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ORIGINAL ARTICLE

Isolation, Identification and Drug Resistance Testing of *Mycobacterium tuberculosis* by Recent Diagnostic Modalities at Teaching Hospital in Moradabad (UP)

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ABSTRACT

Objective: Tuberculosis is the second leading cause of death in developing countries among all infectious diseases. Globally, drug resistance strain of *Mycobacterium tuberculosis* is a public threat. Due to diagnostic delay, inadequate infection control and poor drug supply there is a emergence of MDR- TB and XDR- TB. Our aim was to isolate and identify the drug resistant strain of *M. tuberculosis* by using newer diagnostic modalities.

Methods: Sputum sample and BAL fluid from 70 suspected cases were collected and analysed for *Mycobacterium* by Ziehl – Neelsen staining and liquid culture with molecular detection of drug resistant strain of *M. tuberculosis.*

Result: In our study, among the 70 patients 27 (38.5%) were positive for AFB by microscopy. On testing for *Mycobacterium* by BacT/Alert 3D system, 54 were found to be positive. On performing further identification and susceptibility of 54 isolates towards rifampicin and isoniazid by molecular method, 5 isolates (9.25%) were resistant to both rifampicin and isoniazid confirming as multidrug resistant. 5 isolates (9.25%) were sensitive to rifampicin and resistant to isoniazid and 2 isolates (3.70%) were resistant to rifampicin and sensitive to isoniazid. Whereas 5 isolates (9.25%) found to be negative for *M. tuberculosis.*

Conclusion: Our investigation highlights the importance of newer diagnostic modalities for isolation and identification of drug resistant strain of *M. tuberculosis*. Which ensure early and accurate diagnosis of patients with prevention of further transmission of disease.

INTRODUCTION

Tuberculosis (TB) is the second leading cause of death in developing countries among all infectious diseases. According to World Health Organization (WHO) fact sheet, approximately 9.27 million new cases of tuberculosis detected worldwide, with about 2 million infected crowd from India alone.[1] Globally in 2014, 5% of *Tuberculosis* cases were estimated to have had multidrug-resistant TB (MDR-TB).

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Surveillance data of drug resistance shows that an estimated 480 000 people developed MDR-TB in 2014 and 190 000 people died due to MDR-TB.

Extensively drug-resistant TB (XDR-TB) has been reported by 105 countries in 2014. On average, an estimated 9.7% of people with MDR-TB have XDR-TB.[2] MDR-TB is a form of TB that is difficult and expensive to treat because it fails to respond to two important first line drug, specifically rifampicin and isoniazid.

When MDR strain of *M. tuberculosis* has additional resistance to a fluroquinolone and a second line injectable antibiotic including amikacin, kanamycin or capreomycin, it is designated extensively drug-resistant (XDR- TB).[3] Spontaneous genetic mutation is responsible for the conversion of wild type tuberculosis into drug resistant strain of *M. tuberculosis*.[4] In case of multidrug

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resistance strain, mechanism is still uncertain and has been observed that mutation in different genes are responsible instead of single gene. Genes involved in drug resistance is *KatG*, *inhA* for isoniazid, *rpoB* for rifampicin, *pncA* for pyrazinamide and *embB* for ethambutol.[5]

Due to diagnostic delay, inadequate infection control and poor drug supply there is a emergence of drug resistant strain of *M. tuberculosis* leads to MDR- TB and XDR- TB.[6]

Traditional methods for *M. tuberculosis* detection and isolation are time consuming and century old procedures, in turn patients lose confidence in Laboratories.

WHO's millennium goal of TB disease reduction by 2015 has been effected due to delay in growth of bacilli in Solid culture medium, cumbersome drug sensitivity testing method (DST) and difficulty in speciation of Mycobacteria.

Now, there is a need of an ideal test for active tuberculosis which shows a rapid result with high sensitivity and specificity including drug susceptibility data, low cost, highly automated or easily performed without the need for excessive sample preparation or technical expertise and distinguish between latent tuberculosis infection (LTBI) and active disease.

Emerging Modern Media with accurate detection, are replacing the Egg and Agar based medium. Even several molecular methods are available, but mainly used as research tools are very expensive, require specialist training and equipments, false positive results and cannot differentiate between live and dead bacilli.

MATERIAL AND METHODS

Study Design

The study was conducted among the 70 Tuberculosis suspected patients (cough more than 2 weeks, fever for 1 month or more, blood stained sputum, night sweats and weight loss) attending DOTS microscopy centre in TMMC & RC, Moradabad (U.P) over a period of 15 months from January 2015 to march 2016, after obtaining informed written consent.

Specimen Collection

Two early morning sputum samples or Bronchoalveolar lavage fluid (BAL fluid) were collected in a 25 ml, screw capped, wide mouthened, sterile universal container and transported to the laboratory within 24 to 48 hours.

Processing of Specimen

For screening and prompt diagnosis, direct microscopy from sputum sample and BAL fluid was done with Z. N staining.

Pretreatment of samples i.e homogenization and decontamination by NALC (N-acetyl – L – cysteine)/NaOH method were done according to CDC guidelines.

Detection of *Mycobacterium* was done by using automated liquid culture system BacT/Alert 3D which is based on colorimetric dectection of CO2.

After digestion and decontamination, 0.5 ml processed specimen and 0.5 ml reconstituted fluid added to coctail of lyophilized antibiotic (PANTA – *polymyxin*, *amphotericin B*, *nalidixic acid*, *trimethoprim* and *azlocillin*) was inoculated into a BacT/Alert MP bottle containing 10 ml Middle brook 7H9 broth and loaded in the BacT/Alert 3D system for *Mycobacterium* dectection.

The BacT/Alert 3D process bottles were incubated in the system at 37°C till it was designated positive or up to a maximum of 4 – 6 weeks.

All bottles designated positive were smeared and stained for Acid fast bacilli (AFB) microscopy.

Further identification, speciation and drug sensitivity pattern of Mycobacteria from positive culture bottle alarmed in automated liquid culture system was done by molecular method i.e Genotype ® MTBDR plus assay, a commercially procured multiplex PCR DNA strip assay (Hain Lifescience, Nehren, Germany).

DNA extraction, amplification and hybridization was done according to the manufacturer instruction.[7]

Method for DNA Extraction

- 1 ml of positive liquid cultures were vortexed and centrifuged for 15 min at 13000 RPM
- By pipette supernatant was discarded
- Then resuspended pellet in 100 µl of molecular water
- Mixed well and incubated at 95°C for 20 minutes
- Again incubated in sonicator for 15 minutes
- Centrifuged for 5 minutes in 13000 RPM and 5 µl of supernatant was used for PCR.

Amplification

45 µl of amplification mix was prepared in a room free from contamination.

A master mix containing AMP A - 10 µl + AMP B - 35 µl and 5µl of extracted DNA were sended for amplification on thermal cycler.

95°C for 15 min	1 cycle
95°C for 30 sec 65°C for 2 min	10 cycle

95°C for 25 sec	20 cycle
50°C for 40 sec	
70°C for 40 sec	
70°C for 8 min	1 cycle

Amplification products was stored at -8 to - 20°C.

Hybridization (Figure 1)

1. 20 µl of denaturation solution were dispensed at the corner of wells
2. Then 20 µl of amplified product was added to the denaturation sample and incubated for 5 min
3. 1 ml of pre warmed hybridization buffer was carefully added to each well and gently shake to make homogenous color
4. A strip was placed in the respective wells and incubated at 45°C for 30 min in waterbath (visually checked the each step in twincubator)
5. Hybridization buffer was completely aspirated with pipette
6. To each strip, 1 ml wash solution of stringent (SAT, red) was added and incubated at 45°C for 15 min in water bath
7. Stringent solution was completely aspirated with pipette and taped on tissue paper
8. It was washed once of each strip with 1 ml of rinse solution (RIN) on shaking platform/Twin cubator for 1 min
9. 1 ml of diluted conjugate was added to each strip on shaking platform/Twincubator and incubated for 30 min at room temperature
10. Each strip were washed twice with 1 ml of rinse solution (RIN) for approximately 1 min
11. Washed with 1 ml distilled water and removed the water completely
12. 1 ml of diluted substrate was added to each strip and was protected from light by covering the twincubator with aluminium foil
13. Substrate was removed completely and taped on tissue paper
14. Washed with 1 ml distilled water for 1 min
15. Reaction was stopped as soon as bands were clearly visible
16. Strip was dried between two layers of absorbent paper and results were interpretated.

RESULTS

The study includes 70 patients suspected for tuberculosis. Among the 70 patients 27 (38.5%) were positive for AFB by microscopy. In which 22 (81.4%) were males and 5 (18.5%) were females. On testing for *M. tuberculosis* by BacT/Alert 3D system, 54 were found to be positive. Among the positive, 42 were males and 12 were females. Of the positives majority of patients were classified in age groups 41 - 60 years. More number of males were found in age group 41 – 60 and females were in 21 – 40 years of age group (Table 1) (Figure 2).

On performing further identification and susceptibility of 54 isolates towards rifampicin and isoniazid by *Genotype ® MTBDR plus assay*, multiplex PCR DNA strip assay, it was found that 5 isolates (9.25%) were resistant to both rifampicin and isoniazid confirming as multidrug resistant. Majority of these multidrug resistant strains belong to males and age group between 40 – 60 years.

5 isolates (9.25%) were sensitive to rifampicin and resistant to isoniazid, 2 isolates (3.70%) were resistant to rifampicin and sensitive to isoniazid and rest of the isolates (68.51%) were sensitive to both rifampicin and isoniazid with 5 isolates (9.25%) found to be negative for *M. tuberculosis* (Table 2) (Figure 3).

DISCUSSION

As we all know that the tuberculosis is a serious health concern and emergence of drug resistance strains of

Table 1: Age and sex wise distribution of patients

Age groups	Male	Female
0-20	9	2
21-40	14	7
41-60	19	3

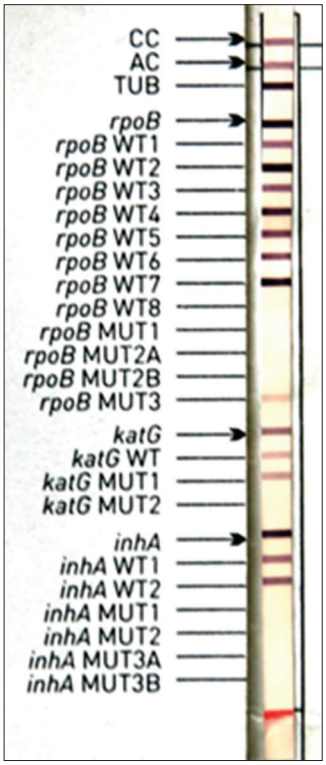

Figure 1: Shows PCR Hybridization strip for detection of *M.Tuberculosis* and having mutation in *rpoB* and *katG* gene

Table 2: Drug sensitivity pattern by *Genotype MTBDR plus assay*, among tuberculosis positive patient (n=54)

Pattern of drug resistance	Male	Female	Total (%)
Multidrug resistance	3	2	5 (9.25)
Mono drug resistance (isoniazid)	5	0	5 (9.25)
Monodrug resistance (rifampicin)	1	1	2 (3.70)
Sensitive to both rifampicin and isoniazid	30	7	37(68.51)
Negative for *Mycobacterium tuberculosis*	5	0	5 (9.25)

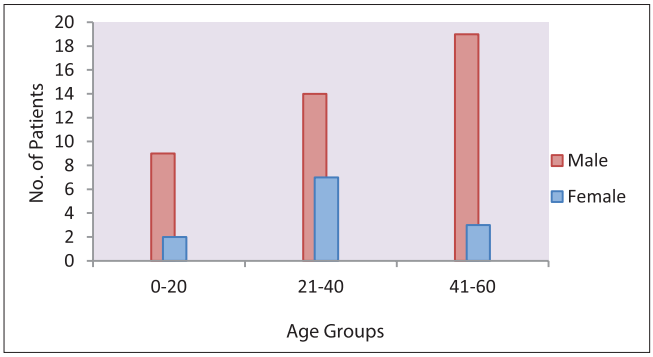

Figure 2: Age and sex wise distribution of patients

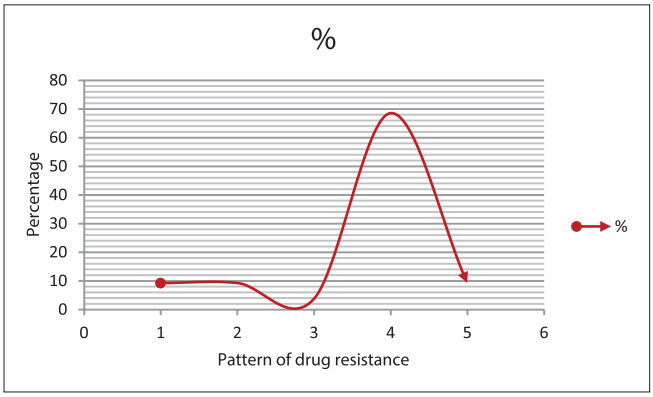

Figure 3: Drug sensitivity pattern by *Genotype MTBDR plus assay*, among tuberculosis positive patient (n = 54)

mycobacterium tuberculosis became a major hurdle to achieve a effective TB control worldwide. Many factors like demography, poverty, illiteracy and smoking may be helpful to tubercle bacilli for engraving their roots into the community. In our study, maximum number of culture positive patient were in age group of 41 – 60 (40.7%) followed by 21 – 40 (38.8%). Kehinde et al study shown similar age group distribution.[8] Males were predominant in our study (77.7%). They have more chances of TB exposure from infected patient in their work areas. A study from south india also reported the higher prevalence of TB among males than females.[9] In our study, 38.7% of *tuberculosis* suspected patient were AFB smear positive and 61.4% were negative for AFB microscopy. Although smear negative TB patient are less infectious than smear positive TB but they also contribute to transmission of disease. A San Francisco study showed that 17% of the TB transmission is seen in smear negative TB cases.[10]

The isolation rate of Mycobacteria among study recruits by liquid culture system was 77.1%. As compared to this study, Damle et al. and Jena et al.observed higher rate of isolation.[11,12]

Conjugation, transformation, transduction and mutation are the four basic mechanism through which bacterium acquired resistance. Among these, genetic mutation makes the MTB resistant to anti tubercular drugs.

In our study, 68% of MTb isolates were sensitive to first line anti tubercular drug. Which is similar to study conducted by Muralidhaar and Srivastava in New delhi, India showed 65.3% sensitivity.[13]

We reported 9.25% of MDR – TB among positive Mycobacterial isolates. Similarly, 4.5% MDR – TB was reported by Malhothra et al.[14] This study evaluated the *Genotype ® MTBDR plus assay* performance on DNA extracted from *mycobacterium* isolates but not from clinical samples (Sputum and BAL fluid). Our study shows, overall resistance was 22.2% detected by molecular method which is higher than different reports from different parts of world.[15,16]

Study Limitation

1. In our study, 9.25% samples were found to be negative for *M. tuberculosis* and cannot differentiated at species level.
2. MDR strains were not further tested by Second line anti – tubercular drugs.

The samples found to be negative for *M. tuberculosis* were preserved for *species* identification in future.

CONCLUSION

The conclusion of our study is that the microscopy for AFB and conventional culture method on egg based solid culture medium is time taking and are responsible for delay in diagnosis which leads to emergence of drug resistant strains of *M. tuberculosis* in the community.

This can be improved by use of newer diagnostic tools, including automated liquid culture system BacT/Alert 3d and *Genotype ® MTBDR plus assay*. Though they are not cost effective but one of the major drawback of PCR that it cannot differentiate between live and dead bacilli is nullified by using liquid culture system, as positive culture bottles contain only live tubercle bacilli and use of liquid media system reduces the turn-around time for isolation of acid-fast bacilli to approximately 10 days compared with 17 days or longer in conventional methods.

This will ensure early and accurate diagnosis of patients with prevention of further disease transmission.

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