

Characterization of *gyrA* and *gyrB* Genes in *Mycobacterium Tuberculosis* Clinical Isolates from Mosul, Iraq

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Abstract Objectives: Tuberculosis (TB) remains among the top 10 causes of death globally. A total of 1.23 million people died from TB in 2024. Accurate and timely identification of TB is essential to initiate anti-TB medication promptly. **Aim:** This study aims to evaluate the diagnostic performance of Ziehl-Neelsen staining and the GeneXpert MTB/RIF assay for detecting *Mycobacterium tuberculosis* in clinical samples and to analyze the phylogenetic relationships of the *gyrA* and *gyrB* genes from a local isolate in Mosul, Iraq, with 15 other global isolates. **Methods:** 190 sputum and other clinical samples were analyzed using Ziehl-Neelsen staining, GeneXpert assay and PCR detection of *gyrA* and *gyrB* genes. **Results:** Of the 190 tuberculosis-suspected patients, 49 samples tested positive by the GeneXpert method and 43 samples tested positive using Ziehl-Neelsen staining method. Sequence analysis of the *gyrA* (OQ955242.1) and *gyrB* (OQ955243.1) genes from the local isolates (Mosul, Iraq) showed limited sequence variation compared with 15 global strains in the NCBI database. **Conclusion:** GeneXpert MTB/RIF is a highly sensitive and accurate method for detecting *Mycobacterium tuberculosis* in clinical samples compared with Ziehl-Neelsen staining. *gyrA* and *gyrB* genes of the local *Mycobacterium tuberculosis* isolate are closely related to global strains.

Key Words Tuberculosis, *GyrA*se, Clinical Isolates, Iraq

INTRODUCTION

In 2024, an estimated 10.7 million cases of TB worldwide were reported [1]. This trend is exemplified in developing countries in South Asia and the Middle East, where TB is increasingly prevalent due to under-resourced healthcare systems, social and cultural factors and political instability.

Iraq is a clear example of how the above factors have led to increased rates of TB. Iraq has a TB incidence rate of about 3% of the worldwide incidence of TB and has potential for high rates of transmission due to extensive years of war and conflict [2]. The number of persons with active TB (approximately 40-45 per 100,000 at the time of writing) is expected to remain high for the foreseeable future unless Iraqis can strengthen their healthcare system. [3,4]. These challenges highlight how systemic and social factors significantly influence disease outcomes.

Screening is crucial for controlling TB, as it helps catch both active and latent infections early, which is key to preventing spread and severe illness. The traditional

Tuberculin Skin Test (TST) remains widely used but has drawbacks, particularly among BCG-vaccinated individuals, as it can yield false-positive results [5]. Tests such as Interferon-Gamma Release Assays (IGRAs) offer better specificity and are less affected by prior vaccination [6].

For active TB, Ziehl-Neelsen staining remains common, especially in resource-poor areas, but it isn't as sensitive as culture on Lowenstein-Jensen medium, which takes weeks to yield results [7,8]. The GeneXpert MTB/RIF assay detects *Mycobacterium tuberculosis* and rifampicin resistance in just a few hours, helping to start treatment sooner and reduce transmission [9]. In countries like Iraq, widespread use of such screening tools is critical for effective TB control.

One serious challenge in TB treatment is Multidrug-resistant TB (MDR-TB), which resists at least two major TB drugs, complicates therapy and leads to poorer outcomes. In Basra province, the rate of MDR-TB increased between 2016 and 2020. The rate of MDR-TB among new cases has ranged from 0.17% to 1.6% [10]. A study in Baghdad found that 69.1% of the isolates were resistant strains [11].

Fluoroquinolones, an important class of second-line TB drugs, work by targeting the DNA *gyrA*se enzyme, a critical bacterial protein encoded by the *gyrA* and *gyrB* genes. Fluoroquinolone resistance typically arises from mutations in specific regions of these genes, particularly *gyrA*. These mutations alter the target site, preventing the drugs from working effectively [12]. Detecting these genetic changes quickly is essential for determining the most effective treatment plans and for controlling the spread of resistant strains [13]. Understanding the types and frequency of these mutations also helps public health officials track and manage resistance patterns [14].

Phylogenetic studies, which examine the genetic relationships among bacterial strains, utilize sequence data from genes such as *gyrA* and *gyrB* to determine the degree of relatedness among TB strains. This helps scientists understand the diversity and evolution of *Mycobacterium tuberculosis* strains worldwide. Phylogenetic trees constructed from *gyr* gene sequences can reveal transmission links and highlight the spread of drug-resistant variants [14,15].

These molecular tools complement other genotyping methods and offer insights that can improve disease surveillance, especially in regions such as Iraq, where comprehensive genetic data are limited. Better knowledge of strains circulating in a particular area helps tailor public health responses and treatment strategies [3].

This study aims to evaluate the diagnostic performance of Ziehl-Neelsen staining, culture on Lowenstein-Jensen medium and the GeneXpert MTB/RIF assay for detecting *Mycobacterium tuberculosis* in sputum samples and to analyze the phylogenetic relationships of the *gyrA* and *gyrB* genes from local isolates in Mosul, Iraq, with 15 other global strains.

METHODS

Ethical Considerations

Current study's ethical approval was granted by the institutional ethics committee of the College of Education for Women, University of Mosul. All patients were fully informed about the nature of the procedure, the purpose of the research and their right to withdraw from the study at any time without affecting their medical care, in accordance with the Declaration of Helsinki and its subsequent modifications.

Sample Collection

About 190 early-morning sputum samples and other clinical samples were aseptically collected from 190 tuberculosis-suspected patients at the Respiratory Infectious Disease Center in Mosul, Iraq, between December 2024 and March 2025.

Smear Preparation and Ziehl-Neelsen Staining

We deposited about 100 µL of sputum onto the slide using a micropipette. Smears were first air-dried at room temperature, then heat-fixed before Ziehl-Neelsen staining. The slide was treated with carbol fuchsin and heated for 5 minutes until steaming. Then, it was immersed in 3% acid-alcohol for 2-3 minutes. After being washed with distilled

water, it was immersed in methylene blue for 1-2 minutes, then washed again with distilled water, air-dried and examined under the microscope. The background appears blue, while acid-fast organisms are stained red [8].

GeneXpert (MTB/RIF) Test

Using the GeneXpert (Smartcycler, Cepheid, USA), Sputum samples were processed according to the manufacturer's protocol. In a Falcon tube (15 mL), the sample reagent was mixed with the raw sputum at a 2:1 ratio, then transferred to the test cartridge using a sterile disposable pipette. Then, it was placed on the GeneXpert recycler (35 cycles, annealing at 54°C). The data interpretation was not user-dependent (software-based) [9].

Culture of *M. Tuberculosis* on Lowenstein-Jensen Medium

Five positive sputum specimens were liquefied and decontaminated for 15 min at room temperature by adding an equal volume of N-acetyl-L-cysteine solution and 1% NaOH, then inoculated into the Mycobacteria Growth Indicator Tube (MGIT) containing Lowenstein-Jensen medium (HiMedia, India), incubated for approximately 1 week and monitored weekly. If there was no observed growth during (1-8) weeks, the result was reported as (no growth) [7].

Genomic DNA Extraction and PCR Detection of *gyrA* and *gyrB*

Total genomic DNA was isolated directly from 5 *Mycobacterium tuberculosis* isolates using a bacterial genomic DNA isolation kit from Foregene (China) according to the manufacturer's instructions. The binding buffer was supplemented with beta-mercaptoethanol (user-provided) to a final concentration of 0.5% (v/v) (500 µL per 100 mL).

Specific primer genes *gyrA* and *gyrB* were detected by PCR [16]. The primers for *gyrA* were *gyrA*-F 5'-CTATGCAATGTTTCGATTCCGGCTTC-3' and *gyrA*-R 5'-ACTGTCTCCTCGTCGATTTCCCT-3'. For *gyrB*, the forward primer was *gyrB*-F 5'-AGAGTTGGTGC GGCGTAAGA-3 and the reverse primer was *gyrB*-R 5'-AACACATGCCCCGTTCTCGAT-3'. These primers were used to amplify a region of the *gyrA* (320 bp) and *gyrB* (375 bp) genes. PCR reactions were performed with 10 pmol/µL of each primer and 100 ng of template DNA, according to the manufacturer's protocol (Foregene, China).

The PCR thermal cycling conditions were as follows: denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 54°C for 45 sec and extension at 72°C for 45 sec, with a final extension at 72°C for 5 min. The PCR products were resolved by agarose gel (2%), stained with Midori Green Advance DNA stain. A molecular weight marker was provided by a 100 bp DNA ladder (New England Biolabs, UK).

The PCR amplicon product was sequenced according to the Sanger method using the (Genetic Analyzer System ABI-310 Macrogen, Korea). The sequence data generated in this study were deposited in the NCBI database under accession numbers OQ955242.1 for *gyrA* and OQ955243.1 for *gyrB*.

Phylogenetic Analysis based on *gyrA* and *gyrB* Sequences

The evolutionary history was inferred using the UPGMA method [17]. The clustering is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer it. The evolutionary distances were computed by the Maximum Composite Likelihood method [18] and are expressed in units of base substitutions per site. This analysis involved 16 nucleotide sequences for each gene. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were deleted. There were 235 positions in the final dataset for *gyrA* and 356 for *gyrB*. Evolutionary analyses were conducted in MEGA6 [19].

Statistical Analysis

The diagnostic performance of Ziehl-Neelsen staining and GeneXpert was evaluated against culture on Lowenstein-Jensen media as the reference standard. Results were presented descriptively. The evolutionary analyses were performed in MEGA X [20].

RESULTS

Mycobacterium tuberculosis Detection

By testing 190 clinical samples from TB-suspected patients, 49 positive results were obtained by GeneXpert (25.7%), compared with 43 positive results by Ziehl-Neelsen staining method (22.6%).

Detection of *gyrA* and *gyrB* Genes

The *gyrA* and *gyrB* genes were recovered directly from 20 of the positive samples. The amplification results for these genes (Figures 1 and 2) showed that *gyrA* was 320 bp and *gyrB* was 375 bp, as expected.

gyrA and *gyrB* Sequence

To further confirm the diagnosis, one member of each gene was sequenced and the results were used to support identification and to construct phylogenetic trees for both genes (Table 1 and 2).

Phylogenetic Tree

The nucleotide sequences of selected *gyrA* and *gyrB* genes were identical to those of the corresponding genes in *M. tuberculosis*

in the NCBI database. The evolutionary relationship was inferred using the Neighbor-Joining method. The phylogenetic distance was computed using the Jukes-Cantor model in Gene 6.

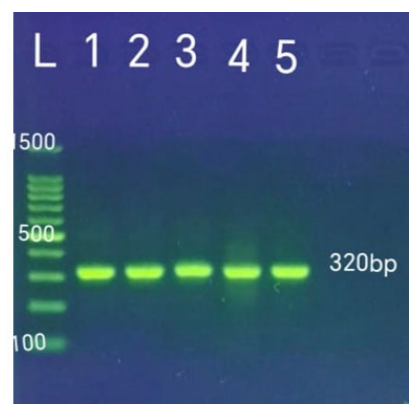


Figure 1: The PCR Product shows the Expected Band Size for *gyrA* (320 bp). The Amplicon Product was Separated by Agarose (2%) Electrophoresis at 5 V/cm in 1x TBE for 1 h. L: 100bp (DNA ladder), 1-5 *Mycobacterium tuberculosis* Local Isolates



Figure 2: The PCR Product shows the Expected Band Size for *gyrB* (375 bp). The Amplicon Product was Separated by Agarose (2%) Electrophoresis at 5 V/cm in 1x TBE for 1 h. L: 100 bp (DNA ladder), 1-5 *Mycobacterium tuberculosis* Local Isolates

Table 1: *gyrA* Gene Sequences of *M. tuberculosis* Local Isolate (Total 235 bp)

Position	Sequence					
1-60	AGTCGGCCCG	GTCGGTTGCC	GAGACCATGG	GCAACTACCA	CCCGCACGGC	GACGCGTCGA
61-120	TCTACGACAC	CCTGGTGC GC	ATGGCCCAGC	CCTGGTCGCT	GCGCTACCCG	CTGGTGGACG
121-180	GCCAGGCAA	CTTCGGCTCG	CCAGGCAATG	ACCCACCGGC	GGCGATGAGG	TACACCGAAG
181-235	CCCGGCTGAC	CCCGTTGGCG	ATGGAGATGC	TGAGGGAAAT	CGACGAGGAG	ACAGT

Purified amplicon products were sequenced using the Genetic Analyzer System ABI-310 Macrogen, Korea)

Table 2: *gyrB* gene Sequences of *M. tuberculosis* Local Isolate (Total 356 bp)

Position	Sequence					
1-60	AGCTGGCCGA	TTGCCGTTCC	ACGGATCCGC	GCAAGTCCGA	ACTGTATGTC	GTAGAAGGTG
61-120	ACTCGGCCGG	CGGTTCTGCA	AAAAGCGGTC	GCGATTTCGAT	GTTCCAGGCG	ATACTCCGC
121-180	TGCGCGGCAA	GATCATCAAT	GTGGAGAAAG	CGCGCATCGA	CCGGGTGCTA	AAGAACACCG
181-240	AAGTTCAGGC	GATCATCAGC	GCGCTGGGCA	CCGGGATCCA	CGACGAGTTC	GATATCGGCA
241-300	AGCTGCGCTA	CCACAAGATC	GTGCTGATGG	CCGACGCCGA	TGTTGACGGC	CAACATATTT
301-356	CCACGCTGTT	GTTGACGTTG	TTGTTCCGGT	TCATGCGGCC	GCTCATCGAG	AACGGG

Purified amplicon products were sequenced using the Genetic Analyzer System ABI-310 Macrogen, Korea)

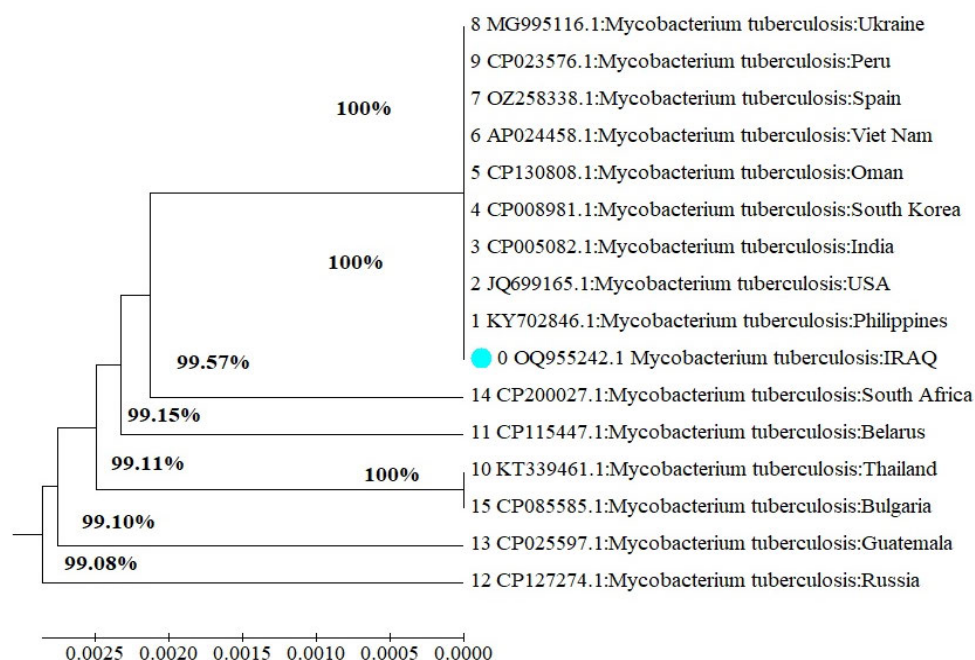


Figure 3: The Maximum-Likelihood Evolutionary Tree of 16 Strains based on *gyrA* Sequences. The Optimal Tree, with a Total Branch Length of 0.01541609, is shown. There were 235 Positions in the Final Dataset

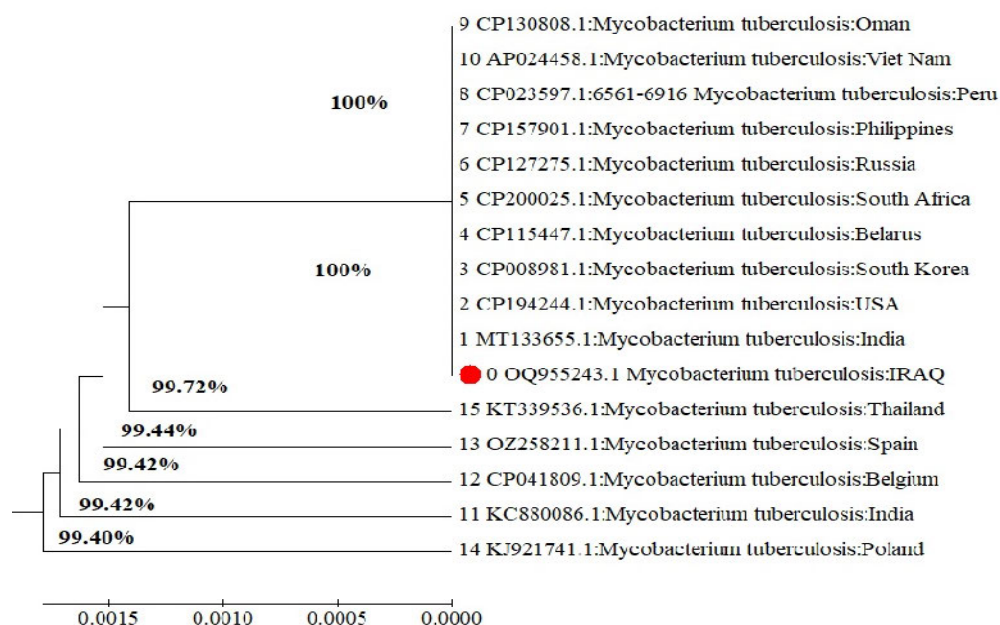


Figure 4: The Maximum-Likelihood Evolutionary Tree of 16 Strains based on *gyrB* Sequences. The Optimal Tree, with a Total Branch Length of 0.00983318, is shown. There were 356 Positions in the Final Dataset

Table 3: Mutation Analysis of *gyrA* and *gyrB* local Genes Compared with Reference Strains

Gene	Sequence ID with comparison	Sequence ID with submission	Identities	Nucleotide change	Nucleotide	Location	Amino acid change	Type of substitution	Predicted effect
<i>gyrA</i>	ID: KY702846.1 Philippines	ID: OQ955242.1 Mosul, Iraq	100%	-----	-----	-----	-----	-----	-----
	ID: KT339461.1 Thailand		99%	GGC\GAC	GVA	281	Glycine\Aspartic acid	Transition	Missense
<i>gyrB</i>	ID: MT133655.1 India	ID: OQ955243.1 Mosul, Iraq	100%	-----	-----	-----	-----	-----	-----
	ID: KC880086.1 India		99%	AAC\ACC	A/C	182	Asparagine\Threonine	Transversion	Missense

To obtain the maximum likelihood phylogenetic tree of 16 strains based on *gyrA* (OQ955242.1) and *gyrB* (OQ955243.1) sequences (Figures 3 and 4). Mutation analysis of *gyrA* (OQ955242.1) and *gyrB* (OQ955243.1) was compared with other strains, as shown in Table 3.

DISCUSSION

The results showed that GeneXpert had higher sensitivity and specificity than Ziehl-Neelsen staining and near-perfect accuracy, similar to the results reported by [21].

Ziehl-Neelsen microscopy cannot distinguish between dead and live bacilli; it is also considered less sensitive and requires a concentration of 5-10 thousand bacilli per ml of sputum to yield a positive result.

Furthermore, the test incorporates a protocol for collecting early-morning sputum samples over three days to enhance its sensitivity. Besides its reduced sensitivity, microscopic examination of sputum smear cannot distinguish *M. tuberculosis* from other species, such as *Mycobacterium bovis* [21,22].

The gold standard for the detection of tuberculosis is culture using Lowenstein-Jensen medium. This technique requires a biosafety level III laboratory and is usually performed in 3-8 weeks with high sensitivity. *M. tuberculosis* culture on Lowenstein-Jensen medium is effective when 10 viable bacilli/ml in sputum are present [23]. The time required to culture *M. tuberculosis* to identify resistant strains is typically 3-8 weeks. Drug sensitivity testing under solid media generally takes 2-4 weeks and under broth media it takes 1 week [24,25].

In contrast, the GeneXpert assay can detect *M. tuberculosis* directly from clinical samples in only 2 h. [26]. Multicenter studies have shown that *M. tuberculosis* was detected in all positive smear cases and in around 3/4 of cases with negative smear results [27]. GeneXpert sensitivities in respiratory specimens with smear-negative and high-positive cultures were reported to be 57-76.9% and 98-100%, respectively, with similar specificity (99%-100%) [26,28].

Analysis of the identity matrix indicates sequence similarity of limited genetic variation, underscoring the highly conserved nature of the *gyrA* gene.

Isolates from Russia (CP127274.1) and Guatemala (CP025597.1) show early divergence, with evolutionary distances of 0.0028-0.0029.

Within *gyrA*, such variations are typically attributed to non-synonymous polymorphisms (e.g., the AGC95ACC mutation) that do not necessarily confer resistance but serve as phylogenetic markers for classification [29].

Sequence analysis of the *gyrB* gene across 16 *M. tuberculosis* global strains revealed distinct evolutionary divergence among geographically isolated strains. The evolutionary branch length of 0.0098 indicates relatively limited sequence variation in this critical drug-targeting gene across global strains [30]. The previous investigations by Kadura *et al.* [31] demonstrated that *gyrB* serves as a

selective target for anti-tuberculosis medications, thereby restricting tolerance to random mutations.

Comparative sequence analysis revealed distinct differences between Asian strains (Thailand, Vietnam, India) and African and European isolates, indicating independent evolution of regionally specific lineages. Eldholm *et al.* [32] suggested that the close clustering of isolates from the same geographic region indicates close genetic relatedness.

The high protein compatibility rates (99.40-100% unpublished data) across isolates indicate the preservation of essential enzymatic structures involved in DNA binding. However, strains with compatibility rates (99.40%) may harbor position-specific mutations associated with fluoroquinolone resistance, warranting further analysis of the Quinolone Resistance-Determining Region [33]. Understanding these genetic variations is fundamental for developing targeted therapeutic options.

The identified genetic mutations underscore the importance of regular molecular surveillance of drug-resistant isolates in high-prevalence regions. DNA *gyrA* gene sequencing can function as a diagnostic tool for rapid detection of fluoroquinolone resistance in resource-limited laboratory settings [34].

CONCLUSION

The results highlight the importance of GeneXpert as a tool for achieving early, accurate diagnoses, demonstrating a clear improvement over Ziehl-Neelsen staining and culture on Lowenstein-Jensen medium. Moreover, since the (GeneXpert MTB/RIF) test can determine possible MDR-TB, tuberculosis patients can receive effective treatment much sooner than they normally would. Sequence analysis of the *gyrA* and *gyrB* genes from 16 *M. tuberculosis* strains revealed relatively low genetic variation in these critical drug-target genes across global strains. Monitoring *gyrA* and *gyrB* mutations is of high clinical importance, as they are primary markers of fluoroquinolone resistance in MDR-TB.

Author's Contribution

Rasmia O. Al-Jobory: Sample collection, methodology, investigation, data curation and writing. Sura I. Khudher: Sample collection, methodology, data curation and writing. Nada F. Al-Rawi: Conceptualization, methodology, writing-review and editing.

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Declaration of Competing Interest

The authors declare that they have no competing interests or personal relationships that influence the work in this paper.

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