence of noncaseous granulomatous necrosis; group 3, histologic evidence consistent with Crohn disease; and group 4, clinical and/or histologic evidence of mycobacterial infection. MTBC includes *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. MAC includes *M avium MAIS2*, *M avium serovar 7*, *M avium WP M21*, *Mycobacterium paratuberculosis* Linda, *M paratuberculosis* C286, and *M paratuberculosis* ATCC 19698.

* The method was applied only to the Ziehl-Neelsen–positive samples.

* Culture identifies these strains as *Mycobacterium tuberculosis* and PCR as *Mycobacterium tuberculosis* complex.

* The microbiologic examination of the samples performed in the hospitals in which the patients were treated allowed identification only to this level.

* Four Ziehl-Neelsen–positive samples were negative by culture and PCR.

* Corresponding clinical material (bronchoalveolar lavage specimens) was used for culture.

* Fresh lymph node biopsy samples were used for culture.

**Determination of Sensitivity and Specificity**

Sensitivity and specificity for all proposed assays were determined as previously described. Briefly, for sensitivity determination, 10 *M tuberculosis*, 10 *M avium*, 5 *M intracellulare*, and 5 *M paratuberculosis* strains were used. The minimum amount of DNA necessary for a positive result was determined by OD measurements of serial DNA dilutions, based on the formula:

\[
\text{OD} \times 50 \mu\text{g/mL} \times D = C
\]

where OD is 260 nm; D, the degree of dilution, and C, the DNA concentration in micrograms per milliliter.

Assay specificity was evaluated on 10 Ziehl-Neelsen–, culture–, and immunofluorescence-negative FFPE samples, together with pure cultures of other mycobacterial and related bacterial species (*Rhodococcus*, *Corynebacterium*, *Nocardia*, *Actinomyces*, *Escherichia coli*, and *Brucella*). The primers were also tested on pure mycobacterial cultures, sterile paraffin samples, mycobacteria-negative FFPE samples, and mycobacteria-negative FFPE samples inoculated with various mycobacterial and related bacterial species (data not shown).

**PCR Assays**

A standard 50-µL PCR reaction was prepared with a 1.5-mmol/L concentration of magnesium chloride, a 200 µmol/L concentration of deoxynucleoside triphosphates, 1 U of Promega (Bioanalytica, Athens, Greece) Taq DNA polymerase, 1× Taq polymerase buffer, 25-pmol concentrations of each of the primers, and, finally, 2.5 pg of DNA in HPLC-quality water. Amplification was performed in a
Perkin Elmer 9600 thermal cycler (PE Applied Biosystems, Warrington, England). To prevent carryover contamination, deoxythymidine triphosphate was substituted by deoxyuridine triphosphate. The reaction mixture was incubated initially at 37°C with 0.5 U of uracil-N-glycosylase (UDG) and then brought to 94°C to inactivate UDG before entering the PCR cycles.\(^2^2\) Amplification started with a 5-minute denaturation step at 94°C, while the Taq DNA polymerase was added after the completion of this step (hot start) to minimize the chance of nonspecific binding.\(^2^0,^2^3\) The specific temperatures selected for each primer pair were adjusted to similar or identical levels to allow the examination of samples in single or, if necessary, in multiplex assays \(\text{Image 1}\). PCR amplification was performed by 30 cycles of 0.5 minute at 94°C, 0.5 minute at 60°C, and 1 minute at 72°C followed by a 7-minute completion step at 72°C. A 15-µL aliquot of the amplified DNA was analyzed by electrophoresis in 2% agarose gels. The DNA products were stained with ethidium bromide and photographed.

The primers were designed with OLIGO 5 software (Primer Analysis Software, National Biosciences Inc, Plymouth, MN). For the detection of \(M\) \(tuberculosis\) complex (\(M\) \(tuberculosis\), \(M\) \(bovis\), \(M\) \(bovis\) bacille Calmette-Guérin, \(Mycobacterium\) \(microti\), and \(Mycobacterium\) \(africanum\)), the primer pair used was as follows: Pr1 (184-197): 5'-GCT CTG TTG TTC GGG TGT GGC GA-3' and Pr2 (404-427): 5'-GAT ATT CAA TTC GTA GGG GGC TTT-3', amplifying a 243-bp fragment (Image 1) of the gene encoding the immunogenic protein MPB64.\(^2^4,^2^5\) For the detection of \(M\) \(avium\) complex (\(M\) \(avium\) MAIS2, \(M\) \(avium\) serovar 7, \(M\) \(avium\) WP M21, \(M\) \(paratuberculosis\) Linda, \(M\) \(paratuberculosis\) C286, and \(M\) \(paratuberculosis\) American Type Culture Collection 19698), we used the following primer pair: Pr3 (1353-1370): 5'-CAG GCA GAG GGT GGC CG-3' and Pr4 (1427-1444): 5'-CGA ATC ACC CCC GAT CA-3', targeted to a 91-bp fragment \(\text{Image 2}\) of the \(IS\) \(111\) element.\(^2^6\) A third pair of primers was used for the amplification of a 504-bp fragment (Image 1) of the \(16S\) ribosomal RNA (rRNA) gene\(^2^7\) of \(M\) \(intracellularre\): \(M\) \(16SD2\) (68-93): 5'-GTG GGC AA T CTG CCC TGC ACT TCG G-3' and \(M\) \(16SU\) (547-572): 5'-GCC CGC ACG CTC ACA GTT AAG CCG T-3', which are also complementary to regions shared by \(M\) \(paratuberculosis\) and \(M\) \(avium\).\(^2^8\)

To detect false-positive results due to carryover effect, 20% of the samples tested in every assay were negative controls. An equal percentage of DNA samples prepared from mycobacteria-free biopsy specimens (normal histologic appearance, Ziehl-Neelsen stain, culture, and PCR negative) were inoculated with standard quantities of mycobacterial DNA and served as positive controls. Control samples were processed identically to the cases under study in regard to fixation, DNA extraction, and preparation for the PCR.

**Dot Blotting**

The probe PRAVMPAR (132-161), 5'-GGA TAG GAC CTC AAG ACG CAT GTC TTC T-3', was designed to complement a region within the highly conserved\(^2^7\) \(16S\) rRNA gene, amplified by the \(M\) \(16SD2\) and \(M\) \(16SU\) primer pair. The amplification product exhibits a degree of interspecies sequence variation, which allowed the differentiation of \(M\) \(paratuberculosis\) from \(M\) \(intracellularre\) and \(M\) \(avium\) directly from FFPE samples. An extended Basic Local Alignment Search Tool (BLAST) search for the probe PRAVMPAR identified no significant homologies to known sequences.\(^2^8\) The chemiluminescent labeling of the latter and of those (data not shown) that were used to verify the specificity of the products amplified by the 3 PCR assays...
Outward-Primed PCR

An OPPCR assay that was described originally as a fingerprinting method has been modified and adjusted to allow easy differentiation of \( \text{M} \) \( \text{tuberculosis} \) from \( \text{M} \) \( \text{bovis} \). As opposed to standard PCR, this procedure involves primers that do not define the area they amplify since they extend outwardly. The primers used in this technique (FINGA \([1507–1531]\): 5’-CGA CCA TCC GCA CCG CCC GCT CAC G-3’ and FINGB \([317–293]\): 5’-ACG CTC AAC GCC AGA GAC CAG CCG-3’) anneal to the edges of the \( \text{IS}6110 \) element of \( \text{M} \) \( \text{tuberculosis} \), extending toward their adjacent genomic regions. The high degree of polymorphism in the size of amplified regions permits the differential detection of species and strains that are almost genetically identical. PCR was performed under the conditions as previously stated. The technique was tested on reference material and selected FFPE samples that had produced a positive PCR result for \( \text{M} \) \( \text{tuberculosis} \) complex (Tables 1 and 2). Positive (FFPE samples spiked with mycobacterial DNA) and negative (FFPE samples with HPLC water) controls were also incorporated in each assay.

Results

Assay Sensitivity and Specificity

The minimum amount of DNA necessary for a positive result was 40 to 50 fg, corresponding to 8 to 10 mycobacterial cells. Amplification with the M16SD2-M16SU pair of primers followed by hybridization of the product with the PRAVMPAR probe allowed the detection of even a single mycobacterium (5 fg of DNA) from pure cultures and 6 to 10 mycobacteria from FFPE samples. However, as mentioned, the total amount of DNA incorporated in the routine PCR examination of the FFPE samples was significantly higher (2.5 pg) in order to increase the chance of detecting the mycobacterial DNA within the sample. The sensitivity of multiplex assay proved slightly inferior to the single ones, most likely owing to antagonist effect in primer binding. The vigorous precautions taken in combination with the use of HPLC-quality water and UDG helped the negative controls to give the expected result to a percentage that reached 97%. For those failing to produce such a result, PCR assays were repeated. Approximately 4% of FFPE samples produced DNA of unacceptable quality, mostly as a result of fragmentation. With few exceptions, careful handling while repeating the process resolved this problem.

The OPPCR assay allowed the examination of samples with only 20 to 30 mycobacterial cells. However, to establish reproducible results readily interpretable by visual observation, samples should preferably contain no less than 1 to 5 pg of mycobacterial DNA, corresponding to 200 and 1,000 mycobacterial cells. This level of sensitivity allowed successful testing of Ziehl-Neelsen–positive specimens (35 samples from group 1 and 22 samples from group 4) (Table 2).

The results obtained on reference material proved that each assay is highly specific for the group of mycobacteria that it was designed to detect. Primers Pr1 and Pr2 produced a positive result in 70 (98%) of 71 strains that belong to the \( \text{M} \) \( \text{tuberculosis} \) complex (Table 1, Images 1 and 2). Such comparison is less straightforward for the Pr3 and Pr4 and the M16SU and M16SD2 primer pairs. This is a result of the differences of traditional microbiologic methods and molecular techniques, which account for conflicting results, especially for the highly heterogeneous group of mycobacteria that constitute the \( \text{M} \) \( \text{avium-intracellulare} \) complex. Nevertheless, Pr3-Pr4 PCR, designed to specifically detect members of the \( \text{M} \) \( \text{avium} \) complex, yielded a positive result. 

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**Image 2** Gel electrophoresis of DNA products obtained with the outward-primed polymerase chain reaction assay from selected samples. Lane 1, DNA marker, pUC19/Sau3AI; Lanes 2 and 8, \( \text{M} \) \( \text{tuberculosis} \) strains showing the species specific bands of 600 and 258 bp (bp) (formalin-fixed paraffin-embedded samples with a strong positive Ziehl-Neelsen reaction); lane 3, \( \text{M} \) \( \text{cobacterium microti} \) strain from a Ziehl-Neelsen–negative sample; lanes 4, 5, 6, and 9, \( \text{M} \) \( \text{cobacterium bovis} \) strains showing the species-specific bands of 580 and 150 bp (pure cultures); lane 7, \( \text{M} \) \( \text{cobacterium microti} \) strain producing a distinct pattern (pure culture); lane 10, negative control.
for all 25 strains that were microbiologically characterized as
*M. avium-intracellularare* complex (Table 1, Image 2). As
expected, these primers detected only 8 (53%) of 15 *M. paratuberculosis* strains, an inadequacy that was resolved by
M16SD2-M16SU primer PCR, followed by hybridization
with the PRAVMPAR probe. This combination detected all
the *M. paratuberculosis* strains and those microbiologically identified as *M. avium-intracellularare* (Table 1, Image 1).
Assays that were performed in samples inoculated with genet-
ically related bacterial species produced no signs of amplifi-
cation, thus excluding the possibility of false positivity.

Use of the OPPCR assay on the reference material
showed that the method is highly specific for *M. tuberculosis* complex strains (Table 1). No positive reactions were
observed with any other mycobacterial or related bacterial species. Electrophoresis of PCR products yielded species-
and strain-specific bands, the latter readily distinguished by
visual observation (Image 2). Analysis by ImagePro Soft-
ware (Media Cybernetics, Silver Spring, MD) identified 2
pairs of bands, one of 625 and 258 bp and the other of 580
and 230 bp, that were the products of *M. tuberculosis* and
*M. bovis* strains, respectively. The small number of strains of
*M. africanum* (2) and *M. microti* (4) available did not provide
enough evidence for a similar differentiation, although the
analysis of these strains produced distinct patterns.

**Mycobacterial Detection in FFPE Specimens**

Among the 75 FFPE samples of group 1 (Table 2) that
showed strong histologic evidence of caseous granulomatous
inflammation, the proposed combination of PCR assays
detected a total of 59 positive cases (79%), of which 38
(51%) were identified as *M. tuberculosis* complex, 7 (9%) as
*M. avium* complex, and 14 (19%) as *M. intracellularare*. All 39
culture-positive samples were identified as PCR-positive,
whereas from the 35 Ziehl-Neelsen–positive samples, 31
(88%) were proven PCR positive, all identified as *M tubercu-
llosis* complex. The observed difference in the results of
Ziehl-Neelsen staining and PCR was verified by repeating both
tests for the cases under question.

The OPPCR assay identified 29 (39%) and 2 (3%) posi-
tive specimens for *M. tuberculosis* and *M. bovis*, respectively,
with the results lying in complete accordance with those
obtained by culture. Discrepancies were recorded between
culture and Ziehl-Neelsen staining, which identified 39 and
35 positive samples, respectively. Furthermore, 1 of the 4
Ziehl-Neelsen–positive, PCR-negative samples was proven
culture negative.

Among the 25 FFPE samples in group 2 with noncaseous granulomatous lesions typical of sarcoidosis (Table 2), PCR assays identified *M. tuberculosis* complex in 6
cases (24%). Ziehl-Neelsen staining performed on the same
specimens, as well as cultivation of corresponding clinical
material, produced negative results.

The use of M16SD2-M16SU primer PCR, followed by
dot blotting with the PRAVMPAR probe, detected the presence
of *M. paratuberculosis* in 7 (35%) of 20 intestinal lesions
with strong histologic evidence of Crohn disease (group 3). Two of these specimens were positive for the
presence of *M. avium* complex DNA by Pr3-Pr4 primer amplifi-
cation. Cultivation and Ziehl-Neelsen staining failed to
produce evidence of mycobacterial infection in any sample
of this group.

Among the 30 FFPE lung biopsy specimens in group 4,
PCR identified 22 cases positive for *M. tuberculosis* (73%),
in complete agreement with cultivation of corresponding
clinical material (bronchoalveolar lavage specimens). However, culture failed to detect 4 cases of *M. avium*
complex infection, which were revealed only by PCR. Ziehl-
Neelsen staining demonstrated the presence of acid-fast bacilli in only 16 (73%) of 22 culture-positive cases. Of the
10 lymph node biopsy specimens, 6 (60%) were positive by
PCR, culture, and Ziehl-Nielsen staining, with OPPCR identi-
fying *M. tuberculosis* as the infectious agent in all Ziehl-
Neelsen–positive cases. In FFPE specimens derived from the
spinal cord (4) and the colon (6), PCR was the only tech-
nique that detected the presence of *M. tuberculosis* in 1
(25%) and *M. paratuberculosis* in 4 (67%) biopsy specimens,
respectively.

**Discussion**

We describe an original combination of PCR and DNA
hybridization assays, providing a definite differential diag-
nosis of infections caused by closely related mycobacterial
pathogens in routinely processed biopsy specimens Table 3.
An initial amplification reaction detects the presence of the
MPB64 gene of *M. tuberculosis* complex, while a subsequent
OPPCR assay, targeted to the IS6110 element, distinguishes
between *M. bovis* and *M. tuberculosis* infections. Amplifi-
cation of a region in the IS1110 element and the 16S rRNA gene,
along with a subsequent dot blotting assay, differentially
detects the genomes of *M. avium*, *M. intracellularare*, and *M.
paratuberculosis*. Because these PCR assays can be

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**Figure 1** Schematic presentation of the outward-primed
polymerase chain reaction assay. FINGA and FINGB are
primers. Arrows indicate the primers 5’-3’ direction.
performed simultaneously in a multiplex reaction, the proposed molecular method allows the identification of mycobacterial species in FFPE tissue material by easily accessible technological means in less than 12 hours of processing.

Several regions of mycobacterial genomes were selected carefully as targets of the described procedure. It has been suggested that the \textit{IS6110} element should constitute the main target for detection of the \textit{M tuberculosis} complex.\textsuperscript{33} However, the \textit{IS6110} element has been reported to account for false-positive or false-negative PCR results, with the latter stemming from the absence of this element in a small number of \textit{M tuberculosis} strains.\textsuperscript{34,35} On the other hand, Ikonomopoulos et al\textsuperscript{16,17} demonstrated that the use of primers, which are targeted to the gene encoding the immunogenic protein MPB64, produces highly sensitive and specific results. As opposed to the diagnostic PCR, the disadvantages of the \textit{IS6110} element were not considered critical for the proposed OPPCR assay, since its aim is not the detection but the differentiation of the main pathogens that constitute the \textit{M tuberculosis} complex, ie, \textit{M tuberculosis} from \textit{M bovis}. Moreover, the fact that \textit{IS6110} is recognized as the main target region for the fingerprinting of \textit{M tuberculosis}\textsuperscript{36} justifies its incorporation in the OPPCR assay for reasons of reliability and compatibility with other studies. The \textit{IS1110} element on the other hand has been incorporated for the detection of \textit{M avium} complex. Its specificity for this group of mycobacteria has been described previously,\textsuperscript{37,38} while a previous study has demonstrated its stability in reference strains and in strains isolated from the same patients several months apart.\textsuperscript{39}

Finally, for \textit{M avium}, \textit{M intracellulare}, and \textit{M paratuberculosis}, we selected the 16S rRNA gene, which is highly conservative\textsuperscript{27} but allows combined detection of the 3 species with PCR and their further differentiation with DNA hybridization.

The sensitivity of the proposed diagnostic procedure allows reliable examination of FFPE samples containing a very small number of mycobacterial cells. Although other molecular methods exhibit comparable sensitivity levels \textit{Table 4}, the described process yields a more descriptive diagnosis with regard to the infectious species. The sensitivity of the applied method reached higher levels when the starting material consisted of pure cultures rather than FFPE specimens, a finding probably attributable to the small mycobacterial/human DNA ratio commonly obtained by the latter samples.\textsuperscript{43} In support of this argument, we noted that sensitivity was improved when we chose sections from the parts of the paraffin block with the characteristic lesions instead of using the entire block. False-positive results have been observed by many researchers, mainly due to carryover mycobacterial contamination.\textsuperscript{19} The use of HPLC-quality water, the incorporation of UDG in the reaction mixtures, and the use of dedicated space and disposable material for DNA extraction and PCR proved effective for eliminating this possibility. False-negative results, most likely due to the presence of paraffin or other PCR inhibitors in extracted DNA, low DNA yield, or DNA fragmentation, were generated by a very small number of samples. Although less commonly compared with sputum preparations,\textsuperscript{17} PCR inhibitors constitute a significant drawback in the processing of FFPE specimens. Fragmentation of DNA often is caused by the fixation procedure.\textsuperscript{44,45} Intense mixing, high-speed centrifugation, and heat shocks may further impair the quality of extracted DNA. Therefore, cautious handling of the samples was practiced during the extraction procedure, whereas DNA quality mainly was assessed with a PCR assay targeted to a relatively large region of the p53 gene.\textsuperscript{18} This method of quality testing was preferred to the use of an internal marker control since this would cause a substantial cost increase for the assay and make the results more

\begin{table}[h]
\centering
\caption{Diagnostic Interpretation of the Combinations of Results Produced by the Proposed Combination of PCR and DNA Hybridization Assays} \label{tab:diag_interpretation}
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\textbf{Mycobacterium Species} & \textbf{PCR} & & & \textbf{Outward-Primed PCR} & \textbf{Dot Blotting} \\
\hline
\textit{M tuberculosis} complex\textsuperscript{1} & + & - & - & + & - \\
\textit{M intracellulare} & - & + & - & - & - \\
\textit{M avium} complex\textsuperscript{1} & - & + & - & - & - \\
\textit{M paratuberculosis}\textsuperscript{1} & - & + & + & - & + \\
\textit{M tuberculosis} & - & + & - & - & + \\
\hline
\end{tabular}
\end{table}

\textsuperscript{1} Includes \textit{M avium} MAIS2, \textit{M avium} serovar 7, \textit{M avium} WP M21, \textit{M paratuberculosis} Linda, \textit{M paratuberculosis} C286, and \textit{M paratuberculosis} ATCC 19698.

\textsuperscript{2} ATCC, American Type Culture Collection: BCG, bacille Calmette-Guérin; PCR, polymerase chain reaction; +, positive; −, negative.

\textsuperscript{3} \textit{PR1-2}, \textit{M16SU-D}, \textit{Pr3-4}, and \textit{FINGA-B} are primers; \textit{PRA VMPAR} is a probe.

\textsuperscript{4} \textit{M. microti} A TCC 19698, \textit{M paratuberculosis}§ C286, and \textit{M paratuberculosis} ATCC 19698.

\textsuperscript{5} Includes \textit{M. avium} MAIS2, \textit{M avium} serovar 7, \textit{M avium} WP M21, \textit{M paratuberculosis} Linda, \textit{M paratuberculosis} C286, and \textit{M paratuberculosis} ATCC 19698.
difficult to interpret, because various oligonucleotide primers are expected to show different sensitivity to inhibition and amplification46 and because the proposed procedure already involves several PCR products.

Previous studies using OPPCR as a typing method for M tuberculosis isolates have reported cross-binding of primers to genomic regions of mycobacteria that do not belong to M tuberculosis complex.29,47 To eliminate this risk, larger primers were designed, capable of specific binding under stringent conditions. As expected,43 this modification caused a slight decrease in the differentiating capacity of the assay, yet only at the strain level, which was not considered inconsistent with the goals of the present study. Because transposition events of IS6110 are rare, OPPCR product patterns are expected to be highly repetitive.25 The quality and the total amount of mycobacterial DNA were proven essential for the results to be reliable (stabilize the specific bands, patterns adequately visible) and, most important, repeatable. Several tests with different amounts of DNA indicated that good DNA quality (as indicated by OD counts, electrophoresis, and PCR) from FFPE samples with at least 1,000 bacterial cells per milliliter was the lower limit for highly repeatable fingerprinting patterns. Therefore, to incorporate a rough measure that would be easily applicable in practice, it was considered preferable to apply this assay only on Ziehl-Neelsen–positive samples to avoid misleading evidence from clinical material.

Evaluation of reference and clinical material was based on the results of molecular assays, Ziehl-Neelsen staining, histologic examination, and microbiologic cultivation. Of the 71 reference M tuberculosis complex strains examined, MPB64-targeted PCR generated a positive result in all but 1 M africanum strain, possibly a mutant one, which yielded no product in OPPCR either. Samples with high mycobacterial load gave almost identical results with all aforementioned techniques. However in group 1, we recorded 4 Ziehl-Neelsen–positive, PCR- and OPPCR-negative, samples (Table 2). This finding could be due to the presence of acid-fast bacteria, other than mycobacteria, a notion supported by the weak Ziehl-Neelsen staining of these specimens. For samples with small numbers of mycobacterial cells, Ziehl-Neelsen staining was the least efficient method of detection, while PCR proved more sensitive than culture for M avium, M intracellulare, and M paratuberculosis (Table 2). As far as these species are concerned, microbiologic and molecular identification resulted in some discrepancies attributable to the genetic diversity of the M avium complex, which also has been the cause of speculation26,27 in accordance with current nomenclature. Thus, the M avium complex includes strains that belonged to M paratuberculosis, while mycobactin dependence that was originally considered an exclusive characteristic of the latter species is now attributed to members of the M avium complex as well as to M avium subsp silvaticum and the wood pigeon bacillus.48 Notably, results of PCR analysis were negative in 16 (21%) of 75 group 1 and 13 (26%) of 50 group 4 samples, despite the existence of strong histologic evidence of tuberculosis. Evidently, only a proportion of the samples with histologic evidence of tuberculosis carry mycobacteria, the presence of which may be confirmed by PCR, Ziehl-Neelsen staining, or culture. A considerable number of samples in groups 1 and 4 were positive by PCR for mycobacteria other than those of the M tuberculosis complex (Table 2), a finding that contrasts with some reports in the literature.49 However, it should be taken into consideration that the samples of our groups were selected as strongly suggestive of tuberculosis based on histologic and clinical examination, and, hence, they are not representative of a random population.

The role of mycobacteria and the species involved in sarcoidosis remains unclear. Some researchers have

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Table 4

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Sample</th>
<th>DNA Target Area</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shah et al, 199540</td>
<td>Q-beta replicase probe</td>
<td>Sputum</td>
<td>IS6110</td>
<td>0.023-0.23 cfu/mL</td>
</tr>
<tr>
<td></td>
<td>PCr</td>
<td>Culture</td>
<td>IS6110</td>
<td>10^5 cfu/mL</td>
</tr>
<tr>
<td></td>
<td>O-beta replicase probe</td>
<td>Sputum</td>
<td>23S rRNA</td>
<td>1-10 cfu</td>
</tr>
<tr>
<td></td>
<td>O-beta replicase probe</td>
<td>Sputum</td>
<td>23S rRNA</td>
<td>10-1,000 cfu/mL</td>
</tr>
<tr>
<td></td>
<td>Amplicof^‡ PCR</td>
<td>Sputum</td>
<td>16S rRNA</td>
<td>100 fg or 5-10 bacteria</td>
</tr>
<tr>
<td></td>
<td>IM-PCR‡</td>
<td>Intestinal content</td>
<td>16S rRNA</td>
<td>100 fg or 5-10 bacteria</td>
</tr>
<tr>
<td></td>
<td>PCR and Southern blotting</td>
<td>Biopsy</td>
<td>IS6110</td>
<td>1 bacterium</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>FFPE</td>
<td>IS6110</td>
<td>1-3 bacteria</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>FFPE</td>
<td>IS1110</td>
<td>1-3 bacteria</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>FFPE</td>
<td>16S rRNA</td>
<td>6-10 bacteria</td>
</tr>
<tr>
<td>Marchetti et al, 199842</td>
<td>PCR and Southern blotting</td>
<td>FFPE</td>
<td>MPB64</td>
<td>8 bacteria</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>FFPE</td>
<td>IS1110</td>
<td>8 bacteria</td>
</tr>
<tr>
<td></td>
<td>OPPCR</td>
<td>FFPE</td>
<td>IS6110</td>
<td>20-30 bacteria</td>
</tr>
</tbody>
</table>

cfu, colony-forming units; FFPE, formalin-fixed paraffin-embedded tissue; OPPCR, outward-primed polymerase chain reaction; rRNA, ribosomal RNA

1 Expressed in the units reported in the reference.
2 Roche Diagnostic Systems, Athens, Greece.
3 Immunomagnetic PCR.
4 Dot blotting/chemiluminescent detection.
suggested the exclusive implication of members of the \textit{M. avium} complex in disease development,\textsuperscript{3,41} while others have proposed a role for pathogens of the \textit{M. tuberculosis} complex.\textsuperscript{4,23,37,50} Based on the former reports, the present study focused on the detection of \textit{M. avium} complex strains from cases with histologic evidence of sarcoidosis. However, the proposed assays allowed the detection of only \textit{M. tuberculosis} complex from patients in the sarcoideal group (group 2). As opposed to PCR-negative cases, those that produced a positive PCR result reacted poorly to immunosuppressive therapy, pointing toward an interference of mycobacterial infection with the applied treatment. In a previous study, Ikonomopoulos et al\textsuperscript{16} detected slowly growing mycobacteria that did not belong to the \textit{M. tuberculosis} or the \textit{M. avium} complexes in biopsy specimens with typical lesions of sarcoidosis. By all means, the data presented in the latter and the present study are inconclusive about the role of mycobacteria in the disease. Further analysis is required to establish the Koch postulates for the implication of a mycobacterial species in sarcoidosis.

Crohn disease of humans seems to have many similarities with Johne disease of animals, for which \textit{M. paratuberculosis} has been identified as the responsible pathogen. Although some reports have implicated \textit{M. paratuberculosis} in the pathogenesis of Crohn disease, its cause remains elusive.\textsuperscript{51,52} In the present study, the results obtained by PCR and DNA hybridization using the PRAVMPAR probe showed that DNA belonging to \textit{M. paratuberculosis} was detected in 35\% (7/20) of the FFPE samples derived from patients with Crohn disease (group 3). From the samples of group 4, we were able to demonstrate the \textit{M. paratuberculosis} DNA in 4 of 6 intestinal biopsy specimens obtained from a patient with ulcerative necrotic colitis (Table 2). This patient originally was given a diagnosis of necrotic angiitis from a patient with ulcerative necrotic colitis (Table 2). This patient originally was given a diagnosis of necrotic angiitis and underwent surgery, after which he was treated with corticosteroids. However, the failure of the corticosteroid treatment that was administered initially to this patient implies that the detection of \textit{M. paratuberculosis} and the improvement in the patient’s health after antibiotic administration were hardly circumstantial.

The proposed molecular method can be applied directly to archival tissue material and allows the differential detection of genetically related mycobacterial pathogens in more detail than most diagnostic procedures commonly used. Thus, the method constitutes a powerful and descriptive tool for the etiologic study of cases of tuberculosis, sarcoidosis, and Crohn disease, producing easily interpretable results within 12 hours of sample processing.

References


