Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN 0976-9595

Research Article

DETECTION OF MYCOBACTERIUM TUBERCULOSIS AND DRUG RESISTANCE IN PEDIATRIC PATIENTS SUSCEPTED OF PLEURAL TUBERCULOSIS

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ABSTRACT

The main purpose of this study is the detection of *Mycobacterium tuberculosis* (MTB) and its drug resistance profile by molecular methods in pediatric patients suspected of pleural tuberculosis. Children ≤ 15 years of age suspected of pleuritis were enrolled in this study. Total 176 pleural fluid samples were collected from January to June 2018 and tested for AFB microscopy, GeneXpert and MGIT culture. Of 176 samples, total 14 (8%) samples were positive for MTB and 1 (7.1%) was observed MDR-TB. Smear microscopy detected AFB in 2 (1.1%) cases only. GeneXpert and MGIT culture showed 6 (3.4%) and 12 (6.8%) positive cases respectively, out of, 1 (16.7%) was found rifampicin resistant by GeneXpert. Of culture positives, 1 (8.3%) was MDR-TB & 1 (8.3%) was found isoniazid mono-resistant by first line LPA. Sensitivity and specificity of GeneXpert by using MGIT culture as gold standard was found to be 33.3% and 98.8% respectively. MDR-TB observed by GeneXpert was 16.7%. Among 12 culture positive isolates, only one (8.3%) isolate was found MDR-TB and one (8.3%) was isoniazid mono-resistant. Results of Geno Type MTBDR*sl* assay showed that only one (50%) isolate was fluoroquinolones resistant and sensitive to second line injectable drugs. None of the isolate was Extensively Drug Resistant (XDR). GeneXpert can provide rapid immediate diagnosis of MTB and rifampicin resistant which can help in giving proper treatment but simultaneous culture also needs to be done as it missed many cases. It's important to look for drug resistance as 16.7% isolates were rifampicin resistant.

Keywords: GeneXpert, MGIT, Pleural tuberculosis, Pleural fluid, MDR-TB.

1. INTRODUCTION

Pleural tuberculosis is the second most infectious form of extra pulmonary TB in India [1]. Tuberculous pleuritis is the major cause of pleural effusion in many countries and is responsible for the worldwide morbidity and mortality [2]. Early diagnosis and efficient management is essential to reduce the mortality rate due to pleuritis. Microbiological diagnosis by AFB stain is rapid but insensitive due to pauci-bacillary nature of the disease and non-uniform distribution of MTB, though MGIT culture is considered as gold standard but takes 2-6 weeks to give results [3].

In 2013 WHO updated the policy and recommended the use of GeneXpert MTB/RIF assay among pediatric and extra pulmonary cases [4]. A rapid, fully automated nucleic acid amplification technology, GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) can simultaneously detect the *Mycobacterium tuberculosis* and Rifampicin resistance in less than 2 hrs. It works on the principal of semi-nested PCR and molecular beacon technology [5]. A number of studies have reported 34-52% range for sensitivity of Xpert MTB/RIF regarding diagnosis of pleural tuberculosis in pediatric patients [6].

Another molecular test available line probe assay Geno Type MTBDR *plus* (Hain Life science, Nehren, Germany) is genotypic drug susceptibility test recommended for both smear positive clinical specimen and smear negative culture positive isolates that targets *rpoB, katG* and *inhA* gene [7] to determine drug resistance against rifampicin & isoniazid drugs and Geno Type MTBDR*sl* targets for *gyrA, gyrB, rrs and eis* gene which determines the drug resistance associated with fluoroquinolones (FQ) and second line injectable drugs (SLID) [8].

The aim of this study was to detect *Mycobacterium tuberculosis* (MTB) and its drug resistance profile by molecular methods in pediatric patients suspected of pleural tuberculosis.

2. MATERIAL AND METHODS

Study was performed at Advance Research & TB laboratory, Department of Microbiology, SMS Medical College, Jaipur. Samples were collected with patient history details in test request form Annexure 15A from pediatric patient's ≤ 15 years of age having symptoms of suspected pleural TB as per the Revised National Tuberculosis Control Program (RNTCP) guidelines during the time period from January to June 2018. This study was approved by Office of the Ethics Committee S.M.S. Medical College & Attached Hospitals, Jaipur.
Received sampled were used for different diagnostic tests so it was fractioned into 3 parts: one for AFB smear, one for GeneXpert and remaining sample for MGIT culture.

2.1. ZN Staining

Ziehl-Neelsen (ZN) or acid fast staining (AFB) was performed on the pleural fluid samples and then smears were graded during microscopy as per RNTCP guidelines [9].

2.2. GeneXpert

GeneXpert MTB/RIF assay was performed for pleural fluid sample as per the protocol [10]. Two ml of sample was added with the sample reagent (2:1 ratio) and shaken vigorously 20 times and then incubated for 15 min at room temperature. After incubation, 2 ml of sample-reagent mixture was poured into the cartridge with the help of sterile dropper and then loaded the cartridge into the GeneXpert system. Results were displayed after 2 hours which detects the presence of MTB and simultaneously detects the resistance to rifampicin.

2.3. Sample processing

Sample processing was performed as per the RNTCP protocol in Mycobacteriology laboratory manual [11]. Pleural fluid samples were digested and decontaminated by N-acetyl-L-cysteine-NaOH method with final concentration of 1% NaOH and concentrated by centrifugation. Final sediment was re-suspended with 1-2 ml of sterile phosphate buffer saline and then used for liquid culture inoculation.

2.4. MGIT culture

Mycobacteria Growth Indicator Tube (MGIT) tube was used for the liquid culture inoculation as per manufacturer's instructions [12]. 800 µl volume of growth supplement with antibiotic mixture (provided with kit) was added to the MGIT tube and then 500 µl processed sample was inoculated in MGIT tube, placed into MGIT 960 instrument and incubated at 37°C. When growth was detected, tubes were flagged as positive by the machine, tubes were then removed from machine and growth was processed for ZN staining, presence of contamination was checked by inoculation on BHI agar plate which was incubated for 2 days at 37°C. Total incubation in MGIT was for 42 days before reporting as negative.

2.5. SD Bioline test

AFB positive and non-contaminated MGIT culture positive growth was tested with SD Bioline test, a rapid immuno-chromatography (ICT) test as per the manufacturer's instructions to differentiate MTB and non-tuberculous mycobacteria (NTM)[13].

2.6. DNA extraction

DNA extraction was performed for culture positive isolates by using GenoLyse extraction kit provided with the GenoTypeMTBDR*plus* kit, as per the manufacturer's instructions [14].

2.7. Genotypic test

Line probe assay was performed by using Geno Type MTBDR *plus* version 2 kit as per the manufacturer's protocol [15]. Smear positives processed samples and culture positive isolates were used for this test. Drug susceptibility pattern in the form of WT and MUT band on the nitrocellulose strip was obtained for the rifampicin & isoniazid drugs (primary anti-TB drugs). Resistant isolates were further subjected to second line drug LPA (procedure similar to first line LPA) by using GenoTypeMTBDR*sl* kit with the help of specific primers to determine the drug susceptibility pattern against fluoroquinolones and second line injectable drugs.

2.8. Statistical analysis

Positivity obtained from GeneXpert and MGIT culture was presented in the form of percentages. Sensitivity and specificity of GeneXpert by using MGIT culture as gold standard and compared in 2*2 table.

3. RESULTS

ZN staining was performed directly for all 176 pleural fluid samples and found 2 (1.1%) samples were AFB positive. GeneXpert was performed for all samples and it was observed that 6 (3.4%) samples were MTB positive and of 6 only 1 (16.7%) sample was found to be rifampicin resistant. Of 176 samples, 12 (6.8%) samples were found culture positive. On comparing the results of

MGIT and GeneXpert, 4 (2.3%) samples were MTB positive by both, 2 (1.1%) were GeneXpert positive but found negative in culture, 8 (4.5%) found positive in culture but negative by GeneXpert. Sensitivity and specificity of GeneXpert was calculated by using MGIT culture as gold standard given in Table 1.

Table 1: GeneXpert sensitivity and specificity profile against MGIT culture (gold standard) in pleural fluid samples of pediatric patients

GeneXpert		MGIT culture	
		Positive	Negative
	Positive	4	2
	Negative	8	162
Sensitivity	33.33% (C.I: 9.92-65.11%)		
Specificity	98.78% (C.I: 95.66-99.85%)		
PPV	66.67% (C.I: 28.91-90.77%)		
NPV	95.29% (C.I: 93.14-96.80%)		

MGIT=Mycobacteria Growth Indicator Tube, PPV=Positive predictive value, NPV=Negative predictive value

All the 12 culture positive isolates were confirmed by MPT64 antigen test as MTB. No non-tuberculous mycobacteria (NTM) were detected. On conducting first line LPA, 8 (66.7%) isolates were found to be sensitive to both RIF & INH drugs but all these were not identified by GeneXpert. One (8.3%) sample was found resistant to both the drugs that is was multidrug resistant (MDR) & INH showed low level of resistance by LPA. This isolate was reported RIF resistant by GeneXpert. Two (16.7%) isolates were found sensitive to both the drugs by LPA and they were also found sensitive to rifampicin in GeneXpert. One (8.3%) isolate was found rifampicin sensitive by GeneXpert and LPA but it had high level resistance to isoniazid. Banding pattern associated with drug resistant mutations is shown in table 2.

Second line drug LPA- Of the 2 resistant isolates (1 was MDR & 1 was INH resistant) one (50%) was found resistant to fluoroquinolones but sensitive to injectable drugs and the other isolate was found to be sensitive to both the drug groups. No XDR case was observed. Banding pattern of mutations associated with second line drug LPA is shown in table 3.

Table 2: Mutation banding pattern for RIF & INH drug in first line LPA

Resistant isolate	Rifampicin		Isoniazid			
	rpoB gene		*katG gene*		*inhA gene*	
	WT probe	MUT probe	WT probe	MUT probe	WT probe	MUT probe
MDR	WT8-	MUT3+			WT1-	MUT1+
INH	-	-	WT+	MUT+	-	-

WT=Wild Type, MUT=Mutant Type, MDR=Multi-drug resistant, INH=Isoniazid

Table 3: Mutation pattern in second line LPA

Resistant isolate	Fluoroquinolones (FQ)		Second line injectable drugs (SLID)			
	*gyrA*gene	*gyrB*gene	*rrs*gene		*eis* gene	
	WT probe	MUT probe	WT probe	MUT probe	WT probe	MUT probe
FQ	WT3-	MUT3C+	-	-	-	-

WT=Wild Type, MUT=Mutant Type

4. DISCUSSION

In pleural tuberculosis, bacillary load in pleural fluid samples is very low so the diagnosis of pleuritis is very difficult. In our study, AFB was detected in 2 (1.1%) samples and GeneXpert detected MTB in 6 (3.4%) samples with rifampicin resistance in 1 (16.7%) sample, our study findings are comparable with the other multi-centric Indian study conducted by Kalra *et al.* [16] which showed smear positivity of 0.6%, GeneXpert positivity 3.9% and 14.4% resistance to rifampicin. Our findings also correlates with another multi-centric Indian study conducted by Raizada *et al.* [17] which observed 2.7% AFB positivity, 3.2% positivity by GeneXpert and 11.1% rifampicin resistance. Low positivity of Gene-Xpert probably reflects the low bacterial load in pleural tuberculosis, although GeneXpert have 3-fold sensitivity than conventional smear microscopy.

MGIT culture positivity was found to be 6.8% in our study; it is in concordance with another Indian study from New Delhi of Sharma *et al.* [18] who observed 5.7% positivity by culture in pediatric patients suffering from pleuritis. In our study, GeneXpert showed 33.3% sensitivity and 98.8% specificity against MGIT culture as gold standard, similar findings have also been

reported by an Italian study of Tortoli *et al.* [19] which reported sensitivity and specificity of 33.3% and 99% respectively for GeneXpert. Culture positive isolates were confirmed for MTB growth by immune-chromatographic assay and no non-tuberculous mycobacteria was found in our study. Hence, it is essential that culture is also done along with GeneXpert as in extra pulmonary specimens there is low concentration of bacilli which may be below the limit of detection of GeneXpert leading to false negative result.

In our study, among 12 culture positive isolates, only 2 isolates had drug resistance. One (8.3%) isolate was MDR and other one (8.3%) had isoniazid mono-resistance. LPA findings of our study are in conjunction with the South Africa study of Seddon *et al.* [20] which observed 8.8% MDR and 5.1% isoniazid resistance for the pleural fluid samples in pediatric patients.

The GenoTypeMTBDR*sl* assay detects the presence of mutations in genes that cause drug resistance to fluoroquinolones, or second-line injectable drugs, or both. It does not report whether there is resistance to individual drugs within these categories (ofloxacin and levofloxacin in the case of the fluoroquinolones; amikacin, kanamycin, and capreomycin in the case of second-line injectable drugs). Of the 2 resistant isolates obtained from first line drug LPA (Geno Type MTBDR *plus*), only 1 (50%) isolate was found to have fluoroquinolones resistance in GenoTypeMTBDR*sl* assay. This finding is in agreement with the Indian study from Mumbai of Shah *et al.* [21] which concluded the increasing trend of fluoroquinolones resistance from 39.1% to 93.7%. It predicts that isolated rifampicin and isoniazid resistance no longer is predominating resistance pattern but the resistance to fluoroquinolones and second-line injectablesare also increasing. None of the patient in our study had XDR, it shows concordance with the other multi-centric Indian study of Dusthackeer *et al.* [22] which reported no XDR case in body fluids& aspirates among drug resistant adults & children.

5. CONCLUSION

GeneXpert and MGIT culture both are important for the diagnosis of pleuritis in pediatric patients. Although GeneXpert have low sensitivity for pleural fluid but the advantage of GeneXpert over MGIT culture is rapid, efficient, easy to handle and results availability within 2 hours including resistance to rifampicin. GeneXpert acts as point of care testing for pediatric tuberculosis. Culture positivity is significantly higher than the GeneXpert, as some of the cases missed by GeneXpert. The increasing burden of drug resistance among extra pulmonary samples is a major threat to control TB in pediatric population. Early diagnosis and rapid management greatly impacts the treatment and it helps in preventing emergence of drug resistance.

6. ACKNOWLEDGEMENT

The authors are greatly thankful to National Tuberculosis Elimination Program (NTEP) and Foundation for Innovative & New Diagnostics (FIND) for supporting this study &financial assistance.

7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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