Molecular detection of multidrug-resistant *mycobacteria tuberculosis* in Al-Muthanna province

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Abstract

Multidrug-resistant tuberculosis (MDR-TB) is a global problem that many countries are challenged with. Rapid and accurate detection of MDR-TB is critical for appropriate treatment and controlling of TB. the aims of study at using Polymerase Chain Reaction for detection of multidrug-resistant Tuberculosis from cultured samples A total of 30 M. tuberculosis isolates from cases with diagnosed TB by GeneXpert, AFB and Culture on L. J media after incubation period from 3-8 weeks, DNA extraction from bacteria colonies. Resistant isolates were tested for characterization of mutations in the rpoB, KatG InhA1 and IhA2 genes by Real Time PCR. The results of the real time PCR showed that mutations of genes (rpoB, katG, inhA1 and inhA2) that were responsible for resistance to rifampicin and isoniazid. The test showed positive results for resistance genes (20%, 10%, 6.6%, 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 Co). Real time PCR results identified three mutations of MDR (rifampicin and isoniazid) resistance genes, whereas there was one MDR mutation of molecular diagnostic results with the GeneXpert MTB/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.

Keywords: Multidrug-resistant tuberculosis (MDR-TB); GeneXpertMTB/RIF; rpoB

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Introduction

Tuberculosis infection is a global problem due to the high-risk of human transmission, morbidity, mortality and most popular health problem in Iraq. Each year around the world about 2 million deaths and more than 9 million cases are reported infection with tuberculosis, making

Mycobacterium tuberculosis the first cause of death from a unique pathogen [1]. Tuberculosis has been present in humans ago antiquity at the latest. It is one of the oldest recognized infectious diseases that has plagued mankind since the extension of the modern human out of Africa [2], Drug resistant tuberculosis (TB) is a global threat and a major public health problem in several countries. World Health Organization (WHO) estimate that 480 000 new cases of multidrug resistant (MDR)-TB and among them cases [3]. Drug resistance in MTBC is classified into three groups based on the way the resistance emerges: primary resistance, acquired resistance and mixed resistance. Primary resistance in particular poses the biggest challenge to control because less than 20% of the estimated drug resistant cases in the world are believed to be properly diagnosed, largely due to the lack of appropriate laboratory infrastructure in low-income endemic areas [4]. This study was aimed at using Polymerase Chain Reaction for detection of multidrug-resistant Tuberculosis from cultured samples in Al-Muthanna province using real time PCR test.

Materials and methods

Setting

All clinical samples were drawn from suspected TB patients submitted to the Center of Thoracic diseases in Al-Muthanna province, a detailed clinical history, sex, location and age were collected from the requisition form that accompanied the samples. Four hundred and fifty patients (sputum samples) were collected from suspected. Infected TB patients age around (11-70) years older during the period extended from 2016 /10 /9 to 2017 / 9/ 8.

DNA extraction

DNA was extracted from the bacterial colonies, A commercial DNA extraction kit (Eurex, Poland) was used for extraction of DNA from clinical specimens.

Molecular detection

The molecular Detection were conducted by using two methods (GeneXpert system MTB/RIF and real time PCR) *According* to Manufacturer's Instructions. Four primer use in this study to detection mutation in Mycobacterium tuberculosis genes, *rpoB gene* were F: 5'-TCACACCGCAGACGTTGATC-3', R: 5'-CGTAGTGCGACGGGTGC-3.' the primer *KatG gene* were F: 5'- GGGCTTGGGCTGGAAGA-3', R: 5'- GGAAACTGTTGTCCCATTTCG-3',

The primer *InhA1gene* were F:5'-TGGTCAGCTTCCTGGCTTCC-3', R: 5'-GACCGTCATCCAGTTGTAG -3', the primer *InhA2 gene* were F: 5'-TGGTGCATTCGATTGGGTTC-3', R: 5'- GGTAACGTTCTCCAGGAAC-3', 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_C° for 1 minutes and 40 cycles of denaturation at 94C° for 20 seconds, annealing at 60_C° for 30 seconds, and extension at 72_C° for 30 seconds, and the final extension at 75_C° for 2 minutes.

Results and discussion

Four hundred twenty-four of them were negative results to AFB test and only twenty-six patients (5.8%) were positive after diagnosis with clinical manifestations. There was [1] specimen each from one patient. The culture test shown thirty positive bacterial isolates (6.7%) while the molecular diagnosis with TB GeneXpert test given twenty-eight (6.2%) positive TB patient results as shown in table (1).

Table 1.

TB screening tests results

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

In total, 450 patients' samples were collected; 28 patients had confirmed TB by use GeneXpert and culture tests. The other two patients who were positive for culture test showed negative results for GeneXpert test. Rifampicin resistance was correctly identified in one (MDR) resistant isolate and 27 patients to sensitive rifampicin. During the past few years, molecular methods have been developed to identify drug resistance causing gene mutations [5]. One of latest techniques is the GeneXpert MTB/RIF, which can detect mutations in the *rpoB*gene only; due to close association of rifampicin resistance and MDR TB, this technique has been used to detect MDR TB cases [6]. Xpert MTB/RIF assay is a new rapid molecular test that disposal many of the current operational difficulties in TB detection, WHO endorsed Xpert MTB/RIF assay for use in TB prevalent resource limited countries [7]. GeneXpert using to detection of MTB complex and RIF resistance by specifically sequence of rpoB gene, which is probed with five molecular beacons (Probes

A-B) for mutation within the rifampicin resistance determining region (RRDR). Xpert assay recommend as subsequent test in patient acid fast negative sputum smear [8]. Resistance estimated was dependent on the particular mutation and required between 65% and 100% mutant DNA to be present in the sample for 95% certainty of resistance detection [9]. Older study [10]. show specificity of genexpert 100% and sensitivity 82.3% with pulmonary specimens, another study shows more sensitivity and specificity values for TB genexpert [11]. the Xpert MTB/RIF assay is a useful tool for the detection of MTBC with high sensitivity in sputum specimens compared with conventional AFB smear microscope [12]. Detection of simultaneous MTBC and RIF resistance are a key advantage of Xpert MTB/RIF over smear microscopy. Rapid detection of RIF resistance is considered crucial for the control of MDR-TB [7]. the false positive result of GeneXpert is expected to be related to the detection by expert of dead *M. tuberculosis* that would not be detection by acid fast smear and culture, so this test has high specificity (99%) [3]. a total of 30 positive culture specimens were 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 fluorescence as 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 tunerculosis*, figures (1.2, 3 and 4) examples of positive PCR results in present of rpoB, katG, inhA1 and inhA2 genes mutants.

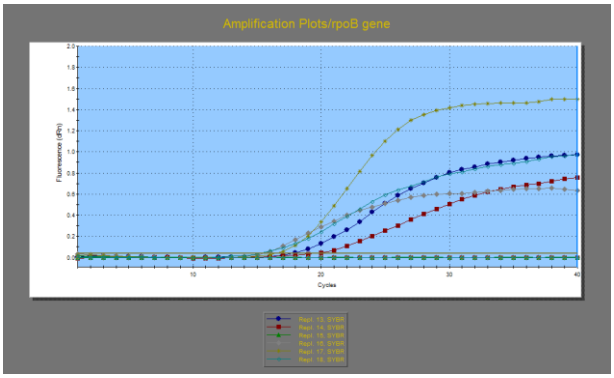

Figure 1.

Two sybr green signals levels of master mix real time PCR that indicate to mutant *M. tuberculosis* with rboB gene from two examinations. (First curve crossed with 14.5 C_t value and the other curves with 15 C_t value

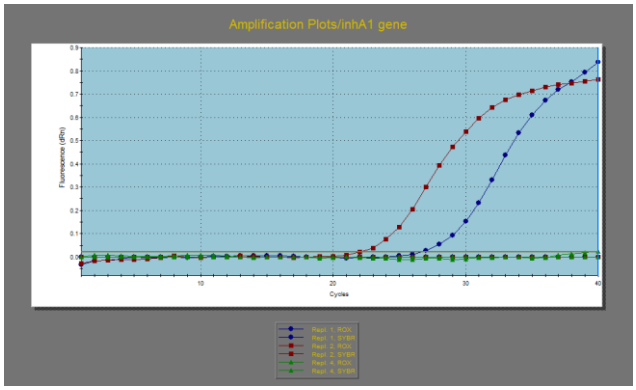

Figure 2.

Two sybr green signals levels of master mix real ti
PCR that indicate to mutant *M. tuberculosis* with inh
gene from two examinations. (First curve crossed v
26.6 C_t value and the other curves with 31 C_t value)

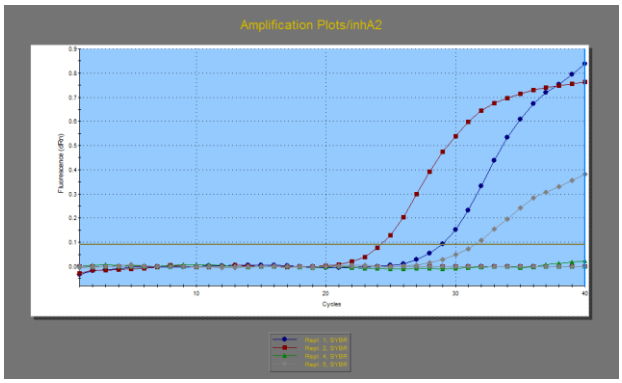

Figure 3.

two sybr green signals levels of master mix r time PCR that indicate to mutant *M. tubercul* with inhA2 gene from two examinations. (F curve crossed with 16.5 C_t value and the ot curves negative C_t valu

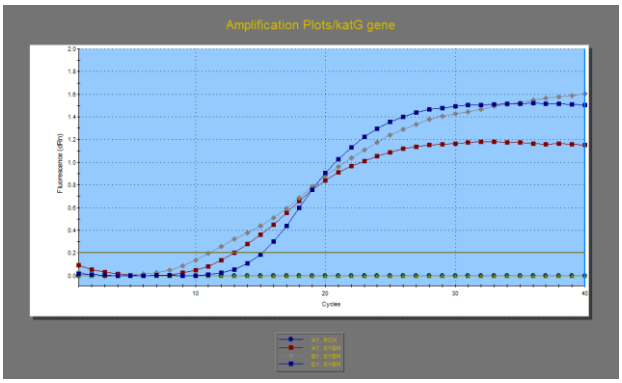

Figure 4.

Three sybr green signals levels of master mix r time PCR that indicate to mutant *M. tubercul* with *katG gene* from two examinations. (First cu crossed with 17 C_t value and the other curves v negative Ct value).

The results were shown there six mutants with rpoB gene, three mutants with katG gene, two mutants with inhA1 gene and three mutants with inhA2 gene in isolates of *Mycobacterium tuberculosis*. The results obtained indicate that three MDR isolates with mutants with both rifampicin resistant isolates (rpoB) and isoniazid resistant isolates katG, inhA1 and inhA2) in different gender and Clinical characteristics of patients. And the positive results on real time PCR as judged by a melting point of 85C^o -88.5C^o. Negative control formed primer with a melting point of 83C^o, figure (5). Explain of dissociation curve of three rpoB mutants' genes with negative control. rifampicin resistance in approximately 95% of the cases is due to mutations in an 81-bp Rifampicin Resistance Determining Region (RRDR) of the rpo B gene.

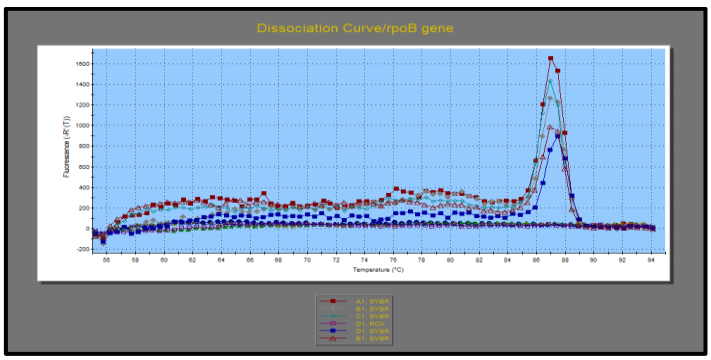

Figure 5.

Syber green mediated real-time PCR Dissociation curve (melting curve) to rpoB gene ,tm 84C^o - 88C^o indicating the present Mutation in rpoB gene.

The rpoB gene encodes the β subunit of bacterial RNA polymerase. Its codes for 1342 amino acids, making it the second -largest polypeptide in the bacterial cell. is the site of mutations that confer resistance to the rifamycin antibacterial agents, such as rifampin. according to Mokrousov *et al*., up to ninety-five per cent of RIF-resistant strains have mutations in codons 531. Mutations in rpoB that confer resistance to rifamycin do so by altering residues of the rifamycin binding site on RNA polymerase, thereby reducing rifamycin binding affinity for rifamycins [13]. RIF resistance is often considered as a surrogate marker for checking MDR-TB [14]. However, Isoniazid (INH) is one of the most effective and specific agents for the treatment of infection with *M. tuberculosis,* Recent increases in INH-resistant (INHr) and multidrug-resistant (MDR) tuberculosis are jeopardizing the continued utility of drug. Furthermore, the development of INH resistance is a common first step in the evolution to MDR, between 40 and 95% of INHr, clinical *M. tuberculosis* isolates have mutations in *katG*, 75 to 90% of which are in codon 315 [15]. The catalase-peroxidase coding *katG* gene is the most commonly targeted, with the majority of mutations occurring at codon 315, Mutations in *katG*315 may be favored because mutations at this location appear to decrease INH activation without abolishing catalase-peroxidase activity, a potential virulence factor [16]. missense mutations in the katG and inhA genes causes to isoniazid (INH) resistance this genes encoding for catalase-peroxidase and inhA, respectively A major role in the development of resistance of Mycobacterium tuberculosis to isoniazid (INH) is attributed to mutations in the katG gene coding for the catalase/peroxidase, an enzyme required for obtaining a pharmacologically active form of the drug. Analysis of mutations in the katG gene in M. tuberculosis strains may contribute to the development of reliable and rapid tests for detection of INH resistance[17].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 [14]. 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 less effective than first-line anti-TB drugs [18]. Many studies used real-time PCR for detection of mutations in rpoB and katG genes [19].

Mutations in *inhA* promoter generally result in low level resistance Drug resistance arises due to improper use of antibiotics in chemotherapy such as inadequate treatment regimens, and failure to ensure that patients complete the whole course of treatment [20]. The polymerase chain reaction (PCR) sequencing-based strategy, designed to detect mutations associated with drug resistance rapidly, is able to provide a “same-day” diagnosis from

culture and even clinical samples with high sensitivity and specificity. This gold standard method can also detect new mutations that could be associated with drug resistance [21]. In the present study, 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 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. The result showed the patient failed treatment has a mutation in rpoB gene detection by two assays, table (2).

Table 2.

Comparison between Real-time PCR (rpoB gene responsible RIF resistance) and Genexpert method (RIF resistance and sensitive) accordance with the category of patients**.**

Category	Real-time PCR		GeneXpert	
	RpoB gene (Positiv	RboB gene(negative)	RIF (resistance)	RIF(sensitive)
New (n=26)	2	24	0	26
RL (n=2)	2	0	0	2
UT(n=1)	1	0	0	1
Fail (n=1)	1	0	1	0

several nucleic acid amplification (NAA) technic have been developed for fast detection and identification of Mycobacterium tuberculosis (MTB) in clinical specimens These methods are attractive because they provide for the direct detection of low MTB genomic copy numbers in specimens. Polymerase chain reaction (PCR) is based on NAA techniques and is generally used for the rapid diagnosis of TB [22].

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 [23]. In our 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.

A total of six rpoB mutants, we traced it within six months by using real-time PCR assay in patients whom positive culture conversion of sputum after treatment. One of the new patient cases/ repoB mutants category were cured within six months but the rest patients were unable to recover from the infection with high Ct values, one patient was recurred in the fifth month but had relapse infection in the sixth moth with 30.8 Ct. the findings were shown one patient was under-treatment but Ct value was elevated in the sixth month, table (3). The threshold cycle (Ct) was higher when mutations were present in the genes. The difference of curve patterns can differentiate the susceptible and resistance isolates. Therefore, real-time PCR detected the all the mutations. In rpoB gene. CT values from 16-39 were considered positive rpoB gene mutation. CT values equal to zero or equal to or greater than 40 were recognized negative, indicating the presence of a drug resistance mutation. [24]. Ct values were inverse to the amount of nucleic acid that is in the sample, and correlate to the number of copies in your sample. Lower Ct values indicate high amounts of targeted nucleic acid, while higher Ct values mean lower (and even too little) amounts of your target nucleic acid. in present study the under treatment and fail treatment samples have a mutation in rpoB gene higher rate than other samples, however the Ct value increases when taking rifampicin during the period of treatment and three sample Ct zero significance of response to treatment.

Table (3).

follow up the values of rpoB Ct during six months of treatment by using real-time PCR assay.

Ct rpoB mutants follow up/months			
Category of patient	2th	5th	6th
New case 1	29	31	31.5
New case 2	29.25	27	No Ct
Relapse infection	24.83	29.2	No Ct
Relapse infection	28.3	No Ct	30.8
Under- treatment	18.56	27.3	No Ct
Fail treatment	12	15.34	30.2

Conclusion

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

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors read and approved the final manuscript.

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