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Detection of Drug Resistance among Suspected Tuberculosis Cases Attending a Tertiary Care Hospital in Western Rajasthan, India

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Abstract: ***Background: In spite of newer modalities for diagnosis and treatment of tuberculosis (TB), unfortunately, it is among the top 10 killer infectious diseases worldwide. India has a high burden of drug-resistant tuberculosis (DR-TB). The National Drug Resistance Survey (2018) results showed that the rates of multidrug resistance (MDR) among new TB patients are 2.84% and that in previously treated to be 11.60%. Methods: Out of total 180 samples received during 2016-2017 for mycobacterium culture, 31 culture samples confirmed for Mycobacterium tuberculosis were randomly selected and 12 smear positive direct samples were also included in the present study and line probe assay (LPA) was performed to detect first line as well as second line anti tubercular drug resistance. Results: Out of total 43 cases subjected to first line drug susceptibility testing, 2 (4.65%) were resistant to only rifampicin, 4 (9.30%) were resistant to only isoniazid and 3 (6.98%) were resistant to both rifampicin and isoniazid. So, according to WHO definition, total 3 (6.98%) cases were diagnosed as multidrug resistance tuberculosis (MDR-TB). One case (2.33%) was detected as extensively drug resistant TB (XDR-TB). Conclusions: LPA plays an important role before initiation of anti-tubercular drug and for early detection of DR-TB cases.***

Keywords: Tuberculosis, MDR-TB, XDR-TB, Line probe assay (LPA), GeneXpert

1. Introduction

In spite of newer modalities for diagnosis and treatment of tuberculosis (TB), unfortunately, it is among the top 10 killer infectious diseases worldwide. India has a high burden of drug-resistant tuberculosis (DR-TB). The National Drug Resistance Survey (2018) results showed that the rates of multidrug resistance (MDR) among new TB patients are 2.84% and that in previously treated to be 11.60%.[1] This global situation of MDR-TB, alert World Health Organization (WHO) to take urgent step for detection of drug resistance amongst TB patients. WHO introduced various techniques among which GeneXpert MTB/RIF and line probe assay (LPA) are widely used for screening of DR-TB.[2]

The present study was conducted to detect drug resistance among clinically suspected and microbiologically confirmed tuberculosis cases attending a tertiary care hospital in Rajasthan.

2. Materials and Methods

A hospital-based prospective study was done in the department of Microbiology, in a tertiary care hospital in western Rajasthan, India.

Out of total 180 samples received during 2016-2017 for mycobacterium culture, 31 culture confirmed *Mycobacterium tuberculosis* samples were randomly selected and 12 smear positive direct samples were also included in the present study. Distribution of samples is shown in Table 1.

Table 1: Distribution of sample

Samples		Liquid Culture (%)	Direct Samples (%)
Pulmonary (%)	29 (67.44)	18 (41.86)	11 (25.58)
Extra pulmonary (%)	14 (32.56)	13 (30.23)	1 (2.33)
Total (%)	43 (100)	31 (72.09)	12 (27.91)

Pulmonary and extra-pulmonary positive liquid cultures for MTB by BacT/ALERT system bio Merieux, France, that is MP (Mycobacteria Process) bottles were further subjected to smear microscopy by Ziehl-Neelsen (ZN) staining method and LPA was done.

Direct specimens, which contains normal commensal bacterial flora, were decontaminated by the standard N-acetyl-L-cysteine-NaOH method.[3,4] Approximately 2-3 ml of specimens from positive liquid culture bottle and from direct specimens with same quantity of samples was collected and further processed.

GenoType® MTBDR*plus* and GenoType® MTBD*sl* assays (LPA)

MTBC confirmed liquid cultures (N=31) and clinical samples (N=12) were subjected to first line anti-tubercular drug resistance, frequency and mutational analysis by a GenoType® MTBDR *plus* assay as per manufacturer's instructions (Hain Life Science, Germany).[5] Samples showing resistance to first line drugs were subjected to GenoType® MTBD*sl* assay second line anti tubercular drug resistance testing.

Procedure

Following steps were carried out in specifically designed rooms of molecular laboratory to minimize contamination.

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DNA Extraction

About 500 µL of liquid culture or direct decontaminated clinical specimen was transferred to the screw cap tube and centrifuged at 12,000 rpm for 15 minutes and the pellet was suspended in 100 µL of lysis buffer (A-LYS). After Vortexing, it was incubated at 95°C for 5-7 minutes, 100 µL of neutralization buffer (A-NB) was added after a brief spin to the lysate and centrifuged at 12000 rpm for 10 minutes, 5 µL supernatant was used for PCR.

Amplification

About 50 µL of PCR Mix for each sample is prepared by adding10 µL AM-A, 35 µL AM-B and 5 µL of DNA solution. Amplification was done in thermo cycler as follows:

MTBDR*plus* (First Line) & MTBDR*sl* (Second Line) PCR Cycling

		For Cultures	For Clinical Samples
15 min	95°C	1 cycle	1 cycle
30 sec 2 min	95°C 65°C	10 cycles	20 cycles
25 sec 40 sec 40 sec	95°C 50°C 70°C	20 cycles	30 cycles
8 min	70°C	1 cycle	1 cycle

Reverse Hybridization

After amplification, hybridization was performed; the biotin-labeled amplicons were hybridized to the single stranded membrane bound probes. After a hybridization buffer and stringent buffer washing, freshly prepared conjugate and substrate were added to the strips and an alkaline phosphatase mediated staining reaction was observed in the bands where the amplicon and the probe have been hybridized. The Geno Type MTBDR plus assay strip contains 27 reaction zones; 21 of them are wild type and mutations probes and 6 are control probes include a conjugate control, and amplification control, *M.tuberculosis* complex-specific control (TUB), *rpoB* locus control, *katG* locus control, and an *inhA* locus control. The absence of any of the wild-type bands or the presence of any mutation bands in each drug resistance-related gene shows resistance to the respective anti-tubercular drugs. The study is approved by hospital ethics committee.

Statistical analysis

Analysis of the data was done by using Microsoft Excel and quantitative data were expressed in numbers and percentages.

3. Results

Out of total 43 cases subjected to first line drug susceptibility testing, 2 (4.65%) were resistant to only rifampicin, 4 (9.30%) were resistant to only isoniazid and 3 (6.98%) were resistant to both rifampicin and isoniazid. So, according to WHO definition, total 3 (6.98%) cases were diagnosed as multidrug resistance tuberculosis (MDR-TB), all the drug resistance cases were pulmonary. The resistant cases (MDR=3 and only rifampicin resistant=2) were subjected to second line anti-tubercular drug susceptibility testing to detect extensively drug resistance tuberculosis (XDR-TB) cases. Out of total 5 cases tested, one showed resistance to aminoglycosides only, one showed resistance to fluoroquinolones only and one showed resistance to both aminoglycosides and fluoroquinolones which was also resistant to both first line drugs. According to WHO case definition one case (2.33%) was detected as XDR-TB. In the present study categorization of resistance among known and unknown mutations of *rpoB* gene, *katG* gene, and *inhA* genes also noted depending upon banding pattern of the isolates. The details of the mutations are shown in Table 2 and Figure 1.

Table 2: Pattern of gene mutations detected by Geno Type MTBDR plus assay in MDR-TB/RR-TB cases

Gene	Case I	Case II	Case III	Case IV	Case V
rpoB gene	MUT3(S531L)	MUT3(S531L)	MUT3(S531L)	MUT3(S531L)	Unknown (absent WT band)
katG gene	MUT1(S315T1)	MUT1(S315T1)	-	-	-
inhA gene	-	-	MUT3A(T8C)	-	-

(MDR-TB: Multidrug resistant tuberculosis, RR-TB: Rifampicin resistant tuberculosis)

Figure 1: Showing banding patterns in Line probe assay

4. Discussion

Early diagnosis and prompt treatment of infectious cases are the only key elements in reducing the spread of DR-TB. Ziehl Neelsen (ZN) smear microscopy has been the mainstay in the diagnosis of pulmonary TB, particularly in resource-limited, high TB-burden countries like India. [6] However, the technique has low sensitivity,[7] observer-dependent and is not capable of distinguishing non tubercular mycobacterium (NTM) strains from mycobacterium TB complex (MTBC). Conventional as well as automated culture and drug susceptibility testing (DST) is a time-consuming process. Molecular diagnostic tools for diagnosis of DR-TB effectively address the issue regarding long turnaround time associated with culture and drug susceptibility testing.[8] With the introduction of line probe assay (LPA) for the rapid diagnosis of TB along with drug susceptibility, there has been a significant reduction in time to initiation of treatment in suspected drug-resistant TB cases.[9] A multi-site validation study from India showed LPA to be a sensitive and specific tool for the detection of rifampicin resistance in AFB-positive sputum specimens.[10] LPAs use multiplex polymerase chain reaction (PCR) amplification and reverse hybridization to identify *M. tuberculosis* complex and mutations to genes associated with rifampicin and isoniazid resistance. In present study multidrug resistant tuberculosis (MDR-TB) was detected in three (6.98%) cases out of total 43 cases tested by using LPA. But if we follow revised national tuberculosis control (RNTC) programme in which Gene Xpert MTB/RIF assay is used to detect DR-TB which can only detect Rif resistance,

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the total MDR-TB cases would have been five (11.63%). Among all the gene mutations conferring RIF resistance, (MUT3) codon S531L was found to be the most frequently encountered mutation in 4 cases (80%). Several other studies also showed high percentage of mutation in above mentioned codon for RIF resistance.[11,12] Unknown mutation in *rpoB* gene was seen in one (20%) case in which wild type band was absent. This could be attributed to mutations in other regions of the *rpoB* gene locus or mutations other than those on the strip. The resistance of INH due to mutation in *katG* gene was found in S315T1 codon region amounting to 66.67% among MDR-TB cases while mutation in InhA was seen in the T8C codon in 33.33% cases. Several other studies showed high percentage (around 90%) of mutation in the S315T1 codon region, for katG gene.[13,14] In high TB-prevalent countries, a high prevalence of *katG* mutations has been reported to attribute for INH resistance and much lower prevalence reported in low TB-prevalent countries. This could be attributed to the on-going transmission of the strains in the high-burden settings.[15] In this study *katG* mutation is seen in 6/9 (66.67%) which suggest high level of INH resistance. In the present study total INH resistance was observed in 16.28% cases out of which 9.3% cases were mono resistant.Between 2003 and 2017, the WHO estimated that the global INH resistance without concurrent RIF resistance were 7.1% in new TB cases and 7.9% in previously treated TB cases.[16] HR*et al* calculated an averageof 39% of INH resistant strains being MDR in their study.[17] In a systematic review and meta-regression of trial data, Menzies D *et al* found that, initial INH resistance increased incidence rates of treatment failure and relapse versus a baseline of pan-sensitive strains with 10.9 and 1.8 respectively.[18] So if in any setting the sole diagnostic test used to detect DR-TB is Gene Xpert MTB/RIF assay, which only tests for rifampicin resistance, the INH resistant cases will be missed by Gene Xpert MTB/RIF assay which may further lead to MDR-TB. In the other hand Gene Xpert MTB/RIF also gives over diagnosis of MDR-TB as in present study five cases were resistant to RIF and among them three cases were also resistant to INH which are considered as actual MDR-TB according to WHO case definition.

5. Conclusion

LPA plays an important role before initiation of anti-tubercular drug and for early detection of DR-TB cases.

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