COMPARATIVE STUDY ON USE OF NESTED PCR AND CONVENTIONAL METHODS IN DIAGNOSIS OF TREATED AND UNTREATED TUBERCULOSIS

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Abstract — *A nested polymerase chain reaction (PCR) assay with two pairs of primers was applied for the detection of M. tuberculosis complex DNA in specimens of 51 patients. Of these, 31 clinically diagnosed patients (group I) recruited from July 1996 to June 1997 were given antituberculous chemotherapy from one to six months with standard daily regimen (Isoniazid, 5 mg/kg; Rifampin, 10 mg/kg; Pyrazinamide, 25 mg/kg., and either Ethambutol or Streptomycin, 15 mg/kg) and returned for subsequent testing. Other 20 persons (group II) were untreated patients suspected of tuberculosis. 24 patients from group I were found to harbor M. tuberculosis before treatment, however, mycobacterial cultures were recovered from specimens of only two patients after treatment. M. tuberculosis complex DNA could be PCR detected in initial specimens from 26 patients, while 2 months after initiation of the therapy, PCR yielded positive results in specimens obtained from three of them, suggesting incomplete treatment. Of 42 cumulated specimens from patients of both groups that grown culture, 41 had positive results on PCR closely matching to that of culture testing. Of other 40 specimens that produced no M. tuberculosis complex colonies, seven were found to be PCR-positive. However, there was a specimen from treated patient which had negatie result on PCR being positive by conventional tests. These results indicate that the PCR assay is highly sensitive and allows for the effective control of the efficacy of antituberculous chemotherapy in patients.*

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INTRODUCTION

The efficacy of antimicrobial therapy in tuberculosis (TB) patients should be monitored carefully during and after the treatment because inadequate therapy may be followed by the recurrence of the disease or drug-related complications. Treatment failure requires a change in antibiotic regimen, however, the control of treatment efficacy in TB patients is often troublesome (1,2). The commonly used smear microscopy for detection of mycobacteria in a specimen is often ineffective to detect negligible amount of pathogen remaining after chemotherapy (3). Mycobacterial culture and biochemical identification are time consuming procedures. The advantages in new laboratory methods of diagnosis, especially a polymerase chain reaction (PCR), have stimulated many attempts of their application for rapid TB diagnosis (4-8). Most of the reports revealed sufficient sensitivity of the PCR strengthened by hybridization of amplified DNA with labeled DNA probe. Such issue has increased the importance of application of PCR to detection of tuberculous bacilli in clinical specimens collected from treated patients.

In this report, we compared results of M. tuberculosis complex-specific nested PCR assay with that of smear microscopy, culture and biochemical tests in the examination of clinical specimens obtained from treated and untreated TB patients. Our purpose was to evaluate the utility of PCR assay as an objective test for treated cases of TB.

MATERIALS AND METHODS

Bacterial Strains

Mycobacterial strains and other bacterial strains were obtained from the American Type Culture Collection (USA, ATCC), Borstel Institute (Canada, SN), and Trudeau Institute Microbial Collection (Canada, TMC), Colindale (England, NCTC) as follows: M. avium (ATCC 25291), M. asiaticum (ATCC 25276), M. bovis (ATCC 19210), M. bovis BCG (ATCC 35734), M. chelonae abscessus (ATCC 19977), M. chelonae chelonae (TMC 1544), M. chitae (NCTC 10485), M. , M fallax (ATCC 35219), M. fortutitum (SN 231), M. gastri (ATCC 15754), gordonae (ATCC 14470), M. intracellulare (ATCC 13950), M. kansasii (ATCC 12478), M. neoaurum (ATCC 25795), M. phlei (ATCC 11758), M. scrofulaceum (ATCC 19981), M. senegalense (TMC 806), M. shimoidei (ATCC 27962), M. szulgai (ATCC 35799), M. thermoresistible (ATCC 19527), M. triviale (ATCC 23292), M. tuberculosis H37Rv (ATCC 27294), M. tuberculosis H37Ra (ATCC 25177), M. vaccae (TMC 1526), E. coli (ATCC 25922), Enterobacter aerogenes (ATCC 13048), Serratia marcescens (ATCC 8100), Staphylococcus aureus (ATCC 25923), Streptococcus pyogenes ATCC (12344), Klebsiella pneumoniae (ATCC 27736).

Patients and Clinical Specimens Studied

We reviewed 31 TB patients (group I) undergoing

antimicrobial drug therapy. All patients had their clinical features, PPD skin test and roentgenography data at the time of consultation. The patients had fluorescent and Ziehl-Neelsen acid fast bacilli (AFB) staining microscopy, culture and PCR tests performed. Specimens were obtained from each patient, before treatment began and afterwards. Specimens were taken from CSF (n=16), sputum (n=10), bone marrow (n=6), urine (n=6), gastric fluid (n=4), pus (n=4), soft tissues (n=10), and other sources. Control specimens were from 20 TB suspected patients (group II) referred to for laboratory testing.

When the results of smear microscopy were obtained patients were prescribed anti-TB drugs and available for fllow-up. Patients had being given four-drug treatment at the frequency of six times weekly with the following regimen: isoniazid (INH), 5 mg/kg; rifampin (RMP), 10 mg/kg; pyrazinamide (PRZ), 25 mg/kg; and streptomycin (SM)/ethambutol (EMB), 15 mg/kg. Each patient was reevaluated during therapy at 1 to 4 months interval.

Specimen Processing and Mycobacterial Isolates

Specimen treatment, auramine-rhodamine fluorescent staining, and bacteriology including species identification were performed following CDC laboratory procedures manual (9). All the specimens, except for those obtained from sterile body fluids, were decontaminated by using N-acetylcysteine method and sedimented. Sediments were resuspended, inoculated onto Lowenstein-Jensen (L-J) media and incubated in an atmosphere of 5% CO_2 at 35°C for 8 weeks. Colonies were distinguished by standard biochemical tests. Antibiotic susceptibility tests were performed with five drugs (INH-0.2 mg/l; SM - 4 mg/l; RMP - 40 mg/l; EMB - 2 mg/l; and ethionamide - 20 mg/l) by the proportional method (10).

PCR Assay

Several precedures including sodium dodecylsulfate-proteinase K lysis and phenol-chloroform extraction method were compared for their efficacy to prepare PCR sample from mycobacterial cells A boiling method was selected in view of its simplicity and low probability of cross-contamination (8,11,12). One-two colonies or 0.5 ml of residual sample from specimen processed for culture were dispersed by vigorous shaking in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM Na-EDTA, pH 8.0) and centifuged. The sediment was washed in 1 ml of TE buffer, suspended in 50 μL TE buffer and incubated at 95°C for 15 min. Then, the sample was frozen at - 20°C, thawed and boiled again. A supernatant fluid was obtained by centrifugation at 10,000 rpm for 15 min and stored until use. This procedure has been found efficient to make DNA from mycobacteria accessible to primers.

A nested PCR was performed by two individual PCR reactions (13) conducted successively with two different pairs of primers. Four nested oligonucleotides relevant to DNA encoding 65 kDa antigen gene described previously were used (14). The outer primers, TB-1 5-GAGATCGAGCTGGAGGATCC and TB-2 5-AGCTGCAGCCCAAAGGTGTT, produced 373 bp amplimer. The inner pair consisted of oligos TB-T 5-GCGGCATCGAAAAGGCCGTG, and TB-B 5-CGAAATCGCTGCGGTGGCCG flanked 101 bp segment. A PCR mix contained 67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM of each dNTP, 0.5 μM of each outer or inner primer, and 3 u Taq DNA polymerase (Biomaster, Russia).The mixture was covered with mineral oil and thermocycling (30 cycles of denaturation at 94°C for 0.9 min, annealing at 56°C for 1min, and extension at 72°C for 1min followed by final extension at 72°C for 5 min) was performed in "Amply-2" apparatus (Biokom, Russia). PCR products were loaded on a 1.5% agarose gel prepared in TBE buffer.

For the second round PCR, 2 μL of ten-fold diluted mixture after the first round PCR was used in reaction with the inner primers at the same thermal/time protocol. The specificity of the PCR assay was checked in 39 M. tuberculosis complex strains, 21 atypical mycobacterial species, and several bacteria commensal of the sites of specimens receiving. A nested PCR product of 101 bp was found in all M. tuberculosis and M. bovis strains but not in non-tuberculous mycobacteria or other bacterial strains tested. The sensitivity of the PCR testing was examined with aliquots of cell suspension prepared according to limited dilution method up to probable injection of five cells into the PCR mixture. Nested PCR produced PCR products for all diluted samples approving high efficiency of the method. All samples were tested in triplicate. Two negative controls were run with each set of samples which contained human DNA as a template or no template at all. To rule out false negative results due to the presence of inhibitors of DNA Taq polymerase within the samples a detectable amount of the control template (approx. 3.0 pg M. tuberculosis DNA) was added to each sample that was negative in the test reaction. Then, the nested PCR was repeated under above conditions and results were compared. The sample reverted to positive was considered as true negative.

RESULTS

A prospective, blinded study was performed with 31 TB patients undergoing therapy by using a four-drug antimicrobial regimen (group I). Ten patients received a 4 to 6 month course of therapy and remaining part was under treatment for 1 to 3 months at the moment of

reevaluation. In addition, 20 patients (group II) with TB symptoms who did not undergo antimicrobial therapy were also monitored. No resistance to the major anti-TB medications was found in isolates grown from all specimens.

Fourteen but one patient in group I who were initially AFB smear positive and were receiving a course of therapy, were converted to smear negative. The initial specimens of 24 patients were positive by culture and PCR; those of two more patients were positive on PCR only (Table 1). Of the specimens obtained from the patients undergoing treatment, three samples gave mycobacteral culture, with two isolates biochemically classified as M. tuberculosis and one as atypical mycobacterium. The results of bacteriology were confirmed by PCR data obatined with samples from the colonies. Isolates of tuberculous bacilli were positive by PCR showing complete agreement with culture method. No positive PCR test was obtained for the atypical organism grown.

When cumulative data of the conventional tests were compared with that of PCR assay in specimens collected from patients before and during treatment, the PCR confirmed 25 of 26 (96.2%) M. tuberculosis culture-positive results. Of the 36 samples negative for tuberculous mycobacteria by culture method, four (11.1%) were positive by PCR (Table 1). The data corresponded to M. tuberculosis complex positivity rate of 46.8% by nested PCR compared to 41.9% by conventional culture.

Table 1. Correlation of PCR finding with detection of M. tuberculosis complex using conventional methods in specimens from patients before and after treatment

	Patients	PCR positive		PCR negative	
	Number	Culture positive	Culture negative	Culture positive	Culture negative
Before treatment	31	24	2	-	5
After treatment	31	1	2	1	27

Although disease was prevented or cured in 27 of 31 patients, laboratory tests were positive in specimens from four patients following antibiotic therapy. Positive AFB of sputum smear and culture result were found after two-month treatment in one of the patients studied. The PCR result remained indicative after one month of anti-TB therapy in two patients, one of whom had become culture negative. In clinical specimens obtained from a patient who had long history and recurrency of TB, PCR still yielded a positive result 4 months after initiation of therapy.

In general, positivity rates were significantly higher in the control group than treated patients. In all patients of group II, either culture of PCR test was positive (Table 2). Of 16 patients with positive culture in group II, 14 had AFB smear positive result. Three other patients had only PCR positive specimens.

Table 2. Examination of PCR finding for untreated patients (group II) using culture of M. tuberculosis complex

	Numbers of	L-J culture		PCR	
	specimens	positive	negative	positive	negative
Smear-positive	14	11	3	14	-
Smear-negative	6	5	1	5	1

DISCUSSION

In accord with recent recommendations of a working group of the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD), the set of disease-specific variables to be collected on each case of TB should include: site of disease, bacteriological status (microscopy and culture), and history of previous anti-TB drug therapy (15). As pathogen absence in specimen might confirm the success of treatment, there is an urgent need for specific, sensitive and quick methods of mycobacteria identification in specimens from TB patients undergoing therapy. Most AIDS patients infected with tuberculous bacilli have unless promptly and appropriately treated, died rapidly. With appropriate treatment even severely immunocompromised patients had culture conversion and prolonged, TB-free survival (16). PCR based methods have been developed for the detection of M. tuberculosis complex in a few hours (4,5,6,7,8,17,18). However, the design and implementation of them for diagnosis are under study yet. A recent innovation of transcription mediated amplification system for direct detection of M. tuberculosis in clinical specimens made a challenge to PCR based methods (19,20).

Detection of PCR product using hybridization with labeled DNA probe could give a good fit to clinical samples but mounting special efforts for procedure (16,17). The advantages of nested PCR is high sensitivity and an ability even with negligible amount of M. tuberculosis DNA to give PCR product detectable in agarose gel. In the present study, the nested PCR assay was applied for detection and identification of tuberculous mycobacteria in specimens from patients undergoing treatment. The samples were fractions easily obtained from the residual specimens processed for mycobacterial culture. The proportions of culture-positive and PCR- positive results were closely matched in contrast to the related data for specimens obtained from untreated patients. In the latter case, there was substantial prevalence of PCR positive results over smear microscopy and culture positive one. Culture results were negative in some cases probably because of decreased cell viability due to decontamination

procedure or inhibition of the cell growth depending on contamination in clinical specimens, reducing sensitivity of culture method. There is considerable evidence that the PCR assay could detect mycobacterial DNA in these specimens. The results of this report suggest that culture tests alone could not provide sufficient information for the control of TB treatment. The PCR assay seems to be more efficient than smear microscopy and cultures method in detecting clearance of M. tuberculosis from specimens. However, use of any method alone is likely to lead to confusion in some cases. Cross-contamination during initial processing of specimens was found to be the most common source of flase-positive culture in laboratory testing (21). PCR results must be validated by the culture.

Durg resistance can be acquired during therapy for drug-susceptible disease. This had led to variations on standard regimen that sometimes include more toxic alternative drugs, including ethionamide, aminosalicylic acid and cycloserine. Regimens used for retreatment usually include the alternative drugs. Success in treating drug-resistant TB varies. Nucleic acid-based monitoring of treatment could be valuable for determining the success of therapy resulting in a cure (19,20). The application of PCR for controlling TB treatment is very promising. Our results and other data show that PCR is highly sensitive and specific when used to confirm the TB diagnosis and to monitor response to therapy. PCR test, though costly, could precisely guide diagnosis and treatment of patients with TB symptoms and, thus, potentially reduce the number of patients inappropriately treated. A positive PCR test result that remains positive despite therapy may be associated with a poor clinical outcome. Recent data suggest that this is likely to be the case (19,20), however, further studies are needed to define the exact use of PCR for diagnosing TB.

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