Comparative Analysis of *mpb-64* and Nested PCR targeting IS6110 for the Detection of Extra Pulmonary Tuberculosis

Narotam Sharma*1, Rakhi Kaushik2, Mukesh Kumar1, Natasha K Ahmed1, Shivani Sailwal1, Satish C Nautiyal1, R.K. Singh1

1Molecular Research Laboratory, Department of Biochemistry, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun-248001(Uttarakhand), India.
2GLA University, Mathura, Uttar Pradesh, India.

* Correspondence Info:
Dr. Narotam Sharma,
Research Scientist,
Molecular Research Laboratory, Department of Biochemistry,
SGRRIM&HS, Patel Nagar Dehradun (U.K)
Email: sharmanarotam5@gmail.com

Abstract

**Introduction:** Extra Pulmonary Tuberculosis can be seen in more than half of the patients with concurrent AIDS and tuberculosis. A high index of suspicion is necessary for timely diagnosis and prompt initiation of therapy. PCR has allowed great progress to be made in the rapid and accurate diagnosis of infections due to organisms for which culture is too insensitive and paubacillary nature of the pathogens in the specimens.

**Aim:** Evaluation of the efficacy of dual targets; *mpb-64* and Nested PCR targeting IS6110 for the detection of Extra Pulmonary Tuberculosis.

**Place and Duration of Study:** Done at Molecular Research Laboratory, Biochemistry Department, SGRRIM&HS, Dehradun, India, in between the duration, 2012-2013.

**Methodology:** A total of ninety clinical specimens, which includes; 14 pus, 32 CSF, 4 ascetic fluid, 6 biopsy, 10 blood, 3 urine, 6 endometrial tissues, 6 pleural fluids, 1 endometrial curetting, and 2 synovial fluid, collected from the various departments of SMI Hospital, Patel Nagar, Dehradun. Specimens were further processed for ZN staining, PCR utilizing; *mpb-64* and nested PCR (N-PCR) using IS6110 as a target for the detection of extra pulmonary tuberculosis cases.

**Results:** Out of 90 clinical specimens processed, 29 (32.2%) came N-PCR positive and 23 (25.5%) are *mpb64* TB PCR positive. When analyzed comparatively for both the molecular targets, it was seen that 20 samples came positive by both the targets i.e. *IS6110* as well as *mpb64* gene, but 9 specimens alone came positive only by *IS6110* NTPCR, and the same came negative for *mpb64* gene. 3 specimens showed positive result status for *mpb64* gene by conventional assay and at the same time they were negative for *IS6110* gene.

**Conclusion:** An essential element in the management of extra pulmonary tuberculosis is the availability of rapid, sensitive and specific identification of the causative agent. The laboratory diagnosis is largely based on direct microscopy and culture for mycobacterium. Direct microscopic examination is used to detect acid-fast bacilli (AFB) & has very low sensitivity. The gold standard for isolation of mycobacterium is not only time-consuming but also has low sensitivity in case of extra pulmonary tuberculosis. TB PCR diagnostics kits must be prepared, targeting *mpb-64* as well as *IS6110* as a targets to avoid false results.

**Keywords:** Extra-pulmonary samples, Copy Number, Molecular targets, Amplimers, Lymph node, Polymerase Chain Reaction.

IJBAR (2013) 04 (10)
1. Introduction

The extra-pulmonary Tuberculosis is much less common than pulmonary disease. However, in individuals with AIDS, extra-pulmonary tuberculosis predominates, particularly with lymph node involvements. Thus the diagnosis become very crucial for the detection of the same. AFB smears, serological tests, molecular tests and cultures can be used for the revealing of the pathogens. In terms of sensitivity, specificity, robustness and time, molecular diagnosis is increasing in Mycobacteriology laboratories. Microscopy with Acid Fast Bacilli (AFB) staining is the most rapid laboratory method for diagnosing tuberculosis. Its drawback is low specificity & sensitivity. Gold standard for laboratory diagnosis of tuberculosis is the isolation of M.Tuberculosis from clinical specimens by culturing. Culture is more specific & sensitive than staining & can detect as few as 10 organism/ml of digested and concentrated material but requires about 2-4 weeks to grow. Numerous targets have been studied as a tool for the detection of this disease. IS6110 is an insertion element found exclusively within the members of the Mycobacterium Tuberculosis complex (MTBC), & because of this exclusivity, it has become an important diagnostic tool in the identification of MTBC species. IS6110 is being used for the identification of TB using procedures such as RFLP and Spoligotyping for several years because this is a genetic insertion sequence (IS) or element that is found exclusively within the members of the Mycobacterium tuberculosis complex (MTBC). Some researchers have found IS6110-like elements in some other mycobacteria such as M. smegmatis is also present, but because smegmatis do not cause TB, its clinical importance is lesser. Only small numbers of bacilli are needed to cause extra-pulmonary tuberculosis cases but PCR amplification for MTB complex in extra pulmonary samples like synovial fluid, CSF, pleural fluid and bone marrow renders the method capable of detecting such small numbers of bacilli in specimens which would otherwise be undetectable using other conventional methods for the diagnosis of tuberculosis. In the last decade, nucleic acid amplification-based techniques (NAAT’s) have become accessible to the clinical mycobacteriology laboratory. PCR protocols amplifying a large variety of chromosomal DNA have concentrated on detection of both genus-specific and M. tuberculosis complex-specific DNA regions. The insertion element IS6110 and the 16S rDNA are the most common targets used. Other regions used for amplification includes rpoB gene encoding the b-subunit of the RNA polymerase, the gene coding for the 32 kD protein, the recA gene, the hsp65 gene, the dnaJ gene, the sodA gene and the 16S-23S rRNA internal transcriber spacer plus. These tests are highly sensitive for clinical samples and studies have shown that sensitivity and specificity ranging as high as 90-100%. NAATs may be tested on any specimen thought to contain bacilli (blood, urine, cerebro spinal fluid (CSF) but there is even less sensitivity reported in extra pulmonary specimens. Sensitivity is improved when multiple sampling are tested, because not all samples necessarily contain detectable nucleic acid.

Most of in house PCR procedures achieve a sensitivity never matched by commercial systems but are often burdened by the high incidence of false positive results due to amplicon cross-contamination of specimens. To minimize the risk of specimen-to-specimen contamination, a physical separation of processes, equipment, and reagents is recommended. Enzyme contamination control systems such as uracil-N-glycosylase (UNG) can be incorporated into the PCR master mix as an added safeguard to sterilize amplified product that may be carried over to subsequent batches of tests. Thus the present study focus on the comparative analysis of molecular markers; IS6110 and mp64 with the utilization of Nested-PCR and Uracil-N-Glycosylase usage (to get rid of amplicon contamination), for the diagnosis of Mycobacterium Tuberculosis complex for extra pulmonary specimens.

2. Materials and Methods

The current research work was carried out at Molecular Research Laboratory, Department of Biochemistry, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun, Uttarakhand. Clinical specimens which includes; 14 pus, 32 CSF, 4 ascetic fluid, 6 biopsy, 10 blood, 3 urine, 6 endometrial tissues, 6 pleural fluids, 1 endometrial curetting and 2 synovial fluid, were collected from various departments of Shri Mahant Indiresh Hospital. Further the Nucleic Acid (DNA) was extracted from all the clinical specimens by spin column based Nucleic Acid Extraction method. Column-based nucleic acid purification is a solid phase extraction method to quickly purify nucleic acids. Further Nested PCR was performed on the DNA template isolated. This test is based on the principles of single-tube nested PCR method, which is a powerful and sensitive diagnostic tool for the identification of Mycobacterium Tuberculosis complex. This assay is a two-step sequential assay. In the first step, the Insertion sequence region of Mycobacterium 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. In this assay, false positive reactions that may be caused by previous amplicon contamination are prevented by the use of uracil DNA glycosylase (UDG) and dUTP instead of dTTP added in the premix. Nested PCR. An amplimer of size 123 bp is indicative...
of infection with Mycobacterium tuberculosis complex. The amplification product of internal control DNA is 340 bp which is used for the validation of the results (as depicted in figure1). Primers utilized for the research work includes; IS6110 and mpb64. IS6110 using Nested primers, yielding, 123 base pairs amplimer of IS6110 repetitive sequence; forward primer, 5’-CCTGAGCGTACCGTGC-3’ (P1) and reverse primers, 5’-CTCTGTCCAGCGCTTCCG-3’ (P2). In case of mpb-64 based PCR, amplification was carried out for all the templates including controls. PCR was performed for the amplification of gene mpb64 using primers Forward primer P1-5’-TCCGCTGCCAGTCTTCCS-3’ and Reverse primer P2 5’-GTCTCGCGAGTCTAGGCCA-3’. A reaction mixture of 50 μl containing 10X PCR buffer (250 mM Tris Hcl, 500 mM KCl), 0.2 mM dNTPS, 25μM primers, Taq polymerase (3 units) and Mg2+ ions (25mM) as MgSO4 was prepared. 25μl of DNA template was added in the 25μl of master mix and amplification was done in thermal cycler (Bench Top) for 40 cycles.

Cycling conditions include; initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 60°C for 1 minute and primer extension at 72° C for 1 minute were provided with final extension of PCR products at 72° C for 7 minutes. Electrophoresis was carried out at 100-150 Volt (5 to 8 V/cm for 20 cm gel) until the bands in the molecular weight marker were resolved. Gel was examined under UV light (302nm) on ULTRA LUM Electronic UV Trans illuminator gel documentation system for the presence of 240 bp PCR product and photographed. The sizes of the amplicons were determined using DNA ladder of 100 bp (Bangalore GENEI Cat # MBD13J). ZN smears for the examination of acid fast bacilli were also prepared and observed under oil immersion.

2.1 Detection of amplified products

Amplification of 240 base pairs DNA fragments against the 240 base pairs target sequence of mpb-64 gene resolving on agarose gel equivalent to positive control and DNA ladder indicate the presence of MTB strain in clinical samples. Where as for N-PCR, 123 base pair amplicon for IS6110 gene was observed, including internal control appearing at 340 base pairs.

3. Results

Out of 90 clinical specimens processed, it was analyzed that 29 (32.2%) specimens are N-PCR positive and 23 (25.5%) specimens are mpb64 TB PCR positive. When analyzed comparatively for both the molecular targets, it was seen that 20 samples came positive for both the targets i.e. IS6110 as well as mpb64 gene. 9 specimens alone came positive only by IS6110 Nested-PCR, and the same came negative for mpb64 gene. 3 specimens showed positive result status for mpb64 gene by conventional assay and at the same time they were negative for IS6110 gene (as depicted in table 2). AFB positive smears were only observed in 5.5 % cases only (as tabulated in table 1).

When analyzed by age wise for positivity rate, higher percent was seen in between 11-40 years. Significantly it was seen that CSF came mostly from children were in age group ranging from 0-10, showing 33.3% positivity rate. Age groups 0-10, 11-20 showed positivity rates of 33.3% and 42.8% respectively, where specimens mostly includes CSF from infants and children.
Table no 1. Specimen wise results interpretation for mycobacterium tuberculosis complex detection by IS6110 and mpb64 genes as targets in PCR with comparative results with AFB smears.

| Sr. No. | Type of Specimen        | PCR Results Positive by N-PCR | PCR Results Positive by mpb64 | AFB Results | Positivity rate AFB (%) |
|---------|-------------------------|-------------------------------|-------------------------------|-------------|-------------------------|
|         |                         | (%)                           | (%)                           | Positive    | Negative                |
| 1.      | Pus (14)                | 7 (50%)                       | 4(4.4%)                       | 4           | 10                      | 4.4                      |
| 2.      | CSF (32)                | 16 (50%)                      | 16(50%)                       | 0           | 32                      | 0                       |
| 3.      | Ascetic fluid (04)      | 1 (25%)                       | 0(0%)                         | 0           | 4                       | 0                       |
| 4.      | Biopsy(06)              | 1(16.6%)                      | 0(0%)                         | 0           | 6                       | 0                       |
| 5.      | Tissue (06)             | 1(16.6%)                      | 0(0%)                         | 0           | 6                       | 0                       |
| 6.      | Blood (10)              | 1(10%)                        | 0(0%)                         | 0           | 10                      | 0                       |
| 7.      | Urine (03)              | 1(33.3%)                      | 0(0%)                         | 1           | 2                       | 1.1                      |
| 8.      | Endometrial Tissue (06) | 1(16.6%)                      | 0(0%)                         | 0           | 6                       | 0                       |
| 9.      | Pleural fluid(06)       | 0(0%)                         | 2(2.2%)                       | 0           | 6                       | 0                       |
| 10.     | Endometrial curetting(01)| 0(0%)                        | 0(0%)                         | 0           | 1                       | 0                       |
| 11.     | Synovial fluid(02)      | 0(0%)                         | 1(1.1%)                       | 0           | 2                       | 0                       |
|         | Total no. of Specimens  | 29(32.2%)                     | 23(25.5%)                     | 5(5.5%)     | 85 (94.5%)              | 5(5.5%)                 |

Table no 2. Comparative results of IS6110 N-TB PCR and mpb64 TB PCR.

| Number of specimens | N-PCR (IS6110) | TB PCR (mpb64) | %   |
|---------------------|----------------|----------------|-----|
| 20                  | +              | +              | 22.2%|
| 9                   | +              | -              | 10%  |
| 3                   | -              | +              | 3.3% |
| 58                  | -              | -              | 64.4%|
| Total positive with individual PCR | 29(32.2%) | 23(25.5%) | 32(35.5%) |
| Total positive with both PCR                  |               |                |      |

Table no 3. Age wise positivity distribution for extra pulmonary tuberculosis.

| Age group (years) | Total no. of Specimens | PCR Positive | PCR Negative | Positivity (%) |
|-------------------|-------------------------|--------------|--------------|----------------|
| 0-10              | 12                      | 4            | 8            | 33.3%          |
| 11-20             | 21                      | 9            | 12           | 42.8%          |
| 21-30             | 24                      | 8            | 16           | 33.3%          |
| 31-40             | 18                      | 6            | 12           | 33.3%          |
| 41-50             | 8                       | 1            | 7            | 12.5%          |
| Above 50          | 7                       | 1            | 6            | 14.2%          |
| Total no. of Specimens | 90 | 29          | 61           | 32.2%          |

Fig.2. Age wise positivity distribution for extra pulmonary tuberculosis.
Table no. 4. Comparative result of IS6110 and mpb64 genes.

| Total no. of specimens | IS6110 (N-PCR) | mpb-64 MTC PCR |
|------------------------|---------------|---------------|
| 90                     | 29 (32.2%)    | 23 (25.5%)    |

4. Discussion and Conclusion

Extra Pulmonary Tuberculosis is a contagious bacterial infection that involves organs other than the lungs. Extra pulmonary tuberculosis is caused by the bacteria *Mycobacterium tuberculosis* (*M. tuberculosis*). Extra pulmonary tuberculosis affects people with weak immune system, diabetes, HIV, or malnourished people, very young or elderly, those undergoing prolonged treatment with Chemotherapy or cortisone. The most common forms of Extra pulmonary tuberculosis are:- Lymph node tuberculosis, pleural tuberculosis, osteo-articular tuberculosis, central nervous system tuberculosis, tuberculosis of other places. Amplification technologies offers the potential for the diagnosis of tuberculosis in a few hours with a high degree of sensitivity and specificity. Polymerase Chain Reaction has been found to be useful for rapid diagnosis of tuberculosis from variety of clinical specimens. Many laboratories around the globe are using primers designed from IS6110 sequence of *Mycobacterium* genome. IS6110 is an insertion sequence specific for *Mycobacterium tuberculosis*.

Timely detection of various forms of extra pulmonary tuberculosis is of great importance for the proper treatment and management of the disease. Novel, rapid and cost effectiveness are the basic features of most of the PCR based techniques, but the technologies which can be either bio molecules, chemicals, antibodies or any recombinant proteins incorporating in the assays to prevent amplicons contamination will be an added advantage. As nested PCR it increases the sensitivity and specificity of an assay and when UNG is being utilized in pre-mix will make the assay a significant molecular diagnostic tool for *Mycobacterium tuberculosis* complex detection. Nested PCR for tuberculosis detection is a better tool when incorporated with an addition of UNG to prevent Amplicons contamination. False positive cases by amplicons contamination can be prevented by UNG and dUTP instead of dTTP.

The skill set required to adequately treat critically ill patients will also require knowledge of molecular biology for better diagnosis and treatment. The foundations of molecular biology and genetics are essential for the understanding of the mechanisms of disease. Correct, novel, significant molecular diagnostic tools are very important for all those laboratories performing routine diagnosis of tuberculosis in PCR based laboratories settings. In addition, particular emphasis should be applied to quality control and quality assurance programs in clinical laboratories which employ any new diagnostic approaches. Amplicons contamination detection and its prevention are of critical importance where the results interpretations are directly involved with patient’s health. Acceptance and implementation of PCR in the diagnostic laboratory requires an understanding of its mechanics, meaning of results, the test’s limitations, and being able to recognize problems and trouble-shoot them as they arise. IS6110 is an insertion element that is found exclusively within the MTBC; the assumption has been that this restriction is a result of the lack of genetic exchange with other Mycobacterial Species. A benefit of this exclusivity is that IS6110 has become an important diagnostic tool in the differentiation of MTBC species from other mycobacteria. Moreover, the element’s presence in multiple copies, and at differing locations in the genome, has provided an excellent method by which strains can be genotyped; because of these characteristics, IS6110 has been used extensively for epidemiological studies. An essential element in the management of extra pulmonary tuberculosis is the availability of rapid, sensitive and specific identification of the causative agent. The laboratory diagnosis is largely based on direct microscopy and culture for mycobacterium. Direct microscopic examination is used to detect acid-fast bacilli (AFB) & has very low sensitivity. The gold standard for isolation of mycobacterium is not only time-consuming but also has low sensitivity in case of extra pulmonary tuberculosis. As an alternative to these classical methods, new nucleic acid-based technologies showed promises as more rapid, sensitive and specific means of detection and identification of mycobacteria. mpb-64 gene has also been found in large copy number in some of the members of *Mycobacterium tuberculosis*, *M. bovis* and some strains of BCG of the Indian subcontinent. Targeting only a single gene can leads to false results as in some of the members of *Mycobacterium tuberculosis* complex, either IS6110 or mpb-64 can be in few copy number which can not be amplified by PCR. This the use of dual targets is of utmost significance for the molecular diagnostics assays designing as well as for studying the epidemiology of tuberculosis in a specific area.

Acknowledgements

The authors are grateful to Honorable Chairman, Shri Guru Ram Rai Education Mission for his kind support and guidance.
References
1. Coros, Abbie, Erin DeConno, and Keith M. Derbyshire. "IS6110, a Mycobacterium tuberculosis complex-specific insertion sequence, is also present in the genome of Mycobacterium smegmatis, suggestive of lateral gene transfer among mycobacterial species." Journal of bacteriology 2008;190, no. 9: 3408-3410.
2. Hance AJ, Grandchamp PB, Vincent-Levy-Frebault V, et al. Detection and identification of mycobacteria by amplification of mycobacteria DNA. Mol microbial 1989;3:843-9.
3. Shawer RM, Zaatari El FAK, Natraj A, Clarndge JE. Detection of Mycobacterium tuberculosis in clinical samples by two step polymerase chain reaction and non-isotopic hybridization methods. Journal of Clinical Microbiology, 1993; 31: 61.
4. Eisenach, K.D., M.D. Sifford, M. Donald Cave, J.H. Bates and J. T. Crawford,. Detection of Mycobacterium tuberculosis in sputum samples using a polymerase chain reaction. Am. Rev. Respir. Dis. 1991,144: 1160–3.
5. Negi SS, Khan SFB, Gupta S, Pasha ST, Khare S, Lal S. Comparison of the conventional diagnostic modalities, Bactech culture and polymerase chain reaction test for diagnosis of tuberculosis. Indian Journal of Medical Microbiology, 2005; 23 (1): 29-33.
6. Banavaliker N, Bhalotra B, Sharma DC, Goel KM, Khandekar PS, Bose M. Identification of Mycobacterium tuberculosis by polymerase chain reaction in clinical Specimens, Indian Journal of Tuberculosis 1998; 45.
7. Barnes, P.F.,A.B. Bloch.P.T. Davidson and D.E. Sinder. Tuberculosis in patients with human immunodeficiency virus infection. New England J. Med. 1991; 324: 1644–50.
8. Dye, C., S. Scheele, P. Dolin, V. Pathania and M.C. Raviglione. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA, 1999; 282: 677–86(WHO/CDS/CPC/TB/99; 264)
9. Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, A. van Soolingen, S. Kuijper and A. Bunschoten,. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J. Clin. Microbiol 1997; 35: 907–10.
10. Gamboa, F., G. Fernandez, E. Padilla, J.M. Manterola, J. Lonca, P.J. Cardona, L. Matas and V. Ausina, Comparative evaluation of initial and new versions of the gene–probe amplified mycobacterium tuberculosis direct test for direct detection of Mycobacterium tuberculosis in respiratory and nonrespiratory specimens. J. Clin.Microbiol.,1998; 36: 684–9.
11. Springer, B., L. Stockman, K. Teschner, G.D. Roberts and E.C. Böttger, Two laboratory collaborative study on identification of mycobacteria: Molecular versus phenotypic methods. J. Clin.Microbiol.1996; 34: 296–303.
12. Eisenach KD, Scifford KD, Cave MD, Bates JH, Crawford JT, 1991. Detection of Mycobacterium tuberculosis in sputum samples using a polymerase chain reaction. American Review of Respiratory Disease, 144: 1160.
13. Cormican, M.G., T. Barry, F. Gannon and J. Flynn, Use of polymerase chain reaction for early identification of Mycobacterium tuberculosis in positive cultures. J. Clin. Pathol.1992; 45: 601–4.
14. Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede and M.Kiekenbeck, Detection and identification of Mycobacteria by nucleic acid sequence determination: report of a 2–year experience in a clinical laboratory. J. Clin. Microbiol. 1993; 31: 2882–9.
15. Fodor, T., Detection of Mycobacteria in sputum smears prepared by cytocentrifugation. Tuber. Lung Dis.1995; 76: 273–4.
16. Herzog, B. H. "History of Tuberculosis", Respiration, 1998, 65:5-15.
17. Wujastyk, Dominic "The Roots of Ayurveda", London, Penguin Books, 2003.
18. Bhishagratna, K. K. L. "An English Translation of the Sushruta Samhita", Calcutta, S. L. Bhaduri, 1916.
19. Meulenbeld, G. J. "The Madhavanidana and its chief commentary", Leiden, E. J. Brill, 1974.
20. Conwell, W. E. "Observations chiefly on Pulmonary Disease in India and an essay on the use of the stethoscope", Mission Press, 1829.
21. Hirsch, A "Handbook of Geographical and Historical Pathology", Vol III Diseases of Organs and Parts, Translated from the second German edition by C. Creighton, London, The New Sydenham Society, 1886.
22. Ewart, J "Scrofula, Tuberculosis, and Phtisis in India", The British Medical Journal, May 21, 1881, 809 www.bmj.com/content/1/1064/808.
23. McLean, A "The history of the Foreign Christian Missionary Society", New York, Fleming H. Revell, 1919 archive.org/details/historyofforeignt00mcle.
24. Stock, E "Beginnings in India", London, Central Board of Missions and SPCK, 1917 anglicanhistory.org/india/
25. Price, F "Story of the Celebration held at Bareilly, India, from December 28th, 1906, to January 1st, 1907, inclusive", IJBAR (2013) 04 (10) www.ssjournals.com
Sharma et al

Calcutta, Methodist Publishing House, 1907, 107

http://openlibrary.org/books/OL23530264M/India_mission_jubilee_of_the_Methodist_Episcopal_Church_in_Southern_Asia

26. Lankester, A "Tuberculosis in India", London, Butterworth, 1920
27. Wilkinson, E "Notes on the Prevalence of Tuberculosis in India", www.ncbi.nlm.nih.gov/pmc/articles
28. "Imperial Malaria Conference October 1909", London, His Majesty's Stationery Office, 1912
29. East India (Sanitary) "Progress of Sanitary Measures in India", London, His Majesty's Stationery Office, 1912
30. Bradfield, E "An Indian Medical Review", New Delhi, Government of India Press, 1938
31. Thierry D, Brisson-Noel A, Vincent-Levy-Freboult V, Nguyen S, Guesdon KL, Gicquel B. Characterizing of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis. J. Clin. Microbiol. 1990; 28: 2668-2673.
32. Asgharzadeh M, Shahbabian K, Majidi J, Aghazadeh AM, Amini C, Jahantabi AR, and Rafi A. IS6110 restriction fragment length polymorphism typing of Mycobacterium tuberculosis isolates from East Azarbaijan province of Iran. Mem inst oswaldo cruz. 2006; 101: 517-521.
33. Asgharzadeh M, Shahbabian K, Kafil HS, and Rafi A (2007a). Use of DNA fingerprinting in identifying the source case of tuberculosis in East Azarbaijan province of Iran. J. Med. Sci. 7: 418-421.
34. Small PM, Shafer RW, Hopewell PC, Singh SP, Desmond E, Sierra MF, Murphy MJ, Desmond E, Sierra MF, Schoolnik GK (1993a). Exogenous reinfection with multidrug-resistant M. tuberculosis in patients with advanced HIV infection. N. Engl. J. Med. 328: 1137-1144.
35. Barnes PF, Cave MD. Molecular epidemiology of tuberculosis. N.Eng. J. Med.2003; 349: 1149-1156.
36. Bauer J, Yang Z, Poulsen S, Andersen AB. Results from 5 years of nationwide DNA fingerprinting of mycobacterium tuberculosis complex isolates in country with a low incidence of M. tuberculosis infection. J. Clin. Microbiol. 1998; 36: 305-308.
37. Barnes PF, Yangn ZH, Preston-Martin S, Pogoda M, Jones E, Otaya M, Eisenah D, Knowles L, Harvey S, Cave MD. Patterns of tuberculosis transmission in central Los Angeles. JAMA 1997; 278: 1156-1163.
38. Burman WJ, Reves RR, Hawkes AP, Rietmeijer CA, Wilson ML, Yang ZH, El-Hajj H, Bates JH, Cave MD. The incidence of false-positive cultures for Mycobacterium tuberculosis. Am. J. Respir. Crit. Care. Med. 1997; 155: 1140-1146.
39. Chin DP, Deriemer K, Small PM, Povce de Leon A, Steinhart R, Schecter GF, Delay CL, Moss AR, Paz EA, Paz A, Jasmer AM, Agasino CB, Hopewell PC. Differences in contributing factors to tuberculosis incidence in U.S. born and foreign-born persons. Am. J. Respir. Crit. Care. Med. 1998;158: 1797-1803.
40. Dooley SW, Villarino ME, Lawrence M, Salinas L, Amil S, Rullan JV. Nosocomial transmission of tuberculosis in a hospital unit for HIV infected patients. JAMA. 1992: 267: 2632-2635.
41. Edlin BR, Tokars JI, Greico MH, Crawford JT, Williams J, Sordillo EM, Ong KR, Kilburn JO, Dudley SW, Castro KG. An outbreak of multidrug resistant tuberculosis among hospitalized patients with a acquired immunodeficiency syndrome. N. Engl.J. Med. 1992 ; 326: 1514-1521.
42. Pena MJ, Caminero JA, Campos-Herrero MZ, Rodrigues-Gullego JC, Garcia-laorden MI (2003). Epidemiology of tuberculosis on Gran Cannria: a 4 year population study using traditional and molecular approaches. Thorax. 58: 618-622.
43. Niobe-Eyangoh SN, Kuaban C, Sarlin P, Cunin P, Thornon J, Sola C. Genetic Biodiversity of Mycobacterium tuberculosis complex strains from patients with pulmonary tuberculosis in Cameroon. J. Clin. Microbiol. 2003; 41; 2247-2553.
44. Asgharzadeh M, Khakpour M, Zahraei T, Kafil HS. Use of Mycobacterial interspersed repetitive unite–variable number tandem repeat typing to study Mycobacterium tuberculosis isolates from East Azarbaijan province of Iran. Pak. J. Biol. Sci. 2007; 10, 3769-3777.
45. Bauer J, Anderson K, Kremer K, and Miorner H. Usefulness of spoligotyping to discriminate IS6110 low copy number mycobacterium tuberculosis complex strains cultured in Denmark. J. Clin. Microbiol. 1999; 37: 2602-2606.
46. Cowan LS, Mosher L, Diem L, Massey JP, Crawford JT. Variable number tandem repeat typing of Mycobacterium tuberculosis. Isolates with low copy number of IS6110 by using Mycobacterial interspersed repetitive units. J. Clin. Microbiol. 2002; 40: 1592-1602.
47. Goguet de la Salmoniere YO, Li HM, Tarrea G, Bunschoten A, van Embden J, Gicquel B. Evaluation of spoligotyping in a study of the transmission of Mycobacterium tuberculosis. J. Clin. Microbiol. 1997; 35:2210-2214.
48. Kremer K, van Soolingen D, Frothingham R, de Hass WH, Hermans PW, Martin C, Palittapongaarnpim P, Plikaytis BB, Riley LW, Yakrus MA, Musser JM, van Embden JDA. Comparison of methods based on different molecular
epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. *J. Clin. Microbiol.* 1999; 37: 2607-2618.

49. Lee AS, Tang LL, Lim IH, Bellamy R, and Wong SY. Discrimination of single–copy IS6110 DNA Fingerprinting of *Mycobacterium tuberculosis* isolates by High-resolution mini satellite based typing. *J. Clin. Microbiol.* 2002; 40: 657-659.

50. Mazars E, Lesjean S, Banuls AL, Gilbert M, Vincent V, Gicquel B, Tibayrenc M, Locht C, Supply P. High–resolution minisatellite based Typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. *Proc. Natl. Acad. Sci.* 2001; USA. 98: 1901-1906.

51. de Boer AS, Bergdorf MW, de Hass PE, Negelkerke NJ, van Embden JD, van Solingen D. Analysis of rate of change of IS6110 RFLP patterns of *Mycobacterium tuberculosis* based on serial patient isolates. *J. Infect. Dis.* 1999; 180: 1238-1244.

52. Niemann ER, Rusch–Gerdes S. Stability of *Mycobacterium tuberculosis* IS6110 restriction fragment length polymorphism patterns and spoligotypes determined by analyzing serial isolates from patients with drug resistant tuberculosis. *J. Clin. Microbiol.* 1999; 37: 407-412.

53. Warren RM, vander Spay GD, Richardson M, Beyers N, Borgdorff MW, Behr MA, van Helden PD. Calculation of the stability of the IS6110 Banding pattern in patients with persistent *Mycobacterium tuberculosis* disease. *J. Clin. Microbiol.* 2002; 40: 1705-1708.

54. Rhee JT, Tanaka MM, Behr MA, Agasino CB, Paz EA, Hopewell PC, Small PM. Use of multiple markers in population based molecular epidemiologic studies of tuberculosis. *Int. J. Tuberc. Lung. Dis.* 2000; 4: 1111-1119.

55. Amdekar, Y. Tuberculosis - persistent threat to human health. *Indian J. Pediatr.* 2005; 72, 333–338.

56. Centers for Disease Control and Prevention (CDC). Trends in tuberculosis-United States, 2005. MMWR Morb. Mortal. *Wkly. Rep.* 2006; (11), 305-308 24; 55.

57. Dye, C., Espinal, M.A., Watt, C.J., Mbiaga, C., Williams, B.G., Worldwide incidence of multidrug-resistant tuberculosis. *J. Infect. Dis.* 2002; 85, 1197-1202.

58. Thierry, D., Brisson-Noel, A., Vincent-Levy-Frebault, V., Nguyen, S., Guesdon, J.L., Gicquel, B., Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. *J. Clin. Microbiol.* 1990; 28, 2668–2673

59. Kirschner, P., Rosenau, J., Springer, B., Teschner, K., Bottger, E.C., Diagnosis of mycobacterial infections by nucleic acid amplification: 18-month prospective study. *J. Clin. Microbiol.* 1996; 34, 304–312.

60. Kox, L.F., van Leeuwen, J., Knijper, S., Jansen, H.M., Kolk, A.H. PCR assay based on DNA coding for 16S rRNA for detection and identification of mycobacteria in clinical samples. *J. Clin. Microbiol.* 1995; 33, 3225–3233.

61. Newton C.R. Setting up a PCR laboratory. In PCR: Essential data. (ed. C.R. Newton), Wiley, New York 1995. p. 216.

62. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature.* 1989; 339, 237-8.

63. Sharma Narotam, Sharma Veena, Nautiyal Satish Chandra, Singh Prem Raj, Rajeev S, Kushwaha, Sailwal Shivani, Ghosh Shayan, Naushad Ahmer, Singh R.K. Conventional PCR usage for the detection of *Mycobacterium Tuberculosis* complex in Cerebrospinal Fluid by MPB64-Target PCR. *International Journal of Drug Development & Research,* 4(4): 201.

64. Sharma, Narotam, Amitab Talwar, Satish C. Nautiyal, Rakhi Kaushik, Amanpreet Singh, Akhil Pratap Singh, Jivesh K. Shaw, Shilpi Dixit, and R. K. Singh. "Nested-PCR and Uracil-N-Glycosylase-significant approach to prevent ampiclon contamination in tuberculosis PCR performing laboratories." *Archives of Applied Science Research* 2013; 5 (2): 177-179.