Detection of Multi-Drug-Resistance (MDR) *Mycobacterium tuberculosis* among Suspected Tuberculosis Patients in Bangladesh using Line Probe Assay

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ABSTRACT: Background: Multi-drug resistant (MDR) Tuberculosis (TB) is one of the most significant concerns in tuberculosis control. Genotyping of *Mycobacterium tuberculosis* helps study evolutionary relationships, its transmission, and molecular epidemiology. The collaborative data from genotyping along with demographic data allows the observation of the current trends of the disease within a population. The molecular tools developed in the past two decades to detect this disease were found to be expensive. Using the Line Probe Assay (LPA) molecular method, it has become simpler to diagnose *M. tuberculosis*. This study aimed to detect the prevalence of MDR-TB in Bangladesh using LPA. **Methods:** LPA was used to identify sensitivity or resistance to the antibiotics Isoniazid (INH) and Rifampicin (RIF) (two out of the five first-line TB drugs.). **Results:** Out of 500 acid-fast smear-positive and LPA-positive sputum samples, the percentages of only RIF, only INH resistant, and resistant to both RIF and INH were 2.2%, 7.6%, and 12.6%, respectively. The majority of the detected MDR-TB cases were from patients within the age range of 21-40 years. This study found the highest number of MDR-TB in the relapse category of patients. **Conclusion:** LPA can be used successfully to identify MDR-TB prevalence in Bangladesh.

KEYWORDS: *Mycobacterium tuberculosis*, Line Probe Assay (LPA), Multi-drug resistant (MDR), Bangladesh

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Introduction

Tuberculosis (TB) is the most frequently encountered bacterial disease globally and the second biggest leading cause of death from infectious diseases after HIV (Onyango *et al.*, 2017). The emergence of MDR strains of *M. tuberculosis* has made treating this communicable disease challenging, especially in developing countries. Bangladesh has been enlisted as the eighth highest TB burden countries by the World Health Organization (WHO) and accounted for two-thirds of the total number of incidences (Tuberculosis, WHO Report 2021). Despite the global fight against TB, the disease is still a significant problem in the public health sector, particularly in developing countries like Bangladesh.

The COVID-19 pandemic was found to threaten to reverse recent progress in reducing the global burden of TB disease worldwide (WHO, 2021). According to Global Tuberculosis Report 2020 by WHO, the number of TB deaths could increase by around 0.2–0.4 million globally in 2020 alone. This might be manifested if the health services are disrupted to such an extent that it decreases the number of TB infected people detected and treated by 25–50% over three months. It has also been reported that in 2017, a total of 233,000 children died of TB infection and approximately 96% of these deaths were due to the lack of access to TB treatment. Although there is no accurate estimate of the prevalence of childhood TB, it is believed that childhood TB is severely underdiagnosed (WHO, 2021).

Anti-tuberculosis drug resistance strain was emerged about 60 years ago and has been almost concurrent with the development of anti-tuberculosis drugs (Caminero *et al.*, 2010). The first major step taken to stop the emergence of drug resistance in *M. tuberculosis* was done by introducing para-aminosalicylic acid (SM) and isoniazid (INH) to reduce the development of streptomycin resistance tuberculosis (Murray, Schraufnagel and Hopewell, 2015). In less than 20 years, resistance to INH and SM has become a major problem in the fight against *M. tuberculosis*. Over time, many INH-resistant *M. tuberculosis* strains showed less susceptibility to rifampicin (RIF) and became multi-drug resistant (MDR) to these two most efficient first-line anti-TB drugs, originally developed and introduced in the 1950s and 1960s (Long,

1958; Sensi, 1983; Vilchèze and Jacobs, 2007; Allué-Guardia, García and Torrelles, 2021).

In terms of global TB control, the emergence of MDR *M. tuberculosis* is one of the major obstacles (Gumbo, 2013; Ba Diallo *et al.*, 2017). MDR-TB is more difficult to treat than drug-susceptible TB and requires second-line drugs. These second-line drugs are not that effective, come with more adverse effects, must be administered for a prolonged period, and are much more expensive (Ramachandran and Swaminathan, 2015).

Line Probe Assay (LPA) is a hybridization-based DNA-strip technology that contains probes specific to the *M. tuberculosis* complex, probes for common RIF resistance-conferring mutations, and a subset of the mutations conferring resistance to INH (Meaza *et al.*, 2017). A banding pattern is obtained on the strip, which provides an easy and fast interpretation of the results for diagnosing the *M. tuberculosis* complex and detecting resistance and sensitivity patterns towards RIF and INH (Rufai *et al.*, 2014).

We hypothesized that the Line Probe Assay is a rapid and reliable diagnostic method for detecting MDR Tuberculosis. This paper studied the effectiveness of LPA as a rapid and reliable diagnostic method for detecting MDR *M. tuberculosis* from the smear-positive for Acid-Fast Bacilli (AFB) sputum samples collected from a tertiary care hospital in Dhaka, Bangladesh.

Methodology

The study was carried out at the National Tuberculosis Reference Laboratory (NTRL) within the National Institute of Disease of Chest and Hospital (NIDCH), Mohakhali, Dhaka, Bangladesh, between July 2016 and December 2016. A total of 530 sputum samples were collected from ages (10-70 years) and gender-matched indoor and outdoor patients in NIDCH. Among them were 498 positive sputa from suspected tuberculosis patients, a few drug-resistant sputum samples were determined by reference laboratories, and the rest were sensitive to RIF and INH. H37Rv was used as a sensitive reference strain. The patients were selected based on several criteria, defined as the following relapse after treatment, treatment failure, delayed converters, and treatment after default (World Health Organization, 2020). Patients with extra-pulmonary TB cases and new pulmonary tuberculosis cases were excluded from the study.

Freshly given 5.0-10.0 ml of sputum samples were collected in a clean, dry, leak-proof plastic container labeled with the patient's name, age, sex, serial number, and sputum collection date and time. Immediately after receipt, samples were processed inside a Class II Safety Cabinet (BSL-2 plus) facilitated laboratory.

Specimen processing and storage

The sputum sample was transferred to a 50.0 mL falcon tube with aseptic precaution. Sputum samples were first digested and then decontaminated by the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method (Kubica *et al.*, 1963). The sediment of processed sputum was used for microscopic examination, culture, drug susceptibility testing (DST) on L-J media (Lowenstein-Jensen Medium), and for the LPA. Briefly, the sputum samples were first decontaminated using an equal volume of 4% NaOH- 2.94% Na-Citrate solution (with 0.5% NALC). The samples were then incubated for 15 minutes, followed by the addition of phosphate-buffered solution to make the total volume of 40.0 ml to stop the decontamination process. The samples were centrifuged at 4°C for 15 minutes at 3000 X g (4000 rpm), and the supernatant was discarded. The pellet containing bacterial cells was collected in microcentrifuge tubes and stored at -20^0 C. One ml of processed sputum sample was used for LPA.

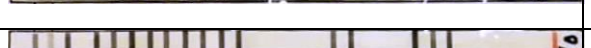
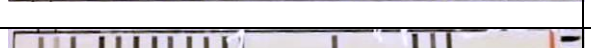
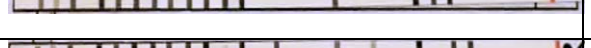
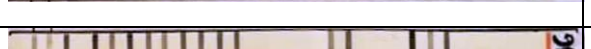
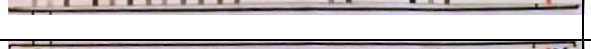
Identification of M. tuberculosis by LED Microscopy

A sputum sample was placed on slides based on size, shape, thickness, oval, and endpoint criteria. The separated samples were air-dried and subjected to the staining procedure. Acid-fast staining was performed according to the Auramine staining technique (Hänscheid *et al.*, 2007) using Auramine-Rhodamine-Fluorochrome staining to visualize Acid-fast bacilli (AFB). Under an LED fluorescent microscope, bacilli were observed, and only samples with positive smears were selected for further analysis using LPA.

Detection of MDR-TB by LPA

In this study, the GenoType MTBDRplus 96 kit (Hain Lifescience, Nehren, Germany) was used to detect *M. tuberculosis* by LPA according to the manufacturer's instructions. The LPA strip was coated with highly specific probes, complementing selectively amplified nucleic acid sequences. The single-stranded amplicons bound specifically to the analog probes during hybridization, while non-specifically bound amplicons were removed in subsequent washing steps. The whole procedure can be divided into three steps, DNA extraction from smear-positive sputum, multiplex amplification (PCR) of extracted DNA with biotinylated primers, followed by reverse hybridization. After a successful reaction, a specific purplish-brown color band was developed on the strip. Strip was removed from the tray by tweezer and dried between two layers of absorbent paper and, the strip was then taped to the LPA worksheet for interpretation. Valid results were accepted based on the presence of conjugate, amplification, and internal control. The results obtained from the LPA are shown in Table 1. The bands developed on the strip correspond to whether MTB was present or absent in the sample tested, and its sensitivity or resistance to rifampicin (RIF) and isoniazid (INH) was also detected (Table 1 & 2).

Table 1. LPA strip showing detection of MTB and sensitivity (S) and resistance (R) patterns to RIF and INH

Serial #	Sample ID	Hybridization Panel	Results										
			TUB	*rpoB* WT	*rpoB* MUT	*katG* WT	*katG* MUT	*inhA* WT	*inhA* MUT	RIF Sensitive	RIF Resistant	INH sensitive	INH Resistant
1	R-3542		-										
2	R-3543		+	-	+	-	+	+	-		R		R
3	R-3580		+	-	+	-	+	+	-		R		R
4	R-4200		+	+	-	+	-	+	-	S		S	
5	R-4296		+	-	+	-	+	+	-		R		
6	R-4583		+	+	-	-	+	+	-	S			R
7	R-4640		-	-	-	-	+	+	-		R		R
8	R-5787		+	-	+	-	+	+	-		R		R
9	CF-3995		+	-	-	-	+	+	-		R		R
10	CF-3008		+	+	-	+	-	+	-	S		S	
11	CF-3370		+	+	+	+	-	+	-	S		S	
12	CF-3782		+	+	+	-	+	-	+		R		R
13	CF-3984		+	-	+	-	+	+	-		R		R
14	CF-4110		+	-	+	+	-	-	+		R		R
15	CF-4704		+	-	-	-	+	-	+		R		R
16	CF-4737		+	+	-	+	-	+	-	S		S	
17	CF-5388		+	-	+	-	+	+	-		R		R
18	CF-5394		+	-	+	-	+	-	+		R		R
19	H37RV		+	+	-	+	-	+	-	S		S	

The LPA strip consists of 27 probes to detect *M. tuberculosis* and identify whether the detected *M. tuberculosis* is resistant to only or both rifampicin and isoniazid. In the LPA strip, the Conjugate Control (CC) band shows the efficiency of conjugate binding and substrate reaction and must be developed. An Amplification Control (AC) band indicates that the DNA extraction and PCR procedures have been carried out successfully. The third band in the LPA strip (TUB) denotes an *M. tuberculosis* complex-specific region that confers *M. tuberculosis* in the sample. The Locus Control gene-specific bands, e.g., *rpoB* for rifampicin, *katG*, and *inhA* for isoniazid, are always positive in TUB band positive samples. Positive bands in *rpoB* WT1-8 for rifampicin or *katG* WT *and inhA* WT1-2 for isoniazid wild-type probes represent that the detected strain is sensitive to rifampicin and/or isoniazid. Similarly, bands in the gene mutation probes *rpoB* MUT1-4 and *katG* MUT1-2 or *inhA* MUT1-2, MUT3A & 3B confers that the detected *M. tuberculosis* strain is resistant to rifampicin or isoniazid or both. (Table 2).

Table 2. Interpretation of LPA result

Corresponding Bands in LPA Strip	Interpretation
Only CC and AC bands are developed	MTB not detected
Band present in 5 control zone (CC, AC, *rpoB*, *katG*, *inhA*) and TUB region	MTB detected
All *rpoB* wild type (*rpoB* WT 1-8) bands present	RIF sensitive
Band in *rpoB* mutant region (*rpoB* MUT 1, MUT2A, MUT2B, MUT3) present, or any band absent in *rpoB* wild type region (*rpoB* WT 1-8) or both	RIF resistant
All *katG* and *inhA* wild type (WT 1-2) bands present	INH sensitive
Band present in *katG* mutant region (MUT1, MUT2) or *inhA* mutant region (MUT1, MUT2, MUT3A, MUT3B) present or both regions, or band absent in any *katG* WT or *inhA* WT or both WT region.	INH resistant

Results and Discussion

Bangladesh ranked the 6th highest among 22 highly TB-burdened countries (*World Health Statistics 2014 - World | ReliefWeb*, 2014). The importance of TB as a significant problem in the public health arena has been dramatically fortified due to the ability to cause co-infection with HIV and the outburst of MDR *M. tuberculosis* strains (Alexander and De, 2007; Eldholm *et al.*, 2016). MDR isolates are one of the major concerns in TB control. MDR-TB usually develops during TB treatment, either due to an interrupted antibiotic course or because the concentration of the active drug is not good enough to kill 100% of the bacteria (Seung, Keshavjee and Rich, 2015). This can happen for several reasons like irregular medication, scarcity of drug supplies, or patients halting their antibiotic course due to feeling better halfway into the course. Another primary reason might be the gene mutation(s) responsible for resistance to certain drugs, which allows the spread of *M. tuberculosis* from person to person (Smith, Wolff and Nguyen, 2013; Palomino and Martin, 2014).

Current research used the LPA method to detect MDR-TB in Bangladesh. H37Rv and specific 22 probes played a crucial role in this study. Positive smear results confirmed the presence of *M. tuberculosis* in the sample. The probes used in LPA were designed to detect the presence of *M. tuberculosis*. Therefore, all the samples were first found positive for *M. tuberculosis.* After confirmation, sample processing was done, and the resistance pattern was analyzed using LPA. A total of 530 sputum samples (395 and 135 samples were collected from male and female patients, respectively) positive for Acid-Fast staining were included in the present research for LPA analysis. The LPA analysis identified 94% of samples (500) as positive for *M. tuberculosis* (Table 3).

Table 3. Number of samples tested positive and negative for *M. tuberculosis* by LPA

Patients	Number of Acid-Fast positive suspected MTB samples	Number of samples tested confirmed positive for MTB by LPA	Number of samples tested confirmed negative for MTB by LPA
Male	395	375	20
Female	135	125	10
Total	530	500	30

The LPA result indicated that the majority of the samples that tested positive for drug resistance were MDR type, resistant to both rifampicin and isoniazid (Table 4, Figure 1). Among the resistant samples, only rifampicin-resistant *M. tuberculosis* was the lowest in both the male and female patients (Figure 1). The percentage of isoniazid resistance has been detected to be higher than rifampicin resistance in both males and females. Our observation also marked that 77.6% of all LPA-positive MTB were sensitive to both rifampicin and isoniazid.

Table 4. Drug resistance pattern of *M. tuberculosis* positive sputum samples detected by LPA as MDR (MDR, resistant to both rifampicin and isoniazid) or Mono-resistant (resistant to either rifampicin or isoniazid)

Resistance pattern detected by LPA	Number of Samples		
	Male	Female	Total
RIF+INH resistance (MDR)	44 (11.73%)	19 (15.20%)	63 (12.6%)
Mono-RIF resistance	8 (2.13%)	3 (2.4%)	11 (2.2%)
Mono-INH resistance	29 (7.73%)	9 (7.2%)	38 (7.6%)
Both sensitive	294 (78.4%)	94 (75.2%)	388 (77.6%)
Total MTB positive samples	375 (100%)	125 (100%)	500 (100%)

A higher proportion of females (15.20%) have been observed to have MDR-TB than males (11.73%) in this study (Table 4). A similar trend was also observed in studies done in India, Pakistan, China, and South Africa (O'Donnell *et al.*, 2011; Ahmad *et al.*, 2016; He *et al.*, 2016). However, another study in Belarus revealed a higher likelihood of having MDR-TB among males than females (Surkova *et al.*, 2012). It shows that the male-to-female ratio in terms of MDR-TB varies across countries.

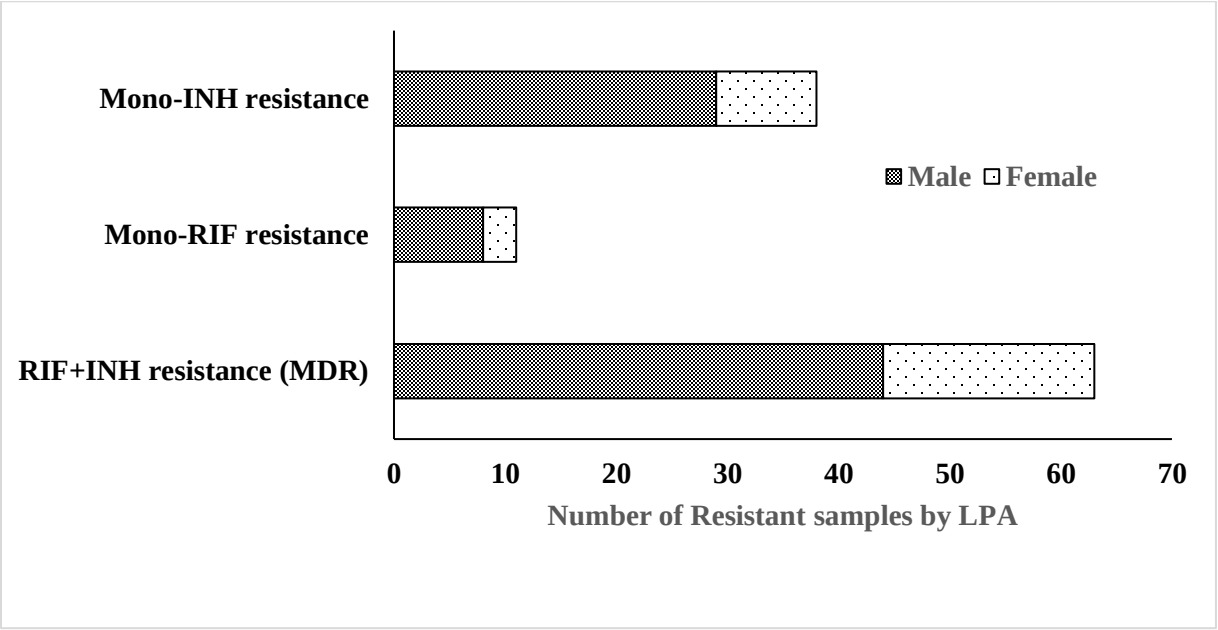


Figure 1. Distribution of LPA positive anti-tuberculosis resistant samples

Among the patients in this study, the largest group comprised 141 individuals aged 21-30 years with sputum samples positive by Acid-fast staining for *M. tuberculosis*, followed by age groups 31-40 and 41-50 with 109 and 100 sputum samples, respectively. Within the age group of 21-30 years, most patients exhibited the MDR phenotype, resistance to both rifampicin and isoniazid (26 out of 141 samples). It has been found that 429 patients who provided sputum samples for the diagnosis of *M. tuberculosis* fell between the active age group of 21-60 years. It is evident from Figure 2 that most patients were infected with MDR *M. tuberculosis* rather than mono-resistant to either rifampicin or isoniazid. The smallest age group used in the study lies within the age of 71≤ years. The lowest proportion of antibiotic resistance to RIF and MDR was observed within this group (both categories comprise 0 out of 12 samples). The high frequency of MDR-TB among the younger age group in the present study might result from inadequate adherence since they frequently move from one place to another for earning and other purposes.

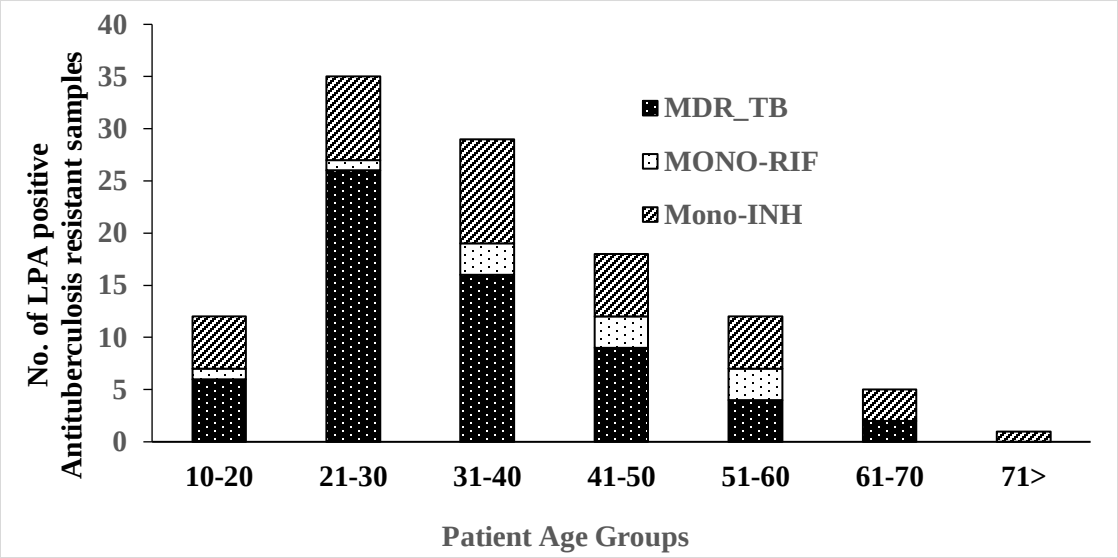


Figure 2. Distribution of Antituberculosis resistance among the patients of different age groups. The total number of samples in individual age groups are 10-20 = 54, 21-30 = 141, 31-40 = 109, 41-50 = 100, 51-60 = 79, 61-70 = 35, 71> 12

This study found the highest number of MDR-TB in the relapse group (~43%) and in patients who were treated after the loss of follow-up (30%), and the lowest number of MDR-TB in the non-converters (1.58%) and close contact with tuberculosis patients (1.58%) (Table 5). The population used for the present study was selected from admitted and outdoor patients of NIDCH.

Table 5. Distribution of antibiotic resistant *M. tuberculosis* in different patient categories

LPA detected Resistance Pattern	Tuberculosis Patient Categories							Total
	Treatment Failures	Non-converters	Relapses	Treatment after loss of follow up	Close contact	Other	HIV infected person with TB	
MDR-TB	11 (17.5%)	1 (1.6%)	27 (43%)	19 (30%)	1 (1.6%)	4 (6.3%)	0	63 (100%)
RIF Resistant	2	0	4	5	0	0	0	11
INH Resistant	6	2	12	16	0	2	0	38
Total of resistant *M. tuberculosis* in different patient categories	19	3	43	40	1	6	0	112

Conclusion

This study attempts to look at the prevalence of MDR-TB using the LPA method. The LPA method might be considered a standard molecular diagnostic tool for diagnosing MDR-TB where facilities are available and suitable. Because of the increasing prevalence of the modern type of *M. tuberculosis* in our country and the preventive effect of BCG vaccine remains low for controlling modern *M. tuberculosis*, it is recommended to make new vaccines by executing various other TB-controlling ways and attempts. The findings of the current study will forge a path towards a better understanding of the MDR-TB scenario in Bangladesh and the mechanisms to combat it.

Declarations

Ethical approval

Ethical approval was obtained from the IERC of The Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh, approval no. BMBDU-ERC/EC/1603.

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Conflicts of Interest

The authors declare no conflict of interest regarding this work.

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