Original Article

Detection of Multi-Drug-Resistance in Extra Pulmonary Tuberculosis by Indirect GenoType MTBDR*plus* Line Probe Assay

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ABSTRACT

Introduction: Approximately 15 to 20 percent of tuberculosis patients have extra-pulmonary tuberculosis (EP-TB), which is often difficult to diagnose. Further multi drug resistant tuberculosis (MDR-TB) in EP samples has not been reported. The aim of the present study was rapid diagnosis of MDR-TB in EP samples by using Mycobacteria Growth Indicator Tube (MGIT) 960 and GenoType MTBDR*plus* Line Probe Assay (LPA).

Methods: A total of 429 extra pulmonary specimens (pleural fluid, lymph node biopsy, cerebrospinal fluid, skin biopsy, urine, faeces) were processed for acid fast bacilli (AFB) smear microscopy and culture by MGIT-960. SD Bioline TB Ag MPT 64 rapid assay was carried out on MGIT positive isolates to differentiate between MTB and Non Tuberculous Mycobacteria (NTM). Growth obtained was tested by Line Probe assay (LPA)- GenoType MTBDR*plus* assay for the identification of Rifampicin and Isoniazid resistance and GenoType CM-AS assay for species identification of NTM.

Results: Among 429 specimens, 58 (13.5%) were AFB positive and 371 (86.5%) were negative on MGIT 960 culture. Amongst 58 positive isolates, 56 were MTB (96.6%) and 2 (3.4%) were NTM, subsequently identified as *M. fortuitum* and *M. intracellulare*. In 56 MTB positive isolates, 35 (62.5%) were sensitive to Rifampicin (RFM) and isoniazid (INH). Two isolates (3.6%) were found mono RFM resistant, 11 (19.6%) were mono INH resistant and 8 (14.3%) were resistant to both (MDR).

Conclusion: Mycobacteria were isolated in 13.5% cases, 14.3% of these were found to be MDR-TB. Overall MDR in EP-TB may be in the same range as pulmonary TB as there was no isolate in 371/429 specimens/cases, this may be due to impact of prior therapy. All these issues need to be investigated by more sensitive molecular methods and correlated microbiologically as well as clinically. Specimens from EP-TB cases must be processed for LPA and DST which will be of help in rapid diagnosis of MDR-TB and institution of right treatment strategy.

INTRODUCTION

Tuberculosis (TB) is one of the leading causes of death in the world. In 2013, 6.1 million TB cases were reported, out of these 5.7 million were newly diagnosed and another 0.4 million were already on treatment. Globally, it's estimated that 3.5% (95% confidence intervals, CI: 2.2%-4.7% of new cases and 20.5% (95% CI- 13.6-27.5% of previously treated cases have MDR-TB. More than half of these patients belong to India, China and the Russian Federation. One fourth of the global incident TB cases occur in India annually[1]. Pulmonary TB is the most important clinical manifestation of *Mycobacterium tuberculosis* however; other organs can be involved, which include the pleura, lymph nodes, abdomen, genitourinary tract, skin, joints, bones, meninges, brain etc. EP-TB has been reported in approximately 15-20% of all TB cases in HIV

negative patients, and 40-50% in HIV positive persons in India[2].

Diagnosis of EP-TB is often based on clinical signs and radiological findings and at times by culture, histology or Polymerase Chain Reaction (PCR) based tests. Major difficulty with EP specimens is that they are paucibacillary, due to which the sensitivity of AFB smear and culture is low. This type of disease therefore, most often remains undiagnosed and, even worse, untreated. The automated liquid culture systems like MGIT 960 have reported better sensitivity[3] for growth of mycobacteria and PCR has also been used for detection of MTB in EP samples with good sensitivity[4]. However, studies reporting MDR-TB in EP-TB samples are very few[5,6,7,8,9]. Gene Xpert has recently been used to detect presence of TB as well as Rifampicin resistance in pulmonary and EP samples but is still under evaluation for EP samples. GenoType MTBDR*plus* LPA (Hain Life science GmbH, Nehren, Germany) has been validated and is routinely being used in TB control program for detection of drug resistance directly in smear positive sputum samples but is not recommended for direct use in EP samples[10]. In this study we detected Rifampin (RFM) and Isoniazid (INH) resistance by using combination of MGIT-960 and indirect LPA to know MDR-TB trends in EP-TB samples.

METHODS

A total of four hundred and twenty nine extra pulmonary specimens (pleural fluid, lymph node biopsy, cerebrospinal fluid, skin biopsy, urine, faeces) from patients suspected of TB were received in Mycobacteriology laboratory of SMS Medical College, Jaipur, India during the January 2013 - March 2015 period. Ethics approval was taken for the broader project on Genotype MTBDR*plus* assay and ethics committee was informed for this specific manuscript.

Sample Processing: All non-sterile and turbid specimens were processed by the standard N-acetyl-L-cysteine and sodium hydroxide (NALC/NaOH) method. While sterile body fluid samples {ascitic fluid, pleural fluid and cerebrospinal fluid (CSF)} etc., which were not turbid, were not processed and directly inoculated into MGIT 960 after centrifugation. One drop was used for AFB smear microscopy, from each sample. Five hundred microlitres (μl) of processed specimens were inoculated into MGIT 960 tube as per the manufacturer's protocol and rest of the deposit were stored at -20°C[11]. Culture positive tubes were further confirmed by smear microscopy and tested by SD Bioline TBAg MPT 64 rapid (MPT 64) test to distinguish *M. tuberculosis* from NTM. *M. tuberculosis* positive growth was further tested by GenoType MTBDRplus assay for detection of RMP and INH resistance. Species identification of Non Tuberculous Mycobacteria (NTM) was done by Genotype CM/AS assays.

SD Bioline TB Ag MPT 64: MPT64, an immuno-chromatographic test was used for characterization and identification of mycobacteria into MTB and NTM, as per the manufacturer's instructions[12]. Briefly 100 μl of the MGIT growth suspension was added in the sample well of cassette and incubated for 15 minutes at room temperature (RT). The presence of only control band (pink color) in the absence of test band was considered as negative for MPT64 antigen (NTM). Presence of both control and test band indicated a positive result and interpreted as presence of MPT 64 antigen thus identity of isolate as a *Mycobacterium tuberculosis* strain.

GenoType MTBDR*plus* assay: All culture positive MTB isolates were subjected to GenoType MTBDRplus assay for detection of RMP and INH resistance as per manufacturer's instructions[13]. DNA was extracted from growth using GenoLyse (Hain Life sciences) DNA extraction kit and subjected to LPA. For amplification 35 μl of a primer-nucleotide mixture (provided with the kit), amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U hot start *Taq polymerase* (QIAGEN, Hilden, Germany) and 5 μl of DNA in a final volume of 50 μl was used. The amplification protocol consisted of 15 min of denaturation at 95°C followed by 10 cycles comprising 30s at 95°C and 120s at 65°C; an additional 20 cycles comprising 25 s at 95°C, 40 s at 50°C and 40 s at 70°C; and a final extension at 70°C for 8 min.

Table 1: Smear and culture positivity in extrapulmonary specimens for mycobacteria

Sample type	Number of samples	Males	Females	Mean Age (years)	AFB Smear	MGIT Positive	Percent Positivity
Pus	72	36	36	32	3(1+), 1(Sc-3)	14	24.14
Blood	3	2	1	28	Negative	0	0
Lymph node	21	7	14	26.6	Negative	11	18.97
CSF	53	35	18	33.7	Negative	3	5.17
Endometrium biopsy	138	0	138	31	1(1+)	7	12.07
Pleural fluid	75	57	18	34.6	1(1+), 1(Sc-6)	14	24.14
Swab	7	4	3	32.3	Negative	0	0
Skin biopsy	28	18	10	39.25	Negative, 1(Sc-4)	5	8.62
Stool	1	0	1	57	Negative	0	0
Urine	24	16	8	33.2	1(Sc-2)	3	5.17
Gastric fluid	4	2	2	27	Negative, 1(Sc-3)	1	1.72
Ascitic fluid	3	0	3	21.5	Negative	0	0
Total	429	177	252		Positive = 10	58	

Table 3: Drug resistance profile in extrapulmonary tuberculosis samples by GenoType MTBDR*plus* assay

Sample Type	No. of samples	MGIT Positive	INH+RFM Sensitive	Mono-rifampicin Resistant	Mono Isoniazid Resistant	INH+RFM	NTM	NTM Species
Pus	72	14	7	1	3	3		
Blood	3	0	0	0	0	0		
Lymph node	21	11	6	0	2	1	2	M. fortuitum, M. intracellulare
CSF	53	3	2	0	0			
Endometrial 138 Biopsy	7	3	0	3	1			
Pleural fluid	75	14	11	1	1	1		
Swab	7	0	0	0	0	0		
Skin Biopsy	28	5	4	0	1	0		
Stool	1	0	0	0	0	0		
Urine	24	3	1	0	1	1		
Gastric Fluid	4	1	1	0	0	0		
Ascitic Fluid	3	0	0	0	0	0		
Total	429	58	35	2	11	8	2	
Percentage		13.5	62.5	3.6	19.6	14.3	3.4	

The amplified product was analyzed by "Reverse Hybridization" using the nitrocellulose membrane strip containing 27 probes, including amplification, hybridization controls to verify the test procedures and TUB band for detection of *M. tuberculosis* complex. For the detection of RFM resistance, wild type and mutant probes (D516V, H526Y, H526D and S531L) targeting *rpoB* locus (encoding amino acids 509-533) which confer resistance to RFM were used. For the detection of INH resistance, probes specific to *katG* gene (S315T) and *inhA* gene (Mutants 15C/T, -16A/G, -8T/C, -8T/A) were targeted. The absence of binding of amplicon to one or more wild-type probe(s) and or binding to mutant probes was considered as indicative of the resistance.

Reverse hybridization and detection was performed in an automated washing and shaking incubator. The hybridization procedure was performed at 45°C for 30 min, followed by stringent washing at 45 C for 15 min and the colorimetric detection of the hybridized amplicons were done by subsequently washing with conjugate (30 min at room temperature), RIN and substrate reagents (5-10 min at room temperature). After the final wash, strips were air-dried and fixed on paper provided with the kit.

GenoType CM/AS assays: GenoType CM/AS assays (Hain Life science GmbH, Nehren, Germany) are also based on reverse hybridization of PCR product to a nitro- cellulose membrane strip with immobilized probes for different mycobacterial species. The GenoType CM is capable of identifying 23 and GenoType AS is identifying 14 species. Assays were performed as per manufacturer's protocol[14], similar to MTBDR*plus* assay described above. Each CM and AS strip contains 17 probes which includes amplification and hybridization controls to verify the test procedures. In CM assay, 15 patterns are observed which identify 23 species, and in AS assay, 16 patterns were observed which identify 18 species.

RESULTS

Amongst the 429 EP specimens, maximum number of samples belonged to patients in the age group of 21 to 40 years (n =264), median age of the patients was 33.0 years. Maximum numbers of specimens were endometrium biopsy followed by pleural fluid, pus and CSF. Ten samples (2.33%) were found to be AFB positive (Table 1). Amongst all specimens, 58 (13.5%) were culture positive, 365 (85.1%) were negative and 6 (1.4%) got contaminated during MGIT 960 culture. The mean turnaround time for detection (TTD) of mycobacteria in smear positive samples was 14.1 days (1+ in 11.6 days, scanty in 16.7 days) and smear negative samples was 18.6 days (Table 2). MGIT positive specimens included 14 (24.1%) pleural fluid, 14 (24.1%) pus, 11 (19.0%) lymph node, 7 (12.1%) endometrium biopsy, 5 (8.6%) skin biopsy, 3 (5.2%) CSF, 3 (5.3%) urine and 1 (1.7%) gastric fluid. Maximum sample positivity was observed in lymph node specimens (52.4%) followed by pleural fluid (18.7%), skin biopsy (17.9%) and pus(9.4%), while none of the swab, blood, stool and ascitic fluid samples were found to be positive. MPT 64 assay was carried out on all 58 MGIT positive isolates. Out of 58 isolates, 56 were identified as *M tuberculosis* (MPT 64 antigen positive) and 2 as NTM (MPT 64 negative). GenoType CM/AS assays were used for species identification of NTM in 2 isolates, which were identified as *M. fortuitum* and *M. intracellulare* (Table 3).

Table 2: Time to detection (TTD) in MGIT-960 for mycobacteria and smear grading in extra pulmonary tuberculosis samples

AFB smear grading	TTD Range (Days)	No of samples	Mean TTD (Days)	
1+	9-15	5	11.6	Positive samples at mean 14.1 d
Scanty	16-21	5	16.66	
Negative	15- 29	48	18.55	

GenoType MTBDR*plus* assay was performed on all 56 MTB isolates. Among them 35 isolates (62.5%) were sensitive to both RFM and INH. The numbers and percentage of Rifampicin resistant, mono INH resistant and resistant to both RFM and INH (MDR-TB) are shown in Table 3. Amongst isolates from 14

pleural fluid, 11 (78.6%) were sensitive, one isolate (7.1%) was INH mono resistant; one isolate (7.1%) was RFM mono resistant and one isolate (7.1%) was MDR. Amid 14 isolates from pus, 7 (50%) were sensitive and three isolates (21.4%) were INH mono-resistant, one isolate (7.1%) was RFM mono resistant and three isolates (21.4%) were MDR. In isolates from 11 lymph node, 6 (54.6%) were sensitive, two isolates (18.2%) were mono INH mono resistant, one isolate (9.1%) was MDR and two isolates (18.2%) were found to be NTM (*M. fortuitum* and *M. intracellulare*). In remaining 19 isolates from CSF, endometrium biopsy, skin biopsy, urine and ascetic fluid, 11 isolates (57.9%) were sensitive, 5 isolates (26.3%) were INH mono resistant and 3 isolates (15.8%) were MDR (Table 3).

DISCUSSION

Diagnosis of EBTB is difficult due to pauci-bacillary nature and difficulty in getting proper sample from the suspected site. As a result it is under diagnosed and may remain untreated/ wrongly treated on many a occasions. Thus data on MDR-TB trends in EPTB is very limited. However, with new molecular methods like Gene Xpert, recently various studies are reporting drug resistance in limited number of EP samples[5-9]. In the present study we have detected drug resistance in EP samples by indirect LPA.

In our study amongst 429 EP specimens 2.3% were found positive by ZN staining, other studies have reported positivity ranging from 5%-16%[15,16,17,18]. In our study 13.5% EP specimens were culture positive, amongst them high positivity was observed in pleural fluid (24.1%), pus (24.1%) and lymph nodes (19.0%). Various other studies have reported positivity ranging from 9%-22%[19,20,21,22] highest in pus (50.8%) followed by spinal aspirate (14.9%), pleural fluid (14.9%), urine (7.5%)20 etc. Isolation rate from bone and Joint tuberculosis (BJTB) specimens was reported to be about 10% by Hillemann et al[22].

Recently Gene Xpert has been used in EP-TB samples with positivity ranging from 8.64% to 37%[23,24,25] Hillman et al reported 9.39% in tissue, 2.67% in gastric fluid, 2.65% in pleural fluid, 21.73% in stool and 6.59 % in urine samples[23]. Sajed et al

reported 51.7% positivity in pus samples, 15.8% in pleural fluid, 6.3% in ascitic fluid and 40.0% in CSF samples[25].In present study maximum sample positivity was observed in lymph node (52.38%) followed by gastric fluid (25%).While Makesh Kumar et al reported 50% positivity in urine, 42.85 % in pus and 36% in CSF, 27 % in ascitic fluid, 22 % in pleural fluids[15]. Maurya and co-workers reported 83.4% positivity in lymph node aspirates and synovial fluid[16]. It is apparent that isolation rates vary a lot among different types of specimens and also among different studies which shows the need of optimizing the procedures/ techniques for this purpose.

The mean turnaround time for detection (TTD) of mycobacteria in smear positive samples was 12.6 days and smear negative samples was 18.66 days. Other studies have reported TTD ranging from 8-18 days depending on smear status[26,27]. In present study amongst the 58 MGIT positive isolates, 3.44% were found to be NTM (*M. fortuitum* and *M. intracellulare* in lymph node samples). Similarly study from Pondicherry also reported four NTM (1.10%) isolates {M. scrofulaceum3 and M. fortuitum1} amongst EPTB suspected patients[20]. The study from Lucknow, however, reported higher positivity of NTM {27.4% (62/227)} amongst EP-TB positive samples, *M. fortuitum* (27.5%) and *M. intracellulare* (20.9%) being the commonest[17]. This also shows the need of optimizing collection and decontamination methods for EP-TB specimens.

In the present study 14.29% isolates were identified as MDR-TB. Other studies have reported varying range; 10% at Delhi[28], 12.5% at Nepa[16], 13.4% from Lucknow[7] (11.4% in new patients and 19.1% in previously treated patients) and 21.4% at Pakistan[5]. In our study 3.57% isolates were mono rifampicin resistant, as per World Health Organization (WHO), Rifampicin resistance is taken as surrogate marker for MDRTB and mono rifampicin resistance in LPA are also treated as MDRTB. Though Gene Xpert assay can also detect MDRTB along with TB in EP samples but very limited data is available. Only one sample each was reported to be Rifampicin resistant

with MDR TB rate ranging from 1.14%[15], 3.4%, and 7.14%[29].While other authors reported their observations on more than one sample as Rifampicin resistant with rate of 2.6% and 3.67%[8,9]. As the use of Gene Xpert to determine RFM resistance in EP samples is still under evaluation[30], all data including ours becomes important for understanding the problems likely to be encountered for its application in different types of EP-TB.

As detection of drug resistance in EP samples is not recommended by direct LPA, therefore indirect LPA was carried out on MTB grown on MGIT-960. Carrying out LPA from growth also ensured presence of live bacilli, which is not possible in the case of Gene Xpert and direct LPA. Moreover time taken for MGIT culture plus indirect LPA was only 12- 15 days (LPA takes only 7-8 hours) in comparison to MGIT culture plus MGIT Drug Susceptibility Testing (DST) which takes total 25 - 30 days and conventional culture and DST takes 8-12 weeks to be completed. Gene Xpert is the most rapid method giving results in 90 minutes but is still under evaluation for detection of drug resistance in EP samples. Thus by use of indirect LPA we could rapidly identify MDRTB in 14.29% isolates from EP-TB specimens.

CONCLUSION

Since diagnosis of EP-TB is very difficult due to low positivity and paucibacillary nature, the main focus remains on the diagnosis of TB as a result detection of MDRTB in EP samples remains under diagnosed. The present study reported MDRTB in 14.29% of MTB isolates which is a significant finding and demands attention. Knowing accurate treatment history of cases will be important to draw inferences of primary or secondary resistance. It may be noted that overall MDR may be in same range as pulmonary cases, as MTB could be isolated in only 58/ 429 specimens. There is thus need to consider direct testing by sensitive molecular / in-situ methods for diagnosis as well as detection of drug resistance in EP samples. Use of MGIT culture and indirect LPA could help in early detection of MDR-TB which, however, should be correlated with microbiological

results and clinical response. Moreover the presence of NTM in these samples should not be overlooked and steps should be taken to speciate them and repeat sample to establish etiological relationship.

ACKNOWLEDGEMENTS

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