Using Bioluminescence Assay to Detect Snps Cause Drug Resistant of *Mycobacterium Tuberculosis* in Iraq

Hassan Kadhim Nemir[1], Ismail Aziz[1], Alaa Kareem Mohammed[2]

[1]Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Iraq,
[2]AL- Khwarizmi Engineering College/Biochemical Engineering department/University of Baghdad, Iraq

Abstract

In this search, a new bioluminescent technique was proved for pyrophosphate which was employed to single- nucleotide polymorphism (SNP) diagnosis using one-base extension reaction. Four *Mycobacterium tuberculosis* genes were chosen (*Rpob, InhA, KatG, GyrA*) 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 procedure of this assay was as follows. A specific primer within each aliquot owning a short 3-OH end of the base of the target gene was hybridized to the single-stranded DNA template. Then, (exo-) Klenow DNA polymerase and one of either α-thio-dATP, dTTP, dGTP, or dCTP were supplemented and incubated for 1 min. Pyrophosphate freed by DNA polymerase is altered to ATP by pyruvate phosphate dikinase (PPDK), and the amount of ATP is measured using the firefly luciferase reaction. This technique, which does not demand expensive equipment, can be applied to rapidly monitor 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: *SNPs, pyrophosphate, ATP, Bioluminescent, genes, drugs.*

Introduction

Mycobacterium tuberculosis constitute a serious threaten to world, this threaten increase when drug resistant of this bacteria occurred.(1).

Traditional method used to detect drug resistant such as Drug Susceptibility test (DST) culture method required long period, of at least two months, that waste time effect on patient treatment(2). Also other method used to detect SNPs such as electrophoresis of single-strand DNA conformation polymorphism (SSCP)(3), cleavage fragment length Polymorphism (CFLP), which combined the restriction enzyme and SSCP)(4), the TaqMan PCR technique(5), amplification refractory Mutation system (ARMS) method(6), the invaders method(7), and the DNA probe method(8) are used. but, there is a problem with these method in that the procedure is complicated and generally demands electrophoresis apparatus, also special analytical apparatus.(9-15)

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 extrapulmoary TB lesions 1820 (61.7%) males and 1125 (38.2%) females, with age range from (1 year – 85 years). Fifty-four specimens which symboled with (S) letter (from S1 to S54 except S26) confirmed as drug resistant were applied in this study, one specimen(S26) was used as negative control.

DNA Extraction: Samples which proved as resistant were isolated and processed with DNA extraction using sonicate bath extraction apparatus(13).

Optimization: Four genes and five SNPs *{rpoB, InhA,* (*gyrA C94, gyr A* C95) and *katG S315T*} were optimized, the gradient annealing was done by a thermal cycler apparatus(9).

Primer Design:

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

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	(14)
inhA gene	5-CGG AAT CAT CAC CGA CTC GTC G-3	Macrogen	(15)
katG gene	5-CGG TAA GGAA CGC GAT CAC CAGT-3	Macrogen	(22)
gyrA gene C94	5-ACG GCG ACG CGT CGA TCT ACC-3	Macrogen	(16)
gyrA gene C95	5-GCG ACG CGT CGA TCT ACG ACT-3	Macrogen	(16)

Material and Method

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

1. **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[17].

2 **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 klenwo DNA pol. And 4 µl of one substrate of either (α- dATP- s, dTTP, dGTP, dCTP) mixed and incubate for 1 min.[15]

3. **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 pyrophosphate (ppi) group to form ATP[18].

Method

Bioluminescence technique Steps:

1. Hybridization reaction: in this reaction primer hybridize to single stranded DNA of target gene, the first base after hybridized was represented target base, for example adenine base (A). 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.
2. Bases added reaction: one substrate of either dATPa-S, dTTP, dGTP, or dCTP.

   α-dATP-S was added to M1 solution in present (exo) Klenow enzyme, the composition form M2 solution. α -dATP-S was used rather than dATP to diminish nonspecific luminescence [10].
3. 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.

Results and Discussion

The new Bioleuminescent Assay for Detection Snps Cause Drug Resistant

The results showed very high variation between amount of ATP between the matching base and mismatching one. These results agree with[10] foundaton.

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

Using Biolumenescent Assay to Diagnosis *Rbop* Gene SNPs: The results showd 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. The output data represented a relative light unit (RLU), which indicated to Adenosine triphosphate compound (ATP)[9].

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 easly 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.

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

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

Rpob genes of DNA Specimense showd an opposite curve when added match and mismatch nucleotide at the same time of bases added as shown in Figure (1).

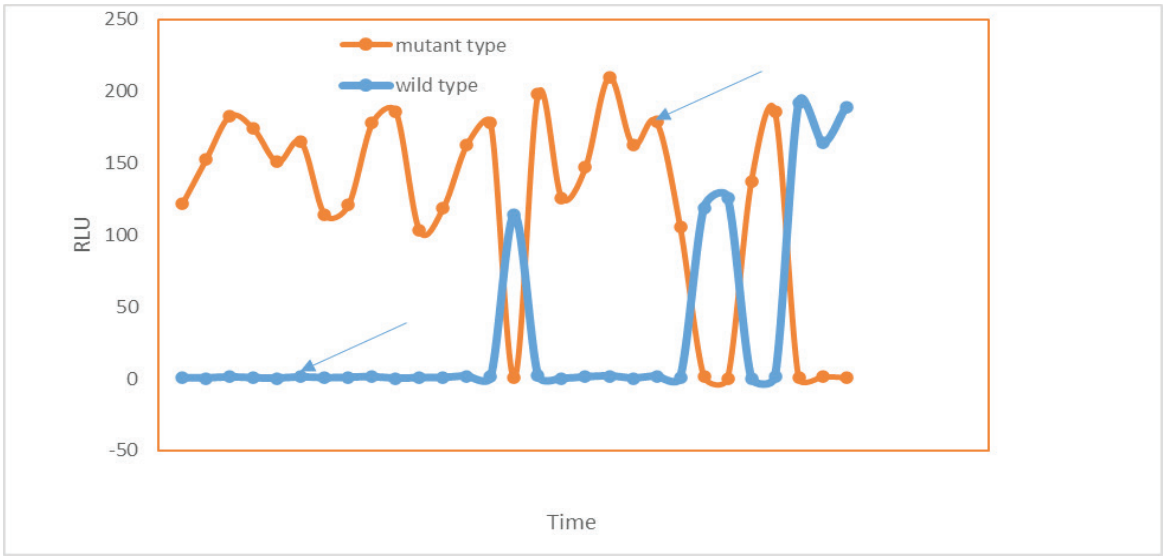

Fig. (1): The biolumenescent assay showed variation in relative light unit (RLU) through matching incorpoation and mismatching bases added at the same time of process.

Using Biolumenescent Assay to Diagnosis Isoniazid Resistant SNPs: Two genes play significant roles in Isoniazid resistant *inhA* and *katG* genes[19]. 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 sample(S3) had XDR behavior(20). *inhA* gene codon 21 ATG converted to GTC, so the amino acid alters as aresult from Leucine to Valine, also within *katG* gene region of codon 315 AGC alter to ACC, then subsequently convert amino acid from serine to therionin (11).As shown in Table (3). The amount of ATP formation with *inh A* gene demonstrated higher sensitivity than that with *rpob* gene that disagree with [21] .

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

Amino acid	Present nucleotide	Biolumencent		Samples no.
		Add C	Add T	
		G	A	
Valine	A to G	210	0.4	S1
		190	1.6	S2
		280	2.3	S3
		189	0.7	S5
		230	1.8	S6
		190	0.2	S7
		214	0.9	S9
		186	1.2	S11
		210	1.7	S12
		194	0.6	S14
		226	0.5	S15
		186	0.7	S18

Amino acid	Present nucleotide	Biolumencent		Samples no.
		Add C	Add T	
		G	A	
Leucine	A	0.5	214	S25
		0.6	198	S26
		1.5	214	S27
Valine	A to G	236	1.5	S35
		229	1.8	S39
Leucine	A	0.9	244	S51

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 [10].

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

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

Using Biolumenescent Assay to Diagnosis Fluoroquinolones Resistant SNPs: *gyrA* codon 94 wild type contained G while mutant type contained C at the identical position of the mutate site of *gyrA* codon 95 as shown in Table (5).

GyrA codon 94 wild type GAC express to Asparginin the mutant GGC produced Glycin, also the mutant with codon 95 convert wild codon AGC to mutant type ACC, subsequently Serin amino acid altered to Therionin[12]. 28.30% (15/53) of specimens established as resistant to

Fluoroquinolones drug by bioluminescent assay, 66.66%(10/15) of specimens had *gyr A* snps within codon 94, (15/15) of specimens had *gyr A* SNPs within codon 95, also 10 of specimens shared both SNPs within C94 and C95.

Table (5): Values of ATP amount for each matching and mismtach bases for *gyrA* codon 95 and codon 94 and amino acid which expressed in each case.

Amino acid expressed	Biolumenescent Amount of ATP as RLU		Wild and Mutant type C94	Amino acid expressed	Biolumenescent Amount of ATP as RLU		Wild and Mutant type C95	Samples
	Add C	Add T			Add G	Add C		
	G	A			C	G		
Glycin	108	1.6	A toG	therionin	190	0.6	G to C	S3
Asprginin	0.4	112	A		0.3	104		S7
	0.6	115		Serine	0.1	111	G	S9
	0.3	131			0.3	135		S11
	2.2	197			0.6	120		S12
	1.8	117			1.3	130		S13
	0.3	102			0.1	154		S14
	2.9	210			0,6	142		S15
Glycin	105	0.3	A toG	therionin	114	0.8	G to C	S25
Asprginin	0.5	116	A	Serine	0.6	142	G	S26
	1.7	103			1.2	177		S27
	1.3	118			132	0.7		S30
	0.5	104			167	0.5		S31
	1.2	113			143	1.8		S32
	0.9	141			123	1.6		S34
	0.4	162		therionin	162	1.7	G to C	S35
Glycin	183	0.8	A toG		176	0.6		S36
	162	1.4			142	0.5		S37
Asprginin	0.4	119	A	Serine	1.5	189	G	S39
Glycin	119	1.3	A toG	therionin	189	0.6	G to C	S40
	1.2	101	A		132	1.3		S41
	0.9	117			145	0.6		S44
	136	1.7	A toG		113	0.9		S47
	151	0.3			164	0.3		S49
	178	1.4			123	1.9		S50
	0.3	193	A		122	0.7		S51
	117	1.2	A toG		135	1.5		S52
	104	0.7			103	0.1		S53

Conclusion

The study proved strongly that bioluminescent assay can dependable in detection of SNPs cause drug resistant in *mycobacterium tuberculosis* genome, rapidly and without need to electrophoresis process as other technique, in addition other method demand time and expensive equipment in contrast with new method. The time factor plays a crucial role in treatment of tuberculosis patients, so new method such as bioluminescent assay required urgently in such disease.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: Self-funding

References

1. Nguyen L, Antibiotic resistance mechanisms in M. tuberculosis. HHS Public Access, 2017;90;1585–1604.
2. Pinto L, Menzies D.Treatment of drug-resistant tuberculosis. Respiratory. Dovepress, 2011; 4;129–135.
3. Rajatileka S, Luyt K, Williams M, Harding D, Odd D, Molnár E, Váradi A. Detection of three closely located single nucleotide polymorphisms in the EAAT2 promoter: comparison of single-strand conformational polymorphism (SSCP), pyrosequencing and Sanger sequencing. BMC Genetics. 2014; 15:80, 1471-2156/15/80
4. Kukita Y, Higasa K, Okazaki Y, Yoshinaga A, Hayashi K. Estimation of SNP Allele Frequencies by SSCP Analysis of Pooled DNA. Springer Science+Business Media. 2009 ;578, DOI 10.1007/978-1-60327-411-1_12.
5. Malkki M, Petersdorf E W. Genotyping of Single Nucleotide Polymorphisms by 5′ Nuclease Allelic Discrimination, NIH Public Access. Springer Science+Business Media. 2012; 882; doi:10.1007/978-1-61779-842-9_10.
6. Suhda S, Paramita D K., Fachiroh J. Tetra Primer ARMS PCR Optimization to Detect Single Nucleotide Polymorphisms of the CYP2E1 Gene. Asian Pacific Journal of Cancer Prevention. 2016; 17; 3065-3069.
7. Olivier M. The Invader® assay for SNP genotyping NIH Public Access. 2009; 3; 573(1-2): 103–110
8. Chen X, Zho D, Shen H,Chen H, Feng W, Xie G. A universal probe design for colorimetric detection of single nucleotide variation with visible readout and high specificity. Scientific Reports. 2016;6;20257, DOI: 10.1038/srep20257
9. Fakruddin M D, Chowdhury A, Hossain M N, Mannan K S, Mazumdar R M. Pyrosequencing-Principles and Applications. Council of Scientific and Industrial Research (BCSIR).2012;2, ISSN 2250-0480.
10. Arakawa H, Karasawa K, Munakata E, Obinata R, Maeda M, Suzuki S, Kamahori M, Kambara H. Development of bioluminescent pyrophosphate assay using pyruvate phosphate dikinase and its application to single-nucleotide polymorphism analysis. Analytical Biochemistry. 2008;379; 86–90.
11. Seifer M, Catanzaro D, Catanzaro A, Rodwel T C. Mutations Associated with Isoniazid Resistance in Mycobacterium tuberculosis. PLOS ONE. 2015; 10(3): e0119628
12. Aubry A, Veziris N, Cambau E, Truffot P C, Jarlier V, Mark F L. Novel Gyrase Mutations in Quinolone-Resistant and –Hypersusceptible Clinical Isolates of Mycobacterium tuberculosis. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. 2006;50; 104–112
13. Aldous W K, Pounder J I, Cloud J L, Woods G L. Comparison of Six Method of Extracting Mycobacterium tuberculosis DNA from Processed Sputum for Testing by Quantitative Real-Time PCR. JOURNAL OF CLINICAL MICROBIOLOGY. 2006;43; 2471–2473.
14. Desikan P, Kharate A, Panwalkar N, Khurana J, Beg- Mirza S, Chaturvedi A, Varathe R, Chourey M, Kumar P, Doshi N, Pandey M. Frequency of mutations in rifampicin and isoniazid resistant isolates of M. tuberculosis. GERMS. 2016; 6(4), 125.
15. Creixell P, Schoof E M, Tan C S H, Linding R. Mutational properties of amino acid residues implications for evolvability of phosphorylaTable residues. The Royal Society. 2012; 367; 2584–2593
16. Ostadhadi S, Rashidi M, Zolfaghari S, Maradeneh J, Nikoui V. Involvement of Mutation in Serine 83 of Quinolone Resistance-Determining Region of gyrA Gene in Resistance to Ciprofloxacin in Escherichia Coli. PHARMACOLOGY & THERAPEUTICS. 2014; 14; 16-21.
17. Riet J, Ramos LRV, Lewis RV, Marins LF. Improving the PCR protocol to amplify a repetitive DNA sequence. Genetics and Molecular Research. 2017; 16; (3), gmr16039796.
18. Yi Y J, Sutovsky M, Kennedy C, Sutovsky P. Identification of the Inorganic Pyrophosphate Metabolizing, ATP Substituting Pathway in

Mammalian Spermatozoa. PLoS ONE., 2011; 7(4): e34524. doi:10.1371.

19. Seifert M, Catanzaro D, Catanzaro A, Rodwel T C. Genetic Mutations Associated with Isoniazid Resistance in Mycobacterium tuberculosis. PLOS ONE. 2015; 10.1371.
20. Torres JN, Paul L V, Rodwell T C , Victor T C, Amallraja A. M, Elghraoui A, Goodmanson A P, Ramirez-Busby S M , Chawla A, Zadorozhny V, Streicher E M, Sirgel F A, Catanzaro D, Rodrigues C, Gler M T , Crudu V , Catanzaro A, Valafar F. Novel katG mutations causing isoniazid resistance in clinical M. tuberculosis isolates. Emerging Microbes and Infections. 2015; 4; e42, 2222-1751.
21. Zheng R , Zhu C, Guo Q, Qin L, Wang J, Lu J, Cui H, Cui Z , Ge B, Liu J, Hu Z. Pyrosequencing for rapid detection of Tuberculosis resistance in clinical isolates and Sputum samples from re-treatment Pulmonary Tuberculosis patients. BMC Infectious Diseases. 2014; 14; 1471-2334.