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Multiplex PCR Assay for Immediate Identification of the Infecting Species in Patients with Mycobacterial Disease

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Rapid identification of infecting mycobacterial species enables appropriate medical care decisions to be made. Our aim was to demonstrate the clinical usefulness of the multiplex PCR assay, a test based on PCR, which permits direct identification of 12 mycobacterial species in clinical specimens. A total of 259 specimens from 177 patients who had clinical symptoms of mycobacterial disease but for whom there were difficulties in diagnosis were tested. Specimens were analyzed within 48 h of receipt of the sample. Mycobacteria were identified in 102 specimens; 66 specimens contained nontuberculous mycobacteria, and 36 specimens contained *Mycobacterium tuberculosis* complex mycobacteria. The PCR assay identified the mycobacterial species in 43 (97.7%) of 44 microscopy- and culture-positive specimens and in 15 (93.8%) of 16 culture-positive, microscopy-negative specimens. It also permitted species identification in infections caused by more than one mycobacterial species. For 56 (96.5%) of the 58 specimens from patients with infections caused by opportunistic mycobacteria, the organisms were identified with the PCR assay. The test was useful also for the identification of fastidious mycobacteria, e.g., *M. genavense*, and those that cannot be cultured, e.g., *M. leprae*. After resolution of discrepant results, the sensitivity of the PCR assay was 97.9%, the specificity was 96.9%, the positive predictive value was 95.0%, and the negative predictive value was 98.7%. For culture these values were 60.8, 100, 100, and 81.0%, respectively. Thus, the multiplex PCR assay enables prompt diagnosis when rapid identification of infecting mycobacteria is necessary.

There is an increase in tuberculosis and mycobacterial disease caused by nontuberculous mycobacteria. Infections by more than one mycobacterial species in immunosuppressed patients are increasingly being reported. The rapid diagnosis of mycobacterial disease relies primarily on the detection of acid-fast bacteria by direct microscopy. The infecting mycobacterial species can usually be identified only after culture of the organism, a time-consuming procedure. However, some mycobacteria, e.g., *Mycobacterium genavense* and *M. ulcerans*, are difficult to grow and some, e.g., *M. leprae*, cannot be cultured in vitro. Since the choice of therapy is dependent on the infecting species (1, 15), identification of the organism(s) at an early phase of disease is required for optimal therapy and medical care decisions, e.g., whether isolation of the patient is necessary. The practice of many clinicians has been to initiate treatment for *M. tuberculosis* upon microscopic detection of mycobacteria in specimens, often several weeks before species identification (12). We developed a PCR assay based on DNA coding for the 16S subunit of rRNA (16S rDNA) (11). We combined this with our IS*6110*-based PCR assay for detection of *M. tuberculosis* (9) in a "multiplex" PCR assay. The PCR product is analyzed in a reverse cross-blot hybridization assay with 13 species-specific probes and a *Mycobacterium*-specific probe. This approach enables detection of infection by a single mycobacterial species or simultaneous infections by more than one species. The objective of this study was to demonstrate the clinical usefulness of the multiplex PCR assay. We used the assay to identify the infecting organism(s) directly in specimens taken from patients.

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MATERIALS AND METHODS

Clinicians sent us samples from patients for whom difficulties with diagnosis were experienced or could be anticipated, e.g., patients with granulomatous disease, patients with suspected extrapulmonary paucibacillary tuberculosis, immunocompromised patients (human immunodeficiency virus [HIV]-positive or AIDS patients), immigrants and refugees from countries where tuberculosis is endemic, and patients in whom mycobacterial infection other than by *M. tuberculosis* was suspected.

Two hundred fifty-nine clinical specimens, consisting of samples of sputum ($n = 31$), tissue obtained by biopsy ($n = 87$), lymph node obtained by biopsy ($n = 37$), feces ($n = 7$), urine ($n = 6$), blood ($n = 10$), cerebrospinal fluid (CSF) ($n = 36$), ascitic fluid ($n = 6$), pleural fluid ($n = 15$), pericardial fluid ($n = 3$), bronchoalveolar lavage fluid ($n = 20$), and gastric lavage fluid ($n = 1$), were obtained from 177 patients who had clinical symptoms compatible with mycobacterial disease. The specimens, which were obtained from various university and teaching hospitals in The Netherlands, were taken before therapy was started. They were divided into two portions, one for conventional mycobacterium detection methods (microscopy and culture) and one for PCR.

Microscopy and culture were performed according to standard methods (6) at the microbiology laboratories of the hospitals to which the patients were referred. Strains were identified by standard methods (6); identifying features included physical characteristics, pigmentation, growth at 25°C, niacin test results, *para*-nitrobenzoic acid test results, and AccuProbe (Gen-probe, San Diego, Calif.) hybridization assay results. The PCR assays were performed at the Royal Tropical Institute, and results were reported to the clinicians within 3 days.

DNA isolation from the clinical specimens was performed as previously described (10). For the amplification of mycobacterial 16S rDNA sequences, we used the 5′-biotinylated primers pMyc14bio (5′-GRGRTACTCGAGTGGCGAAC-3′) (R = A or G) and pMyc7bio (5′-GGCCGGCTACCCGTCGTC-3′). Primer Pt18 (5′-GAACCGTGAGGGCATCGAGG-3′) and the 5′-biotinylated primer INS2bio (5′-GCGTAGGCGTCGGTGACAAA-3′), amplifying the *M. tuberculosis* complex-specific insertion sequence IS*6110*, were included in the PCR mixture. PCR and analysis of the PCR products by reverse cross-blot hybridization were performed as previously described, including the use of DNA glycosylase in combination with dUTP instead of dTTP to break down possible contamination with amplicons and the use of modified *M. smegmatis* 1008 as an internal control for inhibition (8, 11). Briefly, samples of the PCR products were denatured and allowed to hybridize with the specific probes, which were attached to a reinforced nitrocellulose membrane. The assay was performed in a cross format with a cross-blotter (Accutran-Cross ACC 100/0; Schleicher & Schuell, Dassel, Germany), permitting hybridization of the biotinylated DNA strands from one PCR product with all probes simultaneously. The hybridized PCR products on the membrane were detected by incubation with a streptavidin-

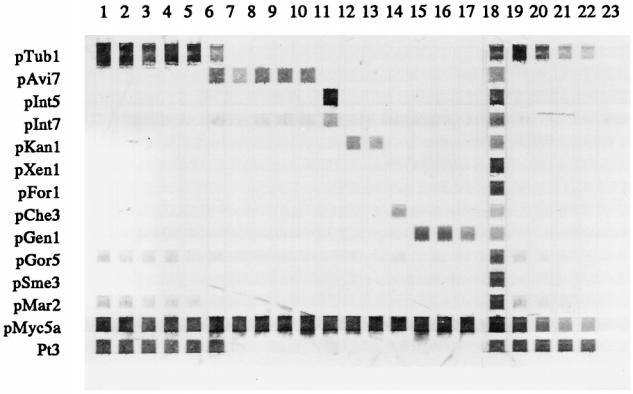

FIG. 1. Analysis of PCR products by reverse cross-blot hybridization. Lanes 1 to 17 show PCR products from clinical specimens. Lanes 1 through 17, samples from patients 1 through 17 as follows: lane 1, sputum; lane 2, brain biopsy tissue; lane 3, lymph node aspirate; lane 4, urine; lane 5, lymph node aspirate; lane 6, spleen biopsy tissue; lane 7, lymph node aspirate; lane 8, spleen biopsy tissue; lane 9, lung biopsy tissue; lane 10, lymph node aspirate; lane 11, sputum; lane 12, sputum; lane 13, sputum; lane 14, sputum; lane 15, liver biopsy tissue; lane 16, liver biopsy tissue; lane 17, feces. Lanes 18 to 23, controls as follows: lane 18, mixture of PCR products from 12 species of mycobacteria (control for the individual probes); lanes 19 to 22, 100 pg, 1 pg, 100 fg, and 10 fg, respectively, of *M. tuberculosis* DNA (positive controls); lane 23, water processed for DNA isolation (negative control). Details of the probes are given in the text.

alkaline phosphatase conjugate and a color substrate (4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate). Probe Pt3 (5′-GAACGG CTGATGACCAAACT-3′) was used to capture the IS*6110* PCR product of *M. tuberculosis*, and pTub1 (5′-ACCACAAGACATGCATCCCG-3′) was used to capture the 16S rDNA PCR product of *M. tuberculosis*. The 16S rDNA PCR products of the following species were captured by the indicated probes: *M. avium*, pAvi7 (5′-CCAGAAGACATGCGTCTTGAG-3′); *M. intracellulare*, pInt5 (5′-C ACCTAAAGACATGCGCCTAA) and pInt7 (5′-CACCAAAAGACATGCGT CTAA-3′); *M. kansasii* complex and *M. scrofulaceum* complex, pKan7 (5′-CAA GGCATGCGCCAAGTGGT-3′); *M. xenopi*, pXen1 (5′-ACCACCCCACATGC GCAGAA-3′); *M. fortuitum* and *M. senegalense*, pFor1 (5′-ACCACACACCAT GAAGCGCG-3′); *M. chelonae*, pChe3 (5′-CCACTCACCATGAAGTGTGTG -3′); *M. genavense*, pGen1 5′-CCACAAAACATGCGTTCCGTG-3′); *M. gordonae*, pGor5 (5′-TGTGTCCTGTGGTCCTATTCG-3′); *M. marinum* and *M. ulcerans*, pMar2 (5′-CGGGATTCATGTCCTGTGGT-3′); *M. leprae*, pLep1 (5′-A CCACAAGACATGCGCCTTG-3′); and *M. smegmatis*, pSme3 (5′-CATGCGA CCAGCAGGGTGTA-3′). pMyc5a (5′-GGGCCCATCCCACACCGC-3′), a general probe for mycobacteria, was also used. The nucleotide sequences of primers pMyc14bio, pMyc7bio, Pt18, and INS2bio and probes Pt3, pTub1, pInt5, pInt7, pXen1, pFor1, and pMyc5a were published previously (9, 11, 13).

RESULTS AND DISCUSSION

Figure 1 shows the results of the PCR assay of 17 representative specimens. Using the multiplex PCR assay we were able to identify mycobacterial species within 48 h. Table 1 shows the PCR and culture results for the 65 microscopy-positive samples. Culture and PCR results were in agreement in most cases. For most of the specimens for which the PCR result was positive but no organism was cultured, the mycobacteria were fastidious (*M. genavense*) or noncultivable (*M. leprae*). There were no false-positive PCR results or microscopy artifacts, and all the microscopy-positive specimens had a positive result by PCR and/or culture.

Table 2 shows the PCR and culture results for the 194 microscopy-negative samples. Most specimens were both PCR and culture negative. A low number of specimens were found to contain the same organisms by PCR and culture. In the majority of patients from whom these specimens were obtained, illness was caused by *M. tuberculosis*. There were discrepancies in the results for other specimens. These results are discussed in detail below.

TABLE 1. Detection of mycobacteria from microscopy-positive samples[a] by PCR compared with culture

PCR result	No. of samples with culture result	
	Positive	Negative
Positive	43[b]	21[c]
Negative	1[d]	0
Total	44	21

[a] $n = 65$.
[b] The samples included 17 sputum samples, 11 biopsy samples, 9 lymph node biopsy samples, 5 fecal samples, and 1 CSF sample. The organisms isolated included 12 *M. tuberculosis* complex strains, 17 MAC strains, 7 *M. kansasii* strains, 3 *M. chelonae* strains, 3 *M. genavense* strains, and 1 *M. marinum* strain.
[c] The samples included 6 sputum samples, 9 biopsy samples, 4 lymph node biopsy samples, and 2 urine samples. The organisms detected by PCR included 4 *M. tuberculosis* complex strains, 1 MAC strain, 1 *M. kansasii* strain, 10 *M. genavense* strains, and 5 *M. leprae* strains.
[d] Bronchoalveolar lavage fluid sample, from which *M. xenopi* was isolated.

Table 3 shows the results of the multiplex PCR assay, microscopy, and culture for 116 specimens from the 64 patients for whom at least one test gave a positive result. Two patients (patients 6 and 17) had double infections with different mycobacterial species. The PCR assay of specimens from patient 6 identified *M. tuberculosis* and *M. avium* in biopsy samples from the spleen and a vertebral body but only *M. avium* in two separate blood samples. Culturing of these four samples yielded only *M. avium*, identified biochemically, by the AccuProbe system, and by our multiplex PCR assay. *M. avium* grows more rapidly than *M. tuberculosis* and so may have obscured *M. tuberculosis* in culture. In the vertebral biopsy sample, in addition to the 16S rDNA target of *M. avium* found by PCR, we identified the IS*6110* target (identifiable with Pt3) of *M. tuberculosis* but failed to detect the 16S rDNA target (identifiable with pTub1) of *M. tuberculosis*. In PCR with genus-specific probes, if more than one mycobacterial species is present in the specimen, there will be competition for primers between the two 16S rDNA targets. When one of the targets is present at an excess of at least 100-fold, only that target will be detected (8). However, with our multiplex PCR *M. tuberculosis* cannot be missed, even in the presence of a large excess of other myco-

TABLE 2. Detection of mycobacteria from microscopy-negative samples[a] by PCR compared with culture

PCR result	No. of samples with culture result	
	Positive	Negative
Positive	15[b]	21[c]
Negative	1[d]	157[e]
Total	16	178

[a] $n = 193$.
[b] The samples included 6 biopsy samples, 5 lymph node biopsy samples, 1 fecal sample, 1 CSF sample, 1 bronchoalveolar lavage fluid samples, and 1 pericardial fluid sample. The organisms isolated included 9 *M. tuberculosis* complex strains, 3 MAC strains, and 3 *M. marinum* strains.
[c] The samples included 1 sputum sample, 9 biopsy samples, 2 lymph node biopsy samples, 1 bronchoalveolar lavage fluid sample, 2 pleural fluid samples, and 6 CSF samples. Organisms detected by PCR included 11 *M. tuberculosis* complex strains, 5 *M. genavense* strains, 1 *M. kansasii* strain, 1 *M. xenopi* strain, 1 *M. fortuitum* strain, and 2 nontuberculous mycobacteria.
[d] Skin biopsy sample, from which *M. marinum* was isolated.
[e] The samples included 7 sputum samples, 51 biopsy samples, 17 lymph node biopsy samples, 17 bronchoalveolar lavage fluid samples, 13 pleural fluid samples, 28 CSF samples, 6 ascitic fluid samples, 1 fecal sample, 1 stomach lavage fluid sample, 4 urine samples, 10 blood samples, and 2 pericardial fluid samples.

TABLE 3. Results of the multiplex PCR assay, microscopy, and culture for 116 specimens from 64 patients for whom at least one test gave a positive result

Patient (sex[a] and age [yr])	Relevant condition[b]	Clinical manifestation(s)	Specimen[c]	Result obtained by:		
				Microscopy	PCR[d]	Culture
1 (M, 36)	AIDS	Persistent cough	Sputum	Positive	Pt3, pTub1	*M. tuberculosis*
			Sputum	Positive	Pt3, pTub1	*M. tuberculosis*
			Sputum	Positive	Pt3, pTub1	*M. tuberculosis*
			Sputum	Positive	Pt3, pTub1	*M. tuberculosis*
2 (M, 63)	Brain tumor	Granulomatous brain infection	Brain biopsy tissue	Negative	Negative	Negative
			Brain biopsy tissue	Positive	Pt3, pTub1	*M. tuberculosis*
3 (M, 35)	AIDS	Cervical lymphadenitis	Lymph node aspirate	Positive	Pt3, pTub1	*M. tuberculosis*
4 (F, 62)		Renal failure	Urine	Positive	Pt3, pTub1	Negative
			Urine	Positive	Pt3, pTub1	Negative
5 (F, 27)		Cervical lymphadenitis	Lymph node aspirate	Positive	Pt3, pTub1	Negative
			Lymph node aspirate	Positive	Pt3, pTub1	Negative
6 (M, 60)		Abscesses in spleen and vertebra	Spleen biopsy tissue	Positive	Pt3, pTub1, pAvi7	*M. avium*
			Vertebra biopsy tissue	Positive	Pt3, pAvi7	*M. avium*
			Blood	Not done	pAvi7	*M. avium*
			Blood	Not done	pAvi7	*M. avium*
7 (F, 2)		Cervical lymphadenitis	Lymph node	Positive	pAvi7	*M. avium*
8 (F, 12)	AIDS	Granulomatous spleen infection	Spleen biopsy tissue	Positive	pAvi7	*M. avium*
9 (M, 14)		Granulomatous lung infection	Lung biopsy tissue	Positive	pAvi7	*M. avium*
10 (F, 79)	MD	Cervical lymphadenitis	Lymph node aspirate	Positive	pAvi7	*M. avium*
11 (F, 91)	COPD	Granulomatous lung infection	Sputum	Positive	pInt5	MAC
			Sputum	Positive	pInt5	MAC
12 (M, 44)	Alcohol abuse	Granulomatous lung infection	Sputum	Positive	pKan7	*M. kansasii*
			Sputum	Positive	pKan7	*M. kansasii*
			Sputum	Positive	pKan7	*M. kansasii*
			Sputum	Positive	pKan7	*M. kansasii*
13 (M, 60)	Previous TB	Granulomatous lung infection	Sputum	Positive	pKan7	*M. kansasii*
14 (M, 74)		Persistent cough	Sputum	Positive	pChe3	*M. chelonae*
			Sputum	Positive	pChe3	*M. chelonae*
			Sputum	Positive	pChe3	*M. chelonae*
			Urine	Negative	Negative	Negative
15 (M, 35)	AIDS	Granulomatous hepatitis and cervical lymphadenitis	Liver biopsy tissue	Positive	pGen1	*M. genavense*
			Lymph node biopsy tissue	Positive	pGen1	*M. genavense*
			Bone marrow	Negative	pGen1	Negative
16 (M, 37)	AIDS	Granulomatous hepatitis	Liver biopsy tissue	Positive	pGen1	Negative
17 (M, 38)	AIDS	Diarrhea	Feces	Positive	pGen1	*M. avium*
			Feces	Positive	pGen1	*M. avium*
			Feces	Positive	pGen1	*M. avium*
18 (F, 27)		Persistent skin wound	Skin biopsy tissue	Positive	pLep1	Negative
19 (F, 30)	Pregnant	Persistent cough	Sputum	Positive	pLep1	Negative
			Sputum	Positive	pLep1	Negative
			Sputum	Positive	pLep1	Negative
			Sputum	Positive	pLep1	Negative
			Nasal swab	Not done	pLep1	Not done
20 (F, 3)		Submandibular lymphadenitis	Lymph node aspirate	Positive	pKan7	*M. kansasii*
21 (F, 11)		Meningitis	CSF	Negative	Pt3, pTub1	*M. tuberculosis*
			CSF	Negative	Negative	Negative
			CSF	Positive	Pt3, pTub1	*M. tuberculosis*
			Brain biopsy tissue	Negative	Pt3, pTub1	*M. tuberculosis*
22 (F, 6)	BCG vaccination 5 mo before	Lymphadenitis	Lymph node aspirate	Positive	Pt3, pTub1	*M. bovis* BCG

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TABLE 3—*Continued*

Patient (sex[a] and age [yr])	Relevant condition[b]	Clinical manifestation(s)	Specimen[c]	Result obtained by:		
				Microscopy	PCR[d]	Culture
23 (M, 33)		Persistent cough	Sputum	Positive	Pt3, pTub1	*M. tuberculosis*
24 (M, 27)			Bone biopsy tissue	Positive	pKan7	Negative
			Sputum	Positive	pKan7	*M. kansasii*
25 (M, 44)	AIDS	Cervical lymphadenitis	Lymph node aspirate	Positive	pGen1	Negative
			Duodenum biopsy tissue	Positive	pGen1	Negative
			Lymph node aspirate	Positive	pGen1	Negative
			Liver biopsy	Positive	pGen1	Negative
			Duodenum biopsy tissue	Positive	pGen1	Negative
26 (M, 33)	AIDS		Duodenum biopsy tissue	Positive	pGen1	Negative
			Duodenum biopsy tissue	Positive	pGen1	Negative
27 (M, 39)	AIDS	Hepatitis	Liver biopsy tissue	Positive	pGen1	*M. genavense*
28 (F, 26)		Peritonitis	Peritoneum biopsy tissue	Positive	Pt3, pTub1	*M. tuberculosis*
29 (M, 31)	AIDS		Bone marrow	Positive	pGen1	Negative
			Sputum	Positive	pGen1	Negative
30 (M, 40)	HIV positive		Feces	Positive	pAvi7	MAC
			Feces	Positive	pAvi7	MAC
31 (F, 2)		Cervical lymphadenitis	Lymph node aspirate	Positive	pAvi7	MAC
			Lymph node aspirate	Positive	pAvi7	MAC
			Auricular aspirate	Positive	pAvi7	MAC
32 (F, 35)	Ascites	Peritonitis	Ascitic fluid	Negative	Negative	Negative
			Peritoneum biopsy tissue	Positive	Pt3, pTub1	*M. tuberculosis*
33 (F, 11)	Severely immunodeficient	Hepatosplenomegaly	Liver biopsy tissue	Positive	pAvi7	Negative
34 (M, 41)	AIDS		BAL	Positive	Negative	*M. xenopi*
			Sputum	Negative	pXen	Negative
35 (M, 42)		Erythema	Biopsy from arm	Positive	pMar1	*M. marinum*
36 (M, 80)		Miliary TB	Bone marrow	Positive	Pt3, pTub1	*M. tuberculosis*
37 (M, 3)		Cervical lymphadenitis	Lymph aspirate pus	Negative	pInt5	MAC
38 (M, 48)	AIDS	Mycobacteriosis	Feces	Negative	pAvi7	MAC
39 (M, 50)		Lymphadenitis	Lymph node pus	Negative	Pt3, pTub1	*M. tuberculosis*
			Lymph node pus	Negative	Pt3, pTub1	*M. tuberculosis*
40 (F, 47)		Pericarditis	Pericardial fluid	Negative	Pt3	*M. tuberculosis*
41 (F, 44)	*M. avium* 1 yr previously in blood	CML, diffuse brain changes	Blood	Negative	Negative	Negative
			Bone marrow	Negative	Not done	*M. avium*
			CSF	Negative	pMyc	Negative
			Isolated strain	Positive	pAvi7	
42 (F, 58)		Chronic arthritis	Hip joint biopsy tissue	Negative	Pt3	*M. tuberculosis*
43 (M, 69)		Aquarium granuloma	Skin biopsy tissue	Negative	pMar1	*M. marinum*
44 (F, 68)		Miliary TB	BAL	Negative	Pt3, pTub1	*M. tuberculosis*
			Urine	Negative	Negative	Negative
45 (M, 36)		Pleurisy	Pleural fluid	Negative	Pt3	Negative
			Lung biopsy tissue	Negative	Negative	Negative
			Lung biopsy tissue	Negative	Pt3, pTub1	*M. tuberculosis*
46 (M, 78)[e]		Prostatic carcinoma, neurological disorder	CSF	Negative	Pt3	Negative
			Blood	Negative	Negative	Negative
47 (F, 14)	Acute lymphatic leukemia	Pneumonia	BAL	Negative	pFor1	Negative

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TABLE 3—*Continued*

Patient (sex[a] and age [yr])	Relevant condition[b]	Clinical manifestation(s)	Specimen[c]	Result obtained by:		
				Microscopy	PCR[d]	Culture
48 (F, 39)		Pleuritis	Pleural biopsy tissue	Negative	Pt3	Negative
			Pleural fluid	Negative	Negative	Negative
49 (M, 40)		Meningitis	CSF	Negative	pMyc	Negative
		Viral meningitis	Pleural fluid	Negative	Negative	Negative
50 (M, 26)		Pleuritis	Biopsy tissue	Negative	Pt3, pTub1	Negative
			Biopsy tissue	Negative	Not done	*M. tuberculosis*
51 (M, 65)		Meningitis	CSF	Negative	Pt3, pTub1	Negative
			CSF	Negative	Pt3, pTub1	Negative
			CSF	Negative	Negative	Negative
52 (F, 62)		Suspected for non-Hodgkin's lymphoma	Lymph node biopsy sample	Negative	Pt3	Negative
53 (M, 10)		Lymphadenitis	Lymph node biopsy sample	Negative	Pt3, pTub1	Negative
54 (M, 10)[f]		Miliary TB				
		Meningitis	CSF	Negative	Pt3, pTub1	Negative
55 (M, 66)	AIDS	Mycobacterial infection	Duodenum biopsy tissue	Negative	pGen1	Negative
		Hepatitis B	Duodenum biopsy tissue	Negative	pGen1	Negative
			Duodenum biopsy tissue	Negative	pGen1	Negative
			Duodenum biopsy tissue	Negative	pGen1	Negative
56 (M, 20)[g]		Uveitis, TB, or toxoplasma	Aqueous humor sample	Negative	Pt3, pTub1	Negative
			Sputum	Negative	Negative	Negative
			Blood	Negative	Negative	Negative
57 (F, 79)		Lymphadenitis	Lymph node biopsy sample	Negative	Pt3, pTub1	*M. tuberculosis*
58 (F, 51)			Biopsy tissue of ulcer on thigh	Negative	pKan7	Negative
59 (F, 38)		Pleuritis	Pleural fluid	Negative	Pt3	Negative
			Biopsy tissue	Negative	Negative	Negative
			BAL	Negative	Not done	*M. tuberculosis*
60 (M, 69)		Aquarium granuloma	Skin biopsy tissue	Negative	pMar1	*M. marinum*
61 (M, 64)		Silicosis	Sputum	Positive	pAvi7	*M. avium*
62 (M, 48)		Aquarium granuloma	Skin biopsy tissue	Negative	Negative	*M. marinum*
63 (M, 46)		Aquarium granuloma	Skin biopsy tissue	Negative	pMar1	*M. marinum*
64 (M, 1)		Lymphadenitis	Lymph node biopsy sample	Negative	pAvi7	MAC

[a] M, male; F, female.
[b] MD, myeloid dysplasia; COPD, chronic obstructive pulmonary disease; TB, tuberculosis; CML, chronic myeloid leukemia; BCG, *M. bovis* BCG.
[c] BAL, bronchoalveolar lavage fluid.
[d] The PCR product hybridized with the indicated probes. Details of the probes are given in Materials and Methods.
[e] Clinical evidence of tuberculosis, but patient was not treated because of his poor condition; he died a few weeks later.
[f] Patient was treated for tuberculosis; his father had had microscopy-positive pulmonary tuberculosis previously.
[g] Patient had had tuberculosis 1 year previously and was noncompliant.

bacteria, since there is no competition for primers between the IS*6110* target of *M. tuberculosis* and the 16S rDNA targets of other mycobacteria. Another advantage of using two targets for *M. tuberculosis* PCR is that the exceptional *M. tuberculosis* bacterium without IS*6110* can easily be identified in the 16S rDNA-based PCR. The IS*6110* PCR has a lower detection limit than the 16S rDNA-based PCR since there are multiple copies of IS*6110* in the genome of *M. tuberculosis* (14) and only one copy of the 16S rDNA sequence. The advantage of this multiplex PCR technique for diagnosing paucibacillary infection is also shown in the results for patients 40 and 42; for these patients, only the IS*6110* probe gave positive results. Patient 40 had pericarditis, although microscopy was negative. One colony of *M. tuberculosis* was identified after 9 weeks of culture. By this time the patient had recovered clinically, although she received consolidating antituberculous therapy when culture confirmed the unexpected PCR result.

Patient 17 had AIDS and double infection with *M. genavense* and *M. avium*. PCR detected only *M. genavense* in the feces, and only *M. avium* was detected by culture of three separate fecal samples. The isolated *M. avium* strain was identified by biochemical methods, by our multiplex PCR assay, and by the AccuProbe system. In general, culturing detects mycobacteria that are easy to grow. Failure to culture *M. genavense* is not surprising, since it is a fastidious mycobacterium that does not grow on solid media and shows only limited growth in broth

medium (3). We postulated that our PCR did not detect *M. avium* in the feces of patient 17 because of an excess of *M. genavense* bacteria, resulting in competition that masked the presence of *M. avium*. This hypothesis was investigated by performing PCR on the fecal samples with a primer set amplifying the multicopy target IS*1245*, which is specific for *M. avium* (5). This *M. avium*-specific PCR was positive, indicating that competition was the cause of the negative results in the original multiplex PCR assay. In the future we will add this third, IS*1245*-specific primer set to the PCR mixture to avoid missing *M. avium* as a result of competition with other nontuberculous mycobacteria. The ability to identify *M. genavense* is important; it has been suggested that the prevalence of *M. genavense* infection in HIV-seropositive patients is underestimated (7). We identified 21 *M. genavense* strains by PCR, but only 3 strains were isolated by culture.

M. tuberculosis complex mycobacteria were detected in 36 specimens; nontuberculous mycobacteria were found in 66 (64.7%) specimens (Tables 1 and 2). Nontuberculous mycobacteria were identified in 49 (75.4%) of the 65 microscopy-positive specimens; nontuberculous mycobacteria were detected in 48 (98%) specimens by PCR and in only 32 (65%) specimens by culture (Table 1). One of the advantages of the multiplex PCR is evident here, since in these cases we were able to exclude tuberculosis and identify the infecting species. This meant that appropriate therapy could be given and isolation of microscopy-positive patients could be stopped, since opportunistic mycobacteria are normally not transmissible (15). The practice of many clinicians has been to initiate treatment for *M. tuberculosis* upon microscopic detection of mycobacteria in specimens, often several weeks before species identification (11). With the multiplex PCR, overtreatment can be reduced. Mycobacteria were identified by PCR and/or culture in 37 of 194 microscopy-negative specimens. In 17 (45.9%) of these specimens, nontuberculous mycobacteria were detected (Table 2).

Mycobacterial infections caused by the *M. avium* complex (*M. avium* and *M. intracellulare*) (MAC) were relatively frequent, occurring in 21 of the patients studied. There is a high rate of treatment failure in disseminated MAC disease because of delayed diagnosis (2, 4). The PCR test described here could be very helpful in improving treatment, since effective therapy depends on early and correct diagnosis.

PCR is particularly useful in cases in which mycobacteria cannot be cultured in vitro. It is the only test available which can rapidly confirm a clinical diagnosis of leprosy. The diagnosis of leprosy can be particularly difficult to make in areas in which the disease is not endemic, where doctors are not familiar with it. For patients 18 and 19, *M. leprae* was identified by PCR. Patient 18 had a persistent skin wound thought on the basis of clinical observations to be caused by either an *M. leprae* or an *M. ulcerans* infection. The final diagnosis was borderline lepromatous leprosy, as the clinical findings and the response to antileprosy treatment were in accord with the PCR result. Patient 19 had a persistent cough and a microscopy-positive sputum sample and had had previous contact with a patient with *M. leprae* infection. The clinician was uncertain whether *M. tuberculosis* or *M. leprae* was the causative organism, since pulmonary infection with *M. leprae* is uncommon. A presumptive diagnosis of tuberculosis was made. An expert opinion from a specialist in leprosy stated that the clinical evidence was compatible with leprosy, and the patient responded to antileprosy therapy.

For 44 specimens there was a discrepancy between the results obtained by culture and those obtained by PCR (Tables 1 and 2). None of the patients received therapy before samples were taken. There were 21 microscopy-positive, PCR-positive, culture-negative specimens (from patients 4 [two specimens], 5 [two specimens], 16, 18, 19 [four specimens], 24, 25 [five specimens], 26 [two specimens], 29 [two specimens], and 33) (Table 3). A second specimen from patient 24 gave a culture result in accord with the PCR result. We concluded that the PCR results for the specimens from this patient represent true-positive test results. The cultures for patients 4 and 5 were repeatedly negative, indicating that the mycobacteria detected by microscopy and identified by multiplex PCR were not viable. These two patients had clinically active tuberculosis, according to class 3 of the diagnostic standards of the American Thoracic Society (1), and responded to antituberculous treatment. Patients 18 and 19 were discussed above. Patients 16, 25, 26, and 29 were AIDS patients; patient 33 was severely immunodeficient. For all these patients, the clinical findings and the granulomatous tissue biopsy samples were in accord with the microscopy and PCR results. Therefore, we regard the PCR results for these patients as true positive.

There were two false-negative PCR results for specimens (from patients 34 and 62) for which culture was positive and PCR was negative (Table 3) (one was microscopy positive [Table 1], and one was microscopy negative [Table 2]). *M. xenopi* was identified by culture in the microscopy-positive bronchoalveolar lavage fluid from patient 34 (Table 3); PCR gave the only positive test result with sputum from the same patient, with the probe for *M. xenopi. M. marinum* was cultured from a microscopy-negative skin biopsy sample from patient

TABLE 4. PCR results compared with culture results and clinical assessments of patients after resolution of discrepancies

Samples (*n*)	No. of specimens with final interpretation		Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
	True positive	True negative				
Microscopy-positive specimens (65)						
Culture						
Positive	43	0	66	100	100	100
Negative	22	0				
PCR						
Positive	64	0	98.5	100	100	100
Negative	1	0				
Microscopy-negative specimens (193)						
Culture						
Positive	16	0	51.6	100	100	91.5
Negative	15	162				
PCR						
Positive	31	5	96.9	96.9	86.1	99.4
Negative	1	157				
All specimens (258)						
Culture						
Positive	59	0	60.8	100	100	81.0
Negative	37	162				
PCR						
Positive	95	5	97.9	96.9	95.0	98.7
Negative	2	157				

62. In both cases sampling error is the most likely explanation for the failure of PCR.

For 21 specimens, PCR was positive but microscopy and culture were negative (Table 2). There were six patients (patients 15, 34, 41, 45, 50, and 59) for whom other specimens were culture positive for the species identified by PCR, so we regard the positive PCR as a true-positive result. The positive PCR results for 10 of these 21 specimens were compatible with the clinical evidence in those patients (from patients 46, 51 [two specimens], 53, 54, 55 [four specimens], and 56) (Table 3), so we regard the PCR results as being correct. For five specimens (from patients 47 to 49, 52, and 58), PCR was positive but culture, microscopy, and, importantly, clinical evidence were not in accord with the PCR results (Table 3). For patient 47 the final diagnosis was aspergillosis; for patient 48, all cultures remained negative and the final diagnosis is pleurisy of unknown origin; for patient 49, who came recently from Suriname, the final diagnosis was viral infection; patient 52 had non-Hodgkin's lymphoma; and patient 58 had a cutaneous streptococcal infection. We regard the PCR results for these specimens as false positive (Table 4).

Taking discrepancies, i.e., specimens which we regard as true positive, into consideration, sensitivity and specificity were calculated and are shown in Table 4. For PCR, the overall sensitivity was 97.9%, the specificity was 96.9%, the positive predictive value was 95.0%, and the negative predictive value was 98.7%. For culture, these values were 60.8, 100, 100, and 81.0%, respectively.

We advise that PCR-based identification be performed on all microscopy-positive specimens when clinical evidence suggests that mycobacteria other than *M. tuberculosis* may be involved. For such specimens, the sensitivity, specificity, and both positive and negative predictive values of this PCR are close to or equal to 100%. This new PCR assay identifies *M. tuberculosis* and the most important opportunistic mycobacteria. Direct identification in clinical specimens is possible, and results are available within 48 h. Mycobacteria that are difficult to culture and are therefore frequently missed, e.g., *M. genavense* and *M. ulcerans*, are easily identified in this PCR assay. Our multiplex PCR is also a tool for investigating the prevalence of multiple infections. Identification of mycobacteria at an early stage of disease can be helpful in the care of patients, enabling the optimal therapy to be given and preventing unnecessary and expensive isolation. The data that we provide here will enable those interested to carry out the test themselves and evaluate it in the context of their own patient populations.

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