

PCR as a Faster Mode of Supportive Tool in Laboratory Diagnosis of *Mycobacterium tuberculosis*

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Conventional methods such as microscopy and culture lack the sensitivity and are time consuming. PCR provides good rate of positive results and better turnaround time than culture (days versus weeks) and smear examination. The present study was to show the sensitivity and time taken for PCR versus positive culture in the diagnosis of tuberculosis. Molecular diagnosis of tuberculosis by PCR has a great potential to improve the clinicians ability to diagnose tuberculosis, so that early treatment to patients can prevent further spread. This study has 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.

Key words: *Mycobacterium tuberculosis*, PCR, Nested PCR.

It is estimated that 2 billion people, that is 1/3 of the people in the world are infected with *Mycobacterium tuberculosis*. Each year nearly 9 million people in the world become sick with *Mycobacterium tuberculosis* disease and almost 1.5 million deaths are attributed to *Mycobacterium tuberculosis*. 95% of deaths due to *Mycobacterium tuberculosis* occur in developing world. The number of people who died from MTB was 1.4 million in 2010 including 350,000 people having HIV. Tuberculosis continues to be a major health problem in both developed and developing countries. Person who is co-infected with *Mycobacterium tuberculosis*- and human immunodeficiency virus has risk of developing tuberculosis 100 times higher than the healthy individuals¹. Tuberculosis primarily affects the

lungs, kidneys being the second target organ². Polymerase chain reaction is an important tool for rapid and accurate diagnosis of tuberculous meningitis, that too in CSF smear negative for AFB, which is quite common in the majority of tuberculous meningitis⁵.

Conventional methods such as microscopy and culture lack the sensitivity and are time consuming. PCR provides good rates of positive results and better turnaround time than culture (days versus weeks) and smear examination⁸. Even with concentrated samples, the sensitivity of microscopy is not appreciable requiring 10,000 acid-fast bacilli per ml of sputum, cultures are quite slow requiring 3-8 weeks for completion¹⁰. The main difficulty with extrapulmonary specimens is presence of very few bacilli resulting in low sensitivity for acid fast bacilli (AFB) smear and culture.

Among the EPTBs, the diagnostic criterion for GUTB is the isolation of MTB from urine, which is not easy to achieve, as the discharge of organisms into the urine is sporadic and, more importantly, involves few organism. The

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conventional method for diagnosing TB using clinical samples by the acid-fast bacilli (AFB) smear has low sensitivity and specificity and culture for MTB is time consuming. Due to the difficulties associated with diagnosing GUTB, there has been considerable interest in applying PCR methods for the detection of these diseases. The presence of an extremely small number of bacteria can thus be detected within 24 to 48 h (2). In nested PCR, two consecutive reactions, has been used; the second reaction amplifies a DNA sequence within the first amplification product. This approach provides a higher sensitivity and specificity when compared⁷.

MATERIALS AND METHODS

Clinical samples received from various diagnostic labs from patients of suspected TB (pulmonary or extra-pulmonary) put up for culture using Bact Alert 3D. ZN staining was carried out for smears. Nested PCR was carried out for 100 samples targeting MPB64 gene, which included samples of all nature, both positive and negative controls were included to assess the sensitivity of PCR.

Sample type

Pulmonary

Sputum, BAL, Pleural fluid, *Extrapulmonary*: Whole blood, Ascitic fluid, CSF, Pus, Peritoneal fluid, Urine and Endometrium.

Decontamination and liquefaction

Sample types are Sputum, Broncho alveolar lavage and Pus. Equal volume of N-acetyl-L-Cysteine (5 mg/ml) solution to liquefy and sodium hydroxide for decontamination, the samples are mixed in a screw cap bottle and incubated at room temperature for 10 minutes with intermittent mixing. The mixture is centrifuged at 8000 rpm for 15 min, the supernatant is discarded and the pellet is re-suspended in 20-30ml of 20 mM Tris buffer and centrifuged at 800 rpm for 10 min.

Microscopy

ZN staining.

Culture

Samples were inoculated into commercial liquid culture bottles from Biomeriux and incubated in BactAlert 3D. Positive growth is indicated by the alarm set by the instrument. Culture bottles were removed and smears made for microscopy and they were subcultured on to L J media, the

culture bottles were incubated up to 8 weeks to after which if there is no growth final report was released. The number samples obtained and processed are shown in table no1 and their results are pictorially represented on Fig. 2.

DNA extraction

20µl of proteinase K is added to 250µl of the processed material, vortexed, and incubated at 65°C for 30 min. The sample was centrifuged at 10000 rpm for 10 min, 200µl of the supernatant was collected in a new tube and 200µl of lysis buffer II containing the internal control DNA is added and vortexed for 15s, and incubated at 70°C for 10 min and 200µl of 96-100% distilled ethanol is added and vortexed. The spin column is kept in 2 ml collection tube and the sample ethanol mixture was added to the column without wetting the rim, centrifuged at 8,000 rpm for 3 minutes. The flow through and the collection tubes are discarded. The spin column is kept in a new 2 ml collection tube and 500µl of wash buffer I is added and centrifuged at 8,000 rpm for 3 minutes. The contents of the 2ml collection tube is discarded and 500µl of wash buffer II is added to the spin column and centrifuged at 14,000 rpm. The flow through is discarded and the empty column is centrifuged at 14000 rpm for 2 minutes for complete removal of the wash buffer. The spin column is placed in a new labeled 1.5 ml tube and 100µl of pre-warmed (50°C) Elution buffer is added and incubated at RT for 5 minutes and centrifuged at 10000 rpm for 1-2 minutes and then the DNA is eluted. Known positive and negative controls are included in every run as QC for samples and the internal controls along with the samples as QC for PCR.

Table 1. Number of positive results obtained in clinical samples by different methods

Sample type	Total no	ZN	Culture	PCR
Pulmonary				
Sputum	70	20	31	31
BAL	4	1	nil	Nil
Extrapulmonary				
Pleural fluid	3	nil	3	3
CSF	3	nil	2	2
Pus	3	nil	nil	
Urine	10	2	2	2
Endometrial biopsy	2	nil	1	Nil
Whole Blood	4	Not done	1	1

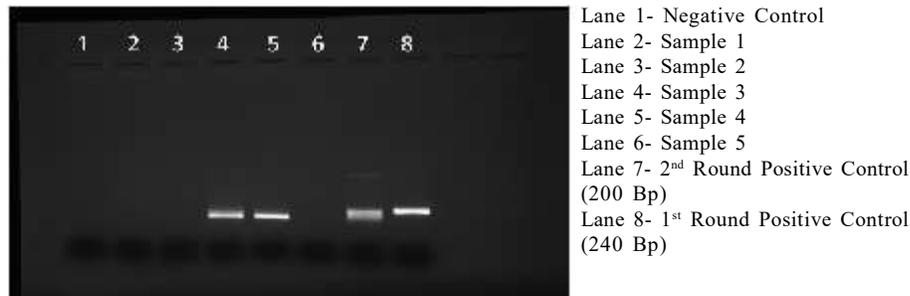


Fig. 1. 2% Agarose gel showing amplified products of *Mycobacterium tuberculosis*

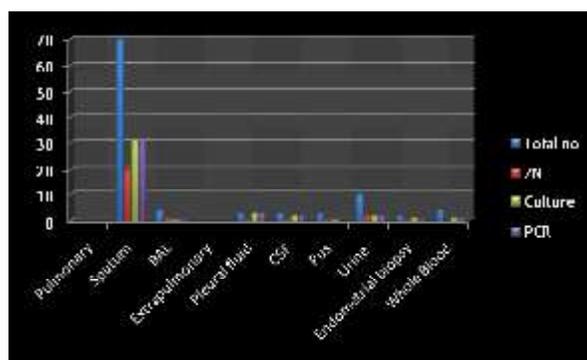


Fig. 2. Number of positive results obtained in clinical samples by different methods

Amplification

Nested PCR was carried out in which after the first round of PCR, primers internal to the first round of PCR were used. 1st round: Thermal cycler was programmed to 22°C for 10 minutes and 94°C for 5 minutes for one cycle, 94°C for 30 sec, 68°C for 1 minute and 72°C for 1 minute for 20 cycles and 72°C for 7 minutes. 2nd round (nested pcr): The tubes of the first round PCR are taken out from the thermal cycler and 15µl of nested pcr master mix is added. The thermal cycler was programmed to 94°C for 5 minutes for one cycle, 94°C for 30 sec, 68°C for 30 secs, and 72°C for 30 secs for 30 cycles and 72°C for 7 minutes.

The Nested primers for MPB64 gene, MPB64-1-5' TCC GCT GCCAGT CGT CTT CC 3' and MPB64-2- 5' GTC CTC GCGAGT CTAGGCCA3' for the first round and MPB64-3- 5' ATT GTG CAA GGT GAACTGAG 3' and MPB64-4- 5' AGC ATC GAG TCG ATC GCG GA 3' for the second round generating 240bp and 200 bp products respectively. The amplified PCR products (10 ul) were run electrophoretically in ethidium bromide stained 2% Agarose gel . The gel is visualized in gel

documentation system (Vilberlourmat , France). Detection of 240bp in the sample shows positive *Mycobacterium tuberculosis* as shown in figure no 1. A negative control should run for each time to check the contamination. A band in negative control shows the contamination , the test to repeated by taking necessary steps to avoid the contamination.

All the culture positive samples turned out positive by PCR also except one endometrial biopsy which was negative by PCR but positive by culture, since all the positive culture showed positive results by PCR, PCR should be considered for faster diagnosis of tuberculosis.

DISCUSSION

The present study was to evaluate the sensitivity of PCR verses positive culture in the diagnosis of tuberculosis. PCR is more sensitive, better turnaround time with respect to result reporting. Confirmation of urinary tuberculosis can be done within 24-48 h, where as automated urine culture takes weeks. 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. Conventional methods such as microscopy and culture lack the sensitivity and are time consuming. Even with concentrated samples, the sensitivity of microscopy is not appreciable requiring 10,000 acid-fast bacilli per ml of sputum. Hain life sciences has come up with speciation and drug sensitivity testing for 1st as well as 2nd line of drugs by DNA PCR in single test which helps in starting treatment early. PCR turnaround time is just 4 – 6 hrs and sensitive than ZN smear examination, the mean detection time for M. tuberculosis was 24 days by LJ medium culture and a minimum of one week for liquid culture for the diagnosis of TB in pulmonary and extra-pulmonary clinical samples. Risk of occupational hazard to the tech person once the DNA is extracted is nil, risk of handling live bacilli is not at all there unlike in conventional method where subculture for identification and sensitivity testing have to be done. Culture bottles are expensive and availability. Commercial culture bottles used for automated culture are expensive and difficulty in procuring at the time of requirement

Molecular diagnosis of tuberculosis by PCR has a great potential to improve the clinicians ability to diagnose tuberculosis, so that early treatment to patients can prevent further spread⁹.

CONCLUSION

PCR plays potentially an important role in strengthening the diagnosis of tuberculosis both pulmonary and extra-pulmonary at the same cutting short the time required for conventional methods of diagnosis. 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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