ORIGINAL ARTICLE

EVALUATION OF SEROLOGICAL AND MOLECULAR METHODS OVER CONVENTIONAL METHODS IN DIAGNOSIS OF PULMONARY AND EXTRA PULMONARY TUBERCULOSIS

Deepika Verma, M. E. Siddique, Vineeta Mittal, Jagadamba Sharan, Sachin Kishore

1. Assistant Professor, Department of Microbiology, Rohilkhand Medical College and hospital,Bareilly, UP.
2. Professor & HOD, Department of Microbiology, Rohilkhand Medical College and hospital,Bareilly, UP.
3. Assistant Professor, Department of Microbiology, Era's Lucknow Medical College and hospital, Lucknow.
4. Assistant Professor, Department of Surgery, Rohilkhand Medical College and hospital,Bareilly, UP.
5. Assistant Professor, Department of Microbiology, Rohilkhand Medical College and hospital,Bareilly, UP.

CORRESPONDING AUTHOR
Dr. Deepika Verma,
C/O Dr. P. C. Verma,
A 74, Vaibhav, Suncity Vistaar,
Pilibit bye pass, Bareilly,
E-mail: dr_dipikaa05@rediffmail.com,
Ph: 0091 9305943057.

BACKGROUND: Despite advances in field of microbiology, diagnosis of Tuberculosis remains a challenge. Diagnosis of Extrapulmonary tuberculosis is more problematic due to low bacillary load in the specimen and difficulty in obtaining the specimen from the site of lesion in many cases. PCR and its modifications are a boon in diagnosis of tuberculosis in such cases. But serological methods like ELISA are still the first choice of small laboratories in India. **AIM**: Comparative evaluation of serological and molecular methods over conventional methods in diagnosis of extrapulmonary and pulmonary tuberculosis. **METHODS**: 170 different clinical specimens suspected of tuberculosis, (100 pulmonary and 70 Extrapulmonary) were examined by PCR using MPB 64 primer, culture and microscopy. All specimens were processed using USP methodology for inhibitors free PCR. TB Ig G, Ig M and Ig A was determined using PATHOZYME MYCO Kit. Response to ATT on clinical follow up was considered as gold standard. **RESULTS:** Total pulmonary specimens found positive by any of the four tests was 87 (out of 100) while that for extrapulmonary samples was 63(out of 70) . For **Pulmonary Specimens** the diagnostic accuracy of microscopy was 88.3%,for culture 88.3%,for ELISA 67.4% and for PCR 94.1%.For **extrapulmonary samples** the diagnostic accuracy of microscopy was 30.1%,for culture 49.2%,for ELISA 44.4% and for PCR 87.3%. **CONCLUSION:** As diagnosis by microscopy and culture are sensitive and specific, for pulmonary specimens, PCR should be kept reserved for clinically probable cases found negative by conventional tests. For Extrapulmonary specimens PCR can be used as an effective screening tool as conventional methods are mostly negative. ELISA was found to have no role in diagnosis of pulmonary TB. For Extrapulmonary TB ELISA can be used as an adjunct tool but results should be interpreted with utmost caution after full evaluation of the patient both clinically and radiologically.
KEY WORDS – ELISA, MPB 64 Primer, PCR, Tuberculosis

INTRODUCTION: The rapid and early diagnosis of tuberculosis is crucial first step for tuberculosis control program world wide especially in the wake of emergence of drug resistant TB and its related implications for HIV infected patients. Although the culture of *Mycobacterium tuberculosis* from the infected specimen remains the gold standard for diagnosing TB, the direct

microscopy for the detection of AFB in sputum continues to be most popular among all the methods currently employed. While the culture of mycobacterium takes 6-8 weeks to become positive [1,] the direct smear microscopy lacks sensitivity in extra pulmonary specimen, due to small number of organism[2] .Therefore there is need for more sensitive and specific tests for the rapid detection of tuberculosis. Many serological methods detecting Antibodies to various Antigens like 38 kDa (PhoS), 30/31 kDa (antigen 85, 19 kDa lipoprotein, 14 kDa, 16 kDa (ACR) and lipoarabinomanan (LAM) have been tested and among these 38 kDa antigen has shown the highest sensitivity and specificity.[3,4] Studies using this purified Ag in an EIA system found a high degree of specificity (91%) and sensitivity (72%) and concluded it to be a useful antigen for the serodiagnosis of tuberculosis.[5]

Recently molecular methods have revolutionized the diagnosis of tuberculosis. PCR is more sensitive as compared to conventional methods especially when the bacterial load is less in the sample and results can be obtained in one to two days. It also has its own drawbacks like false negative results due to PCR inhibitors in samples or absence of the target sequence and false positive results due to cross contamination[6].

The purpose of this study was to evaluate the utility of PCR and serological methods like ELISA over conventional methods like microscopy and culture separately in pulmonary and extrapulmonary samples from a pool of highly probable tuberculosis suspects referred to a tertiary care hospital.

MATERIAL AND METHOD: Research design
The study was conducted in Era's Lucknow Medical college and hospital, Lucknow. Clinical samples were taken from patients attending OPD's and admitted in wards of different departments of Era's Lucknow Medical college. A total of 170 patients suspected of tuberculosis were enrolled in the study, out of which 100 patients were pulmonary tuberculosis suspects while 70 patients were extra pulmonary tuberculosis suspects with no signs of pulmonary tuberculosis clinically. Out of 100 samples of pulmonary origin, only 86 specimens were found to be positive by one of the above tests and out of 70 specimens of extra pulmonary origin, 63 were found positive by one of above tests. Other 21 specimens which were negative by AFB staining, TB culture, ELISA and PCR were excluded from the study.

Two early morning sputum samples or gastric aspirates were collected from patients suspected of pulmonary TB. Extrapulmonary specimens like pleural fluid, ascitic fluid, cerebrospinal fluid, endometrial fluid, lymph node aspirate were collected from patients suspected of extra pulmonary tuberculosis.

SAMPLE PROCESSING, CULTURE AND PCR:
   A) Pulmonary samples (sputum ,gastric aspirate)and pus
After the preparation of smear two samples of sputum or gastric aspirate were pooled and divided into 2 parts. One part was decontaminated by Modified Petroff's method for culture on L J media [7.]The other part was processed as per the protocol described using universal sample processing (USP) methodology for inhibitor free PCR[8.]

EXTRAPULMONARY SAMPLES: (Pleural fluid, Ascitic fluid, Cerebrospinal fluid, Endometrial fluid) After the preparation of smears the sample was divided into two parts. One part was centrifuged at 3000 g for 15 minutes and pellet was inoculated onto two slants of L.J. media and observed upto 12 weeks. No decontamination step was carried out prior to culture as these

ORIGINAL ARTICLE

samples are sterile. The other part was pelleted at a medium speed (5000 - 6000g) for 15 minutes and the pellets were processed in the same manner as that for sputum samples .Cerebrospinal fluid specimens were pelleted at 25000×g and given a USP wash followed by a water wash.

C) BLOOD: Antibodies to mycobacterium in human serum were detected by ELISA using Pathozyme- Myco IgM, IgG, IgA (Omega Diagnostic) and qualitative results were calculated according to manufacturer's protocol.

D) NIACIN TEST: Positive cultures on LJ slants were confirmed by microscopy for AFB and Niacin accumulation test using BBL Taxo Niacin test strips (Becton Dickinson, USA).

E) DNA EXTRACTION AND PCR DNA was extracted, by incubating the pellet in extraction buffer (1mg/ml proteinase K in 10 mM Tris- HCl pH8.0, 1Mm EDTA, 10% SDS). Proteinase K was inactivated by heating at 100^0C for ten minutes. DNA purification was done by addition of equal volume of Phenol: Chloroform (24:1) to the extracted DNA.[9] PCR was done using MPB64 primers (Hysel India (p) ltd) which are specific for Mycobacteria of the tuberculosis complex. Amplification reaction was typically performed in a 50μl reaction mix containing 1μl of forward and reverse primers, 1μl final concentration of dNTP, 0.4μl of Taq polymerase in 10X buffer and 2μl of extracted DNA.

For extrapulmonary samples amplification reaction was performed in a 25 μl reaction mix containing 0.5μl of forward and reverse primers, 0.5μl final concentration of dNTP, 0.4μl of Taq polymerase in 10X buffer and 5μl of extracted DNA .The sequences of the forward and reverse primers used were 5'-TCCGCTGCCAGTCGTCTTCC-3' and 5'-GTCCTCGCGAGTCTAGGCCA-3'. Forty cycles of amplification were performed using an initial denaturation step of 95^0C for five minutes, followed by denaturation at 95^0C for one minute, annealing at 55^0C for one minute and extension of 72^0C for one minute. A final extension was carried out at 72^0C for seven minutes[10].The 240 bp final segment was detected on a 2% agarose gel through ethidium bromide staining. DNA from *M. tuberculosis* strain H37Rv was used as a positive control. Appropriate negative controls were set up for each sample.

STATISTICAL ANALYSIS: Statistical analysis using Mc Nemar's Chi square test was performed for the calculation of sensitivity, specificity, Positive predictive value, negative predictive value and diagnostic accuracy. Response to Anti Tubercular Therapy on clinical follow up was considered as GOLD STANDARD.

RESULTS: A total of 170 samples were examined by microscopy, culture on LJ slants, and PCR using MPB 64 primer. Serum sample from each patient was subjected to serological testing by ELISA.

Response to ATT was found positive in 76 patients of pulmonary TB and 46 patients of extra pulmonary TB on follow up

PULMONARY TUBERCULOSIS: Out of 86, 56 (64.3%) were found to be positive by microscopy, 67(77%) by culture, 54(62%) by ELISA and 77(88.5%) by PCR. The sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of tests under consideration are shown in table 1. PCR was found to be most sensitive test with highest

diagnostic accuracy while ELISA was found to be least sensitive test with least diagnostic accuracy.

EXTRAPULMONARY TUBERCULOSIS: Out of total of 63 positives in clinically suspected patients of extra pulmonary tuberculosis 2(3.2%) were found to be positive by microscopy, 14 (22.2%) by culture, 34 (53.9%) by ELISA and 48(76.1%) by PCR.

The sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of tests under consideration are shown in table 2. Out of all the four microbiological tests performed PCR was found to be most sensitive and its diagnostic accuracy was highest while microscopy was least sensitive and diagnostically least accurate.

DISCUSSION: In search of a need of a simple, sensitive and specific test for tuberculosis which would improve or replace the sputum smear microscopy, demonstration of specific Antibodies has repeatedly been focused. ELISA has been used as a rapid diagnostic Test for TB in low-income countries, which has over 90% of the global burden of TB cases.

Several molecular techniques have emerged during the last decade for the detection of specific DNA of M. tuberculosis in clinical specimens. Not only the molecular methods decrease the turn around time, but also are more patient friendly as result can be obtained by a single specimen. Also probability of confirming diagnosis of TB in paucibacillary cases is higher with PCR than with the conventional techniques. Besides it also helps the clinicians to initiate ATT (Anti Tubercular Treatment) in the initial stages of TB especially in paucibacillary infection. The present study demonstrates the utility of PCR and ELISA in pulmonary and extra pulmonary specimens separately.

Out of 86 samples of pulmonary origin, PCR detected 77(89.5%) , while conventional tests could detect 56(72.7%) by microscopy and 67(87%)by culture. This difference was not found to be statistically significant (P value 0.321) revealing the fact that microscopy and culture still remain the basic tools for diagnosis of pulmonary tuberculosis in a resource constrained country like India and PCR should be kept reserved for probable cases negative by conventional tests. PCR alone was positive in 10 (61.1%) among 18 samples found negative by conventional bacteriological techniques and were declared not to have tuberculosis using the diagnostic paradigm of the RNTCP. This aspect has great potential in the laboratory diagnosis of tuberculosis particularly in paucibacillary cases.

Nine such individuals were traced and clinical information gathered within a year of first presenting the clinic. Eight individuals took ATT and subsequently a clinical improvement was found in them, while one individual who refused to take ATT was found to be immediate family contact of a tuberculosis case. One patient was lost during follow up. (These two cases were not included in evaluation of sensitivity and specificity in our study.)

In our study in patients of pulmonary TB, PCR was found to be more sensitive than culture and microscopy. The sensitivity of PCR was found to be 92.2% and specificity 70% respectively, as compared to that of microscopy whose sensitivity was 73.6% and specificity 100% and culture whose sensitivity was 86.8% and specificity was 100%.The decreased specificity of PCR in our study could be due to the fact that in our study 3 samples which were found positive by PCR were not considered true positive in the criteria of gold standard as they did not take ATT subsequent to our report. However on clinical follow up these patients were found to be highly probable on history due to close family contact. If these 3 PCR positive cases are also considered to be cases of tuberculosis, specificity of PCR would become 100%. The

higher sensitivity and specificity of PCR have also been reported by other workers. While Kavita Modi Parekh et al [10] have found 91.5% sensitivity and 86% specificity over culture and microscopy, Jose Manuel Querol et al [11] have reported 100% sensitivity and 97% specificity.

EXTRAPULMONARY TB: In case of extra pulmonary TB, the sensitivity and specificity of PCR was more (93.4% and 70.5%) than that of microscopy (4.3% and of 100%) and culture (30.4% and 100%). Again, It is worthwhile to mention here that that low specificity in our result as compared to microscopy and culture was due to the fact that 2 samples which were found positive by PCR in our study were not considered true positive in the criteria of gold standard as all these patients did not take ATT (I died and 1 lost during follow up). However examination had shown evident cervical lymphadenitis not responding to usual antibiotics in both patients.

Out of total 63 samples of extra pulmonary origin, in our study, PCR detected 100% of specimens which were positive by conventional methods and 33(68.7%) of the 48 samples negative by conventional methods and were declared not to have tuberculosis using the diagnostic paradigm of the RNTCP. Thus PCR could detect 68.7% more cases as compared to conventional methods. This difference was found to be statistically significant (p value <0.001) indicating the great utility of PCR in extra pulmonary samples. Out of these, 31 individuals took ATT and improved during treatment except one patient with extensive pleural effusion who died during the course of treatment and one patient was lost during follow up. Only 31 cases, who took ATT of the 33 cases found positive by PCR alone were considered as true cases of TB.

Our results were in accordance with the results of several workers B Sekar et al [12], Mandir verma et al [13], Parmeet Kumar et al [14] who have reported similar sensitivity and specificity of PCR in detecting extra pulmonary tuberculosis.

Our in–house PCR assay was based on amplification of a gene MPB 64 which is specific for Mycobacterium tuberculosis complex. MPB 64 gene encodes for the immunogenic protein found only in culture filtrates of *M. tuberculosis* and occasional isolates of M. bovis BCG. Although IS 6110 is the most widely used primers but studies from Tuberculosis research centre, Chennai in south India found 42.7 % isolates either lack or have a few copies of IS 6110. [15,16] . Other workers [17,18] have reported similar results. The PCR tests targeting this sequence will not be applicable to such cases in India and this indicates the need to have alternate gene targets for diagnosis in these situations. Considering the above points, primers targeting the MPB 64 gene were used in our study.

The USP (Universal Sample Processing) Methodology described by Chakravaroty et al [8] was used in the present study. This method is simple and enabled the isolation of inhibitor – free, high quality mycobacterial DNA from a variety of clinical material in an environmentally friendly and inexpensive manner. This solution contains a chaotropic agent GuHCl (Guanidinium hydrochloride). The unique properties of the mycobacterial cell wall renders these organisms selectively resistant to the action of GuHCl while cells of other bacteria and eukaryotic cells are disrupted upon exposure to it.

Several Enzyme-Linked Immunosorbent Assays (ELISAs) have been tried to achieve the rapid and easy diagnosis of pulmonary tuberculosis. The kits used in our study were PATHOZYME-MYCO IgM, PATHOZYME-MYCO IgG and PATHOZYME-MYCO IgA containing recombinant forms of two antigens from the Mycobacterium tuberculosis complex: r38 kDa, expressed in and purified from Escherichia coli and the lipoarabinomanan (LAM; a common lipoglycan component of the mycobacterial cell wall purified from M. tuberculosis.

ORIGINAL ARTICLE

Out of 86 patients of pulmonary TB, ELISA was found to be positive in 51(59.3%) cases out of 68 (79%) patients which were positive by microscopy and culture. In addition ELISA was positive in 5 more patients found negative by conventional tests out of which only one responded to ATT. The other 4 patients were false positives later identified as respiratory diseases other than tuberculosis. Similarly in case of extra pulmonary tuberculosis, out of 15 samples positive by conventional tests, ELISA was positive in 11 samples. In addition ELISA was positive in 23 more patients out of 48 found negative by conventional test. However response to ATT was seen only in 11 patients out of these 23. The other 11 patients were false positives later identified with diseases other than tuberculosis. This may be due to high prevalence of TB in our country where large number of population is exposed to mycobacterial antigen (subclinical specific infection or infection with environmental mycobacteria) which may mount a significant and sustained antibody response. This sort of results has also been reported from other workers from different parts of our country [19,20] .In such a Scenario there can be no utility of the serodiagnostic tests in the diagnosis of pulmonary tuberculosis. In our study also no statistical significance was seen between ELISA and conventional methods (p value 0.321) in diagnosis of pulmonary TB.

However in diagnosis of extra pulmonary tuberculosis, a statistically significant difference (p value < 0.001) was seen between ELISA and conventional methods .But these tests should be interpreted with utmost caution and an extensive evaluation of antibody tests should be made taking into account all the aspects of the disease considering the significant and sustained antibody response caused by sub-clinical specific infection or infection with environmental mycobacteria as mentioned above.

CONCLUSION: As positivity of PCR in comparison to conventional methods was statistically insignificant in our study, we found that the utility of PCR was more for early diagnosis in patients of pulmonary tuberculosis and should be kept reserved for clinically probable cases found to be negative by microscopy. Moreover results of PCR should be interpreted in conjunction with the clinical and radiological data owing to its low specificity. In suspected patients of extra pulmonary tuberculosis PCR can be used as a screening tool as the diagnostic accuracy of PCR was much more than that of conventional methods which are mostly negative.

Determination of TB IgM, IgG and IgA antibody has no role in diagnosis of pulmonary tuberculosis. In diagnosis of extra pulmonary tuberculosis ELISA can be used as an adjunct tool but positive results should be interpreted with utmost caution after full evaluation of the patient both clinically and radiologically.

Activities leading to discovery of new antigen with immunodiagnostic potential need to be intensified and the techniques for antigen detection will continue to have edge over antibody detection methods due to obvious reasons.

Also, more than one PCR system targeting more than one gene should be used in diagnosis of TB by PCR to achieve 100% sensitivity and specificity.
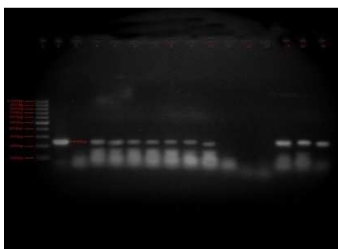
ORIGINAL ARTICLE

Table 1: Analysis of Different Tests on Samples of Pulmonary Tuberculosis Showing Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Diagnostic Accuracy

DIAGNOSTIC TEST	SENSITIVITY% C I (%)	SPECIFICITY% C I (%)	POSITIVE PREDICTIVE VALUE % C I (%)	NEGATIVE PREDICTIVE VALUE % C I (%)	DIAGNOSTIC ACCURACY %
Microscopy	73.6 (65.2-83)	100 (65.5-100)	100 (92.1-100)	33.3 (17.9-52.8)	88.3
Culture	86.8 (79.4-94)	100 (65.5-100)	100 (92.1-100)	50 (27.8-72.1)	88.3
ELISA	68.4 (61.1-81.9)	60 (8-64.6)	92.8 (77.8-88.8)	20 (12.5-35.8)	67.4
PCR	92.2 (83.2-96.7)	70.0 (35.3-91.9)	96.2 (87.8-98.9)	77.7 (26.1-79.5)	94.1

TABLE 2

Analysis of Different Tests on Samples of Extra Pulmonary Tuberculosis Showing Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Diagnostic Accuracy

DIAGNOSTIC TEST	SENSITIVITY % C I (%)	SPECIFICITY % C I (%)	POSITIVE PREDICTIVE VALUE % C I (%)	NEGATIVE PREDICTIVE VALUE % C I (%)	DIAGNOSTIC ACCURACY %
Microscopy	4.3 (7-15.1)	100 (73.2-100)	100 (73.2-100)	27.8 (13.5-35.8)	30.1
Culture	30.4 (17.0-43.4)	100 (73.2-100)	100 (73.2-100)	34.6 (17-43.4)	49.2
ELISA	47.8 (36.5-65.3)	35.2 (3.7-35.8)	66.6 (48.5-79.8)	20.0 (2-22.3)	44.4
PCR	93.4 (87.7-99.8)	70.5 (48.8-94.2)	89.5 (82.7-98.4)	80 (59.7-99.5)	87.3

ORIGINAL ARTICLE

REFERENCES:

1. Forbes B A, Sahm D F, Weissfeld A. Bailey and Scott's Textbook of diagnostic microbiology. 12th ed. St Louis: MOSBY ELSEVIER; 2007.
2. Kulkarni SP, Jaleel MA, Kadival GV. Evaluation of an in-house-developed PCR for the diagnosis of tuberculous meningitis in Indian children. J Med Microbiol 2005 Apr; 54(Pt 4):369-73.
3. Wilkinson RJ, Haslov K, Rappuoli R, et al. Evaluation of the recombinant 38-kilodalton antigen of Mycobacterium tuberculosis as a potential immunodiagnostic reagent. J Clin Microbiol 1997; 35: 553-7.
4. Espitia C, Cervera I, Gonzalez R, Mancilla R. A 38-kD Mycobacterium tuberculosis antigen associated with infection. Its isolation and serologic evaluation. Clin Exp Immunol 1989; 77: 373-7.
5. Palomino JC, Leao SC, Ritacco V. Tuberculosis 2007 - from basic science to patient care. 1st ed. Brazil: BourcillierKamps.com; 2007.
6. Roth A, Schaberg T, Mauch H. Molecular diagnosis of tuberculosis: current clinical validity and future perspectives. Eur Respir J 1997 Aug;10(8):1877-91.
7. C.N. Paramasivan, Pratibha Narang , G. Dakshayani , V. Chandrasekaran and P. Venkataraman. Evaluation of Bacteriological Diagnosis of smear positive Pulmonary Tuberculosis under Programme conditions in three Districts in the context of DOTS Implementation in India. Indian J Tuberc 2006; 53 :196-200.
8. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. J Clin Microbiol 2005 Sep; 43(9):4357-62.
9. Jain Amita, Tiwari Vandana, R.S Guleria, R.K. Verma. Qualitative Evaluation of Mycobacterial DNA Extraction Protocols for Polymerase Chain Reaction. Molecular Biology Today (2002) 3: 43 – 50.
10. Kavita Modi Parekh, Vikas Inamdar, Anagha Jog,Anita Kar. A comparative study of the diagnosis of Pulmonary Tuberculosis using conventional tools and PCR. Indian J Tuberc 2006; 53: 69-76.
11. Jose Manuel Querol ,Maria Ampara Farga, Damiana Granda ,Concepcion Gimeno and Juan Garcia-de Lomas .CHEST 1995 ;107;1631-1635.
12. B sekar, L sevaraj, A Alexis 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.
13. Mandira Varma-Basil, Rakesh Pathak et al. Direct Early identification of Mycobacterium tuberculosis by PCR – Restriction Fragment length Polymorphism Analysis from clinical samples. Jpn. J.Infect.Dis.63, 55-57,2010.
14. Parameet Kumar , Manas K. sen , Devendra S.Chauhan ,Vishwa M. Katoch ,Sarman Singh ,Hanumanthappa K. Prasad et al. Assessment of the N-PCR Assay in Diagnosis of Pleural Fluid and sputum collected in Tandem. PLos ONE 5(4):e10220.doi:10.1371/journal.pone.0010220.

ORIGINAL ARTICLE

15. Das S, Paramasivan CN, Lewis DB,Prabhakar R,Naraynan PR .IS6110 restriction fragment length polymorphism typing of clinical isolates of Mycobacterium tuberculosis from patients with pulmonary tuberculosis in Madras, south India. Tuberc lung disease 1995;76:550-4
16. SujathaN,VijayalakshmiP,FathimaR,ChitraS,GomathyD,KumaraswamiV,et al.Comparative evaluation of PCR using IS 6110 and a new target in the detection of tuberculosis lymphadenitis. Curr Sci 2000; 78 : 1367-70.
17. Shankar P ,Manjunath N,Lakshmi R,Aditi B,Seth P,Shriniwas.Identification of Mycobacterium tuberculosis by Polymerase chain reaction. Lancet 1990; 335:423.
18. Kent L ,Mc Hugh TD , Billington O, Dale JW, Gillespie SH.Demonstration of homology between IS 6110 of Mycobacterium tuberculosis and DNA'S of other Mycobacterium sp. J Clin Microbiology 1995;33 : 2290-3.
19. Sujatha Chandrasekaran, M.M. Chauhan & N. Parimala .Serodiagnosis of Pulmonary Tuberculosis and Evaluation of Two ELISA Kits. Ind J.Tub,1996, 43, 159.
20. Renuka Raju ,Sujai Suneetha, Karuna Sagili et al. Indian Journal of Clinical Biochemistry,2005,20 (1) 123 -128.