UTILITY OF POLYMERASE CHAIN REACTION IN DIAGNOSIS OF TUBERCULOSIS FROM SAMPLES OF BONE MARROW ASPIRATE

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Abstract. Fever of unknown origin (FUO) poses a diagnostic challenge to the clinicians, with a differential diagnosis as varied as neoplastic and infectious diseases. In developing countries, the infectious causes are responsible for more cases of FUO, with tuberculosis as one of the main causes of classic FUO. Disseminated tuberculosis with negative pulmonary findings is a diagnostic problem. This study examines the diagnostic utility of the polymerase chain reaction (PCR) in samples of bone marrow aspirate in 85 patients presenting with diverse clinical symptoms. Using primers specific for Mycobacterium tuberculosis, tubercular etiology was detected in 33% of patients clinically suspected of tuberculosis while culture on Lowenstein-Jensen medium grew M. tuberculosis in only one patient (2.5%). None of these patients had been diagnosed by microscopy. Clinical improvement with ATT was observed in 85% of the patients with positive PCR. PCR demonstrated much higher sensitivity and specificity, thereby facilitating early therapeutic decisions for suspected extrapulmonary tuberculosis.

INTRODUCTION

Tuberculosis (TB) is an infectious disease entailing enormous mortality and morbidity. In India, about 33 million people are infected with Mycobacterium tuberculosis, and 3 million people suffer from pulmonary tuberculosis. Hence, the importance of rapid and accurate diagnosis cannot be undermined to ensure early, appropriate treatment. Early diagnosis of TB is still based on demonstration of acid-fast bacilli (AFB) in secretions and tissue samples. For the diagnosis of disseminated TB, and in samples from extrapulmonary sites that are often paucibacillary in nature, smear microscopy offers low sensitivity (requires 10⁻⁴ bacilli/mL for positivity). Culture is the reference method, but the long incubation time may cause delay in diagnosis and treatment. Histopathological examination also may not prove to be very useful.¹ Epithelioid granuloma and caseating necrosis may occur in diseases other than TB, such as infestations and carcinoma. Response to empirical antituberculous therapy alone, in such cases, has been widely used as a diagnostic tool.

Polymerase chain reaction (PCR) has been shown to be useful in amplifying M. tuberculosis DNA in pulmonary as well as extrapulmonary samples.²⁻³ It has been used in clinical samples, such as sputum, CSF, EDTA-blood, pleural fluid, fluid from fistulas, and pus from wounds.⁴⁻⁵ Few studies are also available on identification of M. tuberculosis in bone marrow material.⁶⁻¹²

Bone marrow aspirate is a valuable sample and is collected when all other routine investigations have failed to elucidate the diagnosis. Availability of a specific diagnostic technique such as PCR may forego the use of trial of anti-tubercular treatment (ATT) for confirmation of diagnoses.

The aim of this study was to evaluate the use of PCR in the detection of M. tuberculosis DNA in bone marrow, in patients presenting with diverse clinical presentations, where disseminated tuberculosis was suspected.

MATERIALS AND METHODS

Study population. In this study, the patients who had FUO alone or associated with cervical lymphadenopathy, ascites, bone marrow transplant as well as those with pyrexia accompanying renal failure, aplastic anemia were investigated for tubercular etiology. Control patients (15) who underwent bone marrow examination, and treatment of conditions unrelated to TB, such as Kala-azar, and Enteric fever, were taken to rule out contamination of specimens at sampling, and during the PCR process.

Samples. Eighty-five samples of bone-marrow aspirate were collected in 10 IU heparin/mL of sample in sterile vials. These were received in the Tuberculosis Laboratory (Department of Microbiology, All India Institute of Medical Sciences, New Delhi) for smear examination (Ziehl-Neelsen (ZN) staining), growth on Lowenstein-Jensen (LJ) medium at 37°C, and PCR. The samples were decontaminated using N-acetyl-1-cysteine-NaOH solution.¹³ About 200 μL of the concentrated sediment was used each for microscopic examination (ZN-stained smear), culture on duplicate LJ slopes, and PCR. Growth was monitored for 8 weeks, and the mycobacterial species was identified in positive cultures. The technicians handling the samples were kept blinded to the clinical details.

PCR. PCR was carried out in a sanitized manner, employing utmost precautions against cross- and carryover contamination as previously reported.¹⁰ The decontaminated sediment/concentrated sample were incubated in a lysis buffer containing 20 mM Tris-HCl (pH 8.3), 0.5% Tween 20, and 1 mg/mL proteinase K for 16 hours at 56°C. The DNA was extracted with chloroform followed by precipitation in 0.3 M sodium acetate and absolute ethanol overnight at −20°C. A 240-bp long region of the MPT64 gene of M. tuberculosis was amplified using primers MPT1 (5’-TCC GCT GCC AGT CGT CCT CC-3’), and MPT2 (5’-GTC CTC GCG AGT CTA GGC CA-3’). The PCR was carried out in 50-μL volumes comprising of 10 mM Tris HCl (pH 8.3), 50 mM NaCl, 1.5 mM MgCl₂, 0.01% (wt/vol) gelatin, 200 μM of each of the four deoxynucleotide triphosphates (Perkin-Elmer, Applied Biosystems Division, CA), 0.4 μM of each primer, 1.25 U of Taq DNA polymerase (Perkin-Elmer), and 10 μL of extracted DNA. Using an Amplitron thermocycler (Barnstead/
Thermolyne, Dubuque, IA), amplification was carried out for 30 cycles, each cycle consisting of denaturation at 94°C for 2 minutes, annealing at 60°C for 2 minutes, and extension at 72°C for 2 minutes. Each series of PCR had a positive control (100 pg of H37Ra DNA), and several negative controls (sterile distilled water) interspersed with the samples. To rule out inhibition, duplicate amplification runs with spiked samples (internal controls) were carried out. The amplified products were analyzed by agarose gel electrophoresis along with the molecular weight marker, 6X174 DNA digested with HaeIII (Promega, Madison, WI). Using UV transilluminator (Chemi-Imager, Alpha Innotech, San Leandro, CA), amplified products having a 240-bp band were identified as positive. DNA from PCR-negative samples were spiked with 100 and 10 ng of DNA from the H37Ra strain of M. tuberculosis and reamplified to check for amplification inhibitors leading to false-negative results.

All patients with the clinical diagnosis of tuberculosis were put on ATT comprising four first-line drugs: rifampicin (RIF), isoniazid (INH), ethambutol (EMB), and pyrazinamide (PZA). Response to therapy was assessed after 8 weeks.

RESULTS

Between January 2003 and July 2004, 85 bone marrow specimens were submitted for mycobacteriological investigations with diverse clinical indications (Table 1).

Subjects. Patients were put into four groups based on clinical diagnosis: (I) definitive tuberculosis, strong clinical suspicion with radiologic or other evidence of tuberculosis, samples sent for microbiological confirmation; (II) probable tuberculosis, high clinical suspicion of tuberculosis with suggestive past history, with no other evidence; (III) possible tuberculosis, tuberculosis as one of the differential diagnosis of the disease presentation; and (IV) undiagnosed, clinical diagnosis not yet established. All samples were negative by smear microscopy. One patient was culture positive for M. tuberculosis. Out of 85 patients, nineteen (22.35%) were positive for M. tuberculosis DNA by PCR. The clinical indications for giving bone marrow biopsies, the clinical categories of patients with respect to tuberculosis, and the PCR results of patients in each category are tabulated (Table 1). All the 15 negative controls, and the in-house controls tested were negative with PCR. The patient positive by culture was also positive for M. tuberculosis DNA (Table 1). To ensure against false-negative reporting duplicate amplification runs with spiked samples (internal controls) were done.

Clinically, among the 8 patients in the category I, 2/3 patients with disseminated tuberculosis and 2/4 of those with cervical lymphadenopathy, fever, and weight loss, and 1 patient on ATT for tubercular osteomyelitis were found to be PCR-positive (Table 1).

All the patients with clinical diagnosis of tuberculosis were given ATT and were observed regularly for a minimum of 3 months for clinical improvement. Improvement consisted of temperature returning to normal, improved appetite, and general well being of the patients. Analysis of the result of PCR assay in relation to response of patients to ATT revealed that 85% patients with positive PCR assay improved with ATT (Table 1). On the other hand, 4.5% patients with negative PCR assay also improved with ATT. In 3 patients, no follow-up data after ATT was available.

DISCUSSION

Disseminated tuberculosis has been reported more in underlying illness such as diabetes, alcohol abuse, parenteral drug abuse, or other conditions with immunosuppression. Disseminated tuberculosis commonly poses diagnostic difficulties as the primary symptoms, and clinical findings are often noncharacteristic. In such circumstances where it is difficult to establish the sites involved with disease, analysis of bone marrow may yield important insights. In a series of 109 patients with miliary tuberculosis, Long, O’Connor, and others established the diagnosis in 19 of 22 patients by bone marrow examination. Similarly, Escobedo-Jaimes used an in-house PCR to diagnose miliary tuberculosis in 30 suspects and found that PCR was positive in 70% suspects, contrasting with only one positive (3.3%) in staining and culture and four with compatible histologic findings (13.3%).

Clinical confirmation of the diagnosis of disseminated tuberculosis is traditionally established by bacteriological, and/or histologic evidence. Cultures are not always rewarding as they are too time consuming in critical situations, precluding their utility for prompt diagnosis and hence delaying appropriate therapy. Studies have highlighted the limitations of associating specific histopathologic features with tuberculosis. In the last decade, PCR has been used in the diagnosis of multi- and paucibacillary pulmonary and extrapulmonary tuberculosis with reasonable success. This study describes the experience using PCR for early diagnosis of tuberculosis in samples of bone marrow from patients presenting with diverse clinical problems.

Studies delineating the utility of PCR for diagnosis of tuberculosis in paucibacillary samples have relied on clinical diagnosis and/or response to ATT as reference standard. Culture of M. tuberculosis is often negative in extrapulmonary tuberculosis, hence it was not considered as a gold standard in the present study for evaluating the PCR technique. Various criteria have been suggested for probabil-
ity-based classification of suspected cases of tuberculosis. PCR results were analyzed vis-à-vis clinical diagnosis of tuberculosis made on the basis of clinical features, CT and other radiologic findings, other supportive evidence of TB, past history, and history of contact with tuberculosis. Clinical categories for suspicion of tuberculosis were used to evaluate the utility of PCR (Table 1).

In the present study of bone marrow aspirates, mostly with culturally undetectable mycobacteria, *M. tuberculosis* DNA was amplified in 33% of clinically suspected patient samples using species-specific primers. Involvement of the reticuloendothelial system may constitute a critical clinical sign of a disseminated disease with bacillary load too low to be isolated by conventional culture procedures but detectable by PCR.

Amongst the patients with “Definite Tuberculosis,” detection of 62.5% cases expeditiously by PCR is in agreement with many studies that have demonstrated high PCR-positivity among cases with clinically and bacteriologically confirmed tuberculosis.1,10,23 Conditions like systemic candidiasis, nocardiosis, cryptococcosis, secondaries in lungs, nontuberculous mycobacteriosis, and certain other conditions can easily simulate clinical presentation of disseminated tuberculosis and could possibly explain the PCR-negative cases.24–26

In the present study, detection of tubercular etiology was fairly good in cases with a probable diagnosis of tuberculosis (40%). Four out of 10 patients with FUO, 1/3 with ascites/hepatomegaly, and 1/2 with a previous history of tuberculosis, presenting with fever and malaise, were positive by PCR. Tuberculosis constitutes the major cause of FUO in both immunocompetent and immunocompromised patients. In a series of 594 episodes of FUO in HIV-infected patients, tuberculosis of pulmonary and extrapulmonary origin accounted for 35.5% cases.27 Among immunocompetent patients, Ritis, Tzoanopoulos, and others11 amplified the IS6110 element of *M. tuberculosis* from bone marrow aspirate in 45.83% of FUO cases, and in 2005,28 they used PCR-based methods to investigate the validity of bone marrow aspirates as an easily accessible alternative samples for molecular analysis in extrapulmonary tuberculosis.

Among the patients in category III,17 with 1/4 cases of renal failure and 1/1 suffering from aplastic anemia, none of the 12 post-transplant patients were positive for PCR. Absence of PCR positivity among renal and bone marrow transplant recipients having severe impairment of cell-mediated immunity and residing in a country with high incidence of tuberculous infections, though surprising, is in conformity with previous studies. The low incidence of mycobacterial infection among patients who underwent bone marrow transplantation has been reported in previous studies.8,14,29

In the present study, the small sample size may also have contributed to low positivity by PCR. The laboratory was also kept blinded to the diagnosis until the end of the study, and hence results obtained were devoid of any bias due to the clinical presentation. Correlation with the clinical data was done after the test results were concluded. In the study, three samples contained inhibitors that could not be removed by additional purification protocols (e.g., using columns). Inhibition to amplification is known to be common, especially in hematogenic samples, resulting in false-negative reports exceeding 20%.22,30–32 Therefore, inclusion of duplicate amplification runs with spiked samples (internal controls) ensured against false-negative reporting. Using varied primers, 42–73% of PCR-positivity has been reported in bone marrow samples collected from patients with clinically/histopathologically confirmed extrapulmonary tuberculosis.1,11,23

All of the patients with the clinical diagnosis of tuberculosis were put on ATT; 85% of the patients with positive PCR showed clinical improvement after ATT. Thus, response to specific ATT supported the positive findings in the PCR. Three of the eight patients in the definite category also did not show response to ATT, hence supporting the PCR findings (Table 1). However, three patients with negative PCR results also improved after ATT. Inhibitory factors,22,30,31 extremely low numbers of mycobacteria in the sample,30 absence of DNA fragment selected for amplification33 or the method of extraction of DNA34 might have been responsible for these false negative results. DNA amplification of tuberculosis does not always mean viable bacilli.35 The PCR result has to be evaluated in the light of other clinical and laboratory findings.36 In our study, the positive PCR result in combination with FUO was taken as a sign of disease in progress and was treated successfully with antituberculous regimens.

In a diagnostic paradigm when patients are given empirical ATT before a confirmed diagnosis because of the presumed delays in laboratory investigations, PCR offers a low risk/benefit ratio that should be favorably considered, especially in regions of high prevalence of tuberculosis. PCR detected 13 of 40 patients (33%) clinically suspected of tuberculosis. Twelve patients improved after starting treatment, thereby demonstrating a high concordance between improvement in response to ATT and PCR positivity.

Reasons for low yield of PCR (33%) and the still lower yield for cultures could be due to absence of organisms in the small amount of sample collected. Furthermore, even if TB is confirmed in a patient, dissemination to the bone marrow may not have occurred at the stage in which the sample was collected from the patient. It is fair to assume that culture results are influenced by therapy, and the fact that PCR relies only on the presence of the genetic material of the organism, and not on its ability to replicate, could be an important advantage. It is also important to consider whether the small number of organisms that PCR is capable of detecting is of clinical significance. In this group of patients, where the diagnosis of disseminated TB was suspected, the finding of even a single organism would probably be significant. Fifteen controls in which TB was not suspected also showed no positive results on PCR. However, larger series done on tissues of healthy persons followed over a long period will be needed to determine the clinical significance of PCR-detected organisms.

In conclusion, PCR appears to be valuable in establishing the diagnosis of tuberculosis among patients with a variety of underlying immunosuppressive pathologies, and manifesting pyrexia, in the absence of any clinching evidence of etiology. In all, PCR offered a 16-fold increase in sensitivity of detection as it gave a definite diagnosis concordant with response to ATT in 16/19 patients with clinical suspicion of TB as contrasted to only one of these by culture. Emphasis should be given to tuberculosis as a common cause of FUO in highly endemic regions. Detecting this treatable cause of FUO more promptly and precisely would assist physicians in better management of FUO patients and limit TB transmission to assist the control of tuberculosis.
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