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Clinical Evaluation of a *Mycobacterium tuberculosis* PCR Assay

J. BEIGE,[1] J. LOKIES,[1] T. SCHABERG,[2] U. FINCKH,[1] M. FISCHER,[3] H. MAUCH,[3] H. LODE,[2] B. KÖHLER,[4] AND A. ROLFS[1]*

Neurochemical Research Laboratory, Klinikum Steglitz, Free University of Berlin,[1] and Department of Pulmonology[2] and Institute of Microbiology and Immunology,[3] Hospital Zehlendorf-Heckeshorn, Berlin, and Institute of Clinical Microbiology, University of Rostock, Rostock,[4] Germany

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On the basis of previously published PCR primer sequences, we have designed a sensitive system for detecting DNA of the *Mycobacterium tuberculosis* complex (MTB) in patient sputum samples which employs a fast and simplified sample preparation method appropriate for routine diagnostic testing. In order to evaluate the accuracy of the PCR assay, we performed a prospective study with 103 patients, comparing PCR results with culture results of samples obtained from a parallel culture assay as well as with subsequent culture results. Using two MTB-specific PCR primer systems, we found 48 of 49 tuberculosis (Tb) patients to be PCR positive (PCR sensitivity, 0.98). Sixteen of 54 presumably non-Tb patients showed amplifiable MTB DNA (specificity, 0.7). The study demonstrates that for diagnostic applications of MTB PCR two MTB-specific primer pairs should be used. MTB infection is extremely unlikely in cases of MTB PCR-negative samples: with our method for the exclusion of active Tb, the validity of one PCR assay seems to be equivalent to those of at least three culturing procedures. Positive PCR results do not necessarily reflect active MTB infection. It remains to be shown whether positive PCR results in Tb-negative patients mean false-positivity, an early laboratory finding which predicts a subsequent reactivation of a prior Tb infection, or whether asymptomatic patients may carry PCR-amplifiable MTB DNA without any clinical relevance. It is important to point out that the validity of PCR results in clinical studies depends on the use of contamination controls parallel to all PCR steps and the simplicity of the DNA extraction method as well as on the specificity of the PCR results.

In recent years the number of tuberculosis (Tb) cases in the United States and Europe has increased by 3 to 6% annually, mainly in high-risk populations such as human immunodeficiency virus type 1-positive patients, chronic alcoholics, the homeless, and drug abusers (2). At present, the diagnosis of Tb due to *Mycobacterium tuberculosis* (MTB) is most commonly made by using microscopy and culture. Microscopy has a low sensitivity and specificity and can provide at best only a preliminary diagnosis. Cultures have to be incubated for 2 to 8 weeks before a final diagnosis can be made. There is thus an urgent need for a rapid, safe, and verifiable method to establish the diagnosis of Tb.

The most promising new approach to this problem is PCR (19). Several research groups have described different PCR systems and/or performed clinical studies based on PCR (3–5, 7–11, 14, 18, 21, 23). The authors reported widely differing results with respect to specificity and sensitivity. One reason for this may be methodological differences concerning sample preparation; another may be the design of amplification (e.g., nested PCR) and detection (e.g., agarose gel detection without further specificity control [4]) procedures. Different clinical parameters were used to evaluate PCR results in relation to the microbiological and clinical findings. These methodological differences complicate the comparison of investigations and estimations of the clinical value of PCR methods. Sample numbers from 7 to 302 included in the studies demonstrate that the method has not yet been sufficiently evaluated in routine diagnostics. All studies reported differing numbers of false-positive PCR results in samples from control patients. Only a few authors performed methodological investigations in their assay before testing clinical samples.

In the present study, we describe a prospective comparison of PCR with conventional diagnostic methods for MTB, using well-characterized nonselected clinical specimens. We established a simple and fast DNA preparation method for routine diagnostic use suitable even for difficult materials like sputum, as well as a nonradioactive, highly specific, and rapid amplicon detection method. We compared PCR results with the diagnosis and laboratory findings of our patients, who were assessed according to standard parameters (1) for MTB diagnosis. Standard procedures demand at least three culture specimens to ensure reliable results, which further lengthens the time required for diagnosis and possible start of therapy. One aim of the study was therefore to investigate the clinical validity of a single PCR assay in comparison to three culture procedures. Another goal was to evaluate three different amplification systems with respect to sensitivity and specificity.

MATERIALS AND METHODS

Patients and samples. In the scope of an open prospective study from August 1992 to March 1993, we consecutively investigated 103 patients who were suspected of having pulmonary MTB on the basis of pulmonary infiltrates on their chest X rays. Of these patients, none were human immunodeficiency virus type 1 infected or suffered from AIDS. Patients with immunosuppression due to medications or radiotherapy were also excluded. The clinical part of the study was done at the Department of Pulmonology, Hospital Zehlendorf-Heckeshorn, Berlin, Germany, a large county hospital serving an urban population. Diagnostic procedures in these patients included the following: (i) sputum analyses by microscopy and culturing (three times for each patient); (ii) tuberculin skin test (0.1 to 100 TU); (iii) computerized axial tomography scan; and (iv) bronchoscopy in all patients showing three negative sputum smears for Tb. Following these diagnostic procedures, patients were classified into five groups (Table 1) according to the recommendations of the American Thoracic Society (ATS) (1) by clinical investigators (T.S. and H.L.) who were unaware of the PCR results. The classification of the ATS described the status of the patients with respect to exposure to or infection with MTB. We merged groups 0 (never MTB infected) and 1 (MTB exposure) from the ATS into one group (group 1); our group 2

* Corresponding author. Mailing address: Department of Neurology, University of Rostock, POB 100 888, Gehlsheimerstr. 20, 18055 Rostock, Germany. Phone and Fax: (49)-381-36031.

TABLE 1. Clinical classification of Tb patients[a]

Tb group	Status	No. of patients
1	History of Tb; negative tuberculin skin test; smear and culture negative; definitive other diagnosis obtained by bacteriological culture or histologically on the basis of specimens obtained by bronchoscopy	30
2	Tb infection; smear and culture negative; not clinically active (positive tuberculin skin and/or history of tuberculosis, definitive other diagnosis)	24
3	Tb infection; smear and culture negative; clinically active (positive tuberculin skin test; history of Tb; clinical, histological, or radiological signs of active disease; exclusion of other definitive diagnosis; improvement under treatment with antituberculotic chemotherapy)	3
4	Tb; smear negative, culture positive	18
5	Tb; smear and culture positive	28

[a] The clinical classification of patients was carried out by the treating physicians (T.S. and H.L.) following the recommendations of the ATS (1). For assessing the microbiological status, three culture assays were carried out by using standard procedures.

corresponds to the ATS group 2 (MTB infection but no disease). In patients belonging to group 3, the diagnosis of MTB infection was based only on clinical data such as history, X-ray findings, present signs of acute disease, and improvement with antituberculotic chemotherapy.

The culture studies for diagnostic classification of patients were performed according to the standard procedures for MTB culturing (16). Up to three different samples were tested in a period between 2 weeks prior to and 2 weeks after PCR. In addition, one sample was divided for culture and PCR was performed in parallel. These (parallel) culture results did not reflect the complete and correct microbiological status of the samples in all cases, because the parallel culture might have been negative even though prior or subsequent culture results may have been positive.

Sample preparation and PCR assay. Pretreatment of sputum samples was performed by using the standard preparation method for mycobacterial laboratory diagnosis (2.5% *N*-acetyl-L-cysteine–NaOH solution and decontamination by Kirchner solution [16] [in a total of 980 ml: 7.5 g of Na_2HPO_4, 2 g of K_2HPO_4, 0.6 g of $MgSO_4$, 2.5 g of Na_3CH_2COOH, 5.0 g of asparagine, 0.012 g of phenol red, 20 ml of glycerol, pH 7.2; 100 IE of polymyxin, 10 μg of trimethoprim, 111.6 μg of azlocillin, 10 μg of amphotericin B per ml]), including concentration by a centrifugation step at 3,000 × *g* for 15 min. After resuspension of the sediment in 1.5 ml of 0.67 M phosphate buffer (pH 5.6), the sediment was used to prepare two smears (Ziehl-Neelsen acid-fast staining) and to inoculate the media. For direct comparison, the sample volume, pretreated as described above, was simultaneously investigated in both culture and PCR. Culture assays were performed with Löwenstein-Jensen tubes (BBL) and Middlebrook 7H11 broth (MB-Check; Roche Diagnostics, Grenzach, Germany) and by inoculating the specimens. The specimen amount for MB-Check was 0.1 to 0.5 (maximum) ml, since the pellet was concentrated in 1 ml and divided in some culture assays. Mycobacterial cultures were incubated for 6 weeks. For PCR investigations, we performed a simplified alkaline DNA preparation method after pretreatment with 2.5% *N*-acetyl-L-cysteine–NaOH solution: the cell pellets were lysed with 100 μl of 50 mM NaOH for 15 min at 95°C under an oil overlay; then material was neutralized with 1 M Tris-HCl (8 μl per 50 μl of NaOH; for details of alkaline lysis, see reference 17). No further purification procedures were necessary.

We tested three amplification systems. Two primer pairs produce MTB-specific amplicons: a 158- (2.4-kb DNA insert, pPH7301 clone) (9) and a 240-bp (protein MBP64) amplification product (21). The third system (3) amplifies a 383-bp fragment from the 65-kDa antigen and was set up to evaluate the presence of MTB as well as mycobacteria other than MTB (MOTT), using a genus-specific primer pair. In a methodological pilot study, we found no differences between alkaline DNA preparation and standard methods (proteinase K, phenol-chloroform, or the sodium dodecyl sulfate method) with respect to the sensitivity of detecting MTB DNA, even with difficult specimens such as sputum. However, the proteinase K method takes about 12 to 24 h and uses toxic substances, whereas alkaline lysis takes only 45 min and is a simple and safe method for preparing samples for PCR. For diagnostic PCR, 1 μl of the prepared biological sample was used in a 50-μl assay; 200 μM (each) deoxynucleoside triphosphate, 0.3 μM each single primer, and 1 U of AmpliTaq (Perkin-Elmer Cetus) were used. The temperature profile for all amplification procedures was as follows: 50 s at 95°C, 40 s at 60°C, 40 s at 72°C; 35 cycles. A beta-actin primer pair (17) served as an internal PCR amplification control for the presence of amplifiable DNA purified from each sputum sample (amplification control). As a contamination control, we used culture-negative sputum samples as well as MTB-positive material (0.5 pg of MTB DNA, corresponding to 100 copies of MTB organisms) in every third PCR tube. We performed negative (one with every three samples) and positive (one with every 10 samples) controls during pretreatment and DNA extraction as well to assess possible contamination at that step. The possibility of PCR contamination was minimized by keeping the amplified products physically separated from starting materials. All pre-PCR handling, such as aliquoting all reagents, packing tips and tubes, etc., was performed in one room. Setting up all PCRs was done in a second room, using a circulation-free, sterile bench with UV lighting. Another room, located on a different floor, was dedicated to the processing and analysis of all amplification products. Equipment in these rooms (positive displacement pipettes, disposable tips, etc.) was used exclusively for PCR. For all reaction steps, gloves were worn and changed frequently. About 40% of all samples were run in duplicate PCRs and demonstrated a congruity of nearly 100% (only two samples that initially tested positive revealed a negative result for one primer pair in the duplicate test). Because of the high level of conformity of the PCR results, about 60% of the samples were not tested further in duplicate. Additionally, after clinical status and PCR data were compared, samples from patients from positive PCR but negative culture were reevaluated completely for all three PCR assays. Furthermore, 7 of 16 patients from groups 1 and 2 were controlled in follow-up samples for culturing and amplification 5 to 14 months after the first samples had been investigated. In order to exclude possible contamination during the PCR process and to examine the specificity of the PCR system in another cohort, sputum samples from 67 healthy subjects with no evidence of mycobacterial disease were investigated.

As a specificity control for all amplification products, we performed Southern blotting (positively charged nylon membranes; Boehringer Mannheim, Penzberg, Germany) (25) followed by a digoxigenin-labelled probe detection (Boehringer Mannheim). To generate sequence-specific probes, we designed an amplicon-nested PCR (6) for the three primer systems, using inner primers. The sequences of the primers used for probe synthesis were as follows: 240-bp amplicon (21; sequence published in reference 27), (+) 5′GGC TTG GAC CCG GTG A3′, (−) 5′CGA TCG CGG AAC GTG G3′; 158-bp amplicon (9), (+) 5′CGA CAT GGA CGG CGG G3′, (−) 5′CCC GCC CAT TCC GGT C3′; 383-bp amplicon (3; sequence published in reference 22), (+) 5′TGC TCA AGG GCG CCA A3′, (−) 5′TGG TCA CCC GCC GAA A3′. For assessing the sensitivity of our assay, we performed a 10-fold serial dilution of whole *M. tuberculosis* cells, ranging from 10^4 to 10^{-3} cells per ml, obtained from liquid culture medium. One milliliter of Tb-negative sputum (confirmed by culture and PCR) was added to 1 ml of each of the serial dilution steps. The sample was divided into two aliquots. One aliquot was added to a liquid culture medium and the second aliquot was used for DNA preparation and testing in PCR. Colony counts following culture growth provided a reference for the exact amount of viable bacteria present in each dilution step. With this procedure, specific hybrids could be detected to a limit of about 10 to 100 MTB organisms (240-bp fragment, 10 to 100 copies; 158-bp fragment, 10 to 100 copies; 383-bp fragment, about 100 copies). The probes react exclusively with the sequences from their intended targets (data not shown).

In assessing the PCR results for clinical diagnosis, a PCR was "positive" if at least one MTB-specific primer system (240- and 158-bp products) revealed specific signals in the hybridization procedure. The 240-bp PCR fragment was semiquantified in seven unselected samples from group 2, 11 from group 4, and 13 from group 5. The amplified 240-bp products were radioactively labeled by incorporation of ^{32}P-dCTP and separated in a denaturing 7% urea–polyacrylamide gel. The semiquantitative analysis of the amplification products was done by measuring the radioactivity incorporated in the specific product cut out of the gels. All PCR assays for semiquantification were repeated five times.

RESULTS

We used 49 samples from 49 Tb patients (groups 3 to 5) for PCR diagnosis and 147 sputum samples from 49 Tb patients for culture. PCR was positive in 48 of 49 samples (sensitivity, 0.98). Culture yielded positive results in 83 of 147 samples (56.5%). One sample not detectable in PCR was culture negative in a concomitant analysis but positive in subsequent cultures. Culture performed in parallel with the PCR samples confirmed the diagnosis of Tb in only 37 of 49 patients

TABLE 2. Comparison of PCR results and clinical evaluation[a]

Clinical group[b]	PCR results	
	No. (%) of samples positive	No. (%) of samples negative
1 (no Tb)	5 (17)	25 (83)
2 (Tb history)	11 (44)	13 (56)
3 (Tb positive)	3 (100)	
4 (Tb and culture positive)	17 (94)	1 (6)
5 (Tb and smear positive)	28 (100)	

[a] PCR was scored positive if a least one MTB-specific primer system (240-bp and/or 158-bp products) revealed specific signals in the hybridization procedure.
[b] The clinical classification is given in Table 1.

(75.5%). Nevertheless, all culture results taken together corresponded to a positive clinical status in 46 of 49 patients (culture sensitivity, 0.93). When we considered the results from all cultures (three analyses of each patient), three Tb patients remained culture negative and therefore were classified in group 3. PCR was positive in all of these three negative culture samples obtained from Tb patients (Table 2). Positive PCR results were obtained in 16 of 54 samples from patients who had no active Tb disease (groups 1 and 2 [Table 2]) (specificity, 0.7). The number of positive PCR results was higher in samples obtained from patients who showed signs of having a history of pulmonary Tb and who demonstrated a positive tuberculin skin test (11 of 24 [45.8%], group 2) than in those obtained from patients with negative tuberculin skin tests (5 of 30 [16.7%], group 1). For these patients, we established a PCR specificity of 0.83.

When considering the PCR results (Table 3), we detected the 240- and 383-bp fragments more often than the 158-bp amplicon (53 of 64 [82.8%] and 47 of 64 [73.4%] versus 46 of 64 [71.8%]). One smear-positive sample was positive exclusively for the 158-bp fragment. In three samples of the Tb-negative group 2, only the 383-bp fragment was detectable; this result may be due to the amplification of MOTT because of the lack of MTB specificity of this primer system.

Table 4 gives detailed information about those patients whose sputum samples were positive in PCR testings but who showed no clinical or culture evidence of active Tb (groups 1 and 2). The amplification results for the 240-bp fragment were extremely stable since nearly all samples collected during the clinical treatment were consistently positive. In contrast, the 158- and 383-bp fragments revealed somewhat inconsistent results. Fifty percent of the patients from groups 1 and 2 had bronchogenic carcinoma, a situation that might cover clinical signs of a concomitant Tb infection. It is very unlikely that the high percentage of positive PCR results in groups 1 and 2, especially of the 240-bp fragment, is due to contamination, since all PCR steps were monitored with contamination controls. Additionally, the sputum samples from 67 healthy subjects were investigated in the same manner as the patient samples. Only two healthy volunteers revealed a positive signal for the 383-bp fragment, which may be due to amplification of MOTT.

Seven of 16 patients from groups 1 and 2 could be reinvestigated 5 to 11 months after the first examination. Nearly all patients with bronchogenic carcinoma had died, and two patients had moved away from Berlin. The sputum samples of six of the remaining seven patients were still PCR positive. Two of these initially culture-negative patients had been treated meanwhile with antituberculotic drugs because of positive culture results for MTB. One patient initially positive in PCR was negative in the follow-up investigations.

The semiquantitative examination of the 240-bp PCR fragments demonstrated an intra-sample variation of 10.2% in repeated measurements. The signal in counts per minute was significantly lower for the 240-bp fragment ($P \leq 0.01$) for samples from group 2 than for those from groups 4 and 5. Seven unselected sputum samples from patients from group 2 revealed a mean amount of 34.560 ± 5.685 cpm (mean ± 2 standard deviations) of ^{32}P-dCTP; sputum specimens ($n = 11$) from patients from group 4 had a signal of 54.385 ± 8.976 cpm, and those from patients belonging to group 5 ($n = 13$) had a signal of 88.317 ± 37.927 cpm.

DISCUSSION

The definitive and rapid diagnosis of Tb in routine clinical work is difficult since culturing takes about 3 to 6 weeks and usually requires at least three samples. Our data demonstrate that PCR can at least help to exclude the diagnosis of Tb as long as a highly standardized protocol for DNA preparation, appropriate precautions for the avoidance of contamination, and a sensitive hybridization assay are used. This is due to the extremely high clinical sensitivity of PCR (0.98), which we demonstrated to be higher than the sensitivity of concomitantly performed cultures (0.75) as well as of subsequent culture analyses (0.93). The clinical sensitivity of the PCR procedure found in our study is in accordance with results of other groups, which are between 0.74 and 1.00 (3, 5, 7, 9, 10). However, some studies report a relatively low PCR sensitivity: e.g., Pierre et al. (14) and Soini et al. (24) found sensitivities for their PCR assays of 63 and 55.9%, respectively. Such low

TABLE 3. Amplification results for all specimens[a]

Group	No. of samples tested	PCR primer results (no. of samples)							
		MTB PCR negative		MTB PCR positive					
		No amplification	Only 383 bp	Only 158 bp	Only 240 bp	240/158 bp	240/383 bp	158/383 bp	240/158/383 bp
1	30	24	1		2	1		1	1
2	24	11	2		3		6	1	1
3	3				1		1		1
4	18	1			1	4	1	5	6
5	28			1	2	2	1	3	19
Total	103	36	3	1	9	7	9	10	28

[a] Because the 383-bp fragment cannot differentiate between MTB and MOTT (for details, see Materials and Methods), cases which were positive for only this fragment were considered MTB PCR negative.

TABLE 4. Detailed amplification data from patients of groups 1 (patients 1 to 5) and 2 (patients 6 to 16) with positive PCR results but negative culture and smear

Patient	Diagnosis	Sputum sample no.	Amplification[a]		
			240 bp	158 bp	383 bp
1	Cold	1	+	−	−
		2	+	−	−
		3	+	+	−
2	Cold	1	+	+	+
3	Thoracic lymphoma	1	+	+	−
4	Acute bronchitis	1	+	+	−
5	Bronchogenic carcinoma	1	+	−	+
		2	+	−	+
		3	+	−	+
6	Fever of unknown origin	1	+	−	+
		2	+	−	−
7	Sarcoidosis	1	+	−	+
		2	+	−	+
		3	+	−	+
8	Bronchogenic carcinoma	1	+	−	+
9	Bronchogenic carcinoma	1	+	+	−
		2	+	+	+
		3	+	−	+
10	Bronchogenic carcinoma	1	+	−	+
		2	−	+	
11	Bronchogenic carcinoma	1	+	−	+
		2	+	−	+
		3	+	−	−
12	Acute bronchitis	1	+	−	+
13	Bronchogenic carcinoma	1	+	−	−
14	Pulmonary fibrosis	1	+	−	+
		2	+	+	+
15	Cold	1	+	+	−
		2	+	−	+
16	Bronchogenic carcinoma	1	+	−	+
		2	+	−	−

[a] +, positive; −, negative.

sensitivities in PCR studies may be explained by suboptimal assay conditions.

With our method to exclude active Tb, the validity of one PCR assay seems to be equivalent to that of at least three culture procedures. The PCR methodology reduces the time period for obtaining results from more than 3 weeks to 1 to 2 days in smear-negative patients. This is of great importance, especially in cases of unknown granulomatous diseases and when rapid diagnosis is necessary.

According to our data, a positive PCR result seems to correspond to active Tb in 70% of cases. We detected MTB DNA in many samples (11 of 24) from patients with a Tb history and a positive skin test result but with no active disease (group 2). Although we found no signs of active disease in these cases, the ability of our assay to detect MTB DNA even in cases with a history of, but no active, Tb is from a clinical point of view a further strong indication for the high sensitivity of PCR diagnosis. In comparing specificity data between different MTB PCR and culture studies, it is important to mention whether the PCR data were compared with the culture results or with the clinical diagnosis of Tb and how the patients were recruited (selected versus unselected recruitment, pulmonary ward or outpatients). When culture is used as the standard technique in a comparison study, specimens containing noncultivable bacteria, which may lead to a positive PCR result, are initially identified as false-positive samples. In the absence of an ideal gold standard, it is not clear what proportion of the specimens with initial false-positive results actually contain noncultivable bacteria. Jonas et al. (10) described 21 specimens which were positive in their Gen-Probe assay but culture negative. Forbes and Hicks (7) investigated two culture-negative patients with positive PCR results. From one patient they were able to reproduce this constellation in five independent sputum samples. At present, there is no adequate comparison to evaluate a new method for MTB diagnosis other than culture results or clinical assessment; culture does not detect nonviable or nonculturable organisms, and clinical assessment is not always reliable. Because of these results, the authors hypothesize that PCR assays not only may prove more sensitive than culture but may modify our present understanding of MTB infection. Schluger et al. (20) assayed 65 inpatients with PCR. When correlated with cultures and clinical history, the sensitivity of PCR was 100%. However, since the PCR assay was also positive in a number of patients with only prior or treated Tb or asymptomatic tuberculosis infection, the specificity for a diagnosis of active Tb was only 70%. Since we too detected MTB DNA in many samples from patients with a history of Tb but no active disease, we know it is not always possible to determine the clinical relevance of a positive PCR result. The fact that PCR correctly detects MTB DNA in such situations is supported by observations about the presence of mycobacteria in patients with diseases other than Tb and the surveillance of PCR-detectable MTB in nonactive Tb and sarcoidosis patients (18, 26), as well as the high percentage of MTB-infected persons without relevant clinical signs (2). In discussing the question of the biological importance and clinical value of methodologically correct PCR-positive results, we have to consider whether the mycobacteriological dogma of an imperative connection between the detection of even a very low number of MTB particles and the diagnosis of Tb is also true for PCR. However, the clinical importance of positive PCR results (5 of 30) in non-Tb patients remains uncertain and is problematic with respect to clinical specificity. From a clinical point of view, these PCR results seem to be false-positive. Suspected false-positive PCR results must be interpreted from several aspects.

First, positive results may be caused by intra- and prelaboratory contamination. This hypothesis is supported by the data from Noordhoek and colleagues (13), who described a blind PCR comparison study for the detection of MTB with a high level of false-positive PCR results (3 to 77%). The authors suspect that this might be due to contamination of buffers and materials used in the pretreatment of the samples and for the

extraction of DNA. In our study, laboratory-associated contamination is unlikely since we rigorously performed negative as well as positive controls during the DNA preparation, amplification, and detection procedures. DNA extraction as done in our laboratory is a single-tube method in which the tube is entered only twice, to add the patient sample for the lysis procedure and to withdraw a sample for the amplification procedure. This simple lysis procedure is especially important for controlling the risk of cross-contamination between specimens. Besides the lower risk of contamination with the one-tube alkaline preparation method, these procedures increase the sensitivity of the PCR test system, because there is no loss of specific DNA as seen in all DNA preparation methods that need multiple pipetting steps and changing of tubes and in precipitation methods. A further hint that the high percentage of positive PCR results, especially from patients of group 2, is not due to contamination is demonstrated by the results of quantification of the mycobacterial copies. There are clearly higher mycobacterial DNA copies in patients from groups 4 and 5 than in those from group 2. Although the group of patients we investigated for PCR quantification is small and the method used for quantification of PCR products is somewhat problematic, the tendency to higher amounts of MTB in more advanced stages of disease might be an interesting marker in the diagnosis of acute versus inactive Tb. In subsequent analyses, 7 of 16 patients from groups 1 and 2 were reexamined 5 to 11 months after the first examination and the sputum samples from 6 of these 7 patients were still PCR positive. Two of these initially culture-negative patients had been diagnosed as Tb culture positive in the meantime and treated with antituberculotic drugs. This also argues against the presumption that prelaboratory contamination inside pulmonary wards or during the DNA preparation steps might have been the cause of the high level of positive results in groups 1 and 2. Beside the importance of contamination controls during all PCR-associated steps, a specificity control for PCR amplicons is also a necessary precondition for the use of PCR in clinical diagnostics. Also, Noordhoek and coworkers (13) reported one laboratory that produced PCR products with a different molecular weight than expected, which, nevertheless, were scored as positive. Especially in the 240-bp primer system, we found in 8% of all samples investigated an amplification band in the agarose gel with about the expected molecular weight; this band did not hybridize in the blot procedure. Since this is a common phenomenon in PCR technology, no diagnostic data should be given without a reliable specificity control. Because of the high risk of further contamination, nested PCR should be used for specificity control only when it is designed as a one-tube procedure.

Second, previously undetectable Tb can be detected by PCR because of its higher sensitivity. This is of special interest to patients with severe bronchial diseases and carcinomas, which made up a major part of the Tb-negative group. Interestingly, about 50% of the patients from groups 1 and 2 who were MTB positive according to PCR had a carcinoma. This could imply that a reactivation of a prior tuberculotic infection has occurred due to possible immunosuppression associated with the carcinoma.

Third, PCR detects DNA of nonviable or quantitatively irrelevant MTB pathogens, which would not cause clinical disease or need to be treated. In the future, PCR quantification of MTB RNA may bring about an improvement in diagnostic accuracy, because active expression of mRNA implies the existence of viable organisms and may imply active infection when present above a certain quantity. The first results of the isothermal enzymatic amplification of MTB rRNA (Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test) from the sputa of patients with a pulmonological diagnosis (10, 12) could be evidence in favor of this argument. However, even this test system revealed a positive reaction in 3.5% (7 of 198) of culture- and clinically Tb-negative patients in a study by Miller and coworkers (12). The group describes the case of a patient with a history of Tb but no actual signs of it. Three culture-negative samples were investigated; one was positive in the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test. Subsequent specimens submitted 6 months later were culture positive for *M. tuberculosis*. This may demonstrate the ability of amplification systems to detect MTB DNA earlier than conventional diagnostic systems.

The fact that the three primer systems tested in the study revealed different results (Table 3) correlates with the different sensitivities of the systems but also exemplifies the problem of PCR failures. Therefore, for the present standardization of sample preparation and processing procedures, the use of at least two MTB-specific primer systems is necessary for a reliable MTB PCR result which could be used in the diagnosis of Tb. In accordance with previous methodological studies from our group (data not shown), the 240-bp primer system (21) is the most sensitive assay in patient specimens. The 383-bp primer systems amplifies MTB as well as MOTT; when the 158- and 240-bp systems were negative but the 383-bp system was positive, it is assumed that a MOTT sequence may have been amplified by the 383-bp primer system. However, since even the most sensitive 240-bp system demonstrates a sensitivity of about 83% and since the clinical interpretation of the 383-bp system depends also on the results of the other two systems, the use of the 383-bp primer system appears to add little information to the analysis of clinical specimens. However, when we started the study, no PCR amplification system which specifically amplifies atypical mycobacteria had been described; during the study, the combination of a positive 383-bp result and negative 158-bp/240-bp fragments might have presaged such a system.

To summarize, our data demonstrate the following findings. (i) Alkaline lysis is a reliable and fast DNA preparation method for reliable MTB DNA amplification. *N*-Acetyl-L-cysteine (2.5%)–NaOH-pretreated samples are also suitable for PCR. Alkaline lysis has the great advantage of being a one-tube reaction that requires only about 45 min and is therefore especially suitable for routine procedures. (ii) If a high grade of standardization (including DNA preparation procedure and contamination controls parallel to all PCR steps) is observed, PCR is a fast and also reliable method for the exclusion of Tb. Nevertheless, because the significance of positive PCR results in Tb-negative patients is unclear, at the moment it is necessary to perform cultures in addition to PCR. It remains to be demonstrated in further prospective studies whether positive PCR results in Tb-negative patients mean false-positivity, an early laboratory finding which predicts subsequent reactivation of Tb, or whether asymptomatic patients may carry PCR-amplifiable MTB DNA without any clinical relevance.

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