REVIEW ARTICLE

Detection of Single Nucleotide Polymorphisms (SNPs) for Genes Cause Drug-Resistant in Iraqi *Mycobacterium Tuberculosis isolates* by new Pyrophosphate Technique.

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ABSTRACT

In this search, a new pyrophosphate technique was proved. The technique was employed to single- nucleotide polymorphisms (SNPs), which diagnosis using a one-base extension reaction. Three *Mycobacterium tuberculosis* genes were chosen (*Rpob, InhA, KatG*) genes. Fifty-four specimens were used in this study fifty-three proved as drug-resistant specimens by The Iraqi Institute of Chest and Respiratory Diseases in Baghdad.; also one specimen was used as a negative control.

The steps of this technique were by used a specific primer within each aliquot that has a short 3-OH end of the base of the target gene that was hybridized to the single-stranded DNA template. Then, the Taq polymerase enzyme and one of either α-thio-dATP, dTTP, dGTP, or dCTP were supplemented and incubated for 1 min. ATP is synthesis by convert Pyrophosphate freed by DNA polymerase using pyruvate phosphate dikinase (PPDK), and the amount of ATP estimates by the firefly luciferase reaction. This technique, which does not demand expensive equipment, can be applied to rapidly monitor a one-point mutation in the gene that causes drug-resistant in *mycobacterium tuberculosis*. The results showed a high variation in values of ATP formation through matching and mismatch bases added. So, this assay (which required only five minutes), enable to find the gene SNP causes resistant for the specific drug.

Keyword: ATP, drugs genes, polymerase, pyrophosphate, SNPs, Taq

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INTRODUCTION

Mycobacterium tuberculosis constitutes a serious threat to the world; this threatened increase when drug-resistant bacteria occurred.[1]

The methods used to detect SNPs such as electrophoresis of single-strand DNA conformation polymorphism (SSCP),[2] cleavage fragment length Polymorphism (CFLP), which combined the restriction enzyme and SSCP),[3] the TaqMan PCR technique,[4] amplification refractory mutation system (ARMS) methods,[5] the invaders method,[6] and the DNA probe method[7] are used. but, there is a problem with these methods in that the procedure is complicated and generally demands electrophoresis apparatus, also special analytical apparatus.[8]

This study aims to install a new rapid method to detect SNPs that cause drug-resistant in Iraqi MT isolates and also to investigate the source of mutant isolates by using bioinformatics tools.

MATERIAL AND METHOD

Sampling

Through the study interval (April 2018- May 2019), with the aid of the Institute of Chest and Respiratory diseases in Baghdad, it was received 2945 patients with suspected pulmonary and extrapulmonary TB lesions 1820 (61.7%) males and 1125 (38.2%) females, with age range from (1year – 85 years). Fifty-four specimens, which symboled with (S) letter (from S1 to S54 except for S26) confirmed as drug-resistant were applied in this study, one specimen(S26) was used as a negative control.

DNA Extraction

Samples that proved as resistant were isolated and processed with DNA extraction using the sonicate bath extraction apparatus.[9]

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Optimization

Three genes *(rpoB, InhA,* and *katG S315T}* were optimized, the gradient annealing was done by a thermal cycler apparatus.[10]

Primer Design

Primers were designed for genes (*rpoB, inhA, katG) gyrA* gene had two SNPs at codon C94 and codon C95 as illustrated in Table 1.

MATERIAL AND METHOD

The procedure was included preparation three solutions M1, M2, and bioluminescent solution (PPDK-luciferase solution) as follow:

- Preparation of mixture1(M1):

1µ of specific primer (75 µM) hybridized to 1 µl of DNA template (1.50 pmol) in 8 µl of 10 mmol/L Tris-acetate buffer containing 2 mmol/L$(CH_3COO)_2Mg$.

The process was included denaturation at 94°C for 20 Sec., then Annealing at 65°C for 2 min.[15]

- Preparation of mixture 2 (M2):

Four µl of (Mix1) was added to another tube containing 2 µl of 100 mmol/L NEB buffer containing 5mmol/L $(CH_3COO)_2Mg$ and 1.6 µl of Klenow DNA pol. And 4 µl of one substrate of either (α- dATP- s, dTTP, dGTP, dCTP) mixed and incubated for 1 min.[14]

- Preparation of Pyruvate, phosphate dikinase (PPDK)-luciferase solution

The composition of solution was contained 2.3 U/ml PPDK, 0.2 mM luciferin, 5.5 U/ml luciferase, 0.0125mM Adenosine monophosphate(AMP), 0.04mM Phosphoenolpyruvic acid (PEP), 0.005U/ml apyrase, 0.05mM Dithiothreitol (DDT), 5% trehalose, 1mM Ethylenediaminetetraacetic acid (EDTA), 7.5 mM MgSo4, 30 mM Beryllium sulphide(Bes). The added (AMP) incorporated with the pyrophosphate (PPI) group to form ATP.[16]

Method

Bioluminescence technique Steps

- Hybridization reaction by the hybridized primer to single-stranded DNA gene of interest, the first base after hybridized was represented target base, for example, adenine base (A) as shown in Figure 1. This reaction included `denaturation at 94 °C for 20 s and then annealing at 65 °C for 2 min [(26)] by added M1 solution to PCR tube, thermal cycler was used for this purpose.
- Bases added reaction: one substrate of either dATPa-S, dTTP, dGTP, or dCTP.

  α-dATP-S was added to the M1 solution in present Taq polymerase enzyme as shown in Figure 2, the composition form M2 solution. α -dATP-S was used rather than dATP to diminish nonspecific luminescence.[11]
- Bioluminescent reaction: the reaction occurred by added MIX2 (extension assay solution) to 10 µL of bioluminescent solution (PPDK- luciferase solution) and 80 µl Luciferin substrate as shown in Figure 3.

Tracking strain isolates

Iraqi DNA isolates were tracked according to NCBI blast genes isolates, that was depended on DNA sequences done by Sanger method. Insilco bioinformatics analysis was used for this purpose.

RESULTS AND DISCUSSION

The new Bioluminescent Assay For Detection SNPs Cause Drug-Resistant

The results showed a very high variation between the amount of ATP between the matching base and mismatching one. These results agree with the[11] foundation.

Optimization of primer optimum temperature for hybridization

The optimum temperature of genes hybridization for (*rpoB, gyrA C94 gyrA C95, Inh A, and Kat G*) genes was 60°C62°C, 58°C, 60°C, 60° C respectively.

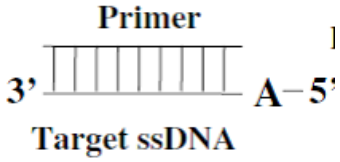

Figure 1: Hybridize primer with single-stranded DNA, the first base after hybridizing represented the target base.

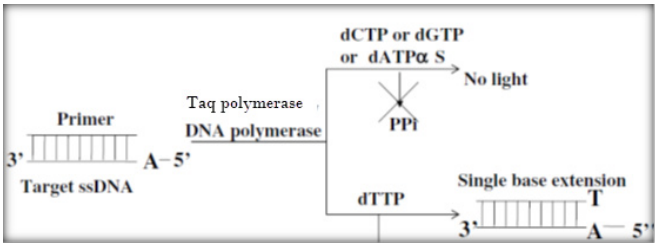

Figure 2: Taq polymerase bonded match base to the first nucleotide after hybridizing zone, PPi released in case of matching incorporation only.

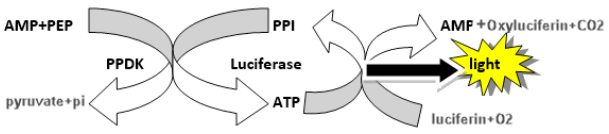

Figure 3: Pyrophosphate reaction using PPDK and the luciferase enzyme.[11]

Table 1: Primers design according to terminal mismatch nucleotide for four genes and five SNPs.

Name of gene	*Primer sequences*	*Company*	*Source*
rpoB gene	5-TGA CCC ACA AGC GCC GAC TGA-3'	Macrogen	(13)
inhA gene	5-CGG AAT CAT CAC CGA CTC GTC G-3	Macrogen	(14)
katG gene	5-CGG TAA GGAA CGC GAT CAC CAGT-3	Macrogen	(20)

Using Bioluminescent Assay to Diagnosis *Rbop* Gene SNPs

The results showed it was possible to clearly determine the wild type containing C and mutant type containing T at the identical position of the mutation site as shown in Table 2. The results of this assay were rapidly shown in the screen of Glomax illuminator after five minutes of insert microplate 96 wells in Glomax apparatus Figure 4. The output data represented a relative light unit (RLU), which indicated to Adenosine triphosphate compound (ATP).[10]

The higher ATP amount was with match base sample S16, the value was (198), while the less value was with mismatch sample S20. The variation between the higher amount and less amount is more than 100 units; this variation enables diagnosis easy to detect the wild base or the mutant one. The negative control (sample S26) shows normal wild type TCG codon; subsequently, the wild amino acid is serine, while mutant samples express leucine amino acid because of wild codon converted from TCG to TTG. The (RLU) value of blank control, which represented luciferase enzyme, only was read, indicating the clear ATP in this blank.

Using Bioluminescent Assay to Diagnosis Isoniazid Resistant SNPs

Two genes play significant roles in Isoniazid resistant *inhA* and *katG* genes.[17] 49.05% (26/53) of all resistant specimens were diagnosis with the bioluminescent assay. The results showed that 53.84%(14/26) had SNPs mutation at codon 21 within *inh* gene region, and 34.61%(9/26) had mutation with *katG* gene region, 15.38%(4/26) of specimens had no mutant within *inh A* or *katG* gens regions .only one sample 3.84%(1/26) exhibited both SNPs mutation within *inh A* or *katG* gens regions this

Figure 4: Glomax illuminator apparatus (10)

Table 2: Values of ATP amount for each matching and mismatch bases for the *rbop* gene demonstrated high variation in values. Amino acid was expressed in each case.

	ATP amount				
	Add G	*Add A*			
Samples No.	*C*	*T*	*Wild type →mutant type*	*Converted base*	*Amino acid*
S1	0.5	122			
S2	0.4	153			
S3	1.2	183			
S4	0.5	174			
S5	1.3	151			
S6	1.2	165			
S7	0.5	114	TCG →TTG	C to T	Leucine
S8	0.9	121			
S9	1.3	178			
S10	2.2	186			
S11	0.6	103			
S12	0.7	119			
S13	1.9	163			
S14	1.2	178			
S15	114	0.6	TCG	C	Serine
S16	2.1	198			
S17	2.3	126			
S18	1.2	147	TCG →TTG	C to T	Leucine
S19	1.9	210			
S20	0.4	163			
S21	1.8	179			
S22	0.7	106			
S25	119	1.6	TCG	C	Serine
S26	126	0.4			
S27	0.3	137	TCG →TTG	C to T	Leucine
S29	1.2	186			
S35	192	0.6			
S39	164	1.7	TCG	C	Serine
S51	187	0.9			
Blank	0.0	0.0	0.0	0.0	0.0

sample(S3) had XDR behavior.[18] *inhA* gene codon 21 ATG converted to GTC, so the amino acid alters as a result from Leucine to Valine, also within *katG* gene region of codon 315 AGC alter to ACC, then subsequently convert amino acid from serine to threonine.[12] As shown in Table 3. The amount of ATP formation with *inh A* gene demonstrated higher sensitivity than that with *rpob* gene that disagrees with Zheng R, *et al*.[19]

As shown in Table 4, the reverse result was obtained. In this manner, SNP analysis for the dGTP gene can be identified clearly and easily by comparison of the luminescence patterns obtained with the addition of dGTP and dCTP, the light released when matched nucleotide incorporate with target base Arakawa H, *et al*.[11]

Rpob Gene Phylogenetic Tree

Phylogenetic tree of *rpoB* gene showed the closing sequence to isolated Iraqi sequences was the stain LCP029, tracking the source of this strain showed that the source of this strain is Philippines country of Asia this foundation agrees with Lishi Q., *et al*.[21] who discovered that the source of *rpob* gene mutation which causes rifampicin-resistant is from Asia Figure 5.

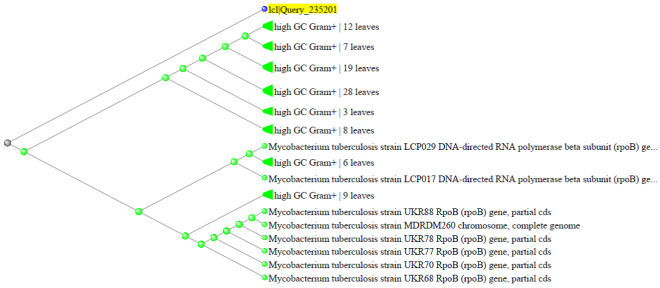

Figure 5: Phylogenetic tree for *rpoB* genes, the yellow strain is Iraqi local query DNA sequences.

Table 3: Values of ATP amount for each matching and mismatch bases for *inhA* gene and amino acid, which expressed in each case.

Samples no.	*Bioluminescent*		*Present nucleotide*	*Amino acid*
	Add T A	*Add C G*		
S1	0.4	210		
S2	1.6	190		
S3	2.3	280		
S5	0.7	189		
S6	1.8	230		
S7	0.2	190		
S9	0.9	214		
S11	1.2	186		
S12	1.7	210	A to G	Valine
S14	0.6	194		
S15	0.5	226		
S18	0.7	186		
S25	214	0.5		
S26	198	0.6	A	Leucine
S27	214	1.5		
S35	1.5	236	A to G	Valine
S39	1.8	229		
S51	244	0.9	A	Leucine

InhA Gene Phylogenetic Tree

Phylogenetic tree of *inhA* gene showed that the closer sequences genotype strain to local isolated colonies are the stain GG-36-11, tracking the source of this strain showed that the source of this strain is Guatemala, the study agrees with a[22] who observed that 85% of the M. tuberculosis strains with *inh A* gene mutant is coming from Mexico, Honduras, Guatemala, Peru. As shown in Figure 6.

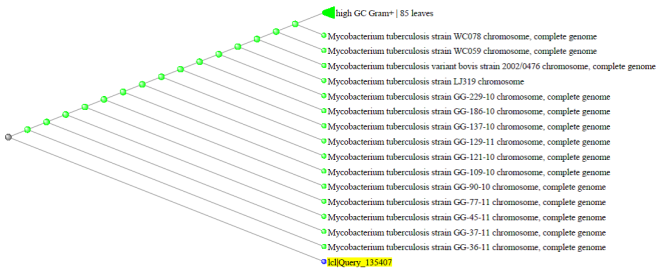

Figure 6: Phylogenetic tree for the *inhA* gene, the yellow strain is Iraqi local query DNA sequences.

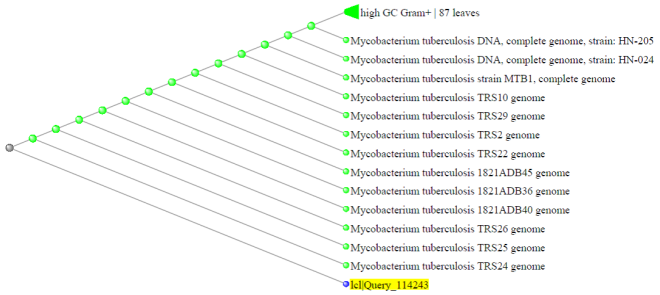

Figure 7: *Kat G* phylogenetic tree, the yellow strain is Iraqi local query DNA sequences

Table 4: Values of ATP amount for each matching and mismatched bases for *katG* gene and amino acid, which expressed in each case.

Samples no.	*Bioluminescent*		*Present nucleotide*	*Amino acid*
	Add C G	*Add G C*		
S3	0.4	112	T to G	Therionin
S4	0.6	145		
S7	117	1.3	T	Serine
S8	1.2	151	T to G	Therionin
S9	130	1.8	T	Serine
S10	1.7	176	T to G	Therionin
S11	189	1.5	T	Serine
S12	114	1.7		
S13	0.3	104	T to G	Therionin
S15	134	0.6	T	Serine
S16	2.1	116		
S17	0.4	107	T to G	Therionin
S19	0.2	101		
S23	1.3	167		
S25	158	0.5		
S26	189	0.6		
S27	116	0.4	T	Serine
S35	136	0.6		
S39	119	1.6		
S51	187	0.9		

KatG gene phylogenetic tree

Phylogenetic tree of *katG* gene found the closer strain sequence to Iraqi isolated was the stain TRS strain, according to NCBI mutant colonies belong to Vietnam that confirms the foundation of [23] who observed tuberculosis epidemic in Asias island, as shown in Figure 7.

CONCLUSION

The study proved strongly that pyrophosphate technique (bioluminescent reaction) can dependable on the detection of SNPs that cause drug-resistant in *Mycobacterium tuberculosis* genome, rapidly and without need to electrophoresis process as other techniques, in addition, other method demand time and expensive equipment, in contrast with a new method. The time factor which plays a crucial role in the treatment of tuberculosis patients, so a new method such as bioluminescent assay required urgently in such disease. The study also found most mutant isolates in Iraq are coming from the Asia continent, particularly from Asia's islands.

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