Iraqi Journal of Biotechnology, May 2018, Vol. 17, No. 1, 51-59

Molecular diagnosis and DNA fingerprinting based on *IS6110* of *Mycobacterium tuberculosis* isolated from patients in Iraq

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Received: December 11, 2017 / **Accepted**: March 26, 2018

Abstract: Polymerase chain reaction assay (PCR) used for the detection of *Mycobacterium tuberculosis* strain and fingerprint study. Ten positive cultures on Lowenstein Jensen (LJ) media were collected from specialized center of chest and respiratory disease\Baghdad .genomic DNA was extracted from all isolates and subjected to genetic characterization by (PCR) methods. two specific primers were used in pcr analysis for detection *16S rRNA* and insertion sequence *IS6110* where commonly used as a target of Mycobacterium. The result of gel electrophoresis showed that all isolates belong to Mycobacterium, and a sequence showed that 99% identity in sequence of *16S rRNA* of *M. tuberculosis* and there is one nucleotide changes, the isolate submission in gen bank NCBI with accession number (MG03060) and 100 identity in sequence of *IS6110 of M. tuberculosis.* The isolate showed there is Ten polymorphism diversity according to copy number of *IS6110* that have, where 80% of *M.tuberculosis* has large copy number (6_10) and 20% have low copy number (1-5) of isolate. This study showed that *M.tuberculosis* isolated in Iraq belong to two groups. Polymorphism groups depended on copy number of IS6110.

Keywords: *Mycobacterium tuberculosis, 16S rRNA, IS6110*- DNA fingerprinting.

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Induction:

Tuberculosis (TB) is a devastating disease that has already been present in the human population since prehistoric times. the majority of cases of human TB are caused by *Mycobacterium tuberculosis* (MTB), a species among the genus *Mycobacterium* that are acid-fast, non-motile, slow-growing aerobic bacilli (1, 2, 3)

This disease is one of the most devastating infectious diseases. Globally, about one-third of the world population is believed to be infected with the tuberculosis bacillus and new carriers occur at a rate of one per second (4). Despite advances and developments in diagnosing and treating TB, TB a major health burden around the globe. An estimated 10.4 million new cases and 1.8 million deaths occurred in 2015 according to report of WHO. Iraq is considered among eight high TB burden countries in Eastern Mediterranean Region .In Iraq, according to a recent report of the Ministry of health (MOH), the incidence rate (45/100.000) inhabitants, (5).

Conventional detection of TB is based on a number of protocols, including microscopic examination of smears stained with the Ziehl-Neelsen stain acid-fast bacilli (AFB) (6). Mycobacterial culture has higher sensitivity and specificity than AFB smear but it takes a long time to verify

the results as a long incubation time is required (colonies are rarely visible before 6-8 weeks) (7,8,9)

A large number of different molecular methods based on DNA fingerprints have been developed. Molecular methods have been increasingly used in the diagnosis of TB (10). Nucleic acid amplification based techniques are potentially the most rapid and sensitive methods for detection, identification, and susceptibility testing. These methods can potentially reduce the diagnostic time from weeks to days (11,12 ,13).

Direct sequencing of the bacterial *16S rRNA* gene) has proven to be a stable and specific marker for mycobacterial identification (14, 15, 16).

Eisenach *et al*. (17, 18, 19) described *IS6110* as a repetitive DNA sequence of the *M. tuberculosis* complex genome, and they used *IS6110* PCR to detect *M. tuberculosis* in clinical samples. Since then, many sensitive and rapid PCR- based assays, most of which use *IS6110* as target for mycobacterial DNA amplification, have been developed for the direct detection of M. tuberculosis in a variety of clinical samples obtained from adults [20.21]. We use developed a rapid PCR assay that specifically types M. tuberculosis isolates by generating distinct DNA band patterns or fingerprints. This assay utilizes sequences in *IS6110,* which occurs as repeated copies in the chromosomes of most M. tuberculosis isolates (22.23.24). The aim of this study using molecular methods for identification of *M.tuberculosis* isolates from Iraqi patients in Baghdad. And the present study was undertaken to determine the suitability of *IS6110* element detection by PCR for the early diagnosis of new TB cases. This assay also has considerable power to differentiate *M.tuberculosis* strains and is highly reproducible by fingerprints.

Materials and Methods:

10 isolates on LJ media previously isolated from patient attended specialized center of chest and respiratory disease/Baghdad. DNA extraction was carried out according to manufactures instruction of Genotype MTBDR pluse kit.

DNA Concentration and Quantification:

Quantity of DNA was confirmed using the NanoDrop ND-1000 spectrophotometer.

PCR for detection of *Mycobacterium tuberculosis*:

Two primers were used in the PCR experiment for the detection of *M. tuberculosis*, and Primer used for fingerprinting, Table(1).

Table (1): sequence of primer used for pcr amplification in this study

Primers name	Sequences(5-3)	Size	Ref
16S rRNA(f)	ACGGTGGGTACTAGGTGTGGGTTTC	542 bp	25
16S rRNA(R)	TCTGCGATTACTAGCGACTCCGACTTCA		
INS-1	CGTGAGGGCATCGAGGTGGC	245 bp	26
INS-2	AAACAGTGGCTGCGGATGCG		
F-1	CGCCAGAGACCAGCCGCC		27
F-2	CGCCAGAGACCAGCCGCC		

PCR mixture was prepared with 25 µl of Green Master Mix 2x (promega), 17µl of nuclease free water, 2µl of each primer at 10 pmol/µl, and 4µl of DNA (equaling 25 to 250 ng). Thermocycling conditions of 95°C (initial denaturation) for 5 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute , and annealing at 60°C for 1 minute and 72°C for 1 minute, then the final extension step at 72 °C for 10 minutes concluded the reaction program.

Products (10µl) and DNA molecular weight marker were electrophoresed on 1% Agarose gel with ethidium bromide staining to verify the size of the amplicon, the remaining of

PCR products of *16S rRNA* and insertion sequence *IS6110* were sequenced at (NICEM-USA, Apparatus: Applied Biosystem).

Results and Discussion:

Ten positive cultures were subject to molecular diagnosis by PCR , the genome of isolates was extracted and purified and The concentration and purity of DNA were measured by nanodrop and the concentration of DNA was ranged between 100 to 420 ng/µl and the purity of DNA (A260/A280) ranged between 1.4 to 1.8

Molecular Identification of *M. tuberculosis* isolates:

Molecular identification of *M. tuberculosis* based on the using of partial *16S rRNA* to prove the extracted DNA was belonged to *Mycobacterium* genus, the product of amplification 542 bp occupying (1472650-1473192), the results are shown that all isolate have *16S rRNA* (Figure 1). The amplification of partial *16S rRNA* gene was used to confirm the presence of Mycobacterial DNA in the sample (28) The *16S rRNA* sequence is an appealing target for the purpose of molecular identification; it is stable property and it contains nucleic acid information allowing the identification of mycobacteria at the genus level as well as the rapid recognition of previously undescribed mycobacterial pathogen (29,30,31).

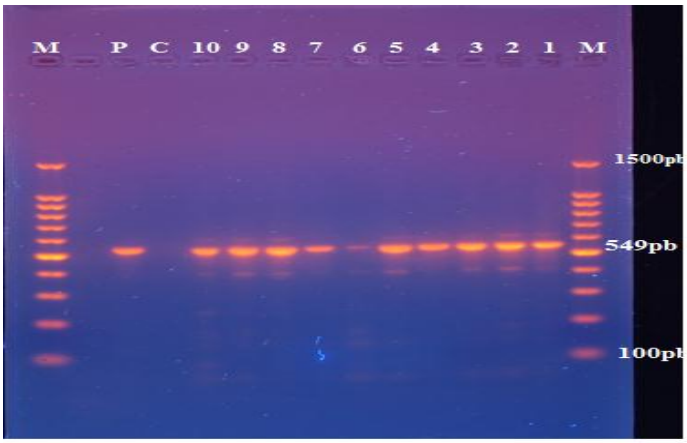


Figure (1): Gel electrophoresis for PCR product of 16SrRNA 543bp using (1.5%) agarose for 90 minutes at 60 volt.

1. **M – 100 bp ladder.**
2. **c- Negative isolates.**
3. **P- Positive isolates.**
4. **(1-10) PCR product of positive isolates from patients**

Presence of insertion sequence *IS6110* in isolates of *M. tuberculosis complex:*

In this study, insertion sequence *IS6110* was used to differeniate *M. tuberculosis complex from* the other of *M. tuberculosis* subspecies, the presence of *IS6110* in *M. tuberculosis* complex collected from different parts of Baghdad and governorates was tested by PCR using specific primer for *IS6110*. The results showed that all isolate contained *IS6110*. These isolates tested for the presence of the sequence contain the *IS6110* suggesting that this sequence is reliable source for the detection and discrimination of *M. tuberculosis* isolated in NRL in Baghdad. (Figure 2). The results indicated that all strains of *M. tuberculosis* carriers *IS6110*. Thus it is possible to conclude that IS6110- based diagnosis is practical regardless of the possible encountering of IS6110- free strains. Other studies (32) have confirmed that *IS6110* can be used for both DNA fingerprinting of *M. tuberculosis* and for diagnosis of tuberculosis disease specifically. The method is more accurate and faster than conventional method for TB diagnosis. Early diagnosis of *M. tuberculosis* disease is crucial in initiating treatment and interrupting the strain transmission. Rapid diagnosis will prevent the development of drug resistant *M. tuberculosis* bacteria.

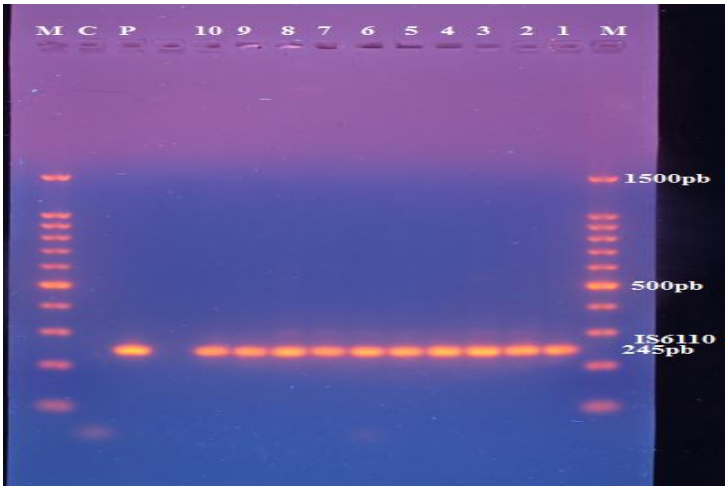


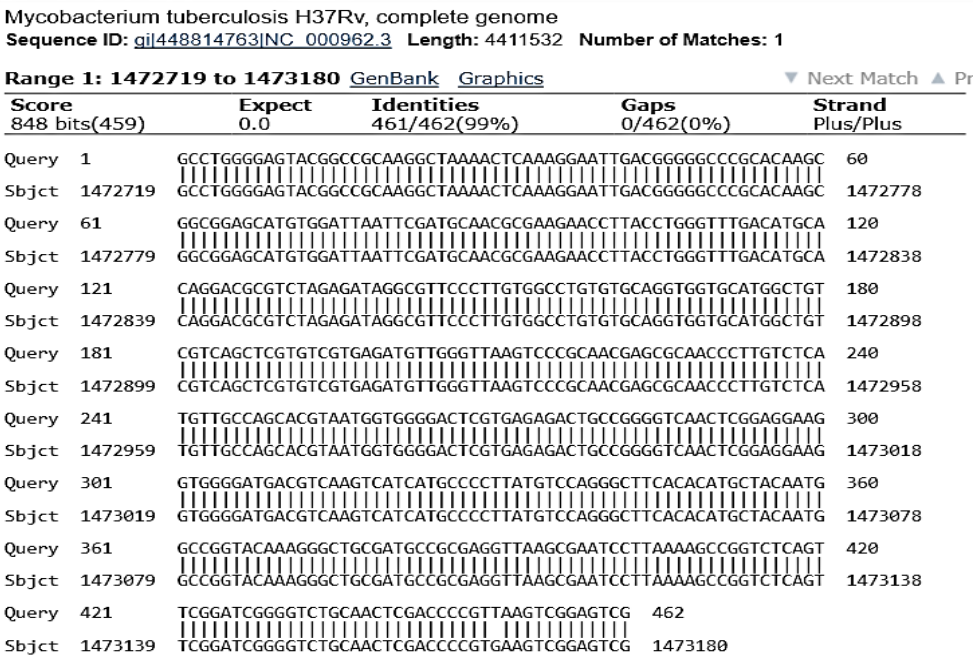
Figure (2): Gel electrophoresis for PCR product of *IS6110* 245pb using (1.5%) agarose for 90 minutes at 60 volt.

1. **M. 100 bp ladder.**
2. **c- Negative isolates.**
3. **P- Positive isolates.**
4. **(1- 10) PCR product of positive isolates from patients**

Sequence of the *16SrRNA* gene and the *IS6110* insertion sequences element:

Sequencing of the *16s rRNA* gene and the *IS6110* gene for M. tuberculosis were analyzed using the genetic analysis tool and compared with the sequencing of the same gene in the NCBI to confirm the molecular diagnosis of Congenital PCR and differentiation *M.tuberculosis* from other types of Mycobacterium, The results of the current study showed that the identity ratio of *16s rRNA* gene

was 99% where the guanin base was replaced with the adenin base in site 1273168 from the genome (figure 3) this sequence of isolation deposited in NCBI with Accesssion number (MG030630) (figure 4) . and the identity ratio of the *IS6110* was 100% (figure5).



Mycobacterium tuberculosis H37Rv, complete genome
Sequence ID: gi|448814763|NC_000962.3 **Length:** 4411532 **Number of Matches:** 1

Range 1: 1472719 to 1473180 GenBank Graphics ▼ Next Match ▲ Pr

Score	Expect	Identities	Gaps	Strand
848 bits(459)	0.0	461/462(99%)	0/462(0%)	Plus/Plus

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Sbjct  1472719  GCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC  1472778

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Sbjct  1472779  GGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCA  1472838

Query  121      CAGGACGCGTCTAGAGATAGGCGTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTGT  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  181      CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTCA  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  1473019  GTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATG  1473078

Query  361      GCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAAGCGAATCCTTAAAAGCCGGTCTCAGT  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1473079  GCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAAGCGAATCCTTAAAAGCCGGTCTCAGT  1473138

Query  421      TCGGATCGGGGTCTGCAACTCGACCCCGTTAAGTCGGAGTCG  462
                ||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1473139  TCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCG  1473180
```

Figure (3): Result of alignment of *16S rRNA* according to NCBI data.

```
LOCUS       Seq1                       451 bp    DNA     linear   BCT 04-OCT-201
DEFINITION  DNA,partial.
ACCESSION   Seq1
VERSION
KEYWORDS    .
SOURCE      Mycobacterium tuberculosis
  ORGANISM  Mycobacterium tuberculosis
            Bacteria; Actinobacteria; Corynebacteriales; Mycobacteriaceae;
            Mycobacterium; Mycobacterium tuberculosis complex.
REFERENCE   1  (bases 1 to 451)
  AUTHORS   Mohammed,k.M.
  TITLE     Mycobacterium tuberculosis
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 451)
  AUTHORS   Mohammed,k.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-OCT-2017) Diyala university, Diyala university,
            Iraq/diyala, diyala, diyala 00964, iraq
COMMENT     Bankit Comment: ALT EMAIL:wahj_dna@yahoo.com.
            Bankit Comment: TOTAL # OF SEQS:1.

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /isolation_source="Sputum"
                     /host="Homo sapiens"
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                     /db_xref="taxon:1773"
                     /clone="16S rRNA-IRQ"
                     /country="iraq"
                     /collection_date="2017"
                     /collected_by="Ahmmed A.mankhi"
                     /note="[cultured bacterial source]"
     gene            <1..>451
                     /gene="16S rRNA"
     rRNA            <1..451
                     /gene="16S rRNA"
                     /product="16S ribosomal RNA"
BASE COUNT      101 a     111 c     144 g      95 t
ORIGIN
        1 acggccgcaa ggctaaaact caaaggaatt gacgggggcc cgcacaagcg gcggagcatg
       61 tggattaatt cgatgcaacg cgaagaacct tacctgggtt tgacatgcac aggacgcgtc
      121 tagagatagg cgttcccttg tggcctgtgt gcaggtggtg catggctgtc gtcagctcgt
      181 gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc cttgtctcat gttgccagca
      241 cgtaatggtg gggactcgtg agagactgcc ggggtcaact cggaggaagg tggggatgac
      301 gtcaagtcat catgcccctt atgtccaggg cttcacacat gctacaatgg ccggtacaaa
      361 gggctgcgat gccgcgaggt taagcgaatc cttaaaagcc ggtctcagtt cggatcgggg
      421 tctgcaactc gaccccgtta agtcgaagtc g
//
```

Figure (4): Accession number of isolate (MG030630)

Mycobacterium tuberculosis H37Rv, complete genome
Sequence ID: NZ_CP009480.1 **Length:** 4396119 **Number of Matches:** 16

Range 1: 888085 to 888267 GenBank Graphics ▼ Next Match ▲ Previous M

Score	Expect	Identities	Gaps	Strand
339 bits(183)	1e-91	183/183(100%)	0/183(0%)	Plus/Plus

```
Query  1       CAAACTCGGCCTGTCCGGGACCACCCGCGGCAAAGCCCGCAGGACCACGATCGCTGATCC  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  888085  CAAACTCGGCCTGTCCGGGACCACCCGCGGCAAAGCCCGCAGGACCACGATCGCTGATCC  888144

Query  61      GGCCACAGCCCGTCCCGCCGATCTCGTCCAGCGCCGCTTCGGACCACCAGCACCTAACCG  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  888145  GGCCACAGCCCGTCCCGCCGATCTCGTCCAGCGCCGCTTCGGACCACCAGCACCTAACCG  888204

Query  121     GCTGTGGGTAGCAGACCTCACCTATGTGTCGACCTGGGCAGGGTTCGCCTACGTGGCCTT  180
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Query  181     TGT  183
               |||
Sbjct  888265  TGT  888267
```

Figure (5): Result of alignment of *IS6110* according to NCBI data

PCR Fingerprinting:

The development molecular methods to genetically differentiate *M. tuberculosis* strains provide a useful epidemiological tool. The first described method for differentiating *M. tuberculosis* isolates used mycobacteriophages or enzymatic characterization(33) Although useful for these studies of specific outbreaks these techniques had very low discriminatory power (34). DNA fingerprinting of *M. tuberculosis* isolates has increasingly been used in epidemiological studies. DNA was compared visually for similarity based on presence or absence of bands and variation in bands intensity were taken to constitute strains differences (35). Our study presents a precise picture of the epidemiology of TB in different regions in Iraq by applying both molecular and classical epidemiological methods. Figure (6) showed fingerprints patterns on agarose gel. The number of copies was distributed within a range of 1-10 copies. These can be classified into two groups, group A, the isolates that have less than 6 copies of *IS6110* and group B, and the isolates that have (more than 6 copies of *IS6110* in their chromosomal DNA (36) Of 10 *M. tuberculosis* isolates (20%) belong to group A and (80%) belong to group B.

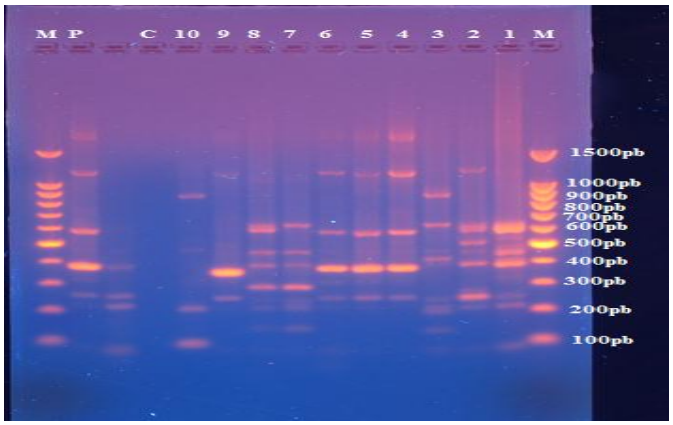

Figure (6) PCR fingerprinting (agarose 1.5%) of *M.tuberculosis* strains based on the number of IS6110 copies:

1. **M – 100 bp ladder.**
2. **c- Negative isolates.**
3. **P- Positive isolates.**
4. **(1- 10) PCR product of positive isolates from patients.**

The result show there was clear diversity banding among the ten isolates. Identification and differentiation of strains of *M. tuberculosis* by molecular technique of targeted insertion elements has provided a better understanding of the epidemiology of infection (37).In the present study different *IS6110*-based patterns were observed for *Mycobacterium tuberculosis* isolates from different regions of Iraq, suggesting differences in copy number and genomic location of the element *IS6110*.the result indicates that there was substantial diversity of *IS6110* banding among isolates of *M. tuberculosis* in Iraq. This may refer to low degree of active transmission of tuberculosis among patients. Cases examined may have arisen from reactivation of previous infection. This study has demonstrated that the majority of isolates have multiple *IS6110* copies (80%). These findings indicate that *M. tuberculosis* strains isolated in Iraq are similar to those found in Iraq and other countries.

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