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Joveria Qais Farooqi
Aga Khan University, joveria.farooqi@aku.edu

Erum Khan
Aga Khan University, erum.khan@aku.edu

Syed Muhammed Zaheer Alam

Asho Ali
Aga Khan University

Zahra Hasan
Aga Khan University, zahra.hasan@aku.edu

See next page for additional authors

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Authors

Joveria Qais Farooqi, Erum Khan, Syed Muhammed Zaheer Alam, Asho Ali, Zahra Hasan, and Rumina Hasan

Line probe assay for detection of rifampicin and isoniazid resistant tuberculosis in Pakistan

Joveria Qais Farooqi,[1] Erum Khan,[2] Syed Muhammed Zaheer Alam,[3] Asho Ali,[4] Zahra Hasan,[5] Rumina Hasan[6]

Department of Pathology Microbiology,[1,4-6] School of Nursing,[2] Aga Khan University Hospital.

Sales Agency Pakistan of Hain Lifescience, Gulistan-e-Jauhar, University Road, Karachi.[3]

Corresponding Author: Rumina Hasan. Email: rumina.hasan@aku.edu

Abstract

Objective: To assess the efficacy of a line-probe assay Δ (LiPA) as rapid diagnostic test for early detection of drug-resistant tuberculosis compared to conventional susceptibility methods in Pakistan.

Methods: Resistance to rifampicin (RIF) and isoniazid (INH) in 108 smear-positive pulmonary tuberculosis samples was detected using a line-probe assay [GenoType® MTBDRplus (Hain Lifescience, GmbH, Nehren, Germany)] at the clinical microbiology laboratory of Aga Khan University Hospital in May, 2009. Results were compared with susceptibilities performed while using agar proportion.

Results: In comparison to the agar proportion method, the detection rate and specificity of resistance using MTBDR plus was 92.5% and 98.2% for rifampicin, and 76.3% and 100% for isoniazid.

Mutations in codons 531 and 533 of rpoB gene (62%S531L) were responsible for 67.9% of rifampicin resistance. S315T mutation of katG gene was detected in 55.9% and inhA promoter mutation at positions -15 (C15T) in 11.9% of isoniazid resistant isolates. Four phenotypically rifampicin-resistant and 14 isoniazid-resistant strains were not detected by MTBDRplus. Sequencing these strains revealed mutations in 4 strains; 2 in rpoB gene S531W, del518 and 2 in katG genesW300L, S315N. Hence, two phenotypic rifampicin-resistant and 13 phenotypic isoniazid-resistant strains were not detected by the commercial line probe assay.

Conclusion: The study showed that MTBDRplus had a high detection rate for rifampicin resistance. However, additional probes need to be included in the assay to improve the detection of isoniazid-resistant mycobacterium tuberculosis strains in Pakistan.

Keywords: Rifampicin, Isoniazid, Tuberculosis, MTBDRplus, Pakistan. (JPMA 62: 767; 2012)

Introduction

Tuberculosis is a major public health problem in Pakistan with an estimated prevalence of 355/100000 and a mortality rate of 33/100000.[1] Multi-drug resistant tuberculosis (MDR-TB), defined as mycobacterium tuberculosis (MTB) resistant to both isoniazid and rifampicin, is a worldwide problem with an estimated 14 million cases in 2009.[1] The rate of MDR-TB in Pakistan is reported to be between 1.8% of the new TB cases.[2]

Rapid molecular methods for the diagnosis of isoniazid and rifampicin resistance in MTB are based on the detection of genetic mutations. Mutation in the rpoB gene encoding β-subunit of RNA polymerase is estimated to be responsible for 95% of rifampicin resistance.[3] Mutations in katG, inhA, kasA, oxyR and ahpC result in isoniazid resistance with 60-90% of mutations attributed to codon 315 of katG, and in the promoter region of inhA.[4]

Commercial assays for rapid detection of isoniazid and rifampicin resistance rely on a selected number of common reported mutations. The World Health Organisation recommended the use of line-probe assays (LiPA) for smear-positive pulmonary specimens in a 2008 policy statement.[5] It also emphasised the need for their evaluation in different epidemiological settings. GenoType® MTBDRplus is one such commercially available assay which has been previously evaluated in Europe, South Africa, Far East and North America.[6-10] While sensitivity of rifampicin resistance detection by this method has been reported to be as high as 94-100%, that for isoniazid has remained low with a reported range of 57-95%.[4] Majority of this data has been reported from regions mentioned above and some from China, Thailand and Vietnam.[8-10] There is, however, no information on its performance in South Asia (Pakistan, Iran, India and Bangladesh). Thus, there is a need for evaluation of this important tool in these TB endemic regions.

In a study of 62 MDR isolates from Pakistan Ali et al. reported that 90% of RIF-resistant mutations were in the mutational hot spot region of rpoB gene, while 77% of INH-resistant mutations were in the hot spot regions of katG gene

with 1.6% in inhA promoter region.[3] Indian reports of MDR strains in Delhi showed that rpoB and katG mutations were variable, with S531L rpoB and S315T katG being predominant.[11]

Apart from rapid detection of MDR isolates, LiPAs play a significant role in the detection of hetero-resistance; whereby both wild type and mutant genes are detected within the same specimen. Hetero-resistance is a preliminary stage towards full resistance.[12] Its detection using conventional drug susceptibility method is labour-intensive. Hence, LiPA is definitely advantageous due to its rapid detection capacity.[12,13]

In the present study, sensitivity and specificity of LiPA (GenoType® MTBDRplus) in detecting RIF and INH resistant MTB strains from smear-positive pulmonary samples from Pakistan were compared with the conventional susceptibility method. Frequencies of mutations in rpoB, katG and inhA genes in MTB detected within the study isolates were analyzed and isolates showing hetero-resistance were identified.

Materials and Methods

The Clinical laboratories of Aga Khan University Hospital (AKUH) receive specimens from all major cities of Pakistan; and the average volume of samples for TB smear and cultures is around 200/week. The present cross-sectional study was conducted after due approval was obtained from the Ethics Review Committee at the Clinical Microbiology Laboratory of AKUH in May 2009, involving 111 pulmonary samples (sputa, tracheal aspirates and bronchoalveolar lavage fluid) submitted for Acid Fast Bacilli (AFB) smear and culture. The samples were randomly selected based on AFB smear positivity. Smear-positive decontaminated pulmonary samples were simultaneously tested for LiPA and cultured for phenotypic drug susceptibility. Phenotypic results were used as the gold standard. Of the total 111 samples, 108 results were included in the final analysis. Three specimens were excluded; one yielding a growth of non-TB mycobacteria, and two due to un-interpretable results.

The pulmonary samples were decontaminated with 1% NALC/NaOH and centrifuged. Auramine-rhodamine fluorescent AFB smear was read and the specimen inoculated onto Lowenstein-Jensen (LJ) slant and MGIT (Becton Dickinson Diagnostic Instruments Systems, Sparks, MD, USA) supplemented with oleic acid-albumin-dextrose-catalase (OADC) and PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin). Positive-signalling MGITs were removed daily and sub-cultured on 7H10 agar while LJ slants were observed weekly for mycobacterial growth. Mycobacterium tuberculosis was identified by susceptibility to PNB 0.5 mg/ml. Cultures positive for mycobacteria were tested for susceptibility to rifampicin (1.0 µg/ml) and isoniazid (0.2 and 1.0 µg/ml) by agar proportion method. Susceptibility results were read at 2 and 3 weeks. All isolates were saved at -80?C.

LiPA was performed using GenoType® MTBDRplus according to the manufacturer's instructions. Briefly, 0.5 ml of decontaminated AFB smear-positive sputum, TA or BAL specimen was centrifuged, heat inactivated, sonicated and re-centrifuged. Supernatant (100µl) was saved as the extracted DNA. Quality and quantity of DNA was assessed on NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific) before running multiplex-PCR on 5µl of DNA using primers and de-oxyribonucleotide precursors provided by Hain Lifescience (Nehren, Germany), as part of the kit. Amplicons were then hybridised to the DNA probe-labelled strip provided in Genotype® MTBDRplus assay. The assay included conjugate, amplification and gene locus control for M. tuberculosis-complex, rpoB, katG and inhA.

While interpretating the data, an isolate was considered sensitive if all wild type probes tested positive and there was no hybridisation with mutation detection probes. The absence of at least one wild type probe indicated resistance of the tested strain to the respective antibiotic. Hetero-resistance was defined when bands for both wild type and mutation probes were detected simultaneously in a specimen.[12]

Gene sequencing for katG and rpoB was also done. For the purpose, isolates giving discordant results between phenotypic susceptibility and LiPA were revived on 7H10 agar for re-testing by phenotypic method and nucleic acid sequencing. A loopful of bacteria was suspended in 300- µl of molecular grade water, heat inactivated, sonicated and centrifuged. Supernatant was used as DNA template and amplification was performed by using specific cycles[14] on a Perkin Elmer thermocycler. Primers (rpoB F: GACGACATCGACCACTTC, rpoB R: GGTCAGGTACACGATCTC, katG F: CCATGGCCGCGGCGGTCGACATT, katG R: GTCAGTGGCCAGCATCGTCGGGGA) were for 81 base-pair hyper-variable region of rpoB, and codon 315 of katG gene. Amplicons were purified using the QIA quick Qiagen PCR purification kit. The purified amplicons were shipped to Macrogen Incorporated, South Korea for DNA sequencing. Sequencing results were analysed upon comparison with MTB wild type strains by BLAST software from the NCBI web link (www.ncbi.nlm.nih.gov/BLAST). Amplicons of the three strains showing rare mutations; W300L of katG, 518 deletion and H526N of rpoB - were re-sequenced to confirm the presence of mutations.

Descriptive analysis was performed using SPSS version 17.0.

Results

A total of 111 AFB smear-positive decontaminated pulmonary samples were tested with GenoType® MTBDRplus. Three specimens were excluded; one yielding a growth of non-tuberculous mycobacteria, and two due to un-interpretable results. This left 108 results for the final analysis (107 sputa, 1 BAL). Of these, 56 were from females (female/male ratio was 1.08). Age ranged from 10 to 86 years (Mean 33.67 ±15.59 years).

While phenotypic proportion method identified 49.1% and 54.6% of the 108 strains as resistant to RIF and INH respectively (Table-1), using the LiPA, the corresponding resistance rates were 47.2% and 41.7%. MTBDRplus failed to detect 3.7% (n=3) of RIF and 23.7% (n=14) INH-resistant strains.

Five strains identified as sensitive to both RIF and INH by MTBDRplus assay failed to grow on culture and could not be verified by the phenotypic method. However, the MTBDRplus findings were supported by the positive response to first-line anti-tuberculous drugs in these patients.

The most common genetic mutation conferring RIF resistance was S531L of rpoB gene, detected in 62.3% of RIF-resistant strains (Table-2). The next most frequent rpoB mutation encountered was D516V which was detected in

5.7% RIF-resistant MTB strains, followed by H526Y (3.8%) and combined H526Y, S531L in 1.9% of the tested strains. Twenty-four percent (13/53) RIF-resistant strains had mutations that could not be identified specifically. Missing bands on the wild type region of these strains but no hybridisation with mutation probes suggested resistance due to mutations other than those included in this assay. One RIF-sensitive strain showed mutation H526Y.

Isoniazid (INH) resistance due to katG mutation S315T (AGC→ACC) was found in 55.9% of INH-resistant MTB; 11.8% had mutation -15 (C→T) of inhA promoter region (Table-2). One strain showed S315T (AGC→AAC) mutation of katG gene.

Four strains failed to show resistance pattern on MTBDRplus assay, but were detected resistant to rifampicin by phenotypic method. Twenty-four percent (14/59) of phenotypically INH-resistant strains did not show any reaction with the mutation probes.

Four samples showed hetero-resistance; two with katG mutations at S315T, one with rpoB mutation at D516V, and one with multiple mutations at H526Y and S531L of rpoB gene. All the four tested resistant by phenotypic drug susceptibility testing.

DNA sequencing analysis (Table-3) of the rpoB gene

Table-1: Comparison of LiPA and phenotypic susceptibility for INH and RIF resistance.

Resistance rates	LiPA n (%)	Phenotypic method n (%)`	Sensitivity* of LiPA (n)	Specificity* of LiPA (n)
RIF	51 (47.2)	53 (49.1)	92.5%	98.2%
INH	45 (41.75)	59 (54.6)	76.3%	100%
Hetero-resistance	4 (3.7)	-	-	-

Note: Overall agreement rate was 84.26% (91/108). (LiPA: Line probe assay, LiPA: Line Probe Assay. RIF: Rifampicin, INH: isoniazid.

Table-2: Frequencies of rpoB, katG and inhA mutations detected through LiPA.

LiPA probes	Mutation Site	n (%)	Specific Mutation detected	n (%)
rpoB				
WT 8	531, 533	36 (67.9)		
MUT 3			S531L	33 (62.3)
WT 3, 4	513-6, 516-8	6 (11.3)		
MUT 1			D516V	3 (5.7)
WT 2, 3	510-3, 513-6	4 (7.5)		
WT 7	526	3 (5.6)		
MUT 2A			H526Y	2 (3.8)
WT 1, 2, 8	505-9,510-3, 531,533	1 (1.9)		
katG				
WT	315	39 (66.1)		
MUT1			S315T (ACG→ACC)	33 (55.9)
MUT 2			S315T (ACG→AAC)	1 (1.7)
inhA regulatory sequence				
WT 1	-15,-16	7 (11.9)		
MUT 1			-15 (C→T)	6 (10.2)

Note: 12 out of 53 RIF-resistant strains showed loss of wild type but no known mutations.

Table-3: rpoB and katG gene mutations identified in hot spot regions of five MTB strains.

Strain Number	rpoB mutations
	Codon number 516
	517 518 519//...//525 526 527……...530 531 532//
	Amino acids
	D Q N N T H K L S A
83 (del518)	C T G G T C ▼ T T G // ... // T G G G T G T T C ...
	GACAGCCGC//.... D Q ▼ N T H K
	L S A
135 (S531W)	C T G G T C T T G T T G // ... // T G G G T G T T C ...
	GACACCCGC//... D Q N N T H K
	L W A
137 (H526N)	C T G G T C T T G T T G // ... // T G G T T G T T C ...
	GACAGCCGC//... D Q N N T N K
	L S A

Strain Number	katG mutations
Codon number	298 299 300 301//...//313 314 315 316//…
Amino acids	L G W K I T S G
139 (S315N)	AACCCGACCTTC//…//TAGTGGTTGCCG//…
	L G W K I T N G
143 (W300L)	AACCCGAACTTC//…//TAGTGGTCGCCG//…
	L G L K I T S G

NOTE: Nucleotides in bold denote mutations found in resistant clinical strains of MTB compared to MTB H37Rv. Nucleotides underlined denote mutations in phenotypically susceptible clinical MTB isolate compared to H37Rv. ▼ = Deletion

Table-4: Discrepant phenotypic and genotypic results based on rpoB and katG testing of strains.

Gene	Mutation	n	LiPA	Phenotypic susceptibility
rpoB	del518	1	Sensitive	Resistant
	H526N	1	Resistant	Sensitive
	S531W	1	Sensitive	Resistant
katG	S315N	1	Sensitive	Resistant
	W300L	1	Sensitive	Resistant

Note: Sequencing established that 2 RIF-resistant and 13 INH-resistant strains did not have mutations in the hot spot regions of rpoB and katG genes covered by LiPA.

illustrated that one RIF-resistant strain had a deletion at codon 518 and another at codon 531. The results were further compared with genotypic and phenotypic data obtained from the strains (Table-4) and it was found that certain resistance was not detected by LiPA. One phenotypic RIF-susceptible strain which revealed rpoB mutation H526Y on LiPA was confirmed on DNA sequencing. katG mutations, S315N and W300L were identified in one INH-resistant isolate each. Thus, in 13 INH-resistant and two RIF-resistant isolates in our study sample, no mutations in katG codon 315 and 81 bp hot spot region of rpoB were found on DNA sequencing.

Discussion

The sensitivity of line-probe assay for detection of rifampicin resistance varies from region to region. In our study, sensitivity was found to be 92.5%, that is, lower than that reported from Europe, Africa, USA and Southeast Asia[4,8,10,15] but comparable to data published from China.[9] This could be due to the prevalence of similar strains within this region as supported by the detection of common mutations among the MTB isolates from these countries.

Mutation in the rpoB gene is responsible for majority of RIF resistance in MTB.[3] Most of our isolates showed mutation at codon S531L of rpoB gene, consistent with previous reports from Pakistan,[3] other countries in the Eastern Mediterranean Region,[16,17] Fareast[9,10] and South Africa.[7]

Mutation rates in the rpoB gene D516V and H526Y were much lower in our study compared to rates of 44% and 15% respectively reported from Europe.[18] Higher mutation rates in rpoB codon 526 are also reported from India (19%), Pakistan (22.5%) and Iran (45.6%).[3,19,20] This difference may be due to smaller sample sizes and different methodologies used to detect mutation in these studies.

The present study identified 11 strains with multiple mutations in the Rifampicin Resistance Determining Region (RRDR). Multiple mutations in rpoB gene have been reported previously from other regions in Asia.[21] Such multiple mutations are associated with high-level resistance to RIF.[20]

S531W mutation of rpoB gene detected through sequencing of RIF-resistant isolate, but not by MTBDRplus, is a rare mutation which has been associated with MDR-TB outbreak.[22] This mutation lies within the hot spot region covered by probe WT 8 of MTBDRplus for rpoB codon 531 and should have been detected by the assay. The fact that it was not detected highlights the limitation of LiPA.

Another rpoB mutation not detected by LiPA was a deletion at rpoB 518. This region of the rpoB gene is situated between two probes (WT 4 and 5) and, thus, can be missed due to their overlap. Deletion at rpoB 518 is a rare mutation and has previously been reported as missed by another line probe test, the Inno-LiPA Rif.TB, (Innogenetics, Belgium).[23] Deletion at 518 has also been reported from other Asian countries.[24] One of our phenotypic RIF-susceptible strains showed an H526Y mutation in rpoB gene confirmed by DNA sequencing. Silent mutations within the RRDR of the rpoB gene have been reported,[21] though codon 526 mutations are very strongly associated with RIF resistance. However, Van Deun et al report mutations at codon 526 in Bangladeshi strains which tested susceptible on growth-based susceptibility method and were classified as probably resistant.[25]

The assay failed to detect 23.7% of isoniazid resistant strains in our samples, lowering the detection rate of MDR-TB, resistant to both RIF and INH, to just 71.7%. Isoniazid resistance detection rate of 76% in our sample is consistent with a sensitivity of 78.5% for the assay reported from China,

though not from South Africa.[7,9] A probable explanation for this could be a difference in the prevalent strains in different regions and their associated mutations. Conversely, high prevalence of Beijing strains in China, as opposed to Central Asian Strain-1 in Pakistan and India,[3,9,11] does not seem to affect the spectrum of rpoB and katG mutations in the region.

Earlier study on INH-resistant strains from Pakistan reported 63% mutation at codon 315 of katG mutation.[3] This is lower than the rate of this mutation detected in our study (66.1%). This difference can be attributed to a smaller sample size and a different methodology used in the previous study. Our rates, however, fell within the wide range of S315T mutation rates (54 to 92%) quoted in studies from China, Vietnam and Egypt.[10,17,26] Additionally, Ali et al detected inhA promoter region mutation in only 1/62 MDR isolates tested, while this study identified 11.9% of INH-resistant isolates with mutation at this locus. Wu et al from China have reported similar rates of inhA mutation at 17%.[26]

Among the phenotypic INH-resistant strains, one was found with W300L and another with S315N mutation upon katG sequencing. Though codon 300 of katG lies beyond the hot spot of mutations, W300L has been reported as a rare mutation in MTB strains from Myanmar.[15] Sequencing for katG mutations in our samples confirmed that 13/14 INH-resistant strains which were not detected by MTBDRplus did not harbour mutations at codon 315, the locus targeted by MTBDRplus. Resistance in such strains may be due to mutations in regions other than codon 315 of katG (W300L in one of our isolates) or positions -15, -16 and -8 in the inhA promoter region; or because of mutations in genes not represented on the test strip, such as ahpC, kasA, oxyR, and ndh, as suggested by Miotto et al and Anek-Vorapong et al.[8,18] Further studies are required to assess the presence and rates of mutations within these genes so that they may be included in rapid assays for the detection of INH resistance.

Conclusion

The line-probe assay, GenoType® MTBDRplus, for rapid detection of anti-mycobacterial resistance detected only 76.3% of INH-resistant MTB strains. The detection rate of RIF resistance was >90%. This indicates the limitation of LiPA for use in this region, and the need for additional probes to improve the sensitivity of the assay for local strains in Pakistan.

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