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MOLECULAR DETECTION OF MULTI DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ALONG WITH IDENTIFICATION OF *NON-TUBERCULOUS MYCOBACTERIUM* SPECIES FROM SPUTUM SAMPLES IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Background: Tuberculosis is the second leading cause of death in developing countries. Multi-drug resistant tuberculosis (MDR) is a form of tuberculosis that is resistant to rifampicin and isoniazid. Extensively drug resistant (XDR) is resistant to isoniazid and rifampicin with resistant to one of the fluoroquinolones as well as resistant to at least one of the injectable drug of second line. **Objectives:** Detection of the MDR and XDR status and NTM identification from smear positive sputum samples by Molecular technique. **Material and Methods:** Study was performed on smear positive sputum samples collected from DOTS centre at TMMC and RC, Moradabad during period of July.15 to Dec.16. Samples were decontaminated by pettrof method and then DNA was extracted by using heating method. Then were subjected to PCR using Applied Biosystem Veriti 96 well Thermal Cycler and LPA by using GenoType MDRplus VER.2.0 kit on Twincubater of HAIN life sciences. **Result:** Total 170 smear positive sputum samples were processed by line probe assay, in which 14 were MDR, 2 were XDR and 10 were detected NTM. **Conclusion:** In this study, we concluded that line probe assay should be used on routine basis for drug sensitivity of *M.tuberculosis* cases, so that appropriate treatment can be given on time.

KEYWORDS: MDR, XDR, NTM, PCR, LPA.

INTRODUCTION

Tuberculosis (TB) is the second leading cause of death in developing countries.[1] Tuberculosis disease is caused by infectious organism *Mycobacterium tuberculosis* which mainly affects lung and respiratory system. According to WHO, in 2016, total number of people infected with tuberculosis was 10.8 million and 1.8 million deaths were recorded. 480000 people developed *Multi-drug resistant tuberculosis.*[2] Multi-drug resistant tuberculosis (MDR) is a form of tuberculosis that is resistant to rifampicin and isoniazid.[3]

Extensively drug resistant tuberculosis (XDR) is a form of tuberculosis that is resistant to rifampicin and isoniazid with resistant to one of the flouoquinolone as well as resistant to at least one of the injectable drug of second line.[1]

Tuberculosis is a global health problem. TB was detected as a global health problem emergency by world health organization (WHO) in 1993.[4] As the incidence of tuberculosis has increased, there has been a corresponding rise in the incidence of drug resistant strain of *Mycobacterium tuberculosis*.[5]

TB control program based on directly observed treatment short course (DOTS) policy is essential for preventing the emergency 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.[6]

Gene involved in drug resistance for first line is katG and inhA for isoniazid and rpoB for rifampicin. For second line drug resistance gene involved is gyrA for fluoroquinolones, rrs for aminoglycosides/cyclic peptides and embB for ethambutol.[7]

Assays based on reversed hybridization of amplicons to immobilized membrane based probe covering wild type sequences of rpoB, katG and inhA gene for first line and gyrA, rrs and embB for second line, their mutation have been developed for detection of drug resistance.[8]

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. NTM have 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.[8]

MATERIAL AND METHODS

Specimen collection

During the period of July 2015 to Dec.2016, 170 smear positive sputum samples were collected from the DOTS centre of Teerthanker Mahaveer Hospital and these samples were processed for the drug susceptibility testing for first line (Rifampicin and Isoniazid) and second line (fluoroquinolones, aminoglycosides / Cyclic peptides and Ethambutol) and species identification in the Molecular laboratory of the Microbiology Department, TMMC and RC, Moradabad, U.P. India.

Decontamination

Sputum specimens were processed for decontamination by mixing N-acetyl-L-cysteine (NALC) and NaOH in the specimen and incubate for 15 min. After that phosphate buffer was added in the specimen and centrifugation was done for 15 minutes at 3000g. Then discard the supernatant and re-suspend the pellet in 1ml phosphate buffer.[6,7]

DNA extraction

500µl of the decontaminated specimen was processed in the micro centrifuge (13000rpm for 15 min at room temp.) The supernatant was discarded and the pellet was re-suspended in 100µl of distilled water and then inactivates the bacteria by incubating in a heating block for 20 minutes at 95°C. After that cells were sonicated in an ultrasonic bath for 15 minutes and centrifuge for 5 minutes at 13000rpm.[6,7]

DNA amplification for first line drug susceptibility

Amplification was performed by combining 35µl of primer nucleotide mix (PNM A) and 10µl of primer nucleotide mix (PNM B). 5µl extracted DNA was mixed in the master mixture (A and B). After that this mixture was kept in the thermocycler for the amplification of the bacterial DNA.[6,7]

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	1 cycle

DNA amplification for second line drug susceptibility

Amplification was performed by combining the following-

35µl 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	30cycle
40 sec	53°C	
40sec	70°C	
8min	70°C	1cycle

DNA amplification for non-tuberculous mycobacterium (NTM)

Amplification was performed by combining the following-

35µl 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 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 prewarmed Hybridization buffer (HYB, green) is added to each well and shake the tray gently until the solution has a homogenous colour.
4. A strip is placed in an each well.
5. Place the tray 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.

RESULT AND OBSERVATION

During this study period total 170 smear positive sputum samples were processed, out of 170, 128 (75.2%) samples were sensitive to first line drug (Rifampicin and isoniazid) as shown in fig 1.1. 08 (4.7%) samples were resistant to isoniazid but sensitive to rifampicin as shown in fig 1.2 and 10 (5.8%) samples were resistant to rifampicin but sensitive to isoniazid as shown in fig 1.3. 14 (8.2%) samples were MDR (resistant to drugs, rifampicin and isoniazid) as shown in fig 1.4 and 10 samples were detected as *Non-Mycobacterium-Tuberculosis.*

Out of 14 (8.2%) MDR samples, 10 (5.8%) samples were sensitive to second line drugs (Fluoroquinolones, Aminoglycosides / Cyclic peptides and Ethambutol) as shown in fig 2.1. One (0.58%) sample is resistant for ethambutol but sensitive to fluoroquinolones, aminoglycosides / cyclic peptides as shown in fig 2.2 and one (0.58%) sample is resistant to fluoroquinolones but sensitive to aminoglycosides / cyclic peptides and ethambutol as shown in fig 2.3 and 2 (1.1%) samples were XDR (resistant to l second line drugs Fluoroquinolones, Aminoglycosides / Cyclic peptides and Ethambutol) as shown in fig 2.4.

Out of 10 (5.8%) NTM samples and 3 species were identified as *Mycobacterium intracellular* as shown in fig 3.1, *Mycobacterium xenopi* as shown in fig 3.2 *and Mycobacterium fortuitum* as shown in fig 3.3.

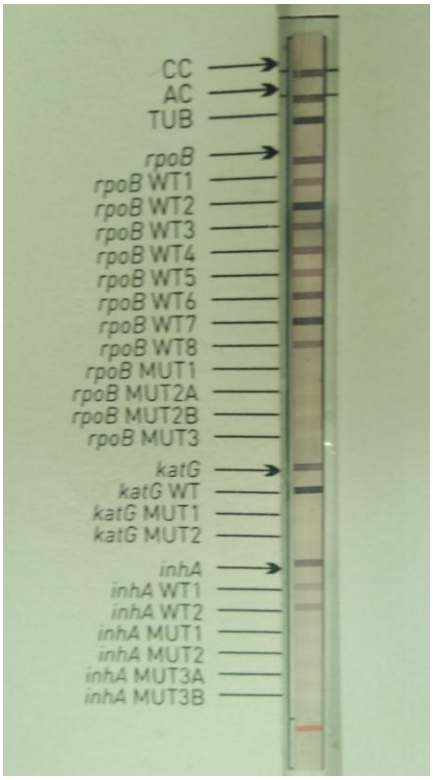

Fig: 1.1

CC (conjugate control)
AC (amplification control)
TUB (Mycobacterium tuberculosis complex) is encoded by antigen 85A and antigen 85B.

All wild type bands are developed for rpoB, katG and inhA with locus control and no mutation band is developed that's mean the test strain is sensitive to rifampicin and isoniazid.

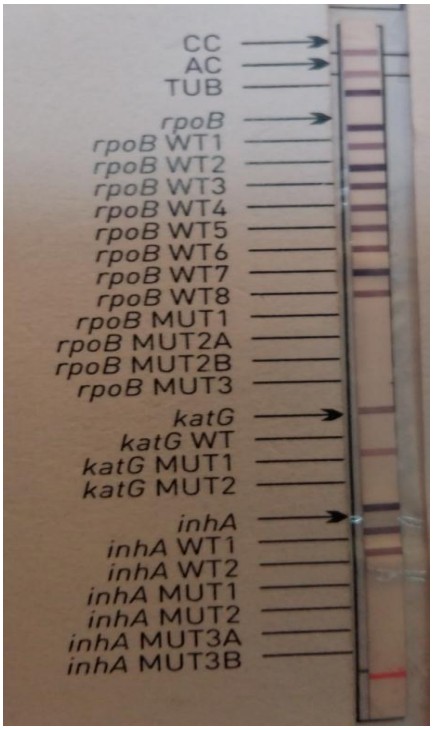

Fig: 1.2

In rpoB gene all wild type bands are developed and no mutation band is developed that's mean the test strain is sensitive to rifampicin. In katG wild type band is absent and mutation 1 is developed that's mean the test strain is resistance to isoniazid.

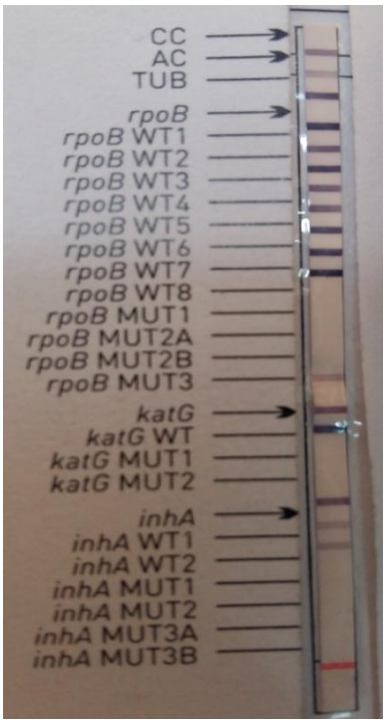

Fig: 1.3

In rpoB gene wild type 8 band is absent, mutation band 3 is developed that's mean the test strain is resistance to rifampicin. In katG and inhA all wild type bands are developed with locus control and no mutation band is developed that's mean the test strain is sensitive to isoniazid.

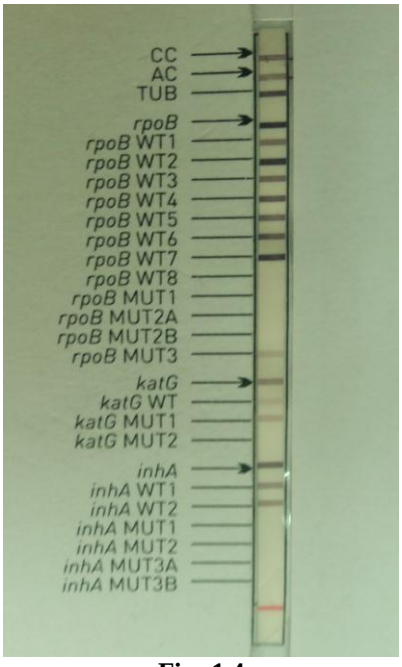

Fig: 1.4

In rpoB gene wild type 8 band is absent and mutation band 3 is developed that's mean the test strain is resistance to rifampicin and in inhA gene mutation band 1 is developed that's mean the test strain is resistance to isoniazid. In this test strain both drugs are reistance that's known as MDR.

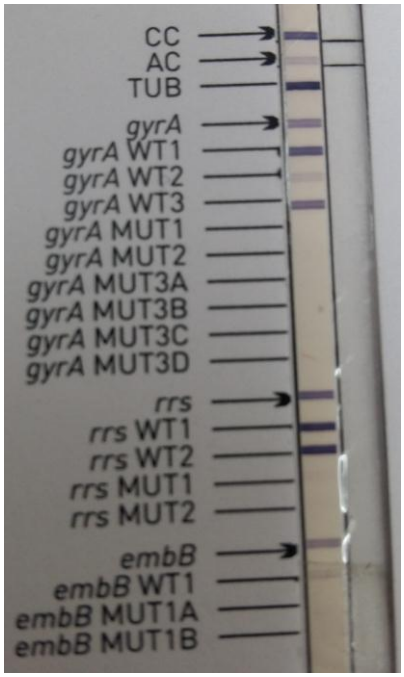

Fig: 2.1

All wild type bands are developed for gyrA, rrs and embB with locus control and no mutation band is developed that's mean the test strain is sensitive to Fluoroquinolones, Aminoglycosides / Cyclic peptides and Ethambutol.

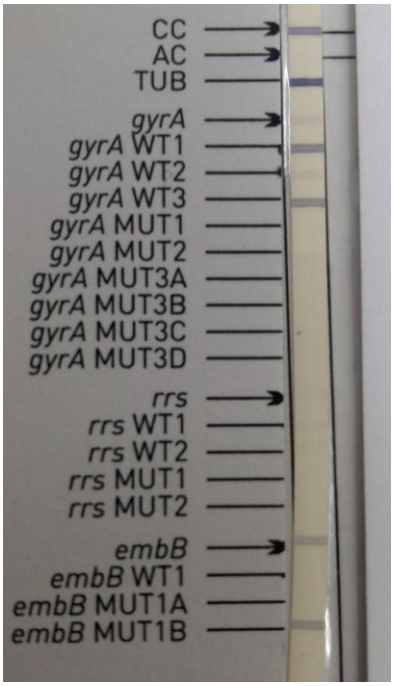

Fig: 2.2

In embB gene wild type 1 and 2 band is absent, mutation band B is developed that's mean the test strain is resistance to ethambutol. In gyrA and rrs all wild type bands are developed with locus control and no mutation band is developed that's mean the test strain is sensitive to Fluoroquinolones, Aminoglycosides / Cyclic peptides.

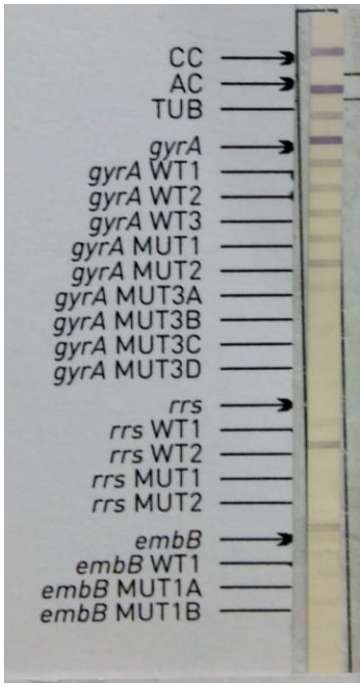

Fig: 2.3

In gyrA gene wild type band is present but mutation band 1 and 2 is developed that's mean 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 that's mean the test strain is sensitive to Aminoglycosides / Cyclic peptides and ehtambutol.

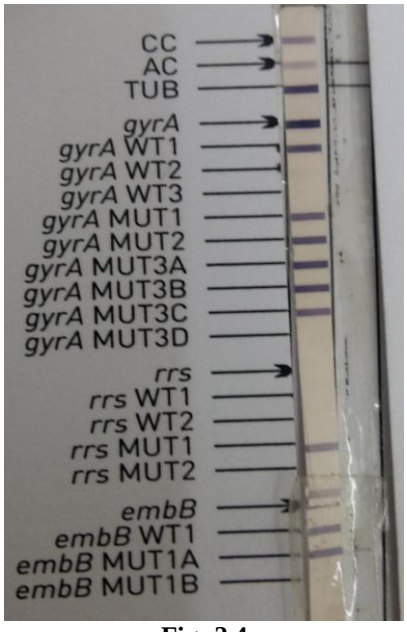

Fig: 2.4

In gyrA gene wild type 2 and 3 band is absent and mutation bands are developed that's mean the test strain is resistance 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 that's mean the test strain is resistant to ethambutol . In this test strain all 3 drugs are reistance that's known as XDR.

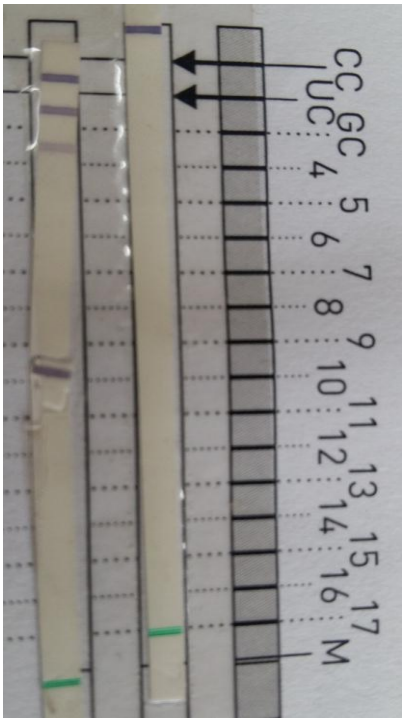

Fig:- 3.1

Mycobacterium intracellular along with Negative Control.

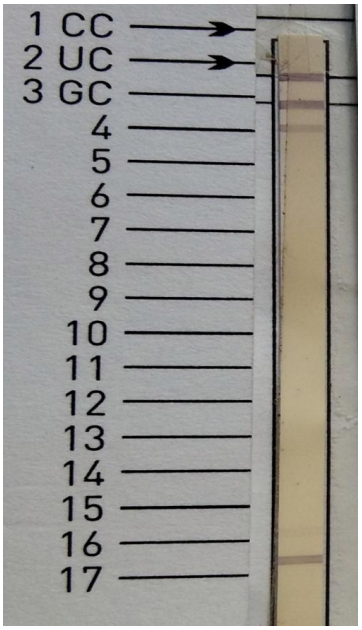

Fig:- 3.2

Mycobacterium xenopi

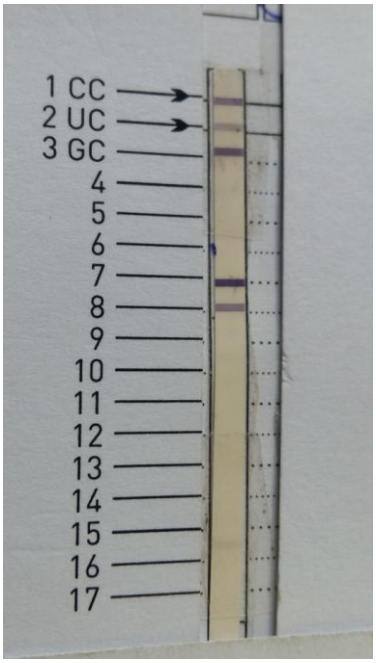

Fig:- 3.3

Mycobacterium fortuitum

DISCUSSION

We all know that tuberculosis is a serious health problem which is distributed worldwide but India is the highest tuberculosis (TB) burden country. Hence, there is a need of introduction of rapid diagnostic tool to detect MDR and XDR tuberculosis.[10]

Line probe assay (LPA) is based on Reverse hybridization that is used to detect *Mycobacterium tuberculosis Complex* as well as drug sensitivity to Rifampicin, Isoniazid, Fluoroquinolones, Aminoglycosides / Cyclic peptides and Ethambutol, detection of NTM also done with LPA.[11]

In our study, we found that MDR occurrence rate is 10.7% which is quite low as compared to the study done by Singhal *et al* in 2014[12] who reported MDR occurrence rate is 55%. We found in our study that rifampicin resistant rate is 5.7% and isoniazid resistant rate is 4.9% which is quite similar to the study done by C.thakur *et al* in 2015[13] 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 by R. singhal *at el* in 2015[14] 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 MDR occurrence rate is 10.7%, out of this 7.4% found to be sensitive to second line drugs. One drug resistant is 0.82% and occurrence of XDR is 1.6% that is quite similar to the study done by A.umubyeyi *at el* in 2008[15] who reported 11.6% XDR and single drug resistant is 1.4%. In this study we found the occurrence rate of NTM is 8.2% which is quite high of the study done by MV jesudason *at el* in 2005[16] who reported occurrence rate is 3.9% of NTM.

CONCLUSION

To conclude, that LPA with GenoType MTBDRplus has revolutionized the MDR and XDR-TB diagnosis. LPA is a rapid diagnostic method with high sensitivity and specificity for detection of *Mycobacterium tuberculosis Complex* along with their mutation pattern.

There is a need for increase in awareness of NTM as pathogen, 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 are quite alarming and there is a need for rapid diagnosis which help in administering appropriate treatment of disease and control over the spread.

REFERENCES

1. Singh S, Farooq U, Nudrat S. Isolation, Identification and Drug resistance testing of *Mycobacterium tuberculosis* by Recent diagnostic modalities at teaching hospital in Moradabad (UP). Acta Med. International, 2016; 3(2): 51-55.
2. Global Tuberculosis Control (WHO) Surveillance, Planning, Financing Geneva Switerzerland WHO 2008.
3. Nudrat S, Farooq U .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 Centre, Moradabad, Uttar Pradesh, India. Inte. Jour. Scientific study. 2016; 4 (2): 228-232.
4. Kharibum S, Farooq U, Nudrat S. Molecular detection of *Mycobacterium tuberculosis Complex* from sputum samples in patients attending tertiary care centre in Uttar Pradesh province of India, 2016; 3(1): 102-106.
5. Dustdar F, Dokhat A, Farnia P *et al*. Mutation in rpoB gene and Genotypes of rifampicin resistant Mycobacterium tuberculosis isolates in Iran. NRITLD Tanaffos, 2008; 7: 11-17.
6. B. Kapadiya, N.B. Ravel, V. Patel *et al*. MDR and XDR Tuberculosis. GMJ / Aug.-2009; 64: 19-24.
7. Genotype MTBDRplus Ver 2.0. Instruction for use. Germany HAIN life sciences Twincubator 1986.
8. R. Singhal, V.P. Myneedu, J. Arora. *et al*. Early detection of multi drug resistance and common mutation in *Mycobacterium tuberculosis* isolates from Delhi using GenoTyppe MTBDRplus assay. India J Med. Microbiol 2014; 140(4): 46-52.
9. Katoch VM. Infections due to Non-tuberculous Mycobacteria (NTM). India J Med Res, 2004; 120(4): 290-304.

10. R. Tripathi, P. Sinha, R. Kumari. *et al*. Detection of rifampicin resistance in *M .tuberculosis* by molecular methods. A report from eastern Uttar Pradesh. India. India. J. Med. Microbiol, 2016; 34(2): 92-4.
11. Barnard M, Albert H, Coetzee G. *et al*. Rapid molecular screening for multidrug resistant tuberculosis in a high volume public health laboratory in South Africa. J. Respir crit care Med, 2008; 177(1): 787-92.
12. Singhal R, Myneedu VP, Arora J. *et al*. Detection of MDR and characterization of mutation in *Mycobacterium tuberculosis* isolates from North Eastern states of India using Genotype MTBDRplus assay. India J. Med. Res, 2014; 140(2): 501-6.
13. C. Thakur, V. Kumar, AK Gupta. *et al*. Detecting mutation pattern of drug resistant *Mycobacterium tuberculosis* isolates in Himachal Pradesh using GenoType MTBDRplus assay. India J. Med. Microbiol, 2015; 33(4): 547-553.
14. R. Singhal, VP Myneedu, J. Arora. Early detection of MDR and common mutation in TB isolates from Delhi using GenoType MTBDRplus assay. India. J. Med. Microbiol, 2015; 33(5): 46-52.
15. Umubyeyi, L. Rigonts, F.portaels. *et al*. Low level of second line drug resistant *Mycobacteria tuberculosis* isolates from Rwanda. Inte. J. Infc. Dies, 2008; 12(2): 152-156.
16. MV Jesudason, P. Glandstone. *Non-tuberculous Mycobacteria* isolated from clinical specimen at a tertiary care Hospital in South India. India. J. Med. Microbiol, 2005; 23(3): 172-175.