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SIGNIFICANCE OF APPROPRIATE SAMPLING IN THE DIAGNOSIS OF TUBERCULOSIS (TB) – A COMPARISON OF DIFFERENT TECHNIQUES

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ABSTRACT

Introduction: *After years of decline, tuberculosis (TB) has re-emerged as a serious public health problem worldwide causing significant mortality and morbidity in developing countries like Pakistan, where the estimated incidence of TB is 181 per 10000. The present prospective study was conducted in Shalamar Hospital Lahore from January 2007 to October 2009. The objective was to compare the PCR results of specific site samples and blood of the same TB patient to see the validity of PCR results based on blood samples.* ***Materials and Methods:*** *Clinical samples obtained from 205 patients of suspected TB (pulmonary or extra-pulmonary) were subjected to ZN smear examination, LJ medium culture, and PCR test by amplifying 541 bp fragment of Mycobacterium tuberculosis complex genome. A highly significant difference was seen in the test results done on samples obtained from specific site according to disease and blood samples of the same patient infected with pulmonary or extra-pulmonary tuberculosis.* ***Results:*** *The sensitivity of different tests was found to be significantly different, which was 67.32 percent for PCR test, 27.81 percent for LJ medium culture and 12.20 percent for ZN smear examination. However, there was no significant difference between different tests as far as specificity was concerned. PCR test sensitivity in pulmonary and extra-pulmonary clinical samples was 77.15 and 61.6 percent respectively, being significantly higher, when compared with sensitivity of other tests. The mean detection time for M. tuberculosis was 24 days by LJ medium culture and less than 1 day by smear examination and PCR test. We concluded that the PCR test is more sensitive than ZN smear examination and LJ medium culture for the diagnosis of TB in pulmonary and extra-pulmonary clinical samples. To get more accurate results PCR for TB diagnosis should be done on specific site samples. Blood samples are not appropriate for the diagnosis of TB by PCR when the PCR is done on TB genomic DNA.*

INTRODUCTION

After years of decline, tuberculosis (TB) has re-emerged as a serious public health problem worldwide causing significant mortality and morbidity in developing countries like Pakistan, where the estimated incidence of TB is 181 per 10000.[1] Factors contributing to this resurgence include the HIV epidemic and immigration of people from countries with a high incidence of tuberculosis. In 1993, the World Health Organization (WHO) declared it to be a global emergency and according to a recent WHO report, there were 7.96 million new cases with 2 million deaths in 1997 alone.[2]

The laboratory diagnosis of tuberculosis is based on microscopy or culture of the clinical samples. These techniques either lack the sensitivity or are time consuming. Even with concentrated samples, the sensitivity of microscopy is not great (sensitivity is in the order of 10^5 acid-fast bacilli per ml of sputum).[3,4] On the other hand, conventional culture methods are quite slow (requiring 3-8 weeks for completion). Once the presence of mycobacterium is indicated additional biochemical testing is required to identify the species. This also requires time and experienced personnel for accurate identification of isolates.[5] However the disease most often remains undiagnosed hence untreated. The main difficulty with extra-pulmonary specimens is that they yield very few bacilli and consequently are associated with low sensitivity for acid fast bacilli (AFB) smear and culture.[6]

The introduction of nucleic acid-based direct amplification tests to target mycobacterium DNA or RNA directly from specimens, is the most exciting milestone in diagnostic mycobacteriology. Among nucleic acid-based techniques, available for the diagnosis of *M. tuberculosis,* Polymerase Chain Reaction is the most widely used, best studied and most widely published technique. An increasing number of laboratories have established PCR as a supplementary test, since PCR provides good rates of positive results and better turnaround time than

culture (days versus weeks) and smear examination.[4,7]

We conducted this study to evaluate the performance of PCR test for the detection of *M. tuberculosis* in different clinical samples obtained from specific site according the disease (pulmonary or extra-pulmonary) by amplifying 541 bp sequence and comparing the result with smear examination and conventional culture using Lowenstein-Jensen (LJ) medium. The PCR results carried out on different samples obtained from specific site according the disease were also compared with PCR results of blood samples of the same patients to see the validity of PCR results done on blood samples in TB diagnosis.

MATERIAL AND METHODS

A total of 205 clinical samples obtained from patients with pulmonary or extra-pulmonary tuberculosis having a strong clinical and radiological evidence of TB, between January 2007 and October 2009 were included in this study. All the necessary clinical details were obtained from the patient in the format developed for this purpose.

Grouping of clinical samples:

The clinical samples (n = 205) included in the present study were divided into two major groups, Pulmonary tuberculosis (n = 80) and extra-pulmonary tuberculosis (n = 125). In first group, 60 samples were sputum from 60 suspected cases of pulmonary TB, 20 broncho alveolar lavage (BAL). In extra-pulmonary TB, 35 pleural fluids, 30 CSF, 25 were from pus, 20 urine and 15 ascitic fluids were received.

Processing of samples:

The present study was conducted at Shalamar Hospital Laboratories and TB Research Center of Pakistan Medical Research Council (PMRC) which is affiliated with King Edward Medical University, Mayo Hospital Lahore. The fresh morning specimens were collected in sterile containers at the Shalamar Hospital Laboratory Lahore, either directly from the patients or from wards and OPD of Shalamar Hospital. Blood samples of all these patients were also collected at the same time for the detection of tuberculosis by PCR. All the samples were equally divided into two separate sterile containers. One of these samples was sent to PMRC for LJ medium culture and other was processed in Shalamar Hospital laboratory for smear examination and Polymerase Chain Reaction (PCR). Direct and concentrated smears were prepared from clinical samples after treating with NALC (N-acetyl-L-cysteine) -NaOH (sodium hydroxide) method.[8,9] Briefly, the NALC-NaOH methods involved the decontamination and digestion of the clinical samples with 2 percent NaOH (final concentration) in 0.5 percent NALC and concentrated by centrifugation at *3000g* for 15 min. Supernatant was discarded and to sediment, 1-2 ml of sterile phosphate buffer of *p*H 6.8 (1 to 2 ml) was added and centrifuged for 15 minutes at 3000g. Deposit was used for smear examination and MTB DNA extraction. Slides for smear examination were stained by Ziehl-Neelsen method.[8]

DNA extraction:

DNA was extracted from the deposit of processed specimens as briefed before, using commercially available DNAzobBD DNA Isolation kit (MRC, USA) with one initial modification step of keeping the preliminary processed materials at 80°C for 15 min for the inactivation of possible Mycobacterium. The material was then processed as per guidelines of the manufacturer of the kit to obtain the DNA.

Amplification of MTB DNA:

PCR was performed on extracted DNA samples using specific primers to amplify a 541bp sequence of MTB complex.[10] Briefly, a 25 ml reaction mixture was set up containing 10.7 ml of double distilled HzO, 2.5 ml of 10X buffer, 1.5 ml of 25 mM MgClz, 300 mM (each) of the four deoxyribonucleoside triphosphate IU of Taq DNA polymerase (Fermentas), 1 µl of forward and reverse primer at final concentration of 10 Pmol, and 5 ml of DNA sample. Positive control DNA from H37Rv and negative controls (distilled water known negative samples) were used for amplification. Amplification cycle used for PCR included one initial cycle of 95°C for 3 min then 35 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min for 72°C adding last one cycle of 72°C for 7 min.

Detection of amplified MTB DNA:

PCR products were electrophoresed on a 2 percent agarose gel in IX TBE buffer containing Ethedium bromide at lo mg/ml concentration; 3µl of 50bp ladder marker was also loaded. The samples were run at 120 V for 40 min. Samples showing the presence of 541bp band under ultraviolet transillumination were considered positive.

RESULTS

The ZN smear examination detected AFB in 25 samples with a sensitivity of 12.20 percent. For LJ medium culture, sensitivity was 27.81 percent by isolating *M. tuberculosis* bacilli in 57 samples. In comparison, PCR test showed a much higher sensitivity of 67.32 percent by showing positive result in 138 clinical samples (Table 1, 2 and 3).

All the 37 negative control samples of sputum showed a negative result in all the tests, thus giving 100 percent specificity for all the tests used. Among the 80 pulmonary samples (60 sputum and 20 BAL), 16 (10 sputum and 06 BAL) were positive for

AFB smear examination (20%), while 61 (45 sputum and 16 BAL) samples were positive for mycobacterium DNA by PCR assay (76.25%). PCR test identified mycobacterium DNA in all 16 smear positive

Table 1: *Comparison of smear examination with PCR.*

Sample name	Smear +ve no. (%)	PCR +ve no. (%)	Smear +ve PCR +ve no. (%)	Smear +ve PCR -ve no. (%)	Smear -ve PCR +ve no. (%)	Smear -ve PCR -ve no. (%)
Pulmonary samples						
Sputum (60)	10 (16.67)	45 (75)	10 (16.67)	--	35 (58.34)	15 (25
BAL (20)	06 (30)	16 (80)	06 (30)	--	10 (50)	04 (20)
Total (80)	16 (20)	61 (76.25)	16 (20)	--	45 (56.25	19 (23.75)
Extra-Pulmonary Samples						
Pleural Fluid (35)	03 (8.57)	27 (77.15)	03 (8.57)	--	26 (68.57)	8 (22.86)
CSF (30)	02 (6.68)	18 (60)	02 (6.68)	--	16 (53.34)	(12 (40)
PUS (25)	02 (8)	17 (68)	02 (8)	--	15 (60)	08 (32)
Urine (20)	01 (5)	08 (40)	01 (5)	--	07 (35)	12 (60)
Ascitic fluid (15)	01 (6.67)	07 (46.67)	01 (6.67)	--	06 (40)	08 (53.34)
Total (125)	09 (7.2)	77 (61.6)	09 (7.2)	--	68 (54.5)	48 (38.4)

Table 2: *Comparison of LJ medium culture with PCR.*

Sample name	LJ +ve no. (%)	PCR +ve no. (%)	LJ +ve PCR +ve no. (%)	LJ +ve PCR -ve no. (%)	LJ -ve PCR +ve no. (%)	LJ -ve PCR -ve no. (%)
Pulmonary samples						
Sputum (60)	23 (38.34)	45 (75)	23 (36.67)	01 (1.67)	22 (36.67)	15 (25
BAL (20)	08 (40)	16 (80)	08 (40)	--	08 (40)	04 (20)
Total (80)	31 (38.75)	61 (75)	30 (37.5)	01 (1.25)	30 (37.50)	19 (23.75)
Extra-Pulmonary Samples						
Pleural Fluid (35)	08 (22.86)	27 (77.15)	08 (22.86)	--	19 (54.29)	8 (22.86)
CSF (30)	04 (13.34)	18 (60)	04 (13.34)	--	14 (46.67)	(12 (40)
PUS (25)	10 (40)	17 (68)	10 (40)	--	07 (28)	08 (32)
Urine (20)	02 (10)	08 (40)	02 (10)	--	06 (30)	12 (60)
Ascitic fluid (15)	02 (13.34)	07 (46.67)	02 (13.34)	--	05 (33.34)	08 (53.34)
Total (125)	26 (20.8)	77 (61.6)	26 (20.8)	--	51 (40.8)	48 (38.4)

pulmonary samples. Among the 64 smear negative pulmonary samples, 45 were also positive by PCR test (56.25%), table 1.

In case of LJ medium culture method, out of 80 pulmonary samples 31 (23 sputum and 08 BAL) were positive (38.75%). PCR test was positive in 30 (96.78%) of these 31 LJ medium positive samples and found negative in one LJ medium positive sputum sample (3.23%). PCR test was also positive in 30 LJ medium culture negative samples (61.23%) out of 49 (Table 2). Comparing the results, PCR test was found to be much more sensitive than AFB smear examination and LJ medium culture.

Among the 125 extra-pulmonary samples only 09 (7.2%) were detected as AFB positive by smear examination. LJ medium culture showed positive result in 26 samples (20.80%). All extra-pulmonary positive samples by smear examination and LJ medium culture were also found positive by PCR.

Comparing statistically, PCR test was found to be more sensitive than the other two tests for the diagnosis of TB in extra-pulmonary samples. The mean detection time for *M. tuberculosis* was 24 days by LJ medium culture and less than 1 day by smear examination and PCR test. PCR sensitivity was high in pulmonary TB specimens as compared to extra-pulmonary, the same situation was also seen in ZN smear examination and LJ medium culture.

It was also interesting to see that the DNA for MTB was detected in 138 (67.32%) out of 205 specimens, obtained from the specific site of TB patients. On the other hand only in 3 (1.46%) serum samples of the same patients DNA for MTB was detected.

Table 3: *Comparison of PCR results done on blood samples and specific site samples of the same patient..*

Sample name	PCR +ve no. (%)	PCR +ve (On Blood Samples) No. (%)	SD	SEM	Comparison
Pulmonary Samples					
Sputum (60)	45 (75)	01 (1.67)	31.11	22.0	0.486
BAL (20)	16 (80)	--	11.31	8.00	0.50
Total (80)	61 (75)	01 (1.25)			0.50
Extra-Pulmonary Samples					
Pleural Fluid (35)	27 (77.15)	00	19.09	13.15	0.50
CSF (30)	18 (600	00	12.72	9.00	0.50
PUS (25)	17 (68)	02 (8)	12.02	8.50	0.50
Urine (20)	08 (40)	00	5.65	4.00	0.50
Ascitic fluid (15)	07 (46.67)	00	4.94	3.50	0.50
Total (125)	**77 (61.6)**	**02 (1.6)**			

DISCUSSION

The present study was designed to evaluate the utility of PCR technology in the diagnosis of tuberculosis. It was our main objective to assess which sample, blood or specific site sample of TB patient according to disease (Sputum, Fluids etc) was better in term of PCR test. Due to lack of knowledge it is a routine practice that for all kinds of tuberculosis (may it be pulmonary or extra-pulmonary) blood sample is sent for the diagnosis of TB by PCR. We also compared different conventional techniques (used for TB diagnosis) with PCR to assess the importance of this technology.

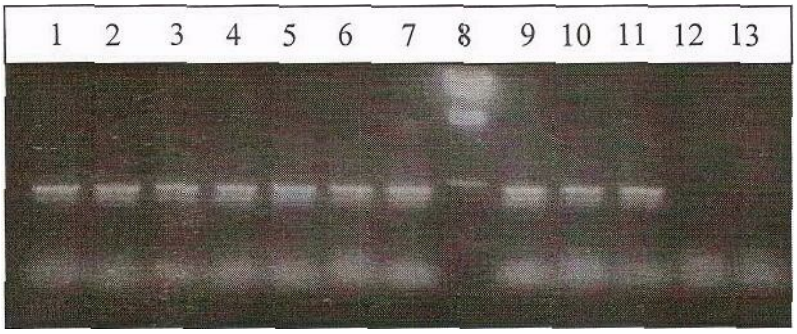

Fig. 1: *PCR amplification of 541-bp fragment from IS986 gene of Mycobacterium tuberculosis complex.*

Lane 1 to 7 and 9 to 10	Positive samples of *Mycobacterium tuberculosis*
Lane 13	Negative samples of *Mycobacterium tuberculosis*
Lane 8	Molecular Weight Marker (λ DNA III digested)
Lane 12	Negative control
Lane 11	Positive controls (541-bp)

According to our findings it is clear that specific site sampling from TB patients according to disease plays a vital role in the diagnosis of tuberculosis by PCR. This is because percentage of blood sample positive results by PCR was only 1.46, In contrast, in specific site samples of the same patients the percentage was 67.32. These results confirm that for accurate diagnosis of TB cases by PCR, specific site sampling is significant. Therefore the idea of TB diagnosis by PCR on blood samples should be discouraged. These findings also illustrate that the application of PCR to the diagnosis of tuberculosis has the potential to resolve one of the foremost challenges faced by a clinician and the diagnostic laboratories.[11-15]

The specificity, sensitivity (100% and 67.32%) and speed (one day result) of PCR test in diagnosis of *mycobacterium tuberculosis* shown in this study should encourage the use of this technique in routine diagnosis of TB. We compared the results of different tests in different clinical samples for the diagnosis of TB. PCR showed the highest sensitivity as compared to other tests as reported by earlier studies.[4] In a total of 180 AFB smear negative samples by ZN staining 113 were positive by PCR. No samples was seen PCR negative and smear positive, while all the PCR negative samples were also negative by smear examination. All these findings indicated that the PCR technique is much more sensi-

tive and specific as compared to AFB smear examination.[16-19]

Culture of mycobacteria is the cornerstone for TB diagnosis. In our findings in of the total of 148 culture negative samples 81 were positive by PCR. During this study one case was also seen smear and PCR positive and culture negative. PCR and ZN smear positive but culture negative result may be due to the presence of nonviable mycobacteria in the samples as of the some subjects were receiving anti-tuberculous treatment. Theraby suggesting that the DNA amplification method could detect even nonviable mycobacteria.[16,20]

We could not detect mycobacterium DNA in one LJ medium culture positive sputum sample, which could be due to the presence of PCR inhibiting substances in the sample or unequal distribution of AFB in these samples.[21] The patient had classical clinical signs of TB infection, thus this sample was regarded as confirmed PCR false negative.

The present study suggests that PCR could make a considerable impact on the diagnosis of TB, particularly extra-pulmonary tuberculosis which is often missed by conventional tests producing negative result or causes an unacceptable delay in diagnosis. This is especially true in tuberculous meningitis cases in which early diagnosis is essential for the outcome of the diseases.[22]

In **conclusion**, PCR has a potentially important role in strengthening the diagnosis of TB both pulmonary and extra-pulmonary. For this specific site sampling according to disease is very important. This study has also demonstrated that MTB PCR assay is rapid, sensitive and highly specific as compared to commonly used conventional techniques. In addition, rapidity of the test allows quick implementation of treatment regimen.

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