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Frequency of multi-drug resistance and mutations in *Mycobacterium tuberculosis* isolates from Punjab state of India

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ABSTRACT

Data regarding prevalence of multi-drug resistant tuberculosis (MDR-TB) and associated common mutations is scarce from Punjab region. The study was designed to determine rate of MDR-TB among presumptive MDR-TB from Punjab and mutation patterns using GenoType MTBDRplus assay. Total of 812 consecutive sputum samples were received from January 2012 to July 2013, from 14 districts of Punjab at the National Reference Laboratory at New Delhi for diagnosis of MDR-TB as hand holding activity. Presumptive MDR-TB patients were identified on basis of criterion B defined by the programme. Smear positive and negatives patients were found to be 636/798 (79.7%) and 162/ 798 (20.3%) respectively. Total of 606 GenoType MTBDRplus tests were conducted and mutations in *rpoB*, *kat G* and *inhA* genes analyzed. Total of 94/606 (15.5%), 43/606 (7.1%) and 40/606 (6.6%) were found to be RIF and INH resistant, mono-RIF resistant and 40/606 (6.6%) mono-INH resistant respectively. Commonest known mutation for RIF in *rpoB* gene and INH in *kat G* gene was S531L (80/ 137; 58.4%) and S315T1 (119/134; 88.8%) respectively. Mutations in *inhA* were found in 21/134 (15.7%) strains. Average turn-around time (TAT) for dispatch of result toPunjab was 4.6 days. Prevalence of RIF resistance in Punjab was found to be 22.6%. Common mutations for RIF and INH were similar to that in other regions of country. GenoType MTBDRplus was found to be useful assay for rapid detection of MDR-TB, responsible for determining better management of MDR-TB patients under the programme.

1. Introduction

Multi-drug resistant tuberculosis (MDR-TB), defined as the resistance to at-least rifampicin (RIF) and isoniazid (INH), continues to be threatening as worldwide, 3.9% of new TB cases and 21% of previously treated cases are estimated to have MDR-TB. In 2015, 580, 000 cases were eligible for MDR-TB treatment. India, China and Russian Federation accounted for 45% of the cases [1]. Almost 10% to 30% of MDR-TB cases result in failure of treatment and death due to prolonged, limited and expensive treatment options.

Considering the numbers described above, it is imperative to perform drug susceptibility testing (DST) for appropriate management of drug resistant cases. The culture based DST methods are based on inhibition of organisms in culture medium containing anti-tubercular agents and take weeks or months to be completed. While culture based DST is ongoing, the undiagnosed drug resistant cases continue to spread resistance [2].

Many molecular techniques for determination of anti-tubercular resistance based on genetic mutation have been introduced in the last decade. Commercial line probe assays (LPA) such as INNO-LIPA Rif TB (Innogenetics, Ghent, Belgium) and Genotype MTBDR plus assay (Hain Life Sciences, Nehren, Germany) based on reverse hybridization of amplicons to immobilized membrane based probes are designed to simultaneously detect *M. tuberculosis* (MTB) isolates and important gene mutations conferring rifampicin (RIF) resistance (*rpoB* gene) and isoniazid (INH) resistance (*inhA, katG*) within 2–3 days [2,3].

Genotype MTBDR plus can be used for detection of resistance directly on the smear positive sputum samples or the culture isolate and has pooled sensitivity and specificity of 98.1% and 98.7% for RIF resistance and 84.3% and 99.5% for INH resistance respectively [4]. The RIF resistance is caused by altered beta-subunit of DNA dependent RNA polymerase, caused by mutations commonly found in 81-bp hot-spot region of *rpoB* gene. Resistance to INH is most frequently associated with a specific mutation S315T in *katG* gene coding for catalase-peroxidase and/ or C15T, A16G, T8A & T8C

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in the promoter region of *inhA* gene coding for nicotinamide-adenine dinucleotide phosphate-oxidase enoyl-acyl carrier protein reductase [5]. These mutations are visible in form of absence of wild type regions and/or presence of specific mutations which in addition provide the knowledge of mutations.

Another path-breaking fully automated molecular assay is Cepheid GeneXpert® MTB/RIF, in which real time polymerase chain reaction technology is used to simultaneously detect MTB and RIF resistant mutation in *rpoB* gene. Xpert requires minimal laboratory expertise, results are available in less than 2 h and sensitivity of detection of MTB is about 130 organisms/ ml [6]. Cepheid is now launching Xpert MTB/RIF Ultra, in which limit of detection is 10–100 organisms/ml. India targets rapid detection of MDR-TB for all possible suspects using LPA as envisioned under Programmatic Management of Drug Resistant Tuberculosis (PMDT), across the country [7]. The patients are referred to the designated Reference Laboratory for diagnosis of MDR-TB. Punjab state is a north-western state of India with population of 27.98 million with 22 districts. Since past few years, Punjab has witnessed very high substance abuse, which has also been epidemiologically linked to the disease [8].

Few studies have reported prevalence of *M. tuberculosis* genotypes and MDR-TB rates using LPA from North India [9,10].Therefore, present study targets to study drug resistance TB and the frequency of various mutations detected among these cases using LPA in Punjab.

2. Materials and methods

2.2. Study setting

The present study is conducted in the National Reference Laboratory (NRL) for TB, Department of Microbiology at National Institute of Tuberculosis and Respiratory Diseases (NITRD), New Delhi. Laboratory is certified as NRL for *M. tuberculosis* DST by Supranational Laboratory (Antewerp Belgium) and recognized as Centre of Excellence by World Health organization.

2.3. Study design

As hand holding activity, samples from 14 different districts of Punjab; Amritsar, Fatehgarh Sahib, Firozpur, Gurudaspur, Hoshiarpur, Jalandhar, Ludhiana, Moga, Mohali, NawanShahar, Patiala, Rupnagar, Sangrur and SAS Nagar were received at the NRL for diagnosis of MDR-TB till state developed complete capacity for the same (Fig. 1). Total of 812 consecutive sputum samples from patients identified as presumptive MDR-TB, received in cold chain from January 2012 to July 2013, were included in the study. The presumptive MDR-TB patients were identified on basis of criterion B of PMDT which include follow up positives on category I and II, smear positive retreatment cases and contacts of MDR-TB. The study is approved by institute's ethical committee (AMS/EC/2012/1028 dated: 5/2012).

2.4. Sample processing

All specimens were screened for presence of acid fast bacilli (AFB) by Ziehl-Neelsen (ZN) staining [11].Samples were processed by N-acetyl-L cysteine – Sodium hydroxide (NALC-NaOH) method of digestion and decontamination [11]. All smear negative processed samples and scanty smear positive (Only when Genotype MTBDRplus assay ver 1.0 was used) were inoculated in MGIT 960 tubes for culture as per PMDT guidelines [7]. Tubes with positive alerts were identified for presence of *M. tuberculosis* by smear microscopy for serpentine cording and rapid immunochromatographic test for detection of MPT64 TB Ag (SD BIOLINE). Cultures positive for *M. tuberculosis* were subjected to LPA. The study workflow is shown in Fig. 2.

2.5. GenoType MTBDRplus assay/line probe assay

Smear positive processed sputum samples were directly processed for GenoType MTBDRplus assay [7].The Genotype MTBDRplus assay ver 1.0 or ver 2.0 (Hain Life Sciences, Nehran, Germany, was carried out on the processed sample as per manufacturer's instructions (http://www.hain-lifescience.de). DNA extraction, master-mix preparation, DNA amplification and hybridization were done after thorough cleaning in dedicated rooms [10,12]. The *M. tuberculosis* H37Rv (ATCC 27294) was run as positive control and sterile molecular grade water was run as negative control, for quality control.

For DNA extraction, 500 μl of NaLC-NaOH processed samples or 1 ml of *M. tuberculosis* cultures from MGIT tubes was taken in cryovial, centrifuged at 10,000*g* for 15 min and bacterial pellets obtained. In version 1.0, pellets were suspended in 100 μl sterile molecular grade water, heat inactivated at 95 °C for 20 min followed by ultra-sonication for 15 min. Supernatant containing DNA was collected after centrifugation at 13,000*g* for 5 min. In version 2.0, Genolyse kit was used and 100 μl of lysis buffer was added to pellet, heat inactivated at 95 °C for 5 min followed by addition of neutralization buffer. Supernatant containing DNA was collected after centrifugation at 13,000*g* for 5 min in fresh tube and stored at - 20 °C (http://www.hain-lifescience.de).

Master-mix was prepared using reagents provided by the kit. In version 1.0, master-mix was prepared by mixing 35 μl polynucleotide mix, 2 μl $MgCl_2$ buffer, 5 μl buffer, 3 μl water, and 0.2 μl taq polymerase. In version 2, the above constituents have been condensed as AM-A & AM-B which are mixed for each sample in ratio of 10 μl and 35 μl respectively. DNA solution is added in volume of 5 μl to PCR tubes and amplified.

Detection of PCR products was done using LPA based hybridization using GenoType MTBDRplus kit. Initially denaturation of amplified DNA was done, followed by hybridization of nucleotides on single stranded DNA strand to LPA strip, followed by conjugate reaction and finally addition of substrate to give the bands on LPA strip (http://www.hain-lifescience.de).

2.6. Reporting

The result of LPA strips was interpreted with the help of reporting card as resistant or sensitive for RIF or INH or invalid based on the kit insert. Resistance to particular antibiotic was considered if one or more wild type (WT) bands were missing or/and one or more mutant bands (MUT) were present. Strains with presence of both wild type and mutant band were termed as hetero-resistance.

The reports were communicated electronically to the District TB Officers (DTO) of the respective districts within 24 h of report generation. Turn-around time (TAT) for each sample was calculated.

2.7. Statistical analysis

Data was presented as frequency tables and mean was calculated wherever required using MS-Excel. Fisher's exact test for statistical significance (Defined as the probability that an effect is not due to by chance alone) was calculated wherever applicable using Graph-pad computer software (https://www.graphpad.com/quickcalcs/contingency1.cfm). The P value less than 0.05 was considered significant.