Original Article

First experience with using simple polymerase chain reaction-based methods as an alternative to phenotypic drug susceptibility testing for *Myobacterium tuberculosis* in Iraq

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Abstract

Context: In Iraq, the time-consuming, phenotypic drug susceptibility testing (DST) on agar is the sole method available for detecting drug resistance in *Myobacterium tuberculosis* (TB). Furthermore, only single laboratory across Iraq is performing it on wide scale. **Aims:** To explore utility of rapid, polymerase chain reaction (PCR)-based systems in detection of drug resistance in under the Iraqi settings. **Settings and Design:** Cross-sectional study. A total of 79 nonduplicated isolates were included in this study. Multiplex allele-specific PCR was used to detect mutations at positions 531, 526, and 516 of the *rpoB* gene. Two simplex PCR systems were used to detect mutations in *katG315* gene and *inhAP-15*. **Statistical Analysis Used:** Chi-square and crosstabs by SPSS v. 20. **Results:** On DST, out of 69 isolates, 55 isolates were found multidrug-resistant (MDR)-TB; six isolates were susceptible to both rifampin (RIF) and isoniazid (INH); two isolates were resistant to RIF but not to INH; and six isolates were resistant to INH but not RIF. RIF and INH resistance mutations were detected in 50 (90.9%), and 43 (78.2%) MDR cases, respectively. Combine resistance mutations to RIF and INH were detected in 40 MDR cases (72.7%). The most frequently mutated codon was the codon 531 in *rpoB* gene, mutated in 42 isolates. *inhAP-15* and *katG315* codons were found mutated in 23 and 25 MDR cases (54.8% and 58.1%), respectively. Among 57 RIF-resistant isolates, 52 (91.2%) were harboring mutations resistance to RIF. **Conclusions:** These PCR-based methods are potential diagnostic and/or screening tools to detect drug-resistance TB in Iraqi settings.

Key words: Drug resistance, isoniazid, multidrug-resistant, mutations, polymerase chain reaction, rifampin, tuberculosis

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Introduction

Control of tuberculosis (TB) transmission is largely dependent on detecting active pulmonary infections and treating them effectively. However, the rapid emerging of multidrug resistance (MDR) *Mycobacterium tuberculosis* (TB) strains is a major challenge of TB controlling programs. MDR-TB is defined as simultaneous resistance to rifampin (RIF) and isoniazid (INH), the two first-line anti-TB drugs and are most effective against *M. tuberculosis*.[1] Drug-resistant *M. tuberculosis* strains impose a global threat. During 2008, there were an estimated 440,000 cases of multidrug-resistant TB (MDR-TB), comprising 3.3% of all new cases of TB, resulting in 150,000 deaths.[2]

Iraq is one of the countries with the highest incidence among the Eastern Mediterranean region with TB accounting for 56/100,000 population in 2006.[3] The estimated MDR-TB cases among new pulmonary TB cases notified in 2010 is 210 (50-380), whereas the estimated number

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among retreated pulmonary cases is 160 (57-260).[4] After receiving accreditation at 2011, the National Reference Laboratory (NRL) started to provide reliable results about the MDR cases. So far, around 210 patients have listed as MDR patients. Unfortunately, NRL is the only accredited laboratory across Iraq performing laboratory identification of MDR cases. Patients suspected to be MDR cases (treatment failure, relapsed, or after default.) are referred from throughout Iraq to the NRL. The method used for testing drug susceptibility in the NRL is phenotypic drug susceptibility testing (DST) using the time-consuming indirect proportion method on Löwenstein-Jensen (ILJ).[5] Wide use of this method in other places is hindered by the lack of laboratories equipped to do this method. Furthermore, DST is even more problematic, because it is difficult to do well, the turn-around time is often measured in months, and some drugs often show discordant results. Therefore, there is a pressing need for more rapid methods that are suitable to be applied in settings with lower infrastructure facilities as in the case of most of the regions in Iraq.

Huge body of molecular studies has explored the mechanisms of drug resistance and showed that point mutations of certain genes are the primary genetic mechanisms responsible for the development of *M. tuberculosis* drug resistance.[6] RIF resistance is mainly caused by point mutations in the "hot spot region" (81 base pairs [bp]) of the *rpoB* gene of *M. tuberculosis* (some references refer to this region as RIF resistance determining region).[7-10] Two point mutations are most commonly reported to be involved in the development of INH resistance: Mutations in codon 315 of the *katG* gene (katG315),[11-13] and in the promoter region (−15) of the *inhA* gene (*inhAP-15*).[14-16] The high accordance between resistance mutations and the phenotypic DST results has encouraged the investigator to develop several molecular techniques to detect these mutations as surrogate of drug resistance in *M. tuberculosis* such as the line probe assays,[17] and the Xpert MTB/RIF assay (Cepheid).[18] However, those techniques require sophisticated equipments that are very expensive. Fortunately, several polymerase chain reactions (PCR)-based systems with simple electrophoresis were evaluated in several settings with promising results. The feasibility of those methods may depend on the frequency of mutations and their patterns in the circulating strains in those settings. However, the prevalence and type of mutations in the *M. tuberculosis* drug resistance genes vary depending on geographical location.[19,20]

Two important considerations in the use of those PCR-based systems in our settings: (1) Are rapid and thus the shortened test turn-around time will have an important effect on the outcome of patients care, (2) may used as screening tools in select cases for DST.

In this study, we aimed to explore the accordance degree between PCR-based systems and the DST in a clinically- and bacteriologically- confirmed MDR cases. We believe that this step is important before deployment of such system in areas where phenotypic DST facility is unavailable. Therefore, the main objective of this study was to figure out the performance of those PCR-based systems in comparison to DST in Iraqi settings.

Subjects and Methods

Specimen collection and patients data

This is a descriptive cross-sectional study conducted from January 2011 to July 2012. The study included non-duplicated culture isolates recovered from 69 active pulmonary TB patients belonging to category II (default, relapsed, and treatment failure) who were attending the National Center of Tuberculosis and Chest illnesses-NTP (National Tuberculosis Program) at Baghdad/Iraq. In addition, the laboratory reference strain H37Rv was used across this study as a control strain within all procedures and protocols. The culturing and identification of MTB complex isolates was based on conventional methods described elsewhere.[5]

DST

Testing of drug susceptibility of these strains to four first-line anti-TB drugs [INH, RIF, streptomycin (SM), and ethambutol] were performed as recommended by World Health Organization (WHO)/International Union Against Tuberculosis and Lung Disease.[21] The method used is the agar proportional method on LJ media. Appropriate controls were used across the study, the controls were tubes of the medium without drugs inoculated by the strains of *M. tuberculosis* being tested. All tubes were incubated at 37°C after 6-8 weeks of incubation, growth was monitored and susceptibility results were then read. As recommended by WHO,[22] drug resistant to the corresponding drug is stated when the growth rate was more than 1% in comparison with bacterial growth in the respective control tube.[5] MDR-TB is declared when strain is reported to resist INH and RIF. All the drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Genomic DNA preparation

Mycobacterial genomic deoxyribonucleic acid (DNA) was extracted from cultured cells as described previously,[22,23] with minor modification. Briefly, by the use of sterile disposal plastic loops, few colonies were harvested from the surfaces of LJ slants and resuspended in 200-300 μL TE buffer [10 mM Tris-HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid (EDTA)]. This suspension was briefly mixed and then inactivated by heating at 95°C for 30 min in a water bath. The heat inactivated mycobacterial

suspension was then centrifuged (4000 RPM for 2 min) and supernatant was removed and stored at –20°C until further use. DNA in the supernatant was quantified by the use of Picodrop instrument following the instruction of the manufacturer (PicoDrop Ltd, Cambridge, UK).

Detection of resistance mutations to RIF

We used multiplex allele-specific (MAS)-PCR assay that is designed to detect mutations in three codons of the *ropB* gene (516, 526, and 531) in *M. tuberculosis* strains.[10] It a simple single-step MAS-PCR assay that is consisted of three independent allele-specific PCRs targeting three *rpoB* codons (codons 516, 526, and 531). The inner forward primers (R516B, R526B, and R531B) are positioned so that their 3´-OH ends pair with the second bases of the respective codons in the case of wild-type allele. Consequently, in the absence of mutation in these positions in *rpoB* 531/526/516, wild-type allele-specific fragments (170 or 185 or 218 bp, respectively) are amplified by the reverse primer RIR and an inner forward primer. If a mutation occurs, this results in mismatch at the 3´-end of the "wild type" inner primer and, under appropriate stringent PCR conditions, in the absence of the allele-specific PCR product.

Each reaction mix includes four primers: *rpoB516* (5'- CAGCTGAGCCAATTCATGGA-3') (20 pmoL in 2 μL), *rpoB526* (5'- CTGTCGGGGTTGACCCA-3') (10 pmoL in 1 μL), *rpoB531* (5'- CACAAGCGCCGACTGTC-3') (30 pmol in 3 μL), RIRm (5'- TTGACCCGCGCGTACAC-3') (60 pmol in 6 μL). The other reagents included in each reaction mix were 1 μL of 50× deoxyribonucleotide mix, 2.5 μL of 10× reaction buffer, 3.5 μL of $MgCl_2$, 0.5 μL DMSO, 0.25 μL Taq polymerase enzyme, 2 μL DNA solution containing 40 ng DNA template, and 13.45 μL PCR-grade water, the final volume was 20 μL. The thermocycling parameters included an initial denaturing at 95°C for 5 min, 35 cycles of 95 8C for 30 s, 70°C for 30 s, and 72 8C for 30 s, and a final extension at 72°C for 7 min. The PCR products were examined for banding patterns by 2.5% agarose gel electrophoresis in 1X Tris-Borate-EDTA buffer.

Detection of resistance mutations to INH

Two are single allele-specific PCR systems were used to detect the mutations in katG315, and *inhAP-15* codons for detecting the resistant strains for INH according to the protocol described by Yang *et al.*,[24] with little modifications. For each PCR reaction, a standard 50 μL reaction mixture was used. Each reaction mix included two primers: In case PCR to detect mutation in katG315 codon, the primers are katGOF (10 pmoL in 1 μL) (5'-GCAGATGGGGCTGATCTACG-3') and katG5R (10 pmoL in 1 μL) (5'-ATACGACCTCGATGCCGC-3'), whereas in case of PCR to detect mutation in *inhAP-15* the primers were *inhAP-15* (10 pmoL in 1 μL) (5'- GCGCGGTCAGTTCCACA-3') and inhAPF2 (10 pmoL in 1 μL) (5'- CACCCCGACAACCTATCG-3'). The other reagents included in each reaction mix were 2 μL of 50× deoxyribonucleotide mix (0.2 mM of each deoxyribonucleotide), 5 μL of 10× reaction buffer, 1 unit of AT taq hot star DNA polymerase (Vivantis, London, England) and 100-200 ng of DNA template. The thermocycling program included an initial denaturing at 96°C for 5 min, 40 cycles of 96°C for 30 s, 62°C (for *inhAP-15*) or 66°C (for katG315) for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were examined for banding patterns by 2.5% agarose gel electrophoresis in 1X Tris-Borate-EDTA buffer.

Ethical approval

Two ethical approvals were obtained from relevant committees belong to the Iraqi Ministry of Health.

Statistics

Statistical analyses between genotype and phenotype were performed in SPSS v. 20 (SPSS Inc.). Chi-square and crosstab analysis were run to identify if the differences between two statistical data were significant, and *P*-value less than 0.05, 0.01, and 0.001 were defined as "significant", "highly significant," and "extremely significant" diversity, respectively.

Results

Patients' data

Table 1 summarizes the results of the DST by using the agar proportional methods. In this study, complete drug susceptibility profiles were obtained for 69 nonduplicating isolates. Among those isolates, 55 isolates were identified as MDR-TB and patients were registered as MDR-TB at NTP-Iraq. This number represents about one quarter of all

Table 1: Results of drug susceptibility testing by agar proportional method

Characteristic	DST*	No. (%)
Combined DST results	"RR" (MDR)	55 (79.7)
	SS	6 (8.7)
	RS	2 (2.9)
	SR	6 (8.7)
DST profile		
Rifampin	Sensitive	12 (17.4)
	Resistant	57 (82.6)
Isoniazid	Sensitive	8 (11.6)
	Resistant	61 (88.4)
Streptomycin	Sensitive	32 (46.4)
	Resistant	37 (53.6)
Ethambutol	Sensitive	32 (46.4)
	Resistant	37 (53.6)

*RR: Combined drug resistance to rifampin and isoniazid (multidrug resistant); RS: Resistance to rifampin but susceptible to isoniazid (regardless other resistance); SR: Susceptible to rifampin and resistant to isoniazid (regardless other resistance); DST: Drug susceptibility testing

registered MDR-TB in Iraq. The DST results of the rest of isolates are as follows; six isolates were susceptible to both RIF and INH; two isolates were resistant to RIF but not to INH; and six isolates were resistant to INH but not RIF.

To simplify the demonstration and discussion of the DST results we used the following acronyms; "RR" phenotype is combined resistance to RIF and INH, "RS" phenotype is resistant to RIF but susceptible to INH, "SR" phenotype, susceptible to RIF but resistant to INH, and "SS" phenotypes, susceptible to both anti-TB drugs.

The highest rate of resistance (88.4%) detected was to INH (61/69) followed by RIF resistance (n = 57/69, 82.60%) and equal rates of resistance were detected to SM and EMB (n = 32/69, 46.37%).

No association was found between DST results and age distribution of the patients; however, statistically significant association was detected between gender and resistance to RIF (P = 0.049) and to INH (P = 0.038), where females were more associated with resistance than males. However, no association could be detected between gender and resistance to SM or EMB.

Molecular test for drug-resistance mutations

Figure 1 showing the electrophoresis mobility of the products of MAS-PCR. As could be seen with the positive control (laboratory strain H37Rv) and clinical isolates no. 2447 and 3234, the primers *rpoB516*, and RIR amplified a 218-bp fragment (representing the wild-type codon 516), primers *rpoB526* and RIR amplified a 185-bp fragment representing the wild-type codon 526), and primer *rpoB531* and RIR amplified a 170-bp fragment representing the wild-type codon 531). Mutation in any codon resulted in absence of the corresponding band.

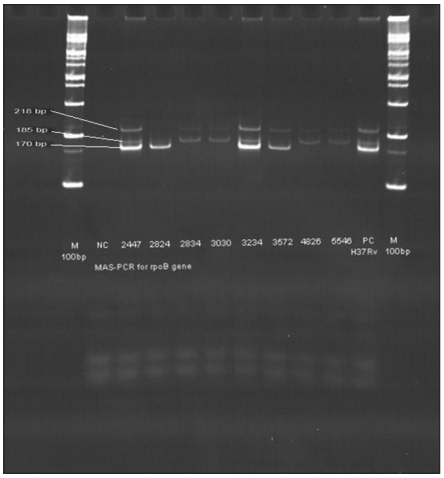

Figure 1: Multiplex allele-specific-polymerase chain reaction for the codons 531, 526, and 516 of the *rpoB* gene. Wild-type 531 is indicated by band of 170 bp, wild-type 526 indicated by band of 185 bp, and wild-type 516 indicated by band at 218 bp. Isolates number H37Rv (positive control strain), 2447 and 3234 revealed wild types of the three codons 531, 526, and 516. Isolate number 2824 harbors mutations at 526 and 516. Isolates 2834, 3030, 4826, and 5546 harbors mutation in codon 531. Isolate number 3572 harbors a mutation in codon 526

As seen in Figure 2, the primers inhAP, inhAPF2 amplified a 270-bp fragment and primers KatGOF and KatG5R amplified a 292-bp fragment. Wild-type codons are revealed by appearance of a specific bands, while mutations revealed by absence of the band.

Table 2 describes the results of the correlations analyses of the phenotypic DST with the results PCR-based genotypes revealed by presence or absence of resistance mutations to RIF and INH. To distinguish the drug-resistance phenotypes from the drug resistance genotypes that are obtained from the results of the allele-specific PCR systems, we designated the genotypes as follows; rr = resistance mutations to both of RIF and INH, rs = resistance mutations to RIF but not to INH, sr = resistance mutations to INH but not to RIF, ss = no resistance mutations could be detected.

An extremely significant association (P = 0.000) was found between RIF resistance mutations and the DST phenotypes. RIF-resistance mutations were detected in 50 out of the 55 (90.9%) MDR cases, the "RR" phenotype. None of the susceptible strains found to harbor RIF resistance mutations, a result that would make the specificity of this PCR system close to 100%. In addition, both of the two isolates having the "RR" phenotype were found to harbor RIF-resistance mutations (100%), whereas three out of six isolates of the "RR" phenotype were found to have resistance mutations to RIF. Therefore, the sensitivity of this MAS-PCR for detecting RIF-resistance mutations is 94.5%, while its sensitivity to predict MDR is 90.9%. Accordingly, these results clearly indicate the usefulness of using MAS-PCR to detect RIF-resistance mutations as a marker of MDR.

Regarding INH resistance mutations, a total of 43 out of

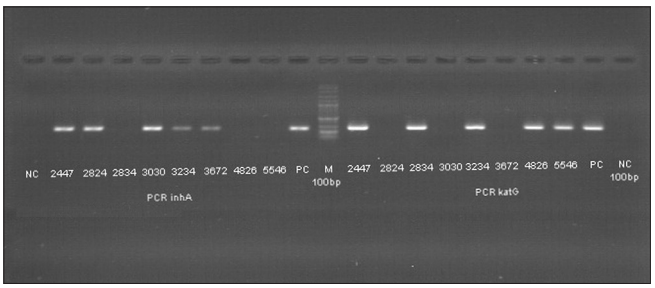

Figure 2: Electrophoresis of the products of allele-specific polymerase chain reaction system targeting *inhAP-15* (left) and *katG315* (right). NC: Negative control (no myobacterial deoxyribonucleic acid addede), PC: Positive control for the technique (deoxyribonucleic acid of H37Rv strain was added), Numbers indicates IDs of isolates. Isolates with bands are harboring the wild-type codons, whereas missing of the corresponding band indicates mutation

Table 2: Correlation between drug resistance mutations and phenotypic drug resistance (drug susceptibility testing)

Attributes of drug-resistance mutations	DST (no./%)‡			
	SS	RR	RS	SR
RIF-resistance mutation				
Wild type	6 (100)	5 (9.1)	0 (0.0)	3 (50)
Mutated	0 (0.0)	50 (90.9)	2 (100)	3 (50)
P value		0.000*		
INH-resistance mutation				
Wild type	6 (100)	12 (21.8)	1 (50)	3 (50)
Mutated	0 (0.0)	43 (78.2	1 (50)	3 (50)
P value		0.001**		
Combined results of RIF-and INH-resistance mutations‡‡				
rr	0 (0.0)	40 (72.7)	1 (50)	2 (33.3)
rs	0 (0.0)	10 (18.2)	1 (50)	1 (16.7)
sr	0 (0.0)	3 (5.5)	0 (0.0)	1 (16.7)
ss	6 (100)	2 (3.6)	0 (0.0)	2 (33.3)
P value		0.000*		

‡SS: Susceptible to rifampin and isoniazid; RR: Multidrug resistant (combined resistance to rifampin and isoniazid); RS: Resistance to rifampin but not to isoniazid; SR: Resistance to isoniazid but not to RIF. ‡‡rr: Resistance mutations to both of rifampin and isoniazid; rs: Resistance mutations to rifampin but not to isoniazid; sr: Resistance mutations to isoniazid but not to rifampin; ss: No resistance mutations could be detected. *extremely significant; **highly significant; DST: Drug susceptibility testing; RIF: Rifampin; INH: Isoniazid

55, (78.2%), MDR isolates were found to harbor INH-resistance mutations. No INH-resistance mutations could be detected in the isolates belonging to the "SS" phenotype. In addition, half of the cases with "RS" (*n* = 1) and "SR" (*n* = 3) were shown to harbor INH-resistance mutations.

We analyzed a combined results of RIF and INH resistance mutations in correlation in with DST results and found an extremely significant association (*P* = 0.000).

A total of 40 out of 55 (72.7%) MDR cases showed the "rr" genotype, whereas 10 (18.2%) and 3 (5.5%) of the MDR cases showed the "rs" and "sr" genotypes, respectively.

Table 3 summarizes the results of distribution of resistance mutation according to the results of traditional DST (resistance phenotypes). Highly significant association (*P* = 0.001) was found between the type of mutation in the *rpoB* gene and results of phenotypic DST. The most frequently mutated codon was the codon 531, mutated in a total of 42 isolates; in form of single mutation in 40 isolates (37 were MDR cases, 1 is RS and 1 is SR) and two in form of combined mutations (531 with 516 and 531 with 526). A total of eight isolates were found harboring mutations in the codon 516, as single mutation in seven isolates (five of them MDR, while two were of "SR" phenotype), and in one isolate in form of combined mutation with codon 531. A total of seven isolates were found harboring mutation in codon 526 (as single mutations in five isolates and combined with mutation in codon 531 in one isolates). It is worthy to note that mutation in codon 526 is only reported in MDR cases, this is in contrast to the mutations in codon 531 and 516 that occurred in different phenotypes. According to these results, for better detection of MDR cases by the use of PCR-based methods, it is better to target the three codons rather than targeting one of them.

No significant association was found between mutations in *inhAP-15* codon and DST results (*P* = 0.123). This may be in part because of the relatively low frequency of this mutation among the MDR isolates (*n* = 23, 54.8%). In addition, mutations in this codon were detected in two of isolates with "SR" phenotype and one isolate with "RS" phenotype. These results clearly indicate that mutations in *inhAP-15* cannot be used alone as a marker of MDR.

A significant association was found between mutations in *katG* codon and the results of DST (*P* = 0.041). A total of 25 MDR isolates were harboring mutation in this codon (58.1%), whereas only one isolate with "SR" phenotype was harboring mutations this codon. These results clearly pinpoint the importance of mutations in this codon as a marker of drug resistance.

Discussion

In last decade, with the rapid emergence and spreading of drug-resistant TB strains of *M. tuberculosis*, especially the MDR strains with resistance to at least RIF and INH, the effect of chemotherapy for TB has been severely depressed. It is very important, therefore, to identify these strains as soon as possible to allow for adjustments treatment and to minimize the transmission of drug-resistant strains. As a result, the drug susceptibility test has become more important than ever. The traditional drug resistance testing is the only available method in Iraq and it is only done as a routine at NRL in Baghdad, the capital of Iraq. This method takes about 4-6 weeks to give the results. The main obstacle preventing its implementation on wide scales to cover other areas in Iraq is the lack of reliable laboratories equipped with bio-safety measures. However, the other major issue is the laboratory expertise to undertake such kind of laboratory work. After several training courses and workshops for the laboratory staff at certain recognized WHO-based supra-national laboratories abroad, the NRL recently (2011), got the accreditation to perform DST locally for the first-line anti-TB drugs by using the agar proportion on LJ media. Accordingly, since 2011, NTP-Iraq has launched a community-based therapy of the MDR cases. So far, 210 patients were confirmed to as MDR-TB, thus, scheduled to receive second line anti-tuberculosis therapy. As Baghdad is 1 out of 18 provinces in Iraq; therefore, there is a pressing

Table 3: Distribution of mutations according to the drug susceptibility testing results

Mutations attributes	DST%			
	SS	RR	RS	SR
rpoB gene				
R516				
Count	0	5	0	2
% within *rpoB gene*	0.0	71.4	0.0	28.6
R526				
Count	0	6	0	0
% within *rpoB gene*	0.0	54.5	0.0	0.0
R531				
Count	0	37	2	1
% within *rpoB gene*	0.0	59.7	3.2	1.6
R531, R516				
Count	0	1	0	0
% within *rpoB gene*	0.0	16.7	0.0	0.0
R531, R526				
Count	0	1	0	0
% within *rpoB gene*	0.0	100.0	0.0	0.0
Wild type				
Count	6	5	0	3
% within *rpoB gene*	13.6	11.4	0.0	6.8
P value		0.001*		
inhAP-15				
Mutated				
Count	0	23	1	2
% within *inhAP-15*	0.0	54.8	2.4	4.8
Wild type				
Count	6	32	1	4
% within *inhAP-15*	6.5	34.8	1.1	4.3
P value		0.123		
katG315				
Mutated				
Count	0	25	0	1
% within *KatG315*	0.0	58.1	0.0	2.3
Wild type				
Count	6	30	2	5
% within *KatG315*	6.6	33.0	2.2	5.5
P value		0.041**		

*highly significant; **statistically significant. DST: Drug susceptibility testing

need for a method for diagnose or screening of MDR cases in the rest 17 provinces. In our study, we aimed to explore the accordance degree between PCR-based systems and the DST in a clinically- and bacteriologically-confirmed MDR cases. We believe that this step is important before deployment of such system in areas, where phenotypic DST facility is unavailable.

In this study, complete drug susceptibility profiles of 69 isolates were obtained. Among those isolates, 55 isolates are from MDR-TB patients whom are already registered as MDR-TB at NTP-Iraq. This number represents about one quarter of all registered MDR-TB in Iraq. We reported a statistically significant association between gender and resistance to RIF (P = 0.049) and to INH (P = 0.038), where females were more associated with resistance than males. Considering the widely accepted hypothesis that resistance in TB is a man-made phenomenon that results from poor compliance with treatment, association of females with resistance, our results may indicate that females have less compliance with TB treatment. However, no association could be detected between gender and resistance to SM or EMB. Furthermore, no association could be found between resistances to any individual anti-TB drugs with the age group.

An extremely significant association (P = 0.000) was found between RIF resistance mutations and the DST phenotypes, RIF-resistance mutations were detected in 50 out of the 55 (90.9%) MDR isolates. Thus, this study reporting a high accordance between phenotypic and genotypic drug resistance assays and this may demonstrate the reliability of the detection of resistance mutations to RIF as surrogate of MDR. Furthermore, none of the susceptible strains found to harbor RIF resistance mutations, a result that would make the specificity of this PCR system close 100%. In addition, the sensitivity of this MAS-PCR for detecting RIF-resistance mutations in any type of resistance was 94.5%, while its sensitivity to predict MDR is 90.9%. The rate of resistance mutation to RIF among the MDR cases is consistent with rate reported globally.[8,9] Accordingly, these results clearly indicate the usefulness of using MAS-PCR to detect RIF-resistance mutations as a marker of MDR for both diagnostics and screening purposes.

Regarding INH-resistance mutations, a total of 43 out of 55, (78.2%), MDR isolates were found to harbor INH-resistance mutations (in katG315 and/or *inhAP-15* codons). No INH-resistance mutations could be detected in the isolates belonging to the susceptible isolates ("SS" phynotype). These results are comparable with that reported in the previous studies which are responsible for 50-80% of INH-resistant (INH-R) isolates,[11-13] and it emphasizes the fact that mechanisms of INH resistance are rather complicated than RIF.

On analysis of the combined results of resistance mutations to RIF and INH and their correlation with results of the traditional DST, we found an extremely significant association (P = 0.000). The percentage of the accordance between the "RR" phenotype and the "rr" genotype was 72.7%. Nevertheless 53 isolates out of 55 MDR isolates (96.4%) were found to harbor a resistance mutation for one or both of RIF and INH. These results would confirm the usefulness use of the rapid PCR-based systems for screening and/or diagnosis of resistance in tuberculosis.

Furthermore, highly significant association (P = 0.001) was found between the type of mutation in the *rpoB* gene and DST results. The most frequently mutated codon was the codon

531, was found mutated in a total of 42 isolates; followed by codon 516 that was mutated in eight isolates. Only seven isolates were found harboring mutation in codon 526. Multiple mutations (combined mutation in more than one codon) were found in low frequencies. It is worthy to note that mutation in codon 526 is only reported in MDR cases, this is in contrast to the mutations in codon 531 and 516 that occurred in different phenotypes. According to these results, for better detection of MDR cases by the use of PCR-based methods, it is highly recommended to target the 3 codons rather than targeting one of them. The results in our study are supporting the previous studies that demonstrated that the main mutations of the *rpoB* gene conferring resistance to INH are correspond to codons 516, 526, and 531.[8,11]

No significant association was found between mutations in *inhAP-15* codon and DST results ($P = 0.123$). This may be in part because of the relatively low frequency of this mutation among the MDR isolates ($n = 23$, 54.8%). In addition, mutations in this codon were detected in two of isolates with "SR" phenotype, and one isolate with "RS" phenotype. These results clearly indicate that mutations in *inhAP-15* cannot be used alone as a marker of MDR. However, a significant association was found between mutations in katG codon and the results of DST ($P = 0.041$). A total of 25 MDR isolates were harboring mutation in this codon (58.1%), whereas only one isolate with "SR" phenotype was harboring mutations this codon. These results clearly pinpoint the importance of mutations in this codon as a marker of drug resistance.

On the basis of our results, we conclude that these methods could be used as simple screening tools to detect drug-resistance TB in setting where DST facility is not available.

Our results are consistent with those obtained in several previous studies that showed that mutations in codon 315 of the katG gene (katG315) are responsible for 50-80% of INH-R isolates.[11-13] However, the frequency of mutations in *inhAP-15* codon was higher than those reported in the previous studies in the world, where the point mutation in the promoter region (–15) of the *inhA* gene (*inhAP-15*) was found to be responsible for 8-20% of INH-isolates.[14-16]

The reason for this high frequency of mutations in *inhAP-15* in our study is possibly due to overrepresentation of *M. tuberculosis* family or cluster (s) that are characterized by harboring this type of mutation. This hypothesis was confirmed based on finding that the largest cluster reported in this study (as you will see in the next sections, T-family) was consisting of 20 isolates belonging of SIT1144 that are characterized by harboring this type of mutations (data not shown).

Conclusion

These methods could be used as simple diagnostic and/or screening tools to detect drug-resistance TB in setting where DST facility is not available. Furthermore, MAS-PCR to detect resistance-mutations to RIF could be used as marker of multidrug resistance and treatment with second-line anti-TB drugs could be started based on its results.

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