PCR based detection of mycobacteria in paraffin wax embedded material routinely processed for morphological examination

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Abstract

Background—The incidence of mycobacterial infections has increased during the past five years. A prompt diagnosis is indispensable for initiating appropriate treatment. Because culturing of mycobacteria takes three to six weeks and sensitivity of microscopic detection of acid fast bacilli is low, amplification methods provide promising possibilities. Recently, the polymerase chain reaction (PCR) has been shown to be useful for confirming a mycobacterial infection, especially in cases with unexpected histological findings or lack of suitable material for culturing.

Aims—To evaluate the impact of PCR based techniques in the detection of mycobacterial infections in uncultured routine histological specimens as an alternative to surgical pathology.

Methods—Two hundred and twenty nine formalin fixed and paraffin wax embedded samples from 141 patients with clinical or histological suspicion of a mycobacterial infection were investigated using three different PCR assays and Southern blotting. PCR results were compared with histology and culture and the patients' clinical findings.

Results—When using culture as the reference method, the sensitivity for the detection of mycobacteria of the tuberculosis complex was 90%, specificity was 92%, the positive predictive value was 81%, and the negative predictive value was 96%. The sensitivity for the detection of non-tuberculous mycobacteria was 100% and specificity was 78%, the positive predictive value was 26%, and the negative predictive value was 100%. The patients' clinical findings supported the PCR positive results, indicating a mycobacterial infection in 11 of 18 initially culture negative cases and in 21 of 35 PCR positive cases without culture results.

Conclusions—These results indicate that PCR based techniques are sensitive, specific, and rapid methods for the detection of mycobacteria in routinely processed paraffin wax embedded and formalin fixed histological samples.

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Keywords: polymerase chain reaction; mycobacteria; paraffin wax embedded samples

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In the past five years, the incidence of infections with mycobacteria of the tuberculosis complex and atypical mycobacteria has increased dramatically, both in industrialised and developing countries. In 1996, three million people died of tuberculosis and there were more new cases (eight million/year) than ever before.[1 2] The main reasons for the growing epidemic of mycobacterial infections in industrialised countries are human immunodeficiency virus (HIV) infections, immigration from countries with a high prevalence of tuberculosis, immunosuppression treatments, and multidrug resistant mycobacteria.[1 2] Rapid, specific, and sensitive methods for the detection of mycobacterial infections are essential for their diagnosis and effective treatment. Culture in liquid and solid media remains the gold standard, but it takes three to eight weeks to obtain a result, and the microscopic detection of acid fast bacilli requires large numbers of bacteria (10^4/ml) in the sample.[3] Particularly in cases with unexpected histopathological suspicion of a mycobacterial infection, there is often no material left for culturing, so that diagnosis depends on a morphological examination and microscopic detection of acid fast bacilli. Therefore, the development of more rapid and reliable methods for the detection of mycobacteria is an important goal.

Hance and colleagues[4] first described the polymerase chain reaction (PCR) based amplification of a 383 bp mycobacterial DNA fragment of the 65 kDa heat shock protein (HSP). Since then, a wide range of molecular amplification techniques has been developed for the detection of mycobacteria, including the amplification of DNA encoding mycobacterial antigens,[5–7] of ribosomal RNA,[8 9] of repetitive sequences of the *Mycobacterium tuberculosis* complex,[10–13] and, recently, of direct repeats.[14]

However, in most cases respiratory specimens, liquid specimens, cultured material, or unfixed tissue samples were examined, and there are few studies on the detection of mycobacteria in paraffin wax embedded, formalin fixed specimens.[15 16] In our study, a combination of three different PCR assays was performed on formalin fixed and paraffin wax embedded material. A 383 bp DNA fragment[4] and an internal 203 bp DNA fragment[17] of the 65 kDa surface antigen gene common to all mycobacteria and a 123 bp DNA fragment of the insertion sequence (IS) 6110, which is specific to mycobacteria of the tuberculosis complex,[10] were amplified.

Table 1 Primer sequences for PCR

Primer	Primer sequence	Gene (product size)	Annealing temperature	PCR cycles	Reference
A1	5'-GAGATCGAGCTGGAGGATCC-3' sense	65 kDa heat shock protein	58°C	30	Hance and colleagues[4]
A2	5'-AGCTGCAGCCCAAAGGTGTT-3' antisense	Primary PCR (383 bp)			
B1	5'TAGCCAAGAAGACCGATGAC-3' sense	65 kDa heat shock protein	54°C	35	Diallo and colleagues[17]
B2	5'-AATCTGCTCCTTGGTCTCGA-3' antisense	Nested PCR (203 bp)			
C1	5'-CCTGCGAGCGTAGGCGTCGG-3' sense	IS 6110 (123 bp)	58°C	35	Eisenach and colleagues[10]
C2	5'-CTCGTCCAGCGCCGCTTCGG-3' antisense				
D1	5'-CAACTTCATCCACGTTCACC-3' sense	β globin (268 bp)	58°C	20	Bauer and colleagues[20]
D2	5'-GAAGAGCCAAGGACAGGTAC-3' antisense				

Since 1992, the PCR based detection of mycobacteria has been firmly established as a diagnostic tool in the routine investigation of histopathological samples suspected of mycobacterial infections at the Gerhard-Domagk Institute of Pathology at the University of Münster.

In our study, a retrospective evaluation of routinely processed cases was performed comparing PCR results with the histological features and clinical data. All 229 samples were derived from routine histomorphological examinations carried out at the institute from 1992 to 1995.

Materials and methods

MATERIALS

Two hundred and twenty nine formalin fixed, paraffin wax embedded samples from 141 patients who, either for clinical (51%) or histological (49%) reasons, were suspected of mycobacterial infection were examined by PCR and Southern blotting. All samples had been processed routinely for morphological examination between 1992 and 1995 at the Gerhard-Domagk Institute of Pathology. For routine histology, samples were stained with haematoxylin and eosin, and with Ziehl-Neelsen for microscopic identification of acid fast bacilli. The results of the microbiological examination of 93 samples taken from 76 patients were available for our study. The samples contained 81 pulmonary and 148 extrapulmonary specimens. The pulmonary samples consisted of 40 bronchus biopsies, 36 bronchoalveolar lavages, and five sputum specimens. The extrapulmonary samples included 45 lymph nodes, 42 skin tissues, 13 samples of the gastrointestinal tract, 11 samples of the nasopharynx and cavity of the mouth, nine bone biopsies, seven samples of the urogenital tract, six abscess punctates, and 15 miscellaneous specimens.

METHODS

Sample preparation for PCR

Formalin fixed, paraffin wax embedded material was cut into 5 μm sections, mounted on glass slides, and dewaxed with xylene. After removing the solvent in a decreasing series of alcohol (99–50% alcohol content),[18] the sample was rehydrated with distilled water and removed from the glass slides with 100 μl digestion buffer (200 μg/ml proteinase K, 50 mM Tris/HCl, pH 8.5, 1 mM ethylenediaminetetraacetic acid, and 0.5% Tween 20). The mixture was incubated at 56°C overnight, followed by inactivation of proteinase K at 94°C for 10 minutes. After the digestion procedure, the sample was submitted to PCR without further purification to prevent loss of DNA.

Primers

Four sets of primers were used for each sample (table 1). Primers A1 and A2 are specific for a 383 bp segment of a 65 kDa mycobacterial surface antigen gene common to all mycobacterial species.[4 19] To increase the sensitivity, internal primers B1 and B2 encoding a 203 bp segment within the 383 bp fragment were chosen.[17] A 123 bp fragment of the repetitive insertion element IS 6110, which is specific to the *M tuberculosis* complex, was amplified using primers C1 and C2.[10] The amplification of a 268 bp fragment of the human β globin gene with the primers D1 and D2 was performed in all samples to test for inhibition and to confirm the suitability of the material for PCR.[20]

Amplification by PCR

The PCR was modified according to the standard protocol of Mullis and Faloona.[21] The reaction mixtures with a final volume of 20 μl included 0.3, 1.0, and 3.0 μl, respectively, of the digested material, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM of each deoxynucleotide trisphosphate, 0.5 U Taq polymerase (AmpliTaq®; Perkin Elmer, Branchburg, New Jersey, USA) and, depending on the amplified fragment, 0.4 pmol/μl of primer pair A and 0.2 pmol/μl of primer pairs B, C, and D. After an initial denaturation step at 94°C for four minutes, amplification was performed under the following conditions. Each cycle consisted of denaturation at 94°C for 30 seconds and primer extension at 72°C for 90 seconds. In the first five cycles, the annealing temperature of the primer pairs A, C, and D was decreased by 1°C every cycle, from 64°C to 61°C, and was followed by 30 cycles for the primer pairs C and D and 35 cycles for the primer pair A, with an annealing temperature of 58°C. In the first nine cycles,

the annealing temperature of the primer pair B was decreased by 1°C every third cycle, from 57°C to 55°C, and followed by an annealing temperature of 54°C for 20 cycles.

Detection of PCR products
To detect the amplified PCR products, 2.5 µl of the reaction mixture was separated electrophoretically on 12% polyacrylamide gels (Gel-
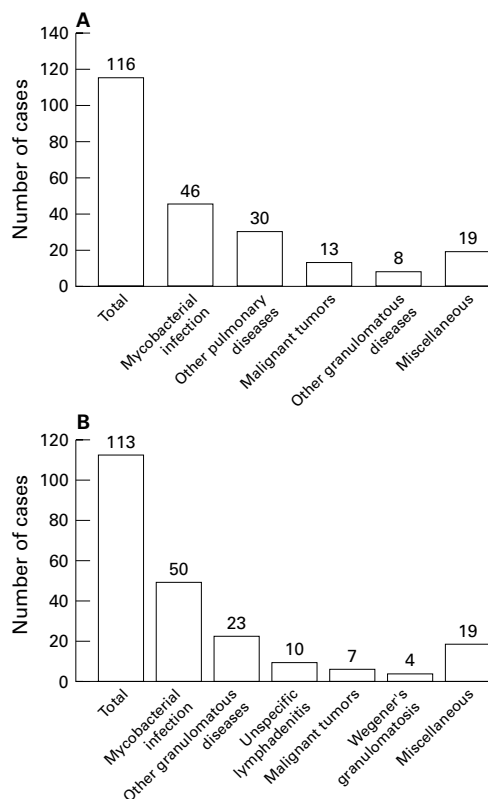

Figure 1 (A) Diagnosis on discharge from hospital of samples with clinical suspicion of a mycobacterial infection on admission to routine pathology. (B) Diagnosis on discharge from hospital of samples with histological suspicion of tuberculosis on admission to routine pathology.
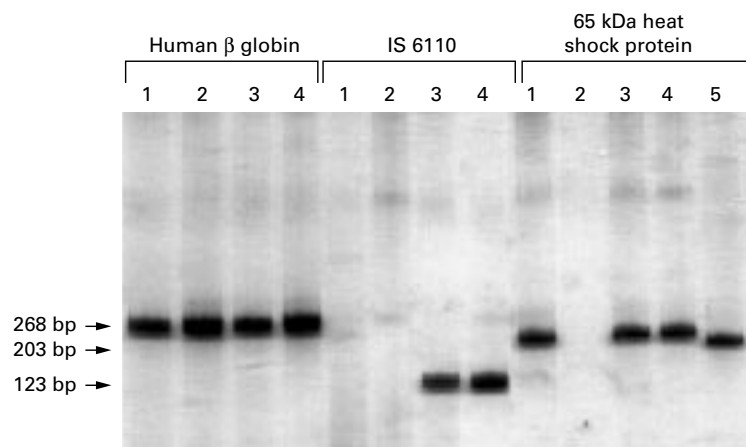

Figure 2 Detection of mycobacterial PCR products, 12% native polyacrylamide gel electrophoresis, silver stained. PCR assays were performed on formalin fixed and paraffin wax embedded lymph nodes from three patients (cases 1–3). Non-tuberculous mycobacterial infection (case 1): detection of 203 bp PCR fragments and no amplification of 123 bp fragments of IS 6110. Mycobacterium tuberculosis complex infection (case 3): detection of fragments of the 65 kDa heat shock protein gene and additional amplification of 123 bp PCR products of IS 6110. No evidence of mycobacterial infection (case 2): no amplification of either 203 bp PCR fragments or 123 bp PCR fragments. The suitability of the material was confirmed by amplification of 268 bp fragments of the human β globin gene.

Bond®-PAG; Bio Products, Rockland, USA) using a thermostatically controlled horizontal electrophoresis system (Pharmacia LKB Multiphor II; Pharmacia, Uppsala, Sweden) and visualised by silver staining.[22]

Southern blotting
The specificity of PCR products was confirmed by Southern blotting, particularly when the PCR amplification products were weak. Negative results in Southern blotting were omitted from the study. PCR products of 123 bp, specific to the *M tuberculosis* complex, and 203 bp PCR products, common to all mycobacteria, were digoxigenin labelled and used as probes.

Aliquots of 3 µl of amplified PCR products were separated electrophoretically on 1% agarose gels containing 20 µl ethidium bromide and visualised by UV transillumination. The DNA was denatured by NaOH and blotted on to nylon membranes (Boehringer, Mannheim, Germany). After prehybridisation (5× saline sodium citrate (SSC), 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulphate (SDS), 1% blocking reagent) at 68°C for one hour, hybridisation with digoxygenin labelled probes was performed at 68°C overnight. Membranes were washed twice with 200 ml wash solution containing 20 ml SSC and 2 ml 0.1% SDS at room temperature for five minutes and at 65°C for 15 minutes. For chemiluminescent detection, antidigoxygenin–alkaline phosphatase (AP) (Boehringer, Mannheim, Germany) was applied, with CSPD as substrate, and membranes were exposed to Kodak scientific imaging film XAR 5 at room temperature for 10–15 minutes

Controls
Each PCR run included positive and negative controls. The positive controls consisted of DNA extracted from cultures of the *M tuberculosis* and *M avium* complexes and a sample of a patient with culture confirmed *M tuberculosis* infection. The negative controls contained the reaction mixture without DNA, which was replaced by the equivalent volume of distilled water.

Results

All the 229 samples were derived from routine histopathological examinations from 1992 to 1995 and belonged to two groups. In 116 of the 229 cases (51%), tuberculosis had been clinically suspected at the time when samples were taken for histopathological examination. In the remaining 113 cases (49%), an unexpected mycobacterial infection had been detected by histology. Figure 1A and B shows diagnoses on discharge from hospital of samples with initial clinical suspicion of mycobacterial infection and of samples with initial histological suspicion of tuberculosis on admission to routine pathology.

All 229 samples were analysed by PCR. Figure 2 shows a silver stained polyacrylamide gel with electrophoretic separation of amplified mycobacterial DNA from three specimens. Figure 3 shows an example of a Southern blot experiment to confirm specificity of a PCR
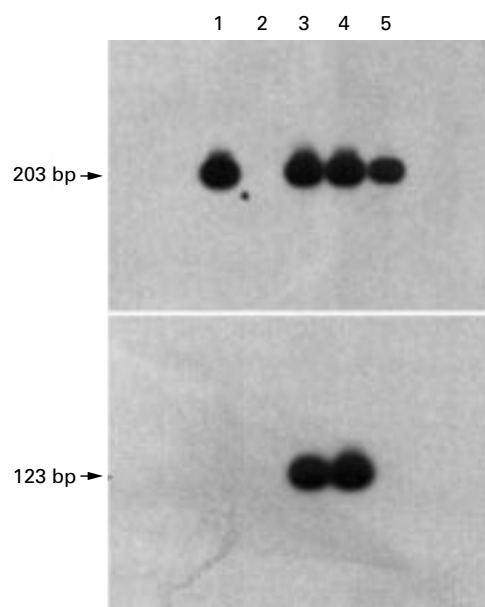
Figure 3 Non-radioactive Southern blot. The specificity of the PCR products obtained from case 1 and case 3 was confirmed by Southern blotting. The probe corresponding to 203 bp fragments of the 65 kDa heat shock protein gene resulting in signals in both cases, whereas positivity with the 123 bp probe was found only in case 3, indicating an infection with mycobacteria of the tuberculosis complex.

product. Nineteen samples lacking amplification of the β globin fragment were not suitable for PCR. In 93 cases, microbiological data from cultures were available. Fifty of those 93 samples were cultured both for the *M tuberculosis* complex and non-tuberculous mycobacteria. The remaining 117 samples were formalin fixed and paraffin wax embedded without further microbiological examination. Clinical findings, treatment results, and follow up data were available from all patients so that they could be compared with the PCR results. Tables 2 and 3 show all the PCR results in correlation with culture results.

Table 2 Results of PCR amplification of 123 bp DNA sequences of IS 6110 in mycobacteria of the tuberculosis complex (contingency table)

	Culture results			
PCR	Positive	Negative	Not cultured	Total
Positive	18	4	13	35
Negative	2	50	82	134
Total	20	54	95	169

Sensitivity, 90% (18 of 20); specificity, 92% (50 of 54); positive predictive value, 81% (18 of 22); negative predictive value, 96% (50 of 52).

Table 3 Results of amplification of DNA fragments of the 65 kDa heat shock protein gene by nested PCR in non-tuberculous mycobacteria (contingency table)

	Culture results			
PCR	Positive	Negative	Not cultured	Total
Positive	5	14	22	41
Negative	0	50	82	132
Total	5	64	104	173

Sensitivity, 100% (5 of 5); specificity, 78% (50 of 64); positive predictive value, 26% (5 of 19); negative predictive value, 100% (50 of 50).

PCR results were positive in 18 of 20 cases with culture confirmed *M tuberculosis* infection. The remaining two culture positive samples, a lymph node and a bone biopsy, were PCR negative.

The mycobacterial cultures were negative in 54 specimens, including 11 cases with strong clinical suspicion of tuberculosis. In 50 of these samples, PCR results were negative. In the remaining four culture negative cases, fragments of the *M tuberculosis* complex could be amplified by PCR and confirmed by Southern blotting. The clinical data of these samples taken from four patients confirmed the suspicion of tuberculosis. Two of those patients who were HIV positive received tuberculostatic treatment, whereas the other two patients were not treated because of their culture negative results. The diagnoses on discharge from hospital of these two patients were bronchial carcinoma and skin sarcoidosis. Further investigations confirmed a squamous non-small cell carcinoma of the right upper lobe in the first patient and extrapulmonary features of sarcoidosis of skin, eyes, liver, and spleen in the second patient.

In all five samples with culture confirmed infections with non-tuberculous mycobacteria, the 383 bp and 203 bp fragments were detected by PCR. The clinical data confirmed an infection with the *M avium* complex in samples from two HIV positive patients.

Fifty of 64 specimens with a culture negative result for non-tuberculous mycobacteria were also PCR negative. In the remaining 14 culture negative cases, DNA fragments of non-tuberculous mycobacteria could be amplified by PCR. These 14 samples were from 14 different patients and consisted of eight bronchoalveolar lavages, three skin specimens, one lymph node, one liver biopsy, and one abscess punctate. In nine of these patients, an infection with atypical mycobacteria was clinically confirmed and treated successfully. The remaining five patients did not show any clinical signs of a mycobacterial infection. Three of the samples were bronchoalveolar lavages and one sample was a skin biopsy from a patient who had been treated for cutaneous tuberculosis for six months. One skin specimen came from a patient with a papulonecrotic tuberculid, yet no infectious tuberculosis has been found.

One hundred and seventeen of the initially formalin fixed and paraffin wax embedded samples could not be analysed by culture, but were suitable for PCR. Eighty two of these samples were PCR negative. In all the remaining 35 samples, DNA fragments of the gene encoding the 65 kDa HSP were amplified. In addition, DNA fragments specific to the *M tuberculosis* complex were detected by PCR in 13 of these 35 specimens. All PCR results of the 117 uncultured samples were compared with the clinical data and the histopathological findings.

In 10 of 13 uncultured samples with PCR positive results for the *M tuberculosis* complex, tuberculosis was suspected during the further assessment of the course of diseases, resulting

in tuberculostatic treatment in all 10 patients. For the remaining three samples, the diagnoses on discharge were bronchial carcinoma in two samples from one patient and Hodgkin's disease in another sample.

An infection with atypical mycobacteria was confirmed in the clinical data of 11 of the 22 uncultured samples that had been PCR positive for non-tuberculous mycobacteria. Five samples came from patients who were immunocompromised as a result either of HIV infections (three patients) or cyclosporin A treatment after heart and renal transplantation (one patient each). All 11 cases were treated successfully with tuberculostatic treatment. An infection with non-tuberculous mycobacteria could not be clinically diagnosed in the remaining 11 cases, although in four of these samples histopathology revealed an infection with atypical mycobacteria. Two specimens came from two patients who were immunocompromised as a result of cyclosporin A treatment.

The contingency tables 2 and 3 show that when culture was used as the reference method for PCR, sensitivity for the detection of the *M tuberculosis* complex by amplification of DNA fragments of IS 6110 was 90% (18 of 20) and specificity 92% (50 of 54). The positive predictive value was 81% (18 of 22) and the negative predictive value was 96% (50 of 52). Sensitivity for the detection of non-tuberculous mycobacteria by nested PCR amplifying DNA sequences of the 65 kDa HSP gene was 100% (five of five) and specificity 78% (50 of 64). The related positive predictive value was 26% (five of 19) and the negative predictive value was 100% (50 of 50).

Discussion

Tuberculosis had been the clinical differential diagnosis in only 51% (116 samples) of the cases submitted to routine histopathological examination. In 49% (113 samples) of the cases, mycobacterial infections had been found unexpectedly by histology. Before admission to routine pathology, these samples had been clinically suspected to be carcinomas, malignant lymphomas, sarcoidosis, or cervical masses with unknown causes. Hence, there was often no material left for mycobacterial culturing. Because microscopic detection of acid fast bacilli by Ziehl-Neelsen staining is often insufficiently sensitive and specific to confirm a histopathological diagnosis, we introduced PCR based techniques, which have become increasingly valuable for the identification of mycobacteria in tissue routinely processed for histopathological examination.

In our study, 229 formalin fixed and paraffin wax embedded samples were analysed by PCR and Southern blotting. Internal control by PCR amplification of the human β globin gene revealed a failure of PCR in 19 samples (8%). All 117 samples without culture information were suitable for PCR. PCR was inhibited in 19 samples. As Chan *et al* have reported previously, inhibitors were detected more frequently in extrapulmonary than in pulmonary specimens.[23] PCR inhibition might be caused

by degradation of DNA by formalin fixation[24 25] of tissue samples and acid decalcification methods for bone biopsies. However, the suitability of 91% of the routinely processed material provides an encouraging basis for molecular genetic analysis.

Mycobacteria of the tuberculosis complex were detected by PCR in 18 of 20 culture positive samples. The two PCR negative samples comprised a bone biopsy and a lymph node. Such a negative PCR result might be attributed to several conditions. As shown for lymph nodes, non-homogenous distribution of mycobacteria results in sections without bacteria.[26] Therefore, in many cases a positive result cannot be attained unless additional sections are analysed. Furthermore, the feasibility of PCR might be hampered by tissue type. In particular, bone specimens (as in the second negative case) are often difficult to process for DNA extraction.

In 50 of 54 cases, PCR results corresponded with negative cultures. The clinical data of the four PCR positive cases confirmed the suspicion of tuberculosis in two HIV positive patients, thus initiating successful tuberculostatic treatment.

The remaining two patients were not treated because of negative cultures and a diagnosis of bronchial carcinoma in one patient and skin sarcoidosis in the other. Positive PCR results in combination with negative cultures in patients with carcinoma have been described previously.[27 28] Mycobacterial DNA might be released from the destroyed tissue.[27] In this case, DNA of non-viable mycobacteria could have been amplified, yielding positive PCR results and negative cultures. The detection of mycobacterial DNA by PCR in culture negative cases with sarcoidosis has been reported previously and it has been suggested that mycobacteria might be involved in the pathogenesis of sarcoidosis.[29–31] Popper *et al* suggested that cell wall defective mycobacteria or persistent intracellular DNA from mycobacteria might be the causual agent in some cases of sarcoidosis.[32]

Although there is compelling evidence that amplification methods are more sensitive than culture,[27] especially in extrapulmonary specimens,[33] culture is still the gold standard.

Therefore, culture was used as the reference method for the sensitivity of PCR, the detection of the *M tuberculosis* complex being 90% and the corresponding specificity 92%.

Culture negative results for atypical mycobacteria corresponded to negative PCR results in 50 of 64 cases, whereas 14 samples were PCR positive for the detection of non-tuberculous mycobacteria. An infection with atypical mycobacteria had been clinically diagnosed and treated with antituberculosis drugs in nine of these 14 patients. The remaining five patients showed no clinical findings indicative of an infectious mycobacteriosis. One of the five patients had been treated for a cutaneous tuberculosis. During tuberculostatic treatment, PCR results often remain positive for several weeks after cultures become negative, which is why amplification of non-viable mycobacteria

could explain the positive PCR result in this sample. On the other hand, mycobacteria in treated tuberculosis may remain viable, even though their growth is inhibited on culture media, whereas mycobacterial DNA sequences continue to be amplified by PCR.

Another patient had a papulonecrotic tuberculid. Degitz reported that mycobacteria might not be cultured from tuberculoids because they do not grow in these lesions, whereas their DNA may still be detected by PCR.[34]

Eight of the 14 samples with positive PCR results for non-tuberculous mycobacteria in combination with negative cultures were bronchoalveolar lavages. The most likely explanation for these results is that because of the high sensitivity of amplification methods, even small numbers of mycobacteria are detectable by PCR. It has also been reported that contamination of bronchoscopes with mycobacteria of the tuberculosis complex and non-tuberculous mycobacteria could lead to positive PCR results.[35][36]

Using culture as a reference method the sensitivity for the detection of non-tuberculous mycobacteria by PCR was 100%. The corresponding specificity of 78% might be explained by the difficulty in culturing non-tuberculous mycobacteria.

In summary, the patients' clinical charts supported the diagnosis of mycobacterial infections in 11 of 18 initially PCR positive results with negative cultures and in 21 of the 35 PCR positive cases without cultures.

Culturing 117 samples was impossible, either because of a lack of suitable material or unexpected histopathological diagnoses. The detection of mycobacteria by PCR in these samples was useful in confirming a histological or clinical diagnosis. In 55 of 229 samples (24%) processed routinely for morphological examination, mycobacterial infections were detected by PCR, 55% of which were caused by the *M tuberculosis* complex and 45% by non-tuberculous mycobacteria. Especially in cases with unexpected histological findings and no suitable material left for culturing, PCR was helpful for confirming a mycobacterial infection. Therefore, in combination with clinical findings, PCR could be of great value for initiating appropriate treatment.

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