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Rapid Diagnosis of *Mycobacterium tuberculosis* Bacteremia by PCR

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A method based on DNA amplification and hybridization has been used for the rapid detection of *Mycobacterium tuberculosis* in blood samples from 38 hospitalized patients (15 human immunodeficiency virus [HIV] positive and 23 HIV negative) in whom localized or disseminated forms of tuberculosis were suspected. In 32 of these patients, the diagnosis of tuberculosis was eventually confirmed by conventional bacteriological or histological procedures. *M. tuberculosis* DNA was detected with the PCR technique in the peripheral blood mononuclear cells from 9 of 11 (82%) HIV-infected patients and in 7 of 21 (33%) HIV-negative patients ($P < 0.01$), while *M. tuberculosis* blood cultures were positive in 1 of 8 (12.5%) and 1 of 18 (5.5%) patients, respectively. PCR was positive in all cases with disseminated disease in both HIV-negative and HIV-positive patients and also in the HIV-positive patients with extrapulmonary tuberculosis. Seven samples from patients with documented illness other than tuberculosis and 12 specimens from healthy volunteers, including seven volunteers with a recent positive purified protein derivative test, were used as controls and had a negative PCR. These results suggest that detection of *M. tuberculosis* DNA in peripheral blood mononuclear cells may be a useful tool for rapid diagnosis of disseminated and extrapulmonary forms of tuberculosis, especially in an HIV-positive population.

With the increased incidence of tuberculosis and the advent of multidrug-resistant *Mycobacterium tuberculosis* strains, the rapid diagnosis of tuberculosis has important public health significance (3, 16). In developed countries, the higher incidence of tuberculosis is related not only to AIDS and homelessness but also to the advancing age of the population (8). Although the lung is the main organ affected, disseminated and extrapulmonary forms of the disease are common, being detected in as many as 77% of human immunodeficiency virus (HIV)-infected patients (7) and in 24% of non-HIV-infected patients (22).

The presence of mycobacteria in blood has been recognized for more than 50 years (6, 27); however, it has never been considered a frequent occurrence until the AIDS epidemic. In the last decade, blood cultures (1, 28) have become a useful tool for the detection of *M. tuberculosis* and other species of mycobacteria in HIV-infected patients, especially those with low CD4 T-cell counts (17). In spite of the experience obtained with AIDS patients, the information about the diagnostic utility of blood culture in non-HIV-infected patients is limited and not very encouraging (4, 26).

PCR is a well-developed technique used extensively for the diagnosis of numerous infectious diseases, including tuberculosis (5, 13). In this study, we have prospectively applied this technique to the detection of *M. tuberculosis* DNA in peripheral blood samples of patients with a wide clinical spectrum of tuberculous infection, ranging from localized pulmonary involvement to miliary disease, in both non-HIV- and HIV-infected patients.

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MATERIALS AND METHODS

Patients. Thirty-eight hospitalized adult patients with a high clinical suspicion of localized or disseminated forms of tuberculosis admitted to our institution, and not receiving antituberculous therapy, were included in this study. High clinical suspicion implied that tuberculosis was the first diagnosis on admission, established by clinical, epidemiological, and imaging studies. Fifteen of these patients were infected with HIV. The diagnosis of HIV infection was established on the basis of two positive enzyme-linked immunosorbent assays confirmed by Western blot (immunoblot) analysis.

Pulmonary tuberculosis was defined as disease confined to the lungs, pleura, and mediastinal lymph nodes. Disease outside these sites was considered extrapulmonary. Disseminated tuberculosis was defined as the involvement of two or more noncontiguous extrapulmonary organs.

Blood samples from two HIV-infected and five non-HIV-infected patients with documented illness other than tuberculosis were used as controls. The purified protein derivative (PPD) test was positive in one of the two HIV-infected patients and in four of the five non-HIV-infected individuals. We have also tested 12 blood specimens from healthy volunteers, including seven health care workers with positive PPD tests.

Bacteriological techniques. Blood cultures for detection of mycobacteria were obtained in 31 patients. Five milliliters of blood was inoculated into a vial of Middlebrook 7H13 broth containing ^{14}C-labeled substrate (BACTEC 13A; Johnston Laboratories, Towson, Md.), and the vial was incubated for 6 weeks.

Depending on the suspected organ involvement, specimens from other sites were obtained for conventional mycobacterial culture. These specimens were prepared by standard procedures (24), processed for direct examination by auramine-rhodamine stain, inoculated into Lowenstein-Jensen medium, and incubated for 8 weeks. Nucleic acid probes (Gen-Probe Inc., San Diego, Calif.) were used for identification of *M. tuberculosis* complex and *M. avium* from samples that demonstrated mycobacterial growth.

Preparation of samples for PCR. In all cases, 5 ml of peripheral blood was collected in EDTA anticoagulated tubes and the contents of the tubes were processed immediately after sampling. The entire contents of the tube was carefully layered over a solution of Ficoll-Hypaque (specific gravity, 1,077; Sigma Chemical Co., St. Louis, Mo.) and centrifuged ($400 \times g$) for 30 min at room temperature. Peripheral blood mononuclear cells (PBMC) were removed from the serum–Ficoll-Hypaque interface and washed twice with phosphate-buffered saline (10 mM phosphate buffer [pH 7.2], 150 mM NaCl). Cells, 10^6, were centrifuged at $9,500 \times g$ for 15 min, and the pellet was incubated in 100 μl of lysis buffer (50 mM Tris-HCl [pH 8], 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P-40) (16) containing 100 μg of proteinase K per ml at 56°C for 3 h and then heat inactivated at 95°C for 10 min.

All samples were analyzed with a primer pair (PCO4-GH20) that amplifies a region of the β-globin gene, as previously described (2), with the following modifications: the PCR reactions were performed in a final volume of 25 μl

TABLE 1. Characteristics of the 32 patients in whom diagnosis of tuberculosis was established by conventional procedures

Characteristic	HIV-positive patients	HIV-negative patients
No. of patients studied	11	21
Demographics		
Age (yr.), median (range)[a]	27 (21–36)	48 (19–78)
Sex, no. male/no. female	9/2	13/8
HIV risk factor, no. (%)		
Intravenous drug abuse	10 (91)	
Heterosexual contact	1 (9)	
Previous diagnosis of AIDS, no. (%)	4 (36)	
CD4 cell count (cells per ml),[b] median (range)	278 (12–648)	ND[c]
Other underlying disease, no. of patients (%)		
None	9 (82)	17 (80)
Other disease[d]	2 (18)	4 (19)
PPD test, no. of positive tests/no. of tests performed (%)	4/9 (44)	8/13 (61)
Means of diagnosis		
Positive AFB smear,[e]/no. (%)	8 (73)	15 (72)
Positive culture for *M. tuberculosis*,[f] no. (%)	11 (100)	19 (90)
Histological findings only,[f] no. (%)	0 (0)	2 (9)

[a] Ten patients were older than 65 years.
[b] In all cases, a CD4 cell count was available within 3 months of the date at which a positive culture for *M. tuberculosis* had been obtained.
[c] ND, not determined.
[d] One of each; lymphoma, diabetes, renal transplant, liver transplant, silicosis, and periarteritis nodosa.
[e] AFB, acid-fast bacillus.
[f] In six HIV-positive patients (54%) and eight HIV-negative individuals (38%), invasive diagnostic procedures (bone, spleen, liver, bone marrow, or ganglionar biopsies) were performed to obtain samples for culture and histology.

containing 1.5 mM $MgCl_2$ and 12.5 μl of sample lysate. This procedure allowed for the evaluation of the integrity of cellular DNA and for the presence of inhibitors of *Taq* polymerase.

PCR procedure. Amplification was performed as previously described (13), using the hot start technique (12). The assay detects a 123-bp region from the *M. tuberculosis* complex-specific insertion sequence IS*6110*. PCR was carried out in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The amplification reactions were performed in a final volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM (each) deoxynucleoside triphosphate, primers (1 μM each) (10), and 100,000 PBMC. After the reaction mixture reached 82°C, 0.625 U of *Taq* polymerase (Promega, Madison, Wis.) was added. Then the samples were denatured at 94°C for 5 min, and 30 amplification cycles were performed as follows: 2 min of denaturation at 94°C, 2 min of annealing at 68°C, and 2 min of primer extension at 72°C. The extension time was increased by 5 s with each subsequent cycle.

All procedures for sample preparation and PCR included measures to avoid cross-contamination between samples and contamination of reagents or samples with PCR-amplified products or positive controls (18). DNA extraction and reaction mixture preparation were performed under a UV light-equipped hood in different laboratories; another room, located in a different building, was dedicated to the processing and analysis of all products following amplification. Aerosol-guarded tips were used during all steps. For each PCR, batch samples from patients were interspersed with tubes containing reaction mixture but not target DNA. The samples were divided into aliquots and were processed in duplicate throughout the sample preparation step, the PCR itself, and the detection procedure.

Detection of PCR products. After amplification, 10 μl of the reaction mixtures was electrophoresed on ethidium bromide-containing 2% agarose gels (Nusieve GTC agarose; FMC BioProducts, Rockland, Maine), and visualized by UV transillumination. The DNA was transferred to nylon membranes (ZetaProbe; Bio-Rad, Richmond, Calif.) by alkaline blotting (23). Hybridization was done in 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate [pH 7.7], 5 mM EDTA)–1× Denhardt's solution–1% sodium dodecyl sulfate (SDS)–10% dextran sulfate at 55°C overnight with a ^{32}P-labeled 5′ probe (14). The specific activity of the probe was about 10^8 cpm/μg, and approximately 10^7 cpm was used in each hybridization. Subsequently, the membranes were washed in 2× SSPE–0.1% SDS at 55°C and exposed to radiographic films in cassettes containing amplifying screens for 2 and 24 h at −70°C.

A sample was regarded as positive when DNA with a molecular weight expected for the amplified product was seen in the autoradiograph. In all cases, the results obtained from duplicate samples were identical.

Statistical analysis. Statistical significance for comparisons of proportions was determined by the Fisher exact test.

RESULTS

In 30 of 38 patients with an initial clinical suspicion of tuberculous infection, *M. tuberculosis* was isolated from one or more samples. Granulomatous lesions compatible with tuberculosis were demonstrated in two additional patients who responded to antituberculous therapy; thus, in 32 of the 38 originally suspicious patients, a diagnosis of tuberculosis was established by conventional procedures. The characteristics of these 32 patients (11 HIV infected and 21 non-HIV infected) with confirmed diagnosis of tuberculosis are given in Table 1.

Blood cultures were performed in 26 patients with documented tuberculosis; *M. tuberculosis* was recovered in only 2 patients (7.7%). Both were patients (one with and one without HIV infection) with disseminated disease. The other five blood cultures performed were from patients in whom tuberculosis was not documented.

In order to rule out PCR false-negative results due to the presence of enzymatic inhibitors in the samples, an amplification of a region of the β-globin gene was performed in all specimens studied. All specimens had a positive PCR, showing the integrity of cellular DNA and the absence of inhibitors of *Taq* polymerase (Fig. 1).

The frequency of *M. tuberculosis* DNA detection in PBMC by PCR according to the presence or absence of HIV infection and the extent of tuberculous infection is shown in Table 2. Nine of 11 (82%) HIV-infected and 7 of 21 (33%) non-HIV-infected patients with documented tuberculosis had positive PCR results ($P < 0.01$). All patients with disseminated infection were PCR positive. In 3 of 3 (100%) HIV-infected patients and in 3 of 11 (27%) non-HIV-infected patients with extrapulmonary tuberculosis, PCR was positive ($P = 0.055$). If we compare the proportion of PCR-positive results in HIV-positive patients with that in HIV-negative individuals who had extrapulmonary or disseminated disease (9 of 9 versus 5 of 13), the difference is statistically significant ($P < 0.04$). Two non-HIV-infected patients who presented with pulmonary tuberculosis had positive PCR. One of these patients had a renal allograft and was receiving immunosuppressive therapy.

In all samples, visualization of PCR-amplified fragments was only possible after hybridization (Fig. 1).

In all six patients in whom *M. tuberculosis* infection was not documented by bacteriological or histological procedures, PCR was negative. In three of the four HIV-positive cases of this group, *M. avium* was isolated from blood. None of the 19 blood samples from controls were positive by PCR, including specimens from two HIV-positive patients, three HIV-negative patients with immunosuppressive therapy, and seven health care workers with positive PPD tests.

DISCUSSION

A rapid initial diagnosis of *M. tuberculosis* infection is problematic if the techniques of direct visualization are negative. The definitive diagnosis depends on culture of the mycobacteria, a technique that is time-consuming and not always sensitive enough. Furthermore, the performance of inconvenient invasive procedures is often necessary, especially when dissemina-

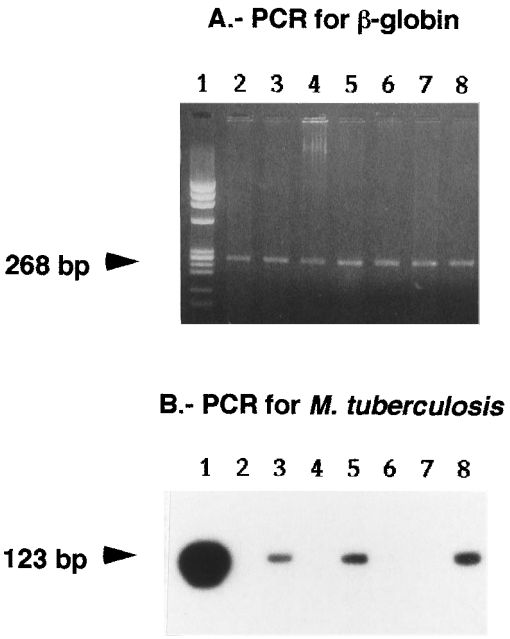

FIG. 1. (A) Representative results of amplification analysis of β-globin in PBMC samples. A specific band of 268 bp (indicated by arrowhead) represents a positive reaction for β-globin. The presence of inhibitors of *Taq* polymerase was ruled out in the samples studied, since all specimens were positive when tested for β-globin. Lane 1, ϕX174 hydrolyzed with *Hae*III; lanes 2 to 8, reactivities of some samples. (B) Autoradiograph of the transferred DNA hybridized with the internal oligonucleotide probe. In all PCR-positive samples the visualization of amplified fragments was made after hybridization. Lane 1, positive control, 10 pg of purified *M. tuberculosis* DNA, equivalent to about 2,000 copies of the *M. tuberculosis* chromosome; lane 2, negative buffer control; lane 3, positive sample from AIDS patient with extrapulmonary disease; lane 4, negative sample from AIDS patient in which *M. avium* grew; lane 5, positive sample from non-HIV-infected patient with disseminated disease; lanes 6 and 7, samples from control group; lane 8, positive sample from AIDS patient with disseminated disease. The 123-bp amplified fragment is indicated by the arrowhead.

tion is suspected, in order to obtain appropriate specimens for culture (9, 21).

Because of the high sensitivity of the PCR to detect *M. tuberculosis* DNA from clinical samples (5, 13, 14), we have investigated the potential use of this technique as a rapid diagnostic procedure for *M. tuberculosis* bacteremia. In this study, we detected *M. tuberculosis* DNA in PBMC samples from 9 of 11 (82%) HIV-infected patients and in 7 of 21 (33%) non-HIV-infected patients with different forms of tuberculosis. Additionally, our data suggest that PCR detects *M. tuberculosis* DNA in PBMC only in cases with active tuberculous infection, as samples from 12 controls with positive PPD tests were PCR negative.

TABLE 2. Summary of PCR results in PBMC from 38 patients with high suspicion of tuberculous infection[a]

Disease process	Total no. of patients	HIV-infected patients		Non-HIV-infected patients	
		No. studied	No. PCR positive (%)	No. studied	No. PCR positive (%)
Documented TB	32	11	9 (82)	21	7 (33)
Pulmonary alone	10	2	0 (0)	8	2 (25)
Extrapulmonary	14	3	3 (100)	11	3 (27)
Disseminated disease[b]	8	6	6 (100)	2	2 (100)
No documented TB	6	4[c]	0 (0)	2	0 (0)

[a] $P < 0.01$ when HIV-infected and non-HIV-infected patient groups were compared. TB, tuberculosis.
[b] Positive cultures from at least two noncontiguous extrapulmonary organs.
[c] *M. avium* was isolated in three cases.

The presence of *M. tuberculosis* in peripheral blood has been demonstrated occasionally in the past by animal inoculation and culture (6, 27); these findings were important to establish the accepted concept that bacteremia is the natural way that tuberculosis is spread in disseminated infections. However, until the advent of the HIV epidemic, blood cultures were not considered a useful diagnostic technique in mycobacterial infections. We had a very low recovery of *M. tuberculosis* from blood cultures in our patients, as only 2 (one from a HIV-infected patient and one from a non-HIV-infected patient) of the 26 cultures performed in tuberculous individuals were positive. Positive blood cultures for *M. tuberculosis* have been reported in up to 42% of HIV-infected patients with tuberculosis (19). The yield of blood culture correlates well with the severity of immunosuppression and the extent of tuberculous dissemination (17, 26). Some authors (17) have reported that blood cultures are only sensitive enough in HIV-positive patients with <100 CD4 T cells per μl, suggesting that CD4 lymphocytes play a critical role in the containment of hematogenous dissemination. Most of our HIV-infected patients had >200 CD4 cells per μl, and this could explain our low yield of positive cultures. The fact that in our tests the PCR-amplified fragments could only be detected after radiolabeled hybridization (Fig. 1) is compatible with the presence of very low amounts of mycobacteria in blood, probably amounts below the limit of blood culture detection.

Information about *M. tuberculosis* detection in blood cultures in HIV-negative patients is limited. The published reports (4, 26) show a very low positive rate, as is also the case in the group of patients included in our study. The demonstration of *M. tuberculosis* DNA in the blood of 7 of 21 (33%) of these patients underlines the fact that the incidence of hematogenous dissemination is probably underestimated in the non-HIV-infected population and that it is worthwhile to try to improve our ability to use blood as a convenient specimen for the diagnosis of tuberculosis. The PCR technique is a good candidate for use in the routine diagnosis of mycobacterial infections from blood samples once standardization is accomplished (20).

In summary, the results presented in this study confirm previous reports (11, 25) that indicate that a great improvement in the rapid diagnosis of tuberculosis, especially in HIV-infected patients, is possible by the direct amplification of *M. tuberculosis* from blood samples.

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