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PREVALENCE STUDY OF *M.TUBERCULOSIS* GENOTYPES BY USING SPOLIGOTYPING TECHNIQUE

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Abstract

AIMS/OBJECTIVE: To genotype *Mycobacterium tuberculosis* isolates and assess the magnitude of their clustering.

STUDY DESIGN: The present study was aimed at genotyping of *M. tuberculosis* isolates from Ghatampur area by spoligotyping. Thirty five isolates were typed by spoligotyping during 1 year period. Isolates were taken from Ghatampur area where NJIL &OMD is carrying out epidemiological studies on TB.

MATERIAL & METHODS: DNA of these thirty five isolates was extracted by physiochemical process for genotyping. Spoligotyping – DR region was amplified according to the instruction of manufacturer **(Isogen, Bioscience, Maarsen, The Netherland) and** hybridized DNA was detected by chemiluminescence's detection system (ECL).

RESULTS: Twenty seven spoligotypes were obtained. Six isolates were clustered into two shared type ST 26(CAS1_Del) and ST11 (EAI3_IND. Thirteen isolates showed orphan profile and further analysis with "Spotclust" showed that- 10 belonged to CAS family and one each toH3, EAI3, EAI5 family.

CONCLUSION: Our study showed spoligotyping to be fast and reliable system both for typing of *M. tuberculosis* from culture isolates. CAS & few EAI was found to be prevalent family in this area. Large number of samples from particular area and time period needs to be studied. In future spoligotyping may be used as a best first line tool to identify and genotype *M. tuberculosis* especially from field setting.

Keywords: Spoligotyping, *Mycobacterium tuberculosis*, Ghatampur, NJIL&OMD, DNA Extrauction, Culture

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Introduction

Tuberculosis is an ancient disease. Tubercle bacilli has been detected by molecular analysis in a mummy dated circa 1550-1080 B.C. The disease has been called the 'white plague' and 'the captain of all the men of death'. **(Ananthanarayan & Panicker 2001).** Tuberculosis (TB) is a contagious disease. Like the common cold, it spreads through the air.Tuberculosis is one of the leading infectious causes of death worldwide **(Frieden et al 2003).**

Someone in the world is newly infected with TB bacilli every second. Overall one third of the world's population is currently infected with the TB bacillus. 5-10 % of people who are infected with TB bacilli (but who are not infected with HIV) become sick or infectious at sometime during their life. People with HIV infection are much more likely to develop TB.The World Health Organization (WHO) estimates that more than 80% of TB patients are between the ages of 15-49 years. About 30 % tuberculosis patients were reported from India and 33% from South- East Asia **(WHO 2004).**

The only currently available vaccine is *M.bovis* BCG for tuberculosis, it is alive attenuated mycobacterial strain first developed in 1921 **(Behr et al 1999).** In India the overall figures for MDR vary from 0-6% and primary MDR is considered to be less than 4 % **(Paramasivan et al 1998).**Various types of tests are used for mycobacterial disease detection such as radiological examination, tuberculin test sputum examination and microscopy for acid fast bacilli in clinical samples. Various other methods include biochemical test **(Vestal 1997)**, composition lipid based

characterization **(Buller et al 1998).** But these are time consuming and may not give specific result.

Recent technological advances have lead to many improvements in the diagnostic methods, Mycobacterial growth indicator tube, fluorescent tubes etc, have been developed for early detection of growth. Besides the time tested biochemical methods, several newer techniques like gene probes, HPLC (High performance liquid chromatography), protein/isoenzymes patterns, ELISA has been developed for characterization of mycobacteria **(Katoch and Sharma 1997).** Recent development in DNA technology and Molecular Biology have led to method for rapid detection of mycobacterial DNA or RNA in clinical specimen, such as ribosomal RNA gene **(Cox and Katoch 1986).**

In the past strain specific characters used to distinguish strains were mainly antibiograms **(Collins et al 1984)** or susceptibility to mycobacteriophage **(Bates and Fitzhugh 1967)**. The discovery of a variety of repetitive DNA elements in *M.tuberculosis* genome has led to development of restriction fragment length polymorphism (RFLP) **(Eisenach et al 1988)** for differentiation of clinical isolates of *M. tuberculosis*. DNA fingerprinting of *M. tuberculosis* has gained increase acceptance for useful tool for epidemiology and phylogenetic investigation of *M. tuberculosis* **(van soolingen embden et al 1993).**

Various DNA fingerprinting methods used are IS6110 RFLP **(van Embden et al 1993, PGRS Ross et al 1992, Poulet and Cole 1994)**,(GTG)5 **(wiid et al 1994)** etc, of these methods IS6110 RFLP has been recognized as gold standard and extensively used worldwide to establish outbreaks and strain discrimination. But this method suffers from several disadvantages like method is not applicable to those stains having either too high or too low copy number of IS6110**(Cowan et al 2002,Lee et al 2002)**, requires availability of luxuriant growth on solid culture medium and method is slow, cumbersome, labour intensive and technically demanding.

Various PCR based strain genotyping method including RAPD **(Abed et al 1995,Linton et al 1984,Singh et al 2002)**,AFLP(Amplified Fragment Length Polymorphism)**(Vos et al 1995,Ahmed et al2003),**DR based methods **(Herman's et al 1992),**VNTR typing **(Frothingham and Meeker O' Connell 1998)**MIRU- VNTR typing **(Supply et al 2000)** and spoligotyping **(Hermens et al 1991, Kamerbeek et al 1997)** are now available.
Spoligotyping (spacer oligotyping) in which the direct repeats (DRs) are used as a target for in vitro DNA amplification. The size of direct repeat in direct repeat locus is 36 bp. It is multiple and well conserved ,interspersed with non repetitive spacer sequences 34to 41 bp long **(Kamerbeek et al 1997)**and which is exploited to obtain different hybridization patterns of the amplified DNA with multiple synthetic spacer oligonucleotide which are covalently bound to membrane**(Kamerbeek et al 1997).**

Although its level of discrimination is lower than obtained with restriction fragment length polymorphism associated with IS6110 **(Goyal et al2001, Baueret al1998)** in most strains, the performance of the technique regarding the degree of differentiation and reproducibility is good **(Kamerbeek et al1997).** Spoligotyping is suitable, rapid and robust and PCR based method for simultaneous detection and typing of *M. tuberculosis* specimen. It has been extensively used alone or in conjugation with other techniques for tracking epidemics **(Sola et al 1999).** For description of highly prevalent familes such as Beijing family **(van Soolingen et al 2000)** etc.

There are limited numbers of studies on epidemiology/genetics diversity of human tuberculosis using spoligotyping of Indian strain of *M. tuberculosis* **(Singh et al 2002,Bhanu et al 2004).** The study is being undertaken to identify genotypes of *M. tuberculosis* isolates from Ghatampur area and to gain knowledge about the prevalent spoligotyping based family in this region.

MATERIALS AND METHODS:

This study was conducted with *M. tuberculosis* strains isolates. Isolates were taken from Ghatampur area where NJIL &OMD is carrying out epidemiological studies on TB. During 1 year period of study, 35 ,*M. tuberculosis* strains were isolated. Only one isolate was included per patient. Samples included in this study were from patients presenting to our laboratory units from the Ghatampur area, East Uttarpredesh, India.

DNA method. Mycobacteria were cultured on Middlebrook 7H10 agar. DNA extraction from mycobacterial colonies was carried out by the cetyltrimethylammonium bromide method. DNA extraction was performed by using **van Embden et al (1993)** method.

Spoligotyping. Spoligotyping was carried out with a commercially available kit from Isogen Bioscience BV, Maarssen, The Netherlands, according to the manufacturer's instructions. Spoligotyping based on the 43 spacers of the direct-repeat region of the *M. tuberculosis* complex was carried out with primers DRa (5_GGTTTTGGGTCTGACGAC 3_) and DRb (5_CCGAG AGGGGACGGAAAC 3_) as originally described by **Kamerbeek et al (1997)**.

RESULTS

In this study spoligotyping was applied to identify and genotype of thirty five *M. tuberculosis* isolates from Ghatampur

area . Figure 13 and Table-1 show the spoligotypes of *M.tuberculosis* from Ghatampur area.

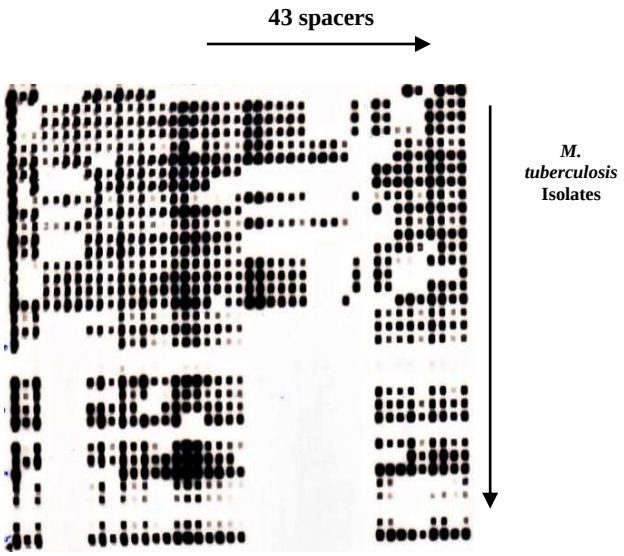

Fig: 13 Spoligotype of different *M. tuberculosis* isolate from Ghatampur area

The following table describes various spoligotypes of *M. tuberculosis* isolates from Ghatampur area; their octal code and their shared type in the international data base spol DB4.0 **(Brudey et al 2006).**

Table: 1- Spoligotypes from *M.tuberculosis* isolates from pulmonary TB cases from Ghatampur area

S.No.	Isolate	Octal code	ST/Family
	KMW-122	703760000000331	ST1120/CAS
	KMW-167	477777777413071	ST11/EAI3_IND
	KMW-175	477777777413071	ST11/EAI3_IND
	KMW-177	777777777413671	ST256/EAI5
	KMW-186	777766777760771	ST221/X1
	KMW-238	776377777760771	ST34/S
	KMW-205	703777740003771	ST26/CAS1_DEL
	KMW-341	501777400003771	ORPHAN/CAS
	KMW-353	577777677413771	ORPHAN/EAI5
	KMW-370	703777740002771	ST1091/CAS1_DEL
	KMW-392	777777777760771	ST53/T1
	KMW-404	703777740000771	ST357/CAS
	KMW-426	703777740003731	ST429/CAS1_DEL
	KMW-474	477777777413671	ST1369/EAI5
	KMW-491	477777777413001	ORPHAN/EAI3
	KMW-495	477777777413071	ST11/EAI3_IND
	KMW-521	777777577420771	ORPHAN/H3
	KMW-559	700367740003771	ORPHAN/CAS
	KMW-565	703777740003771	ST26/CAS1_DEL
	KMW-202	400377740003771	ORPHAN/CAS
	KMW-227	NA	
	KMW-236	NA	
	KMW-256	703777740000000	ST1264/CAS
	KMW-313	700357740003671	ORPHAN/CAS
	KMW-334	703717740003771	ORPHAN/CAS
	KMW-337	703774740003771	ORPHAN/CAS
	KMW-348	NA	
	KMW-350	703767740003771	ST141/CAS
	KMW-460	703777700000371	ST1345/CAS
	KMW-466	400377740003771	ORPHAN/CAS
	KMW-514	503347740003011	ORPHAN/CAS
	KMW-524	503347700003071	ORPHAN/CAS
	KMW-561	NA	
	KMW-582	503367740003061	ORPHAN/CAS
	KMW-583	703777740003771	ST26/CAS1_DEL

NA-Not Amplified

Twenty seven spoligotypes were obtained. Six isolates were clustered into two shared type

ST 26(CAS1_Del) and ST11 (EAI3_IND. Thirteen isolates showed orphan profile and further analysis with "Spotclust" showed that- 10 belonged to CAS family and one each to H3, EAI3,EAI5 family.

DISCUSSION

Tuberculosis (TB) is a global problem **(Raviglion et al 1995).** As estimated 1.7 billion people, nearly one third of the world population, is infected with *M. tuberculosis* **(Brudey et al 2004).** Each year, there are 8.4 million new cases and 2 to 3 million deaths **(STP 2006).** The risk of infections is proportional to the intensity of exposure. Infection does not usually lead to disease. WHO estimated that if the effectiveness of TB control programmes does not improve substantially the number of TB cases will pass the 200 million **(WHO 2007).**

HIV (Human Immunodeficiency Virus) is the strongest known risk factor for the development of TB. HIV breaks down the immune system and makes patients highly susceptible to tuberculosis. These patients, in turn, can spread TB to others **(Rajasekaran** et al 2000).

TB can be controlled and treated. Various types of tests are used top diagnose TB. Key factors in the control of tuberculosis are rapid detection, adequate therapy and contact tracing to arrest further transmission. A complete medical evaluation for TB must include a medical history, a chest X-ray, and a physical examination. Tuberculosis radiology is used in the diagnosis of

TB. It may also include a Mantoux tuberculin skin test, a serological test, microbiological smear and cultures.

Various other methods used for identification includes biochemical test **(Vestal 1977),** chemical composition/lipid based characterization **(Butler et al 1998),** but these methods are time consuming and may not give specific result. Recent development in DNA technology and molecular biology have led to methods for rapid detection of mycobacterial DNA or RNA in clinical specimens.

DNA fingerprinting of *M. tuberculosis* has gained increase acceptance as a useful tool for epidemiology and phylogenetic investigation of *M. tuberculosis***.** Restriction fragment length polymorphism (RFLP) typing with insertion element **(Hermans 1990)** IS6110 as a probe, has become the most widely used method for differentiating the strains of *M. tuberculosis* isolates **(Behr et al 1997).** This technique is used for the discrimination of *M. tuberculosis* strain **(Cave et al 1991)** and identification of the transmission chain .

Spoligotyping (also known as spaceroligotyping), a new method for simultaneous detection and typing of *M. tuberculosis* complex bacteria, has been recently developed **(Groenen et al 1993 Hermans et al 1991).**

In this method, direct repeats (DRs) are used as a target for in vitro DNA amplification and the variation in the spacer is exploted to obtain different hybridization patterns of the amplified DNA with multiple synthetic spacer oligonucleotides which are covalently bound to membrane. In Spoligotyping DRs (direct repeats) are used as a target to find polymorphism.Polymorphism in these region appear to comprise mainly the presence or absence of single discrete DVRs (direct variable repeat) or stretches of contiguous DVRs **(van Embden et al 2000)** with in the direct repeat locus of *M.tuberculosis.*

Results of spoligotyping can be obtained from a *M. tuberculosis* culture within one day. Thus the clinical usefulness of spoligotyping is determined by its rapidity, both in detecting causative bacteria and in providing epidemiologic information on strain identities **(Embden et al 1993)**. It has advances over IS6110 typing **(Chauhan et al 2004)** in that it is faster and easier to perform reproducible and it requires only small quantities of DNA.

It is the method for simultaneous detection and typing of *M. tuberculosis* strain with acid-fast bacilli positive slides from clinical specimens or mycobacterial cultures. Spoligotyping may be used alone or with other techniques for tracking epidemics **(Sola et al 1999).**

In Indian settings, the application of this PCR based technique for studying epidemiology of tuberculosis is lacking particularly in field settings. In this study the usefulness of this technique both for genotyping of *M. tuberculosis* from culture isolates from Ghatampur area was evaluated. Isolates from this area were clustered into two families CAS1_del and EAI 3_Ind family. Orphan isolates on analysis with 'spotclust' showed that-10 belonged to CAS family and one each toH3, EAI3,EAI5 family.

Further study will include large number of isolates so that it can be used in field settings and can provide useful data to clinician in different settings.

"CAS-family" & EAI was found to be predominant, because sample size was low, distribution of other families like Beijing, MANU, etc needs to be studied and correlated with the drug resistance profile.

CONCLUSION

Our study showed spoligotyping to be fast and reliable system both for typing of *M. tuberculosis* from culture isolates. CAS & few EAI was found to be prevalent family in this area. Large number of samples from particular area and time period needs to be studied. In future spoligotyping may be used as a best first line tool to identify and genotype *M. tuberculosis* especially from field settings.

References

Ahmed N, Alam M, Abdul MA et al (2004). Genomes sequence based comparative analysis of the Amplified Fragment Length Polymorphism of tubercle bacilli from seals provides molecular evidence for new species within the *Mycobacterium tuberculosis* complex. Infect Gent E;vol2:193-199.

Ananthanarayan R and Panicker C K G (2001). *Mycobacterium tuberculosis* In Textbook of Medical Microbiology,(VIth) Orient Longman Ltd. Annasulai, Chennai;pp: 324-338.

Ananthanarayan R and Panicker C K G (2001). *Mycobacterium tuberculosis* In: Textbook of Medical Microbiology,(VIth) Orient Longman Ltd. Annasulai, Chennai;pp: 240-245

Arnold C, Methrell, L, Clewley IP et al (1999). Predictive modeling of fluorescent (AFLP). A new approach to the molecular epidemology of *E.coli. Res. Microbiol.* (1999); 150: 33-44.

Barreto AMW, Areuju JVM, Medeiros PFM et al (2003). Evaluation of indirect susceptibility testing of *Mycobacterium* tuberculosis to the first and second line alternate drug by the newer MB/BACT system. By dissolve CO. *Mem Inst Oswlado Cruz*:827-830.

Bauer J, Andersen AB, Kremer K,et al(1998). Usefulness of spoligotyping to determinate IS6110 low copy number of

Mycobacterium tuberculosis complex strains cultured in Denmark. *J. Clin. Microbiol.*(1998); 37: 2602-2606.

Bhanu,N VD van Soolingen, D van Embden J D et al(2004). Predominance of a novel *Mycobacterium tuberculosis* genotype in the Delhi region of India. *Tuberculosis (*2002); 82:105-112.

Bifani PJ, Mathema B, Lin Z, et al (1999). Identification of a variant out break of *Mycobacterium tuberculosis* via population based molecular epidemiology. *JAMA***;**282:2321-2327.

Brisson-Noel A, Gicquel B, Lecossier D, et al(1989). Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. Lancet;2:1068-1070.

Brudey K,Gordon M,Monstrom P,et al(2004). Molecular epidemiology of *Mycobacterium tuberculosis* in western Sweden.J Clin Microbiol ;7:3046-3051.

Butler WR, Thibert I and Kilburn JO(1992). Identification of *Mycobacterium avium* complex strains and some similar species by lipid characterization. *J. Clin. Microbiol* ;30:2698-2704.

Cave MD, Eisenach KD, Mc denmoll PF, et al(1991).IS6110: Conservation of sequence in *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. Mol cell probes;5:73-80.

Chauhan A, Chauhan DS, Prashar D et al(2004). DNA fingerprinting of *Mycobactreium tuberculosis* isolates from Agra region by IS6110. *Indian J Med Microbiol* 22: 234-304.

Chauhan D S, Sharma V D, Parashar et al (2007). Molecular typing of *Mycobacterium tuberculosis* strains isolates from different part of India based on IS6110 element polymorphism using RFLP analysis. Indian JMed. Res; (Under Publication).

Clark curtiss JE(1989). Genome structure of mycobacteria in molecular biology of mycobacteria .J Mc Fadden(ed) surrey: Surrey University Academic press. London;77-96.

Cole S.T., Brosch R., Parkhill J et al. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature (London); **393**: 537-544.

Collins DM and de Lisle (1985). DNA restriction endonuclease analysis of *M. bovis* and other members of tuberculosis complex. *J Clin Microbiol* 1985; 130:562- 564.

Coro nado VG, Beek–sague CM, Hutton MD , et al(1993). Transmission of multidrug resistant *Mycobacterium tuberculosis* among persons with HIV infection in an urban hospital, epidemiologic and RFLP analysis . *J Infect Dis;*168:1052 -1055.

Cowan Ls. Mosher L, Diem L,et al (2002). Variable number tandem repeat typing of Mycobacterium tuberculosis isolates with low copy number of IS6110 by using mycobacterial interspersed repetitive units. *J Clin Microboil*;40:1592-1602.

Cox (2004). Quantitative relationships for specific growth rates and macromolecular composition of *Mycobacterium tuberculosis Streptomoyces coelicolor* A3(2) and *Escherichia coli* Blr, an integrative theoretical approach.*J Clin microbial*;150:1413-1426.

Dale J. W, Brittain D, Cataldi AA et al (2001). spacer oligo nucleotide typing of bacteria of the *Mycobacterium tuberculosis* complex recommendation for standardized nomenclature Int J Tuber Lung Dis ;4:216-219.

Davies PDO,Yew WW, Ganguly D, et al(2006). The epidemiological association and pathgenesis of tuberculosis in HIV patients. Trop med; HIV00:291-298.

Duffey PS, Erans Ge (1996). Improved rapid identification of mycobacteria by combining solid phase high performance liquid chromatography analysis of BACTEC cultures. *J. Clin microbiol* ;1996; 34:1939 – 1943.

Goria A, Bandera A, Marchetti et al. (2005). Spoligotyping and *Mycobactreium tuberculosis. Emerging Infectious Dis;* 1242- 1248.

Goyal M, Lawn S, Affered B,et al(1999). Spoligotyping in molecular epidemiology of tuberculosis in Ghana. *J. Infect* (1999); 38: 171-175.

Groenen PMA, van Soolingen D and van Embden JDA(1993). Nature of DNA polymorphism in the direct repeat cluster of *M.tuberculosis:* application for strain differentiation by a novel method. *Mol. Microbiol;* (1993); 105: 1057-1065.

Hermans PW, van Soolingen, D, Dale JW, Mc et al(1990). Insertion elements for diagnosis and epidemiology of tuberculosis. *J. Clin. Microbiol.* (1990); 28: 2051-2058.

Hermans PW, van Soolingen D et al. (1992). Characterization of a major polymorphic tendom repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology. *J Bacteriol*, 174: (1992); 4157- 4165.

Isenberg H D, Amato R F, Heifeta (1991). Collaborative feasibility study of a biphasic system (roche septicheck AFB) for rapid detection and isolation of mycobacteria . J. Clin. Microbiol.; 29 : 1719-722.

Kamerbeek J, Kolk A, van Soolingen D, et al(1997).Simultaneous detection and strain differentiation of *M.tuberculosis* for diagnosis and epidemiology by direct repeat *J Clin Microbiol* (1997); 35: 907-914.

Katoch V M (1997). Recent advances in the development of techniques for diagnosis and epidemiology of tuberculosis. Indian J. Microbiol; 63:362-370.

Katoch VM, Parashar D, Chauhan et al (2007). Rapid identification of Mycobacteria by gene amplification restriction analysis technique targeting 16s-23s ribosomal RNA internal transcribed spacer and flanking region. Indian J. Med. Res;125: pp 155-162.

Katoch VM and Sharma VD (1997). Advance in diagnosis of mycobacterial disease .*Indian Med .Microbiol*.15:49:55 .

Katoch VM(2004). Infections due to non-tuberculosis mycobacteria (NTM). *Indian J Med Res*.; 120: 290-304.

Katoch VM(2004). Newer diagnostic techniques for tuberculosis. *Indian J Med Res;* 120: 418-428.

Katoch VM, Shivannaver CT, Datta et al. (1987). Immunological relatedness of superoxide dismutase of mycobacteria, a new parameter ELISA as a taxonomic identification and classification. A CJIL publication coronation press, Agra 1987: 219- 226.

Kilburn (1973). A Co-operative numerical analysis of rapidly growing Mycobacteria .*J Gen Microbial*;73:52-70.

Kirschner R, Springer B, Vogel U, et al. (1993). Genotypic identification of mycobacteria by nucleic acid sequence determination. A report of a 2 year experience in clinical laboratory. J. Clin Microbial; 3 : 2882 – 2889.

Kubicak G.P, Beamk,E, Palmer J. W. et al (1996). The isolation of unclassified(atypical) acid fast bacilli from soil using catalase activity.Amer rv Resp Dis; 84:135-136.

Linton CJ, Jalal H, Luming JPet al(1994). Rapid discrimination of *M.tuberculosis* strain by Random polymorphic DNA analysis. *J Clin Microbiol.* (1994); 32: 2169-2174.

Lipsky BA, Gates J, Tenover FC et al(1984). factors altering the clinical value of microscopy for acid fast bacilli. *Rev Infect Dis* ,1984; 6: 214- 222.

McFadden J J, Kunze Z and Seechurn P (1990). DNA probes for detection and Surrey University identification; *In Molecular Biology of Mycobacteria.* (ed) Academic Press, Surrey University ; McFadden J : 173 – 198.

Middle Brook G, Reggiareoz. Tiger 11 WD (1977). Automatic radiometric detection of growth of M. tuberculosis in selective media. Am Rev Pir Dis; 115 : 1066 – 1069.

Minnikin DS and Goodfellow M(1980). Mycobacterial lipid composition fast growing bacteria. *Microbiological classification & identification Goodfellow and RG Board* (Eds) Academic Press; London. (1980); 9: pp 184-200.

Mistry N F, Iyer A M, D'souza T B, et al(2002). Spoligotyping of *M.tuberculosis* isolates from multiple drug resistant tuberculosis from Bombay. Indian. J. Clin. Microbiol.; 40: 2677-2680.

Narayanan S, Parandaman V, Narayanan P et al(2001). Evaluation of PCR using TRC4 and IS6110 primers in detection of tuberculous meningitis. J. Clin. Microbial.; 39: 2006-2008.

Narayanan S, Parandaman V, Vencatesan P et al. (2001). Evaluation of PCR using TRCH and IS6110 primers in detection of tuberculosis. *J Clin Microbiol*; 39: 2006- 2008.

NIAID. National Institute of Allergy and Infectious Disease (2006). Microbes in Sickness and in health.

Piersimoni C, Scarparo C, Callegaro A,(2001). Comparison of MB/BacT Alert 3D system with Radiometric BACTEC system and LJ medium for recovery and Identification of mycobacteria from clinical specimens. A multicenter study. *J. Clin. Microbiol*; 39: 651-657.

Rajasekaran S, Uma A, Kamakshi S, et al(2000). Trend of HIV infection in patients with tuberculosis in rural South India. *Indian J Tub* 2000; 47: 223-26.

Rattan A, Kalia A and Ahmed N(1999). Multidrug Resistant *M. tuberculosis*: Molecular Perspective. *Emerging Infections Diseases*; 4: 195-209.

Raviglione MC, Snider Jr DE, Kochi A(1995). Global epidemiology of tuberculosis. *JAMA* 1995; *273:* 220-227.

Reddi P P, Talwar G P, and Khanderkar P S(1993). Molecular cloning and characterization of continuously located repetitive and single copy sequences of *Mycobacterium tuberculosis* development if PCR based diagnostic assay. Int. J. Lepr.; 61: 227-235.

Roberts M C, Mc Millan C and Coyl M B(1987). Whole chromosomal DNA probes for rapid identification of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. J.Clin.Microbiol;25:1239-1243.

Runyon E. H. (1959). Anonymous mycobacteria in pulmonary disease. Med. Clin. North Amer. 43:273-299.

Sahadevan R, Narayanan S, Paramasivan CN, et al(1995).Restriction fragment length of polymorphism typing of clinical isolates of *M.tuberculosis* in Madras. *J Clin Microbiol.* (1995); 33: 3037-3039.

Sharma RK, Katoch K , Shivannaver CT et al (1995). Comparation of sensitivity of probes targeting RNA as DNA in tuberculosis cases. Indian J med microbiol; 14:99-104.

Sharma VD, Katoch VM, Shivannaver CT et al. (1995). Protein and Isoenzyme Patterns of mycobactreia . Their role in indication of rapidly growing mycobacteria. *Indian J Med Microbiol*;13: 115- 116.

Singh VB, Suresh, N, Bhanu et al (2004). Predominant tuberculosis spoligotype Delhi India. Emer infect bis; 10:1130-1142.

Singh HB, Chauhan DS, Das S, et al(2002). Rapid discrimination of Indian isolates of *Mycobacterium tuberculosis* by Random amplified polymorphic DNA analysis *Indian J. Med. Microbiol* (2002); 20: 69-71.

Sola C, Horgen L, Filliol(1999). Spoligotyping followed by double repetitive elements PCR as rapid alternative to IS6110 fingerprinting for epidemiological studies of tuberculosis. *J Clin microbial;36:1122-1124.*

Standford and Grange JM(1974). The meaning and structure of species as applied to mycobacteria tubercle 1974. 55: 143-152.

STP –Stop Tuberculosis partnership (2006). London tuberculosis rates now at third world population. PR new swire Europe Ltd://www.Prenwswier.co.uk

Supply P, Mazars E, Lesjean S et al(2000). Variable human minisatellite-like region in the *Mycobacterium tuberculosis* genome. *Mol. Microbiol.* (2000); 36: 762-771.

Swaminathan S, Ramachandra R, Baskaran G et al. (2002). Risk of development of tuberculosis in HIV infected patients. *Int J Tuber- Lung Dis; H(2002):* 839 .

Tortolis E, Mandler F, Tronicm et al(1997). Evolution of mycobacterial growth indicator tube (MGIT) compare with the BACTEC radio metric method BBL biphasic growth medium and LJ medium. J micro infect; 1997:3468.

van Soolingen (2001). Molecular epidemiology of tuberculosis and other mycobacterial infection, main methodologies and achievements.*J inter med;249:1-26.*

vander Zanden, G:M, Hoentjen AH, Hielmann FG et al. (1998). Simultaneous detection and strain differentiation of *Mycobactreium tuberculosis* complex in paraffin wax embedded tissue and in stained microscopic preparation.

van Embden JDA, Kremer K, Jansen R et al. (2000). Genetic variation and evolutionary origin of direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. *J Bacteriol;*182: 2393- 2401.

van Soolingen D, Hoogen Boezem T, de Hass et al. (1991). A novel pathogenic taxon of the *Mycobacteruim tuberculosis* complex; characterization of an exceptional isolate from Africa. *Int J Syst Bacteriol* 1991; 47: 1236- 1245.

Vestal AL (1977). In Procedure for the Isolation and Identification of *Mycobacteria*, US department of health, education and welfare.. Center for disease control, Atlanta, Georgia; (1977) Pub no. (CDC 77 – 8230).

Vestal AL,(1977). Procedure for isolation and identification of Mycobacterium. US dept of Health Education and welfare *Pub. No. CDC Atlanta: Georgia*, (1977); 15: 77-8230.

Warren R.M. Streicher EM, Sampson et al (2002). Micro evolution of the direct repeat region of *Mycobacterium tuberculosis* implication for interpretation of spoligotyping complex . *J Med microbial* (2002);*bio*:562-564.

Wayne LG and Kubica GP (1986). Genus *Mycobacterium In: Bergey's manual of systematic bacteriology*. Sneath PHA, Mair, NS Sharpe ME and Holt JG (eds) Baltimore: Williams and Wilkins: pp 1436 - 1457.

WHO (World Health Organisation in WHO weekly epid. Record (2006).

WHO Report (2004). Report of Tuberculosis Epidemic. World Health Organization.

WHO (2007). Tuberculosis Fact sheet N104; Global and Regional Incidence. http://www.who.int/entity/mediacentre/factsheets/southeastasia.

Woolinsky E (1979). Nontuberculosis Mycobacteria in soil and their relation of disease associated strains .Amer. Rev. Resp. Dis.; 97:1032-1037.

Zhang Y,Mazurek H, Cave MD et al (1992). DNA polymorphism in strains of *Mycobacterium tuberculosis* analysed by pulsed field gel electrophoresis a tool for epidemiology. *J Clin microbial 1992;30:1551-1556.*