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MOLECULAR DETECTION OF THE MUTATION IN RPOB GENE RESPONSIBLE TO RIFAMPICIN RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT : Pulmonary Tuberculosis (TB) is caused by the bacterium *Mycobacterium tuberculosis* which represents an important cause of mortality from all infectious diseases worldwide. Fast detection of MDR, XDR Mycobacterial strains is very essential to prevent the spread of tuberculosis. RT-PCR tests can identify the whole changes exist in the *81*-bp central sequence of the *rpoB* gene responsible for Rifampicin resistance. A total of 120 confirmed pulmonary tuberculosis sputum samples were collected from TB centers in Babylon-Hilla from the first of October 2017 until the end of June 2018. All clinical samples were diagnosed with Tuberculosis by traditional and up to date procedures (traditional zihel-Nelseen staining technique, molecular procedure technique, Gene-Xperte and cultivation of the bacteria). All the TB positive samples exposed to drug susceptibility testing (DST) by consuming the proportion method and GeneXpert. The presence of *rpoB* was confirmed by RT-PCR. Totally the 120 pulmonary tuberculosis positive clinical sputum samples were exposed to measurethe resistance to rifampicin via molecular diagnosis of GeneXpert MTB/RIF, Real-time PCR and drug susceptibility testing in a medium containing anti-tuberculosis drug. The ages of patients ranged from 19-70 years where pulmonary tuberculosis increase in the age of group (61-70) years old by percentage (35%) compared with other age groups. Two male in (31-40) age group resistance to rifampicin, whereas one female in (41-50) age group resistance to rifampicin. The results showed that (3) tuberculosis samples were resistant to Rifampcin by using Genexpert molecular method, (5) samples by using real time PCR by detection the mutation in *rpoB* gene and (3) samples by using medium containing anti-tuberculosis drug. These included 100(83.3%) new cases, 12(10%) cases under the treatment but not respond to treatment, (6%) patients were relapsed and (2%) patient was under treatment. Older persons more infection with tuberculosis than other groups. DNA extraction direct since sputum sample. Resistant isolates were confirmed for account of mutations in *rpoB* gene by Real Time PCR. The values of Ct for this examination were vacillated from (12-38.25) and the melting points of the genes were among (84-89.5°C). The current study advises that drug-resistant strains of *M. tuberculosis* can be identified by melting curve or Ct without Taq man and MGB in real-time PCR. SYBR Green was mediated RT-PCR procedure definite, is quick and simple to achieve and recognizing variance of vital genes responsible in the resistance to rifampicin and causes MDR.

Key words : *M. tuberculosis*, q-RT-PCR, -RIF resistance, *rpoB* gene.

INTRODUCTION

TB has remained an emergent international health issuance. WHO (2012) mentioned that there were 8.7 million TB cases in 2011. Also, 1.4 million dead because of tuberculosis (990,000 losses inpatient free from HIV and 430,000 inpatients with HIV) (WHO, 2014). Resistance of *Mycobacterium tuberculosis* to anti TB treatments is the main communal health difficult that threatens the development prepared in TB control worldwide. Drug resistance increases due to inadequate use of antibiotics in chemotherapy of drug subject organisms (WHO, 2013).

The mechanism of resistance to RIF comprises missense mutations, short deletions or insertions in the *rpoB* gene encoding the â-subunit of RNA polymerase. Studies from numerous countries have determined that 95–96% of all RIF-resistant isolates have mutations inside 81 bp 'core region' of *rpoB* (Cavusoglu *et al*, 2002). Also rifampicin resistance can be used as a surrogate marker for MDR (Zhang, 2005). Resistance to anti-TB drugs is due to spontaneous chromosomal mutations that happen at almost low frequency, 10^6 to 10^8 mycobacterial replications. This is a natural phenomenon and it could occur at any time during bacterial replication. Single nucleotide variations (point mutations) confer resistance to single drugs and totally mutations that happen in that section effect in RIF resistance (Soini and Musser, 2001).

Since of the treatment of MDR-TB is hard and its

diffusion is fast, the requisite for developed a fast analytical examination to discover MDR-TB and efficiently avoid the extent of MDR-TB in the public is principal to evade an epidemic. New molecular diagnostic techniques distinguish a possibly quick and sensitive different to conventional diagnostics. There has been an aggregate concentration in the advance of fast molecular techniques for finding of those mutations connected with drug resistance to substitute the conservative phenotypic drug-susceptibility testing (DST). RT-PCR analyze is quicker and can notice the whole changes that arise in the 81-bp*rpoB* gene (Boehme *et al*, 2010).

MATERIALS AND METHODS

Collection of sputum sample

A total of (120) sputum sample collected from pulmonary TB patients. The primary susceptibility to RIF was determined by molecular detection (GeneXpert), moreover determined via conventional absolute concentration technique on (L-J) medium holding the have rifampin conferring to (WHO) guideline (Zhang *et al,* 2014). The RIF concentration in the medium was 40mg/L.

Processing of the clinical sample

All sputum sample was disinfected through a 2–4 fold size of(4%) NaOH plus (60mg) N-acetyl-L-cysteine. The solution was incubated in 37C^{o} for 15 min, afterward that mixed through Ice vortex for 15-30 seconds. Sputum samples were centrifuged at 3000 rpm/15 min and the supernatant was discarded leaving the sediment in the tube (Forbes *et al*, 2007).

Genomic DNA extraction

A size of 1.0 ml of dissolved sputum was transference to a 1.5 ml tube and centrifuge at 6000 rpm for 15 min at room temperature. The supernatant was waste warily and the sediment was washed with 0.9% NaCl solution and re-centrifuged as earlier. The tube was washed over again and 50ul lysis solution was additional to the administered sediment in 1.5 ml (Eppendorf tube), mixed well and incubated at 37°C for 1 hr. The lysate was stored at 4–8 °C while waiting for DNA extraction was achieved by using (Genomic DNA Extraction Kit. Eurx -Poland).

GeneXpert

The sputum was collected from all patients and eventually mixed and shaken strongly for 15-20 time. The resultant sample was keep warm at 37^{o}C for 15 minutes. Later eliminating the MTB/RIF cartridge from the Xpert device (Cepheid, USA) the liquid sample was extracted by sterilized transfer pipette. The cartridge cover was exposed and 2mlof sample was removed in to exposed harbor of cartridge. Lastly the cartridge was positioned on the Xpert stratagem and results was well-known in 2 hours

Real Time PCR

The primer for rpoB gene was designed to discover the mutation responsible for rifampicin resistance in *M. tuberculosis*. The primer has the following sequence: Forward: 5'TCACACCGCAGACGTTGATC'3, Reveres:5' CGTAGTGCGACGGGTGC'3, tm(67.2), which used in this study. The PCR reaction mixture was DNA template 2mM, Nuclease-free water 10mM, 2x SYBR Green qPCR mix7mM and complete primer 1mM. Amplification was completed in a Master cycler Gradient (Eppendorf, Germany) doing the next program: initial denaturation at 95^{o}C for 1 minutes and 40 cycles of denaturation at 94^{o}C for 20 seconds, annealing at 60^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds, and the last extension at 75^{o}Cfor 2 minutes.

The major advantages of qPCR are that it can be performed in a very short time, does not require electrophoretic analysis and avoids contamination and q-PCR monitors the amount of amplicon as the reaction occurs. Commonly, the quantity of product is directly connected to the fluorescence of a reporter dye. For the reason that it discovers the amount of product as the reaction developments, qPCR provides a wide linear dynamic range, determines great sensitivity, and is very quantitative. The values of Ct from 18-39 were measured (+ve), suggesting that no changes were found on the gene fragment presence confirmed and considerliability. The values of Ct equivalent to (nil) or >(40) were measured (-ve), demonstrating no resistance exist to rifampicin (George and Ray, 2010).

RESULTS

A total of 120 *Mycobacterium tuberculosis* sputum samples were subjected to molecular detection (GeneXpert MTB\RIF). Out of these isolates, 113 isolates (94.1%) were positive by GeneXpert, whereas 7(5.8%) were negative to GeneXpert, but positive by using AFB & culture, 3 samples (2.5%) were resistant to RIF and 110 (91.6%) were susceptible to RIF this show as in Tables 1, 2. proportion analysis of RIF-resistant MTB isolates was achieved by real time PCRused for the discovery of the mutation in *rpoB gene* and the results showed that 5(4.1%) isolates were positive for the mutation in rpoB gene, whereas 115(95.8%) isolates were negative to rpoB gene mutation as showed in Table 2.

In the current study, real-time PCR and GeneXpert test were conducted on positive *rpoB* diagnosed mutant genes. The results showed that there are two positive

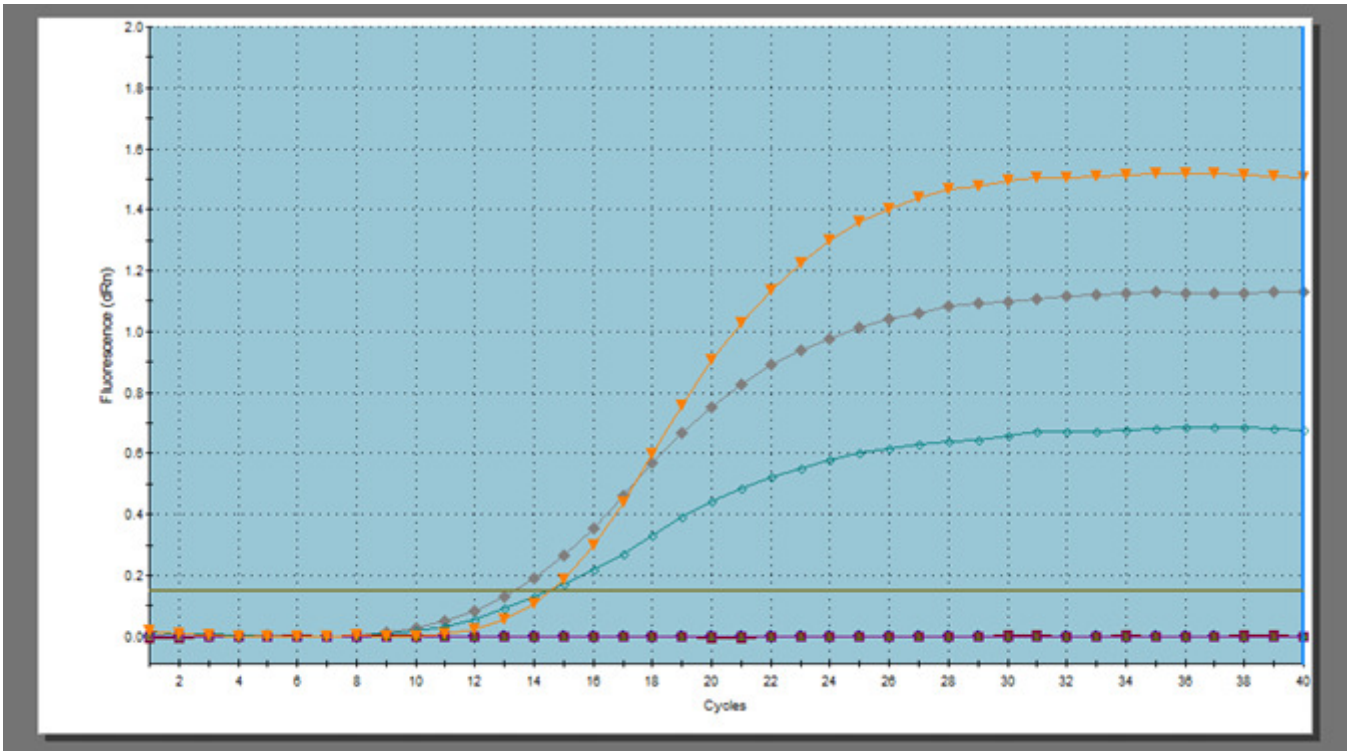

Fig. 1 : Real-Time PCR amplification plots for *rpoB* gene in *M. tuberculosis*, that display a positive and negative DNA samples by using SYBR Green 1 RT-PCR amplification.

Table 1 : Numeral and percentage distribution of studied sample according to GeneXpert assay.

Test	Positive			Negative		
GeneXpert	No	%	Total	No	%	Total
	113	94.1	120	7	5.8	120

Table 2 : Pattern of drug (rifampicin) susceptibility testing (DST) using different laboratory methods.

Method of testing drug(RIF) susceptibility	Resistance		Sensitive		Total positive
	No	%	No	%	
Solid media	3	2.5	117	97.5	120
GeneXpert	3	2.5	110	91.6	113
Real time PCR	5	4.1	115	95.8	120

rpoB mutant genes with real-time PCR assay in new case-patients while the GeneXpert test diagnose only one mutation of those genes. In case of relapse tuberculosis, Real-time PCR can detect the mutation in *rpoB* gene and could not detect *rpoB* mutation in the patient under treatment. The two techniques coincided with the diagnosis of two *rpoB* mutant genes in fail treatment patient, the result show as in Table 3. In the present study, the result showed that the patient with treatment failure has a mutation in *rpoB* gene detection by two techniques.

Total 120 patients were involved in this study, patients were grouped according to age and gender, the patients were categories into 5 groups of patients according to age,the age of patients ranged 19-70 years and the study was demonstration that the pulmonary tuberculosis increase in the age of group (61-70) years old by percentage (35%) compared with other age groups. Two male in (31-40) age group resistance to rifampicin, whereas one female in (41-50) age group resistance to rifampicin. This result as shown in the Table 4.

DISCUSSION

Since *M. tuberculosis* taking long time to grow on culture media, great dead occurringuntil the identification of TB is accomplished.Culture vulnerability test is used (obligatory period is Two-four weeks), additional fast methods which based on genomic examination for the uncovering of mutations connected with resistance to drug have been defined and the RT-PCR test requires as an another method to identify rifampin-resistant bacteria (Boehme *et al*, 2010). In our work, we calculated testified RT-PCR tests to evaluate the resistance of *M. tuberculosis* isolates to rifampin.

One of modern approaches is the GeneXpert MTB/RIF, which can discover mutations in the *rpoB* gene single; owing to nearby documentation of rifampicin resistance and MDR TB. This method has been used to identify MDR TB cases (WHO, 2015). GeneXpert is functioning to discovery of MTB complex and RIF resistance by a specifically sequence of *rpoB* gene, which is probed with five molecular beacons (Probes A-B) for mutation inside

Table 3 : Comparison between GeneXpert and real time PCR of rpoB gene mutation (Sensitive and Resistance) according to group of patients.

Category	Real-time PCR		GeneXpert	
	***rpoB* gene (Positive)**	***rpoB* gene (negative)**	**RIF (resistance)**	**RIF (sensitive)**
New N in gene x = 93N in RPCR=100	2	98	1	92
*RL (n=6)	1	5	0	6
*UT(n=2)	0	2	0	2
*Fail (n=12)	2	10	2	10
Total	5	115	3	110

*RL=Relapse, *UT=Under Treatment, *Fail=Treatment failure.

Table 4 : Anti-tuberculosis rifampicinresistance (MDR-TB) with gender and age of patients.

Age group	Total	Male	Female
		R	R
19-30	9	0	0
31-40	3	2	0
41-50	26	0	1
51-60	40	0	0
61-70	42	0	0
Total	120	2	1

the rifampicin resistance determining the region (RRDR).

Drug resistance happens owing to indecorous custom of drug in chemotherapy such as insufficient treatment regimes and failure to ensure that patients finished the complete course of treatment (Dorman, 2010). Drug resistance in MTBC is deliberated through 'persisters' (bacterial cells that phenotypically tolerate excessive levels of drug concentration, prolongs the normal lifetime of bacteria simple to treatments), or supposed confident chromosomal mutations and promoted each through conservational/extrinsic influence or bacterial elements. These elements can also be a effects of extended time in identification, insufficient or constant drug amount, patient non-adherence to treatment (NTPC, 2011).

Rifampicin resistance in about 95% of the cases in worldwide have mutations within the 81-bp Resistance Determining Region (RRDR) of the *rpoB* gene, this site of mutations that converse resistance to the rifampicin antibacterial agents, such as rifampin. This gene encodes the â subunit of bacterial RNA polymerase. It codes for 1342 amino acids, charitable it the second-largest polypeptide in the bacterial cell, 95% of RIF-resistant strains have mutations in codons 531. The machine of RIF resistance comprises missense base change, short deletions or insertions in *rpoB*, which give rifampicin resistant by shifting the RIF attaching site on RNA polymerase, thusdropping rifampicin binding affinity for rifampicin, the increasing percentage of RIf resistance explains that incomplete use of this anti- mycobacterial drug which imposes high selective pressure to select the resistant cells, in addition that the SSR might help in changing the cell phenotypes and pushing them toward a high virulence (Daniela *et al*, 2012).

CONCLUSION

The current study advises that drug-resistant strains of *M. tuberculosis* can be identified by melting curve or Ct without Taq man and MGB in real-time PCR. SYBR Green was mediated RT-PCR procedure definite, is quick and simple to achieve and recognizing variance of vital genes responsible in the resistance to rifampicin and causes MDR.

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