MOLECULAR DIAGNOSIS OF DRUG RESISTANCE TUBERCULOSIS IN THE DISTRICTS OF TAMILNADU

SURESHBABU.R[1,2]. LAKSHMI MURALI. A[2] AND PALANISWAMY.M[1]*

[1.] *Department of Microbiology, Karpagam University, Coimbatore, Tamil Nadu, India.*
[2.] *Intermediate Reference Laboratory/State Tuberculosis Training and Demonstration Centre, State TB Office,Tamil Nadu, India.*

ABSTRACT

To diagnose the multi-drug resistant *Mycobacterium tuberculosis* by using molecular methods from pulmonary specimens of presumptive TB suspects belonging to the districts of Tamil Nadu. Two thousand three hundred and thirty five (2335) clinical specimens of presumptive MTB patients were collected from 23 districts of Tamil Nadu between the period of January and March 2015. Smear microscopy was performed by LED fluorescent microscopy. All smear positive samples were tested using Line Probe Assay (LPA) and smear negative samples were tested by Xpert MTB/RIF (Xpert) to detect the percentage of drug resistance pattern and to identify MTB complex. Among 1235 smear positives subjected to LPA method; 116 (9.4%) MTB was not detected and 3 (0.2%) showed invalid result; 1116 (90.4 %) strains showed MTB positive; 896 (80.3 %) were sensitive for both rifampicin (RIF) and isoniazid (INH) drugs; 128 (11.5 %) were resistance for INH; 18 (1.6 %) resistant for RIF and58 (5.2 %) were resistance for RIF and INH and smear negative specimens were subjected to Xpert, Out of 1090 specimens; 647 (59.4 %) MTB was not detected; 30 (2.8 %) showed an invalid/error results; 413 (37.9 %) strains showed MTB positive of which 379 (91.8 %) were sensitive for RIF, 24 (5.8 %) showed resistant for RIF and 09 (2.2 %) showed MTB detection and RIF Indeterminate. LPA and Xpert molecular technology are the rapid, feasible and reliable methods for the detection of multi drug resistant (MDR) mutation.

Key Words: Multi Drug Tuberculosis, LPA, Gene Xpert, Rifampicin and Isonizid.

PALANISWAMY.M
Department of Microbiology, Karpagam University, Coimbatore, Tamil Nadu, India

INTRODUCTION

World Health Organization (WHO) estimated, nine million people fell ill with TB and 4,80,000 people developed Multi Drug Resistant TB (MDR-TB) and 2,10,000 deaths were estimated in 2013.[1] Isolation, identification and drug susceptibility testing (DST) of MTB take several weeks, because of its slow growth rate. Many methods have been developed which can potentially reduce the diagnostic time from days to hours.[2] MDR-TB resistance to both RIF and INH is a global problem with an estimated death of 1,90,000 cases in 2014.[3] Detection of tuberculosis and drug resistance pattern are slow by means of phenotypic method. Availability of molecular diagnosis has made possible the early detection of drug-resistant tuberculosis (DR-TB).[4] Genotypic rapid molecular methods for the diagnosis of isoniazid and rifampicin resistance in MTB are based on the detection of genetic mutation in the *rpoB* gene, which encodes RNA polymerase enzyme of MTB and *katG* gene encoding the mycobacterial catalase peroxidise which is the only enzyme in MTB capable of activating the pro-drug INH to its active form.[5] Mutation in the *rpoB* gene is responsible for majority of RIF's resistance in MTB and the *inhA* shows low level isoniazid resistance. GenoType®MTBDRplus is one such commercially available assay which has been previously evaluated in Europe, South Africa, Far East and North America.[6-9] INH and RIF are the key first-line anti-tuberculosis drugs and resistant to these drugs denotes MDR-TB.[10-11] This multiplex Polymerase Chain Reaction (PCR) based reverse hybridization genotype MTBDR rapid line probe assay is to detect the most common mutations of *katG* and *rpoB* from smear positive sputum specimens and MTB growth positive cultures, either from liquid or solid, and it has the potential to shorten the overall turnaround time from specimen receipt to reporting of results of susceptibility testing.[12] Xpert MTB/RIF is an automated molecular test for MTB and to identify the resistance to RIF, uses real-time PCR assay to amplify an MTB specific sequence of the *rpoB* gene. The Xpert assay utilizes molecular beacon technology to detect DNA sequences amplified in a hemi-nested RT-PCR assay.[13-14] Five different nucleic acid hybridization probes are used in the same multiplex reaction.[15-16] Each probe is complementary to a different target sequence within the *rpoB* gene of rifampicin susceptible MTB and is labelled with a differently coloured fluorophore. Together, these overlapping probes span the entire 81 bp core region of the *rpoB* gene. The assay utilizes single-use plastic cartridges with multiple chambers that are preloaded with liquid buffers and lyophilized reagent beads necessary for sample processing, the Xpert cartridge is then inserted into the Xpert device, DNA extraction and heminested RT-PCR , this provides results within 2h.[17-20] Foundation for Innovative New Diagnosis - FIND initiated and signed MOU with India and Tamil Nadu on 2010 for implementation of rapid diagnosis. As per Central TB Division (CTD) report in 2013, 23289 and 1548 MDR cases were diagnosed in India and Tamil Nadu respectively. The present study is to detect the percentage of mutation in *rpoB* (Rifampicin), *katG* and *inh A* gene (Isoniazid) in MTB isolates from Tamil Nadu.

MATERIALS AND METHODS

Clinical specimens

A total of 2335 specimens of MDR suspects were received for culture and drug susceptibility testing for MTB from 23 District TB centre (DTC) of Tamil Nadu from January to March 2015. These specimens were collected, packed and transported as per standard operating procedure from DTC's to Intermediate Reference Laboratory (IRL). Ten specimens were rejected due to leakage and for 2325 specimens, the smear microscopy was performed by fluorescent microscopy method (Auromine-O)-LED FM.[21] The smear positive specimens were subjected to LPA genotypic method. Genotype MTBDR line probe assay (LPA) (Hain Life science GmbH, Nehren, Germany) was carried out according to the manufacturer's instruction.[22] Smear negative specimens were processed by Xpert automated molecular method.[23-24]

Specimen digestion and decontamination

Collected specimens were processed by NALC–NAOH (4%) method (N –acetyl –L cystine – sodium hydroxide with sodium citrate 2.9 %) and neutralized with Phosphate buffer solution pH 6.8. Centrifuged at 3000g for 20 min and the supernatant was discarded and the pellet was reconstituted with 1 ml of PBS, after discarding the supernatant .[25]

Genotype MTBDR Assay

Rapid genotypic method Line Probe Assay (LPA) was used for the detection of MTB and their drug resistance pattern to RIF and INH, from various districts of Tamil Nadu smear positive specimens were processed by GenoType® MTBDR plus V2.0 (LPA method) according to the manufacturer's instruction.[2-22]

Xpert MTB/RIF technology

Smear negative specimens were processed by Xpert MTB/RIF, an automated molecular test for MTB to identify the organism as well as resistance to rifampicin. Xpert uses real-time polymerase chain reaction (PCR) assay to amplify an MTB specific sequence of *rpoB* gene.[23-24]

RESULTS

A total of 2335 specimens were received in the study period at the IRL,Chennai, Tamil Nadu. Of these,2325 specimens were processed by NALC-NAOH method. Based on smear microscopy, 1235 were observed as smear positive and 1090 as smear negative. Smear positive specimens were processed by LPA method and the results are shown inflow chart. Out of the 1235 smear positive subjected to LPA method; in 116 (9.4%) specimens MTB was not detected and 3 (0.2%) showed invalid results.1116 (90.4 %) specimens showed MTB positive of which 896 (80.3 %) were sensitive for both Rif and INH drugs. 128 (11.5 %) were resistant to INH, 18 (1.6 %) were resistant to RIF and 58 (5.2 %) were resistant to both Rif and INH. Smear negative specimens were subjected to Xpert, Out of 1090 specimens; MTB was not detected in 647 (59.4 %) and 30 (2.8 %) showed invalid/error results. 413 (37.9 %) specimens showed MTB positive of which 379 (91.8 %) were sensitive to RIF. 24 (5.8 %) showed resistance to RIF and 09 (2.2 %) showed RIF indeterminate results.

Figure1
*Total TB suspects tested and the outcome of results
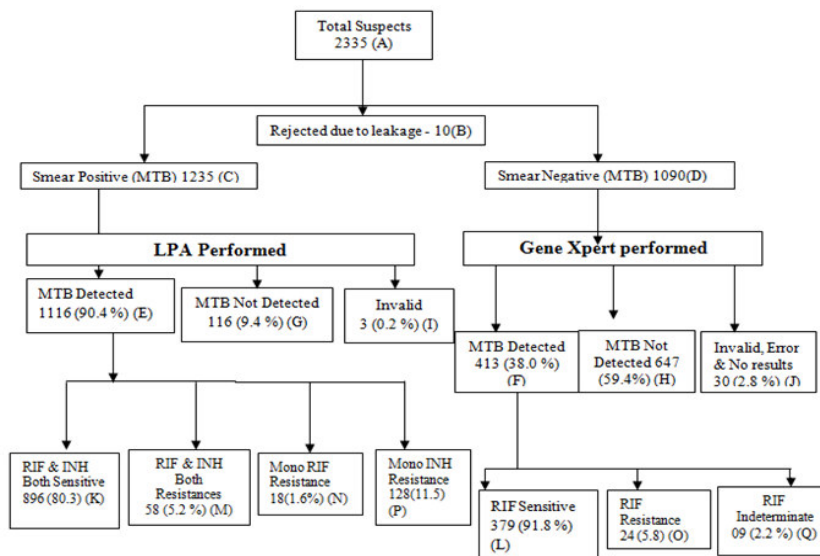by molecular technology*

Table 1
*Age and gender wise distribution of presumptive TB cases
with MTB and MDR detection*

Age (Yrs)	Gender	Total suspects tested (A-B)	MTB detected (E+F)	MTB detected (%)	Mono INH Res (P)	Mono INH Res (%)	Mono RIF Res (N+O)	Mono RIF Res (%)	Both INH & RIF res (M)	Both INH & RIF res (%)	MTB Not detected (G+H)	MTB Not detected (%)
0-15	Male	7	1	14.3	0	0.0	0	0.0	0	0.0	6	85.7
	Female	9	3	33.3	1	11.1	0	0.0	0	0.0	6	66.7
15 - 30	Male	211	153	72.5	11	5.2	7	3.3	8	3.8	48	22.7
	Female	137	101	73.7	4	2.9	2	1.5	5	3.6	38	27.7
31- 45	Male	629	457	72.7	39	6.2	11	1.7	20	3.2	159	25.3
	Female	166	83	50.0	5	3.0	4	2.4	1	0.6	82	49.4
46 - 60	Male	682	489	71.7	52	7.6	13	1.9	14	2.1	206	30.2
	Female	153	63	41.2	1	0.7	2	1.3	5	3.3	70	45.8
> 60	Male	276	159	57.6	12	4.3	3	1.1	5	1.8	113	40.9
	Female	55	20	36.4	3	5.5	0	0.0	0	0.0	35	63.6
	Total	2325	1529	65.8	128	5.5	42	1.8	58	2.5	763	32.8

Figure 2
*The difference between suspects, MTB detected, RIF and INH resistance
among male and female patients*
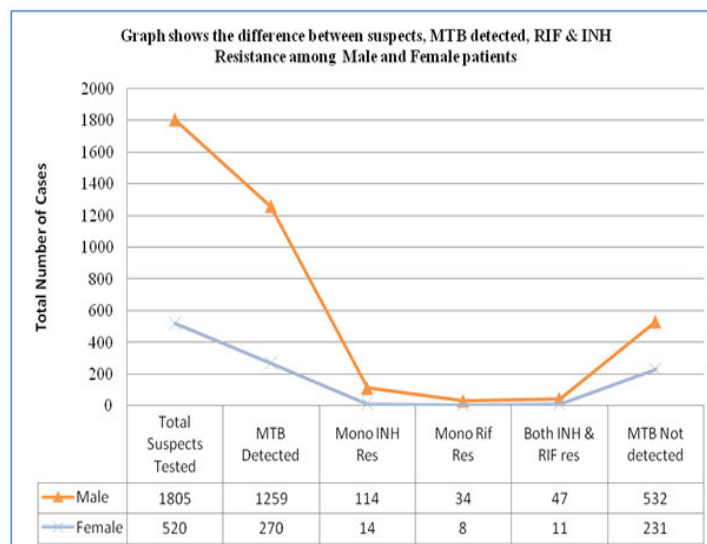
DISCUSSION

There is an absolute necessity for application of new technology for rapid diagnosis of tuberculosis on day to-day basis, which is the need of the technology in developing countries like India where the prevalence of TB is high. It is based on methods that permit recognition of mycobacterium products in clinical specimens. Genotypic testing is useful for rapid identification of *M. tuberculosis* in pulmonary samples, and results may be generated within a few hours. In addition, this technique has a higher sensitivity than sputum AFB smear microscopy. Genotypic approach based on amplification of MTB DNA by PCR in clinical specimens is a rapid and valuable screening method to overcome the limitations of the conventional techniques and it may have high impact on patient health and further will be in control of transmission of the disease. The current study is the first study to be carried out in the state of Tamil Nadu with such a large sample size (2335) for detection of Mycobacterium and INH and RIF resistance. In our study 2335 specimens were transported from 23 districts of Tamil Nadu of which 10 (0.42 %) samples were rejected due to inappropriate quality sample collection and packing .The smear positive specimens were processed by LPA method. Of the 1235 smear positive subjected to LPA method; 1116 (90.4 %) specimens were positive for MTB; 11.5 % were identified to be mono resistant to INH; 1.6 % were identified to be mono resistant to RIF and 5.2 % were identified as MDR (resistant to both Rif and INH). The resistance patterns of these MTB strains using LPA for detection for RIF and INH resistance were observed to be 6.2% and 11.5% respectively this percentage has been observed to be uniform for both the drugs across districts in Tamil Nadu and which is lesser than other published study, ie MDR 9 % and Mono RIF resistant11 %.[26] Smear negative specimens were subjected to Xpert technology, Out of 1090 smear negative specimens;37.9 % specimens were showed MTB positive; 5.8 % showed resistance to RIF, as per study conducted across India the positive predictive value for detecting rifampicin resistance among smear negative using Xpert MTB/RIF is 5.1 % .[27] Using XPERT technology MTB was detected in413 (37.9 %).This percentage is low, since the samples from people living with HIV (PLHIV)patients and smear negative patients were tested even though our study shows higher percentage of MTB detection compared to Gidado *et al* study.[28] Challenges in the amplification of mycobacterial DNA on extraction from biological specimens such as sputum are due to several inhibitors in these specimens, which may lead to several errors.LPA invalid results were very minimal (0.2 %) and Xpert error/invalid was observed in 2.8 % which is lesser than other study ie 4 %.[24] In our study, it has been observed that the number of female suspects are very less compared to male suspects (Fig.2),which is similar to the other study ie, only 28 % females were suspected themselves for testing, which may be due to several reasons such as ignorance, social stigma towards the disease, and reluctant attitude towards self-

health and also the high prevalence of MTB (63.6%) between the age group of 30 and 60 yrs (Table.1), this is similar to that described by Holmes et al.[29] This lower notification rates are reported among women, whereas men having high rates of notification are at older adolescence. The limitation of this study is related to the Socio-economic characteristics. Clinical information details were available in DTC and not documented in IRL; Only LPA and Xpert technique for 2325 samples have been performed and not compared with other techniques to detect INH and RIF resistance. Studies have shown that solid culture from smear negative specimens for MTB diagnosis and susceptibility testing, may take up to 84 days. The application of molecular methods in the study has enabled early diagnosis (2h) from smear negative cases using Xpert technology by 38 %. This technology is rapid for diagnosis of MTB/RIF resistance, which in-turn has reflected in early treatment and thus reduction in transmission of MDR in the society. Hence, these methods are simple and easy to use.

CONCLUSION

Based on the molecular methods, the diagnosis of MDR TB from presumptive TB suspects of 23 districts in TN, the following pattern of resistance have been observed in our study: among the smear positives 11.5 % have been observed to be resistant to INH, 6.2 % resistant to RIF and 5.2 % resistant to both INH and RIF using LPA facility. Among the smear negative 5.8 % were observed to be resistant to RIF using Xpert facility. It is also observed that the presumptive TB suspects were less in females 22.4 % than male patients 77.6 % and MTB infection is more common 73% in the male adults in the age group 30 to 60 Yrs. This study also clearly demonstrates the rapid diagnosis of MTB from smear negative patients, which in-turn would help in appropriate treatment and thus reduction in transmission of MDR-TB in the society.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

Conflict of interest declared None.

REFERENCES

1. Global Tuberculosis Report , World Health Organization, WHO Press, Switzerland: 2014.p.13-15 Available from; http://www.who.int/tb/publications/global_report/gtbr 14_main_text.pdf

2. Hillemann D, Weizenegger M, Kubica T, Richter E, Niemann S.Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in Mycobacterium tuberculosis complex isolates. J Clin Microbiol 2005;43:3699-3703.

3. Global Tuberculosis Report, World Health Organization, WHO Press, Switzerland: 2015.p.13-15Availablefrom;http://apps.who.int/iris/bitstream/10 665/191102/1/9789241565059_eng.pdf

4. Anals Sharma SK, Mohan A. Multidrug-resistant tuberculosis. Indian J Med 2004; 120: 354-376

5. Ali A, Hasan Z, Moatter T, Tanveer M, Hasan R. M. Tuberculosis Central Asian Strain MDR isolates have more mutations in rpoB and katG genes compared with other   genotypes.   Scand   J Infect Dis; 2009; 41: 37-44.

6. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 2006; 368:1575–1580.

7. GenoType MTBDRplus, Molecular Genetic Assay for Identification of Resistance to Rifampicin and/or Isoniazid of the Mycobacterium tuberculosis complex,version1.0[Internet]2007,(Feb) p.23Availablefrom:http://www.biocentric.com/notices /304xx_pbl.pdfBarnard, M., H. Albert, G. Coetzee, R. O'Brien, and M. E. Bosman. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am. J. Respir. Crit. Care Med. 2008;177:787-792.

8. Miotto P, Piana F, Penati V, Canducci F, Migliori GB, Cirillo DM. Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in Mycobacterium tuberculosis clinical strains isolated in Italy. J Clin Microbiol 2006;44: 2485-2491.

9. Orenstein EW, Basu S, Shah NS, Andrews JR, Friedland GH, et al. Treatment outcomes among patients with multidrug-resistant tuberculosis: systematic review and meta-analysis. Lancet Infect Dis.2009; 153–161

10. Johnston JC, Shahidi NC, Sadatsafavi M, Fitzgerald JM, Treatment outcomes of multidrug-resistant tuberculosis:a systematic review and metaanalysis,2009;4:6914.

11. Inderlied, C. B., and M. Salfinger. Antimycobacterial agents and susceptibility tests,. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken, Manual of Clinical Microbiology, 7th ed. ASM Press, Washington, D.C,p.1999;1601–1623

12. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. Nat.Biotechnol, 1996; 14:303–308.

13. Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination. Nat. Biotechnol. 1998; 16:49–53.

14. Piatek AS, Tyagi S, Pol AC, et al. Molecular beacon sequence analysis for detecting   drug resistance in mycobacterium tuberculosis. Nat. Biotechnol. 1998;16:359–363.

15. Piatek AS, Telenti A, Murray MR, et al. Genotypic analysis of mycobacterium tuberculosis   in   two distinct populations using molecular beacons: implications   for   rapid   susceptibility   testing. Antimicrob. Agents Chemother. 2000; 44:103–110.

16. Hillemann D, Rusch-Gerdes S, and Richter E, Application of the Genotype MTBDR assay directly on sputum specimens. Int J Tuberc Lung Dis. 2006;10: 1057-1059.

17. Helb D, Jones M, Story E, et al. Rapid detection of mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J. Clin. Microbiol. 2010; 48:229–237.

18. Blakemore R, Story E, Helb D, Evaluation of the analytical performance of the Xpert(R) MTB/RIF assay. J. Clin. Microbiol. 2010; 48:249–251.

19. S.R.Beenish, S.Sarman et al., Comparison of Xpert MTB/RIF with Line Probe Assay for Detection of Rifampicin-Monoresistant   Mycobacterium tuberculosis. J. Clin. Microbiol.2014;52:1846–1852.

20. M.Bhalla, Z.Sidiqi P.P. Sharma, R.Singhal, V.P.Myneedu,R. Sarin, Performance of light-emitting diode Fluorescence microscope for diagnosis of tuberculosis, Int.J.Mycobacteriol. 2013;174-178.

21. Barnard M, Somoskovi A, Molecular Detection of Drug- Resistant Tuberculosis By Line Probe Assay, laboratory manual.2012;22:65 – 104.

22. Bang, D., A. B. Andersen, and V. O. Thomsen. Rapid genotypic detection of rifampin- and isoniazid-resistant Mycobacterium tuberculosis directly in clinical specimens. J. Clin. Microbiol.2006;44:2605–2608.

23. Miotto, P, F. Piana, V. Penati, F. Canducci, G. B. Migliori, and D. M. Cirillo, Use of   Genotype MTBDR assay for molecular detection of rifampin and isoniazid   resistance in Mycobacterium tuberculosis clinical strains isolated in Italy. J. Clin. Microbiol. 2006;44:2485–2491.

24. Kent   PT,   Kubica   GP..   Public   health mycobacteriology: a guide far a level III laboratory. Centres for Disease control and prevention, Emerging Infectious Diseases 2006;12:752-759.

25. Johnson R, Helden V, Victor CT. Drug susceptibility testing using molecular techniques can enhance tuberculosis diagnosis, J Infect Countries 2008; 2:40-45

26. Sachdeva K.S, Paramasivan C.N, Use of Xpert MTB/RIF in decentralized public health settings and its effect on pulmonary TB and DR-TB case finding in India. PLoS ONE 2015;10(5):1-18.

27. M.Gidado, O Onazi , J O Obasasanya, N Chukwueme Strategic approach for Xpert MTB/RIF implementation and results from Nigeria. The International Journal of Tuberculosis and lung Disease.2013;3

28. Holmes.C.B, Hausler.H, Nunn.P A review of sex differences in the epidemiology of tuberculosis, INT J Tuberculosis Lung Diseases1998; 2(2):96-104.