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Original Research Article

Evaluation of Nested Polymerase Chain Reaction for the Diagnosis of Pulmonary and Extrapulmonary Tuberculosis

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ABSTRACT

Utility of Polymerase Chain Reaction (PCR) test for diagnosis of pulmonary and extrapulmonary specimens was evaluated in 91 clinically diagnosed cases of tuberculosis (50 pulmonary & 41 extrapulmonary). All samples were also processed for microscopy by Ziehl-Neelsen (ZN) staining and culture on Lowenstein-Jensen medium (LJ). PCR was positive in all microscopy and culture positive specimens. There were 11 samples positive by PCR but failed to grow in culture both in the pulmonary and extrapulmonary specimens. With respect to culture the sensitivity (SN), specificity (SP), Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of PCR for the pulmonary specimens was 100%, 57%, 68% and 100% respectively. Whereas the values for EPS were SN- 100%, SP- 65%, PPV- 45% and NPV- 100%. The values for PCR on final evaluation taking into consideration clinical, radiological, microbiological evidence and response to antitubercular treatment were SN-100%, SP-88%, PPV-94% & NPV-100% for the PS and SN- 95%, SP- 100%, PPV- 100 & NPV- 95% for the EPS. PCR detects *M. tuberculosis* complex with greater sensitivity and should be useful for rapid diagnosis of tuberculosis.

Key words: *Mycobacterium tuberculosis*, Polymerase Chain Reaction, IS 6110, Sensitivity, Specificity.

INTRODUCTION

Tuberculosis (TB) is an increasing public health problem in developing countries. India is the highest TB burden country accounting for fifth of global incidence. Reported global annual incidence of TB case estimate is 9.4 million cases, out of which it is estimated that 1.8 million cases are from India. [1] Studies involving immunocompetent adults have revealed that Extrapulmonary Tuberculosis (EPTB) constitutes about 15 to 20 percent of all cases of tuberculosis. With global rise of Human Immunodeficiency Virus infection EPTB accounts for more than 50 percent of all cases of TB among HIV positive patients. [2] Conventional methods available for diagnosis namely tuberculin test, radiological examination, smear microscopy and culture have their own limitations. Sputum smear microscopy requires more than 10,000 organisms per ml be present in the sample and has low sensitivity. [3-6] In case of EPTB microscopy is still less sensitive. Culture is more sensitive than microscopy and is still considered the "gold

standard" being 100% specific. [3-5] But culture is time consuming requiring 3 to 8 weeks for growth to appear. [3,6,7] Great progress have been made in reducing the time required for detecting the growth of mycobacteria using various culture systems like BACTEC, MGIT, MB/BACT etc, still however, on an average 2 to 3 weeks are needed to detect the growth. [3,6,7] Another recent approach to culture of *M. tuberculosis* is TK medium (Salubris, Inc.MA USA) with average time to detection of two weeks as compared to four weeks on LJ medium. It promises to be a practical, low cost, simple test. [8] A new test being introduced by WHO in national tuberculosis control programs is Xpert MTB / RIF which provides sensitive detection of *M. tuberculosis* and rifampicin resistance detection in less than two hours. This is being implicated in a phased manner. [9]

Recent advances in DNA amplification using Polymerase Chain Reaction has allowed great progress to be made in the rapid and accurate diagnosis of infections due to organisms that are not cultivable by in vitro means, that require complex media or cell cultures and prolonged incubation times, or for which culture is too insensitive. [10] Nested PCR (nPCR) for diagnosis of tuberculosis has been found to be useful in a number of studies. [6,10-12] This study reports the application of nPCR for the diagnosis of tuberculosis by amplification of IS6110, an IS-like element of *M. tuberculosis* complex. As the IS6110 sequence has been shown to be repetitively present in *M. tuberculosis* genome, it helps to increase the sensitivity of the test. [6, 11,13-15] This study evaluates nPCR for diagnosis of pulmonary and EPTB along with culture and microscopy. This is the first comprehensive study comparing conventional methods with nPCR for the diagnosis of tuberculosis from region of Marathwada of Maharashtra State, India.

MATERIALS AND METHODS

Clinical specimens and conventional methods:

A total of 50 pulmonary specimens (sputum 32, pleural fluid 14, bronchoalveolar lavage (BAL) 2, intercostal drain (ICD) 2) and 41 extrapulmonary specimens (ascetic fluid11, pus 12, blood 5, tissue biopsy 4, aspirates 3, CSF 3) were obtained from patients clinically suspected to have tuberculosis. Microscopic examination of the specimens was done by Ziehl-Nelsen staining and if negative staining was repeated on the specimens after concentration and decontamination using 4% sodium hydroxide (Except: CSF, blood and tissue). The decontaminated material was also used for culture on Lowenstein Jensen's medium in duplicate. The cultures were incubated at 37^0C for 8 weeks. All isolates of acid fast bacilli on LJ medium were further identified to species level by colony characteristics including the speed of growth and pigmentation and a battery of biochemical tests (niacin, nitrate reduction, catalase and growth on medium containing p-nitrobenzoic acid).

nPCR Assay:

nPCR assay was carried out using Genei ™ Amplification Reagent Set MTB-25 for *M. tuberculosis*, manufactured by Bangalore Genei Bangalore, India. This test is based on the principle of single tube nested PCR method. The assay is a two-step sequential assay. In the first step in the IS region of *M. tuberculosis* complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers are added to further amplify a 123 bp amplification product. A band of 123 bp was indicative of infection with *M. tuberculosis* complex. In this test false positive reactions that may be caused by previous amplicon contamination are prevented by the use of uracil DNA glycolase (UDG) and dUTP instead of

dTTP. DNA extraction was carried using Proteinase K according to the manufacturer's instructions. DNA extraction was done in a separate room. DNA amplification was carried out on the same day of receiving the sample. PCR inhibition was identified using an amplification internal control. Amplification product of 340 bp was indicative of successful amplification and DNA extraction. Its absence indicated inhibition of amplification or DNA amplification failed. (Figure-1)

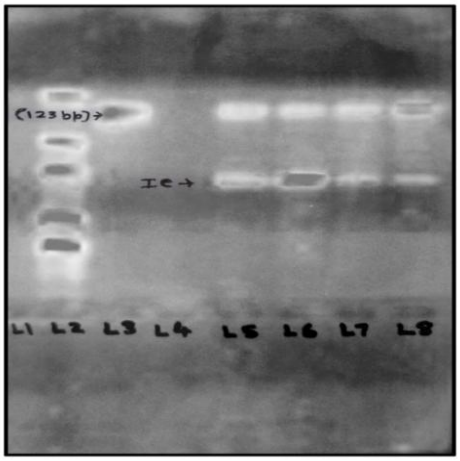

Figure - 1: Agarose gel electrophoresis analysis for DNA amplification products.
Lanes : L1 empty, L2 Molecular weight marker, L3 positive control (123 bp),
L4 negative control.
L5, L6, L7, L8 specimen positive for *M.tuberculosis* complex (123 bp).
IC: Internal control band (340 bp).

DNA Amplification:

A) First amplification:

Master Mix I was prepared, so that for each specimen the master mix I contained Amplification Premix I8.2 µl, Gene Hotstart Taq DNA Polymerase 0.33 µl and uracil DNA glycolase (UDG) 0.5 µl. To each 0.2ml PCR tube 9 µl of Master Mix-I was taken and 3 µl of extracted DNA was added. Positive and Negative controls were also included. DNA amplification was carried using the thermal cycler MJ Research using the calculated mode as suggested by the manufacturer. The first amplification profile was as follows: Step-1 was 22^0C for 10 minutes and initial denaturation at 94^0C for 5 minutes (No. of cycles -1). Step-2 was denaturation at 94^0C for 30 second, annealing at 68^0C for 1 minute and extension at 72^0C for 1 minute (No. of cycles - 20). Step-3 was final extension at 72^0C for 1 minute and storage at 4^0C (No. of cycles – 1).

B) Nested or Second Amplification:

For each specimen Master Mix-II contained Amplification premix-II 14.7 µl and Genei Hot Start Taq DNA polymerase 0.33 µl. Fifteen µl of the Master Mix-II thus prepared was added to the same PCR tubes used in the first amplification. Nested Amplification was performed using the following profile: Step-1 was initial denaturation at 94^0C for 5 minutes (No. of cycles-1). Step-2 was denaturation at 94^0C for 30 seconds, annealing at 68^0C for 30 seconds and extension at 72^0C for 30 seconds (No. of cycles 30). Step-3 was final extension at 72^0C for 7 minutes and storage at 4^0C (No. of cycles 1).

Analysis of amplified product:

Analysis of the amplified product was done using submarine electrophoresis using 2.5% agarose gel containing 10 µl of 10 mg per ml ethidium bromide dye solution for 100 ml gel. The gel was visualized under UV Transilluminator.

Nested PCR was also carried out on MOTT (Mycobacteria Other Than Tubercle) strains namely *M.chelonei, M.szulgai, M.phlei, M.avium and M.intracellulare* and also *M. tuberculosis* H37Rv obtained from Department of Microbiology, MGIM's Sewagram, district Wardha Maharashtra, India, to check the specificity of the primers.

Sensitivity, Specificity, Positive predictive value and Negative Predictive value were calculated as per Parks text book of Preventive and social medicine. [16]

RESULTS

We have analyzed 50 pulmonary and 41 extrapulmonary specimens obtained from patients with suspected tuberculosis. Ziehl-Neelsen staining for AFB was positive in 19 (38%) pulmonary and 8(19.51%) extrapulmonary specimens. Culture was positive in 24 (48%) pulmonary and 9 (22%) extrapulmonary specimens. This leaves 26 specimens negative on culture in pulmonary group and 32 in extrapulmonary group. All culture isolates were identified as *M. tuberculosis*. nPCR gave a positive amplification result in 35 (70%) of pulmonary and 20 (48.78%) extrapulmonary specimens. nPCR was positive in all smear and culture positive specimens. There were 16 pulmonary and 12 extrapulmonary specimens which were nPCR positive but smear negative. nPCR gave a positive amplification in 11 out of 26 culture negative pulmonary specimens and 11 out of 32 extrapulmonary specimens (Table-1). As culture is the gold standard, with respect to culture these specimens would be false positive. Therefore with respect to culture for nPCR in pulmonary specimens the sensitivity (SN), specificity (SP), positive predictive value (PPV) and negative predictive values (NPV) are 100%, 57%, 68% and 100% respectively. Similarly for extrapulmonary specimens the values are SN- 100%, SP - 65%, PPV - 45%, and NPV -100%.

Table 1: Correlation between smear, culture and nPCR

nPCR	Smear +ve (n=19)	Smear -ve (n=31)	Culture +ve (n = 24)	Culture -ve (n = 26)
Pulmonary [N = 50]				
nPCR +ve (n=35)	19	16	24	11
nPCR -ve (n=15)	00	15	00	15
Extrapulmonary [N = 41]				
nPCR +ve (n=20)	8	12	9	11
nPCR –ve (n=21)	00	21	00	21

n = Number of specimens

As the culture results in the present study were low we did a final evaluation of results by studying each case on the parameters of clinical, microbiological, radiological evidence and response to anti-tubercular treatment to prove the presence of disease. We thought this to be a better way of evaluating the results. On this basis we found of the total 50 pulmonary cases, 33 to be diseased and 17 not diseased (Table-2). All 33 diseased were positive by nPCR (True positive). Of the 17 non diseased patients 15 were negative for amplification (True negative) and 2 were amplification positive (False positive). Thus the final values for nPCR were SN-100%, SP-88%, PPV-94% and NPV-100%. For microscopy and culture there were no false positives, but there were 14 and 9 false negatives respectively. Accordingly for microscopy and culture the final SN, SP, PPV & NPV were 57%, 100%, 100%, 54% and 72%, 100%, 100% and 65% respectively.

For the extrapulmonary specimens, of the total 41 specimens 21 proved to have disease. Out of these nPCR was positive in 20 specimens (True positive) and negative in one specimen (False negative). For all the 20 not diseased nPCR gave a negative amplification so there were no false positives (Table-2). For microscopy and culture there were no false positives. The final results for the three tests were, for microscopy SN, SP, PPV, & NPV were 40%, 100%, 100%, and 63% respectively. For culture the values were 45%, 100%, !00% & 65% respectively. For nPCR they were 95%, 100%, 100% & 95%

respectively. nPCR gave a negative amplification result for all the MOTT strains tested and a positive result for *M. tuberculosis* H37Rv.

Table 2: Final results for various tests after correlation with evidence of disease and response to treatment

	Pulmonary [N=50]		Extrapulmonary [N=41]	
	Disease present	Disease absent	Disease present	Disease absent
Smear +ve Smear –ve	19 (TP) 14(FN)	0 (FP) 17 (TN)	8 (TP) 12 (FN)	0 (FP) 21(TN)
Culture+ve Culture-ve	24 (TP) 09(FN)	0 (FP) 17 (TN)	9 (TP) 11 (FN)	0 (FP) 21(TN)
nPCR+ve nPCR -ve	33 (TP) 0 (FN)	02 (FP) 15 (TN)	20 (TP) 1 (FN)	0 (FP) 20(TN)

n = Number of specimens, TP = True Positive, FN= False Negative, FP = False Positive, TN= True Negative

DISCUSSION

In this study we explored the usefulness of nPCR for diagnosis of Tuberculosis. Smear microscopy for acid fast bacilli is an important test for diagnosis of tuberculosis. In this study smear positivity was 38% for pulmonary tuberculosis and 19.51% for extrapulmonary pulmonary tuberculosis. For pulmonary and extrapulmonary tuberculosis positivity of culture was 48% and 22% respectively. One reason for culture results to be low was slightly higher contamination rate of 8% and these specimens were considered as culture negative. It has been suggested that digestion and decontamination procedures should be as gentle as possible, with no more than an overall contamination rate of 5%. [17] nPCR was positive in all smear and culture positive specimens. In both pulmonary and extrapulmonary there were 11 specimens which were culture negative but nPCR positive. After the final evaluation of the 11 culture negative but nPCR positive pulmonary specimens, 9 proved to have disease as they responded to anti-tubercular treatment and 2 specimens were false positive as these patients responded to routine antibiotic treatment (Table-3). These were also negative in microscopy. The 2 false positive nPCR may be because of contamination somewhere during sample processing. This must not be because of previous amplicon transfer as we had used uracil DNA glycolase (UDG) and dUTP instead of dTTP, which eliminates previous amplicon transfer. [18,19] Of the nine specimens from patients proved to have disease, microscopy was positive in 3 specimens. Out of which 2 had culture contamination (thus considered culture negative) and one was negative in culture. This culture negative specimen was from a patient who was already on anti-TB drugs. This is consistent with the fact that patients can still harbor mycobacteria long after culture for mycobacteria have become negative. This may suggest that DNA amplification method could detect mycobacteria that are unable to grow in vitro. [20]

In the extrapulmonary specimens after the final evaluation of the 11 nPCR positive but culture negative specimens all proved to have disease. Of these 2 were positive in smear. Of these 2 one was culture contaminated (thus considered culture negative) and other was negative in culture and was from a patient on anti-TB therapy, thus harboring dead bacilli picked up by microscopy and nPCR. There was 1 false negative result for nPCR (tissue specimen) also negative in smear and culture. This patient responded to anti-TB treatment. This may be because of very low no of bacteria in the specimen. This is not because of inhibitors of nPCR as we had used amplification controls (IC) which can detect

inhibition and the DNA extraction did not fail. Obtaining a positive signal from the second target (IC) demonstrates successful amplification, there by validating the result for the primary target. Increased sensitivity is achieved because false negative result is avoided and because additional positive results are detected by retesting inhibitory specimens. When introduced into the unprocessed specimen, the IC can also monitor nucleic acid recovery during specimen preparation. [21]

Table 3: Comparison of culture and nPCR with TB

Pulmonary N=[50]	Culture +ve (n=24)		Culture –ve (n=26)	
	nPCR		nPCR	
	+ve	-ve	+ve	-ve
Disease present (n=33)	24	0	9	0
Disease absent (n=17)	0	0	2[a]	15
Extrapulmonary [N=41]	Culture +ve (n=9)		Culture –ve (n=32)	
	nPCR		nPCR	
	+ve	-ve	+ve	-ve
Disease present (n=21)	9	0	11	1[b]
Disease absent (n=20)	0	0	0	20

n = Number of specimens, a = False positive specimens,
b = False negative specimen

The comparison of nPCR and disease with specimens was interesting (Table-4). Of the 32 sputum specimens nPCR was positive in 24. Twenty four of these proved to have disease resulting in a SN & SP of 100% & 80% respectively. Pleural fluid, BAL & ICD also were good specimens for amplification with 100% SN & 100% SP for nPCR. In extrapulmonary specimens the best results were for pus, 11 out of 12 being positive for nPCR. All these were diseased resulting in SN & SP of 100%. For tissue there was one false negative resulting in a SN of 0% and SP of 100%.

Table 4: Specimen wise results for the three tests.

Pulmonary					
Specimen	No	Smear +ve	Culture +ve	nPCR +ve	Disease present
Sputum	32	15	17	24	22[a]
Pleural fluid	14	2	5	8	8
BAL	2	1	1	1	1
ICD	2	1	1	2	2
Extrapulmonary					
Ascitic fluid	11	2	3	5	5
Pus	12	5	5	11	11
Aspirates	3	1	1	1	1
Urine	3	0	0	1	1
CSF	3	0	0	0	0
Tissue	4	0	0	0	1[b]
Blood	5	0	0	2	2

n = Number of specimen, a = Two false positive
b = One false negative

nPCR in the present study with respect to culture showed a 100% SN and NPV confirming the usefulness of the technique. Specificity and PPV was less because a large number of specimens were culture negative but nPCR positive. After the final evaluation microscopy and culture had no false positive and showed 100% specificity. nPCR had two false positives because of contamination this pointing towards the importance of good laboratory practices. nPCR gave a sensitivity of 100% for

pulmonary and 95% for extrapulmonary specimens which was much higher that the values for microscopy and culture, ascertaining the usefulness of nPCR in the diagnosis of tuberculosis.

In this study nPCR gave a negative amplification result with the MOTT bacteria and a positive amplification with *M. tuberculosis* H37Rv indicating the specificity of the IS6110 target for *M.tuberculosis* complex as was found by other workers. [4,18,22] We found no difficulty in detecting *M.tuberculosis* using this target, and the nested format suggesting it to be suitable for the diagnosis of tuberculosis confirming the findings of other workers. [23-25]

CONCLUSION

In conclusion, nPCR was found to be efficient in the diagnosis of tuberculosis. It correlates well with conventional techniques and is useful in the diagnosis of tuberculosis in specimens, where conventional techniques fail. The method is fast with results available in 10-12 hours. At the same time the importance of microscopy and culture can not be overlooked from the findings of the present study. Disadvantage of nPCR is that if MOTT is present in the specimen they will be missed by nPCR using primers targeting the *M.tuberculosis* complex. To conclude we find nested nPCR targeting IS6110 to be useful for the rapid diagnosis of tuberculosis.

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