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Diagnosis of Mycobacterial Infections by Nucleic Acid Amplification: 18-Month Prospective Study

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We have investigated the use of DNA amplification by PCR for the detection of mycobacteria in clinical specimens, with the gene encoding the 16S rRNA as a target. Following generic amplification of mycobacterial nucleic acids, screening was done with genus-specific probe; this was followed by species differentiation by use of highly discriminating probes or nucleic acid sequencing. In a prospective 18-month evaluation, criteria to select specimens for PCR analysis were defined. Of a total of 8,272 specimens received, 729 samples satisfied the criteria and were subjected to DNA amplification. Clinical specimens included material from the respiratory tract (sputa and bronchial washings), aspirates, biopsies, and various body fluids (cerebrospinal, pleural, peritoneal, and gastric fluids). After resolution of discrepant results, the sensitivity of the PCR assay was 84.5%, the specificity was 99.5%, the positive predictive value was 97.6%, and the negative predictive value was 96.4%. The sensitivity and negative predictive value of culture (with a combination of broth and solid media) were 77.5 and 94.8%, respectively. In conclusion, this PCR assay provides an efficient strategy to detect and identify multiple mycobacterial species and performs well in comparison with culture.

The most common means of laboratory diagnosis of mycobacterial infections relies on the microscopic examination of a stained specimen followed by culturing (17, 28). The recognition of acid-fast bacilli in smears is by far the most rapid and cost-effective detection method, but because of the lack of both specificity and sensitivity, any suspected mycobacterial infection has to be confirmed by culture. Because of the fastidious growth of mycobacteria, culture-based detection methods may require several weeks (28).

Nucleic acid-based amplification strategies offer great promise for the rapid detection of mycobacteria in clinical specimens. Most reports describing the use of molecular amplification techniques for detection of mycobacterial infections have suffered from two restrictions: the clinical samples investigated were mainly limited to material obtained from the respiratory tract, and the detection assays were restricted to *Mycobacterium tuberculosis* (1, 4, 7, 9, 10, 12, 16, 20, 23, 24).

During the last 2 decades, infections with nontuberculous mycobacteria have become increasingly important (5, 8, 11, 30, 31). In addition, mycobacterial infections are not limited to the respiratory tract but may involve other organs, e.g., the lymph nodes, meninges, skin, liver, and kidneys (31). Given the existence of multiple mycobacterial pathogens which may cause similar diseases, e.g., lymphadenitis in children, pneumonia, and disseminated infections in AIDS patients, a species-specific assay for the detection of mycobacterial infections would be cumbersome. Likewise, a molecular assay which allows the detection of *M. tuberculosis* only and not other pathogenic mycobacteria would be of limited value.

The gene encoding the 16S rRNA has been identified previously as a target which allows the detection and identification of multiple mycobacterial pathogens by using a single amplification reaction. The 16S rRNA gene is a suitable target for amplifying mycobacterial nucleic acids at the genus level, confirming correct amplification by genus-specific probes, and differentiating at the species level with a set of species-specific probes or by sequence analysis (3, 18).

Given the current bottlenecks of gene amplification techniques, i.e., significant costs and the amount of labor necessary, these techniques have to be restricted to selected samples. Criteria which will help to implement this type of methodology as a valuable adjunct in the clinical laboratory have to be developed and defined.

Using a variety of clinical samples, including, e.g., sputum, bronchoalveolar lavage, cerebrospinal fluid, and biopsies, we conducted an 18-month prospective evaluation of a molecular assay which targets the 16S rRNA gene, allowing the detection of multiple mycobacterial pathogens in a single amplification reaction. The purpose of this study was to establish criteria for PCR analysis of clinical samples and to evaluate the performance and clinical usefulness of both the molecular assay and the selection criteria.

MATERIALS AND METHODS

Clinical samples. Samples were obtained from the mycobacteriology laboratory of the Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover. Samples included sputum, tracheal aspirate, bronchial washing, cerebrospinal fluid, urine, pleural fluid, gastric fluid, aspirate, peritoneal fluid, and biopsy specimens (Table 1).

Decontamination and preparation of samples. With the exception of cerebrospinal fluid and biopsies, all samples were decontaminated for culture; spinal fluid, urine, and other fluids were first centrifuged and then resuspended in 1 ml of the respective fluid. For PCR analysis all samples except biopsies were decontaminated. Samples were processed for decontamination within 24 h of receipt; samples for PCR were stored at −20°C until analysis following the decontamination procedure.

Samples were processed by the *N*-acetyl-L-cysteine (NALC)-NaOH method (28). An equal volume of NALC-NaOH solution (2% NaOH, 1.45% sodium citrate, 0.5% NALC) was mixed with the specimen and incubated at room temperature for 20 min. Phosphate buffer (67 mM, pH 6.8) was added, and the mixture was centrifuged (3,500 × *g*) for 25 min. Excess fluid was poured off, and the sediment was resuspended in 1.0 ml of the phosphate buffer; 0.8 ml of this suspension was used for microscopy and routine culture, while 0.2 ml was used for PCR.

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TABLE 1. Specimen sources and culture and PCR results

Specimen	Study part	No. of:				
		Specimens	Culture-positive specimens	PCR-positive specimens	False-negative culture results	False-negative PCR results
Upper respiratory tract (e.g., sputa, tracheal aspirates, bronchial washings)	A	208	49	62	18	6
	B	198	17	21	8	4
	Total	406	66	83	26	10
Cerebrospinal fluid	A	24	3	3	1	0
	B	12	0	0	0	0
	Total	36	3	3	1	0
Biopsy[a]	A	90	14	14	1	2
	B	65	7	9	3	2
	Total	155	21	23	4	4
Urine (n = 29), pleural fluid (n = 35), gastric and duodenal fluids, (n = 23), peritoneal fluid (n = 5), bone marrow aspirates (n = 9), other aspirates[b] (n = 32)	A	90	13	11	1	4
	B	42	7	3	0	4
	Total	132	20	14	1	8

[a] Skin (n = 35), bone marrow (n = 22), lymph node (n = 20), gastrointestinal tract (n = 17), lung (n = 11), pleura (n = 7), liver (n = 6), necropsy material (n = 16), and others (n = 21).

[b] mainly from the lymph node, subcutis, liver, bone, and joint.

Biopsies were split in half; for culture the material was homogenized with a glass pestle and processed without decontamination, and for PCR homogenization was achieved by cutting the material into short pieces and subsequently using glass beads in combination with guanidium isothiocyanate (see below). Similarly, cerebrospinal fluid specimens were split in half and centrifuged if appropriate. One half was used to inoculate a culture directly, while the other half was subjected to decontamination and resuspended in 0.2 ml of phosphate buffer for PCR analysis.

Microscopy and routine culture. All samples were microscopically examined by auramine-rhodamine fluorochrome staining (magnification, ×400) after being concentrated (28). Slides were air dried, passed through a burner flame as for other bacteriological smears, and incubated at 120°C for 10 min. Positive results were confirmed by Ziehl-Neelsen staining (magnification, ×1,000), the results of which were reported as ± (1 or 2 acid-fast bacilli [AFB] in 300 fields), + (1 to 9 AFB in 100 fields, ++ (1 to 9 AFB in 10 fields, or +++ 10 or more AFB in 10 fields).

Culture was performed according to standard techniques (28) by inoculating a Löwenstein-Jensen slant (Bio-Merieux) and a liquid radiometric broth (BACTEC 12B broth supplemented with PANTA PLUS; Becton-Dickinson) with 0.2 and 0.5 ml of the material, respectively. Cultures were incubated at 37°C for 8 weeks. Cultured mycobacteria were identified from the BACTEC 12B broth or the Löwenstein-Jensen slants by sequence analysis of the 16S rRNA gene (19). All positive BACTEC broths were subcultured on slants to allow for recognition of mixed infections.

Selection criteria. The following criteria were defined to select samples for PCR analysis: (i) smear positivity, to allow for rapid differentiation as well as recognition of microscopy artifacts; (ii) defined risk groups, i.e., immunocompromised patients (immunodeficiency induced by drug use, transplantation, or human immunodeficiency virus infection), immigrants and refugees from countries with a high morbidity of tuberculosis, elderly patients with a history of tuberculosis, and drug abusers and homeless people; (iii) sample origin, i.e., samples which were difficult to obtain, such as biopsies and aspirates; and (iv) clinical criteria, i.e., probability of disease, defined as clinical suspicion as judged by the physician in charge (not investigations which were performed routinely). Materials investigated included sputum, tracheal aspirate, bronchial washing, pleural fluid, gastric fluid, urine, cerebrospinal fluid, aspirate, peritoneal fluid, and biopsy specimens (Table 1). Blood and stool specimens were excluded from this study because these materials frequently contain inhibitors of the amplification reaction.

Purification DNA from clinical samples. The decontaminated material (0.2 ml) was inactivated by being heated for 10 min at 80°C. Nucleic acids were prepared essentially as described previously (18). In brief, following centrifugation, the pellet from the decontaminated sample or from biopsies which were previously reduced into small pieces by the use of sterile scissors was resuspended in 400 μl of guanidium isothiocyanate solution prepared as described previously (18). After the addition of glass beads (100-μm diameter; Sigma, Deisenhofen, Germany) and 400 μl of Tris-EDTA-saturated phenol-chloroform-isoamyl alcohol (25:24:1), the sample was placed in a tissue disintegrator (H. Mickle, Gomshall, Surrey, United Kingdom) for 3 min at the maximum speed to disrupt the cells. Following centrifugation and reextraction with phenol-chloroform-isoamyl alcohol (25:24:1), the aqueous phase was transferred to another tube. Nucleic acids were precipitated by adding 0.05 volume of cold 3 M sodium acetate (pH 5.2) and 1.0 volume of cold isopropanol. After being mixed by vortexing, samples were placed for 2 h at −20°C before centrifugation in a microcentrifuge for 20 min. The resulting pellet was washed two times with 70% ethanol (800 μl each), dried, and resuspended in 30 μl of aqua dest. For PCR analysis, a volume of 10 μl was used.

During the second part of the study, a more simple protocol for sample preparation was evaluated by using the Amplicor *M. tuberculosis* Sputum Preparation Kit (Hoffmann LaRoche, Grenzach-Wyhlen, Germany). Essentially the manufacturer's instructions were followed, except that 75 μl each of sputum lysis reagent and neutralization reagent was used (instead of 100 μl as recommended by the manufacturer). For PCR analysis, a volume of 30 μl was used.

Samples prepared with the Amplicor Sputum Preparation Kit and showing the presence of inhibitors of the amplification reaction (see "Controls" below) were subjected to further purification as follows: to 100 μl of sample solution prepared by use of the Amplicor Sputum Preparation Kit, 300 μl of the guanidium isothiocyanate solution (18) was added, and the sample was further processed as described for the guanidium isothiocyanate procedure (see above).

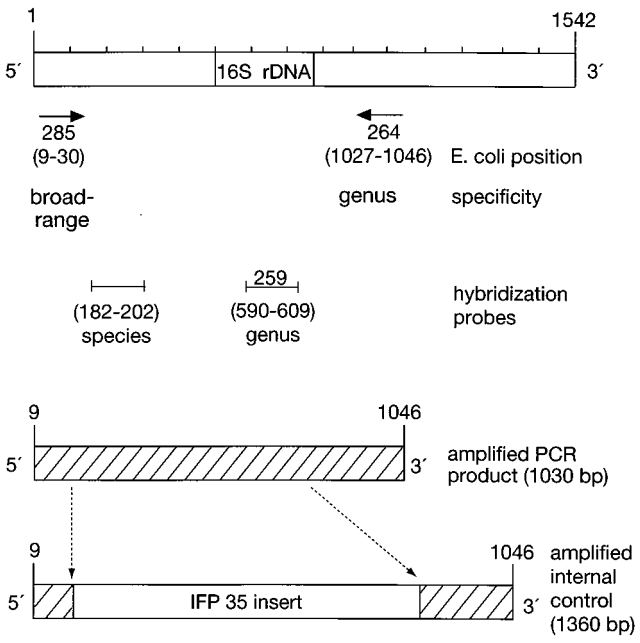
PCR for clinical samples. The PCR was performed with a total volume of 60 μl with 1.5 U of *Taq* polymerase (Perkin-Elmer, Überlingen, Germany), 50 mM KCl, 10 mM tris hydrochloride (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 24 pmol of primer 264 (5′ TGCACACAGGCCACAAGGGA 3′), and 6 pmol of biotinylated primer 285 (5′ GAGTTTGATCCTTGGCTCAGGA 3′). The mixture was covered with 100 μl of light mineral oil (Perkin-Elmer). DNA was always added last while the reaction mixture was kept at 70°C (3). The thermal profile involved 40 cycles of denaturation, primer annealing, and extension.

Following preferential amplification of mycobacterial nucleic acids, screening at the genus level was performed by hybridization to an appropriate probe (oligonucleotide 259 [5′ TTTCACGAACAACGCGACAA 3′] (Fig. 1). Subsequently, positive samples were differentiated by hybridization to species-specific probes (for *M. tuberculosis* complex and *Mycobacterium avium* [3]) or by sequence determination (19).

Hybridization and analysis of amplified samples. Aliquots of amplified samples (5 μl) were electrophoresed through 0.8% agarose gels, and the DNA was visualized by UV fluorescence after it was stained with ethidium bromide (3). For slot blot analysis, 10-μl aliquots of amplified samples were denatured by the addition of 100 μl of 0.5 M NaOH–25 mM EDTA and further processed as described previously (3).

For nonradioactive detection, a microtiter plate format and modifications of previously described procedures (15, 22) were used. Dynabeads (Dynal, Hamburg, Germany) were employed for preparation of a single-stranded DNA template. The Dynabeads (7.5 μl [10 mg/ml] per sample) were prepared according to the manufacturer's instructions, resuspended in 50 μl of binding and washing buffer (Dynal), and added to a 10-μl aliquot of the amplification reaction mixture. The single-stranded DNA template was prepared by alkaline hydrolysis of the double-stranded DNA template essentially as described by the manufacturer. Following washing with 100 μl of binding and washing buffer, the beads were resuspended in 50 μl of hybridization solution containing 6 pmol of digoxigeninated oligonucleotide (custom synthesized by Biometra, Göttingen, Germany). The hybridization solution consisted of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 0.1% Tween 20, and 100 μg of tRNA per ml. Following hybridization for 1 h at 50°C, washes were done by using a magnetic particle separator for microtiter plates (Dynal). Washes were done one time with 4× SSC–0.1% Tween 20 at room temperature (100 μl per well) and two times with 4× SSC–0.1% Tween 20 for 15 min each at 56°C (100 μl per well). Following the final wash a 50-μl solution of 1% bovine serum albumin in phosphate-buffered saline (PBS) containing antidigoxigenin antibody (Boehringer, Mannheim, Germany) was added, and the mixture was incubated for 30 min at room temperature with shaking. Antibody incubation was followed by washing (two times) with PBS–0.05% Tween. Finally, 50 μl of the substrate solution (40 μg of 4-methylumbelliferyl phosphate [Sigma] per ml in 100 mM Tris-HCl [pH 9.6]–100 mM NaCl–50 mM $MgCl_2$) was added, and after 1 h, fluorescence units were determined on a microtiter plate fluorometric reader (Titertek Fluoroscan II; Flow Laboratories, Meckenheim, Germany). To determine the cutoff value, the mean values for four negative controls (sputa negative for mycobacteria which were processed through the whole procedure of nucleic acid extraction, amplification, and hybridization) and the respective standard deviations were calculated. Values greater than the means plus five standard deviations were considered positive.

Controls. Every fifth sample was a negative control sputum, which was subjected to whole procedure (nucleic acid extraction, amplification, and hybridization). The efficiency of DNA extraction was monitored by preparing nucleic acids from serial dilutions of mycobacteria corresponding to 5, 25, and 50 CFU. Each



285: 5′ GAGTTTGATCCTGGCTCAGGA 3′
259: 5′ TTTCACGAACAACGCGACAA 3′
264: 5′ TGCACACAGGCCACAAGGGA 3′
M.tb complex: 5′ ACCACAAGACATGCATCCCG 3′
M.avium: 5′ ACCAGAAGACATGCGTCTTG 3′

FIG. 1. Physical locations and nucleotide sequences of oligonucleotides used. For explanations, see the text.

amplification run was monitored for correct execution by amplification of a serial dilution of mycobacterial nucleic acids corresponding to 1, 10, and 100 CFU. During this study, a control to test for the presence of inhibitors of the amplification reaction was constructed. This control plasmid was constructed by cloning a PCR-amplified 16S rRNA gene fragment from *M. tuberculosis* (positions 10 to 1534) into the pT7 Blue vector (Novagen, Madison, Wis.). Following digestion with *Apa*I and blunt ending with T4 DNA polymerase, restriction with *Xho*I was performed and the desired fragment (3,580 bp) was gel purified. Digestion with *Apa*I and *Xho*I will remove a fragment extending from position 94 to 926 from the 16S rRNA gene fragment. An 1,161-bp gene fragment from IFP 35 (2), which was generated by restriction of a full-length clone with *Hin*dIII, blunt ending, and digestion with *Xho*I, was cloned into the gel-purified gene fragment described above, resulting in the control plasmid pIK18. Amplification with primers 285 and 264 will result in a gene fragment containing 1,360 bp. Five hundred copies of the control plasmid per PCR reaction were used, and the success of the amplification reaction was controlled either by agarose gel electrophoresis or by hybridization to an IFP 35-specific oligonucleotide (5′ ACTGGGTGGGCAG CAAGT 3′).

Precautions to prevent contamination. Stock solutions (including solutions necessary for the routine preparation of specimens [e.g., the NALC-NaOH procedure]), were prepared in a separate room, aliquoted, autoclaved two times whenever this was compatible with the chemical composition, stored apart from the mycobacteriology laboratory, and used only once. Chemical decontamination of surfaces (daily) and equipment (weekly) was done with 0.5% sodium hypochloride (27). Prevention of DNA contamination was further accomplished by physically separating the different steps in the PCR procedure, using different sets of pipettes and aerosol-free tips, wearing separate coats and gloves in each laboratory, and appropriately organizing the daily work load (doing clean work first).

RESULTS

Specimens investigated. Specimens that were submitted daily to the clinical laboratory were cultured for growth of mycobacteria and, if appropriate, were lysed to prepare nucleic acids. A total of 8,272 specimens were received during the study period; 4.1% of them were positive by culture.

The first part of the study (part A) involved the period from September 1993 to September 1994. During this period, a total of 5,889 samples were received; 412 of them satisfied the selection criteria and were subjected to PCR analysis. The assay was performed as follows: (i) regardless of the origin, sample DNA was prepared by the guanidium isothiocyanate method, and (ii) detection was performed by using either the radioactive detection procedure or the nonradioactive enzyme-linked immunosorbent assay (ELISA) format. The second part of the study (part B) involved the period from October 1994 to April 1995. During this period, a total of 317 samples were investigated by PCR (of a total of 2,383 samples received), with the following modifications. (i) Except for biopsies, sample DNA was prepared by using the Amplicor Sputum Preparation Kit; for biopsies, the guanidium isothiocyanate procedure was used. (ii) An internal control to test for the presence of inhibitors of the amplification reaction was added to each sample DNA. (iii) Samples prepared by the Amplicor procedure and showing the presence of inhibitors were further processed by the guanidium isothiocyanate method. (iv) Detection was done by using the nonradioactive ELISA format.

The two parts of the study gave similar results for assay performance (see Tables 1, 2, and 3).

Analysis of smear-positive specimens. Of a total of 104 smear-positive specimens, 26 were not investigated by PCR because they were from patients with known mycobacterial infection. Table 2 shows the overall PCR and culture results for the remaining 78 samples. Fifty-six specimens were positive by culture and PCR analysis, 17 specimens were negative by both methods, and 5 specimens gave discrepant results (all 5 were negative by culture and positive by PCR).

Of the 56 samples which were positive by culture and PCR, 33 (58.9%) were identified as *M. tuberculosis*, 14 (25.0%) were identified as *M. avium*, 5 (8.9%) were identified as *Mycobacterium chelonae*, 3 (5.4%) were identified as *Mycobacterium intracellulare*, and 1 was identified as *Mycobacterium fortuitum*. Except for two specimens (one *M. chelonae* and one *M. fortuitum*), the finding of nontuberculous mycobacteria was clinically significant and related to disease.

Of the 17 smear-positive specimens negative by both culture and PCR, none was moderately or heavily positive by smear

TABLE 2. Detection of mycobacteria from smear-positive samples (n = 78) by PCR compared with detection by culture

PCR result	Study part	No. with culture result	
		Positive	Negative
Positive	A	43	2
	B	13	3
	Total	56[a]	5[b]
Negative	A	0	13
	B	0	4
	Total	0	17[c]

[a] *M. tuberculosis* complex (n = 33), *M. avium* (n = 14), *M. chelonae* (n = 5), *M. intracellulare* (n = 3), and *M. fortuitum* (n = 1).

[b] *M. tuberculosis* complex (n = 3), *M. genavense* (n = 1), and *M. chelonae* (n = 1).

[c] These samples were judged to represent microscopy artifacts (see text).

TABLE 3. Detection of mycobacteria from smear-negative samples (n = 651) by PCR compared with detection by culture

PCR result	Study part	No. with culture result	
		Positive	Negative
Positive	A	23	22
	B	9	8
	Total	32[a]	30[b]
Negative	A	12	302
	B	10	265
	Total	22[c]	567

[a] *M. tuberculosis* complex (n = 21), *M. avium* (n = 6), *M. intracellulare* (n = 3), *M. chelonae* (n = 1), and *M. simiae* (n = 1).

[b] *M. tuberculosis* complex (n = 15), *M. chelonae* (n = 3), *M. avium* (n = 5), *M. genavense* (n = 3), *M. intracellulare* (n = 1), *M. simiae* (n = 1), and nontuberculous mycobacteria (n = 2)

[c] *M. tuberculosis* complex (n = 18), *M. avium* (n = 2), *M. marinum* (n = 1), and *M. simiae* (n = 1).

analysis, but rather smear positivity was of the ± type (one or two AFB in 300 fields). Clinical findings did not correlate with the smear results for these specimens; a retrospective analysis revealed that the majority of these smear results (n = 10) were reported by one of the technicians performing the smear analysis. Taken together, the absence of clinical findings, the ± type of smear positivity, and the negative results of culture and PCR analyses suggest that these specimens represent microscopy artifacts.

The five discrepant PCR-positive, culture-negative specimens were identified as containing *M. tuberculosis*, *Mycobacterium genavense*, and *M. chelonae*. None of the four patients from which these specimens were obtained had received prior therapy for tuberculosis. Other specimens from three of the four patients (with the exception of the patient with *M. genavense* infection) were positive by culture for the respective pathogen. We conclude that the results for these five specimens represent true-positive test results.

Considering these corrected data, the PCR sensitivity for smear-positive specimens was 100%, with a specificity and positive and negative predictive values of 100% each.

Analysis of smear-negative specimens. A total of 651 smear-negative samples were investigated by PCR. Table 3 shows the overall PCR and culture results. PCR results were accurate for 32 specimens which grew mycobacteria and for 567 specimens which were culture negative for mycobacteria. In total, there were 52 discrepant results: 22 specimens were positive by culture and negative by PCR, and 30 specimens were negative by culture and positive by PCR.

Of the 32 specimens which were positive by PCR and culture, 21 (65.6%) were identified as containing *M. tuberculosis*. Eleven specimens (34.4%) contained nontuberculous mycobacteria, which were identified as containing *M. avium* (n = 6), *M. intracellulare* (n = 3), *M. chelonae* (n = 1), and *Mycobacterium simiae* (n = 1). For six samples the findings of *M. avium* and *M. intracellulare* were clinically significant.

The 22 specimens which were positive by culture and negative by PCR analysis were identified as containing *M. tuberculosis* (n = 18), *M. avium* (n = 2), *Mycobacterium marinum* (n = 1), and *M. simiae* (n = 1). The findings of *M. marinum* and *M. avium* were clinically significant and related to disease. The results for these 22 samples were categorized as false-negative PCR results.

When the specimens with results which were false negative by PCR were analyzed not on a per-sample basis but considering the results for all specimens examined from a single patient in total (accumulative), it was found that no patient who had three or more specimens tested would have been misdiagnosed by PCR (Table 4).

Thirty samples that were positive by PCR and negative by culture were identified as containing *M. tuberculosis* (n = 15), *M. chelonae* (n = 3), *M. avium* (n = 5), *M. genavense* (n = 3), *M. intracellulare* (n = 1), *M. simiae* (n = 1), and two nontuberculous mycobacteria which could not be further differentiated. When the clinical data were not suggestive of a mycobacterial infection and other specimens from the same patient did not grow the respective pathogen, the result was considered to be a false-positive PCR result (n = 3); false-positive PCR results included *M. tuberculosis* (n = 1) and nontuberculous mycobacteria (n = 2). Twenty-seven of the discrepant specimens were considered true positives (Table 5); these organisms included *M. tuberculosis* (n = 14 [8 specimens were obtained from patients on tuberculosis medication]), *M. chelonae* (n = 3), *M. avium* (n = 5), *M. genavense* (n = 3), *M. intracellulare* (n = 1), and *M. simiae* (n = 1). For 13 samples the finding of nontuberculous mycobacteria (*M. avium*, *M. genavense*, and *M. chelonae*) was clinically significant.

In our hands, for 75% (15 of 19) of the PCR-positive, culture-negative specimens, other specimens from the same patient grew the respective pathogen, when apparent reasons for culture failure, i.e., tuberculosis medication and fastidious growth requirements such as those required for *M. genavense*, were excluded. Likewise, as discussed above, for PCR-negative, culture-positive specimens, when several specimens from the same patient were investigated, other specimens gave positive PCR results. After resolution of discrepancies, i.e., by taking all specimens which represent true positives into consideration, the sensitivity of the PCR was 72.8%, the specificity was 99.5%, the positive predictive value was 95.2%, and the negative predictive value was 96.3%. The corresponding values for culture (sensitivity and negative predictive value) were 66.7 and 95.5%, respectively (Table 6). Overall, the sensitivity of PCR for the detection of mycobacterial pathogens in smear-negative specimens is similar to that of culture, i.e., a combination of the BACTEC technique with Löwenstein-Jensen slants.

Presence of inhibitors of the amplification reaction. During the second part of the study, it was observed that nucleic acids prepared with the Amplicor Sputum Preparation Kit frequently showed the presence of inhibitors of the amplification reaction. Of a total of 252 samples prepared with this procedure, 21.0% revealed the presence of inhibitors (specimens obtained from the upper respiratory tract, 17.8%; from cerebrospinal fluid, 54.5%; and from urine, gastric fluid, peritoneal fluid, and pleural fluid, 25.0%). After the nucleic acids prepared with the Amplicor procedure were subjected to the guanidium isothiocyanate method, the inhibitors were eliminated in 96% of the specimens. For biopsies, inhibitors of the amplification reaction were found in 11.5% of the specimens when the guanidium isothiocyanate procedure was used for extraction of nucleic acids.

Analysis of smear-negative, culture-positive samples not investigated by PCR. We were concerned that because of the defined selection criteria, most of the smear-negative, culture-positive samples might not have been subjected to PCR analysis. We thus wanted to address the clinical significance of the finding of mycobacteria in these patients. In other words, what would have been the clinical benefit if all samples received in the mycobacteriology laboratory had been investigated by PCR analysis?

During the 18-month prospective evaluation, a total of 218

TABLE 4. Analysis of 22 culture-positive specimens with false-negative PCR results

Patient no.	Specimen type	Specimen no.	Date specimen obtained	PCR result	Culture result	Species	Disease related to mycobacteria
1	Urine	1739	4/25/94	−	+	*M. tuberculosis*	Tuberculosis
2	Urine	735	3/2/95	−	+	*M. tuberculosis*	Tuberculosis
	Urine	736	3/2/95	−	+	*M. tuberculosis*	Tuberculosis
3	Urine	783	2/23/94	−	+	*M. tuberculosis*	Tuberculosis
4	Lymph node	1001	3/23/95	−	+	*M. tuberculosis*	Tuberculosis
5	Lymph node	2545	6/16/94	−	+	*M. tuberculosis*	Tuberculosis
6	Skin biopsy	1866	5/4/94	−	+	*M. marinum*	Skin infection
7	Bone marrow aspirate	5565	12/16/93	−	+	*M. avium*	Disseminated *M. avium* infection in an AIDS patient
8	Bone marrow biopsy	18	1/3/95	−	+	*M. avium*	Disseminated *M. avium* infection in an AIDS patient
9	Pleural fluid	96	1/9/95	−	+	*M. tuberculosis*	Tuberculosis
	Pleural fluid	97	1/9/95	−	+	*M. tuberculosis*	Tuberculosis
10	Ascites	3359	10/17/94	−	+	*M. tuberculosis*	Tuberculosis
11	Sputum	3218	8/5/94	−	+	*M. tuberculosis*	Tuberculosis
12[a]	Bronchial lavage	1656	4/20/94	−	+	*M. simiae*	None
	Bronchial lavage	1853	5/4/94	+	+	*M. simiae*	
13[a]	Bronchial lavage	1732	4/25/94	−	+	*M. tuberculosis*	Tuberculosis
	Bronchial lavage	1733	4/25/94	−	+	*M. tuberculosis*	
	Sputum	1734	4/25/94	+	+	*M. tuberculosis*	
14[a]	Sputum	1226	3/22/94	+	+	*M. tuberculosis*	Tuberculosis
	Sputum	1286	3/28/94	−	+	*M. tuberculosis*	
	Sputum	1564	4/14/94	+	+	*M. tuberculosis*	
	Sputum	1565	4/14/94	−	+	*M. tuberculosis*	
15	Bronchial lavage	464	2/9/95	−	+	*M. tuberculosis*	Tuberculosis
	Sputum	465	2/9/95	+	+	*M. tuberculosis*	
	Sputum	466	2/9/95	+	+	*M. tuberculosis*	
	Sputum	467	2/9/95	−	+	*M. tuberculosis*	
16	Transbronchial biopsy	1313	4/20/95	+	+	*M. tuberculosis*	Tuberculosis
	Sputum	1314	4/20/95	−	+	*M. tuberculosis*	
	Bronchial lavage	1315	4/20/95	−	+	*M. tuberculosis*	

[a] This patient also had PCR-positive, culture-negative samples (see Table 5).

samples, from 112 patients, which were smear negative and culture positive did not correspond to the selection criteria defined and were thus not investigated by PCR (Table 7). For 56 patients the laboratory finding of mycobacteria had no clinical relevance (e.g., isolation of *Mycobacterium gordonae* and *Mycobacterium xenopi*), 19 patients corresponded to known cases, and for 4 patients the materials investigated were necropsy specimens. For 33 patients an earlier diagnosis of mycobacterial infections would have been clinically significant. Twenty-two of the patients, however, had disseminated disease due to nontuberculous mycobacteria (isolates were recovered mainly from blood cultures).

DISCUSSION

The steady decline of tuberculosis seen in industrialized countries since the beginning of this century has been followed by an increase in nontuberculous mycobacterial infections during the past decade, most notably, but not limited to, disseminated *M. avium* or *M. genavense* infection in late-stage AIDS disease (6, 14). The increasing importance of nontuberculous mycobacteria as significant pathogens necessitates the timely detection of mycobacterial infections and identification of the respective pathogens because of the need to make decisions regarding management, such as isolation precautions, drug therapy, and surgical procedures.

One of the intriguing features of the 16S rRNA molecule is the presence of conserved as well as variable regions, allowing the amplification of nucleic acids on the genus level followed by species differentiation by using highly discriminating probes or nucleic acid sequencing (3). During this 18-month prospective evaluation of a PCR assay for detection of mycobacterial infections, a total of 8,272 specimens were received in the mycobacteriology laboratory. Of these, 729 clinical specimens

TABLE 5. Analysis of 32 PCR-positive, culture-negative specimens[a]

Patient no.	Specimen type	Specimen no.	Date specimen obtained	PCR result	Culture result	Species	Disease related to mycobacteria
17	Sputum	4438	10/06/93	+	+	*M. tuberculosis*	Tuberculosis[b]
	Sputum	4600	10/18/93	+	+	*M. tuberculosis*	
	Sputum	5301	12/01/93	+	+	*M. tuberculosis*	
	Sputum	5423	12/08/93	+	+	*M. tuberculosis*	
	Bronchial lavage	816	2/24/94	+	−	*M. tuberculosis*	
	Sputum	1095	3/14/94	+	−	*M. tuberculosis*	
	Sputum	1107	3/15/94	+	−	*M. tuberculosis*	
	Sputum	1611	4/18/94	+	−	*M. tuberculosis*	
13[c]	Sputum	1392	4/5/94	+	+	*M. tuberculosis*	Tuberculosis[b]
	Bronchial lavage	1936	5/10/94	+	−	*M. tuberculosis*	
	Sputum	1939	5/10/94	+	+	*M. tuberculosis*	
	Bronchial lavage	2514	5/20/94	+	−	*M. tuberculosis*	
	Sputum	2516	5/20/94	+	−	*M. tuberculosis*	
18	Bronchial lavage	77	1/9/95	+	−	*M. tuberculosis*	Tuberculosis[d]
	Sputum	78	1/9/95	+	−	*M. tuberculosis*	
	Sputum	79	1/9/95	+	−	*M. tuberculosis*	
19	Sputum	1237	4/7/95	+	−	*M. tuberculosis*	Tuberculosis[e]
	Sputum	1238	4/7/95	+	−	*M. tuberculosis*	
14[c]	Urine	902	3/2/94	+	+	*M. tuberculosis*	Tuberculosis[b]
	Urine	1288	3/28/94	+	+	*M. tuberculosis*	
	Urine	1648	4/19/94	+	−	*M. tuberculosis*	
20	Liver biopsy	1629	4/14/94	+	−	*M. tuberculosis*	Disseminated tuberculous infection in an AIDS patient[e]
21	Cerebrospinal fluid	2093	5/24/94	+	+	*M. tuberculosis*	Tuberculous meningitis
	Cerebrospinal fluid	2152	5/26/94	+	−	*M. tuberculosis*	
22	Bronchial lavage	3319	8/17/94	+	−	*M. tuberculosis*	Tuberculosis
	Sputum	3320	8/17/94	+	+	*M. tuberculosis*	
23	Bronchial lavage	263	1/18/94	+	−	*M. tuberculosis*	Tuberculosis[e]
24	Sputum	319	1/21/94	+	+	*M. chelonae*	Chronic lung disease
	Sputum	466	2/1/94	+	+	*M. chelonae*	
	Sputum	519	2/2/94	+	−	*M. chelonae*	
	Sputum	520	2/2/94	+	−	*M. chelonae*	
	Sputum	521	2/2/94	+	+	*M. chelonae*	
	Sputum	532	2/4/94	+	−	*M. chelonae*	
	Sputum	1055	3/10/94	+	+	*M. chelonae*	
	Sputum	1926	5/10/94	+	−	*M. chelonae*	
25	Sputum	240	1/24/95	+	−	*M. genavense*	Disseminated *M. genavense* infection in an AIDS patient
	Sputum	241	1/24/95	+	−	*M. genavense*	
	Sputum	242	1/24/95	+	−	*M. genavense*	
26	Bronchial lavage	5362	12/3/93	+	+	*M. avium*	Chronic lung disease
	Sputum	5363	12/3/93	+	+	*M. avium*	
	Sputum	1117	3/15/94	+	−	*M. avium*	
27	Bronchial lavage	4724	10/26/93	+	+	*M. avium*	Disseminated *M. avium* infection in an AIDS patient
	Sputum	1532	4/12/94	+	−	*M. avium*	
	Bronchial lavage	1534	4/12/94	+	+	*M. avium*	
	Blood	1603	4/16/94	Not done	+	*M. avium*	
28	Sputum	5666	12/23/93	+	+	*M. avium*	Chronic lung disease
	Sputum	306	1/20/94	+	−	*M. avium*	
	Sputum	2651	6/26/94	+	+	*M. avium*	
29	Bone marrow biopsy	372	2/2/95	+	−	*M. genavense*	Disseminated *M. genavense* infection in an AIDS patient
30	Lymph node	4434	11/22/94	+	−	*M. avium*	Cervical lymphadenitis
31	Vertebral biopsy	4474	11/28/94	+	−	*M. avium*	Spondylodiscitis
12[c]	Bronchial lavage	1853	5/4/94	+	+	*M. simiae*	None
	Sputum	1854	5/4/94	+	−	*M. simiae*	
32	Sputum	3592	9/7/94	+	−	*M. intracellulare*	None
	Sputum	3597	9/8/94	+	+	*M. intracellulare*	
	Sputum	3598	9/8/94	+	+	*M. intracellulare*	

[a] Twenty-seven smear-negative and five smear-positive samples.
[b] The patient was on tuberculosis medication.
[c] This patient also had PCR-negative, culture-positive samples (see Table 4).
[d] Clinical signs and lung X-ray were highly suggestive of tuberculosis; there was a prompt response to antituberculosis treatment.
[e] Cultures not investigated in our laboratory were positive for *M. tuberculosis.*

TABLE 6. PCR compared with culture and clinical assessment of patients after resolution of discrepant results

Samples	Final interpretation (no. of specimens)		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
	True positive	Negative				
Smear positive (n = 78)[a]						
Culture:						
Positive	56	0	91.8	100	100	77.3
Negative	5	17				
PCR:						
Positive	61	0	100	100	100	100
Negative	0	17				
Smear negative (n = 651)						
Culture:						
Positive	54	0	66.7	100	100	95.5
Negative	27	570				
PCR:						
Positive	59	3	72.8	99.5	95.2	96.3
Negative	22	567				
All (n = 729)						
Culture:						
Positive	110	0	77.5	100	100	94.8
Negative	32	587				
PCR:						
Positive	120	3	84.5	99.5	97.6	96.4
Negative	22	584				

[a] Includes 17 microscopy artifacts.

from a variety of sources, including sputum, bronchial washing, pleural fluid, urine, cerebrospinal fluid, aspirate, and biopsy specimens, fulfilled the selection criteria defined and were subjected to PCR analysis.

Smear-positive samples were investigated by PCR to allow rapid species identification and recognition of microscopy artifacts. Seventeen of 78 (22%) smear-positive test results were identified as microscopy artifacts. The overall unexplained false-positive rate for smear analysis has been reported to vary between 10 and 20% (28). Most of the false-positive smear results in our study (10 of 17) were in all likelihood due to the inexperience of one technician. Although with the use of strict procedures and experienced laboratory personnel this error may be reduced (17), it is clear that false-positive smear results occur. Most of these are of the ± type (one or two AFB in 300 fields). The reasons for false-positive smear results include, e.g., nonspecific staining including partially acid-fast microorganisms such as rhodococci (28). PCR is perfectly suited for the rapid recognition of microscopy artifacts due to nonspecific staining, as all 61 true smear-positive specimens were reliably detected and identified by PCR.

PCR has been used extensively for the detection of *M. tuberculosis* in clinical specimens (1, 7, 9, 11, 20, 24). However, a surprisingly high variation in sensitivity and specificity has been reported for such assays (25). A review of the recent literature indicates that amplification procedures show a sensitivity of 60 to 80% for smear-negative samples (1, 4, 9, 12, 16, 23, 26). After resolution of discrepant test results, the sensitivity, specificity, and positive and negative predictive values of our PCR assay for smear-negative samples were 72.8, 99.5, 95.2, and 96.3%, respectively. The sensitivity and negative predictive value for culture with a combination of broth and solid media were 66.7 and 95.5%, respectively.

On the basis of these data, the performance of our PCR assay compares well with that of culture. When results for smear-positive and smear-negative specimens were combined, there was a 92% agreement (672 of 729 samples) between PCR and culture. The sensitivity, specificity, and positive and negative predictive values of the PCR assay were 84.5, 99.5, 97.6, and 96.4%, respectively.

Discrepant test results (culture-positive, PCR-negative specimens and culture-negative, PCR-positive specimens) may be explained by (i) a nonuniform distribution of microorganisms in the test suspension, since mycobacteria are known to form clumps (28), and (ii) the presence of possible amplification inhibitors in the sample. The discordant results between culture and amplification assay (PCR-positive, culture-negative constellations as well as PCR-negative, culture-positive constellations) for a single specimen from an individual patient indicate that under paucibacillary conditions, sample inhomogeneity represents a methodological problem. Likewise, the difference in sensitivity between analysis per sample and analysis per patient reflects the often paucibacillary nature of mycobacterial infections. This necessitates the well-known practice of investigating at least three samples per patient to most probably exclude a mycobacterial infection (9, 23). A prerequisite for this procedure is a specificity of the test assay of ≥99%, as otherwise the likelihood of a false-positive diagnosis will increase significantly. For the patients who had at least three samples subjected to PCR analysis, the PCR assay found all patients positive for mycobacterial infections (Table 4). Our recommendation for the analysis of patients with smear-negative specimens would be to test three specimens from the patient before reporting a negative amplification reaction.

In this study we have constructed and successfully used an internal control which is coamplified with the primers used for the mycobacterial 16S rRNA gene. This control has been chosen to be slightly larger than the target sequence in order not to interfere with the amplification of the target sequence. By carefully choosing a small amount of the control target, the presence of possible inhibitors affecting the efficiency of the amplification reaction can be controlled for each individual sample. The frequent presence of inhibitors, especially when more-simple DNA extraction procedures, such as the Amplicor Sputum Preparation Kit, are used, illustrates the need to use appropriate controls. On the more positive side, we note that these inhibitors can easily be eliminated in 96% of the cases simply by processing the leftover nucleic acids by the guanidium isothiocyanate procedure.

The diagnostic utility of any laboratory test for the detection

TABLE 7. Analysis of 218 smear-negative, culture-positive samples, from 112 patients, not investigated by PCR

Group	No. of patients
Known cases of mycobacterial infections	19
Postmortem investigations	4
No clinical relevance	56
Clinical relevance	33
M. tuberculosis	11[a]
M. avium	20[b]
Mycobacterium spp.	1[b]
M. chelonae	1[c]

[a] Two patients had already been treated because of clinical suspicion.
[b] AIDS patients with disseminated mycobacterial infection.
[c] Cystic fibrosis patient with *M. chelonae* pneumonia.

of mycobacterial infections is influenced by factors related to the performance of the test itself as well as by factors determined by the patient population: the distribution of positive and negative specimens, the percentage of smear-positive (multibacillary) versus smear-negative (paucibacillary) cases, and tuberculous versus nontuberculous infections. Of the 729 selected specimens investigated in this study, 89 were positive for *M. tuberculosis*. Fifty-three specimens were positive for nontuberculous mycobacteria, and 70% of these were considered clinically significant. The defined selection criteria missed 33 patients for whom an earlier diagnosis of mycobacterial infection would have been of clinical benefit. However, of these 33 patients, 22 had infections with nontuberculous mycobacteria, mostly disseminated *M. avium* infections in AIDS patients. These findings point to two issues: (i) a molecular assay which detects *M. tuberculosis* only, and not other mycobacterial pathogens, would be of limited value for the patient population we serve (patients at a large university hospital in central Europe), and (ii) the selection criteria for PCR analysis need to be enforced with respect to AIDS patients. Disseminated mycobacterial infections in these patients are most frequently diagnosed by blood cultures. Such specimens, however, are difficult to investigate by PCR analysis, as (i) 30% of AIDS patients have low-level *M. avium* bacteremia with less than 100 organisms per ml of blood (13), and (ii) blood specimens frequently contain inhibitors of the amplification reaction. Until reliable sample preparation methods for this type of specimen are developed, a laboratory compromise would be to focus on specimens which are suitable for PCR analysis, i.e., sputum and bone marrow biopsy specimens.

We are well aware of the fact that the homemade PCR assay described in this report is a prototype assay which requires highly specialized and well-trained laboratory personnel. To facilitate routine application, we have included a versatile nonradioactive detection method in our procedure. We have found it extremely useful to treat samples which are normally not decontaminated for culture because they represent primarily sterile body fluids, e.g., cerebrospinal fluid, with NALC-NaOH prior to PCR analysis. For reasons which probably relate to the dilution and washing steps during decontamination with NALC-NaOH, this procedure seems to effectively remove inhibitors of the amplification reaction which are otherwise present in these materials (12).

So far we have explicitly avoided the use of post-PCR sterilization procedures (29), such as uracil-DNA-glycosylase (21), for financial reasons but also to be able to rapidly detect faulty laboratory execution of the PCR assay. Until now, the strategy of careful education and training has paid off, as we have not experienced problems with PCR product contamination.

To implement PCR as a valuable adjunct in diagnostic mycobacteriology, we recommend (i) the definition of criteria for its application, (ii) the use of an assay which simultaneously allows screening at the genus level and differentiation at the species level, (iii) controlling for test performance by methodological procedures (controls for contamination, inhibition, nucleic acid extraction, and efficiency of the amplification reaction), and (iv) interpretation of PCR results in conjunction with culture and acid-fast smear test results. For the moment we suggest that PCR analyses should be done only by laboratories which simultaneously perform culture and microscopy to allow for a rapid control of the PCR assay performance.

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