Peripheral Blood PCR For Detection Of Mycobacterium Tuberculosis In Patients With HIV/AIDS In Mumbai, India

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Citation

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Abstract

Background:
Since the co-infection of Mycobacterium tuberculosis (MTB) and HIV is recognized as a lethal combination, there is need for a reliable diagnostic test that can be conducted on a readily available specimen such as peripheral blood to periodically screen HIV-infected individuals for MTB at an early stage. Methods:
A study was designed to assess the diagnostic value of PCR targeted to IS 1081 in peripheral blood of HIV-infected individuals because of ease of obtaining periodic samples. A cohort of 129 individuals was recruited for this purpose. It contained of adult, non-pregnant HIV sero-positive as well as HIV sero-negative individuals who were naïve to anti-Koch's treatment at two teaching hospitals in Mumbai. Results: The cohort of 129 individuals was categorized into 5 groups based on their clinical TB and HIV status. The mean CD4 count for TB+HIV+(Groups 1,2,3) ranged between 381 and 525 cells/cmm suggesting early to moderate immune suppression.TB PCR assay was compared with the 'gold' standard, namely the LJ culture in each of the 5 groups. Overall, the sensitivity of PCR was 83.3% and specificity was 97.1%. PCR+ LJ-were subjected to sequential TB PCR tests at intervals of two weeks after initiation of AKT.TB PCR+ patients converted to TB PCR negative between 6-8 weeks. Conclusions: The study established that PCR targeted to IS 1081 is a valuable test for early diagnosis of TB from peripheral blood at an early point of TB activation when most patients (>85%) did not produce other traditional specimens such as the sputum and/or pleural fluid.

BACKGROUND

The World Health Organization (WHO) estimates that over 2 billion individuals globally are infected with Mycobacterium Tuberculosis (MTB) (1). While MTB continues to be a major cause of morbidity and mortality in developing countries, there has been a resurgence even in developed countries where it was successfully contained (2). The situation is further compounded by emergence of MDR and XDR-TB (3,4). With the advent of the HIV/AIDS pandemic and its consequent immune suppression, the coinfection of TB/HIV is now recognized as a lethal combination (5). Such co-infection occurs in at least 60-70% of HIV-infected individuals during their lifetime leading to their rapid HIV-disease progression and death (1, 5).

The existent diagnostic tests for MTB in developing countries such as microscopy that lacks sensitivity, LJ culture that takes 6-8 weeks to produce a result, or Bactec 460 assay that takes 10-14 days are not very helpful. Consequently, the authors felt that there was a need for a reliable diagnostic test that can be conducted on a readily available specimen such as peripheral blood to periodically screen HIV-infected individuals for MTB at an early stage of infection (6, 7). Polymerase Chain Reaction (PCR) assay is a more sensitive, specific and quicker method for detection of MTB in clinical specimens (8, 9). Early diagnosis of TB co-infection will help reduce morbidity, mortality and further immunologic damage in HIV-infected individuals.

PATIENTS AND METHODS

PCR targeted to insertion sequence (IS) 6110 has been successfully used for detection of Mycobacterium tuberculosis(MTB) in cerebro-spinal fluid in India (10). The authors designed a study to assess the diagnostic value of PCR targeted to IS 1081 in peripheral blood of HIV-infected individuals. An earlier study from India reported that IS 1081 to be prevalent in >80% in the study group (7). The study used peripheral blood because of ease of obtaining periodic samples. Between February 2006 and January 2008, a cohort of 129 individuals was recruited for this purpose

(Table I). It contained of adult, non-pregnant HIV sero-positive as well as HIV sero-negative individuals who were naïve to anti-Koch's treatment (AKT) and attending the Department of Infectious Diseases (DID) MGM Medical College, Navi Mumbai and KJ Somaiya Medical College, Mumbai. All HIV+ patients were tested for CD4 counts using flowcyto-meter at the point of recruitment (Guava technologies, USA).

Five milliliters of peripheral blood was collected from study participants in EDTA vacutainers (Becton Dickinson, USA) after obtaining the individual informed written consent. LJ medium slants (Hi-Media laboratory, Mumbai) were inoculated with 0.2 ml of sediment from isolator lysis tubes was inoculated on LJ slants (11). The remaining sample was processed for PCR in the following manner: Leukocytes were pelleted after lysis of erythrocytes using chilled hypotonic erythrocyte lysis buffer. These were then re-suspended in 3 ml of erythrocyte lysis buffer. This was followed by lysis with 20% SDS and digestion with proteinase K (20mg/ml) at 37 °C overnight. The DNA was extracted by Phenol:Chloroform extraction method. DNA was precipitated after adding 2 volumes of chilled ethanol. DNA was further washed thrice with 70% ethanol. DNA was then dissolved in 150µl of Tris-EDTA buffer. The concentration of DNA was measured from the absorbance at 260nm using UV-light Spectrophotometer (Mitsubishi, Japan). A 5µl aliquot of total DNA (~200ngm) was used for a 25µl PCR cocktail. Primers BW-6 [5' CGA CAC CGA GCA GCT TCT GGC TG 3'] and BW-7 [5' GTC GGC ACC ACG CTG GCT AGT G 3'] aimed at 306-bp region of the multicopy insertion sequence (IS) 1081 were used to amplify mycobacterium DNA.

For amplification, reaction was performed in a volume of 25µl of mixture which contained 50pmol of each primer, 200µM of each dNTP, 3 units of Taq DNA polymerase

(Bangalore Genei, Bangalore) and 5µl of extracted DNA containing ~200 ng of DNA.

The amplification parameters included an initial denaturation step at 94°C for 5 minutes followed by 35 cycles each of denaturation at 94°C for 1 minute, annealing at 68°C for 1.5 minutes (12), extension at 72°C for 2 minutes. After the final extension at 72°C for 10 minutes, the samples were shifted to 4°C for storage. The PCR products were detected by Polyacrylamide Gel electrophoresis followed by silver staining (13). In each run, genomic DNA of MTB was used as a Positive control and a known negative DNA was used as a negative control. Reagent control without DNA was also included. Markers with known bands between 80 and 400 base-pairs were included (Sigma, USA). The areas for template preparation, PCR setting and detection of PCR products were separated to prevent any carry over contamination. Only the results of a reaction set, where positive showed a good amplification product, with good band density and both negative controls showed clean negative results were considered valid.

Wherever possible, additional samples of sputum, CSF and pleural fluid were obtained from the 15 patients and PCR assay were performed. Guarded CSF taps were performed on 2 patients who presented with neck stiffness.

An effort was made to determine the accuracy of PCR in samples which were TB PCR+ LJ- in Groups 1 and 2. All TB PCR+ patients were prescribed presumptive anti-Koch's therapy consisting of Rifampicin, isoniazid, pyrazinamide and ethambutol (AKT) according to the national guidelines of RNTCP/DOTS therapy (14).

Sequential PCR tests were performed on peripheral blood at two-week intervals among PCR+LJ-till the point of PCR conversion.

RESULTS

The cohort of 129 patients was categorized into 5 groups based on their clinical TB and HIV status (Table 1). The mean age of TB+HIV+ (Group 1,2,3) patients ranged between 32 and 37 years, that of TB+ HIV-(Group 4) was 48 years and that of healthy volunteers who were TB-HIV-(Group 5) was 30 years. HIV infected individuals (Group 1,2,3) had a mean CD4 count ranging between 381 and 525 cells/cmm suggestive of early to moderate immune suppression (Only 10/113 had CD4 count of <200 cells/cmm). Of 113 HIV+ patients, 55/113 (48.6%) had weight loss of >10% in 1 month, 58/113 (51.3%) had recurrent fever> 1month, 40/113 (35.4%) had recurrent cough > 1 month, 5/113 (4.4%) had an abdominal lump and 1/113 (0.89%) had neck stiffness suggestive of meningitis.

Table 2 presents the sensitivity and specificity of TB PCR assay in comparison with the 'gold' standard, namely LJ medium culture in each of the 5 groups. Overall, the sensitivity was 83.3% and specificity was 97.1%.

Tables 3 and 4 present the results of sequential TB PCR of patients who were discordant i.e. PCR+ LJ- to determine

accuracy and validity of PCR as a superior diagnostic test. All sequential TB PCR assays in group 1 and 2 were reproduced twice. TB PCR+ patients converted to TB PCR- between 6-8 weeks after initiation of AKT (DOTS).

Figure 1

Table 1: Demographics and clinical features of the cohort.

CATEGORY	N	MEAN AGE (in years)	MALE	FEMALE	Mean Absolute CD4 count	WEIGHT LOSS >10% in 1 mo (%)	RECURRENT FEVER >1mo (%)	RECURRENT COUGH >1 mo (%)	ABDOMINAL LUMP SUGGESTIVE OF TB (%)	NECK STIFFNESS (%)
Group 1 Presumptive diagnosis of TB. Symptoms suggestive of clinical TB AND confirmed by conventional laboratory and radiologic findings HIV Status = Positive	32	31.6 sd+/- 4.6	24	08	426.1 sd+/- 244.7	17 (53.1)	20 (62.5)	18 (56.3)	4 (12.5)	0
Group 2 Suggestive diagnosis of TB. Symptoms suggestive of clinical TB OR indicative by conventional laboratory and radiologic findings HIV Status = Positive	64	37.0 sd+/- 8.5	55	09	381 sd+/- 227.6	38 (59.4)	38 (59.4)	22 (34.4)	1 (1.6)	1 (1.6)
Group 3 Asymptomatic for clinical TB HIV Status = Positive	17	35.1 sd+/- 8.4	11	06	524.6 sd+/- 270	0	0	0	0	0
Group 4 Symptomatic for clinical TB HIV Status = Negative	06	48.5 sd+/- 22.6	05	01	ND	0	4 (66.7)	1 (16.7)	0	1 (16.7)
Group 5 Healthy health-care workers as volunteers HIV status = Negative	10	30.5 sd+/- 4.2	03	07	ND	0	0	0	0	0

ND = not done; Conventional methods of diagnosis for clinical TB (21)

Figure 2

Table 2. Comparison of TB PCR assay with LJ culture: Sensitivity and specificity

GROUP	N	PCR+/ CULTURE+	PCR+/ CULTURE-	PCR-/ CULTURE+	PCR-/ CULTURE-	SENSITIVITY (%)	SPECIFICITY (%)
1.	32	16	07	01	08	69.5	88.9
2.	64	23	03	0	38	88.5	100
3.	17	07	0	01	09	100	90
4.	06	04	0	0	02	100	100
5.	10	0	0	0	10	NA	100
Overall	129	50	10	02	67	83.3	97.1

Figure 3

Table 3. Sequential TB-PCR Of patients in Group 1 who were PCR+ LJ-

No.	0 Week	AKT started	2nd week	4th week	6th week	8th week	10th week	12th week	14th week	16th week	18th week
1.	P	Yes	P	P	N	N	N	ND	ND	ND	ND
2.	P	Yes	P	P	P	N	N	N	ND	ND	ND
3.	P	Yes	P	P	N	ND	ND	ND	ND	ND	ND
4.	P	Yes	P	P	N	N	P	N	N	ND	ND
5.	P	Yes	P	P	P	N	N	N	N	ND	ND
6.	P	Yes	P	P	P	N	N	N	N	N	ND

P=Positive; N=Negative; ND=Not Done

Figure 4

Table 4. Sequential TB-PCR of patients in Group 2 who were PCR+ LJ -

No.	0 week	AKT started	2nd week	4th week	6th week	8th Week	10th week	12th week	14th week
1.	P	Yes	P	P	N	N	N	N	N
2.	P	Yes	P	P	N	N	N	ND	ND
3.	P	Yes	P	P	P	N	N	N	N

P=Positive; N=Negative; ND=Not Done

DISCUSSION

It is now fairly well established that 60-70% of HIV-infected individuals develop active clinical TB during their lifetime (1,5). With activation of TB, the HIV disease progression is accelerated; hence TB-HIV is considered a lethal combination (5). The study underscores the importance of quick diagnosis of TB using PCR assay with primers such as those with IS1081 that are prevalent in India (7). The study has established the value of early diagnosis of TB from peripheral blood at an early point of activation when most patients did not produce other traditional specimens such as the sputum and/or pleural fluid. In fact, only 15/113 (13.3%) TB+ HIV+ patients had readily accessible specimens such as sputum and/or pleural fluid. In other words, over 85% of early TB infection in HIV infected individuals would have been missed if the peripheral blood was not tested with PCR assay. In fact, recent studies in other parts of the worlds have also shown that with intensified screening strategies, the incidence of tuberculosis was two times lower in the first four months of Anti-retroviral therapy (ART) in a cohort based study in South Africa (15). Thus, the value of early diagnosis of TB using PCR cannot be over emphasized.

Published evidence suggests that the efficiency and utility of PCR assays using a number of sequences in target DNA of MTB, from a variety of body fluids, depends on the sequence targeted for amplification as that sequence should be present in the strain of MTB involved in the infection (16-22). Moreover, the prevalence of specific strains differ from region to region. For example, high prevalence of IS1081 has also been reported from Iran (23) and Poland (24). It has been reported that insertion sequence IS 6110 which was found to be useful in many PCR assays elsewhere was found to be less prevalent in MTB strains in India [8]. However, the study itself suggested that the reason for low sensitivity and specificity using LJ culture as gold standard was because IS6110 was less prevalent in India and it used conventional biological specimens such as sputum. Both the choice and design of primer pair are critical determinants of

efficiency of the PCR assay.

By comparison, our study using primer IS1081 established a sensitivity and specificity of 83% and 97%, respectively. However in real terms the sensitivity would be higher considering that PCR+ LJ- discordant patients showed PCR conversion after AKT began. All 9 patients with discordant results at recruitment responded well to AKT comprising of rifampicin, isoniazide, pyrazinamide and ethambutol (Table 3 and 4). The patients showed clinical improvement such as weight gain, disappearance of cough and

fever within weeks of initiation of AKT and no deaths occurred. The average time for PCR conversion to negative was 6-8 weeks. Thus, even in cases of mild infection, PCR is a more sensitive assay compared to LJ medium culture which is likely to fail or take more time to produce a positive result with respect to this slow-growing organism. Various factors such as immune competence, time required for clearance of bacilli from the blood and other factors may differ from individual to individual; hence there is a difference in the time required for PCR conversion. Our data suggests that both live and dead bacilli were cleared from blood in about 6-8 weeks of AKT. This is an important finding that clinicians can use to determine the therapeutic response or the emergence of resistance (MDR/XDR TB) during treatment.

PCR assay for TB costs between $15-20 in India. It can be rapidly deployed in major cities with high burden of TB because of affordability (of cost) by the local population, large hospitals that have PCR infrastructures, and trained manpower which is able to perform quality PCR testing. The risk of over diagnosis of TB using peripheral blood can be reduced by instituting a good quality assurance program and linking PCR diagnostic laboratories to research institutes.

AUTHORS' CONTRIBUTIONS

Rajiv S. Hira contributed by thinking of the topic, doing thorough background research, managed and analyzed the data, obtained reference articles and wrote the manuscript

Vishwas C. Sarangdhar contributed in the design of the study, conducted and supervised the PCR and other laboratory tests and participated in writing the manuscript.

Subhash K. Hira contributed in the design of the study, recruitment of the patients in the study, and participated in data analysis and preparation of the manuscript.

Herbert L. DuPont contributed in the design of the study and in preparation of the manuscript.

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