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MOLECULAR ASPECTS

High degree of multi-drug resistance and hetero-resistance in pulmonary TB patients from Punjab state of India

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SUMMARY

Line Probe Assays (LPAs) have been recommended for rapid screening of MDR-TB. Aims of this study were (1) to compare the performance of LPA with standard Bactec MGIT 960 system and (2) to ascertain the pattern of genetic mutations in the resistance isolates. In phase I, a total of 141 *Mycobacterium tuberculosis* isolates from our routine laboratory were tested by LPA and Bactec MGIT 960 for DST. In phase II, 578 sputum specimens of suspected DR-TB patients were received from the Punjab state of India. Of them 438 specimens or their cultures were subjected to LPA. The presence of mutant bands with their corresponding wild type band was identified as "hetero-resistance". In phase I, LPA showed high concordance with 96.4% positive agreement and 97.6% negative agreement with Bactec MGIT 960-DST. In phase II, 12 (2.7%) specimens were detected as invalid by LPA. Of the remaining 426 specimens, 184 (43.1%) had resistance to RIF and 142 (33.3%) to INH while 103 (24.1%) specimens showed resistance to both INH and RIF (MDR-TB) by LPA. Of the 142 INH resistant, 113 (79.5%) showed mutations in *katG* and 29 (20.4%) in *inhA*. A high rate of hetero-resistance pattern was observed in *rpoB* gene (28.8%) and *katG* gene (9.8%). The most frequent mutation was S531L (81.1%) in *rpoB* region and S315T1 (100%) in *katG* gene.

1. Introduction

Multi-drug resistant tuberculosis (MDR-TB) is considered when the causative agent *Mycobacterium tuberculosis* (*M. tuberculosis*) becomes resistant to at-least two first line anti-tubercular drugs – the isoniazid (INH) and rifampicin (RIF). India has an estimated 63,000 cases of notified MDR-TB, the highest in South-East Asia Region [1]. Though MDR-TB is seen in untreated cases also, the rate of MDR-TB in treated cases is very high [2]. The Global Plan to Stop TB 2011–2015 targets that all patients who have been previously treated for TB should be tested for MDR-TB using rapid tests by 2015 [3].

Drug susceptibility testing (DST) is conventionally performed using solid medium and is based on visible appearance of growth on the medium containing anti-tubercular drugs but these methods take longer time and are cumbersome. During this period patients may be inappropriately treated, drug-resistant strains may continue to spread and amplifying the resistance [4]. Given the number of people at risk the detection of drug susceptibility pattern of the pathogen must be rapid, inexpensive and easy to perform. Though in the last 10 years liquid culture based DST has reduced this time, still it is in weeks. Since resistance in *M. tuberculosis* is conferred by genetic mutation, molecular techniques (*i.e.* sequencing based) are preferred [5]. However, these techniques are cumbersome and costly for routine use. In the last 10 years two molecular methods have been endorsed by World Health Organization (WHO), in addition to the liquid culture based DST for rapid diagnosis and DST. These are Bactec MGIT 960 (*Mycobacterium* Growth Indicator Tube 960) liquid culture system [6], Line Probe Assay (LPA) [7] and Xpert MTB/RIF [8]. Government of India in its revised national TB control programme (RNTCP) has rolled out Bactec MGIT 960 and GenoType MTBDR*plus* in its national programme while Xpert MTB/RIF is yet to be taken up by the national programme.

Currently, two commercial LPA have been developed: INNO-LiPA Rif TB (LiPA) by Innogenetics, Zwijndrecht, Belgium and GenoType MTBDR*plus* by Hain Life Science GmbH, Nehren, Germany. The LiPA test can simultaneously detect *M. tuberculosis* and the presence of a mutation in the *rpoB* gene, which confers resistance to

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RIF [9]. Several studies had reported the sensitivity of 95% or greater, and nearly 100% specificity for RIF resistance [10]. It has been seen that more than 95% of RIF resistant isolates harbor a mutations within the 81-bp hot spot region of the *rpoB* gene [11]. The GenoType MTBDR*plus* assay (referred here after as LPA) has an additional advantage over the LiPA because it can detect mutations in *rpoB* gene as well as mutations in *katG* gene and *inhA* gene [12]. Moreover, LPA is able to detect hetero-resistance, which is defined as the co-existence of the susceptible and resistant bacteria in the same specimen/culture [13,14]. Hetero-resistance is a preliminary stage towards full resistance which is difficult to detect using conventional DST methods. Nevertheless, liquid culture based DST remains the most reliable and standard method.

However, very few studies have been carried out in India, that too on a very few number of patients to evaluate the performance of LPA vis-à-vis Bactec MGIT 960 liquid culture for DST [15]. Moreover, to best of our knowledge we have not found even a single publication from Punjab state of India related to the prevalence of various genotypes of *M. tuberculosis* and their drug susceptibility pattern. Hence, the present study was aimed (I) to compare the performance of LPA and Bactec MGIT 960-DST on a significantly larger sample size of mycobacterial isolates made from various clinical samples obtained from the patients belonging to Delhi region and (II) applying LPA directly on sputum samples from suspected MDR patients of a study naïve north-western region (Punjab) of India. The study also deciphers the pattern of mutation in *M. tuberculosis* obtained from these two different pools of patients.

2. Materials and methods

2.1. Study settings

The study was carried out in two phases, after certification of laboratory by Government of India. In phase I, standardization of LPA in our local settings was done. For this, we tested 141 *M. tuberculosis* isolates with known resistance patterns by Bactec MGIT 960 first line DST. The isolates were obtained from clinical specimens of patients from Delhi region of India. These specimens were submitted to our TB reference laboratory for culture and DST. All study participants gave informed consent, as a standard routine diagnostic procedure of the laboratory. All Bactec MGIT 960-DST results were blinded to the microbiologists performed the LPA. In phase II, the prospective study was carried out directly on clinical specimens for rapid screening of MDR cases under the programmatic management of drug resistant TB (PMDT), plan B of RNTCP, India.

2.2. Specimens

A total of 578 sputum specimens of suspected drug resistant-TB (DR-TB) patients from designated microscopy centers (DMCs) of eight districts (Amritsar, SAS Nagar, Ludhiana, Sangrur, Patiala, Gurdaspur, Jalandhar and Rupnagar) of Punjab state were sent to our TB Laboratory for LPA testing. The specimens were transported from the DMCs to the laboratory in the cold chain within 72 h of collection as per the guidelines of PMDT [16]. Only smear positive specimens (1+ to 3+) or Bactec MGIT 960 positive cultures were used for LPA testing as per the manufacturer's protocol (HAIN Lifescience, MTBDR*plus*V2_0212_304A-02-02.pdf) [17]. The detailed algorithm of the study protocol is shown in Figure 1.

2.3. Processing of specimens

The sputum specimens were processed using the modified Petroff's method [18]. In brief, about 3–4 ml of specimen was mixed with an equal volume of 0.5% NALC-4% NaOH mixture in 50 ml ridge capped round bottom processing tube. The mixture was vortexed and incubated at 37°C for 10 min. Then mixture was neutralized with phosphate buffer (pH 6.8) up to a total volume of 50 ml and centrifuged at 10,000 rpm for 10 min. The pellet was re-suspended in 2 ml of the phosphate buffer and smears were prepared for Zeihl–Neelsen staining. The smears were graded for acid fast bacilli (AFB) positivity as per WHO standard guidelines routinely adopted in all TB laboratories, as 1+, 2+ or 3+. If the entire smear examination (100 oil fields) demonstrated 1–9 AFB, the specimen was considered scanty+ and subjected to Bactec MGIT 960 culture and, if culture flashed positive it was subjected to LPA.

2.4. Bactec MGIT 960 culture and DST

After processing, 500 μl of the decontaminated specimens were inoculated in Bactec MGIT 960 culture tubes containing 800 μl mixture of oleic acid, albumin, dextrose and catalase (OADC) and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin (PANTA) supplement as per the manufacturer's instructions (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA). The left over sediments of the decontaminated specimens were stored at −20°C. All the manipulation of the specimens was performed under the Class II-B biosafety cabinet in a BSL-2B laboratory. In phase I, mycobacterial isolates obtained in Bactec MGIT 960 culture, were identified as *M. tuberculosis* by *in-house* developed multiplex PCR assay [18] before subjecting them to 4 first line (streptomycin, isoniazid, rifampicin and ethambutol) SIRE DST by Bactec MGIT 960 system and the results were continuously monitored by BD Epi-centre® software V 5.75A [19].

2.5. GenoType MTBDRplus (LPA) testing

The LPA was performed according to the manufacturer's protocol (MTBDR*plus*V2_0212_304A-02-02.pdf) [17]. The test is based on the DNA hybridization on strip technology and has three steps: DNA extraction, multiplex polymerase chain reaction (PCR) amplification, and reverse hybridization. All the three steps of LPA testing were performed as per the WHO recommendations [7]. In brief: DNA extraction was performed in the BSL-2 laboratory, master mix preparation in a second room with UV chamber, and PCR amplification and hybridization were performed in another room. Five hundred microliter of decontaminated specimen and 1 ml of Bactec MGIT 960 culture was used for the DNA extraction using the GenoLyse kit (GenoLyse_1011_51610-08-02.pdf) [20]. Amplification of the targets was performed by adding 35 μl of PNM mix, 10 μl of reaction buffer and 5 μl of template DNA in each reaction mix except the negative control. PCR reaction was run in an MTC-100 thermal cycler (MJ Research, USA) at the amplifying conditions of initial denaturation at 95 °C for 15 min and 10 cycles of 95°C for 30 s, 58°C for 2 min and additional 30 cycles of denaturation at 95°C for 25 s, annealing at 53°C for 40 s, elongation at 70°C for 40 s; and a final extension at 70°C for 8 min. After amplification, the hybridization was carefully performed in the TwinCubator as per the manufacturer's instructions. Briefly, 20 μl of biotin labeled amplicon with 1 ml of hybridization buffer was hybridized on the strip. After the washing with stringent buffer, streptavidin-alkaline-phosphatase conjugate was added to the strip's membrane. An alkaline phosphatase mediated staining reaction was allowed to occur and the strips were allowed to air dry for the interpretation of the results. During the evaluation of LPA on mycobacterial isolates and clinical samples, the Bactec MGIT 960-DST results were blinded to the person who carried out LPA (PK & VB). For quality control, *M. tuberculosis* H37Rv (TMC-102) was run as positive control and sterile molecular grade water was used as a

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