Polymerase chain reaction versus culture in the diagnosis of extrapulmonary tuberculosis-A comparative study

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Abstract

Introduction: Extrapulmonary tuberculosis (EPTB) accounts for approximately 40% of tuberculosis cases and is a significant health problem in both developing and developed countries. Extrapulmonary TB represents a greater diagnostic problem than pulmonary TB because it presents with less frequency and occurs with little liberation of bacilli, as well as the fact that it is localized in sites that are difficult to access.

Materials & Methods: Samples from 60 clinically diagnosed cases of extrapulmonary TB were included in the study. All tissue samples were homogenized in a homogenizer. Thick viscous samples were subjected to NALC-NaOH method and all fluid samples were centrifuged. For DNA extraction, 200µl of the homogenized tissue or 200µl of centrifuged deposit from fluid samples were taken. PCR testing was done using IVD approved commercial kits. Deposit was also inoculated on LJ medium and Middlebrook-7H9 medium (MB7H9) aseptically.

Results: A total of 60 samples from clinically diagnosed patients of extrapulmonary tuberculosis (EPTB) were studied. Maximum EPTB positivity was seen among pleural fluid samples followed by pus, CSF, tissue samples, ascitic fluid, synovial tissue, synovial fluid and pericardial fluid. The comparison of PCR versus culture was done and the sensitivity, specificity, PPV and NPV of PCR were 68%, 62.9%, 56.66% and 73.33% respectively considering culture as the gold standard. The comparison of duration taken for positivity by LJ and MB7H9 media showed that the mean duration of isolation on LJ and MB7H9 was 4.8 and 3.5 weeks respectively. Liquid culture showed growth earlier than the growth on solid medium. The period of maximum isolation on both media was 3rd week followed by 4th week.

Conclusion: As PCR is a rapid method, and when used along with the conventional diagnostic methods increases the overall isolation rate of M. tuberculosis in the diagnosis of extrapulmonary tuberculosis.

Keyword: Extrapulmonary tuberculosis; EPTB; PCR; LJ medium; MB7H9 medium.

Introduction

Tuberculosis is a disease of great antiquity and has almost certainly caused more suffering and death than any other infection.(1) According to WHO; more than one-third of the world's human population has been infected by tubercle bacillus. It causes ill health among millions each year and ranks as the second leading cause of death from an infectious disease worldwide after the human immunodeficiency virus which caused an estimated 1.8 million deaths in 2008.There are almost 9 million new cases in 2011 and 1.4 million TB deaths; 9, 90, 000 among HIV negative people and 44, 30, 000 HIV associated TB deaths.(2)

Extrapulmonary tuberculosis (EPTB) accounts for approximately 40% of tuberculosis cases and is a significant health problem in both developing and developed countries. Extrapulmonary TB constitutes about 15-20% of all cases of tuberculosis in immuno-competant patients and accounts for more than 50% cases in HIV positive individuals.(3) Though not communicable, it is a significant cause of morbidity. Extrapulmonary TB represents a greater diagnostic problem than pulmonary TB because it presents with less frequency and occurs with little liberation of bacilli, as well as the fact that it is localized in sites that are difficult to access.(4)

The laboratory diagnosis of TB is generally established by microscopy for demonstration of AFB and mycobacterial culture on clinical specimens. Demonstration of AFB by ZN (Ziehl-Neelsen) stain and Auramine (Fluorescent) stain are simple and rapid methods but lack sensitivity and fail to detect large number of cases especially in extrapulmonary tuberculosis. Sensitivity of ZN method varies from 20-40 %.(5) Under these circumstances, cultivation of mycobacterium provides a sensitive and specific means for diagnosis of TB. Conventional culture methods such as Lowenstein-Jensen medium (LJM) requires 2-4 weeks for isolation plus additional 1-2 weeks for speciation. Such a prolonged turn-around time in the diagnosis is unacceptable as rapid detection and identification of *M .tuberculosis* is essential for both medical and epidemiological purposes.(6) Thus, there is an evident need of a culture method that is reliable and has shorterturnaround time. Middlebrook 7H9 (MB7H9) medium with ADC supplement is relatively inexpensive, does not involve use of radiometric materials, can be used with minimum infrastructure and technical skills.

Nucleic acid amplification techniques notably Polymerase chain reaction (PCR) is being increasingly used to diagnose TB. This is largely due to the ability of

PCR to increase the sensitivity, specificity and rapid turn-around time. However, even this technology has its own drawbacks e.g., high cost and considerable demand in terms of technical expertise.[7] The technique of DNA amplification by PCR has been used successfully to detect thepresence of extremely small quantities of *M. tuberculosis* in clinical samples and has been suggested as a successful tool for diagnosis of extrapulmonary tuberculosis.[8] This study was done to compare the sensitivity and specificity of PCR and culture in the diagnosis of EPTB.

Materials & Methods

This present study was carried out in department of Microbiology in a tertiary care hospital which included samples from 60 clinically diagnosed cases of extrapulmonary TB. All tissue samples were homogenized in a homogenizer. Thick viscous samples were subjected to NALC-NaOH method and all fluid samples were centrifuged. For DNA extraction, 200µl of the homogenized tissue or 200µl of centrifuged deposit from fluid samples were taken. PCR testing was done using IVD approved commercial kits. DNA extraction was performed by column method using QIAamp DNA extraction mini kit and amplificationwas done according to manufacturer's instructions using Seeplex MTB/NTM ACE detection kit and Amplisen's MBT-Eph PCR kit. Seeplex MTB/NTM ACE detection kit was used to target ***IS6110*** **and MPB64** genes and Amplisen's MBT-Eph PCR kit was used to target ***IS6110***gene. Amplified product was detected by agarose gel electrophoresis and gels were visualized under ultraviolet transilluminator (Zenith Research, Bangalore). Seeplex MTB/NTM ACE detection kit amplicon information displayed internal control band at 800bp, Mycobacteria at 491bp and MTB band at 366pb.Amplisen's MBT-Eph PCR kit information displayed internal control band at 750bp and MTB band at 390 pb. Positive PCR bands by Seeplex MTB/NTM ACE detection kit and Amplisen's MBT-Eph PCR kit are displayed in Fig. 1 and Fig. 2 respectively.

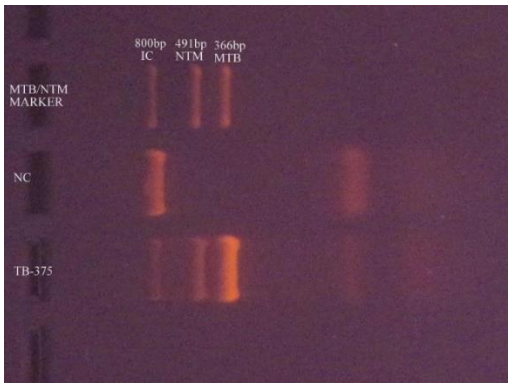

Fig. 1: PCR results by Seeplex MTB/NTM ACE detection kitshowing positive bands at 366bp (MTB) and 491bp (NTM)

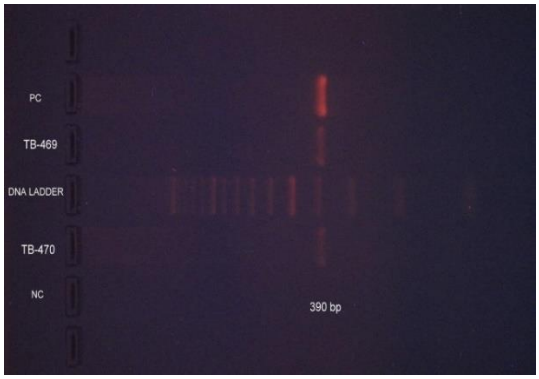

Fig. 2: PCR results by Amplisen's MBT-Eph PCR kit showingpositive bands at 390 bp

LJ medium with Gruft mycobacterial supplement (HiMedia, Mumbai) was used for isolation of mycobacteria. Two loops full of sediment was inoculated on the entire surface of two LJM slopes in a BSC level 2 taking necessary aseptic precautions. Date of inoculation was noted. The slopes were incubated at 37ºC for a maximum period of 8 weeks. The slopes were inspected once a week for the growth. In case of growth of mycobacteria, date of appearance of first colony was noted and slopes were further incubated for more growth. Commercially obtained Middlebrook 7H9 broth base as well as ADC (Albumin Dextrose and Catalase growth supplement) was used for study (HiMedia). The medium was prepared as per the manufacturer's instructions and wasdispersed in 5ml aliquots in sterile screw capped bottles.Tubes were inoculated with deposit aseptically. The date of inoculation was noted. Tubes were incubated at 370C for a maximum of 8 weeks. They were observed twice a week for the appearance of turbidity. Growth of tubercle bacilli on LJ medium was confirmed by observing the presence of characteristic acid fast bacilli in the ZN stained smears made from colonies. Growth on Middlebrook 7H9 medium was confirmed by observing rise in turbidity of medium and presence of characteristic acid fast bacilli in the ZN stained smear. Growth on LJ medium and Middlebrook 7H9 medium obtained during the study is demonstrated in Fig. 3 and Fig. 4.

Fig 3: LJ medium showing MTB growth

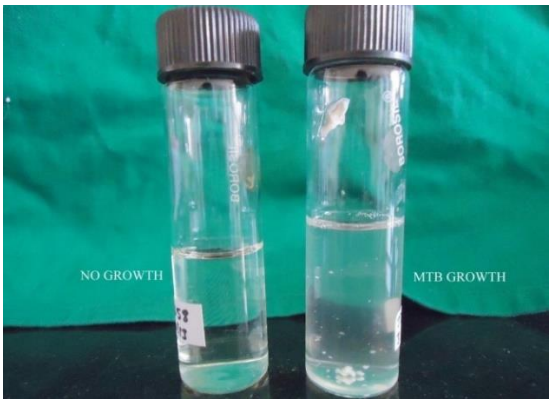

Fig 4: Middlebrook 7H9 medium showing MTB growth

Results

A total of 60 samples from clinically diagnosed patients of extrapulmonary tuberculosis (EPTB) were studied. In this study, EPTB positive patients were most common in the age group from 31-40 years followed by the age group of 41-50 years comprising 8 patients and the 21-30 years group having 7 patients. Only one EPTB patient was in more than 70 years age group.(Graph 1)

Out of 60 patients, 31 were males (51.66%) and 29 were females (48.33%). Among 31 males, 19 were EPTB positive and among 29 females 18 were EPTB positive. Maximum EPTB positivity was seen among pleural fluid samples followed by pus, CSF, tissue samples, ascitic fluid, synovial tissue, synovial fluid and pericardial fluid. None of the blood and endometrial tissue samples showed EPTB positivity as represented in Table 1.

Table 1: Distribution of extrapulmonary samples among EPTB positive and EPTB negative patients

Samples	EPTB positive	EPTB negative	Total
Pleural fluid	12 (32.4%)	4 (17.39%)	16
Pus	7 (18.9%)	1 (4.3%)	8
CSF	7 (18.9%)	6 (26%)	13
Tissue	6 (16.2%)	5 (21.7%)	11
Ascitic fluid	2 (5.4%)	1 (4.3%)	3
Synovial fluid	1 (2.7%)	2 (8.6%)	3
Synovial tissue	1 (2.7%)	1 (4.3%)	2
Pericardial fluid	1 (2.7%)	-	1
Blood	-	2 (8.6%)	2
Endometrial tissue	-	1 (4.3%)	1
Total	37 (61.7%)	23 (38.3%)	60 (100%)

The comparison of PCR versus culture was done and the sensitivity, specificity, PPV and NPV of PCR were 68%, 62.9%, 56.66% and 73.33% respectively considering culture as the gold standard. (Table 2)

Table 2: Comparison of Culture and PCR

N=60	Culture +ve	Culture –neg	Total
PCR +ve	17	13	30
PCR –neg	8	22	30
Total	25	35	60

A comparison of culture positivity rate in solid medium and liquid medium was done and among 60 samples tested, 25 were positive by both or one of the culture methods. Among the culture-positive samples, 20 were positive by both LJ and MB7H9. Three samples grew MTB only on LJ whereas two only in MB7H9 medium.Combination of LJM and MB7H9 gave us the increased rate of isolation by 8.3 %. (Table 3)

Table 3: Comparison of solid culture by LJ against liquid culture by MB7H9

N=60	LJ +ve	LJ –neg	Total
Liquid culture +ve	20	2	22
Liquid culture -neg	3	35	38
Total	23	37	60

The comparison of duration taken for positivity by LJ and MB7H9 media showed that the mean duration of isolation on LJ and MB7H9 was 4.8 and 3.5 weeks respectively. None of the strains was isolated on either medium in the first 2 weeks of incubation. Hence, liquid culture showed growth earlier than the growth on solidmedium. The period of maximum isolation on both media was 3rd week followed by 4th week. (Table 4)

Table 4: Comparison of duration taken for positivity by LJ and MB7H9

Duration	LJ Medium	MB7H9
1st week	-	-
2nd week	-	-
3rd week	7	14
4th week	4	3

5th week	3	5
6th week	4	-
7th week	5	-
Total	23	22

Discussion

Extrapulmonary TB poses a great challenge for diagnosis than pulmonary TB as it occurs with little liberation of bacilli and it is localized in sites that are difficult to access. Moreover, it places an enormous burden on the society as the youth are affected more compared to the older age group. In this study, EPTB positive patients were most common in the age group from 31-40 years followed by the age group of 41-50 years comprising 8 patients and the 21-30 years group having 7 patients. Only one EPTB patient was in more than 70 years age group. A study done by S. Rama Prakasha discusses the trends in extrapulmonary TB and concludes that the burden of extrapulmonary TB is among the productive age group which is very similar to the findings of our study.[9]

Out of 60 patients, 31 were males (51.66%) and 29 were females (48.33%). Among 31 males, 19 were EPTB positive and among 29 females 18 were EPTB positive. Maximum EPTB positivity was seen among pleural fluid samples followed by pus, CSF, tissue samples, ascitic fluid, synovial tissue, synovial fluid and pericardial fluid. None of the blood and endometrial tissue samples showed EPTB positivity.The comparison of PCR versus culture was done and the sensitivity, specificity, PPV and NPV of PCR were 68%, 62.9%, 56.66% and 73.33% respectively considering culture as the gold standard. Several other studies showed variable PCR results with variations in sensitivity and specificity. A study done by Ghoolobi A showed slightly lower sensitivity (66.67%) with higher specificity (77.78%).[10] In another study done by Park et al which reported higher sensitivity and specificity of 85% and 99% respectively, the reason for high sensitivity and specificity could be because of large sample size and different PCR methods.[11] A comparison of culture positivity rate in solid medium and liquid medium was done and among 60 samples tested, 25 were positive by both or one of the culture methods. Among the culture-positive samples, 20 were positive by both LJ and MB7H9. Three samples grew MTB only on LJ whereas two only in MB7H9 medium.Combination of LJM and MB7H9 gave us the increased rate of isolation by 8.3 %. A study done by Bhargava et al showed similar results.[12] Liquid medium is economical and less cumbersome method and when used with LJ media increases the overall isolation of mycobacteria.The comparison of duration taken for positivity by LJ and MB7H9 media showed that the mean duration of isolation on LJ and MB7H9 was 4.8 and 3.5 weeks respectively. A study done by Smaoui S et al showed similar findings as our study.[13] None of the strains was isolated on either medium in the first 2 weeks of incubation and liquid culture showed growth earlier than the growth on solid medium. The period of maximum isolation on both media was 3rd week followed by 4th week. All other studies show that none of the culture methods could isolate all of the mycobacterial strains.[9,10,12,13] Hence combination of both solid and liquid media could be justified for maximum recovery.

Conclusion

Molecular methods like PCR are more sensitive and specific than conventional methods and being improved continuously in the diagnosis of extrapulmonary tuberculosis. However as of today, available diagnostic tests should be combined with molecular methods and the results need to be correlated with the clinical findings to diagnose the disease. PCR is a rapid method, and when used along with the conventional diagnostic methods increases the overall isolation rate of *M. tuberculosis* in the diagnosis of extrapulmonary tuberculosis.

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