Evaluation of polymerase chain reaction,ziehl-neelsen stain, auramine stain and culture in the diagnosis of extrapulmonary tuberculosis- A Comparative Study

Dr. Harish Thummala, Dr. P. Vijayalakshmi

1 &2. Micro Lab, Guntur, Andhra Pradesh, India.

Corresponding author: Dr. Harish Thummala,

Abstract

Background and Objectives: Tuberculosis continues to have the highest number of TB cases in the world with over 2 million active TB cases every year. Extrapulmonary TB (EPTB) constitutes about 15 to 20% of all cases of TB. The conventional diagnostic methods are either slow or less sensitive in diagnosing EPTB. So there is a need for rapid and accurate diagnostic tests like molecular techniques such as RT-PCR. These tests would facilitate early diagnosis of potentially infectious patients and prompt institution of anti tubercular therapy.

Aim & Objective: The present study intends to evaluate the diagnostic efficacy of RT-PCR, conventional AFB microscopy by ZN and Auramine stain, and culture by LJM and MB7H9 in the diagnosis of different forms of extrapulmonary tuberculosis taking Culture as gold standard.

Methods: Clinically diagnosed patients of EPTB were subjected to RT-PCR (commercial kits). Out of these, 25 RT-PCR positive samples and 25 PCR negative samples were evaluated by microscopy and culture. A total of 24 DNA samples from PCR-positive cases tested using commercial kits, were subjected to an in-house RT-PCR test (IS6110 - 123 bp).

Results: In the present study, the sensitivity, specificity, positive predictive value and negative predictive value of microscopy were found to be 37.5%, 94.44%, 81.8% and 69.38% respectively against gold standard culture. Sensitivity and specificity of RT-PCR was found to be 70.83%, 63.88%. Positive predictive value of RT-PCR was found to be 56.66% and negative predictive value was 76.66% against gold standard culture.

Conclusion: EPTB is an important cause of morbidity and mortality. Laboratory diagnosis has many problems. Molecular techniques are rapid, sensitive and specific. However, all the available diagnostic methods should be utilized as RT-PCR is not 100% sensitive. The results need to be carefully correlated with clinical findings, to diagnose this disease.

Key words: Extra pulmonary tuberculosis; EPTB; PCR; ZN; Auramine; 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. According to WHO; more than one-third of the world''s human population has been infected by Tubercle bacillus. Every year more than eight million new cases occur among them and more than two million die.[1]

According to WHO report, 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. 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. Each of these diagnostic methods has its own merits and demerits and varies in terms of sensitivity and specificity.
Demonstration of AFB by ZN (Ziehl-Neelsen) stain and Auramine (Fluorescent) stain are simple and rapid but lack sensitivity and fail to detect large number of cases especially in extrapulmonary tuberculosis. Sensitivity of ZN method varies from 20- 40%. Fluorescent method is easy to interpret and the sensitivity is higher and recommended for large programmes.[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 a manifest need of a culture method that is reliable and has shorter turnaround 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 RT-PCR has been used successfully to detect the presence of extremely small quantities of *M. tuberculosis* in clinical samples and has been suggested as a successful tool for diagnosis of extrapulmonary tuberculosis.[8]

The present study intends to evaluate the diagnostic efficacy of RT-PCR, conventional AFB microscopy by ZN and Auramine stain, and culture by LJM and MB7H9 in the diagnosis of different forms of extrapulmonary tuberculosis.

AIMS AND OBJECTIVES

1. To compare the sensitivities of ZN stain, auramine stain, culture and RT-PCR in

the diagnosis of extrapulmonary tuberculosis.

2. To compare „in-house PCR" with commercial RT-PCR test.

3. To know the extent of problem of extrapulmonary tuberculosis in the cases attending our institute.

METHODOLOGY

Source of data: Samples from clinically diagnosed cases of extrapulmonary tuberculosis positive by RT-PCR assay at our laboratory were included in the study.

Study design: Comparative study

Inclusion criteria:
Clinically suspected EPTB patients who were positive by RT-PCR (25 samples) and an equal number of clinically suspected EPTB patients with negative RT-PCR result (25 samples) were included in the study. All these 50 samples were subjected to ZN staining, Auramine staining and culture on solid and liquid media.

Specimen collection: Sterile body fluids were aseptically collected in sterile containers using aspiration techniques or surgical procedures.

Any tissue was collected aseptically into sterile container without fixatives or preservatives. Sterile saline was added to prevent drying of the specimens.

All aspirations, in syringe or sterile containers. Storage at 4-8 Degree centigrade.

Details of the history and clinical examination of the cases and controls included in the study were recorded in a proforma.

Transportation: All specimens collected in appropriate sterile containers were transported to the laboratory as soon as possible preferably within one hour of collection. If any delay is anticipated in processing, they were stored at 4^0C.

All tissue samples and thick viscous samples were homogenized and decontaminated by NALC-NaOH method and all fluid samples were centrifuged. 200µl of the homogenized tissue or 200µl of centrifuged deposit from fluid samples were subjected to RT-PCR.

Smears were prepared from the deposit and were stained by

- ZN stain [9]

- Auramine O stain [10]

Deposit was also inoculated on LJ medium and Middlebrook-7H9 medium.

PCR

    I.   DNA extraction kit for microscopy, NALC-NAOH method. (QIAGEN DNA Extraction kit)

   II.   DNA amplification

  III.   Identification of amplified products by gel Electrophoresis (RT-PCR)

Seeplex MTB/NTM ACE detection kit:

Target gene: IS6110 and MPB64

Principle: Seeplex MTB ACE detection kit is a multiplex assay that permits theAmplification of target DNA of mycobacterium tuberculosis (MTB). It uses a Multitarget (IS6110 and MPB64) PCR. To prevent false negative results, Seeplex MTB ACE detection carries out both IS6110 and MPB64 PCR.

Genei (MERCK) kit:

Principle: This is a single-tube nested PCR. In the first step, 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.

RESULTS

This study was carried out at the Microlabs, Guntur. A total of 50 samples (25 RT-PCR-positive and 25 RT-PCR- negative) from clinically diagnosed patients of extrapulmonary tuberculosis (EPTB) were studied.

Table 1: Age group wise distribution of patients

Age group	No of patients	EPTB positive	EPTB negative
1-10	1 (2%)	1 (2%)	0
11-20	7 (14%)	3 (6%)	5
21-30	12 (24%)	**5 (10%)**	5
31-40	8 (16%)	**7 (14%)**	2
41-50	12 (24%)	**8 (16%)**	4
51-60	5 (10%)	3 (6%)	2
61-70	4 (8%)	3(6%)	1
>70	1 (2%)	0	1
Total	50 (100%)	30 (60%)	20 (40%)

1.   EPTB positive – positive by at least one of the laboratory tests.

2.   EPTB negative – Negative by all the laboratory tests.

In this study, EPTB positive patients were most common in the age group from 41-50years followed by the age group of 21-30 years comprising 17 patients and the 31-40 years group having 15 patients. Only one EPTB patient was in >70 years group.

Table 2: Gender wise distribution of patients

Sex	No of patients (%)	EPTB positive	EPTB negative

Males	30(60%)	17	11
Females	20 (40%)	13	9
Total	50 (100%)	30	20

Out of 50 patients, 30 were males (60%) and 20 were females (40%). Among 30 males, 17 were EPTB positive and among 20 females 13 were EPTB positive.

Table 3: Distribution of extrapulmonary samples among EPTB positive and EPTB negative patients

Samples	EPTB positive	EPTB negative	Total
Pleural fluid	11 (36.66%)	3 (15%)	14
Pus	5 (16.6%)	1 (5%)	6
CSF	5 (16.6%)	5 (25%)	10
Tissue	4 (13.3%)	4 (20%)	08
Ascitic fluid	2 (6.6%)	1 (5%)	3
Synovial fluid	1 (3.3%)	2 (10%)	3
Synovial tissue	1 (3.3%)	1 (5%)	2
Pericardial fluid	1 (3.3%)	-	1
Blood	-	2 (10%)	2
endometrial tissue	-	1 (5%)	1
Total	30 (60%)	20 (40%)	50 (100%)

Out of 60 extrapulmonary samples, 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.

Table 4: Comparison between ZN and Auramine:

N=50	ZN +ve	ZN -neg	Total
Auramine +ve	7	1	8
Auramine -neg	2	40	42
Total	9	41	50

Out of 50 samples studied by two microscopic methods, seven were positive by ZN which also showed positive result by Auramine. Two samples were positive by ZN but negative by Auramine. Similarly, two samples were positive by Auramine but negative by ZN. Totally, 10 samples (7 both + 2 ZN only + 1 Auramine only) were positive by microscopy.

Table 5: Comparison of Microscopy and RT-PCR

N=50	PCR +ve	PCR -neg	Total
Microscopy +ve	10	1	11
Microscopy -neg	20	19	39
Total	30	20	50

Out of 50 samples, 10 were positive by both microscopy and RT-PCR. However, 20 were negative by microscopy but positive by RT-PCR. One sample was microscopy positive but PCR negative.

Table 6: Comparison of Microscopy and culture

N=50	Culture +ve	Culture –neg	Total
Microscopy +ve	6	2	8
Microscopy -neg	14	28	42
Total	20	30	50

This table shows the comparison between microscopy and culture. The sensitivity, specificity, PPV and NPV of microscopy against culture were 37.5%, 94.4%, 81.8% and 69.38% respectively considering culture as gold standard.

Table 7: Comparison of solid culture by LJ against liquid culture by MB7H9

N=50	LJ +ve	LJ -neg	Total
Liquid culture +ve	18	2	20
Liquid culture -neg	2	28	30
Total	20	30	50

Among 50 samples tested, 20 were positive by both or one of the culture methods. Among the culture-positive samples, 18 were positive by both LJ and MB7H9. Two samples grew MTB only on LJ whereas only one in MB7H9 medium.

Table 9: Comparison of Culture and RT-PCR:

N=50	Culture+ve	Culture-neg	Total

RT-PCR +ve	15	10	25
RT-PCR - neg	5	20	25
Total	20	30	60

This table shows the comparison between Culture and PCR. Out of 50 tested samples, 15 were positive by both PCR and culture, 10 were positive by PCR but negative by culture and 5 were negative by PCR but were picked up by culture.

Table 10: Distribution of HIV positive cases among EPTB positive and EPTB negative patients

N=50	HIV +ve	HIV -neg	Total
EPTB +ve	3	30	33
EPTB -neg	0	17	17
Total	3 (6%)	47 (94%)	50

This table shows the association of HIV among the EPTB positive patients and EPTB negative patients. Out of 50 patients, only 6% of the individuals were known HIV positives and remaining 94% were HIV negatives.

Table 12: Clinical outcome among EPTB positive patients

Outcome	Number (n=33)	%
Recovered	32	96.9%
Expired	1	3.03%
Total	33	100%

This table shows the clinical outcome of EPTB positive patients. One case ended in fatality. 32 cases were recovered. Three cases were discharged against medical advice.

Table – 14: Positivity rate of individual tests

Total positives* (n = 30)		
RT-PCR	25	83.3%
Culture	20	66.6%
Microscopy	10	33.3%
***Positive by at least one diagnostic test**		

Highest positivity was shown by the molecular method (83.3%) followed by culture (66.6%). The least positivity rate was seen in microscopy (33.3%).

Table-15:Results of in-houseRT- PCR test

Samples positive by commercial RT-PCR	Samples positive by in-house RT-PCR	Samples negative by\ in-house RT-PCR
26	4	24

Out of 26 clinical samples which were positive by commercial PCR test, four samples gave positive results by in-house PCR test.

DISCUSSION

Tuberculosis is one of the most serious infectious diseases and an increasing public health problem in developing countries. Burden of tuberculosis is enhanced because of the HIV epidemic which predisposes to infection with *M. tuberculosis* and mycobacteria other than *M. tuberculosis* (MOTT). [11]

Extrapulmonary TB is a significant health problem in both developing and developed countries. EPTB accounts for more than 50% of all cases of TB among HIV positive patients[3].Early diagnosis followed by adequate treatment is essential to prevent both morbidity and mortality.

In the present study, total of 50 samples from clinically diagnosed EPTB patients were analysed. Clinically suspected cases of EPTB were tested by RT-PCR. Clinically suspected EPTB patients positive by RT-PCR were placed in one group and equal number of clinically suspected EPTB patients which were RT-PCR negative were included in the other group. All the samples of both groups were tested by microscopy (ZN and Auramine) and culture (LJ medium and Middlebrook 7H9).

Age wise distribution of cases is shown in table 1. The age groups of 21-30 yrs, 31-40 yrs and 41-50 yrs were the most commonly affected age groups. Together, 64.9 % of the laboratory-positive EPTB cases were between 21-50 yrs of age. In a study by Maurya AK et al (2012), maximum cases affected were in the age group of 25-44 yrs which accounted for 43% of total cases.[12] In a study done by Kumar et al, mean age of affected adults was 40.7 yrs.

In our study out of 50 patients, 30 were males (60%) and 20 were females (40 The other studies have also shown male preponderance.[12]

Of the 50 extrapulmonary samples, maximum EPTB positivity was seen among pleural fluid samples followed by pus, CSF and then tissue samples. Sekar B*et al*, showed maximum EPTB positivity in lymph node aspirates followed by CSF, ascitic fluid and was least in pleural fluid.[13] Kolk A.H.J *et al* (1998), in their study, maximum EPTB positivity was seen in pus followed by biopsy material and pleural fluid and was least in pericardial fluid.[14]

We evaluated the performance (sensitivity and specificity) of microscopy and RT-PCR over culture on all suspects of Extrapulmonary TB considering culture (growth on either or both of liquid and solid media) as the gold standard.

We have tested all the samples by two staining methods viz. ZN staining and auramine staining. In the results, shows that nine out of 50 (14%) samples gave positive result by ZN staining.

The results are comparable to that of Sekar B *et al*.[13] Intheir study, 34 out of 191 EPTB cases were positive for AFB smear examination (18%).

By Auramine O method, 7 out of 50 (14%) samples gave positive results. Our study showed no difference in the results between ZN staining and Auramine staining. To avoid the bias, both the smears (ZN and Auramine) were also screened by RNTCP laboratory technician of our center.

The sensitivity of PCR was found to be 70.83% and specificity was 63.88% against gold standard culture. So far, the theoretical sensitivity of PCR has never been achieved in the diagnosis of tuberculosis. This might have made WHO and other health agencies to recommend culture as the gold standard as of today. Variations in DNA extraction could be a reason for the false negativity of PCR. Many different methods for extraction are available. Their sensitivity varies and no single extraction method is accepted as a „gold standard" especially for clinical specimens.[15,16]

Six percent of EPTB cases were HIV seropositive in the present study. According to WHO report 2009, the prevalence of HIV seropositives among tuberculosis patients was 5.3% in India. According to Sharma SK (2004), EPTB accounts for more than 50% of all cases of TB among HIV positive patients. In a clinical study by Maurya AK et al (2012), HIV positivity was seen in 3 (1.8%) cases. Another study by Nandagopal *et al* (2010), HIV positivity was seen in 6.8% cases.[3,12, 11]

A total of 30 cases (60%) were positive by at least one of the laboratory tests used in the study. Utilizing all the available tests in combination increases the laboratory detection rates of *M. tuberculosis* from clinical samples. Maximum cases could be identified because of applying all the available diagnostic methods.

When individual tests are considered, RT-PCR detected the maximum number of cases followed by culture. This emphasizes the importance of including the molecular methods, along with other tests, on a regular basis in the diagnosis of EPTB. The low sensitivity of in-house PCR in the study could be because of several reasons. The extracted DNAs were stored at -20^0C for a long time and all the samples were subjected to RT-PCR testing when the in-house PCR was standardized.

CONCLUSION

Tuberculosis is globally a leading cause of adult mortality. EPTB is a big problem in our area also where adult males in the age group of 21-50 years were most affected with tubercular pleural effusion being the commonest form.

Laboratory diagnosis relies on microscopic examination of smears and culture of specimens. Smear microscopy although rapid and inexpensive, lacks sensitivity and can detect AFB, if the smear contains > 10,000 bacilli per ml. AFB culture remains the gold standard and is sensitive but time consuming.

Molecular techniques being improved continuously and rapidly appear to be the future tests of choice for laboratory diagnosis of tuberculosis. They are rapid, more sensitive and specific than the conventional methods. These tests will ensure early evidence based treatment to patients and prevent further transmission of disease. However, as of today, as many as available diagnostic tests should be utilized. The results need to be carefully correlated with clinical findings, to diagnose this disease.

Conflict of Interest

None

REFERENCES

1.Pfyffer GE, Vincent V. Mycobacterium tuberculosis complex, Mycobacterium Leprae, and other slow going mycobacteria. in: Borriello SP, Murray PR, Funke G editors. Topley and Wilson''s Microbiology and Microbial infections. 10th edition vol 2, London:Hodder Arnold; 2005; 1181-1203.

2.WHO global tuberculosis report 2012.

3.Sharma S.K and Mohan A. Extrapulmonary tuberculosis. Ind J Med Res 2004; 120:316-353.

4.Garcia-Elorriaga G, Gracida-Osorno C, Carillo-Monster G, Gonzalez-Bonilla

C. Clinical usefulness of the nested polymerase chain reaction in the diagnosis of extrapulmonary tuberculosis. Salud Publica Mex 2009; 51:240-245.
5.Suria Kumar J, Chandrasekar C, Rajasekaran S. Comparison of conventional and fluorescent staining methods in diagnosis of Pulmonary tuberculosis among HIV seropositive individuals. Journal of Evolution of Medical and Dental Sciences 2012; 4:463-466.

6.Park C, Hixon D, Furguson C. Rapid recovery of mycobacteria from clinical specimens using automated radiometric technique. Am. J. Clin. Path. March. 1984; 81(3):341-345.

7.Patwardhan SA, Bhargava P, Bhide VM, Kelkar DS. A study of tubercular lymphadenitis: A comparison of various laboratory diagnostic modalities with a special reference to tubercular Polymerase chain reaction. Indian Journal of Medical Microbiology 2011; 29(4):389-94.

8.Negi SS, Khan SFB, Gupta D, Pasha ST, Khare S, Lal S et al. Comparison of various microbiological tests including Polymerase Chain Reaction for the diagnosis of Osteoarticular tuberculosis. Indian Journal of Medical Microbiology 2005; 23(4):245-8.

9.Laboratory services   to   tuberculosis   control   part   II: Microscopy.

WHO/TB/98.258(1998).

10.Revised National TB Control Programme.Manual for sputum smear fluorescent microscopy central TB division, Directorate General Of Health Services, Ministry Of Health And Family Welfare.

11.Nandagopal B, Sankar S, Lingesan K, Appu KC, Sridharan G, Gopinathan AK et al. Evaluation of a nested PCR targeting IS6110 of Mycobacterium Tuberculosis for detection of the organism in the leukocyte fraction of blood sample. Indian Journal of Medical Microbiology 2010;28(3):227-32.

12.Maurya AK, Kant S, Nag VL, Kushwala R, Dhole TN. Detection of 123 bp fragment of insertion element IS6110 *Mycobacterium tuberculosis* of extrapulmonary tuberculosis. Indian Journal of Medical Microbiology 2012; 30(2):182-6.

13.Sekar B, Selvaraj L, Alexis A, Ravi S, Arunagiri K, Rathinavel L et al. The utility of IS6110 sequence based Polymerase chain reaction in comparison to conventional methods in the diagnosis of Extrapulmonary tuberculosis. Indian Journal of Medical Microbiology 2008; 26(4):352-5.

14.Kolk AHJ, Kox LFF, Van Leeuwen J, Kuijper S, Jansen HM. Clinical utility of the Polymerase chain reaction in the diagnosis of extrapulmonary tuberculosis. Eur Respir J 1998; 11:1222-1226.

15.Jain A, Tiwari V, Guleria RS, Verma RK. Qualitative Evaluation of Mycobacterial DNA Extraction Protocols for Polymerase Chain Reaction. Molecular Biology Today 2002; 3:43-50.

16.Aldous WK, Pounder JI, Cloud JL, Woods GL. Comparison of Six Methods of Extracting Mycobacterium tuberculosis DNA from Processed Sputum for Testing by Quantitative Real-Time PCR. J Clin Microbiol 2005; 43:2471-3.