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Rapid Identification of *Mycobacterium tuberculosis* and Non Tuberculous *Mycobacterium* Isolates from Pulmonary and Extra Pulmonary Samples using MGIT320 Liquid Culture System and MPT64 Antigen Test

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A B S T R A C T

Tuberculosis (TB) is a major public health problem in India and a leading cause of death in adults, especially among the economically productive age group. Historically TB has been associated with significant morbidity and mortality and remains a major global health problem. The present study was initiated to determine the prevalence of *Mycobacterium tuberculosis, Non Tuberculous Mycobacterium* and its resistance to first line Anti-Tubercular drug from both pulmonary and extra pulmonary samples A total of 583 properly collected samples (226 pulmonary and 357 extra pulmonary) from patients with clinical/radiological suspicion of Tubercular infection were included in this study. All the samples were screened by Zeihl-Neelsen AFB microscopy, and subjected to liquid culture using Mycobacterium Growth Indicator Tube (MGIT-320). Positive cultures were differentiated into *Mycobacterium tuberculosis* complex (MTBc) or non-tubercular *mycobacterium* (NTM) by immunochromatography assay using MPT-64 antigen. Further it was followed by drug susceptibility testing of MTBc isolates thereby identifying multi-drug resistant strains. Out of 583 samples, 141 strains were isolated on MGIT-320 (81 pulmonary, 60 Extrapulmonary) and the detection time was 15 days. *Mycobacterium* complex isolates were 116 and Nontuberculous Mycobacteria were 25. Among *Mycobacterium tuberculosis* complex isolates 92(56 pulmonary, 36 Extrapulmonary) were sensitive to all the drugs and 24(16 pulmonary, 8 Extrapulmonary) were resistant to one or more drugs. Multiple drug resistant (MDR) isolates were 7(6 pulmonary, 1 Extrapulmonary). MDR-TB is gradually increasing due to improper diagnosis and inadequate treatment. Differentiating *mycobacterium* as MTBc and NTM supported by sensitivity testing by using liquid culture has proved to be helpful in early decision for chemotherapy in MDR-TB patients.

Introduction

Tuberculosis (TB) is a major public health problem in India and a leading cause of death in adults, especially among the economically productive age group. Historically TB has been associated with significant morbidity and mortality and remains a major global health problem. India accounts for one- fifth of the global burden of TB. It is estimated that about

40% of Indian population is infected with TB bacillus.(1) The prevalence and mortality due to TB in India were estimated to be 249 and 26 respectively per100,000 population.(2)The importance of early diagnosis and correct etiological identification of pulmonary tuberculosis need not be over-emphasised, since treatment is different for *Mycobacterium tuberculosis* and atypical Mycobacteria (non-tuberculous Mycobacteria, NTM). World Health Organization has given guidelines for low and medium income countries for use of liquid culture systems and drug sensitivity testing for tuberculosis work. (3) The emergence of anti‑ tubercular drug resistance is an increasing public health problem and TB control programmes in industrialized and developing countries alike. (4) Drug resistance arises due to improper and irrational use of anti-tubercular drugs (ATDs) in chemotherapy of drug-susceptible TB patients. This improper use is a result of a number of actions including administration of improper treatment regimens and failure to ensure that patients complete the whole course of treatment. Essentially, drug resistance indicates a weakness in TB control program in that area. A patient who develops active disease with a drug-resistant TB strain can transmit this form of TB to other individuals. Strategies used for the clinical management of patients infected with drug-resistant *Mycobacterium tuberculosis* scomplex (MTBC) are different, therefore, prompt detection, isolation, and implementation of alternate anti-tubercular treatment regimens are necessary for suitable management (5) (6). Moreover, early detection of such cases is of utmost importance in preventing spread of resistant bugs in the community. Automated non-radiometric systems for accelerated isolation of *Mycobacterium tuberculosis* complex (MTBC), being expensive, are available only in selected centres in India and third-world countries. However, most laboratories still depend upon conventional techniques, thus resulting in an extended reporting time of 4-5 weeks. The MGIT is a liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. In addition to Middlebrook 7H9 liquid media, the MGIT tube contains an oxygen-quenched fluorochrome. It detects oxygen consumption induced by growing micro-organisms (7). There are a few published reports on the evaluation of Bactec MGIT 960 on extrapulmonary samples. An innovative rapid kit, MPT64-ICT, to detect an established marker of MTBC, the MPT64 antigen, by immune chromatography test (ICT)developed by Japanese scientists(8) found universal acceptance due to its simplicity, accuracy and rapidity. (9) (10) (11) Indian reports on EPTB in general and the use of rapid kits for confirmation of MTBC in particular are few.

The present study was initiated to determine the prevalence of *Mycobacterium tuberculosis, NonTuberculous Mycobacterium* and its resistance to first line Anti-Tubercular drug from both pulmonary and extra pulmonary samples among patients attending a tertiary care hospital in Hyderabad.

Materials and Methods

Study design

The study was carried out in the clinical Microbiology laboratory of a tertiary care hospital in Hyderabad during the period January 2013 to December 2015. Our Institutional Human Ethics Committee scrutinized and approved this research. Patients' informed consent was obtained before collection of specimens.

Study population

A total of 583 properly collected samples (226 pulmonary and 357 extra pulmonary) from patients with clinical/radiological suspicion of

Tubercular infection were included in this study. We included both pulmonary (like deeply expectorated freshly collected sputum samples, free of saliva, blood and food contamination and bronchial alveolar lavage samples) and extra-pulmonary samples (such as all body fluids, tissue, urine, pus, aspirates etc.). Samples were included irrespective of the treatment status of the patients (e.g. both new suspected cases as well as post-treatment cases).

Patients were finally included on the basis of availability of consent forms. Any patient without consent was excluded from the study. All samples showing evidence of contamination with saliva (determined by Bartlett's grading system) (12) were excluded from our study. We excluded the whole blood samples as well as swab samples for TB diagnosis in this study as per standard guidelines.

Inclusion criteria

Both pulmonary (like deeply expectorated freshly collected sputum samples and bronchial alveolar lavage samples) and extra-pulmonary samples (such as all body fluids, tissue, urine, pus, aspirates etc.) were included. All samples were selected on the basis of availability of consent.

Exclusion criteria

Swabs, Blood, salivary samples were excluded from our study.

Materials and Methods

Acid fast bacilli smears

Smears were prepared from each sample, stained by Ziehl Neelson method and examined for presence of AFB with a light microscope.

Decontamination and processing of the samples

All specimens were liquefied and decontaminated by the standard N-acetyl-L-cysteine, sodium hydroxide method (NaOH-NALC). After 15 min holding at room temperature, specimens were neutralized with phosphate buffer saline (PBS, pH 6.8) and centrifuged in cold centrifuge at 4500 rpm for 20 min at 10°C. The pellets were resuspended in 1.5 ml of sterile phosphate buffer and collected for further analysis.

BACTEC MGIT 320 liquid media

The BBL MGIT tube was inoculated by 0.5 ml of the decontaminated and concentrated specimen suspension. It contained 7 mL of modified middlebrook 7H9 broth enrichment with albumin, dextrose and catalase (BBL MGIT OADC) and an antibiotic mixture consisting of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL MGIT PANTA). After inoculation, the tubes were loaded in the BACTEC MGIT 320 instrument and incubated up to 42 days at 37°C. Culture vials are monitored hourly by the instrument. The positive tube was further confirmed by ZN staining, subculturing on blood agar plate. The TTD (Time to Detection) of mycobacteria was based on the date of the earliest instrumental indication of positivity.

Morphological and biochemical identification

For differentiation of M. tuberculosis complex and NTM, a commercially available kit was used, the BD MGIT MTBc identification test (TBc ID). It is a rapid chromatographic immunoassay for the qualitative detection of M. tuberculosis complex antigen from AFB smear-positive BD MGIT tubes. The assay is performed

according to the manufacturer's instructions. Briefly, 100µl of mixed and vortexed culture fluid from AFB positive MGIT tubes were transferred to sample window of the cassette. The results of ICT were read within 15 minutes. Positive test had two red to purple bands, one for internal control and the secondline for the test.

Negative had only one band in internal control slot. Strong or light bands with any intensity were considered to be positive. MGIT tubes showing non-acid fast bacilli and/or fungi were excluded from MPT64 Ag test.

BACTEC MGIT 320 liquid media DST

MTBc isolates was further tested for the first line drugs in BACTEC MGIT 320. Conc. of various drugs used was – streptomycin (STR)- 1µg/ml, isoniazid (INH)- 0.1 µg/ml, rifampicin (RIF)- 1 µg/ml, ethambutol (ETB)- 5 µg/ml. Drug susceptibility was reported when the growth control units reached 400 as indicated by the instrument.

Control strains

Reference strains of H37Rv and *Mycobacterium fortuitum* were included as positive and negative controls, respectively.

Results and Discussion

583 clinical samples (226 pulmonary and 357 extra-pulmonary) were analyzed during the period of our study (Figure 1).

Number of Positive and Negative samples, screened through Ziehl-Neelsen AFB Staining procedure and culture by liquid media MGIT 320 are given in Table1. Out of these 583 samples, 257 were male patient and 326 were females, in which 63 and 78 were positive respectively, summarized in Table 2. There was no much difference in gender distribution among positive pulmonary samples whereas females were predominant in case of Extra pulmonary positive samples (Figure 2 and 3).

The results of age wise distribution among positive cases in both pulmonary and Extra pulmonary samples shows majority of case in the age group of below 40 years, summarized in Table 3. Distribution of various samples is given in the Table 4.

The results show that, out of 226 pulmonary samples, 81 were MGIT culture positive, of which 47 were positive for AFB by ZN staining and out of 357 Extra pulmonary samples, 60 were MGIT culture positive, of which 19 were positive for AFB by ZN staining (Table 5 and 6). Out of these 81 culture positive isolates from pulmonary samples 71 were MPT64Ag test positive and 9 were negative samples. This was considered as *Non-Tubercular Mycobacterium sp.* (Speciation not done).

Similarly, Out of these 60 culture positive isolates from Extra pulmonary samples 44 were MPT64Ag test positive and 16 were negative samples. This was considered as *Non-Tubercular Mycobacterium sp.* (Speciation not done) (Table 7).

The average TTD was 15 days for MGIT 320 with the extremity from 6 to 38 days. Among pulmonary positive cases, resistance to any drug was found in16 cases (19.75%), to S in 5(6.17%), to I in 13(16.04%), to R in 7(8.64%) and to E in 2(2.46%). Multidrug resistance rate was6 (7.40%) (Figure 4).

Similarly among Extra pulmonary positive cases, resistance to any drug was found in 8 cases (13.3%), to I in 2(3.33%), to R in 3(5.00%) and to E in 1(1.66%) and no mono resistance in S. Multidrug resistance rate was1(1.66%) (Figure 5).

Table.1 Distribution of culture positive cases

No. of cases studied	No. of positive cases	No. of negative cases
583	141(24.18%)	442(75.18%)

Table.2 Gender distribution of patients and percentage of positive samples

Gender	No. Of collected samples (%)	Positive isolates (%)
Male	257 (44.08)	63 (10.80)
Female	326 (55.92)	78 (13.38)
Total	583	141

Table.3 Age and Sex distribution of positive cases

Age Distribution	Pulmonary		Extra Pulmonary	
	Male(n=41)	Female(n=40)	Male(n=22)	Female(n=38)
20 and below	9(22%)	13(32%)	6(27%)	8(21%)
21 – 40	11(27%)	16(40%)	9(41%)	13(34%)
41 – 60	15(36%)	8(20%)	4(18%)	17(45%)
61 and above	6(15%)	3(8%)	3(14%)	0

Table.4 Sample distribution in patients

Type of sample		No of cases	Positive
Pulmonary n=226 (38.77%)	Sputum	195	76
	BAL	31	5
Extra pulmonary n= 357(61.23%)	Pleural fluid	199	27
	Pus	41	11
	Ascitic fluid	29	3
	ET Secretion	11	4
	CSF	19	2
	Urine	8	1
	Pericardial fluid	7	0
	Lymph Node Aspirates	31	12
	Peritoneal fluid	3	0
	Semen	1	0
	Synovial fluid	8	0
Total		**583**	**141**

Table.5 Distribution of positive cases sample wise

Sample Type	Total Samples	AFB Culture Positive	AFB Culture Negative
Pulmonary	226	81	145
Extra pulmonary	357	60	297

Table.6 correlation between stain and culture

	Pulmonary			Extra Pulmonary		
	Stain +ve	**Stain -ve**	**Total**	**Stain +ve**	**Stain -ve**	**Total**
Culture Positive	47	34	81	19	41	60
Culture Negative	0	145	145	0	297	297

Table.7 MTBC and NTM positive samples

Sample Type	MTBC	NTM
Pulmonary	72	9
Extra Pulmonary	44	16
Total	**116**	**25**

Fig 1 Distribution of sample type

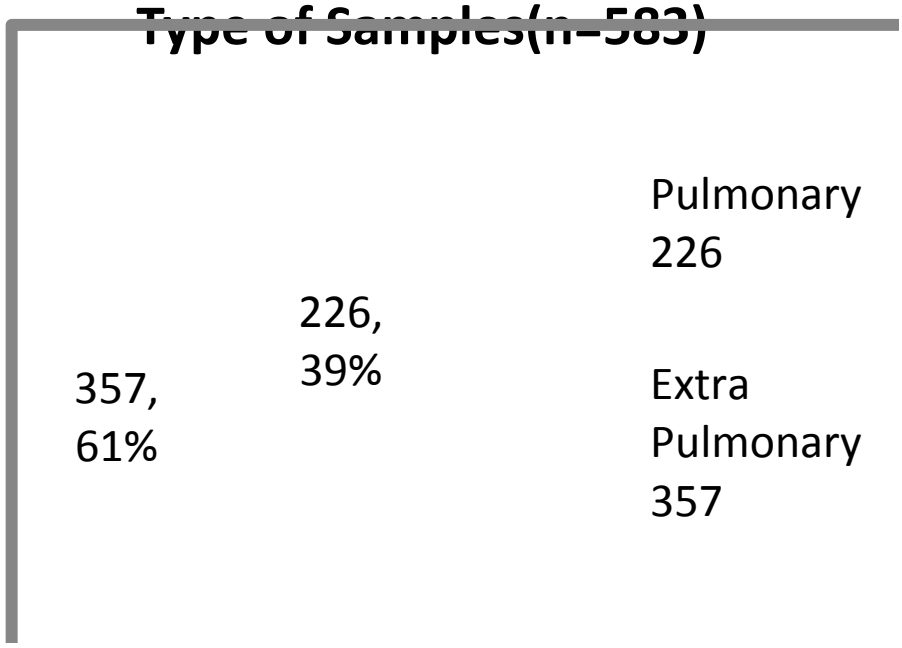


Fig.2 Sex wise distribution of MTB positive pulmonary samples

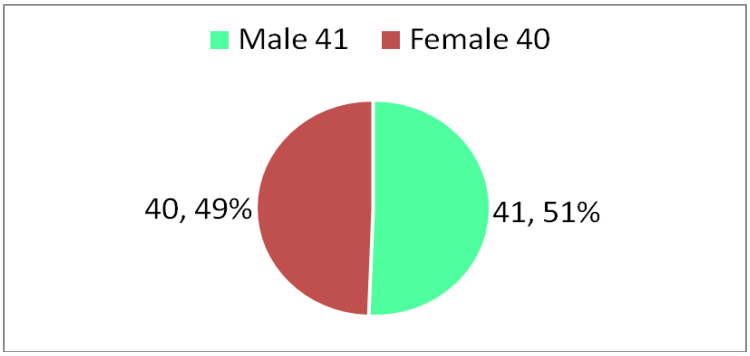


Fig.3 Sex wise distribution of MTB positive extra pulmonary samples

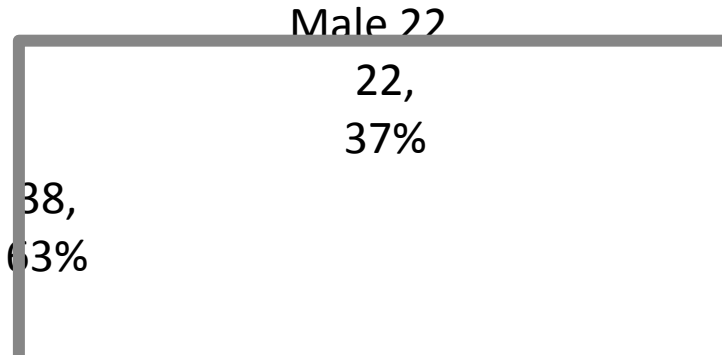


Fig.3 Results of Drug susceptibility testing in pulmonary samples

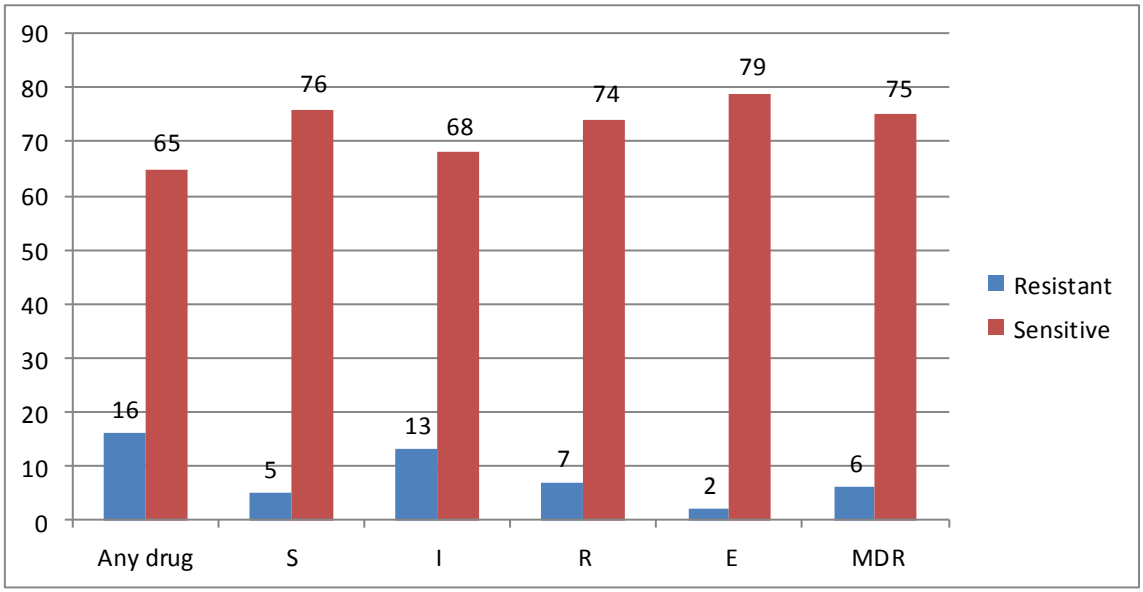
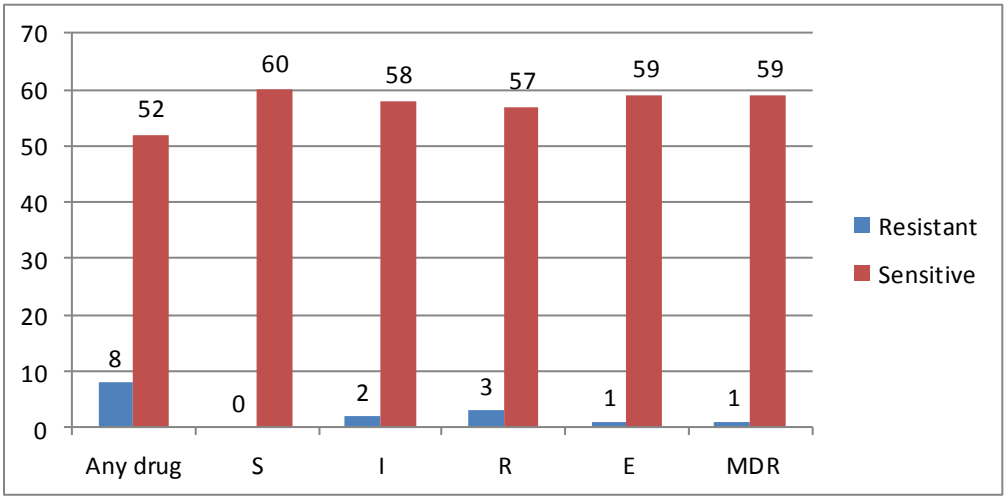


Fig.4 Results of Drug susceptibility testing in extrapulmonary samples



Early diagnosis of *Mycobacterium tuberculosis* infection is pre-requisite to achieve WHO's target to end Global TB epidemic. A definitive diagnosis of TB can only be made by culturing *Mycobacterium tuberculosis* organisms from a specimen obtained from the patient. Therefore, techniques which shorten the time for detection of *Mycobacterium* deserve attention.

In our study, out of the 583 clinical samples (both pulmonary and extra pulmonary), 141(24.18%) were culture positive. The importance of early diagnosis and correct etiological identification of Tuberculosis need not be over-emphasised, since treatment is different for *Mycobacterium tuberculosis* and atypical Mycobacteria (non-tuberculous Mycobacteria, NTM). In our study, out of 141 positive isolates 116(82.2%) were *Mycobacterium tuberculosis* (MTBc) and 25(17.7%) isolates were NonTuberculous *Mycobacterium* (NTM) using MPT64Ag test. Similar results were given in various studies like Kannade *et al.,* (13) from Bombay (Mumbai) who examined 165 isolates (125 MTB; 30 NTM; 10 Non-Mycobacterial species) and observed sensitivity of 99.19% and 100% values for specificity, positive predictive value (PPV) and negative predictive value (NPV) for the rapid MPT64 antigen detection kits in comparison to conventional methods. Vadwai *et al.,* (14) from Bombay analysed 394 strains from 280 pulmonary and 114EPTB samples (388 MTB; 6 NTM) with similar result, i.e. 99.4% sensitivity and 100% specificity. Kumar *et al.,* (15) from Mysore, Karnataka, analysed 77 isolates (55 MTB; 10 NTM; 12 Non-Mycobacterial species) recorded 100% results for all four parameters.

In our study majority of the pulmonary MTB infected male patients were within the age group of 20–40 years and female patients, within the age group of 10-40 years. In the case of extra pulmonary samples too both the males and females were from the age group of 20 - 40 years. This is in correlation with the study done by Kandhakumari *et al.,* (16).

The prevalence of drug-resistant TB was found variable in different studies from around the world and in our country. In our study, out of the 583 clinical samples, among pulmonary samples the prevalence of resistance to any drug was found in 16 cases (19.75%), to S in 5(6.17%), to I in 13(16.04%), to R in 7(8.64%) and to E in 2(2.46%). Multidrug resistance rate was6

(7.40%). Similarly among Extra pulmonary positive cases, resistance to any drug was found in 8 cases (13.3%), to I in 2(3.33%), to R in 3(5.00%) and to E in 1(1.66%) and no mono resistance in S. Multidrug resistance rate was 1 (1.66%). Multidrug-resistance is the independent factor for morbidity and mortality due to tuberculosis (17) (18). Treatment of MDR-TB is difficult and drugs used for treatment are less potent, more toxic and more expensive than firstline drugs (18) (20). Many studies published from different parts of India have reported high MDR-TB prevalence, but mostly among first-time re-treatment patients with relapse, treatment after default, and treatment after failure (21) (22). The possible reasons of a higher prevalence of drug resistance in our study can be, mixing of new as well as retreatment cases and smaller sample size. Although many Indian studies have reported lower prevalence of Rifampicin mono-resistance from various parts of the country, in our study the higher rate can be due to a possible co-existence of INH resistance and the rate may be acting as a proxy to the local MDR-TB prevalence. Various Indian studies have reported MDR rates to be varying from 17.4% to 53% among re-treatment cases.(23,24) World-wide surveillance of MDR in re-treatment cases ranged from 9.4% to 36.5%, from 1994-2000 across the world.(25) Previous exposure to anti-tuberculosis agents is the most common cause of developing MDR. In 2008, the WHO reported a worldwide resistance rate to INH of 5.9%. INH resistance rates higher than 10% can predict the development of MDR TB according to the WHO (26). The higher resistance rate of INH according to other first line drugs may be resulted by both its wide use in the chemoprophylaxis and latent TB (27).

According to WHO in 2014, 220,000 people died from TB in India, which is the highest in the world. The same report says that 2.1% cases in this emerging percentage are due to MDR-TB.

Thus early detection of MDR-TB cases and initiation of appropriate treatment based on drug resistance testing can lower the burden of this deadly disease.

In conclusion to conclude, globally the prevalence of Tuberculosis is on the increase. Due to prolonged time taken for positive culture and drug susceptibility report by conventional methods in suspected cases, the clinicians in developing countries empirically initiate anti-tuberculosis treatment (ATT) with first-line drugs. However, if the etiology happens to be NTM, this would be a burden to the patients and can promote emergence of drug resistance in Mycobacteria. The isolates must be checked for drug sensitivity in this era of increasing drug resistance. Thus rapid isolation of *Mycobacterium* species using automated MGIT320 system is more beneficial when combined with rapid ICT kit which detects MPT64 Ag in 15 minutes and also differentiates MTBC from NTM isolates. Notification of the DST results with clinical data is a key element to get valid and representative information on drug resistance. As a study of prevalence of drug resistance in TB from Hyderabad, we believe that this study can help in the control of TB at the national level and probably can help us in the mapping drug resistant TB cases in this part of the country.

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