

Rapid detection of Multi Drug Resistant- Tuberculosis using Line Probe Assay (LPA) in Sudan

MUATAZ M. ELDIRDERY

Tropical Medicine Research Institute
National Center for Research, Sudan

INTISAR E. ELRAYAH

Tropical Medicine Research Institute
National Center for Research, Sudan

Faculty of Applied Medical Science, Shaqra University, KSA

MONA OA. AWAD ELKARIM

Blood Transfusion Service, National Blood Transfusion Center, Sudan

FATIMA A. KHALID

Tuberculosis Research Center, University of Kassala, Sudan

ASRAR M A/SALAM ELEGAIL

NUHA Y. IBRAHIM

EMAN O M. NOUR

RAHMA H. ALI

National Tuberculosis Reference Laboratory
National Laboratory of Public Health, Sudan

HAMDAN M. HAMDAN

Consultant of Tuberculosis control program, Sudan

NIHAD M A. ELHAJ

ATIF A. ELAGIB

Tropical Medicine Research Institute
National Center for Research, Sudan

Abstract:

Tuberculosis (TB) remains an important community health problem; emergence of multidrug-resistant tuberculosis (MDR-TB) poses a major threat to global TB control. This study was designed to test the sensitivity and specificity of LPA towards DST. Molecular Line Probe Assay (LPA) (Genotype MTBDRplus) have been recently

approved for use in low income settings and can be used to screen smear-positive sputum specimens for rapid detection of Rifampicin and Isoniazid resistance in 1-2 days. A total of 300 smear positive sputum specimen were collected from Pulmonary Tuberculosis patients ,182 specimens from new cases and 118 from previously treated TB patients in a national reference TB laboratory in Khartoum, Sudan used in this study. The performance of a commercial line probe assay (Genotype MTBDRplus) was assessed for rapid detection of rifampicin and isoniazid resistance directly on smear-positive sputum. Sudan. Results were compared with LJ culture and drug susceptibility testing (DST). The overall prevalence of MDR in all study groups was 38% by DST and 37.3% by LPA. Results of LPA were compared with DST, out of 300 smear-positive sputum specimens only 112 samples were found MDR by (DST) and (LPA), 186 sample sensitive by both and 2 sample were MDR by DST but sensitive by LPA. No significant differences were found between the drug sensitivity test (DST) and Line Probe Assay (LPA) technique, (P = 0.50). The sensitivity of Line Probe Assay was (98.3%) and the specificity was 100% while the positive predictive value was (100%) and negative predictive value was (98.9%). Out of 112 specimen screened by LPA, 94(83.9%) of RIF Resistant samples were found linked with RpoB gene, codon 530-533. On the other hand, all of INH Resistant samples were linked with KatG gene, codon 315 (S 315T1). Line Probe Assay rapid technology and provide results in 2 days, comparing by DST it provide results in 14 weeks; therefore it can be useful diagnostic method in high TB burden countries.

Key words: Line Probe Assay, *Mycobacterium tuberculosis*, Sudan.

INTRODUCTION:

Tuberculosis (TB) remains a major public health problem worldwide due to its high risk of person-to-person transmission, morbidity and mortality [1]. It is an important community health problem. In 2013 there were 9.0 million incident cases of

TB (range, 8.6 million–9.4 million) globally, equivalent to 126 cases per 100 000 population [2]. Furthermore the emergence of multidrug-resistant tuberculosis (MDR-TB) and more recently, extensively drug-resistant TB (XDR-TB) is a major threat to global TB control [3]. Sudan is a country has a high burden of tuberculosis (TB) with an estimated 50,000 incident cases during 2009, when the estimated prevalence was 209 cases per 100,000 of the population. Few studies have been undertaken on TB in Sudan and the prevalence of drug resistant disease is not known yet [4]. The rapid identification of MDR-TB is essential for proper patient management [5, 6]. The World Health Organization (WHO) recommended the use of molecular line probe assays (LPAs) for rapid screening of MDR-TB in low and middle income settings [7]. LPA is 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. LPA can be performed directly from acid fast bacilli (AFB) smear-positive sputum or from culture isolates, and provide results in 1-2 days, it is highly sensitive and specific for detection of Rifampicin resistance ($\geq 97\%$ and $\geq 99\%$) and Isoniazid resistance ($\geq 90\%$ and $\geq 99\%$) on culture isolates and smear-positive sputum. Overall agreement with conventional DST for detection of MDR-TB was 99% [8].

MATERIALS AND METHODS:

Study setting:

Sputum samples were collected from new cases and previously treated Pulmonary Tuberculosis patients who attended the hospitals and National Reference TB laboratory and consecutively screened for acid fast bacilli (AFB) using Ziehl-Neelsen (ZN) smear microscopy, the line probe assay (LPA) testing and LJ culture and DST were carried out at the

National Reference Tuberculosis Laboratory Khartoum, Sudan. Samples were collected from the patients after obtained their written informed consent. The study was approved by Tropical Medicine Research Institute (TMRI) Ethics committees.

Specimen collection and processing:

Smear-positive sputum was collected from patients at risk of MDR-TB at the National Reference Tuberculosis Laboratory were consecutively screened for acid fast bacilli (AFB) using Ziehl-Neelsen (ZN) smear microscopy, All consenting smear positive patients were enrolled. Two or three sputum samples (spot or morning) were collected per patient in 50 ml sterile conical centrifuge tubes. All manipulations with potentially infectious clinical specimens were performed in a Class II safety cabinet in a BSL 2 or 3 Laboratory. Sputum was processed with sodium hydroxide (NaOH) method 4%. Any processed specimen remaining was stored at 2-8°C for the duration of the study to allow for re-testing of specimens giving discrepant results.

Conventional laboratory testing:

Conventional laboratory testing sputum specimens submitted for smear, culture and DST was treated using 4% sodium hydroxide (NaOH) method, for decontamination because the specimens susceptible to contamination by more rapidly growing normal flora. Two or three drops of the decontaminated sample were inoculated in the Lowenstein-Jensen (LJ) solid medium. and were incubated at 37°C for 8 weeks [9]. Drug susceptibility test was done for 300 isolates, LJ media contain drugs (Rifampicin, Isoniazid, Streptomycin and Ethambutol), for drug susceptibility test of *Mycobacterium tuberculosis* strains was prepared as mention by National Tuberculosis Programme briefly; LJ media containing a drugs with the different concentrations (0.2µg/ml for INH, 40 µg/ml for RIF, 4 µg/ml for SM and 2.0 µg/ml for ETH). Plain LJ medium were

prepared without drugs as a control (10). The suspension was prepared by transferring of a certain amount of bacterial growth from the culture by 10 μ L loop into the suspension bottle (Peugeot bottles contains glass beads). Homogenization was made by vortexing the bottle, the turbidity of suspension was adjusted to McFarland standard No.1. Then four serial dilutions were made from the original suspension of each sample (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}). Two to three drops was inoculated from dilution 10^{-2} in media which contain drugs and 10^{-4} in plain media, then was incubated at 37°C, the bottles was tightening loosely for 24 hours to allow evaporation and then well tighten. Contamination was examined for 72 hours and the contaminated bottles were discharged. The first examination for growth of *Mycobacterium tuberculosis* was done after two weeks from inoculation, and the second examination after month from the first examination. The resistance was calculated as the ratio of the number of colonies on the drug containing medium and those of control media, the strain was considered as resistant if the ratio is greater or equal to 1% [11].

Line probe assay (LPA)

Line probe assays (LPA) have been recently approved for use in low income settings and can be used to screen smear-positive sputum specimens for resistance to Rifampicin and Isoniazid in 1-2 days. LPA testing was performed in three separate rooms, according to WHO recommendations [12]. DNA extraction was performed in the BSL3 laboratory, master mix preparation in a second room, and PCR and hybridization were performed in a third laboratory. Five hundred microlitres of processed sediment was used to perform the Genotype MTBDRplus (Hain Lifescience GmbH) assay, according to the manufacturer's instructions [13]. LPA was performed without knowledge of conventional DST results. Residual processed specimens were

refrigerated at 2-8°C overnight after DNA extraction to allow repeat testing if required. All steps were done in National Reference TB laboratory - Sudan.

STATISTICAL ANALYSIS:

Statistical Package for Social Science (SPSS) version 16.0 (Inc. Chicago, IL,USA) was used to analyze the results. Mc-Nemar test was used to compare between two techniques (DST and LPA), P. value less than 0.05 was considered statistically significant.

Sensitivity, specificity, positive predictive value and negative predictive value were calculated according to the follow statistical formula

Sensitivity = True positive / True positive + False negative X100

Specificity = True negative / True negative + False positive X100

Positive Predictive Value: = True positive / True positive + False positive X100

Negative Predictive Value: = True Negative / True Negative + False Negative X100

RESULTS:

Drug susceptibility Test (DST):

Drug susceptibility test was done for three hundred positive culture specimens. According to WHO guidelines the MDR-TB was recorded according to at least resistance to RIF and INH. In this study 114 cases were found MDR cases and 186 were sensitive. Out of 186 sensitive cases 7 (3.8%) were resistant just for INH and 1(0.5 %) for RIF.

Line Probe Assay (LPA):

Line probe assay was done for three hundred positive sputum specimen, 112 cases were found MDR and 188 were sensitive. Out of 112 specimen screened by LPA, 94(83.9%) of RIF Resistant samples was found linked with *Rpob* gene, codon 530-533 and the remaining linked with other codons; 510-517, 513-519, 518-525 and 526-529, 1(.89%), 2(1.8%), 3(2.7%), 12(10.7%) respectively. On the other hand, all of INH Resistant samples were linked with *KatG* gene, codon 315 (S 315T1).

INTERPRETATION OF LPA RESULTS:

Results of LPA were compared with drug susceptibility testing (DST). Out of 300 smear-positive sputum specimens only 112 was found MDR by conventional method (DST) results and Molecular (LPA), 186 sample sensitive by both and 2 sample were MDR by DST but sensitive by LPA. No significant differences were found between the drug sensitivity test (DST) and Line Probe Assay (LPA) results, $P = 0.50$. The sensitivity of Line Probe Assay was (98.3%) and the specificity was 100% while the positive predictive value was (100%) and negative predictive value was (98.9%), table (1).

Table 1 Performance of line probe assay (LPA) in smear positive sputum specimens compared with conventional drug susceptibility testing (LJ DST).

Techniques	LPA				
		INH ^R RIF ^R	INH ^R RIF ^S	INH ^S RIF ^R	INH ^S RIF ^S
DST	INH ^R RIF ^R	112	0	1	1
	INH ^R RIF ^S	0	0	0	7
	INH ^S RIF ^R	0	1	0	0
	INH ^S RIF ^S	0	0	2	176

LPA, line probe assay

DST, Drug Susceptibility Test in LJ Media

Muataz M. Eldirdery, Intisar E. Elrayah, Mona OA. Awad Elkarim, Fatima A. Khalid, Asrar M A/Salam Elegail, Nuha Y. Ibrahim, Eman O M. Nour, Rahma H. Ali, Hamdan M. Hamdan, Nihad M A. Elhaj, Atif A. Elagib- **Rapid detection of Multi Drug Resistant-Tuberculosis using Line Probe Assay (LPA) in Sudan**

RIF^S, rifampicin susceptible; RIF^R, rifampicin resistant
 INH^S, isoniazid susceptible; INH^R, isoniazid resistant.

Table 2 Performance of line probe assay (LPA) in detecting rifampicin, isoniazid and multidrug-resistance from smear positive sputum specimens

	Rifampicin	Isoniazid	Multi-drug resistance
No. resistant/No. susceptible strains	115/185	113/187	112/188
Sensitivity, % (95% CI)	98.3% (93.86% 99.79%)	92.6% (86.35% to 96.54%)	98.3% (93.81% 99.79%)
Specificity, % (95% CI)	98.9% (96.15% 99.87%)	99.4% (96.93% to 99.99%)	100% (98.04% to 100.00%)
Overall accuracy, % (95% CI)	98.7%	96.7%	99.3%
PPV, % (95% CI)	98.3% (93.86% 99.79%)	99.1% (95.17% to 99.98%)	100% (96.76% to 100.00%)
NPV, % (95% CI)	98.9% (96.15% 99.87%)	95.2% (91.06% 97.78%)	98.9% (96.21% to 99.87%)

Table 3 Pattern of gene mutations detected by Line Probe Assay (LPA) in drug resistant *M. tuberculosis* strains.

Gene	Band	Gene region or mutation	MDR strains (n = 112)	RIF resistant (n = 115)	mono strains	INH resistant strains (n = 121)	mono
rpoB							
	WT1	506 509	2*				
	WT2	510 513		1			
	WT3	513 517	1				
			5*				
	WT4	516 519		2			
	WT5	518 522	3	3			
	WT6	521 525					
	WT7	526 529	12	12			
WT8	530 533	94	97				
katG	MUT1	D516V	1				
	MUT2A	H526Y	4*				
	MUT2B	H526D					
	MUT3	S531L	10*				
WT		315					
	MUT1	S315T1	112			113	
	MUT2	S315T2					

Muataz M. Eldirdery, Intisar E. Elrayah, Mona OA. Awad Elkarim, Fatima A. Khalid, Asrar M A/Salam Elegail, Nuha Y. Ibrahim, Eman O M. Nour, Rahma H. Ali, Hamdan M. Hamdan, Nihad M A. Elhaj, Atif A. Elagib- **Rapid detection of Multi Drug Resistant- Tuberculosis using Line Probe Assay (LPA) in Sudan**

InhA					
	WT1	15/ 16			
	WT2	8			
	MUT1	C15T			
	MUT2	A16G			
	MUT3A	T8C			
	MUT3B	T8A			

1 MDR strain had both rpoB WT2 and WT3 mutations, 2 MDR strain had both rpoB WT3 and WT4 mutations, 3 MDR strain had both rpoB WT5 and WT6 mutations and 12 MDR had both rpoB WT6 and WT7 mutations.

* More than one mutation in rpoB Gene.

DISCUSSION:

In this study 182(60.7%) of the Pulmonary TB patients were diagnosed for the first time while 118 (39.3%) had history of P-TB reflecting the active transmission of TB. The findings of the present study indicated that the molecular methods were highly consistent with the conventional culture and DST method. In this study (41) patients were new cases for MDR-TB and (73) patients were retreatment cases for MDR-TB. There was no XDR-TB case recorded in this study. This is the first study in Sudan to ascertain levels of drug resistant tuberculosis by LPA.

Result of Drug susceptibility test (DST) showed 114 cases were MDR and 186 were sensitive. LPA result showed that 112 cases were MDR TB and 188 cases were sensitive. The sensitivity and specificity of LPA for the detection of MDR TB for clinical isolates was 98.3% and 100% respectively.

This results in agreement with findings reported in India, south Africa, and Bangladesh {14,15,16,17}, they compared the result of LPA with the DST results and found that high sensitivity(95.5%-98.8%) and high specificity(96.9-100%) of LPA for detection of MDR-TB. findings of this study also agrees with study done in Uganda, [18] which assessed the performance of a commercial line probe assay (Genotype

MTBDRplus) for rapid detection of Rifampicin and Isoniazid resistance, and the results were compared with (DST). The Sensitivity and specificity of LPA were 92.3%, 96.2% for detection of MDR compared with DST.

In this study the RIF resistance was associated with mutation in the region of *rpoB* 530-533, mostly S531L mutation, this agrees with the study of Telenti *et al.*, [19] and Barnard *et al.*, [20], they found that this mutation was more frequently in MDR strains in South Africa. The present findings provide the basis for rapid detection of Rifampicin resistance, and it is considered a marker of multidrug-resistant tuberculosis. On another hand all of INH resistant samples in this study were linked with KatG gene, codon 315 (S 315T1) as was shown in many high TB burden countries presumably related to ongoing transmission of these strains [21].

In DST it was noticed that 73% of re-treatment cases and 15% of new patients were found to have strains resistant to streptomycin thus suggests that the efficacy of this drug is limited in this setting. It is an injectable drug that necessitates regular attendance at a health clinic and the high levels of resistance observed suggest its role in the treatment of TB in Sudan should be reappraised. This study agrees with study done by Zaki and Ibrahim [22], as out of the 45 patients testing revealed that 48.9% (n=22) were resistant to streptomycin, also agrees with study by Magzoub [23] who used Lowenstein Jensen proportion method to test the antimicrobial sensitivity of 200 isolates of *Mycobacterium tuberculosis* complex against streptomycin, 43 (21.5%) were resistant to STM, it recorded the highest proportion of resistant comparing with all drugs.

CONCLUSION:

Line Probe Assay (LPA) was a highly accurate, reliable and rapid screening tool for MDR TB, it provide an early diagnosis

of resistance to Rifampicin and Isoniazid with highly sensitivity and specificity. It achieves a substantial reduction in diagnostic delay and it has the potential to revolutionize MDR TB diagnosis, therefore it can be useful diagnostic method in high TB burden countries.

ACKNOWLEDGEMENTS:

This study was funded by International Atomic Energy Agency (IAEA) RAF 60/40, National Center for Research and National TB Control Program. Also Extends thanks to Tropical Medicine Research Institute (TMRI) staff and National Reference Laboratory Staff.

Competing Interests

The authors have no competing interests.

REFERENCES:

- 1- Martin G and Lazarus A (2000). Epidemiology and Diagnosis of Tuberculosis. Recognition of At-risk Patients is Key to Detection, *Postgrad. Med.* 108:42-44, 47-50, 53-54.
- 2- WHO Global Tuberculosis Report 2014.
- 3- Shah NS, Wright A, Bai GH. (2007). Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg Infect Dis.* 13:380–7.
- 4- Sharaf Eldin GS, Fadel-Elmula I, Ali MS, Ali BA, Abdel Latif GA, Mallard K, Bottomley C and McNerney R (2011) Tuberculosis in Sudan: a study of Mycobacterium tuberculosis strain genotype and susceptibility to anti-tuberculosis drugs *BMC Infectious Diseases*, 11:219 doi:10.1186/1471-2334-11-219.

- 5- American Thoracic Society (1994). Treatment of tuberculosis and tuberculosis infection in adults and children. *Am J Respir Crit Care Med.* 1359-1374.
- 6- British Thoracic Society-Tuberculosis Committee (1998). Chemotherapy and management of tuberculosis in the United Kingdom. *Thorax.* 53:536-548.
- 7- WHO (2008) Policy Statement. Molecular Line Probe Assays for Rapid Screening of patients at risk of multidrug resistant tuberculosis (MDR-TB), [.http://www.who.int/tb/dots/laboratory/lpa_policy.pdf](http://www.who.int/tb/dots/laboratory/lpa_policy.pdf).
- 8- Ling D, Zwerling A and Pai M (2008). GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: a meta-analysis. *Eur Respir J*, 32:1165-1174.
- 9- WHO (1998). Laboratory Services in Tuberculosis Control Culture Part III PP (57-75).
- 10- National TB Control Programme (2009). Manual of Standard Operating Procedures (SOPs). Culture of *Mycobacterium tuberculosis* and Drug Susceptibility Testing on solid Medium Intermediate Reference Laboratory for Tuberculosis - Central TB Division, (Ministry of Health & Family Welfare) New Delhi-Version No. 01.01 Date: 01/04.
- 11- Sethi S, Sharma S, Sharma SK, Meharwal SK, Jindal SK and Meera S (2004). Drug susceptibility of *Mycobacterium tuberculosis* to primary anti- tubercular drugs by nitrate reductase assay. *Indian J Med Res.* 120, PP 468-471.
- 12- World Health Organisation: Policy Statement. Molecular Line Probe Assays for Rapid Screening of patients at risk of multidrug resistant tuberculosis (MDR-TB), 2008. http://www.who.int/tb/dots/laboratory/lpa_policy.pdf.
- 13- Hain Lifescience. Genotype® MTBDRplus product insert. Version 1 http://www.hain-lifescience.de/en/products/microbiology/mycobacteria/genoty_pemtldrplus.html.

- 14- Sharma M, Sethi S, Mishra B, Sengupta C, Sharma SK (2003). Rapid detection of mutations in *rpoB* gene of rifampicin resistant *Mycobacterium tuberculosis* strains by line probe assay. *Indian J Med Res.*117:76-80.
- 15- Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME (2008). Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *Am J Respir Crit Care Med*, 177(7):787-792.
- 16- Aurin TH, Munshi SK, Kamal SM, Rahman MM, Hossain MS, Marma T, Rahman F, Noor R (2014). Molecular approaches for detection of the multi-drug resistant tuberculosis (MDR-TB) in Bangladesh. *PLoS One*. 9(6):e99810.
- 17- Yadav RN, Singh BK, Sharma SK, Sharma R, Soneja M, Sreenivas V, Myneedu VP, Hanif M, Kumar A, Sachdeva KS, Paramasivan CN, Vollepore B, Thakur R, Raizada N, Arora SK and Sinha S. (2013). Comparative evaluation of GenoType MTBDRplus line probe assay with solid culture method in early diagnosis of multidrug resistant tuberculosis (MDR-TB) at a tertiary care centre in India. *PLoS One*. ;8(9):e72036. doi: 10.1371/journal.pone.0072036.
- 18- Albert H, Bwang F, Mukkada S, Nyesiga B, Ademun JB, Lukyamuzi G, Haile M, Hoffner S, Joloba M and O'Brien N (2010). Rapid screening of MDR-TB using molecular Line Probe Assay is feasible in Uganda. *AlbeBMC Infectious Diseases*. 10:41.
- 19- Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K and Bodmer T. (1993). Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet*. 341, 647–650.
- 20- Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. (2008). Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *Am J Respir Crit Care Med*, 177(7):787-792.

- 21- Van Rie A, Warren R, Mshanga I, Jordaan A, Spuy van der GD, Richardson M, Simpson J, Gie RP, Enarson DA, Beyers N, van Helden PD, Victor TC (2001). Analysis for a limited number of gene codons can predict drug resistance of *Mycobacterium tuberculosis* in a high-incidence community. *J Clin Microbiol*, 39:636-641.
- 22- Zaki AM and Ibrahim N Y (2004). A study on Prevalence of Drug Resistance in Drug Default Pulmonary Tuberculosis. *Trop Med Int Health*. 9(12):1305-11.
- 23- Magzoub EMN (2008). Isolation, Identification and Antimicrobial Sensitivity of *Mycobacterium tuberculosis* from Patients with Pulmonary Infection. Thesis for M.Sc degree University of Khortoum.