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Amplification of *Mycobacterium tuberculosis* from Peripheral Blood

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To determine the value of the PCR assay of lymphomonocytic blood cells for the diagnosis of pulmonary tuberculosis, we compared, in a prospective study, PCR results with results of conventional diagnostic methods of *Mycobacterium tuberculosis* detection. Clinical investigators who were unaware of the PCR data classified 162 immunocompetent patients, who had been hospitalized because of suspected pulmonary tuberculosis, in accordance with the recommendations of the American Thoracic Society (ATS). By testing blood samples from these patients for mycobacterial DNA with three different PCR systems, we were able to demonstrate that nonquantitative PCR of peripheral blood leukocytes is of little value for the specific diagnosis of pulmonary tuberculosis. However, semiquantitative PCR assays might have some significance since we found an increasing level of mycobacterial DNA copies in blood from patients classified in ATS groups 2 to 5.

The most common routine methods for establishing a diagnosis of pulmonary tuberculosis are still microscopic inspection and sample culturing. Microscopy has the disadvantage of low sensitivity and specificity, and cultures have to be incubated for up to 8 weeks before a final diagnosis can be made. The most promising new approach for rapid, safe, and reproducible determination of *Mycobacterium tuberculosis* infection is the PCR. The several different PCR systems that have been described have produced widely differing results with regard to specificity and sensitivity (2, 4, 7, 8). Handling of sputum samples for the DNA preparation steps is especially laborious and time-consuming and is often the origin of PCR-inhibiting substances in sputum samples (3). Recently, Schluger and colleagues (12) reported that use of PCR techniques with phenol-chloroform-purified DNA from a buffy coat layer of cells makes the diagnosis of pulmonary tuberculosis easier. In tests on blood cells using a primer pair which was specific for repetitive insertion element IS*6110*, Schluger et al. (12) found all patients with active pulmonary infections to be PCR positive and all controls, as well as all tuberculin skin test-positive cases, to be negative. In contrast, Kolk and colleagues (6) have pointed out that PCR assay of peripheral blood lymphocytes may only be useful for diagnosis of tuberculosis in immunocompromised patients. Here we describe a prospective comparison of PCR assay of peripheral blood leukocytes with conventional diagnostic methods of *M. tuberculosis* detection.

MATERIALS AND METHODS

Study design. Within the previous 28 months, 162 patients suspected of having pulmonary tuberculosis were examined in a prospective, double-blind study in which the clinical investigators were unaware of the PCR results and the laboratory workers were unaware of the clinical diagnoses. None of these patients was infected with human immunodeficiency virus type 1 or had AIDS. Patients with immunosuppression due to medication or radiotherapy were excluded. Diagnostic procedures included sputum analysis by standard microscopy and culture techniques, tuberculin skin tests, and tomography or computerized tomography scanning. Bronchoscopy of about 35% of the patients was carried out. The patients were classified into five groups in accordance with the modified recommendations of the American Thoracic Society (1). Group 1 had no history of tuberculosis, negative tuberculin skin tests, negative mycobacterial smear and culture tests, and positive identification of a carcinoma or of viral, fungal, or bacterial (other than mycobacterial) pathogens as responsible for the pulmonary disease. Group 2 had a history of pulmonary tuberculosis, positive tuberculin skin tests, negative mycobacterial smear and culture tests, and positive identification of a carcinoma or of viral, fungal, or bacterial (other than mycobacterial) pathogens as responsible for the pulmonary disease. Group 3 had positive tuberculin skin tests, histological and radiological signs of pulmonary tuberculosis activity, negative mycobacterial smear and culture tests, and no other definitive diagnosis and showed a clear clinical benefit from therapy with antituberculosis drugs. Group 4 patients had definite pulmonary tuberculosis and were smear negative but culture positive. Group 5 patients had definite pulmonary tuberculosis and were smear and culture positive. The culture studies were performed in accordance with the standard procedures for *M. tuberculosis* culturing (9).

PCR assays. We used three amplification systems designed to reveal *M. tuberculosis*-specific amplicons: 158-bp (2.4-kb DNA insert, pPH7301-clon) (5), 240-bp (protein MBP64) (13), and 244-bp (IS*6110* insertion element) amplification products defined in accordance with the primer sequences given by Schluger et al. (12). PCR amplification was achieved as described elsewhere (2). To check that DNA samples were suitable for amplification, an internal amplification control of the pyruvate dehydrogenase gene (10) was included in our study. The specificity of all amplicons was assessed by using the Southern blot hybridization method (positively charged nylon membranes; Boehringer GmbH, Mannheim, Germany), followed by digoxigenin-labelled probe detection. To generate sequence-specific probes, we designed an amplicon-nested PCR for the 244 bp from the IS*6110* insertion element: positive, 5′ ATG ACC AAA CTC GGC C 3′; negative, 5′ GCC CAG GTC GAC ACA TAG 3′. The sequences for the other PCR-generated probes are given in reference 2. A PCR result was considered as positive if there was a clear, distinct band in the Southern blot with a size we expected to see. Furthermore, the 240-bp PCR fragment was semiquantified as PCR positive by incorporation of [^{32}P]dCTP in the samples from all 39 patients (2). All samples were tested, twice, and the data given in Table 1 are means ± the standard deviations for all 78 samples.

For EDTA-blood sample (2 ml) preparation, we used a simplified alkaline DNA preparation method. After lysis of the erythrocytes, leukocytes were pelleted by means of a centrifugation step; the resulting cell pellets were lysed with 100 μl of 50 mM NaOH for 15 min at 95°C under an oil overlay, and then the material was neutralized with 1 M Tris-HCl (8 μl/50 μl of NaOH; for details of alkaline lysis, see reference 10). No further purification procedures were necessary.

Each assay for the investigation of one sample included the patient's sample with the three different mycobacterial primer pairs (tubes 1 to 3), the patient's sample with the pyruvate dehydrogenase primer pair (tube 4), a negative control for detection of potential contamination during sample pretreatment and the DNA extraction procedures (tube 5), a second negative control for detection of

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TABLE 1. Amplification results of all blood samples in accordance with the clinical classification recommended by the American Thoracic Society[a]

Group (no. of patients)	No. (%) of patients		No. (%) of patients PCR positive with:			Mean cpm (10^3) obtained with 240-bp primer ± 2 SD[b]
	PCR negative	PCR positive	244-bp primer	240-bp primer	158-bp primer	
1 (47)	47	0	0	0	0	
2 (29)	24 (83)	5 (17)	4	4	2	8.9 ± 6.1
3 (10)	7 (70)	3 (30)	1	2	2	9.4 ± 8.5
4 (31)	16 (52)	15 (48)	13	12	9	30.3 ± 17.1
5 (45)	29 (64)	16 (36)	14	14	11	36.2 ± 18.2
Total no.: 162	123 (76)[c]	39 (24)[c]	32 (20)[c]	32 (20)[c]	24 (15)[c]	

[a] The patients were classified into five groups in accordance with the modified recommendations of the American Thoracic Society (for details, see Materials and Methods).
[b] For details, see Materials and Methods.
[c] The percentages given are in relation to the sum of all of the patients investigated.

potential contamination during pipetting of the PCR mixture (tube 6), a positive control with 100 copies of *M. tuberculosis* DNA for detection of potential problems during the DNA extraction procedure (tube 7), and a second positive control with 1 ng of purified mycobacterial DNA (tube 8). The chance of PCR contamination was minimized by physical separation of the amplified products from the starting materials. All pre-PCR handling of materials was performed in a room separate from the PCR site. All PCRs were set up in a second room with 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. The equipment handled in these rooms (positive-displacement pipettes, disposable tips, etc.) was used exclusively for PCR. To further check the possibility of artifactual contamination of blood samples, we reinvestigated original blood materials which were known to be positive for the 244-bp product (n = 32) and which had never before been in the PCR laboratories. This PCR assay was done with the uracil *N*-glycosylase (UNG) system (11), which allows safe pre-PCR enzymatic decontamination of a dU-containing PCR product.

RESULTS

All patients in group 1 (tuberculin skin test negative) tested completely negative in all PCR assays. In groups 2 to 5, the percentages of patients found to be positive in one or more PCR amplification systems varied between 17 and 48% (Table 1). However, the highest percentage of PCR-positive samples was found in groups 4 and 5. This result correlated quite well with the semiquantitative PCR values for the 240-bp product, which increased from 8,900 cpm in group 2 to 38,200 cpm in group 5; however, these differences were not significant. Of a total of 39 patients, the blood samples of 21 were positive for all three primer pairs. Also, 10 of the 39 patients were positive by only one PCR assay (4 patients for the 244-bp product, 3 for the 240-bp product, and 3 for the 158-bp product). This somewhat erratic correlation between the amplification assays of different gene regions is a known phenomenon and has also been seen in previous studies (2) of sputum samples. However, such a lack of conformity in the different PCR assays cannot be explained by differences in sensitivity, since we found the 244-bp and 240-bp PCR assays to be identical in sensitivity, whereas the 158-bp PCR system was less sensitive. The lower sensitivity of the 158-bp system than the 240-bp system in the present study confirms earlier results obtained by our group (2).

The sensitivity of the blood PCR assay for the diagnosis of proven pulmonary tuberculosis ranged from 0.30 (group 3) to 0.48 (group 4) and 0.35 (group 5). Thus, the assay was less sensitive than the system described by Schluger et al. (12) but much more sensitive than that reported by Kolk and coworkers (6).

For potential decontamination of possible PCR amplicon-generated false-positive results, we retested the 244-bp-positive blood samples with the UNG system. This procedure completely confirmed the original data: the UNG results correlated in all cases with the first assays processed without the UNG system. This supports our claim that positive results obtained with the 244-bp PCR product are not the result of sample contamination with PCR products.

DISCUSSION

Nonquantitative PCR assay of peripheral blood leukocytes seems to be of little value for the diagnosis of pulmonary tuberculosis in immunocompetent patients. In patients who were PCR positive, we found increasingly higher numbers of *M. tuberculosis* DNA copies circulating in the blood of groups 2 to 5. However, while the quantitation gave a higher value for group 5, a lower percentage of samples in group 5 gave a positive PCR result (36% in group 5 versus 48% in group 4). We do not believe that the higher rate of positive PCR results than that reported by Kolk and coworkers (6) is due to PCR-generated contamination, since we routinely performed negative and positive controls during DNA preparation, amplification, and detection procedures and all of the UNG PCR assays with completely new and separately prepared DNA samples confirmed our original results. Furthermore, the extremely simple DNA extraction method which we employed in our study is a single-tube method in which the reaction cup is opened twice only, once to add the chemicals for the lysis procedure and then a second time to withdraw an aliquot for the PCR. This one-tube alkaline preparation method might increase the sensitivity of the PCR test system, since there is no loss of specific DNA, which always occurs with DNA extraction methods which require tube changes. Comparison of our data with those of Schluger and coworkers (12) indicates that it is unlikely that we used a less sensitive assay since we were able to demonstrate a detection limit in our assay of about 10 copies of *M. tuberculosis* DNA per ml of EDTA-blood.

In conclusion, we believe that nonquantifying PCR methods which use blood for the diagnosis of pulmonary tuberculosis are of only limited value—at least for immunocompetent patients—because we found patients who definitely had no pulmonary tuberculosis (group 2) to be up to 17% PCR positive. On the other hand, only 40% of patients with proven pulmonary tuberculosis had positive PCR results. Even the investigation of three different gene regions of the *M. tuberculosis* genome did not improve the positive detection rate of *M. tuberculosis* DNA in the peripheral blood. A more detailed investigation of reliable quantitative PCR assays in a greater

group of patients is necessary to estimate the usefulness of quantitative PCR systems for the clinical diagnosis of mycobacterial infection. Nevertheless, the fact that the blood of patients with no trace of extrapulmonary disease or a miliary pattern were PCR positive for *M. tuberculosis* supports the hypothesis that *M. tuberculosis* escapes from the alveolar space to the blood circuit more often than previously thought.

REFERENCES

1. **American Thoracic Society.** 1990. Diagnostic standards and classification of tuberculosis. Am. Rev. Respir. Dis. **142:**725–735.
2. **Beige, J., J. Lokies, T. Schaberg, U. Finckh, M. Fischer, H. Mauch, H. Lode, B. Köhler, and A. Rolfs.** 1995. Clinical evaluation of a *Mycobacterium tuberculosis* PCR assay. J. Clin. Microbiol. **33:**90–95.
3. **Buck, G. E., L. C. O'Hara, and J. T. Summersgill.** 1992. Rapid, simple method for treating clinical specimens containing *Mycobacterium tuberculosis* to remove DNA for polymerase chain reaction. J. Clin. Microbiol. **30:**1331–1334.
4. **Eisenach, K. D., D. Cave, J. H. Bates, and J. T. Crawford.** 1990. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J. Infect. Dis. **161:**977–981.
5. **Hermans, P. W. M., A. R. J. Schuitema, D. Van Soolingen, C. P. H. J. Verstynen, E. M. Bik, J. E. R. Thole, A. H. J. Kolk, and J. D. A. van Embden.** 1990. Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. J. Clin. Microbiol. **28:**1204–1213.
6. **Kolk, A. H. J., L. F. F. Kox, S. Kuijper, and C. Richter.** 1994. Detection of *Mycobacterium tuberculosis* in peripheral blood. Lancet **344:**694.
7. **Kox, L. F. F., D. Rhienthong, A. M. Miranda, N. Udomsantisuk, K. Ellis, J. van Leeuwen, S. van Heusden, S. Kuijper, and A. H. J. Kolk.** 1994. A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. J. Clin. Microbiol. **32:**672–678.
8. **Noordhoek, G. T., A. H. J. Kolk, G. Bjune, D. Catty, J. W. Dale, P. E. M. Fine, P. Godfrey-Faussett, S.-N. Cho, T. Shinnick, S. B. Svenson, S. Wilson, and J. D. A. van Embden.** 1994. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. J. Clin. Microbiol. **32:**277–284.
9. **Roberts, G. D., E. W. Koneman, and Y. K. Kim.** 1991. *Mycobacterium*, p. 304–339. *In* A. Balows, W. J. Hausler, Jr., K. L. Hermann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
10. **Rolfs, A., I. Schuller, U. Finckh, and I. Weber-Rolfs.** 1992. PCR: clinical diagnostics and research, p. 74–77. Springer-Verlag KG, Berlin.
11. **Rys, P. N., and D. H. Persing.** 1993. Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. J. Clin. Microbiol. **31:**2356–2360.
12. **Schluger, N. W., R. Condos, S. Lewis, and W. Rom.** 1994. Amplification of DNA of *Mycobacterium tuberculosis* from peripheral blood of patients with pulmonary tuberculosis. Lancet **344:**232–233.
13. **Shankhar, S., N. Manjunath, K. K. Mohan, K. Praasad, M. Behari, G. K. Shriniwas, and K. Ahuja.** 1991. Rapid diagnosis of tuberculosis meningitis by polymerase chain reaction. Lancet **337:**5–7.