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REAL TIME PCR DETECTION MULTIDRUG-RESISTANCE *MYCOBACTERIUM TUBERCULOSIS* IN AL-SAMMAWA CITY

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Abstract

Multidrug-resistant tuberculosis (MDR-TB) is a global problem that many countries are challenged. Rapid and accurate detection of MDR-TB is critical for appropriate treatment and controlling of TB. The aims of study at using repaid real time PCR based assay to detection multidrug-resistant tuberculosis from culture samples, mutation in *rpoB* and *katG* genes *Mycobacterium tuberculosis* that responsible resistance to rifampicin and isoniazid, respectively. A total of 30 *M. tuberculosis* isolates from cases with diagnosed TB By GeneXpert, AFB and Culture on L.J media. DNA extraction from bacteria colonies. Resistant isolates were tested for characterization of mutations in the *rpoB*, *katG* genes by Real Time PCR. The test showed positive results for resistance genes (20%, 10%, respectively) as well as note that the values of Ct for this test ranged from (12-38.25) and the melting points of the genes were between (85-88.5^{o}C). Real time PCR results identified three mutations of MDR (rifampicin and isoniazide) resistance genes, whereas there was one MDR mutation of molecular diagnostic results with the GeneXpertMTB / RIF test for rifampicin. When comparing the results of the Real time PCR and GeneXpert tests at the level of the genetic mutation with rifampicin, the real time PCR test showed four resistance mutations for the *rpoB* gene for both new cases and relapse tuberculosis as well as one *rpoB* mutant for under treatment patient. Both molecular tests have agreed to identify one *rpoB* mutant in the case of failure TB treatment.

Key words : *Mycobacterium tuberculosis*, RT-PCR , (*rpoB, KatG* genes) and Multidrug resistanttuberculosis (MDR-TB).

Introduction

Tuberculosis (TB) especially multidrug resistance is a global problem due to the high-risk of human transmission, morbidity, mortality and most popular health problem worldwide (Ahmed, 2013). MDR is define the bacillus resistant to least two drug including rifampicin (RIF) and isoniazid (INH). *Mycobacterium tuberculosis* acquires resistance to drug through mutations in specific genes. In a population of *M. tuberculosis,* resistance to anti-TB drugs is due to spontaneous chromosomal mutations that happen at an 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 the sequential accumulation of these mutations in different genes included results within multi-drug resistance (Soini and Musser, 2001).

Resistance to rifampicin is mainly occur to missense mutation in the β subunit of DNA – dependent RNA polymerase encoded by specifically gene (rpoB gene). Each mutation in 81bp variable region in the rpoB gene results in failure of binding and subsequently (Piatek *et al*., 2000). Different study shown that more than 95% of mutations are found in 81bp core region of rpoB gene. Isoniazid resistance in *M. tuberculosis* is more complex and it involves more than one genes (katG, inhA1 and inhA2).

Mutation in katG have been reported to be associated with high level of isoniazid resistance (WHO, 2014). As per the WHO Global TB Report 2014, (9.6) million people died sick with TB in 2014, 1.5 million people died from this microbe (WHO, 2014). In 2015, there were a determined 480000 new cases of MDR-TB (WHO, 2016).

The development of drug resistance is the result of selection of random genetic mutations in genes associated with mutation confers resistance to a certain antibiotic several new laboratory techniques have been produced by world health organization (WHO) for faster detection of drug-resistant tuberculosis. Currently new molecular

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diagnostic methods sophisticated represent a potentially rapid and sensitive alternative to conventional diagnostics. There has been an increasing importance in the development of fast molecular techniques for detection of those mutations linked to drug resistance GeneXpert and real time PCR (WHO, 2013).

Materials and Methods

Collection of sputum sample and isolation *M. tuberculosis*

Sputum samples were obtained from 30 (n = 30) patients from Center of Thoracic diseases in Al-Smawwa city in Iraq. In 30 of these cases independent to smear microscopy for confirmation of acid fast bacilli (AFB), GeneXpert assay and were culture on Lowenstein-Jenson (LJ) media and incubate for 8 week at 37^0C. Biochemical identification of MTB colonies was done basis of niacin production and other biochemical test.

Extraction of Mycobacterium DNA

DNA was extracted from each bacterial colonies isolates were extraction by using a commercial DNA extraction kit (Eurex, Poland). DNA stored at -20^0C to analysis by real time PCR.

Real Time PCR

For detection mutation responsible to multidrug resistance, two primer use in this study to detection mutation in *Mycobacterium tuberculosis* genes, *rpoB* and *KatG* genes. The PCR reaction mixture was DNA template 2mM, Nuclease-free water 10mM, 2x SYBR Green qPCR mix7mM and total primer 1mM. Amplification was performed in a Mastercycler Gradient (Eppendorf, Germany) using the following program : initial denaturation at 95^0C for 1 minutes and 40 cycles of denaturationat 94^0C for 20 seconds, annealing at 60^0C for 30 seconds and extension at 72^0C for 30 seconds, and the final extension at 75^0C for 2 minutes.

Target gene	Primers	Nucleotide sequence	Tm
RpoB	*rpoB F*	TCACACCGCAGACGTTGATC	62°C
	rpoB R	CGTAGTGCGACGGGTGC	58°C
KatG	*katG F*	GGGCTTGGGCTGGAAGA-	56°C
	katG R	GGAAACTGTTGTCCCATTTCG	62°C

Results

Four hundred fifty (450) sputum specimens were collected from suspected TB patients in thoracic diseases hospital of Al-Sammawa city, Iraq during for the period from October, 2016 to August, 2017. Four hundred twenty four of them were negative results to AFB test and only twenty-six patients (5.8%) were positive after patients diagnosis with clinical manifestations, there was one specimen each from one patient. The culture test was showed thirty positive bacterial isolates (6.7%) while the molecular diagnosis with TB GeneXperttest was give twenty-eight (6.2%) positive. TB patient the results as show in table 1.

Correlation of AFB test with culture

Out of 30 positive culture specimens, there were 3:30(10%) positive cultures with scanty positive AFB smears, 21:30 numerous positive AFB smears were given positive results on culture test (70%), the remaining 6:30 (20%) positive cultures were false negative to AFB, but these isolates were able to grow on (LJ medium). This media represented different cultivation systems, both are rich to enhance the growth even of small number of bacilli, and it would be essential to remember that *M. tuberculosis* isolates are very slow grower and needs about 17 hr for doubling *in vitro*, correlation of AFB test with culture in this study give specificality of (99.5%) and sensitivity (80%) the result shown in table 2.

Relationship between age and pathological condition of patient

In the present study, the patients were categories into 6 groups of patients according to age started from 11-20 years to 61-70 years and the study is show the pulmonary tuberculosis increase in the age of group (21-30) years old by percentage (26.6%) compared with other age groups (26) new case infection with tuberculosis. This result as shown in the table 3.

Molecular Detection

Overall positive TB cases in this study were 30 positive cultured cases 26 (87%) patients were new cases, 1 (3.3%) patients with failure treatment infection, 2 (6.6%) patients were relapsed cases and 1(3.3%) patient was under treatment, a total of 30 positive culture specimens are tested by using sybr green real-time PCR Master Mix and the accumulation of sybr green PCR product was monitored by measuring the level of fluorescenceas described in methods and the final products of real-time PCR gave Ct rang (12-38.25) on sybr green signals shift that indicate truly positively of *Mycobacterium tuberculosis*, three MDR isolates with mutants with both rifampicin resistant isolates (*rpoB*) and isoniazid resistant isolates (*katG*), 6 patient have mutation in rpoB gene and patient have mutation in katG. Real-time PCR and geneXpert assay were conducted on positive *rpoB* diagnosed mutant genes. The results showed there two positive *rpoB* mutant genes with real-time PCR assay in new case-patients while the genXpert test did not diagnose

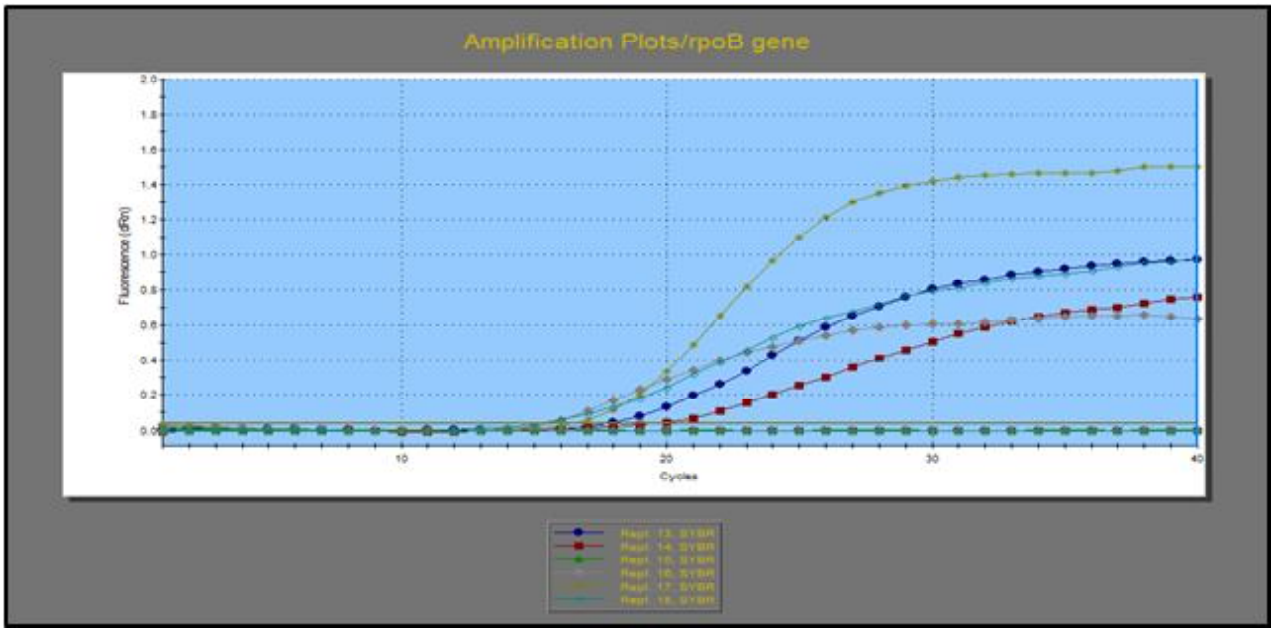

Fig. 1 : Two sybr green signals levels of master mix real-time PCR that indicates to mutant *M. tuberculosis* with the rboB gene from two examinations. (First curve crossed with 14.5 C_T value and the other curves with 15 C_T value).

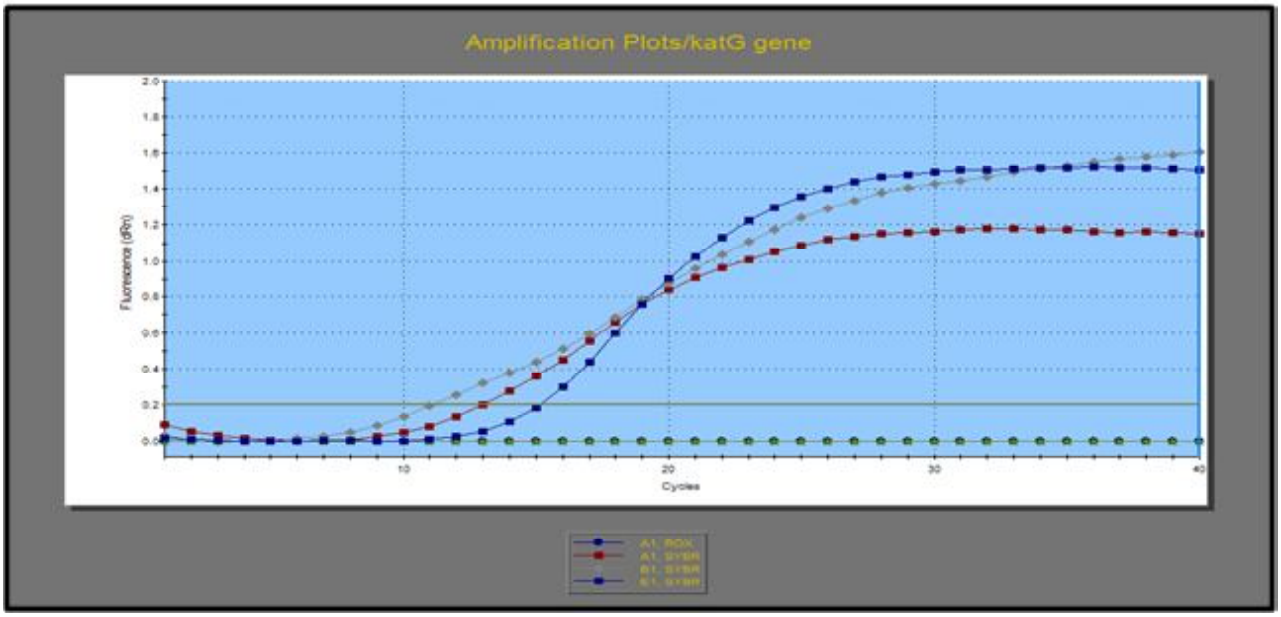

Fig. 2 : Three sybr green signals levels of master mix real time PCR that indicate to mutant *M. tuberculosis* with *katG* gene from two examinations. (First curve crossed with 17 CT value and the other curves with negative CT value).

those genes. in relapse TB infection, real-time diagnosed two positive *rpoB* mutant gene and other one *rpoB* mutant in the under-treatment patient. The two tests coincided with the diagnosis of one *rpoB* mutant genes in fail treatment patient (table 5). In the present study, the result showed the patient failed treatment has a mutation in *rpoB* gene detection by two assays.

Discussion

Tuberculosis is a global public health problem. In recent years, various approaches have been employed to develop effective diagnosis and vaccines for the management of TB and other important mycobacterium infection. Real time PCR have high sensitivities and specificities have been developed during the last few years. However, Acid fast smear (ZN) stain is of low sensitivity in diagnosis of TB than culture but it stills an essential approach to diagnosis of TB, only patients with high bacillary load, smear would be positive for sputum .Culture requires fewer bacilli (10-100/ml) for detection, but AFB requires 5,000 to 10,000 bacilli/ml allow to detection bacilli.

Table 1 : TB screening tests results.

AFB test results	No. of AFB (%) tests	Culture (LJ medium)		TB GeneXpert	
		+ve (%)	-ve (%)	+ve (%)	-ve (%)
(+ve) results	26 (5.8)	24(5.3)	2(0.4)	26(5.8)	0(0)
(-ve) results	424 (94.2)	6(1.3)	418(93)	2(0.4)	422(94)
Total	450	30(6.7)	420	28 (6.2)	422

Table 2 : Correlation of AFB smear test results with culture.

AFB smear test	Culture		Total
	+ve	-ve	
Scanty AFB	3	0	3
Numerous AFB	21	2	23
Negative	6	418	424
Total	30	220	450

Sensitivity = 80%
Specificity = 99.5%

Solid media (LJ) is most popular media in clinical laboratories and allows the determination of characteristic features of colonial morphology and pigment production, but it takes a very long time. A false negative on AFB defined by laboratory errors such as (serious microscope failure, grossly inadequate technique, poor staining reagents and incorrect reading or carelessness) (WHO, 1998). The results of these table 3 shows that young people are at high risk of T.B infection, the cause of this may due to the nature of the disease and the direct association with bacilli due to take on life expense older people may not withstand the severity of the disease and they may pass away as a tool of the disease. Accordingly the number of old people included in this study was fewer than young people (Bhatt *et al*., 2012).

Table 3: Relationship between age and pathological condition of patient.

Age	Total number No(%)	Positive new Case TB (%)	Positive old Case TB (%)	Total of positive Case (%)
≥ 20	23(5.1%)	3(11.5%)	0(0%)	3(10%)
21-30	96(21.3%)	7(26.9%)	1(25%)	8(26.6%)
31-40	33(7.3%)	2(7.6%)	0(0%)	2(6.6%)
41-50	54(12%)	4(15.3%)	1(25%)	5(16.6%)
51-60	106(23.5%)	5(19.2%)	0(0%)	5(16.6%)
61-70	138(30.6%)	5(19.2%)	2(50%)	7(23.3%)
Total	450	26	4	30

Table 4 : Number of cases diagnosed for Drug Sensitivity by RT-PCR.

Drug Response	No. of Cases
	RT-PCR Diagnosis
RIF-R / INH-R (MDR)	2
RIF-R / INH-S	4
RIF-S / INH-R	1
RIF-S / INH-S	23
Total	30

The reported that young people at higher risk for received tuberculosis. Due to the involvement of young people in increase the habit of smoking, alcohol, air contamination, dangerous jobs and loss of awareness Failure treatment infection occurs when the patient

Table 5 : Comparison between real time pcr (rpoBgene responsible RIF resistance) and GeneXpert method (RIF resistance and sensitive) accordance with category of patients.

Category	*rpoB* gene		*KatG*gene		Genexpert	
	+ve	-ve	+ve	-ve	Sensitive	Resistance
New case (n=26)	2	24	1	26	24	0
Fail treatment (n=1)	1	0	1	0	0	1
Relapse TB (n=2)	2	0	1	1	2	0
Under treatment (n=1)	1	0	0	0	1	0
Total	6	24	3	27	27	1

doesn't take their TB drug therapy properly, this bacteria can start to the development of drug-resistant, also actions of doctors in prescribing incorrect management, difficulties with the drugs being delivered (either when they are delivered or the quality) and the patients don't have an adequate intake of the drugs. Patients, who experience only a low improvement whilst on TB therapy or who never react to treatment, are said to have failed their TB treatment. Sometimes treatment failure is defined as the continued presence of positive sputum or culture (positive result to a culture test) or positive sputum, during the course of a patient's anti-tuberculosis drug treatment. Relapsed infection must be considered a very possibility that the person has drug-resistant TB. This may either be because of reactivation of the person's previously latent TB or because they have continued re-infected (Annabel, 2017).

Due the slow growth of bacteria in drug susceptibility test un acceptable delays in diagnosis of drug resistance tuberculosis, so in last few years use molecular detection to rifampicin isoniazid resistance because the real time PCR have several advantages. The major advantages of qPCR are that it can be performed in a very short time, does not require electrophoretic analysis, and avoids contamination Q-PCR monitors the amount of amplicon as the reaction occurs. Usually, the amount of product is directly related to the fluorescence of a reporter dye. Because it detects the amount of product as the reaction progresses, qPCR provides a wide linear dynamic range, demonstrates high sensitivity, and is very quantitative. The initial amount of template DNA is inversely proportional to a parameter measured for each reaction, the threshold cycle (Ct). While not requiring post-reaction processing (such as characterization by agarose gel electrophoresis), RT- PCR does require dedicated and expensive equipment. The method is expensive to set up initially, but it becomes rather cost-effective once used routinely particularly when employing SYBR Green as the fluorescent reporter. SYBR Green-based detection is the least expensive and easiest method available for qPCR. SYBR Green specifically binds double-stranded DNA by intercalating between base pairs, and fluoresces only when bound to DNA. Detection of the fluorescent signal occurs during the PCR cycle at the end of either the annealing or the extension step when the greatest amount of double-stranded DNA product is present. However, SYBR Green detects any double-stranded DNA non-specifically. Therefore, the reaction must contain a combination of primers and master mix that only generates a single gene-specific amplicon without producing any non-specific secondary products (Geolrge and Ray, 2010). The current study used SYBR Green I, as one of the most commonly used DNA binding dyes, binds to total amounts of DNA generated during PCR, so it can induce specific and non-specific amplification. The Ct is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The Ct is used to calculate the initial DNA copy number because the Ct value is inversely related to the starting amount of target. Melting curve analysis can only be performed with RT-PCR detection technologies in which the fluorophore remains associated with the amplicon. Amplifications that have used SYBRR Green I or SYBR GreenER™ dye can be subjected to melting curve analysis. The level of fluorescence of SYBR Green dye significantly increases upon binding to dsDNA. By monitoring the dsDNA as it melts, a decrease in fluorescence will be seen as soon as the DNA becomes single-stranded and the dye dissociates from the DNA. The specificality of a RT- PCR assay is determined by the primers and reaction conditions used. However, there is always the possibility that even well designed primers may form primer-dimers or amplify a nonspecific product melting curve analysis can identify the presence of primer-dimers because they exhibit a lower melting temperature than the amplicon (Bonab *et al*., 2015). MDR-TB is defined as resistance to isoniazid and rifampicin, with or without resistance to different first-line drugs (Laurenzoa and Mousa, 2011). Drug resistance is occur due to use of the wrong antibiotics in chemotherapy of drug-susceptible organisms ,therefore, the multidrug resistance of tuberculosis is a major public health problem that threatens the progress made in tuberculosis control and stops spread worldwide (WHO, 2013). Spontaneous chromosomal mutations that happen during *M .tuberculosis* replication, this phenomenon occur any time during bacterial replication Single nucleotide variations (point mutations) confer resistance to single drugs and the sequential accumulation of these mutations in different genes included results within multi-drug resistance (Soini and Musser, 2001). Therefore, isolates resistant to INH have a comparative disadvantage, as patient harboring such a strain will, on average be transmitters for longer period of time than patients with fully susceptible isolates. Thus, one would expect an increase in the prevalence of primary resistance to INH (Jagielski *et al.*, 2013). At the present time, it is well known that there is an incredible increasing in multidrug resistant *M. tuberculosis* (MDR-TB). Tubercle bacilli considered as MDR- TB if they are resistant to at least two drugs among the 6 drugs used in most treatments, and especially INH and R since these drugs considered the most important chemotherapy for TB . Therefore,

resistance to RIF can be used as a marker for MDR-TB, because it is usually preceded by that to INH, isolated resistance to RIF being extremely rare (Diacon *et al*., 2012).

Most strains of bacteria in this study have a mutation to isoniazid and rifampicin back to patients be the clinical diagnosis of their flair or relapse treatment, MDR TB cases threaten the effectiveness of chemotherapy for both treatment and control of TB and require the use of second-line drugs that are more expensive, toxic, and few effective than first-line anti-TB drugs (Prammananan *et al*., 2005). Drug resistance occurs due to improper use of antibiotics in chemotherapy such as inadequate treatment regimens, and failure to ensure that patients finished the whole course of treatment (Dorman, 2010). Drug resistance in MTBC is conferred by 'persisters' (bacterial cells that phenotypically tolerate high levels of drug concentration, prolongs the average lifetime of bacteria exposed to drugs), or throught specific chromosomal mutations and promoted either through environmental/extrinsic effect or bacterial factors. These factors can either be a results of delay in diagnosis, inadequate or interru Rapid identification of drug resistance particularly MDR-TB is most important to help decrease the spread of disease. Xpert MTB/RIFis a simple fast method assay has been widely used in routines identification of MTB and mutation resistance to rifampicin. The fast detection of *M. tuberculosis* resistance continues (Zeka *et al*., 2011). In present study, the use of RT-PCR to determine *M. tuberculosis* resistance to RIF was also very promising and its application in clinical samples will be explored in future studies an assay is a powerful tool because it is simple to perform and readily automatable.

Corruppted drug supply, patient non-adherence to treatment (NTCP, 2011). Rapid identification of drug resistance particularly MDR-TB is most important to help decrease the spread of disease. Xpert MTB/RIF is a simple fast method assay has been widely used in routines identification of MTB and mutation resistance to rifampicin. The fast detection of *M. tuberculosis* resistance continues (Zeka *et al*., 2011). In present study, the use of RT-PCR to determine *M. tuberculosis* resistance to RIF was also very promising and its application in clinical samples will be explored in future studies an assay is a powerful tool because it is simple to perform and readily automatable.

The polymerase chain reaction (PCR) sequencing-based strategy, designed to recognize mutations associated with drug resistance rapidly, is able to provide a "same-day" diagnosis from culture and even clinical samples with high sensitivity and specificality. This gold standard test can also discover new mutations that could be associated with drug resistance (Choi *et al*., 2010).

Conclusion

1. The present study suggests that drug-resistant strains of *M. tuberculosis* can be detected by melting curve or Ct without TaqMan probes and MGB in real-time polymerase chain reaction (PCR).

2. The optimized SyBR green mediated Real time PCR procedure described is rapid and simple to perform and could assist in identifying and differentiation of importance genes responsible to resistance of drug and causes multi drug resistance.

3. The rate of occurrences of mutation are widely found in Rifampicin Resistance Determination Region (81bp) of rpoB gene was highest to other mutations in katG, inhA1 and inhA2 genes.

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