EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 8
59-65
Year: 2017

USE OF GENOTYPE MTBDR ASSAY FOR MOLECULAR DETECTION OF RIFAMPIN AND ISONIAZID RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*CLINICAL STRAINS ISOLATED IN SUDAN

Ahmed Musa Abdallah*, Mohammed Ahmed Mohammed and Najwa Juda Adam

Department of Microbiology, Faculty of Medical Laboratory Sciences, University of Khartoum, Sudan.

***Corresponding Author: Ahmed Musa Abdallah**
Department of Microbiology, Faculty of Medical Laboratory Sciences, University of Khartoum, Sudan.

Article Received on 10/06/2017 **Article Revised on 29/06/2017** **Article Accepted on 20/07/2017**

ABSTRACT
Introduction: The most effective first line drug against *Mycobacterium tuberculosis* are rifampicin and isoniazid, mutations in several gene determinant resistance *Mycobacterium tuberculosis* to isoniazid and most common gene target is katG and resistance rifampicin due to mutation in rpoB. The aim to assess to mutation in the gene region related to rifampicin and isoniazid. **Methodology:** A total of 200 smear positive samples were subjected to line probe assay using genotype MTB DR plus assay (HainLifescience Germany). **Result:** Out of 220 sputum samples, The smear positive were 200(90%) and smear negative were 20(10%), positive smears were subjected to MTBDR plus assay, Valid MTBDR plus assay results were obtained for 156 of 200 (78 %) . Of the 156, 80 (51.3%) tests were found to be resistant and 68(85%) of 80 were resistant to both RIF and INH, 76(95 %) of 80 were resistant to RIF only, 78(97.5%) of 80 was isoniazid resistant were resistant to INH only. **Conclusion:**The high percentage of mutations in the *rpo*B and *kat*G genes highlights their importance, and should be further investigated in order to gain a more comprehensive of the geographical distribution of resistant strains, as well as of the mechanisms underlying them.

KEYWORDS: Mycobacterium Tuberculosis, Rifampicin, Mutations, Isoniazid, line Probe Assay, Sudan.

INTRODUCTION

Tube [rculosis (TB), caused by *Mycobacterium tuberculosis*(*M. tuberculosis*), is an infectious disease with a high morbidity and mortality in humans.[1] In 2014, according to the World Health Organization (WHO) report estimated that 9 million people developed tuberculosis and 1.5 million people died as a consequence of the disease.[2]

Most of these cases were found in Africa (4 million new cases), and Sudan one of the African country with high prevalence of tuberculosis[2,3], the disease is responsible from 19.226 million new cases, equivalent to 151 cases per 100,000 inhabitants and nearly 1.5 million deaths annually.[2-4]

Effective TB control is affected by HIV infection and multidrug-resistant (MDR)-TB. WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) reported that the MDR-TBin new cases ranged from 0 to 22.3%.[5] The highest proportion of MDR-TB reported was 60% among previously treated cases. It has been estimated that 489, 139 cases of MDR-TB emerged in 2006, and the global proportion of resistance among all cases was equivalent to 4.8%.[5] However, MDR-TB has emerged as a new challenge, especially in developing countries. This is mainly due to lack of funding to support the treatment of MDR-TB with second line anti-TB drugs.[6]

MDR-TB is defined as the resistance to at least two main first line TB drugs: Rifampicin (RIF) andIsoniazid (INH).[7] INH is the most widely used first-line anti-tuberculosis drug.[8] Requiring activation through oxidation by a mycobacterial catalase-peroxidase enzyme encoded by katG gene.[9] Activated isoniazid interferes with the biosynthesis of essentialmycolic acids through inhibition of nicotinamide adenine dinucleotide hydrogen (NADH) dependent enoylacylcarrier protein reductase, which is encoded by inhA promoter. Mutations incodon 315 of *kat* Ggene and inhA promoter were strongly associated with isoniazid resistance.[10-13] *Kat*G accounts for 50-95% of INH-resistant clinical isolates. Mutations in *inh*A or its promoter region are usually associated with low-level resistance and are less frequent than *kat*G mutations.[14,15] RIF is also an important first-line drug for the treatment of TB. The most majority of RIF resistance is caused by mutations located in the 81-base pair (bp) region of the *rpo*B gene. Mutations at positions 531, 526 and 516 are among the

most frequent mutations in RIF-resistant strains.[16] The most commonly mutations associated with drug resistancescreened in the present study were describedby previous investigations.[14,17,18] In order to allow the detection of the majority of mutations causing RIF resistance and also a high proportion of mutationscausing INH resistance in *M. tuberculosis* isolates, a DNA strip assay was developed (Genotype MTBDR; Hain Lifescience, Nehren, Germany). It is based on a multiplex PCR incombination with reverse hybridization to identify either wildtypesequences or specific mutations. Therefore in this study we aims to detectthe mutation pattern in the various genomic regions of MDR-TB using MTBDR plus assay.

MATERIALS AND METHODS

Study population

A descriptive cross-sectional study was carried out during the period between January to march 2015 at the National public health laboratory, Khartoum, Sudan. A total of 200 pulmonary TB cases were subjected to treatment based on the laboratory diagnosis and/or clinical symptoms and X-ray findings were enrolled in the present study. From each patient sputum specimen was obtained for direct microscopy and line probe assay (LPA).

Sample processing

From each sputum specimen, smear was prepared, stained with Ziehl-Neelsen (ZN) stain and examined microscopically for acid fast bacilli (AFB). For every slide, more than 50 fields were examined. Then all Specimens were decontaminated by 5% sodium hydroxide (5% NaOH) method of digestion and decontamination.Only positive smears were subjected to LPA after 5% NaOH processing.

DNA isolation from clinical specimens

DNA directly from the clinical specimen was extracted by thermal lysis and sonication, and the Genotype MTBDR assay was performed in accordance with themanufacturer's instructions. In brief, for direct detection from clinical specimens, 500 µlof decontaminated sample were centrifuged at 10,000 xg for15 minutes room temperature; the pellet was then resuspended in 100 µlof sterile distilled water and then inactivated by incubating the bacteria in a heat block for 20 minutes at 95°C. Cells were sonicated in an ultrasonic bath for 15 minutes, and concentrated for an additional 5 minutes. The supernatants were transferred to a new tube to be stored for PCR.

DNA amplification

Amplification was performed by combining 35 µl of primer nucleotide mix (PNM) with 5 µl of 10× PCR buffer (containing 15 mM MgCl2), 2 µl MgCl2 (25 mM MgCl2), 3 µl molecular grade H2O, 0.2 µl (1 unit) HotStarTaq polymerase (QIAGEN, Hilden, Germany), and 5 µl of the bacterial suspension for a total final volume of 50.2 µl.

The amplification was performed in a GeneAmp system 2400 (Perkin-Elmer) thermal cycler with a protocol consisting of 1 cycle at 95°C for 15 min (*Taq*activation cycle), followed by 10 cycles of denaturation at 95°C for 30 s and primer annealing at 58°C for 2 min, 30 cycles of denaturation at 95°C for 25 s, primer annealing at 53°C for 40 s, and extension at 70°C for 40 s, followed finally by extension at 70°C for 8 min.The product was visualized by electrophoresis on 2% agarose gels (Hispanagar, Sphaero Q, Leiden, The Netherlands).

Hybridization

Hybridization was performed manually using a shaking water bath/Twincubator® pre-heated to 45°C. After denaturation, the single-stranded biotin-labeled amplicons are hybridized to membrane-bound probes at 45°C for 30 min on a shaking water bath. The strips are washed for 15 min at 45°C according to the manufacturer's instructions, and hybridization is detected by the addition of a streptavidin-alkaline phosphatase conjugate, followed by an alkaline phosphatase substrate reaction. Then the reaction was stopped by rinsing twice with distilled water. The test strips were allowed to dry, and then taped to the Geno Type MTBDR plus assay worksheet for interpretation according to the manufacture's recommendations. Strips were interpreted for susceptibility or resistance to RIF and INH according to the manufacturer's instructions. The run was considered valid if the negative controls (molecular biology grade water used in master-mix and DNA extraction areas) showed the presence of conjugate control (CC) and amplification control (AC) bands only. Each strip contains 17 probes, including five amplification and hybridization controls to verify the test procedures, five *rpoB*wild-type and four mutant probes, and one *katG*wild-type and two mutant probes (Figure 1 and 2).The presence of a mutation is indicated by the lack of hybridization on one or more of WT probes with or without hybridization on the MUT probes. The presence of rpoB gene locus predicts rifampicin resistance, while KatG predicts high level and inhA low level INH resistance. Absence of wild type and /or presence of mutant band signify resistance of a particular drug. There are also a universal control (UC) that verifies the presence of amplicons derived from gram-positive bacteria with a high percentage of G-C genomic content and a specific control (TUB) for the *M. tuberculosis* complex isolates.

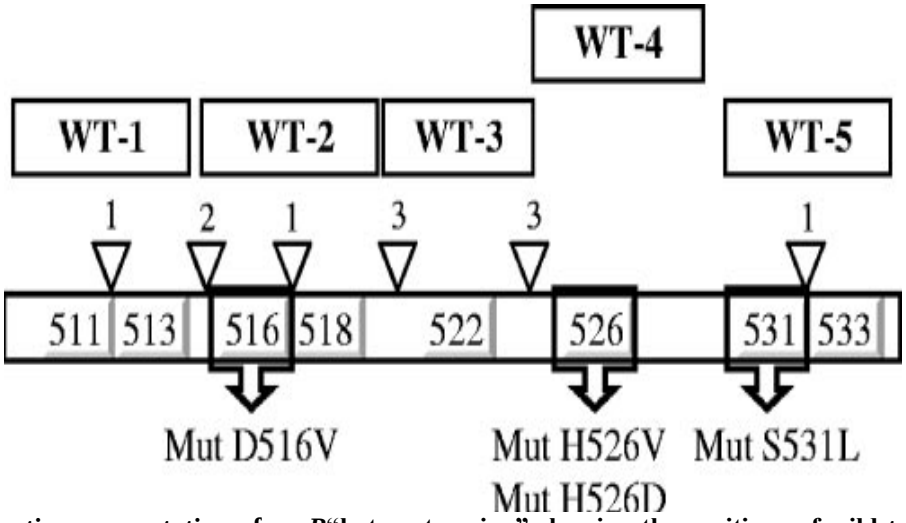

Figure. 1: Schematic representation of *rpoB*"hot-spot region" showing the positions of wild-type and mutated probes present in the strip. The average size of the probes is 20 bases. The arrowheads indicate the numbers and positions of omitted codons.

Ethical approval

The study protocol was approved by the institutional review board of faculty of medical laboratory sciences, university of Khartoum,and participants informed consent was done.

RESULTS

Out of 220 sputum samples, The smear positive were 200(90%) and negative were 20 (10%) positive smears were subjected to MTBDRplus assay, Valid MTBDR plus assay results were obtained for 156 of 200 (78%), Of the 156, 80(51.3%) tests were found to be resistant and 68(85%) of 80 were resistant to both RIF and INH, 76(95 %) of 80 were resistant to RIF only, 78(97.5%) of 80 was isoniazid resistant were resistant to INH only . The sensitivity and specificity of the test for detecting INH and/ or RIF-resistant strains from clinical specimensare reported in table 1.

Table. 1: Sensitivity and specificity of the Genotype MTBDR test

No. of positive strains/no. of strains tested (%)				
Type	**Sensitivity**		**Specificity**	
	Strains	**Specimens**	**Strains**	**specimens**
RIF monoresistant	76(95%)	80	76(100%)	76
INH monoresistant	78(97.5%)	80	76(100%)	76
MDR-TB (resistant to RIF and INH)	68(85%)	80	68(100%)	68

For RIF, codon 531 was the most frequently affected, being mutated in 43 of the 76 RIF-resistant strains (57%), followed by 17(22.4%) strains had mutations located in codons 530 to 533 (table -2). Analysis of INH resistance (table- 3) showed that the *kat G*codon 315 mutation was observed with a much higher frequency in INH-mono-resistant isolates(88.4%,69/78), and for *InhA*promoter it was C15T (22%17/78).

Table. 2: MTBDR test results for the detection of mutations conferring RIF resistance of 80 MDR *M. tuberculosis* strains isolated in Sudan

MTBDR result (location of mutation)	Affected codon(s)	Mutation detected	number of RIF isolates%(n=76)
rpoB			
mut1	516	D516V	4(5.3)
mut2A	526-529	H526Y	8(10.5)
mut2B	526-529	H526D	1(1.3)
mut3	531	S531L	43(57)
wt1	505-509		1(1.3)
wt3,4	513-519		3(4)
wt5,6	518-525		2(3)
wt7	526-529		2(3)
wt8	530-533		17(22.4)

Table. 3: MTBDR test results for the detection of mutations conferring INH resistance of 80 MDR*M. tuberculosis* strains isolated in Sudan

MTBDR result (location of mutation)	Affected codon(s)	mutation detected	number of INH isolates %(n=78)
katG			
mut1	315	S315T1	69(88.5)
Wt	315		3(3.8)

Concerning 68 isolates that were resistant to both RIF and INH s, mutation pattern of these isolates were found to be varied with all 16 different patterns of mutation, the most prominent was observed in rpoB MUT3, KATG MUT1 36(53%) (table4).

Table. 4: Pattern of multiple gene mutations observed in MDR TB isolates using Genotype MTBDRplus assay.

Pattern of gene mutations in MDR-TB STRAINS	Number of isolates%(n=68)
rpoB MUT3, katG MUT1	36(53)
rpoB wt8, katG MUT1	10(14.7)
rpoB mut3, katGmut2	1(1.6)
rpoB wt5,6 katG MUT1	1(1.6)
rpoB mut3, inhAWT1	3(4.4)
rpoB wt3, 4 inhA WT1	2(2.9)
rpoB wt3, 4 katG mut1	1(1.6)
rpoB mut2A, katGMUT1	6(8.8)
rpoB mut2B, mut 3, kat G mut 1	1(1.6)
rpoB WT7, inh Awt1, 2	1(1.6)
rpoB mut3, inh Awt1, 2	1(1.6)
rpoB wt8,inh Awt1	1(1.6)
rpoB wt1, rpo B MUT2a katG MUT 1	1(1.6)
rpoB mut3, kat Gmut1, inhAmut 1, 2	1(1.6)
rpoB mut3, kat Gmut1, inhAWT 1	1(1.6)
rpoBwt8, kat Gmut1. inhAwt1, 2	1(1.6)
Total	**68**

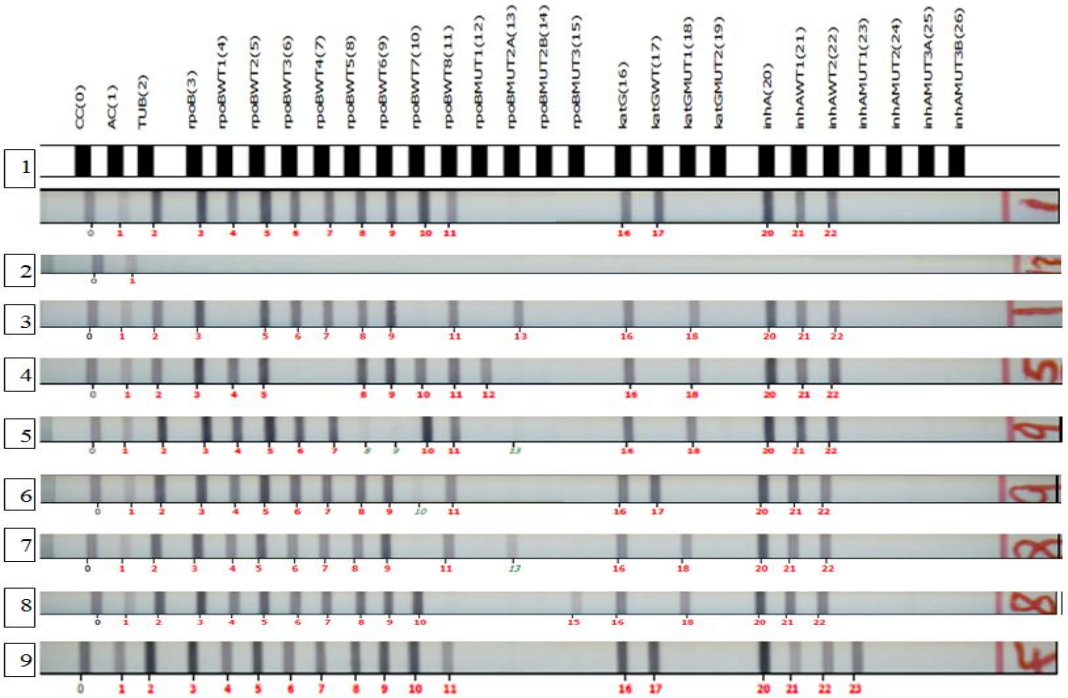

Figure. 2: Representative results for patterns obtained with the Genotype MTBDR assay.

Strep1: shows control of line probe assay, **Strep2**: negative control **Strpe3**: shows MDR-TB strain with mutation pattern in the rpoB gene locus (absence of WT1) with no corresponding MUT band +rpoB MUT2A (H526Y) codon and aKatG (S315T1 codon) mutation. **Strep4**: shows MDR-TB strain with mutation pattern in

the rpoB gene locus (absence of WT3 and WT4) with corresponding MUT band rpo BMUT1 (D516V) and aKat G (S315T1 codon) mutation. **Strep5**: shows MDR-TB strain with mutation pattern in the rpoB gene locus (absence of WT5 and WT6) with no corresponding MUT band and aKatG (S315T1 codon) mutation. **Strep6**: shows MDR-TB strain with mutation pattern in the rpoB gene locus (absence of WT7) with no corresponding MUT band. **Strep7**: shows MDR-TB strain with mutation pattern in the rpoB gene locus (absence of WT7) with corresponding MUT band rpoB MUT2B (H526D) codon and aKatG (S315T1 codon) mutation. **Strep8**: exhibits MDR-TB strain with common mutation pattern in the rpoB (S531Lcodon) and aKatG (S315T1 codon) mutation. **Strep9**: shows mutation in inhA MUT1.

DISCUSSION

RIF and INH are key drugs in tuberculosis treatment. RIF resistance is rarely observed as mono-resistance and for this reason it is often considered a marker for MDR isolates. Previous studies underline the relevance of molecular analysis indicating that isolates harboring mutations in codons 526 and 531 show both high-level resistance to RIF and cross-resistance between rifabutin and RIF.[19-21] INH is the most widely used first-line anti-tuberculosis drug.[8]

Is the drug of choice for treatment of latent TB infection in close contacts of infectious cases. For these reasons it is important for the clinician to know as soon as possible the susceptibility to these two key drugs. Several genotypic assays for resistance detection have been developed in the past few years.[22-27] Most of them requires equipment and human skills. The MTBD Rassay, a molecular method based on the reverse hybridization principle, became commercially available a few years ago. The Genotype MTBDR test allows rapid and specific detection of most mutations conferring resistance not only to RIF but also to INH in *M. tuberculosis* isolates. This test combines the probes targeting the 81-bp "hot-spot" region in the *rpoB* gene, with two probes targeting two different mutations at position 315 of the *katG*gene. Mutations affecting this codonare responsible for INH resistance in 60% of the cases worldwide.[28,29] The detection of resistance to both RIF and INH allows an early diagnosis of an MDR case. Previous reports[22,30-34] suggested that the frequency of particular mutations and the correlation of RIF resistance to the MDR phenotype could be different in selected populations. As reported in table 1, our results indicate that 95% (76 of 80) of the RIF-resistant strains investigated were correctly identified as RIF resistant by the test, and in 156 of the cases the mutation was identified by specific hybridization with a mutated probe. The MTBDR test was able to easily identify the presence of mutations in positions 530 -533 and 531 and could be used as a predictor of MDR in 68cases. As expected, the S531L mutation was the most frequently observed in Sudanese MDR strains and was demonstrated in 43 cases (57%), followed by the H526Y in 8 cases (10.5%). A high frequency of the S531L mutation has also been reported in studies performed in other countries.[35-38] A mutated codon 315 in *katG*was responsible for INH resistance in 97.5% (78of 80) of the resistant strains included in the study and all 78 strains were correctly detected by the LPA assay. Regarding the 80 MDR strains, the *rpoB*and *katG*probes selected in the test proved to be a sufficient tool to detect the relevant mutations conferring resistance to both RIF and INH of 68of 80 of the cases, with a sensitivity of 85%. If we consider RIF resistance alone an accurate marker for MDR, the sensitivity of the test in MDR detection increases to 95%, with a specificity of 100%.

Performing the test on selected clinical specimens may give critical information needed for appropriate treatment in a very short time and can be crucial for avoiding the transmission of drug-resistant *M. tuberculosis* strains. Indeed, the results are available in the same day. Due to the high prevalence of MDR cases in some areas in Africa and to the high numbers of immigrants from these regions, the MDR-TB should be screened in all new cases or retreatments in which MDR-TB is suspected and the results of the screening could allow appropriate management of the patients. of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Hungary.

CONCLUSION

The detection of mutations in resistance determining regions plays a crucial role for the rapid detection of anti-TB resistance and could aid strategies to further explore the mechanisms of resistance. The high percentage of mutations in the *rpo*B and *kat*G genes highlights their importance and should be further investigated in order to gain a more comprehensive of the geographical distribution of resistant strains, as well as of the mechanisms underlying them.

ACKNOWLEDGEMENT

We acknowledge the national public health laboratory for technical support.

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