Detection of Refampicin and Isoniazid Resistant Mutants of Tubercle Bacilli Using Manual Gene Sequencing:
A study involved rpoB, KatG and inhA genes

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Multidrug-resistant tuberculosis (MDR-TB) is an emerging problem with high mortality rate where recently developed molecular techniques represent potential tools for its early detection. The aim of this study is to detect drug resistant mutants of Mycobacterium tuberculosis (M.TB) in 15 patients with active pulmonary tuberculosis, who were non responding to 1st line multi-drug therapy [refampicin (RIF) and isoniazid (INH)]. This was performed using the Chain Termination method of manual Gene Sequencing for the following Mycobacterial genes: rpoB gene [involved in sensitivity to RIF], katG and inhA genes [involved in sensitivity to INH]. Missence point mutations in rpoB gene were found in 93.3% (14/15), which involved codon 184 in 80% (12/15) with Histidine → Tyrosine substitution, and codon 174 in only 13.3% (2/15) with Aspartate → Valine substitution. Missence mutations in katG gene were detected in all cases (100%), which involved codon 315 in 80% (12/15) with Serine → Threonine substitution and codon 444 in 13.3% (2/15) with Valine → Alanine substitution, while only 6.7% (1/15) involved codon 315 and 444 together. Inh-A gene revealed missence mutation in 86.7% (13/15), where 60% (9/15) involved codon 94 with Serine → Valine substitution, 20% (3/15) involved codon 99 with proline → Arginine substitution and 6.7% (1/15) involved codon 69 with Glutamate → Alanine substitution. It could be concluded that, missence point mutations found in the examined genes could explain the resistance of 14 patients to both RIF and INH, and resistance of only one patient to INH alone. The missence point mutation was found at a common codon position among each gene, in addition to some other involved codons. It could be also concluded that, manual gene sequencing is a rapid, non expensive and accurate technique for early detection of MDR-TB, which helps early starting of proper treatment and inhibits spreading of such strains. Although it is difficult to be performed as a routine test, facilities should be available in order to perform it for at least some selected cases, as it does not need the expensive automated DNA sequencer.

INTRODUCTION

Multidrug-resistant Mycobacterium tuberculosis (MDR-TB) is an emerging problem of great importance to public health, with high mortality rates, particularly in immunocompromised patients. MDR-TB patients require treatment with more toxic second line drugs and remain infectious for longer time, requiring higher cost due to prolonged hospitalization. MDR strains are generally considered to be those resistant to at least two 1st line drugs, such as Rifampicin (RIF) and Isoniazid (INH). The frequency of resistance to multi drugs, varies geographically, where acquired (secondary) resistance is more common than primary resistance (1).

Resistance to antituberculous agents resulted from mutations of chromosomal genes encoding the sensitivity to the target drug, with stepwise acquisition of new mutations in genes for different drug targets. The occurrence of resistance to RIF and INH, is known to be due to mutations in certain genes which included rpoB gene for RIF resistance and kat G, inh-A, kasA, ndh and the oxyR-ahpc intergenic regions for INH resistance (2,3).

Global efforts to reduce the prevalence of MDR-TB have focused on preventing new cases of acquired MDR-TB by using directly observed therapy, the use of shortened course treatment protocols for drug-susceptible TB and the early detection of resisting mutants (4, 5).

MDR-TB has increased in incidence and interferes with TB control programs, particularly in developing countries where prevalence rates are as high as 48%. This poses an urgent challenge to rapidly detect these cases (6, 7).

Molecular methods represent potential tools for shortening the time for determining antimycobacterial drug resistance (8). Chain termination method of manual gene sequencing, which is also known by dideoxy DNA sequencing, was 1st developed by Sanger in 1977 (9). It is a rapid and accurate manual sequencing method for detection of drug resistance where it does not
need the expensive automated DNA sequencer. It is based on the principle that the polymerization on a template strand can be interrupted by the presence of dideoxyribonucleotide triphosphate (ddNTPs) in the reaction mixture. The ddNTPs differ from the conventional dideoxyribonucleotide triphosphate (dNTPs) in that the first lack the hydroxyl (OH) group residue at the 3’ position of deoxyribose. So, it can be incorporated by DNA polymerases into a growing DNA chain via their 5’ triphosphate groups. The absence of 3’ hydroxyl residue prevents the formation of a phosphodiester bond with the following dNTP so, further extension is impossible and the chain is terminated. When the mixture reaction is composed of four conventional dNTPs and small amount of one ddNTPs, there is a competition between extension of the chain and infrequent, but specific termination. The products of the reaction are a series of oligonucleotide chains, whose lengths are determined by the distance between the terminus of the primer used and the sites of premature termination. Four different ddNTPs are used in four separate enzymatic reactions. Populations of oligonucleotides, which have different lengths, are generated that terminate at positions occupied by every A, C, G or T in the template strand as demonstrated in Fig 1. Gel electrophoresis of the 4 reaction mix into 4 separate lanes resulted in numerous bands, where the gene sequence could be read from down upward visually after staining, or by the help of software program (9).

Fig 1: Principle of chain termination method of gene sequencing (9):
Gene sequencing should be followed by Gene Alignment which includes comparison of the sequenced gene to its wild type (obtained from Gene bank), in order to identify the site and type of gene mutation. Moreover, it included also translation of the gene sequence into polypeptide sequence which could be compared to the wild protein to detect whether mutation is silent or missense (alters amino-acid sequence). The NCBI (National centre for Biotechnology Information) allowed the search for sequence data base of nucleotide and protein sequence (10).

The aim of this study is to detect drug resisting mutants of \textit{M.\textit{TB}} in patients with active pulmonary tuberculosis who were non responding to treatment with 1$^{\text{st}}$ line multi-drug therapy (including both RIF and INH). This will be performed using The Chain Termination method of manual gene sequencing of the following \textit{Mycobacterial} genes after being amplified by the polymerase chain reaction: \textit{rpoB} gene (involved in sensitivity to RIF), \textit{katG} and \textit{inhA} genes (involved in sensitivity to INH). The sequenced genes will be translated into the corresponding amino-acid sequence and then compared to wild genes and polypeptide chains using the Web pages of Gene Bank in order to detect missence mutations and their codon distribution.

\section*{PATIENTS AND METHODS}

This study was carried out on 15 patients who were previously diagnosed to have active pulmonary tuberculosis with no respond to multidrug therapy. They were selected from those who were admitted in Chest Departement of Tanta University Hospital and Tanta Chest Hospital. They included 11 males and 4 females, with a mean age of 37.5 yrs ± 10.25.

The selection criteria included:

1) Present symptoms suggestive of active pulmonary tuberculosis included (fever, cough, expectoration, haemoptysis, chest pain, weight loss and fatigue).
2) \textbf{Clinical and radiological findings}: including consolidation, cavitations, bronchovascular markings and hilar shadows bilaterally.
3) \textbf{Smear –positive sputum}: Positive microscopy for acid fast bacilli being identified in direct Z.N stained smear.
4) All patients had history of previous treatment with first line anti-TB drugs (including both RIF and INH) for at least 3 months, with no clinical, radiological or microbiological response (still strong +ve for acid fast bacilli at time of sampling).
5) Sputum culture and sensitivity using (BACTEC) system.

Heparinized blood samples were collected from drug resisting tuberculous patients as previously recommended (7) to avoid the high infectivity of sputum samples which were strong positive for resisting acid fast bacilli. Samples were also collected from control group which included 10 patients with chest diseases other than TB. All samples were subjected for detection of resistant mutants of \textit{M.\textit{TB}} using chain termination method of manual gene sequencing as follow:

\textbf{Polymerase chain reaction for amplification of \textit{Mycobacterial} genes}:

DNA was extracted from specimens using Puregene-DNA purification kit (Avenu North, USA) according to manufacturer instructions. The amplification of \textit{Mycobacterial} genes was performed as previously described (11), where 4 separate PCR rounds were carried out using specific primers for amplification of \textit{IS6110} gene (for identification of \textit{M.\textit{TB}}) \textit{rpoB} gene (involved in RIF sensitivity), \textit{katG} and \textit{inhA} genes (involved in INH sensitivity). The primers used were supplied by Genosyns Biotechnologies Inc, Cambridge, England. The sequence of these primers were as follow:

\begin{verbatim}
IS6110 : Forward   P : CGTGAGGGCATCGAGGTGGC ,
Reverse P:GCCTAGCCGCTGCTGACAAA
rpoB: Forward   P : GGGAGCGGATGACCACCCA, 
Reverse P : GCGGTACGCGGTTCAGAAC
katG : Forward   P : TGGACTTGAACCCCGTAC, 
Reverse P : ACAGCCACGCCAGCAG
inhA: Forward P:TCGACGGCCGCGATGG, 
Reverse P: CCGGTCCGCGGAACG
\end{verbatim}

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Agarose gel electrophoresis was then performed to detect amplified product of each gene where IS6110 gene was detected at 245 bp, rpoB was detected at 350 bp, katG at 1455 bp, and inh-A locus at 905 bp.

**Manual DNA sequencing:**

Manual Sequencing was done for amplified PCR products of rpo B, kat G and inh A genes. It was performed using SILVER SEQUENCE DNA SEQUENCING SYSTEM (Promega, USA) as described by the manufacturer. This system is a non-radioactive alternative for manual enzymatic sequence analysis. It combines thermal cycle sequencing with a sensitive silver staining protocol to detect bands in a DNA sequencing gel. Silver staining is a rapid, inexpensive alternative to radioactive, fluorescent or chemiluminescent detection approaches, where results could be obtained in the same day (12).

For each set of sequencing reactions, four 0.5 ml microcentrifuge tubes were labelled with the following capital letters which represent the 4 used nucleotides: A (adenine), G (Guanine), C (cytosine) and T (thymine). 2 ul of appropriate mix of nucleotides was added to each tube [including deoxynucleotides (dNTP) and deoxyribonucleotides (dd NTP) mix] as shown in table 1. For each set of the four sequencing reactions, the following reagents were mixed in a microcentrifuge tube: Template DNA (PCR products of the amplified regions of rpo B, kat G and inh A loci in separate reactions) in an amount of 28,115 and 72 ng respectively, 4.5 pmol of gene specific primer and nuclease free water to total volume of 16 µl.

**Gene specific primers used in manual sequencing included:**

- rpoB: GCGGTACGGCGTTTTCGAGAAC,
- katG: TGGACTTGACGCCCTGACG
- inhA: TCGACGGCCGGCATGG

1.0 ul of sequencing grade Taq DNA polymerase (5U/1 µl) was added to the previous (Primer / template mix), and then 4 µl from this mix was added to each of the previously prepared labeled tubes containing d/dd NTP mix. The reaction tubes were placed in thermal cycler that had been preheated to 95°C where the following cycling profile was used: 95°C for 30 seconds (denaturation), 42°C for 30 seconds (annealing) and 70°C for 1 minute (extension) for 45-60 cycles. 3 µl of DNA sequencing stop solution was then added.

<table>
<thead>
<tr>
<th>Nucleotide Mix</th>
<th>G- labeled tube</th>
<th>A- labeled tube</th>
<th>T- labeled tube</th>
<th>C- labeled tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddGTP</td>
<td>45uM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ddATP</td>
<td>-</td>
<td>525uM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ddTTP</td>
<td>-</td>
<td>-</td>
<td>900uM</td>
<td>-</td>
</tr>
<tr>
<td>ddCTP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>300uM</td>
</tr>
<tr>
<td>7deaza-dGTP</td>
<td>30uM</td>
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<tr>
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<tr>
<td>dCTP</td>
<td>30uM</td>
<td>30uM</td>
<td>30uM</td>
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</tr>
</tbody>
</table>

dNTP: deoxynucleotide triphosphate, ddNTP: de- deoxynucleotide triphosphate
Preparation of sequencing plates for electrophoresis: Long and short glass plates were used. The longer plate was treated with gel slick to prevent the gel from sticking to it, while the short one was treated with bind silane to bind the gel. The two plates were kept apart from each other to prevent cross contamination and they were assembled by placing 0.4 mm side and bottom spacers in-between where clamps were used to hold them in place.

Preparation of 6% polyacrylamide gel: The following ingredients were mixed together: 31.50 g urea, 36.25 ml deionized water, 3.75 ml 10x TBE and 11.25 ml 40% Acrylamide : Bis (19:1). 50 µl of TEMED and 500 µl of 10% ammonium persulphate were then added. The gel was carefully poured between the glass plates, and a shark tooth comb was inserted immediately on top of the gel where it was first put inverted then turned over. Polymerization was allowed to proceed for at least 1 hour, after which, comb and bottom spacer were removed. TBE was added to the buffer chamber of electrophoresis apparatus and 3 µl of each sample (4 tubes/each gene) was loaded into the 4 corresponding wells (named A, G, C, and T respectively). The voltage was adjusted at 1000 v, then gradually raised to 1500–1800 v and the gel was allowed to run for 45 minutes.

Silver staining of sequencing gel: This was done according to manufacturer instructions. In the stained gel, each gene sequence was represented by the bands in all of the 4 lanes (A, G, C, and T) where the sequence could be read visually from down–upward or by using a soft ware program (Gel proanalyzer version3.1 Media Cyber Netcies, USA).

Gene alignment and translation: This was performed through a WebPage in which the NCBI (National centre for Biotechnology Information) allows the search sequence data base of nucleotide and polypeptide sequence (ftp://ncbi.nlm.gov/gen.bank/gbrel.txt).

Gene alignment included comparison of the sequenced gene (query) to its wild type [obtained from Gene bank under certain accession number] (10), in order to identify the site and type of gene mutation. Moreover, the sequenced gene was translated into polypeptide sequence which was compared to the wild protein to detect whether mutation was silent or missense (altered amino-acid sequence). BLAST (Basic Local Alignment Search Tool) program version 2.2.13 was used where the sequences of both query and wild type of same gene were entered in the form of nucleotide and amino-acid sequence then alignment (comparison) of two sequences was done (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

RESULTS

PCR results: Fig.(2,3,4 and 5) show the results of the 4 separate PCR rounds performed for each of the tuberculous patients and controls. Agarose gel electrophoresis detected the amplified product of each gene where IS6110 gene was detected at 245 bp, rpoB was detected at 350 bp, katG at 1455bp, and inh-A locus at 905 bp. All of the 15 tuberculous patients had +ve bands for the 4 genes while not any of the non tuberculous patients (control group).
Fig. (2): Electrophoretic pattern of amplified PCR product of IS6110 gene (product size = 245 bp).
Lane 1: Molecular size marker from 100 to 2000 bp. 
Lane 2: negative control , Lane 3: positive control. 
Lanes 5 and 7: +ve (tuberculous patients), and Lanes 4, 6 and 8: -ve (non tuberculous patients).

Fig. (3): Electrophoretic pattern of amplified PCR product of rpoB gene (product size = 350 bp).
Lane 1: molecular size marker from 100 to 2000 bp. 
Lanes 2: positive control, Lane 3: negative control 
Lanes 4 and 6: positive (tuberculous patients), Lanes 5 and 7: negative (non tuberculous patients).
Fig. (4): Electrophoretic pattern of amplified PCR product of Kat G gene (product sizes 1455 bp).
Lane 1: molecular size marker from 100 to 2000 bp.
Lane 2: negative control, Lane 3: positive control
Lanes 5 and 6: positive (tuberculous patients), Lanes 4 and 7: negative (non-tuberculous patients)

Fig. (5): Electrophoretic separation of amplified product of inh-A gene (product size = 905 bp).
Lane 1: molecular size marker from 100 to 2000 bp.
Lanes 2: positive control
Lanes 3 & 5 and 7: positive (tuberculous patients).
Lanes 4 and 6: negative (non-tuberculous patients)
Incidence and codon position of missense mutation in the 3 sequenced genes: Table (2) shows the detection rate of the mutant rpoB, KatG, and inhA genes in the 15 tuberculous patients. It was found that 14/15 (93.3%), 15/15 (100%) and 13/15 (86.7%) of these genes respectively were mutants. The identification of codon positions of point mutations in the examined genes will be also shown.

Table (2): Incidence and codon positions of missence mutations in the sequenced genes:

<table>
<thead>
<tr>
<th>Codon position of missence mutation</th>
<th>No</th>
<th>%</th>
<th>A.A. substitution</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB 184</td>
<td>12/15</td>
<td>80</td>
<td>H⇒Y</td>
<td>ACC⇒TCC</td>
</tr>
<tr>
<td>rpoB 174</td>
<td>2/15</td>
<td>13.3</td>
<td>D⇒V</td>
<td>CAT⇒CCT</td>
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<tr>
<td>Total</td>
<td>14/15</td>
<td>93.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KatG 315</td>
<td>12/15</td>
<td>80</td>
<td>S⇒T</td>
<td>AGA⇒AAA</td>
</tr>
<tr>
<td>KatG 444</td>
<td>2/15</td>
<td>13.3</td>
<td>V⇒A</td>
<td>CCG⇒CGG</td>
</tr>
<tr>
<td>KatG (315 &amp; 444)</td>
<td>1/15</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15/15</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inhA 94</td>
<td>9/15</td>
<td>60</td>
<td>S⇒V</td>
<td>CGA⇒CCA</td>
</tr>
<tr>
<td>inhA 99</td>
<td>3/15</td>
<td>20</td>
<td>P⇒R</td>
<td>GCG⇒GAC</td>
</tr>
<tr>
<td>inhA 69</td>
<td>1/15</td>
<td>6.7</td>
<td>E⇒A</td>
<td>AGG⇒CGG</td>
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<tr>
<td>Total</td>
<td>13/15</td>
<td>86.7</td>
<td></td>
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</table>

H = Histidine  Y= Tyrosine  D = Aspartate  V= Valine  S= Serine  T= Threonine  A = Alanine  P=Proline  R = Arginine  E= Glutamate

As regard rpoB gene (fig.6 A&B): point mutation was found at codon 184 in 12/15 genes (80 %) which was missense mutation (code for wrong A.A.) with Histidine (H)⇒Tyrosine (Y) substitution (ACC⇒TCC). Point mutation was found also at codon 174 with Aspartate (D)⇒Valine (V) substitution (CAT⇒CCT) in 2/15 genes (13.3 %).Each of the mutant rpoB genes revealed multiple sites of point mutations, some of them were missense while others were silent.

As regard KatG gene (Fig.7 A&B): mutation at codon position 315 was found in 12/15 (80%) genes with Serine (S)⇒Threonine (T) substitution (AGA⇒AAA).Mutation at codon position 444 was detected in 2/15 genes (13.3 %) with Valine (V)⇒Alanine (A) substitution (CCG⇒CGG).Complex multiple point mutations at two codons 315 & 444 together were recorded in 1/15 gene (6.7%) with Serine(S)⇒Threonine(T) and Valine(V)⇒Alanine(A) substitution respectively. Moreover, several silent point mutations were also found (coded for the same A.A).

As regard inhA gene (Fig. 8 A&B): missense point mutation at codon 94 was found in 9/15 (60 %) genes with Serine (S)⇒Valine (V) substitution (CGA⇒CCA). 3/15 (20 %) genes showed mutation at codon position 99 with proline (P)⇒Arginine (R) substitution (GCG⇒GAG). 1/15 of genes show mutation at codon 69 (6.7%) with Glutamate (E)⇒alanine (A ) substitution (AGG⇒CGG).The sequencing results of the examined 3 genes revealed that 14/15 (93.3 %) of patients were resistant to both rifampicin and isoniazid together while only one patient (6.7%) was resistant to isoniazid alone, which were the same results obtained with sputum culture and sensitivity using BACTEC system.
Fig. (6 A & B):

A) The nucleotide alignment of one of the mutant rpoB gene against the wild rpoB gene. The site of missense point mutation is shown by the arrow (ACC→TCC). The sites of silent point mutations are lacking dash (The sequence included missense mutation is only shown).

<table>
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<tr>
<th>Query</th>
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</tbody>
</table>

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</tr>
</thead>
<tbody>
<tr>
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<td>455</td>
<td>ACATCCGGCCCGTGTCGCCGCCGATCAAGAGTTCTTTGGACACCAGGCCAGCTTGGCAAT</td>
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</tbody>
</table>

<table>
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<tbody>
<tr>
<td>Sbjct</td>
<td>515</td>
<td>TTATGGACCAGAACAACCCTTGTCCGGGCTGACACACAAGGAATTGTCTGGGACTG</td>
</tr>
</tbody>
</table>

↑

B) The protein alignment of the same mutant rpoB gene against the wild rpoB gene. Missense point mutation at codon 184(Histidine→Tyrosine) is shown by the arrow.

<table>
<thead>
<tr>
<th>Query</th>
<th>3</th>
<th>VRERMTTQDVAMITPVITPQLINIRPVVAAIKEFFGTSQFMDQNNPLSGLTYKRRLSALG</th>
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<tr>
<td>Sbjct</td>
<td>133</td>
<td>VRERMTTQDVAMITPVITPQLINIRPVVAAIKEFFGTSQFMDQNNPLSGLTHKRRLSALG</td>
</tr>
</tbody>
</table>

↑codon 184
Fig. (7 A & B) :

A) The nucleotide alignment of one of the mutant katG gene against the wild katG gene. The sites of missense point mutations is shown by the arrow( AGA → AAA and CCG → CGG ) . The sites of silent mutations are lacking dash ( The sequence included missense mutation is only shown ) .

B) The protein alignment of the same mutant katG gene against the wild kat G gene. Complex missense point mutations at codon positions 315 (Serine → Thereoine) and codon 444(Valine → Alanine) are shown in-between the arrows ( The sequence included missense mutation is only shown ) .
A) This figure represents the nucleotide alignment of one of the mutant inh A gene against the wild type of inh A gene. The site of missense point mutation is shown in-between the arrows (CGA → CCA). The sites of silent mutations are lacking dash (The sequence included missense mutation is only shown).

B) This figure represents the protein alignment of the same mutant inh-A gene against the wild inh-A gene. Missense mutation at codon 94 (Serine → Valine) is shown by the arrow (The sequence included missense mutation is only shown).

**DISCUSSION**

Multi-drug resisting strains of TB are considered to be serious threats to the success of TB control programs. They lead to higher mortality rates and patients require treatment with more toxic second line drugs and remain infectious for longer time, requiring higher cost due to prolonged hospitalization (13, 14).

Manual DNA sequencing is a rapid and accurate method for early detection of drug resistance which is also much less expensive than automated one. It shortens the time needed for the start of treatment and it reduce the risk of transmission of resistant strains (11).

In this study, we used manual gene sequencing to detect missense mutations in the amplified regions of the following M. TB genes: rpoB gene (involved in resistance to RIF), katG and inh-A genes (involved in INH resistance) (15). The study involved 15 patients who were previously diagnosed to have active pulmonary tuberculosis with previous treatment with first line anti-TB drugs (including both RIF and INH) with no response for 3 months.

The present results revealed that, missense mutations in rpoB gene were found in 93.3% of tuberculous patients which could explain the failure of therapeutic response to RIF in such patients. RIF is a semisynthetic derivative of rifamycin that is used as a first line anti-TB drug. It binds to the B-subunit of RNA polymerase encoded by rpoB gene and inhibit transcription initiation (16).
In a similar study (17), it was found that 96% of RIF resistant isolates in India have point mutation in rpoB gene and these mutations are absent in susceptible isolates making them an ideal target for development of molecular drug susceptibility testing method.

Our results were also in accordance with Lee et al., 2005 (18), who found that the frequency of rpoB gene mutations among RIF resistant isolates in Singapore was 98% and 96.1% respectively. Moreover, Yeun et al., 1999 (19) analyzed rpoB gene in 21 RIF resistant M.TB isolates in Brazil where they found 100% point mutation while Karahan et al., 2004 (20) found that the percentage of the mutations among rpoB gene in 80 RIF resistant isolates in Turkey was 90%.

On the other hand, Ahmad and Mokaddas, 2005 (21) identified a rare mutation, namely insertion mutation 514TTC in 9% of DNA sequenced rpoB genes in 32 RIF resistant M.TB isolates in Kuwait.

In our study, we found that the sites of missense point mutations in rpoB gene were in codon 184 (80%) and codon 174 (13.3%). In some other studies, mutations were noticed in other codons such as codon 531, 526 and 516 (18), codon 507 and 533 (17) and codon 531, 526, 516 and 513 (20). The variation in frequencies and codon position of point mutations in different genes was previously explained by the different geographical distribution (22,23). Moreover, Bartafai et al., 2001 (24) reported that in certain geographical areas, less common or novel mutations occur more frequently.

In the present study, it was also found that the incidence of missense mutation of kat G genes was 100% which could explain the resistance of all patients to INH. These results were in accordance with Kapur et al., 1995 (25) who estimated mutant kat G genes in100% of 34 INH resistant isolates. On the other hand, Hoffling et al., 2004 (22) found less frequent mutations in INH resisting isolates (61.4%). They stated that the absence of mutation in some of the INH resistant isolates was explained by the fact that resistance to INH could be mediated by mutations in genes other than kat G gene, as it has been proven that a complex array of mutations involving kat G, inhA, ndh and oxyR-ahpC intergenic regions are associated with INH resistance. However, they also reported that extensive research revealed that katG, which encodes a catalase-peroxidase enzyme involved in the activation of the pro drug INH into its active form, is the main site of mutation.

Missense point mutation at codon position 315 represented 80% of mutations in kat G gene in our study. This result was in agreement with Mokrousov et al., 2002 (26) who found this mutation in 93.6% of INH-resistant strains. On the other hand, Hofling et al., 2004 (22) and Ozturk et al., 2005 (3) found lower rate of mutation in kat G gene at codon position 315 (in 47.9% and 60% respectively). Our results were also in agreement with Kapur et al., 1995 (25) who identified mutations in kat G genes involving codon 315 and 463.

Mutations in katG gene outside codon 315 were also recorded in the present study and some others, as we found mutation at codon position 444 in 13.3% and in both codon 315 and 444 in 6.7% of genes. Similarly, Mokrousov et al., 2002 (26) recorded mutation at codon 314.

On the other hand, inh-A gene revealed 86.7% missense mutation which was at codon 94 in 60%, codon 99 in 20% and codon 69 in 6.7%. Our results disagreed with Kapur et al., 1995 (25) who made automated sequencing of entire inh-A genes from 37 INH resistant isolates and found no missense point mutations. This could be explained by the heterogeneity of genes linked to INH resistance (22,27).

The sequencing results of the examined 3 genes revealed that 14/15 (93.3%) of patients were resistant to both rifampicin and isoniazid together while only one patient (6.7%) was resistant to isoniazid alone as a result of missense mutation in the corresponding genes. This was in concordance with the results obtained by sputum culture and sensitivity for these patients using BACTEC system.

In the present study, DNA of tubercle bacilli was isolated from peripheral blood of the patients as previously recommended (7) in order to avoid the high infectivity of sputum samples which were strong positive for resisting acid fast bacilli. It was suggested that handling of sputum samples for DNA preparation is laborious,
time consuming, highly infectious and could be the origin of PCR-inhibiting substances. On the other hand, patients with active TB infection were found to harbor mycobacterial DNA in their peripheral macrophages, not only in immunosuppressed as previously thought, but also in immunocompetent. So, the use of blood based PCR was suggested to make the diagnosis of pulmonary TB more safer and easier as regards; sampling, handling and processing (7,28).

- **Conclusion**: It could be concluded that, manual DNA sequencing is a rapid and accurate technique that could be of a high value in rapid detection of mutant genes for drug resistance. This could improve the chance to optimize early treatment and improve the prognosis in TB. It could be also concluded that inspite of the presence of common codon position mutation among each gene, there was a wide range of variation between codon position mutation among genes from our locality compared to others, which indicated that geographical regions have important effect on distribution of such mutations.

- **Recommendation**: More facilities for manual DNA sequencing should be available as it is a rapid and accurate method for detection of drug resistance. It shortens the time needed for the start of treatment and it reduce the risk of transmission of resistant strains. Although it is difficult to be done as a routine test for each patient, it could be done for selected cases where it does not need the expensive automated sequencer.

**REFERENCES**


The study investigated the presence of ‘flora’ in the blood and tissue samples collected from patients. The samples were analyzed for the presence of ‘flora’ and the results were compared with the ‘flora’ present in the tissues of the patients. The study also examined the survival rate of ‘flora’ under different conditions.

It was observed that the survival rate of ‘flora’ was significantly higher in the blood samples compared to the tissue samples. This could be due to the presence of certain compounds in the blood that are not present in the tissues.

The study also examined the effects of different treatments on the survival rate of ‘flora’. It was found that treatment with antibiotics significantly reduced the survival rate of ‘flora’.

Overall, the study provides valuable insights into the behavior of ‘flora’ in blood and tissue samples and the effects of different treatments on their survival.