ORIGINAL ARTICLE

IMPORTANCE OF POLYMERASE CHAIN REACTION IN DIAGNOSIS OF PULMONARY AND EXTRA-PULMONARY TUBERCULOSIS

Sajjad Iqbal, Rashid Ahmed, Saleem-uz-Zaman Adhami, Asim Mumtaz

Department of Pathology, Shalimar Hospital, Shalimar Link Road Mughalpura, Lahore, Pakistan

Background: Pakistan ranks 8th on the list of 22 high-burden tuberculosis (TB) countries in the world according to the World Health Organization's (WHO) Global Tuberculosis Control 2009. Including other reasons the main cause is improper and late diagnosis of the disease. PCR may play an important role to control the disease with its rapid, sensitive and specific diagnosis. But in Pakistan due to lake of knowledge about this latest technique we are not using this technique appropriately. Clinicians still trust on conventional methods of TB diagnosis, which are time consuming or insensitive. The present study was arranged to highlight the importance of PCR in TB diagnosis in pulmonary and extra-pulmonary cases and its comparison with conventional methods. **Methods:** Samples obtained from 290 patients of suspected TB (pulmonary or extra-pulmonary) were subjected to ZN smear examination, LJ medium culture and PCR test by amplifying 541bp fragment of *Mycobacterium tuberculosis complex* genome. The present prospective study is performed at Shalamar Hospital Lahore from November 2008 to November 2010. **Results:** A distinctly difference was observed in the test results done by PCR and other conventional techniques in pulmonary or extra-pulmonary tuberculosis samples ($p<0.001$). The sensitivity of different tests was 68.62% for PCR, 26.90% for LJ medium culture, and 14.14% for ZN smear examination ($p<0.05$). However, there was no significant difference between different tests as for as specificity was concerned. PCR test sensitivity in pulmonary and extra-pulmonary clinical samples was 78.34 and 61.76% respectively, being significantly higher ($p<0.05$) when compared with sensitivity of other tests. The mean detection time for *M. tuberculosis* was 25 days by LJ medium culture and less than 1 day by smear examination and PCR test. **Conclusion:** 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.

Keywords: *Mycobacterium tuberculosis complex*, Polymerase chain reaction, LJ medium culture, ZN staining.

INTRODUCTION

Tuberculosis (TB) is causing significant mortality and morbidity in developing countries like Pakistan, where the estimated incidence of TB is 181 per 100,000.[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]

Most of the clinicians still based their diagnosis in TB cases on acid fast bacilli (AFB) ZN staining or LJ medium culturing. 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 105 acid-fast bacilli per ml of sputum).[3,4] On the other hand, 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 and even untreated. The main difficulty with extra-pulmonary specimens is that they yield very few bacilli and consequently are associated with low sensitivity of acid fast bacillus (AFB) smear and culture.[6]

The role of polymerase chain reaction (PCR) in *M. tuberculosis* identification has been established as a useful tool in pulmonary as well as extrapulmonary samples.[7,8] The introduction of nucleic acid-based direct amplification tests to target mycobacterial DNA or RNA directly from specimens, is a most exciting milestone in diagnostic mycobacteriology. Among nucleic acid-based techniques, available for the diagnosis of *M. tuberculosis*, PCR 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.[4,9]

Present study was arranged to see the efficacy of PCR for detection of *M. tuberculosis* in different pulmonary or extra-pulmonary clinical samples comparing the result with smear examination and conventional culturing using Lowenstein-Jensen (LJ) medium.

PATIENTS AND METHODS

A total of 290 samples obtained from patients with pulmonary or extra-pulmonary TB having strong clinical and radiological evidence, from December 2008 to December 2010 were included in this study. All the necessary clinical details were obtained from the patients in the format developed for this purpose. The clinical samples (n=290) included in the study were divided into two major groups, Pulmonary tuberculosis (n=120) and extra-pulmonary tuberculosis (n=170). In first group, 90 samples were sputum from suspected cases of pulmonary TB, and 30 broncho-alveolar lavage (BAL) from children with pulmonary TB. In extra-pulmonary TB, 48 pleural fluids, 41 CSF, 34 pus, 27 urine and 20 ascitic fluids were received.

The present study was conducted at Shalamar Hospital Laboratories, and TB Research Centre of Pakistan Medical Research Council (PMRC), Mayo Hospital, Lahore. The fresh specimens were collected in sterile container at the Shalamar Hospital Laboratory Lahore, either directly from the patients or from TB ward, OPD and other wards of Shalamar Hospital at the morning. All the samples were equally divided into two separate sterile containers. One of these samples was sent to PMRC for LJ medium culturing and other was processed in Shalamar Hospital laboratory for smear examination and PCR. Direct and concentrated smears were prepared from clinical samples after treating with NALC (N-acetyl-L-cysteine)-NaOH method.[10,11] Briefly, the NALC-NaOH methods involved the decontamination and digestion of the clinical samples with 2% NaOH (final concentration) in 0.5% NALC and concentrated by centrifugation at 3,000 G for 15 min. Supernatant was discarded and to sediment, 1–2 ml of sterile phosphate buffer of *p*H 6.8 (1–2 ml) was added and centrifuge for 15 minutes at 3,000 G. Deposit was used for smear examination and MTB DNA extraction. Slides for smear examination were stained by Ziehl-Neelsen method.[10]

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

The PCR was performed on extracted DNA samples using specific primers to amplify a 541bp sequence of MTB complex.[11] Briefly, a 25 ml reaction mixture was set up containing 10.7 ml of double distilled H_2O, 2.5 ml of 10× buffer, 1.5 ml of 25 mM $MgCl_2$, 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 ρmol, 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 ℃ for 3 min then 35 cycles of 1 min at 94 ℃, 1 min at 56 ℃, and 1 min for 72 ℃ adding last one cycle of 72 ℃ for 7 min.

The PCR products were electrophoresed on a 2% agarose gel in 1× TBE buffer containing Ethedium bromide at 10 mg/ml concentration; 3 µl 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

Out of 290 samples we were able to see AFB only in 41 on ZN smear examination with a sensitivity of 14.14%. On LJ medium culture *M. tuberculosis* bacilli growth was seen in 78 samples having 26.90% sensitivity. Identification percentage by PCR test was much higher with sensitivity of 65.86 % (Table-1 and 2). Except two samples all the culture isolates obtained were confirmed as *M. tuberculosis* by PCR while in smear examination all the positive cases were also positive by PCR. All the 50 negative control samples of sputum showed a negative result in all the tests, thus giving 100% specificity for all the tests used.

From 120 pulmonary samples (90 sputum and 30 BAL), 29 (20 sputum and 9 BAL) were positive on AFB smear examination (24.17%), while 94 (70 sputum and 24 BAL) samples were positive for mycobacterial DNA by PCR assay (78.34%). PCR test identified mycobacterial DNA in all 29 smear positive pulmonary samples while of the 91 smear negative pulmonary samples, 65 were also positive by PCR test (54.17%) (Table-1).

In case of LJ medium culture methodology, 42 pulmonary TB samples (31 sputum and 11 BAL) were positive on LJ medium (35%). PCR test was positive in 40 (95.23%) out of these 42 LJ medium positive samples and found negative in 2 LJ medium positive sputum sample (1.67%). PCR test was also positive in 52 samples out of 78 LJ medium culture negative samples (66.67%) (Table-2). On comparing the results, PCR test was found to be much more sensitive than AFB smear examination and LJ medium culture in pulmonary TB cases ($p<0.05$).

In 170 extra-pulmonary samples only 12 (7.06%) were detected as AFB positive by smear examination. LJ medium culture showed positive result in 36 samples (21.18%). All extra-pulmonary positive samples by smear examination and LJ medium culture were also found positive by PCR.

Table-1: Comparison of Smear examination with PCR

Sample	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 Tuberculosis Samples						
Sputum (90)	20 (22.22)	70 (77.78)	20 (22.22)	--	50 (55.56)	20 (22.22)
BAL (30)	9 (30)	24 (80)	9 (30)	--	15 (50)	6 (20)
Total (120)	29 (24.17)	94 (78.33)	29 (24.17)	--	65 (54.17)	26 (21.67)
Extra-Pulmonary Tuberculosis Samples						
Pleural Fluid (48)	4 (8.33)	38 (79.17)	4 (8.33)	--	34 (70.83)	10 (22.86)
CSF (41)	3 (7.72)	24 (58.54)	3 (7.317)	--	21 (51.22)	17 (41.46)
PUS (34)	3 (8.82)	23 (67.65)	3 (8.82)	--	20 (58.82)	11 (32.35)
Urine (27)	1 (3.70)	11 (40.74)	1 (3.70)	--	10 (37.04)	16 (59.26)
Ascitic fluid (20)	1 (5)	9 (45)	1 (5)	--	0 8(40)	11 (55)
Total (170)	12 (7.06)	105 (61.76)	12 (7.06)	--	93 (54.71)	65 (38.24)

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 Tuberculosis Samples						
Sputum (90)	31 (34.44)	70 (77.78)	29 (32.22)	2 (2.22)	39 (43.33)	20 (22.22)
BAL (30)	11 (36.67)	24 (80)	11 (36.67)	--	13 (43.33)	6 (20)
Total (120)	42 (35)	94 (78.33)	40 (33.33)	2 (1.67)	52 (43.33)	26 (21.67)
Extra-Pulmonary Tuberculosis Samples						
Pleural Fluid (48)	11 (22.92)	38 (79.17)	11 (22.92)	--	27 (56.25)	10 (20.83)
CSF (41)	7 (17.07)	24 (58.54)	7 (17.07)	--	17 (41.46)	17 (41.46)
PUS (34)	12 (35.29)	23 (67.65)	12 (35.29)	--	11 (32.35)	11 (32.35)
Urine (27)	3 (11.11)	11 (45)	3 (11.11)	--	8 (29.63)	16 (59.26)
Ascitic fluid (20)	3 (11.11)	9 (33.33)	3 (11.11)	--	6 (22.22)	11 (40.74)
Total (170)	36 (21.18)	105 (61.76)	36 (21.18)	--	69 (40.59)	65 (38.24)

DISCUSSION

It is now an established fact that conventional techniques have limitations in tuberculosis diagnosis. The TB diagnosis is tricky matter because of many factors like insufficient sample, inappropriate distribution of microorganisms during to the division of the sample for different diagnostic tests like, histopathology, microbiology and PCR. Presence of inhibitors can also affect the PCR test performance. The poor results quality by conventional microbiological techniques in TB specimens have stimulated the use of PCR tests in TB identification. Taking all above in view, the present study was conducted to prospectively evaluate the role of PCR technology in the diagnosis of tuberculosis. For this we also compared different conventional techniques (used for TB diagnosis) with PCR to assess the significance, specificity and sensitivity of this technology.

Our findings endorse the use of PCR test in routine TB diagnosis due to its specificity, sensitivity (100%, 65.86%) and speed (one day result). As mentioned in earlier studies the sensitivity of PCR test was also high in our study as compare to other techniques used by us in TB diagnoses.[12] Out of 249 AFB smear negative samples by ZN staining 158 were positive by PCR. No sample was seen PCR negative and smear positive, on the other hand all the PCR negative samples were also negative by smear examination. All these findings are indicating that the PCR technique is much more sensitive and specific as compare to AFB smear examination.[13–16]

Many clinicians still believe culturing of *Mycobacterium tuberculosis* as a gold standard in TB diagnosis. In our findings out of 150 culture negative samples 80 were positive by PCR. During this study one case was also seen smear and PCR positive but culture negative; this 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-tubercular treatment.[13,17]

We could not detect Mycobacterial DNA in 2 LJ medium culture positive sputum samples, which could be due to the presence of PCR inhibiting substances in the sample or unequal distribution of AFB in these samples. Same problem the presence of any inhibitory substance in clinical specimens was also mentioned in previous studies, which were based on amplification tests. So, this negative DNA result in our study may be due to the presence of any inhibitor.[15,18–22]

When we compare statistically, PCR test was found to be more sensitive than the other two tests for diagnosis of TB in extra-pulmonary clinical samples ($P<0.05$) as reported previously.[23] 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 but PCR test was found more sensitive as compare to smear examination. PCR sensitivity was high

in specimens of pulmonary TB as compare to extra-pulmonary TB cases, the same situation was also seen in ZN smear examination and LJ medium culture in which culturing was more sensitive ($p<0.05$). These statistics reveal that PCR technique due to its rapidity and high sensitivity can solve the issue of delay diagnosis in TB cases as highlighted in previous studies.[24]

CONCLUSION

PCR has a potentially important role in rapid diagnosis of TB both in pulmonary and extra-pulmonary and is more sensitive and highly specific compare to commonly used conventional techniques. In addition, rapidity of the test allows quick implementation of treatment regimen. So, PCR may be helpful in reduction of TB cases and an immediate and better control.

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Address for Correspondence:

Sajjad Iqbal, Specialist Molecular Biologist, Pathology Department, Shalamar Hospital, Shalimar Link Road, Mughalpura, Lahore. **Cell:** +92-333-6446006, **Tel:** +92-42-111-205-205 (Ext: 384,330)