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Blood and urine samples as useful sources for the direct detection of tuberculosis by polymerase chain reaction

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Abstract

The aim of the study was to assess the utility of the polymerase chain reaction (PCR) assay in blood and urine for the diagnosis of tuberculosis (TB). We prospectively evaluated the usefulness of PCR performed in blood and urine samples from patients with proved or probable TB compared with a control group of patients. The PCR technique was performed using IS*6110* primers. We included in the study 57 patients (43 with definite TB and 14 with probable TB) and 26 controls. Blood and urine samples were drawn at the time of microbiologic diagnosis and 3, 6, 9, and 12 months later. Cultures were positive in the early period (<1 month after treatment) in 11 of 57 patients (19%) with probable or definite TB, in comparison with 42% of patients (24/57) who yielded a positive PCR ($P = 0.02$). Urine samples increased the sensitivity of PCR determination in blood samples by 10%. The PCR in blood and/or urine was positive in 41% of patients with pulmonary TB, in 36% of patients with extrapulmonary TB, and in 50% of patients with disseminated TB. *Mycobacterium tuberculosis* was still detectable by PCR in 5 of 13 patients with cured TB after 1 or more months of antituberculous treatment. The PCR detection of *M. tuberculosis* in blood and urine samples is useful for the diagnosis of different clinical forms of TB, mostly in those patients in which sample extraction is difficult or requires aggressive techniques. The sensitivity of this technique could be improved studying more than 1 sample in each patient, even after initiating an antituberculous treatment.

Keywords: Tuberculosis; Blood; Urine; PCR

1. Introduction

Tuberculosis (TB) is still a major diagnostic challenge. An optimal microbiologic diagnostic approach to this disease would depend on the collection of appropriate samples—which is not easy in most patients with nonproductive cough or those with disseminated clinical forms—and the performance of a test that could provide an early diagnosis. Detection of DNA of *Mycobacterium tuberculosis* by polymerase chain reaction (PCR) assay in samples easy to obtain such as blood and urine could provide an early diagnosis of TB in some patients (Ahmed et al., 1998; Nakatani et al., 2004; Aguado et al., 1996; Condos et al., 1996; Kafwabulula et al., 2002; Folgueira et al., 1996), although the sensitivity of PCR assay in blood and urine has yielded widely differing results depending on the PCR system used (Aguado et al., 1996; Condos et al., 1996; Folgueira et al., 1996). On the other hand, prospective studies evaluating these tests in a clinical basis are scarce and include only few patients.

The aim of the present study was to evaluate the usefulness of the PCR technique for the diagnosis of TB using samples of easy extraction as blood and urine.

2. Methods

2.1. Study samples and setting

We prospectively selected for the study 57 patients admitted to University Hospital "12 de Octubre" with the diagnosis of TB, 43 patients with the diagnosis of definite TB (with growth of *M. tuberculosis* in any clinical sample), and 14 patients with the diagnosis of probable TB (clinical

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diagnosis with response to antituberculous treatment and/or compatible histopathologic findings). In the group of patients with TB, blood and urine samples were obtained previously to starting antituberculous treatment and 1, 3, 6, and 12 months after initiating treatment. We also collected serial samples from 13 control patients suffering from other diseases included in the differential diagnosis of TB and from 13 healthy volunteers (5 of them with a positive purified protein derivative [PPD] test) without evidence of TB.

Pulmonary TB was defined as a disease confined to the lungs, pleura, and mediastinal lymph nodes. Disease outside these sites was considered extrapulmonary. Disseminated TB was defined as the involvement of 2 or more noncontiguous extrapulmonary organs.

All patients provided written informed consent to participate in the protocol approved by the ethics committee and review board of the University Hospital "12 de Octubre".

2.2. Bacteriologic techniques

2.2.1. Mycobacterial culture

2.2.1.1. Blood cultures. 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. Urine samples and other specimens from other sites depending on the suspected organ involvement were obtained for conventional mycobacterial culture. These specimens were prepared by standard procedures (Roberts et al., 1991), processed for direct examination by auramine–rhodamine stain (Truant et al., 1962), inoculated into Lowenstein–Jensen medium and liquid medium (BACTEC MGIT 960 liquid medium), and incubated for 8 weeks. Nucleic acid probes (Gen-Probe, San Diego, CA) were used for identification of *M. tuberculosis* complex from samples that demonstrated mycobacterial growth.

2.2.1.2. Polymerase chain reaction procedure. For the extraction of peripheral blood mononuclear cells (PBMCs), 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 were carefully layered over a solution of Ficoll–Hypaque (specific gravity, 1077; Sigma Chemical, St. Louis, MO) and centrifuged (700 × *g*) for 30 min at room temperature. PBMCs were removed from the serum–Ficoll–Hypaque interface and washed twice with 10 mL phosphate-buffered saline (10 mmol/L phosphate buffer [pH 7.2], 150 mmol/L NaCl). Urine samples were first decontaminated (Kubica et al., 1963) previously to the lysis procedure.

For the lysis procedure, a 500-μL aliquot of decontaminated urine was first centrifuged at 9500 × *g* for 15 min. The resultant pellet and a 10^6 PBMC aliquot were resuspended in 50 μL of lysis buffer (10 mmol/L Tris–HCl [pH 9], 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.45% Tween 20, 0.1% Triton X-100, 0.45% Nonidet P-40 [NP40; Sigma]) containing 100 μg of proteinase K per milliliter. The samples were incubated 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 268 bp of the β-globin gene (Bauer et al., 1991). The PCR reactions were performed in a final volume of 25 μL containing 10 mmol/L Tris–HCl (pH 9), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L each deoxynucleotide (deoxyadenosine triphosphate [dATP], deoxyguanosine triphosphate [dGTP], deoxycytidine triphosphate [dCTP], and deoxyuridine triphosphate [dUTP]), 0.1 μmol/L each primer (PCO4 and GH20), 0.625 U Taq DNA polymerase, 0.5 U uracil–DNA–glycosilase (UNG), and 5 μL of sample lysate. This procedure allowed the evaluation of the integrity of cellular DNA and the presence of inhibitors of Taq polymerase. The reaction mix was incubated at 20 °C for 10 min to make UNG enzyme act (to prevent carryover contamination) and then heat inactivated at 95 °C for 5 min. Amplification was performed using the hot-start technique (Erlich et al., 1991), in a 9600 Perkin Elmer thermal cycler (Perkin Elmer, Ramsey, MN) as follows: 35 cycles of 15 s denaturation at 94 °C and 1 min annealing and extension at 60 °C. Final extension was at 72 °C for 9 min.

The assay to detect TB amplifies a 123-bp region from the *M. tuberculosis* complex-specific insertion sequence IS*6110*. PCR was carried out in a DNA thermal cycler (480 PTC-100, MJ Research). The amplification reactions were performed in a final volume of 25 μL containing 10 mmol/L Tris–HCl (pH 9), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each deoxynucleotide (dATP, dGTP, dCTP, and dUTP), primers IS1 and IS2 (1 μmol/L each) (Eisenach et al., 1990), 0.625 U Taq DNA polymerase, 0.5 UNG, and 5 μL of the lysate. Then the samples were incubated at 20 °C for 10 min to make UNG enzyme act and then heat inactivated at 95 °C for 5 min. The amplification was performed as follows: 35 cycles of 2 min denaturation at 94 °C, 2 min annealing at 68 °C, and 2 min extension at 72 °C. The extension time was increased by 5 s with each subsequent cycle. After IS*6110* amplification, the reaction mixtures were electrophoresed on ethidium bromide-containing 2% agarose gels (Nusieve GTC agarose; FMC BioProducts, Rockland, ME) and visualized by UV transillumination. The DNA was transferred to nylon membranes (ZetaProbe; Bio-Rad, Richmond, CA) by alkaline blotting. Prehybridization was done in 10 mL rapid-Hyb buffer (Amersham Biosciences, Basking Ridge, NJ) at 56 °C for 90 min, and hybridization was done in the same buffer with a ^{32}P-labeled 5′ probe (Folgueira et al., 1994) for 3 h at least; 10^7 cpm was used in each hybridization. Subsequently, the membranes were washed once in 50 mL 5× SSC–0.1% SDS at 56 °C for 30 min and twice in 50 mL 1× SSC–0.1% SDS at 56 °C each. The washed membranes were exposed to radiographic films in cassettes containing amplifying screens at least 16 h at −80 °C. A sample was regarded as positive when DNA with a molecular weight