DIRECT DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS* IN BLOOD SAMPLES OF HIV INFECTED PATIENTS BY POLYMERASE CHAIN REACTION.

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ABSTRACT

We have developed a simple, economical and reproducible method for processing blood samples from HIV infected patients for diagnosis of tuberculosis. The procedure was validated on 55 samples selected for tuberculosis based on clinical criteria. 52 patients had radiological changes indicative of pulmonary tuberculosis of which only 28 were positive for AFB in sputum (sensitivity 54%) and 27 for tuberculin (sensitivity 52%). 26 HIV positive patients who showed positive X-ray did not react to tuberculin. The genus PCR probe missed 3 samples (sensitivity 94%) compared to X-ray. *M.tuberculosis* was detected in the blood of all X-ray positive cases by PCR using TB400 probe (sensitivity 100%) and another probe for *M.tuberculosis*, IS6110, missed 6 of them (sensitivity 88% compared to X-ray and 89% compared to TB400). It is proposed that this simple sample processing method could be used to screen all blood samples quickly for mycobacteremia using the genus PCR and only those positive for mycobacteria need to be tested for *M.tuberculosis*. This would save the scarce resources and time by reducing significantly the number of samples to be screened for species confirmation.

KEY WORDS: Polymerase Chain Reaction, mycobacteremia, tuberculosis diagnosis in AIDS, DNA probes, *Mycobacterium tuberculosis*.

INTRODUCTION

Evidences indicate an increasingly close association between HIV and *Mycobacterium tuberculosis* infections. An estimated 4.4 million people are co-infected with HIV and *M. tuberculosis* globally of which half a million have active tuberculosis (1). Persons infected with tubercle bacilli and the human immuno deficiency virus have an increased risk of developing active tuberculosis. Conventional methods not only have poor sensitivity but also take several weeks and this delay could be fatal in AIDS patients. Many AIDS patients show anergy (2); hence tuberculin testing has only limited value in the diagnosis of tuberculosis in these patients. Sputum analysis, though very simple, apart from having poor sensitivity,

poses difficulties due to contaminating microorganisms (including mycobacteria other than *M.tuberculosis*) and non-availability of sputum in some AIDS patients. Polymerase Chain Reaction (PCR) based nucleic acid probes potentially hold the key for this problem (3). Blood has been reported to be useful in the diagnosis of pulmonary tuberculosis (4). Blood samples are easily obtainable from patients and detection of *M.tuberculosis* in blood is definite indication of active infection. Several PCR based assays have been reported for the detection of *M.tuberculosis* directly from the clinical samples (5-11). The processing of blood samples for PCR in these reports has always been tedious, involving gradient centrifugation on

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expensive polymers or enzymatic digestion or organic extraction or all of the above steps. The objectives of this study were (i) To validate the simple sample processing method for PCR analysis on HIV positive blood (ii) To check if the TB400 probe, developed by the corresponding author at Boston, is applicable to Indian samples (iii) To do a preliminary comparison of the sensitivities of TB400 (11) and IS6110 (6) probes on Indian samples and (iv) To validate in India the sensitivity of the genus probe pMAv17 on samples positive for HIV and tuberculosis. We are reporting the results of the PCR assay, which includes the simplest of sample processing methods reported so far, for the direct detection of *M. tuberculosis* in blood samples based on our TB400 probe (11). The results of our validation in three different PCR assays from HIV infected patients are reported. We have also compared the usefulness of the conventional methods against PCR.

MATERIALS AND METHODS

Blood samples (0.5 ml) were collected in an eppendorf tube (with EDTA upto 5mM final concentration as anticoagulant) from patients (who showed symptoms of pulmonary tuberculosis) attending the YRG Foundation for AIDS Research, Madras, India. Routine clinical investigations were done on these patients for tuberculosis and HIV infection. All steps in the processing of blood samples for PCR were done at room temperature. The blood samples were centrifuged at 12,000 rpm for 15 minutes in a microfuge, the cell pellet was lysed by vortexing briefly in (0.5 ml) lysis buffer (0.01M Tris, 5.0 mM EDTA, pH 8.6 and 1% Triton X-100) and centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded. The above step was repeated (on an average about 4 times) till the supernatant became

colourless. The control tubes (to check for contamination during processing) contained just 0.5 ml of lysis buffer and processed like blood samples. The pellet was re-suspended finally in 100 µl of lysis buffer and boiled for 30 minutes. This lysate when stored at 4°C was found to be stable for several months for PCR analysis.

Genus (pMAv17, product size 310 bp) and *M. tuberculosis* (TB 400, product size 383 bp) specific primers as reported by Sritharan and Barker (11) and *M. tuberculosis* specific primers (IS6110, product size 123 bp) developed by Eissenach et.al (6) were used in this study. The sequences of the primers used are given below. Amplification reactions were performed in 30 µl reaction mixture consisting of 0.2 mM dNTP, 30 ng of each primers, 0.3 units of Taq DNA polymerase (Amersham International plc., London, UK.), PCR buffer (70mM Tris, pH 8.8; 20mM (NH₄)₂ SO₄, 2.5 mM MgCl₂, 1mM DTT, 100 g ml⁻¹ BSA and 0.1 % Triton X-100) and appropriate amount of target DNA (1-3 µl of processed blood lysates). The samples were denatured at 95°C for 5.0 minutes. Subsequently amplification was done either at 55°C for 1.0 min, 72 °C for 2.0 min and 94 °C for 1.0 min through 30 cycles for Genus (A1, B1) and TB400 primers (C1, D1) or at 68 °C for 2 min, 72 °C for 3 min and 94°C for 1 min for IS6110 primers (A, B). *Mycobacterium tuberculosis* specific homologous 383 bp and 123 bp DNA fragments and mycobacterium genus specific 310 bp DNA fragments were resolved on low melt agarose by electrophoresis. They were used as targets and labelled with digoxigenin-11 dUTP (Boehringer Mannheim) by PCR under conditions as mentioned above except that dTTP was replaced by a mixture of digoxigenin -11 dUTP and dTTP in the molar ratio of 0.35: 0.65.

	Probe	Primer Sequence	Product Size
1)	Genus primers:		
	pMAv17:	A1 GATCAGGACCGCTGCCGCG	310 bp
		B1 CACTCGTCGGGAATGCCCTGC	
2)	<i>M. tuberculosis</i> primers:		
	a) TB 400	C1 TACGGATTCCGTCCACCGTCATGG	383 bp
		D1 CACCGGCGGAACACTGCAGG	
	b) IS6110:	A CCTGCGAGCGTAGGCGTCGG	123 bp
		B CTCGTCCAGCGCCGCTTCGG	

The amplified products were resolved on 1% agarose gel and confirmed by Southern blotting after hybridisation to specific probes. Hybridisation was performed as described by Boehringer Mannheim (Genius™ kit, Boehringer Mannheim). Digoxigenin labelled probe was detected using anti-digoxigenin antibody alkaline phosphatase conjugate and AMPPD (Tropix Inc., Bedford, USA) as substrate and exposing the membrane to X-ray film (Hyper Film™, Amersham) for 30-45 min at room temperature.

RESULTS

We had earlier (11) described a method in which a simple direct lysis procedure was used for PCR identification of mycobacteria in unprocessed sputum samples. Unlike the sputa, blood samples can not be used for PCR amplification directly because they contain inhibitors for PCR. Processing of blood samples required a higher concentration of EDTA because in blood samples processed with 1mM EDTA, significant degradation of DNA was noticed when stored at 4°C compared to samples processed with 5mM EDTA, while EDTA concentrations higher than 5mM were inhibitory to PCR amplification (data not shown). Sputum samples need only be washed twice with the lysis buffer to get consistently inhibition free amplification. However, the blood lysates obtained after only two washes could not be amplified under the same conditions of PCR. Therefore the number of washings was increased to at least 4 times or till supernatant was colourless. When the colour of the supernatant was kept as an indicator all samples processed were found to be PCR ready. This marks an improvement over all the methods that have been published so far for processing blood. This method is simple, straightforward and involves a visible indicator (the red colour), which is, the disappearance of the colour of contaminating haemoglobin from the processed sample. This procedure, thus eliminates, the need for extraction of DNA using organic solvents, use expensive enzymes to lyse the bacteria, adsorbing the DNA onto the expensive columns or inert matrices like silica gel, glass milk etc., separating the DNA on expensive gradients by centrifugation or precipitation of DNA. The sensitivity (data not shown) of the procedure was the same as reported before for sputum (11).

55 patients presented the radiological changes suggestive of pulmonary tuberculosis. Sputum acid

fast bacilli (AFB) were negative in 24 patients. The sensitivity of each method was calculated as the percentage of the number of samples positive by both methods divided by the total number of samples detected by the reference method. The sensitivity of the AFB method compared to X-ray chest was 57%, that of tuberculin test was 31.4% and that of tuberculin test compared to AFB was only 64%. The results of these conventional methods of analysis are presented in Table 1 (a, b, c).

Table 1

(a)

		AFB			
		+	-		
X ray + Chest	+	25	19	44	Sensitivity of AFB = 57%
	-	1	6	7	
		26	25	51	

(b)

		Mx			
		+	-		
X ray + Chest	+	11	24	35	Sensitivity = 31.4%
	-	2	9	11	
		13	33	46	

(c)

		Mx			
		+	-		
AFB	+	9	14	23	Sensitivity of Mx vs AFB = 64%
	-	8	15	23	
		17	29	46	

52 blood samples contained mycobacteria based on the genus probe analysis, a sensitivity of 94% compared to X-ray chest results (Table 2a).

Table 2(a).

		PCR Genus			
		+	-		
X ray + Chest	+	44	0	44	Sensitivity of Genus PCR =100%
	-	3	4	7	
		47	4	51	

Products of the expected size (310bp) were amplified from the processed blood samples, (Fig. 1a) demonstrating that our protocol yields lysates that can be readily used in PCR. When analysed for *M. tuberculosis* by two PCR assays (Fig. 1b) each one detecting distinct, species specific sequences, all 55 specimens contained TB specific DNA sequences as detected by TB 400 Table 2(b), while, only 49 were detected as *M. tuberculosis* by IS6110 probe Table 2 (c).

Fig. 1 (a). Analysis of PCR product. Analysis of Genus PCR product by agarose gel electrophoresis under ethidium bromide staining (upper half) and after Southern hybridisation (lower half). Hybridisation was done with digoxigenin labelled 310 bp genus probe. M = Marker, Lanes 3,4,6,7,9,11 = samples from HIV patients with pulmonary tuberculosis. Lanes 1 = Positive Control, *M. tuberculosis* DNA. Lane 2 = Blank (contamination control); lanes 5,8,10 = sample numbers 13,36 and 45 from Table 3 which were negative in Genus PCR but positive in *M. tuberculosis* PCR assay.

Table 2(b)

		PCR TB400		
		+	-	
X ray +	Chest	44	0	44
-		3	4	7
		47	4	51

Sensitivity of TB400 = 100%

Fig. 1 (b). Southern Analysis for *M. tuberculosis*. Southern Hybridisation analysis of some samples after PCR amplification for *M. tuberculosis*. Hybridisation was done with digoxigenin labelled *M. tuberculosis* specific 383 bp (TB400, upper lanes) and 192 bp (IS6110, lower lanes) probes. Lane 1 = Positive control, *M. tuberculosis* DNA. Upper Lanes 3, 4, 5, 7, 10, 11 and lower lanes 3, 4, 5, 6, 9, 10 are samples from HIV positive patients with pulmonary tuberculosis. Samples analysed in the same order in the lower lanes also except that lanes 9, 10 corresponds to lanes 10, 11 of the upper half. Lane 2 in both upper half and lower half = contamination control (blank). Upper lanes 8, 9 and lower lanes 8, 11 = Negative samples.

Table 2 (c).

		PCR TB400		
		+	-	
X ray +	Chest	41	6	47
-		0	4	4
		41	10	51

Sensitivity of IS6110 = 87%

When compared to the X-ray chest results, this represents a sensitivity of 100 % and 87 % respectively, for TB400 and IS611. The summary of the results of the study are presented in Table 3.

Table 3. Consolidated Results of Comparison of Laboratory and Clinical Investigations

No.	HIV	X-ray	APB	Mx	Genus	TB400	IS6110
1	+	I	+	NA	+	+	+
2	+	F	+	NA	+	+	+
3	+	C&I	+	-	+	+	+
4	+	F	+	-	+	+	+
5	+	I	+	-	+	+	+
6	+	NA	+	NA	+	+	+
7	+	F	+	+	+	+	+
8	+	PE	+	-	+	+	+
9	+	I	-	NA	+	+	+
10	+	I	-	-	+	+	+
11	+	I	-	NA	+	+	+
12	+	I	+	-	+	+	+
13	+	I	+	-	-	+	+
14	+	I	-	+	+	+	+
15	-	C&I	+	NA	+	+	+
16	-	I	-	-	+	+	-
17	+	I	+	-	+	+	+
18	+	I	+	-	+	+	+
19	-	I	+	-	+	+	+
20	-	C&I	-	-	+	+	+
21	-	F&I	-	+	+	+	+
22	-	F	-	+	+	+	+
23	-	C	+	+	+	+	+
24	-	F&I	+	+	+	+	+
25	-	F&I	+	+	+	+	+
26	+	F&I	-	+	+	+	+
27	+	PE	-	NA	+	+	-
28	+	NAD	-	-	+	+	+
29	+	NAD	+	+	+	+	+
30	+	I	-	-	+	+	+
31	+	I	+	-	+	+	+
32	+	I	+	+	+	+	+
33	-	I	-	NA	+	+	+
34	-	I	-	-	+	+	-
35	+	I	-	-	+	+	+
36	-	I	+	-	-	+	+
37	+	I	+	NA	+	+	+
38	+	I	+	-	+	+	+
39	+	I	+	-	+	+	+
40	+	I	+	+	+	+	+
41	+	I	-	+	+	+	-
42	+	NAD	-	+	+	+	+
43	+	NA	-	NA	+	+	+
44	-	NA	-	NA	+	+	+
45	+	PE	-	-	-	+	+
46	+	I	-	NA	+	+	-
47	-	C	-	NA	+	+	+
48	-	I	+	NA	+	+	+
49	+	NA	-	+	+	+	+
50	+	NA	-	+	+	+	-
51	+	NA	-	-	+	+	+
52	+	I	-	-	+	+	+
53	+	I	-	NA	+	+	+
54	+	NA	+	+	+	+	+
55	+	NA	-	NA	+	+	+
56	+	NAD	-	-	+	-	-
57	+	NAD	-	-	+	-	-
58	+	NAD	-	-	-	-	-

I = Infiltration. C = Consolidation. F = Fibrosis. PE = Pleural Effusion. HIV = HIV antibody ELISA positive. Mx = Montoux. NA = Not Available. NAD = No Abnormality Detected.

DISCUSSION

Presence of *M. tuberculosis* in the blood normally indicates active extra-pulmonary tuberculosis and in those without any extra pulmonary tuberculosis it may indicate pulmonary tuberculosis. Immunological evidences have been presented to demonstrate the presence of *M. tuberculosis* cell components in the blood of patients who had no extra-pulmonary tuberculosis (12, 13) though the presence of the bacilli had never been demonstrated directly. Mattar et al (14) showed that HIV infected patients having sepsis or sepsis syndrome, had *M. tuberculosis* DNA in peripheral blood and that blood samples could be used to detect *M. tuberculosis* by PCR for the diagnosis of pulmonary tuberculosis in patients who have no evidence for extra-pulmonary tuberculosis (4). The results of this study substantiate this recent report.

Contamination either with target (genomic) DNA or the amplicons and inhibition of the amplification reaction are some constraints in PCR assay. One simple and very effective way to minimise contamination with target genomic DNA is to minimise the number of steps required to process the samples for PCR analysis. All the methods reported for processing blood samples so far, are tedious, expensive and involve enzymatic digestion and often organic extraction. This is not desirable particularly when, hundreds of highly infectious samples have to be processed. As the number of steps in the processing increases the chances of cross contamination in the samples also increases. If PCR has to be adopted routinely in clinical microbiology laboratories, particularly in developing countries, not only the cost but also the complexity of the process should be greatly reduced.

Blood is easier to obtain than sputa from AIDS patients and children. Further, sputa obtained from AIDS patients are often

contaminated with non-tuberculous opportunistic pathogens and other microorganisms. Primary isolation and biochemical characterisation is inevitable in almost all such cases. Therefore, blood is an ideal sample for diagnosis of TB in such patients. Many laboratories in the developing countries, which have only limited resources, are not equipped to handle blood samples from HIV infected patients for culture confirmation. The dramatic increase in the association between tuberculosis and HIV infection in recent years has made the need for simple, yet rapid and specific methods for direct detection of *M. tuberculosis* in clinical samples very important and urgent. In this study we have demonstrated a simple technique for processing blood samples. Our sample processing yielded lysates free from any inhibitors as all samples were amplified in PCR irrespective of the primers used.

The Genus primers and probe are being used for the first time in India since they were developed and validated (15). This genus PCR missed 3 samples out of 55 which were positive for *M. tuberculosis*. The genus primer sequences are part of multiple repeats present in the genome of several mycobacteria and it is possible that these three strains lacked these sequences. Three separate PCR experiments, each done with increasing amounts of target DNA did not yield any amplification (data not shown) indicating the absence of these primer sequences in these strains. None of the several hundreds of samples screened in Boston (USA) from the Massachusetts State Laboratory tested negative in Genus PCR assay (15). This is the first time that variation in the Genus primer sequence in *M. tuberculosis* is being observed. On the contrary, geographical variation in the IS6110 sequences have been reported (16) and therefore lack of amplification in 6 samples could

be due to absence of primer sequence in the IS6110 target of the genome. However, this needs to be confirmed.

The results of this study have shown that the sample processing procedure is applicable to blood samples from HIV infected patients and our probe TB400 and pMAv17 may be used for the diagnosis of TB in such patients. The TB probe IS6110 shows less sensitivity on Indian samples compared to our earlier reports from Boston. This probably indicates variation in the probe sequence in the *M. tuberculosis* genome in Indian strains.

In a reference laboratory where culture facility is available, the need to cut down the cost is very important. The cost of PCR based diagnosis has decreased dramatically now. Culture though, less sensitive, is still considered as the gold standard. If culture isolation has to be done for testing the drug sensitivity pattern of the organisms, the genus PCR could be used to quickly screen and select mycobacteria positive samples for culture. Further, short course multi drug therapy is being administered to all patients clinically positive to TB even before culture results become available. In this context we would like to recommend the usefulness of PCR to initiate and monitor the effectiveness of the therapy.

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