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Identification and Drug Resistance Pattern of *Mycobacterium Species* among Patients Attending DOTS Microscopy Center

Sudhir Singh[1], Shewtank Goel[1], Umar Farooq[1], Shweta R Sharma[1], Prachi Singh[2], Abhishek Singh[3]
[1]Department of Microbiology, TMMC&RC, Moradabad (U.P), India
[2]Department of Pathology, TMMC&RC, Moradabad (U.P), India
[3]Department of Community Medicine, SHKM Govt. Medical College, Mewat

Original Research Article

***Corresponding author**
Shewtank Goel

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Abstract: Tuberculosis is a chronic infection, potentially of lifelong duration, caused by two species of mycobacteria *M. tuberculosis* and rarely, *M. bovis*. Today one third of the world's population is infected with this disease. Emergence of drug resistant tuberculosis is another public health quandary. Our study aims to detect drug resistance cases by using combination of automated culture system and molecular method for rapid and accurate diagnosis. The study was conducted over a period of 16 months from January 2016 to June 2017, among 210 clinically suspected patients having infection with Mycobacterium species. Sputum and BAL fluid from all clinically suspected cases of Tuberculosis among all age groups and both sexes attending DOTS microscopy center and TB chest department were collected for further identification. Total 210 suspected cases for pulmonary tuberculosis were included. Among 210 patients 38.5% were AFB smear positive, 77.1% clinical samples were culture positive and 15 sample were found to be MDR. In our study we concluded that, liquid culture methods and Line Probe assay are costly but the test is rapid and more accurate, which helps in rapid detection of drug resistant strains of Mtb.
Keywords: Tuberculosis, *M. tuberculosis*, Mtb, MDR, XDR.

INTRODUCTION

Tuberculosis (TB) is one of the world's deadliest diseases and leading cause of death in developing countries like India, which remains a major cause of morbidity and mortality worldwide. Today one third of the world's population is infected with this disease.

According to the World Health Organization (WHO) data, there were an estimated 8.6 million cases of TB in 2012 and 1.3 million deaths were attributed to the same. In children more than half a million cases occurred and 320,000 deaths were reported among AIDS patients [1].Emergence of drug resistant tuberculosis is another public health quandary. Which may be Multidrug-resistant tuberculosis (MDR-TB) or extensively drug-resistant (XDR).

Multidrug-resistant tuberculosis (MDR-TB) is a form of rifampicin-resistant TB that has additional resistance to isoniazid, which is a valuable anti-TB drug. When MDR *M. tuberculosis* has additional resistance to a fluroquinolone and a second line injectable antibiotic (i.e. amikacin, kanamycin or capreomycin), it is designated extensively drug-resistant (XDR)

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. Genotype MTBDRplus Ver 2.0. Instruction for use. Germany HAIN life sciences Twincubator 1986. Drug resistant TB was difficult and expensive to treat and became an economical burden.

An estimated 450,000 cases of multidrug resistant (MDR-TB) and 170,000 deaths reported in 2012. 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 [2,3].

Early detection of drug resistant strain of MTb is a key factor to reduce the spread of these resistant

strains. A better knowledge about the mechanisms of action of anti-TB drugs and the development of drug resistance will allow identifying new drug targets and help in better way to detect drug resistance. The HIV/AIDS pandemic have been attributed to upsurge of drug resistant TB globally and attack rate is heavy on the wage earning age group of 15-49 years[4].

For initiating effective treatment and preventing its transmission in the community, rapid diagnosis of TB is critical [5] Recent advances and upliftment in molecular methods have shortened the turnaround time for the Mycobacterium tuberculosis identification; however, culture is still gold standard and essential for phenotypic drug susceptibility testing and improving the case detection of smear negative patients[6].

Now a days, the BacT/Alert 3D MB system, which is fully automated and nonradiometric liquid culture system, has been recommended for faster mycobacterial isolation from clinical samples [7]. Traditional methods for MTb detection and isolation are time consuming and are age old procedure, by which patient lose confidence in Laboratories.

This study aims to detect drug resistance cases by using combination of automated culture system and moleculer method for rapid and accurate diagnosis.

MATERIAL AND METHODS

Study design

The study was conducted over a period of 16 months from January 2016 to June 2017, among 210 clinically suspected patients having infection with Mycobacterium species. Patients with symptoms of Tuberculosis were evaluated as per the study protocol. Written informed consent was taken from the patient/ relatives.

Inclusion Criteria

All clinically suspected cases of Tuberculosis among all age groups and both sexes attending DOTS microscopy center and TB chest department were included in the present study.

Exclusion Criteria

Previously diagnosed cases and recovered patients having complete course of ATT (Anti tuberculer drugs) were excluded from the study. Details like age, gender, smoking habbit, duration of cough, etc. were included in a case record form.

Specimen collection

Two early morning sputum samples and one BAL (Broncho Alveolar Lavage) fluid obtained at least eight hours apart. Sputum collected in the morning before any meal or anti tubercular therapy and patient should not use oral antiseptics. The quantity of sputum is atleast 5 mL in Single use Universal containers are made of plastic which is clean, wide mouthed, and leak proof specimen containers of 50 ml capacity.

The bronchial aspiration procedure was applied to the patients in whom a positive smear of acid-fast bacilli could not be obtained from sputum or a qualified sputum sample could not be collected. All specimens were subjected to direct smear microscopy by a standard Ziehl – Neelson (Z. N) staining method in Mycobacteriology section of Microbiology laboratory.

Mycobacterial cultivation and identification

After Z.N staining, all sputum sample were subjected to digestion and decontamination by Modified petroff's method (N-acetyl-L-cysteine–Sodium hydroxide method)

Subsequently, an equal volume of the analysis solution was added to the sample, and mixed by whirling for 30 **s,** inverting the tube in the process to obtain improved homogenization. This mixture was incubated at room temperature for 15 min, after which 20-30 mL of phosphate-buffered saline was added, followed by mixing and centrifugation at *3000g* for 15 min. The top layer was poured out andthe sediment used to prepare a smear for microscopy [8, 9].

Finally, the sediment was resuspended in 1 mL of 0.2% bovine albumin and the suspension inoculated into the the liquid medium of the BacT/Alert 3D MB system (BIOMERIEUX ® North Carolina, USA) which is based on colorimetric dection of carbon dioxide. BALfluid is inoculated directly in liquid culture bottle.

Culturing of Mycobacteria in the BacT/Alert 3D MB automated culture system was achieved by inoculating 0.5 mL of the decontaminated sputum sample or the BAL fluid in culture bottles containing liquid culture media i.e. Middle brook 7H9 along with coctail of lyophilized antibiotic mixture (PANTA – polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin). Addition of antibiotics is recommended to avoid growth of contaminant microorganisms.

Then all the bottles were loaded in the automated continuous-monitoring system (BacT/Alert 3D MB) for Mycobacterium detection. When the system indicated the presence of growth, then all positive culture bottle were smeared and stained by Z.N method.

Geno Type MTBDR plus assay

Further processing of culture positive and smear positive samples was done by commercially

available multiplex PCR DNA strip assay kits (Genotype ® MTBDR plus assay [Hain Lifescience GmbH, Nehren, Germany]) according to manufacturer's instruction[10].

In brief, 1 ml of positive culture broth was re suspended in 300 µl molecular biology-grade water. Heat killing was done at 95°C for 20 min followed by sonication for 15 min. The samples were then centrifuged at 13000g for 5 min and 5 µl of the supernatant was used for the PCR.

Amplification mixture consisted of 35 µl primer nucleotide mixes, 5 µl of PCR buffer with 20 mM MgCl2, 2 µl of 25 mM MgCl2, 1 U of HotStar Taq DNA polymerase from Qiagen, 3 µl of molecular biology grade water and 5 µl supernatant in a final volume of 50 µl. Amplification was done in a thermo cycler (MyCycler, Bio-Rad Laboratories) using the amplification profile: denaturation of 15 min at 95°C, followed by 10 cycles of 30 sec at 95°C and 2 min at 58°C, and 20 cycles (for culture isolates) of 25 sec at 95°C, 40 sec at 53°C and 40 sec at 70°C and the extension step of 8 min at 70°C. Hybridisation was performed using a pre-programmed Twin Cubator (Hain Life science, Nehren, Germany).

After denaturation, the biotin-labelled amplicons were hybridized to the single stranded membrane-bound probes. After a stringent washing, as streptavidin-alkaline phosphate conjugate was added to the strips, an alkaline phosphatatase mediated staining reaction was observed as bands where the amplicon and the probe had hybridized. Then, MTBDR *plus* strips were interpreted according to manufacturer's guidelines.

RESULTS

In our study, 210 suspected cases for pulmonary tuberculosis were included. Among 210 patients 81(38.5%) was AFB smear positive. In which 66 (81.4%) were males and 15 (18.5%) were females. 162 (77.1%) clinical samples were positive by automated liquid culture system (BacT/Alert 3D MB) for *Mycobacterium species*. Among culture positive samples, 126 (77.7%) were found to be male and 36 (22.2%) were females. From the positive samples, most of the patient were classified in age group of 41 to 60 years with more number of males in same age group i.e. 126 (77.7%) and females were in 21 to 40 years of age group.

Table-1: Age wise and Sex distribution of patients positive by automated liquid culture system:

Age Group	Male	Female	Total
0-20 yrs	8	2	10
21-40 yrs	66	18	84
41-60 yrs	52	16	68
Total	126	36	162

Out of 162 positive samples 15 (9.2%) were negative for *M. tuberculosis* and their drug sensitivity pattern by using Genotype® MTBDR plus assay shows 111 (68.5%) samples were sensitive to both Rifampicin and isoniazid, 15(9.2%) samples were resistant to isoniazid but sensitive to rifampicin, 6 (3.7%) isolates were resistant to rifampicin but sensitive to isonizid and 15 (9.2%) isolates were found to be Multidrug resistant (MDR) i.e resistant to first line drugs (Rifampicin and isoniazid).

Table-2: Drug sensitivity pattern by Genotype MTBDR plus assay among tuberculosis positive patients:

Pattern of drug resistance	Male	Female	Total
Multidrug resistant	9	6	15
Mono drug resistant (Isoniazid)	15	0	15
Mono drug resistant (Rifampicin)	3	3	6
Sensitive to both Rifampicin and Isoniazid	90	21	111
Negative for M tb	15	0	15
Total	**132**	**30**	**162**

All MDR samples were further processed for second line drug sensitivity and out of 15(9.2%) samples, 11(6.7%) samples were sensitive to second line drugs (Fluoroquinolones, aminoglycosides / cyclic peptides and ethambutol), 3(1.8%) samples were resistant for ethambutol but sensitive to fluoroquinolones, aminoglycosides / cyclic peptides and 1(0.61%) sample is resistant to fluoroquinolones but sensitive to aminoglycosides / cyclic peptides and ethambutol.

DISCUSSION

All forms of Tuberculosis (TB) are still number one public health problem in many countries. Diagnosis and treatment of many forms of TB is still causing lot of hardship to several governments and private agencies across the world. Yet it is completely curable and is (relatively) inexpensive to cure but there are the challenges in TB care like low health priority, limited resources (human and financial), low case detection and drug resistance, stigma and discrimination, poverty, mobile population and migration.

As Teerthankar Mahaveer medical college and Research centre is a tertiary care teaching hospital in Moradabad, to which most of the patient from western U.P came for medical advice. This study helps to show the current trend and drug resistance pattern of Mtb from different region of western U.P.

Among 210 pulmonary tuberculosis suspected cases 38.5% were AFB smear positive and 61.4% were smear negative. Although they are less infectious than smear positive but are caplable of transmitting disease in the same manner. Smear-negative TB often requires review of the findings of radiological investigations and other diagnostic tests to evaluate the causative agent of pulmonary tuberculosis. In our study smear negative cases were quite high as compared to study done by Ber MA *et al.* in 1999[11], who showed 17% of the TB transmission from smear negative cases.

The rate of Mycobacterium isolation by automated liquid culture system was 77.1% in our study. Which is compared to the study done by Damle *et al.* who observed high rate of isolation [12].

In our study, the occurrence rate of MDR –Tb is 9.2%. Which is quite similar to the study done by Nudrat S *et al.* in 2016[13]. Who reported MDR occurrence rate is 10.7%. In our study, we found that the rifampicin and isoniazid resistant rate is 9.2%, which is quite similar to study done by C. Thakur *etal.* In 2015. Who reported rifampicin resistant rate is 6.1% and isoniazid resistant rate is 8.6% [14].

When all MDR – Tb samples were further processed for second line drugs sentivity , we found that out of 15(9.2%) samples, 6.7% samples were sensitive to second line drugs. Single drug resistance i.e etambutol is 1.8% and fluoroquinolones is 0.61%. Similar data shown by A. umubyeyi *at el.* in 2008, who reported occurrence rate of single drug resistance, is 1.4 %. We didn't found any XDR sample in our study.

CONCLUSION

From our study we concluded that the microscopy and solid culture methods are age old procedure had reasonable cost. Which is in favor of community but are time taking and have high contamination rate. Liquid culture methods and Line Probe assay are costly but the test is rapid and more accurate. Early diagnosis and treatment leads to prevention of drug resistance, which is beneficial for the patient and more importantly for the society.

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