ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Molecular Line Probe Assay: Genotype MTBDR *plus* for Rapid Detection of Primary Drug Resistance in *Mycobacterium tuberculosis* Isolates From A Tertiary Care Hospital in Western India.

***Patil Seema[1], Angadi Kalpana[2], Modak Meera[3] and Bodhankar MG[4]**

[1]Department of Medical Microbiology, Bharati Vidyapeeth Deemed University Rajiv Gandhi institute of Information technology and Biotechnology, Pune; Maharashtra-India.
[2]Department of Microbiology, Pad. Dr. D.Y.PatilMedical College, Hospital and Research centre (D.Y.Patil vidyapeeth Pune) Pimpri Pune-411018.
[3]Department of Medical Microbiology,Bharati Vidyapeeth Deemed University Medical college and Research center, Pune; Maharashtra-India.
[4]Department of Microbiology, Bharati Vidyapeeth Deemed University Yashwantrao Mohite College, Pune; Maharashtra-India.

ABSTRACT

It is reported that in spite of availability of anti mycobacterial drugs, tuberculosis (TB) remains one of the major health problems facing mankind particularly in developing countries. There is a rise in the trend of drug-resistant TB, especially multi drug resistant (MDR), in different parts of world and India being one of the high burden countries. This study was undertaken to assess the pattern of initial drug- resistance among confirmed pulmonary tuberculosis patients. To detect the primary drug resistance patterns among confirmed pulmonary tuberculosis patients and to identify the mutations in the genes responsible for drug resistance by using Geno Type MTBDR plus technique. This study was undertaken in a urban tertiary care medical college and hospital in Maharashtra over a period of one year in the department of microbiology. Study period: June 2011 to June 2012. A total of 118 pulmonary tuberculosis cases were studied. For each TB suspected patient, sputum smear microscopy and culture on Lowenstein- Jensen's (L.J) media were performed according to standard conventional procedures. Drug sensitivity testing(DST) was carried out by phenotypic gold standard proportion method and by a rapid molecular Genotype MTBDR plus assay which detects the resistance to INH (Isoniazid) and RMP (Rifampicin) by showing the mutations in the *katG* and /*inhA* and *rpoB* genes respectively. All 118 smear positive sputum samples were cultured successfully on L.J. media. Out of the 118 B cases, 5isolates (4.2%) showed resistance to 1st line anti-tuberculosis drugs by phenotypic DST method. Resistance pattern was noticed according to the bands of mutations in the *rpoB* genes (RMP-resistance) and *katG* and/*inhA* genes (INH-resistance) by using Genotype MTBDR plus method. Commonest (80%) mutations were observed inS531L region of *rpoB* gene while mutations in *katG* and *inhA* were observed in 60% and 40% isolates respectively. GenoType MTBDRplus assay is an appropriate tool for rapid screening for MDR-TB. In the present study, overall rate of primary drug resistance in *Mycobacterium tuberculosis* is higher as compare to other studies.

Key words: Line-probe assay (LPA), Primary drug resistance, *Mycobacterium tuberculosis, rpoB gene*

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Tuberculosis (TB) has been associated with significant morbidity and mortality. It still remains a major global health problem [1]. Worldwide 9.2 million new cases of TB and 1.7 million deaths from TB occurred per year [2]. India ranked first in terms of total numbers of TB cases in the world. India, China and Russian federation accounted for 62% of the estimated global burden of multi-drug resistant-TB (MDR-TB) [3]. MDR-TB is defined as TB that is resistant to at least to rifampicin (RMP) and isoniazid (INH) the two most important first-line anti-TB drugs [4]. MDR-TB is a challenge to TB controlled to its complex diagnosis and obstacles in treatment. In 2008, there were an estimated 440000 cases of MDR-TB globally [5]. Many mutations have been identified in different regions of these drug resistance genes and resistance to rifampicin has been shown to be associated with mutations in the 81bp rifampicin resistance-determining region (RPDR) of the RNA polymerase β-subunit (*rpoB*) gene[6] . The prevalence of MR-TB among new and previously treated cases is increasing all over the world as well as in India. As long as, MDR-TB is not verified, use of inadequate and hence ineffective antibiotics may lead to further spread of resistant bacteria and amplification of resistance therefore, rapid diagnosis and identification of MDR-TB is a prerequisite for appropriate treatment. New rapid molecular tests for 1st line drug resistance can be used to reduce the delays to detect MDR-TB and adapt the treatment. In 2008, the good performance of molecular line probe assays: Genotype MTBDR plus (Hain Lifescience GmbH, Nehren, Germany) have led WHO to approve their use for rapid screening of patient at risk of MDR-TB [7].

The Genotype MTBDR plus test is a PCR-based amplification and reverse blotting assay that employs specific probes hybridized to nitrocellulose strips to detect RMPand INH resistance. The assay detects mutations in the *rpoB* gene for RMP resistance, in the *katG* gene for high-level INH resistance and in the *inhA* regulatory region gene for low-level INH resistance [8,9].

In India, the burden of TB remains high and it is one of the leading causes of death in adults. In 2012, the prevalence of all forms of TB was estimated at 232/100,000, the incidence at 185/100000 and the mortality at 26/100000 [8-10]. In this context, this study was aimed to provide a preliminary data of rate of *Mycobacterium tuberculosis* resistance to INH,RMP in a tertiary care hospital, Pune; using a phenotypic proportion method and presence of mutations in the MDR-strains of *Mycobacterium tuberculosis* isolates by molecular line probe assay 'Genotype MTBDR plus'.

MATERIALS AND METHODS

Study design

This was a prospective, hospital-based clinic-microbiological observational study conducted at a tertiary care hospital in Maharashtra, India. The study was approved by the institutional ethics committee. Written informed consent was taken from patients.

Study population

All the patients attending the chest and TB units with symptoms suggestive of TB (cough for two weeks or blood stained sputum) were included regardless of their HIV status.

Inclusion criteria

New cases – never previously treated or beginning the treatment within the last month prior inclusion,

Exclusion criteria

Retreatment cases- a previously treated patient who was declared cured or completed treatment becomes sputum- positive again, and defaulters- who has taken treatment for at least 4 weeks and comes after interruption of treatment for 2 months and has active disease.

Specimen processing

Two sputum samples were collected for each patient, the 1^{st} during the consultation and the II^{nd} next day early in the morning. Screening for acid-fast bacilli (AFB) was performed by Ziehl-Neelsen stained smears. Specimens were digested and decontaminated by Petroff's method, then inoculated on two slants of L-Jmedium and incubated at 37^0c. Cultures were examined once in a week for 80 days. Any suspect colony confirmed as AFB by microscopy was identified by biochemical tests [6-10] and tested for antibiotic resistance by proportion (phenotypic) method and Genotype MTBDRplus technique. The details of these two techniques used for MDR detection are as follows:

Proportion method

All the culture positive strains, after sufficient growth were tested to detect drug susceptibility testing (DST) against primary anti-tuberculosis drugs -Isoniazide:0.2µg/ml, Ethambutol: 2µg/ml, Streptomycin: 4µg/ml and Rifampicin: 40µg/ml by economic variant of proportion method. All the isolates resistant to Isoniazid and Rifampicin was taken as multidrug resistant (MDR). For inoculation on to a pair of plain and drug containing L.J. media, the opacity/turbidity of inoculums was matched to McFarland no.1, against a black background the slopes were incubated at 37°C and examined weekly for 8 weeks for the growth. The definition of resistance is based on the amount of growth seen on the drug-free and drug-containing medium. The proportion of resistant bacilli existing in the strain was calculated by dividing the number of colonies in drug-containing media by that in drug free media. Below a certain value- the critical proportion- the strain is classified as sensitive; above that value, it is classified as resistant. The proportions were reported as percentages [10-15].

Quality control

For each batch of testing, a standard strain of H37RV of *Mycobacterium tuberculosis* (sensitive strain) was tested and validated.

In the present study out of 118 isolates of *Mycobacterium tuberculosis* 5 were MDR-TB strains which shows resistance to and INH and RMP.

Line Probe Assay (LPA): GenoTypeMTBDR plus

The GenoTypeMTBDRplus detects resistance to INHand RMP in *Mycobacterium tuberculosis* based on the detection of the most common mutations in *katG, inhAand rpoB* genes. All the 5 MDR strains of *Mycobacterium tuberculosis* were tested to detect mutations in *katG,inhAand rpoB* genes. The Genotype MTBDRplus (Hain life science, Gmbh, Germany) was used for *Mycobacterium tuberculosis* complex identification and INH and/ or RMP resistance detection according to manufacturer's instructions.

Briefly it consists of 3 steps: 1) DNA extraction from the *Mycobacterium tuberculosis*, 2) PCR-based DNA amplification and 3) Reverse hybridization.

1) DNA extraction: Growth of *Mycobacterium tuberculosis* was suspended in a 300µl of Mol.bio.Grade water and homogeneous suspension was prepared by using vortex mixing for 30 seconds, which is added in a 1.5ml micro centrifuge tube and spinning was carried out at 10000g for 15 seconds. Pellet was resuspended in 200µl of Mol.bio. Grade water and homogenization was carried out by vortex mixing for 30 seconds. It was then incubated in a dry bath at 95°c for 20 minutes and then for 15 minutes in an Ultrasonicator (to increase the quantity of DNA). Spinning of it was carried out at full speed for 5 mins and 5µl of the supernatant was used for PCR.
2) PCR- based DNA amplification: The amplification mixture contained 35µl of the primer nucleotide mix, 5µl of 10x polymerase incubation buffer, 5µl of 25 Mm $MgCl_2$,1µl of AmpliTaqGold polymerase (5U/µl; Biomerix), and 5µl of the supernatant of the cell lysate, for a final volume of 50µl. The amplification protocol consisted of denaturation at 95°C for 5 mins, followed by 10 cycles comprising 30 sec at 95°c and 2 mins at 58°C, an additional 20 cycles comprising 25 sec at 95°c, 40 sec at 53°C and 40 sec at 70°C, and then a final extension at 70°C for 8 mins.
3) Reverse hybridization: Hybridization and detection were performed with a Twincubator (Hain Lifescience GmbH, Nehren, Germany). The hybridization procedure included the following steps: chemical denaturation of the amplification products at room temperature for 5 min, hybridization of the single-stranded biotin-labled amplicons to membrane-bound probes at 45°C for 30 mins, stringent washes, addition of a streptavidin-alkaline phosphatase (AP) conjugate at room temperature for 30 mins, and an AP staining reaction to detect colorimetric bands.

Results were read by lining strips up to code provided with kit. In order for results to be valid, CC(Conjugate control) and AC(Amplification control) bands appeared for every sample. The presence of TUB band indicated that *Mycobacterium tuberculosis* complex in the sample. When all WT (wild type) probes showed positive staining for an isolate and

mutant probes demonstrated no staining, the isolate was considered susceptible. In contrast, the isolate was considered resistant when either any one of the WT probes was absent or any one of the mutant probe was present. The *rpoB, katG* and *inhA* each had a control band which was present in order to interpret the results. Mutation in *rpoB* predicts RMP resistance, *katG* predicts high level INH resistance and *inhA* predicts low level INH resistance. For the results to be valid the bands were of intensity equal to or greater than the intensity of the AC band. In order for a batch of results to be valid, the negative control strip had a CC and AC band present, but no other bands were visible. The product insert was referred for interpretation of banding patterns and troubleshooting [15-22].

RESULTS

118 *Mycobacterial* isolates were identified as belonging to the *Mycobacterium tuberculosis* complex. Characteristics of the patients: majority of patient's i.e 22.8% were found in the age range of 21-30 yrs, followed by 16.3% in 41-50 yrs range and 13.7% were in the age group of 31-40 yrs. Sex ratio(M/F) : 1.7:1

Table-1: Age and gender wise distribution of cases

AGE GROUP	MALE	FEMALE
0-10	01	01
11-20	08	10
21-30	15	12
31-40	12	04
41-50	13	06
51-60	13	06
61 onwards	09	06
Total	75	43

Drug resistance

Among 118 *Mycobacterium tuberculosis* isolates, 5 (4.2%) were MDR-TB strains showing resistance to both and INH and RMP: first-line anti-tuberculosis drugs by phenotypic gold standard proportion method on L.J. All these strains were subjected to line probe assay (LPA) to detect mutation pattern in the respective genes. Table no.-2 and shows the different patterns of mutations in the genes of these 5 MDR-TB strains. In 3 strains *rpoB WT8* gene was missing while missing of *rpoB WT7, 8 and rpoB WT4,5* was observed in two different strains. High level INH resistance was observed in 2 strains showing presence of *katG* MUT1 band while low level INH resistance was observed in 3 strains showing presence of *inhA* MUT3A in 2strains and *inhA* MUT3B in one strain. LPA was fond to be 100% sensitive for detection of MDR-TB strains.

ISSN: 0975-8585

Table no.-2: Pattern of gene mutations detected by LPA in *Mycobacterium tuberculosis* Isolates

Sample number	Banding patterns of MDR-TB isolates
1	Negative control
3	*rpoB WT8 missing and rpoB MUT3, katG MUT1 present*
4	*rpoB WT8 missing and rpoB MUT2A, inhA MUT3A present*
5	*rpoB WT8 missing and rpoB MUT3, inhA MUT3B present*
6	*rpoB WT7and8 missing and rpoB MUT3, inhA MUT3A present*
7	*rpoB WT4and5 missing and rpoB MUT3, katG MUT1 present*

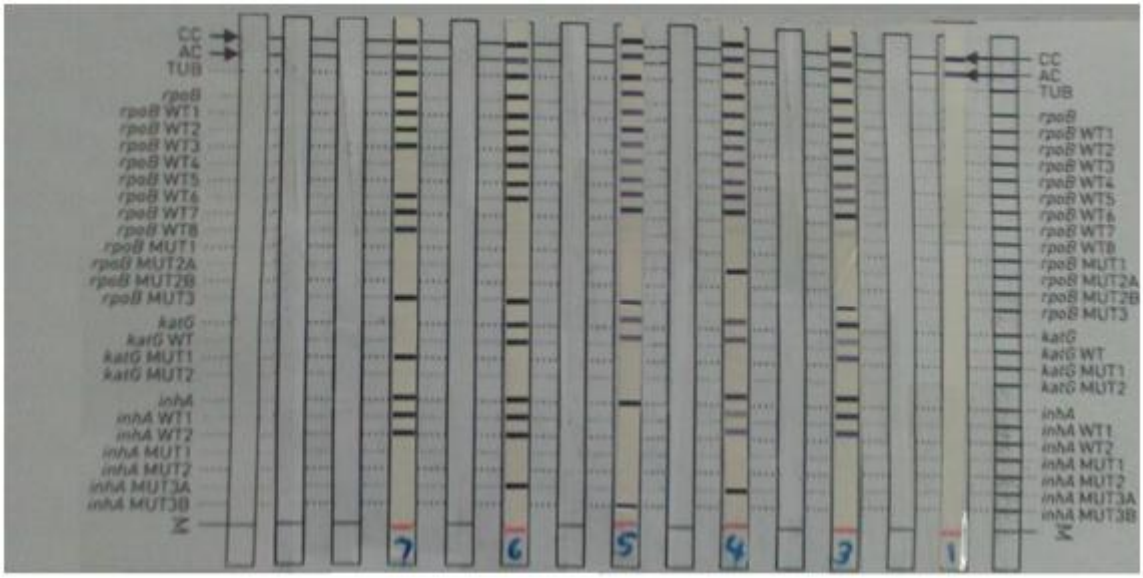

Figure 1 Representative DNA strip patterns obtained with the genoType MTBDR plus assay.

DISCUSSION

This study aimed to provide preliminary data on the rate of resistance to first-line anti-tuberculosis drugs, especially INH and RMP and pattern of mutations in the genes responsible for drug resistance. For studying this aspect, the phenotypic proportion method and genotypic method (LPA) were used.

Genotypic (LPA) Vs Phenotypic (Proportion)

It is reported that in recent years substantial progress has been made in our understanding of the molecular basis of *Mycobacterium tuberculosis* drug resistance. Molecular based assays are potentially the most rapid and sensitive methods for the detection of drug resistance. These assays detect all common drug resistance mutations. Some of these techniques include direct sequencing of PCR products, SSCP analysis, heteroduplex analysis, dideoxy fingerprinting, an RNA/DNA duplex, base-pair mismatch assay, Lucifer's mycobacteriophage strategy, a rRNA/DNA- bioluminescence-labeled probe method, a reverse hybridization- based line probe assay, add end other strategies [18-23] . In the present study GenoType MTBDR*plus* (Line probe assay) was used which detects

resistance to INH and RMP in clinical isolates based on the detection of the most common mutations in *rpoB, katG* and *inhA* genes. It uses PCR and reverse-hybridization to probes immobilized on a assay strips [23-28]

All the 5 MDR strains detected by phenotypic methods have shown the mutations in the rpoB gene (RMP-resistance) and *katG /inhA* (INH- resistance) by GenoType MTBDR*plus* molecular method (Table No.2). Thus the sensitivity for MDR, RMP, INH detection was found to be 100%. The same 100% sensitivity for MDR-TB detection by this method was reported by Kembhavi et.al. While lower sensitivity (92.5%) was reported by Farroqui *et.al.* and 97.7% by Maurya *et.al*. [17] . The commonest pattern seen was missing of WT8 and presence of MUT3 in *rpoB* in 4 (80%) strains while presence of *rpoB MUT2A* was seen in one strain. Low level INH resistance was observed in 60% strains while high level resistance was observed in 40% strains. The similar pattern of mutations was reported by Kembhavi *et.al*., Farroqui *et.al*., Maurya *et.al*. Details of these studies are given in Table no.3.

Table No. 3: showing comparison of results of studies based on MTBDR plus

Study	Technique used	MDR sensitivity	Mutations RMP	Mutations INH
Farroqui *et.al*.2012	MTBDR*plus*	92.5%	531,533 S531L	*S315T:katG C15T:inhA*
Maurya *et.al*. 2013	MTBDR*plus*	97.7%	S531L	*S315T1:katG*
Kembhavi *et.al.* 2013	MTBDR*plus*	100%	531,526,516 , S531L	*S315T1:katG C15T:inhA*
Present study	MTBDR*plus*	100%	S531L	*S315T:katG C15T:inhA*

Rifampicin resistance (RMP)

Rifampicin is a powerful bactericidal agent used as a first line anti-tubercular drug. Rifampicin resistance arises due to mutations in *rpoB* gene DNA dependent RNA polymerase. The nature and frequency of mutations in the gene of RMP resistant isolates vary considerably according to geographical location. Analysis of approximately 500 rifampicin resistant strains from global sources has found that 96% of rifampicin resistant clinical isolates of *Mycobacterium tuberculosis* have mutations in the *81- bp core region of rpoB* gene, which encodes the β-subunit of RNA polymerase. Detection of mutations in the 81 bp core region correlated (100%) very highly in their study. Similar findings have been reported by Barnard *et.al.* and Ravindran *et.al*. The sensitivity of RMP can be decreased if mutations are outside the 81-bp region of *rpoB* gene which cannot be detected by this assay. We found a higher (80%) proportion of RMP resistance due to S531L mutations. Kembhavi *et.al*.also found higher proportion (89.65%) of RMP resistance due to S531L in their study but the lower proportion (62.3%) and (62%) of RMP resistance was reported by Maurya *et.al*. and Farooqi *et.al*. respectively [25-30].

INH résistance

INH is also a bactericidal agent used as a first line drug for TB. Resistance to INH arises due to mutations in different genes including *katG, inhA, ahpc* andand other genes that remain to be established. In the present study, *katG* mutations were found in 60% and

40% in *inhA* genes. Kembhavi *et.al*. had reported higher (75.86%) *katG* mutations and lower (20.66%) *inhA* mutations. Maurya *et.al.* had also reported commonest INH mutations in *katG* (93.3%) and lower (28.9%) in *inhA* genes. Similarly Farooqi *et.al.* had reported the higher proportion (66.1%) of mutations in *katG* genes and lower proportion (1.9%) of mutations in *inhA* genes. High prevalence of *katG* mutations has been reported to confer resistance in high prevalence countries [24] and for a much lower proportion in lower TB prevalence settings presumably due to ongoing transmission of these strains [25-27].Geographical profile of *rpoB* mutations in *Mycobacterium tuberculosis* was studied by Lingala *et.al.* reported that74% of RMP resistant isolates in their study includes common mutations at 531,526 and 516 region of rpoB gene. They have also reported the multiple silent mutations between 145-184 (outside the hot spot region) in their study [23-25].

The main objective of our study is detection of MDR rate in new cases (Initial drug resistance).It is defined as the patients who have never been treated previously or treated for less than one month. The present overall rate of MDR-TB in India is 12-17% in among retreatment cases and 2-3% in new cases. In the present study the MDR rate in new cases was 4.2%. The increase in MDR rate can be attributed to the emergence of HIV co infection, lack of awareness about treatment among the infected, non-availability of well-equipped diagnostic laboratory and family commitments. In this study we found that LPA is an appropriate tool for rapid screening for MDR-TB and has the potential to substantially reduce the turnaround time of DST results by phenotypic methods. However, WHO recommendations on infrastructure, training, assurance and other requirements should be followed to ensure high quality results.

CONCLUSION

Use of automated, molecular assays will help in early detection of drug-resistant cases and also helps to detect mutations. But these systems should be made affordable as the prices are higher than the conventional tests.

Competing interests

There are no competing interests.

REFERENCES

[1] Swaminathan S,Narendran G, Journal of Biosciences, 2008; 33(4): 527-37.
[2] Gillespie SH. Antimicr Agent Chemother 2002; 46(2): 267-274.
[3] Sam I, Drobniewski F, More P, Kemp M, Brown T. Emerging Infectious Diseases 2006; 12(5): 752-759.
[4] Hirano K, Abe C, Takahashi M. J Clin Microbiol 1999; 37: 2663-2666.
[5] Cavusoglu C, Hilmiioglu S, Bilgic A. J Clin Microbiol 2002; 40: 4435-4438.
[6] Bartfai Z, Somoskovi A, Kodmon C, Szabo N, Puskas E, Kosztolanyi L, Farago E, Mester J, Parsons LM, Salfinger M. J Clin Microbiol 2001; 39(10): 3736-3739.
[7] Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. J Clin Microbiol 2006; 44(10): 3659-3664.
[8] otto P, Piana F, Penati V, Canducci F, Migliori GB. J Clin Microbiol 2008; 46: 393-394.

[9] http://www.hain-lifescience.com/pdf/304xx_pbl.pdf
[10] Skenders G, Holtz TH, Toma A, Prokopovica I, Broka L, Wells CD, Leimane V. Int J Tuberc Lung Dis 2007; 11(11) Suppl 1.
[11] Whiting P, Rutjes AW, Reitsma JB, Bossuyt PM, Kleijnen. BMC Med Res Method 2003; 3: 25.
[12] Baron EJ, Finagold SM. Mycobacteria. In: Nancy, editor. Baily and Scott's Diagnostic Microbiology. 9th ed. St. Luis: The CV Mosby Company; 1994. p. 590-633.
[13] Pitchenik AE, Fertel D, Bloch AB. *Clin Chest Med* 1988; *9* : 425-41.
[14] Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. Am J Resp Crit Care Med 2008; 77: 787-792.
[15] Siddiqi N, Shamim MD, Jain NK, Rattan A, Amin A, Katoch VM,et.al. Mem Inst Oswaldo Cruz, ARio de Janeiro. 1998;93(5):589-594.
[16] Ahmad S, Araj GF, Akbar PK, Fares E, Chung TD, Mustafa AS. Dian Microbiol Infect Dis.2000;38:227-32.
[17] Valim A, Rossetti M, Ribeiro M et.al. J Clin Microbiol. 2000:3119-22.
[18] Ravindran R, Wattal C, Oberoi JK, Goel N, Datta S, Prasad KJ. Indian J Medical Microbiol. 2012;30:58-63.
[19] Ramswamy S, Musser J. Tubercle and Lung disease. 1998;79:3-29.
[20] Zhuang Y, He X, Zhang X. Zhonghua Jie He Hu Xi Za Zhi 2000;23:711-4.
[21] Mokrousov I, Narvskaya O, Otten T, Limeschenko E, Steklova L, Vyshnevskiy B. Antimicrob Agents Chemother 2002;46:1417-24.
[22] Lingala MAL, Srikantam A, Jain S, Rao KVSM, Rao PVR. J Microbiol Antimicrob. 2010;2(2):13-18.
[23] Molecular Line Probe Assays for rapid Screening of patients at risk of Multidrug-resistnat Tuberculosis (MDR-TB): WHO policy statement. 27 June, 2008. Available at http://www.who.int/tb/laboratory/line_probe_assays/en/index.html.
[24] Morgan M, Kalantri S, Flores L, et al. BMC Infect Dis 2005;5: 62.
[25] Heifets, L. B., and R. C. Good. 1994. Current laboratory methods for the diagnosis of tuberculosis, p. 85–110. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
[26] Chain, K. 1995. Clinical microscopy, p. 33–51. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
[27] Maurya AK, Singh AK, Kant S, Umrao J. Kumar M, Kushwaha RAS,Nag VL, Dhole TN. Ind J Med Microbiol,2013;31(3):230-236.
[28] Savita V.Jadhav, Chanda R.V., Nabamita C., Neetu S.G., Nageswari R.G., Misra R.N. Journal of clinical and diagnostic research 2013; 7(9):1996-1998.
[29] http://www.who.int/tb/dots/laboratory/lpa_policy.pdf. [Last accessed on 2012 Aug 18.
[30] Telenti A, Honore N, Bernasconi C, March J, Ortega A, Heym B, et al. J Clin Microbiol 1997;35:719-23.