Indian J.Sci.Res. 8(1) : 131-136, 2017

ISSN : 0976-2876 (Print)
ISSN : 2250-0138 (Online)

UTILITY OF LINE PROBE ASSAY FOR IDENTIFICATION OF MDR-TB AND NTM IN SMEAR POSITIVE SPUTUM SAMPLES FROM A TERTIARY CARE HOSPITAL OF WESTERN U.P. INDIA

SANA NUDRAT[1a], UMAR FAROOQ[b] AND SHIVENDRA MOHAN[c]

[ac]Department of Microbiology, Teerthankar Mahaveer Medical College, Moradabad, UP, India
[b]Department of Microbiology, Teerthankar Mahaveer Medical College, Moradabad, UP, India

ABSTRACT

Detection of the MDR & XDR status and NTM identification from smear positive sputum samples by Line Probe Assay Study was performed on smear positive sputum samples collected from DOTS centre at TMMC & RC, Moradabad during period of July 2015 to March 2017. Samples were decontaminated by Pettrof method and then DNA was extracted by using heating method, and then were subjected to PCR using Applied Biosystem Veriti 96 well Thermal Cycler and Line Probe Assay by using GenoType MTBDRplus on Twincubater of HAIN life sciences.Total 200 smear positive sputum samples were processed by line probe assay, in which 16 were MDR, 2 were XDR and 10 were detected as NTM. In this study, we concluded that, on routine basis we should use line probe assay for drug sensitivity of *M.tuberculosis* cases, so that appropriate treatment can be given on time to the patients.

KEYWORDS : MDR-TB, XDR-TB, NTM, PCR, LPA

In developing countries the second cause of death is Tuberculosis (TB). (Singh S. et al; 2016) *Mycobacterium tuberculosis* is an infectious organism, which causes Tuberculosis that mainly affects lungs and respiratory system. According to W.H.O, in 2016, number of people infected with tuberculosis was 10.8 million and 1.8 million deaths were recorded. 480,000 people developed resistant to anti-tubercular drugs that are Multi-drug resistant tuberculosis. (WHO Surveillance Report, 2008) Multi-drug resistant tuberculosis (MDR) is a form of tuberculosis that is resistant to rifampicin and isoniazid. (Nudrat and Farooq, 2016) Extensively drug resistant tuberculosis (XDR) is a form of tuberculosis that is resistant to isoniazid and rifampicin with resistant to one of the flouoquinolone as well as resistant to at least one of the second line injectable drug. (Singh S. et al; 2016) In 1993 Tuberculosis was declared as a global health problem by World Health Organization (WHO). (Khairbam et al*.,* 2016) There, has been a corresponding rise in the incidence of drug resistant strain of *Mycobacterium tuberculosis* just because of the increased incidence of tuberculosis. Tuberculosis was one of the top ten causes of death in 2015, worldwide. (Dustdar et al; 2008)

TB control program based on directly observed treatment short course (DOTS) policy is essential for preventing the emergence of MDR-TB. Management of MDR-TB is a challenge which should be undertaken by experienced clinicians at centres equipped with reliable laboratory service for detection of MDR and XDR. (Kapadiya et al; 2009) Gene involved in drug resistance for first line is rpoB for rifampicin and katG & inhA for isoniazid. For second line drug resistance gene involved is rrs for aminoglycosides/cyclic peptides, gyrA for fluoroquinolones and embB for ethambutol. (Genotype MTBDR Ins. For use) Line Probe Assay is based on reverse hybridization detects wild type sequences of rpoB gene for rifampicin, katG & inhA gene for isoniazid in first line and gyrA gene for fluoroquinolones, rrs for aminoglycosides / cyclic peptides & embB gene for ethambutol in second line and detect their mutation pattern, that is responsible for drug resistance. Most of other mycobacteria are present in the environment as saprophytes. Their pathogenic potential has been recognized since the beginning of last century. These organisms in the past have been called atypical mycobacteria. Also known as Non-Tuberculous Mycobacteria (NTM) has been observed to be an important cause of morbidity and mortality in western countries. NTM are widely distributed in nature and there is a tendency to ignore such isolates as contaminants. (KatochV.M; 2004)

SPECIMEN COLLECTION

During the period of July 2015 to March 2017, 200 smear positive sputum samples were collected from DOTS centre of Teerthankar Mahaveer Hospital and these samples were tested for the drug susceptibility pattern for first line (Isoniazid and Rifampicin) and second line (fluoroquinolones, aminoglycosides/Cyclic peptides and Ethambutol) and species identification was also done in the

[1]Corresponding author

Molecular section of the Microbiology Department, TMMC & RC, Moradabad, U.P. India.

Decontamination

Sputum samples were processed for decontamination in falcon tube by mixing NaOH and N-acetyl-L-cysteine (NALC) in the sputum sample and incubate for 15 minutes at room temp. After that phosphate buffer was added in the sputum sample and centrifugation was done at 3000g for 15 minutes. Then discard the supernatant and re-suspend the pellet in 1ml phosphate buffer

DNA Extraction

500µl of decontaminated sample was processed in appendrof tube and kept in micro centrifuge at 13000rpm for 15 min at room temp. The supernatant was discarded with the help of micro pipette and the pellet was re-suspended in 100µl of distilled water and then bacteria were inactivated by incubating in a heating block for 20 minutes at 95°C. After that cells were sonicated in an ultrasonic bath for 15 min. and centrifuge at 13000rpm for 5 min.

DNA Amplification For First Line Drug Susceptibility

For amplification, mix 35µl of primer nucleotide mixture (PNM A) and 10µl of primer nucleotide mixture (PNM B). 5µl extracted DNA was mixed in the master mixture (PNM A and PNM B). After that this mixture was kept in the thermocycler for the amplification of the bacterial DNA.

Amplification Cycle

15min	95°C	1 cycle
30sec	95°C	
2min	65°C	20 cycles
25sec	95°C	
40sec	50°C	
40sec	70°C	30 cycles
8min	70°C	1cycle

DNA Amplification For Non-tuberculous Myco bacterium (NTM)

For amplification mix35µl PNM, 5µl 10× PCR buffer for HotStarTaq, 2µl 25mM $Mgcl_2$, 0.2µl HotStarTaq, 3µl molecular grade water. 5µl extracted DNA solution was mixed in the master mixture. After that this mixture was kept in the thermocycler for the amplification of the bacterial DNA.

Amplification Cycle

15 min	95°C	1cycle
30 Sec	95°C	10cycle
2 min	58°C	
25 sec	95°C	20cycle
40 sec	53°C	
40sec	70°C	
8min	70°C	1cycle

Hybridization for first line, second line and non-tuberculous mycobacterium (NTM)

Hybridization was performed manually using Twincubater / shaking water bath at 45°C.

PROCEDURE FOR HYBRIDIZATION

1. 20µl of Denaturation solution (DEN blue) is dispense in a each corner of the wells used.
2. 20µl of amplified samples are added to the denaturation solution by the help of pipette up and down and incubate for 5 min. at room temp.
3. 1ml of pre-warmed Hybridization buffer (HYB, green) is added to each well and shakes the try gently until the solution has a homogenous colour.
4. A strip is placed in an each well.
5. Place the try in shaking water bath/Twincubator and incubate at 45°C for 20 min.
6. Completely aspirate Hybridization buffer by pipette.
7. Add 1ml of Stringent Wash Solution (SAT, red) to each strip and incubate at 45°C for 15 min in shaking water bath/Twincubator.
8. Work at room temp. from this step forwards and removes completely Stringent Wash Solution.
9. Wash each strip with 1ml of Rinse Solution (RIN) once for 1min on shaking platform/Twincubator (pour out RIN after incubation).
10. Add 1ml diluted Conjugate to each strip and incubate on shaking platform/Twincubator for 30 min.
11. Remove the solution and wash each strip twice with 1ml of Rinse solution (RIN) for 1 min and once with approx. 1ml of distilled water for 1 min. on shaking platform.
12. 1ml of diluted Substrate is added to each strip and incubate protected from light without shaking.

13. As soon as bands are clearly visible, stop the sreaction by briefly rinsing twice with distilled water.
14. By using tweezers remove strips from the try and dry them between two layers of absorbent paper.

RESULTS AND OBSERVATION

During this study period total 200 smear positive sputum samples were processed, out of 200, 144 (72%) samples were sensitive to first line drugs (isoniazid and Rifampicin) among which 104 were from male and 40 samples were obtained from female patients (Table 1). Strip representing first line sensitivity is shown in (Figure 1.1) 14 (7%) samples were resistant to isoniazid but sensitive to rifampicin (Figure 1.2) and 17 (8.5%) samples were resistant to rifampicin but sensitive to isoniazid (Figure 1.3) 15 (7.5%) samples were MDR i.e., resistant to rifampicin and Isoniazid (Figure 1.4) and 10 (5%) samples were detected as Non-Mycobacterium tuberculosis. Out of 15 (7.5%) MDR samples, 10 (5%) samples were sensitive to second line drugs Fluoroquinolones, Aminoglycosides / Cyclic peptides and Ethambutol (Figure 2.1) 2 (1%) samples were resistant for ethambutol but sensitive to Fluoroquinolones, Aminoglycosides/Cyclic peptides (Figure 2.2) and 1 (0.5%) sample was resistant to fluoroquinolones but sensitive to aminoglycosides / cyclic peptides and ethambutol (Figure 2.3) and 2 (1%) samples were XDR (resistant to 1 second line drugs Fluoroquinolones, Aminoglycosides/Cyclic peptides and Ethambutol) (Figure 2.4) Out of 10 (5%) NTM samples and 3 species were identified as *Mycobacterium intracellular* (Figure 3.1*) Mycobacterium xenopi* (Figure 3.2*) Mycobacterium fortuitum* (Figure 3.3)

DISCUSSION

In 2015 16, there were an estimated 10.4 million new TB cases worldwide of which 5.9 million (56%) were male and 3.5 million (34%) were female. 1.0 million (10%) children were infected with tuberculosis. People living with HIV accounted for 1.2 million (11%) of all new TB cases. In, Top 3 countries which have highest rate of tuberculosis, one is India. So, there is a need to introduce a rapid diagnostic tool for detection of MDR and XDR tuberculosis with drug sensitivity pattern (Katoch V.M; 2004 and Singhal et al; 2014) in 92.5% cases resistant to isoniazid, gene mutation found in codon 315 of katG. These finding support the utility of the GenoType MTBDR*plus* assay for detecting the majority of genetic mutation. (Lee et al, 2001) Genotype MTBDR*plus* assay has excellent specificity (100%) for detection of Rifampicn and Isoniazid resistance *M. tuberculosis*, while sensitivity of the assay has some lesser accuracy for detection of Rifampicin (89.4%) and Isoniazid (91.4%) resistance. (Lee et al, 2001) Line probe assay (LPA) is based on multiplex polymerase chain reaction and used to detect *Mycobacterium tuberculosis* Complex as well as drug sensitivity pattern to Rifampicin, Isoniazid for first line and Fluoroquinolones, Aminoglycosides/Cyclic peptides and Ethambutol for second line and also helpful for detection of Non tuberculous mycobacterium.(Sharma et al, 2014) In our study, we found that MDR occurrence rate is 7.5% which is quite low as compared to the study done by K.S. Sharma (Sharma et al, 2014) who reported MDR occurrence rate is 51%. Fahmiya L. Y. (Fahmiya et al, 2016) reported the MDR occurrence rate 1% which is quite low to our study and we found rifampicin resistant rate 8.5% and isoniazid resistant 7% which is also quite low to same study they reported rifampicin resistant rate 1% and isoniazid resistant rate 1.5%. Our rifampicin and isoniazid resistant rate is quite similar to the study done by C. thakur (Thakur et al, 2015) who reported rifampicin resistant rate is 6.1% and isoniazid resistant rate is 8.6%. Our study is also quite similar to the study done byR. singhal (Singhal et al, 2015) who reported MDR is 17.9%, resistant to rifampicin is 4.6% and resistant to isoniazid 7.3%. In our study, we found that MDR occurrence rate is 7.5%, out of this 5% found to be sensitive to second line drugs. One drug resistant is 0.5% and occurrence of XDR is 1% that is quite low to the study done by Umubyeyi (Umubyeyi et al, 2008) reported 11.6% XDR and single drug resistant is 1.4%. M.V Jesudason (Jesudasan et al, 2005) reported occurrence rate of NTM is 3.9% which is quite low with our study, we found NTM rate is 10%.

CONCLUSION

To conclude, LPA with GenoType MTBDR*plus* had a rapid turnaround time and high specificity and sensitivity for detection of MDR and XDR TB with their mutation pattern and it should be used as a routine diagnostic tool for MDR Tuberculosis. There is a need for

increase in awareness of NTM as pathogens; the clinical index of suspicion should be high, especially in AIDS patients with low CD_4 count. Increased rate of MDR-TB, emergence of XDR cases and NTM species isolated from clinical samples. These observations arequite alarming and there is a need for rapid diagnosis which helps in administering appropriate treatment of disease and control over the spread.

Table 1 Sex- wise distribution of drug resistance pattern obtained

Pattern of drug Resistant	Male	Female	Total
Multi drug Resistant	11	04	15
Mono drug Resistant (Rifampicin)	10	07	17
Mono drug Resistant (Isoniazid)	09	05	14
Sensitive to Rifampicin and Isoniazid	104	40	144
NTM	07	03	10
Total	141	59	200

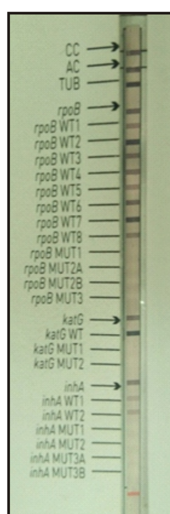

Fig: 1.1

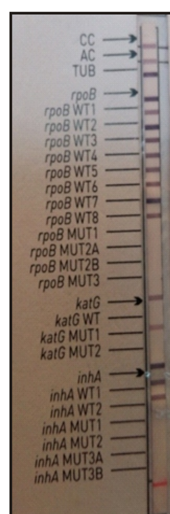

Fig: 1.2

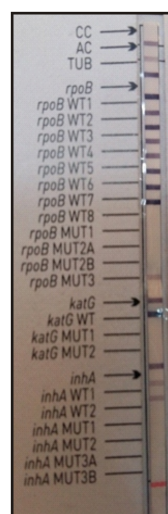

Fig: 1.3

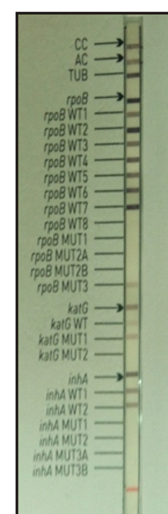

Fig: 1.4

Fig: 1.1
CC (conjugate control)
AC (amplification control)
TUB (Mycobacterium tuberculosis complex) is encoded by antigen 85A & antigen 85B
All wild type bands are developed for rpoB, katG and inhA with locus control and no mutation band is developed which indicates test strain is sensitive to rifampicin and isoniazid.

Fig: 1.2
In rpoB gene all wild type bands are developed and no mutation band is developed indicating the test strain is sensitive to rifampicin. In katG wild type band is absent and mutation 1 is developed means the test strain is resistance to isoniazid.

Fig: 1.3
In rpoB gene wild type 8 band is absent, mutation band 3 is developed which shows test strain is resistant to rifampicin. In katG and inhA all wild type bands are developed with locus control and no mutation band is developed which shows test strain is sensitive to isoniazid.

Fig: 1.4
In rpoB gene wild type 8 band is absent and mutation band 3 is developed which represent test strain is resistant to rifampicin and in katG gene mutation band 1 is developed that means the test strain is resistant to isoniazid. i.e. both first line drugs are resistant.

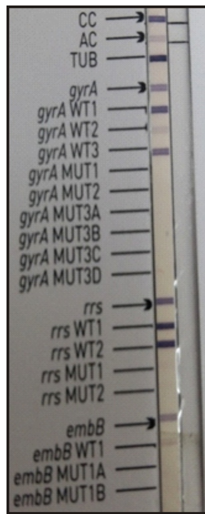

Fig: 2.1

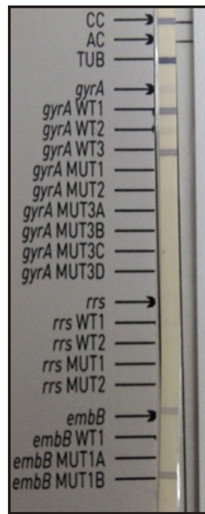

Fig: 2.2

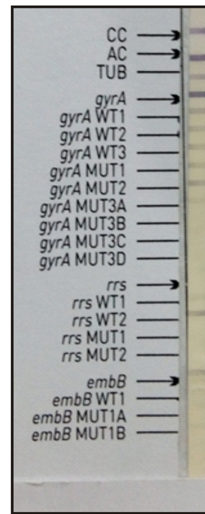

Fig: 2.3

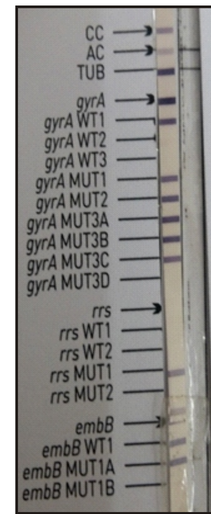

Fig: 2.4

Fig: 2.1 : All wild type bands are developed for gyrA, rrs and embB with locus control and no mutation band is developed which indicates the test strain is sensitive to Fluoroquinolones, Aminoglycosides / Cyclic peptides and Ethambutol.

Fig: 2.2 : In embB gene wild type 1 band is absent, mutation band 1B is developed which shows the test strain is resistant to ethambutol. In gyrA and rrs all wild type bands are developed with locus control and no mutation band is developed which shows the test strain is sensitive to Fluoroquinolones, Aminoglycosides / Cyclic peptides.

Fig: 2.3 : In gyrA gene wild type band is present but mutation band 1 & 2 is developed i.e.; the test strain is resistance to fluoroquinolones. In rrs and emB all wild type bands are developed with locus control and no mutation band is developed which represent test strain is sensitive to Aminoglycosides / Cyclic peptides and ethambutol.

Fig: 2.4 : In gyrA gene wild type 2 & 3 band is absent and mutation bands are developed which means the test strain is resistant to fluoroquinolones, in rrs gene mutation band 1 is developed that's mean the test strain is resistance to Aminoglycosides / Cyclic peptides and in embB wild type 1 is developed along with mutation A band i.e.; test strain is resistant to ethambutol. In this test strain all 3 drugs are resistant.

Fig: 3.1

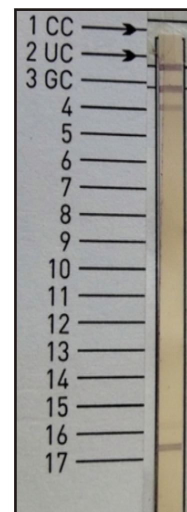

Fig: 3.2

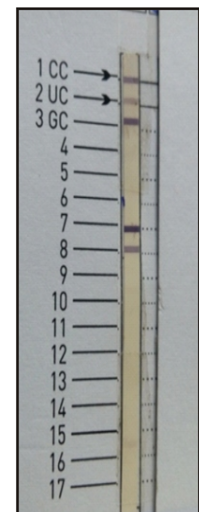

Fig: 3.3

Fig: 3.1
Mycobacterium intracellular along with Negative Control.

Fig: 3.2
M*ycobacterium xenopi*

Fig: 3.3
M*ycobacterium fortuitum*

REFERENCES

Acta Medi. Int. **3**(1):102-06Lee AS., 2001 Novel Mutation in Isoniazid resistant Mycobacterium tuberculosis isolates Antimicrob. Agent Chemo. **45**(7):2157-59**.**

Dustdar F et al; 2008 Mutation in rpoB gene and genotypes of rifampicin resistant Mycobacterium tuberculosis isolates in Iran NRITLD Tanafoss **7**:11-17.

Fahmiya, 2016 Primary Multidrug Resistant Tuberculosis and Utility of Line Probe Assay for its Detection in smear positive sputum samples in a tertiary care hospital in South India. J. Pathogens article ID 6235618.

Global Tuberculosis Control (WHO) surveillance, planning, financing report 2008 Geneva, Switzerland.

Genotype MTBDRplus Ver2.0 Instriction for use Germany HAIN Lifesciences Twincubator.

Jesudasan MV., 2005. Non tuberculous Mycobacteria isolated from clinical specimen at a tertiary care hospital in South India. Indian j Med. Microbiol. **23**(3):172-75.

Kapadiya, 2009 MDR and XDR tuberculosis GMJ **64**(8): 19-24.

Katoch VM., 2004 Infections due to Non Tuberculous Mycobacteria (NTM). Indian J. Med. Res. **120**(4) : 290-304.

Kharibum S., 2016 Molecular Detection of Mycobacterium tuberculosis complex from sputum samples in patients attending tertiary care centre in Uttar Pradesh province of India.

Nudrat and Farooq 2016 Molecular Detection of Rifampicin and Isoniazid Resistance in multidrug resistant Mycobacterium tuberculosis complex from sputum samples in patients attending Teerthankar Mahaveer Medical College and Research College Moradabad India. **4**(2): 228-32

Sharma B.K., 2014 Rapid detection of rifampicin and isoniazid resistant Mycobacterium tuberculosis using Genotype MTBDR plus Assay in Nepal Int. Scholarly Res. Notices Art. ID 648294: 1-6.

Singhal R et al; 2014 Early detection of multidrug resistance and common mutations in Mycobacterium tuberculosis isolates from Delhi using genotype MTBDRplus assay Indian j Med. Microbiol. **33**(Supp.1):46-52

Singhal R., 2015 Detection of multidrug resistance and characterization of mutations in Mycobacterium tuberculosis isolates from North Eastern states of India using Genotype MTBDR plus assay Indian J Med. Res. **140**(4):501-506

Thakur C., 2015 Detecting mutation pattern of drug resistant Mycobacterium tuberculosis isolates in Himanchal Pradesh using Genotype MTBDR plus assay. Indian J Med Microbiol **33**(4):547-53

Umubyeyi A., 2008 Low level of second line drug resistant mycobacterium tuberculosis isolates from Rwanda. Int J Inf Dis **12(**2):172-75