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Research Article

Analysing the Impact of Bedaquiline Resistance in Fluoroquinolone-Resistant Clinical Isolates and Investigating the Molecular Interactions of BDQ with the atpE Gene

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ABSTRACT

The progress of the tuberculosis eradication programme is hindered by the global health issue of drug-resistant tuberculosis, which is a significant threat to the population. Resistance-associated mutations vary geographically, so investigate the prevalence of resistance to fluoroquinolone (anti-TB) drugs and its association with resistance-related mutations in clinical isolates of Tamilnadu (north zone) and Puducherry was carried out. And the resultant fluoroquinolone drug-resistant tuberculosis strains will be subjected to the molecular analysis of bedaquiline resistance to study the mutation in the atpE gene. The study includes 430 *specimens received at Intermediate Reference Laboratory, State TB training and Demonstration Centre in the Government Hospital for Chest Diseases for Molecular testing between January 2020 and December 2021. Samples were analysed by GenoType MTBDRslV.2 for the molecular detection of mutations in the gyrA and gyrB genes. FQ resistance was observed in 32 samples (7.4%), most of which were naïve and previous treatment failure cases. Twenty-five isolates had mutations in DNA gyrase subunit -A (gyrA), while mutations in gyrB were observed in only 7 isolates. Mutational analysis revealed that the mutations mainly alter codon 94 (replacing aspartic acid with glycine, D94G), and 90 (replacing alanine with valine, A90V). In Isoniazid Resistance, MDR, and treatment failure cases, the resistance to Fluoroquinolones was most commonly associated with the D94G mutation. A molecular stimulation study evaluates the effect of BDQ on atpE gene by docking, using AutoDock 4.2, showing hydrogen, hydrophobic and electrostatic interactions.*

Keywords: *Mycobacterium Tuberculosis, Molecular Docking, gyrA, Fluoroquinolones, Bedaquiline.*

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INTRODUCTION

Tuberculosis (TB) is a significant public health concern in many countries with a high disease burden and mortality rate. TB was the leading cause of death from a single infectious agent, ranking above HIV/AIDS (WHO report, 2022). A total of 13,068 cases of XDR-TB were reported by 81 countries. The latest Global TB report states that the estimated number of new TB cases in India was 2.7million and the mortality number is 440,000 cases (WHO report, 2019). India also has a high burden of drug-resistant TB, most of them are undetected and continue to transmit infection. Which is considered to be a serious threat to people in India.

High-efficiency diagnostic tools for early diagnosis, clinical trials for new medications, vaccinations, and genomic studies for potential TB control are the main areas of focus for the National Strategy Plan (2017–25) (NSP, 2017). Due to spontaneous mutation in these target genes such as rpoB, katG, inhA, embB, gyrA, gyrB, rrs, eis, tlyA and pncA results in MDR, Pre-XDR, and XDR-TB (Seung, 2015). The rate of TB decline is too slow to meet the 2030 Sustainable Development Goals (SDG) and End TB targets. To combat this, the National Tuberculosis Elimination Program (NTEP) emphasis on expanding the scope of TB services and implementing cutting-edge strategic interventions by adhering to the "Detect -Treat -Prevent -Build" model (DTPB) approach (NSP, 2017).

On the front lines of disease control, antibiotic resistance is an issue that is constantly changing and requires diverse treatment strategies. In this study we aim to examine and characterize the single nucleotide variants (SNVs) associated with Fluoroquinolone resistance in the clinical isolates of North-zone of Tamilnadu and Puducherry. We analyse the resistance patterns developed to BDQ by atpE gene among the chosen isolates.

The Food and Drug Administration has authorised a novel drug-Bedaquiline, a diarylquinoline agent, for the treatment of multidrug-resistant tuberculosis. Bedaquiline has a unique and specific mechanism of action, it inhibits the ATP synthase's proton pump by binding to the subunit c of the protein [Deshkar, 2022 & Fox, 2013). Bacterial ATP synthesis is decreased as an outcome of inhibition of ATP-synthase activity by BDQ **(**Segala, 2012). One analysis revealed that it suppresses dormant cells in latent TB infection at a low dosage and that it inhibits both actively replicating and non-replicating mycobacteria (Koul, 2008). It shortens the duration of culture conversion when combined with other MDR-TB medications, suggesting it may be possible to reduce the course of treatment.

The currently reported targets and non-target mutations for BDQ resistance include (i) BDQ target atpE gene mutations; (ii) mutations in the transcriptional repressor Rv0678, which controls the MmpS5-MmpL5 efflux pump; (iii) mutations in pepQ (Rv2435c), encoding cytoplasmic peptidase pepQ (Ghajavand, 2019). The analysis of molecular affinity between the BDQ drug against these genes (atpE, Rv0678 and pepQ) were conducted. So that it may be used as potential diagnostic and therapeutic targets for the creation of new anti-drug resistance strategies (Sharma, 2018).

Here, we provide the findings from the initial phase of this study, which was conducted to assess the resistance pattern for BDQ produced by the atpE gene. Hence, the results of our study will benefit for the appropriate regimens and precise diagnosis.

MATERIALS AND METHODS

Bacterial Sample collection and processing

430 drug-resistant TB isolates of M. tuberculosis which was MTBDRplus assay (v.2.0) positive, were collected from State TB Training and Demonstration Centre (Intermediate Reference Laboratory) between January 2020 to December 2021. In this study, we have selected clinical isolates of patients who were not yet exposed to BDQ in their regimen. All the investigations associated with culture-positive specimens were processed in

biosafety cabinet Class-II in a biosafety level III laboratory. The NALC-NaOH decontamination procedure was employed to process the sputum samples in accordance with WHO recommendations (final NaOH concentration, 1%). Using phosphate-buffered saline (pH 6.8), the decontaminated and digested samples were neutralised and properly homogenized. The samples were centrifuged at 3000g for 15 minutes and the supernatant discarded and pellet used for further investigation. All treated samples were kept at −20 °C for the course of the investigation (Muthuraj, 2017).

GenoType MTBDRplus V.2.0 assay

A GenoLyse kit (Hain Lifescience, Nehren, Germany) was used for DNA extraction from all specimens. The Genotype MTBDRsl V.2 assay was performed according to the manufacturer's protocol. The test is based on DNA strip technology and has three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization. All three steps were performed as per the WHO recommendations. And the interpretation for Genotype MTBDRsl V.2 was performed according to the manufacturer's instructions (Hain Life Science Gmb, Nehren, & Germany, 2009).

PCR Amplification of Drug Target Genes

The DNA from the clinical M. tuberculosis isolates were extracted using standard protocols of CTAB method (Somerville, 2005). PCR program was performed in an authorized thermal cycler (Eppendorf Gradient Cycler). The isolated template DNA was amplified using gyrA and gyrB primers, as mentioned (Dauendorffer, 2003).

The PCR amplification of the DNA fragments corresponding to the gyrA and gyrB QRDRs was performed as follows using the primers for Mycobacterium gyrA and gyrB. The degenerated oligonucleotides Pri9 (5'-CGCCGGGTGCTCTATGCAATG-3') and Pri8 (5'-CGGTGGGTCATTGCCTGGCGA-3')

and for gyrB by using gyrB-A (5'-GAGTTGGTGCGGCGTAAGAGC-3') and gyrB-E(5'-CGGCCATCAA/GCACGATCTTG-3').

The primer for atpE is designed using primer-BLAST available at NCBI and TB database (http://tbdb.bu.edu/tbdb_sysbio/MultiHome.html). The oligonucleotides atpE F (5'-GGAGCTCGAAGAGGAACACC-3') and atpE R (5'-ACTTGACGGGTGTAGCGAAG-3') at the concentration of 10 pmol/µl is used for the overall reaction.

The PCR cycling parameters were 94°C for 5 minutes; followed by 35 cycles of 94°C for 1 minute, 57–62°C (according to the optimal primer annealing temperature) for 1 minute and 72 °C for 1 minute; and a final extension of 72°C for 10 minutes. The PCR product was then kept at hold at 4°C for 15 minutes. The amplified PCR product was run on a 2% Agarose gel in Tris–borate–EDTA buffer. The ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc. The size of the amplified fragment for gyrA, gyrB and atpE was 216 bp, 322bp and 317bp(Guillemin, 1998 & Wu, 2021).

Sanger Sequencing and Analysis

The amplified PCR products of gyrA and gyrB genes were further purified using PCR purification kit (Invitrogen). The purified PCR product was directly sequenced in an automated DNA Sequencer (Immu Genix Biosciences Pvt Ltd, Chennai, India). The nucleotide sequences obtained were analyzed using BLASTn and BLASTx and ClustalW 2.0 bioinformatics tools available at National Centre for Biotechnology Information (NCBI) compared with wild type M. tuberculosis (H37Rv).

Molecular docking

The present in silico study evaluates the effect of BDQ on atpE by docking. Molecular docking of the BDQ with these genes was studied by AutoDock 4.2, and the properties were analysed by PreADMET and Biovia Discovery Studio visualizer (Panikar, 2021).

Protein preparation

The structure for atpE from *Mycobacterium tuberculosis* structure was not available in the

PDB database. The sequence of atpE was retrieved from Uniprot database and uniport Id was P9WPS0, respectively (Boyle, 2011).

Protein structure prediction using Swissmodel and Validation using Procheck

The structure of atpE was predicted using Swissmodel using the template 4v1g.1.G F0F1 ATP C Crystal structure from *Mycolicibacterium phlei* with 89.87% sequence identity. Ramachandran plot predicted using SAVES-Procheck server shows 93.3% residues on most favoured regions and only no residues on disallowed region **Figure.4**.

Ligand preparation

ACD/ChemSketch is a chemical structure depicting tool that allows us to draw compound structures. The SMILES format of the compounds, namely Bedaquiline from PubChem database. The 2D structure was generated through SMILES structure, converted to 3D structure, and 3D optimization was also carried out and saved in MDL-MOL File format and converted in to the PDB format using converter program (open babel) (Morris, 2009).

Docking analysis and interpretations

Molecular interaction analysis was carried out to understand the molecular affinity between the drug Bedaquiline and against atpE gene of *M. tuberculosis* using AutoDock 4.2.

Docking visualization

The protein–compound interactions such as bonded and other non-bonded energies of the drug against the target genes of MTB were depicted by utilizing Biovia Discovery Studio visualizer. This software visualizes the molecular interaction such as hydrogen bond, hydrophobic interactions and van der Waals interactions.

RESULTS

The total number of FQ suspects enrolled for the study from first line drug-resistant was 430 and analysed to find mutations in the gyrA and gyrB genes using MTBDRplus V.2.0 assay. There were 320 affected males and 110 affected females out of 430 samples and it is presented in **Figure.1.** In which 245 males were affected with Isoniazid Mono-resistant TB and 85 females were affected. Among Rifampicin resistant 3o males and 5 females were affected. In MDR-TB cases 45 males and 20 females were affected.

The prevalence study for Fluoroquinolone states that 32 samples (7.46%) showed resistance among the total selected population, in which most of them were naïve and previous treatment failure cases. Twenty-five isolates had mutations in DNA gyrase subunit -A (gyrA), while mutations in gyrB were observed in only 7 isolates.

The observation of clear bands at 216-bp and 322-bp regions in agarose gel confirmed the amplified products of gyrA and gyrB genes of M. tuberculosis clinical isolate respectively **Figure.2.**

Mutational analysis revealed that the mutations mainly alter codon 94 (replacing aspartic acid with glycine, D94G), and codon 90 (replacing alanine with valine, A90V). In Isoniazid Resistance, MDR, and treatment failure cases, resistance to FQs was most commonly associated with the D94G mutation. The results of resistance pattern analysis of gyrA and gyrB genes from Isoniazid Resistance, Rifampicin resistance and MDR-TB clinical isolate are presented in **Table.1.** and **Table.2**. We found nucleotide substitutions and insertions leading to change in the corresponding amino acids.

Molecular docking analysis of Bedaquiline with atpE, Rv0678 and pepQ

The docking analysis of of Bedaquiline with atpE by utilizing AutoDock software 4.2 version. The docking simulation generated different conformations of the protein–compound complex which are displayed based on least binding free energy (ΔG). The best conformations were chosen based on the least energy and minimal solvent accessibility. The interactions such as hydrogen, hydrophobic, and other non-bonded terms between BDQ and

drug target genes are visualized using Biovia Discovery Studio Visualizer software (Panikar, 2021).

Bedaquiline showing hydrogen, hydrophobic, van der Waals interactions within active binding sites of atpE are shown in **Figure.5.**

Binding interaction of BDQ with atpE

The illustration of molecular interaction of BDQ in the key residues of drug target gene are shown in **Table.5.** The docking simulation of atpE with BDQ showed two hydrogen bonds with GLY38 and ALA31; seven hydrophobic bonds with ALA34, LEU49, ILE55, ALA34, PRO52 and ALA34 residues; shown in **Figure.5.** with a binding energy value of –5.23 kcal/mol and 147.43 µM inhibitory constant.

Table.5. shows the least energy values, vdW + Hbond + desolv energy, torsional energy, intermolecular energy, electrostatic energy, unbound energy, inhibition constants, and refRMS value of BDQ with atpE. All the energy values are given in Kcal/mol.

atpE structure prediction

ATPE sequence from Mycobacterium tuberculosis was retrieved from Uniprot database and it uniport ID was P9WPS0. The structure of ATPE was predicted using Swissmodel using the template 4v1g.1.G F0F1 ATP C Crystal structure from Mycolicibacterium phlei with 89.87% sequence identity. Ramachandran plot predicted using SAVES-Procheck server shows 93.3% residues on most favoured regions and only no residues on disallowed region **Figure.4.**

DISCUSSION

This study on fluoroquinolone resistance among smear-positive pulmonary TB cases. Fluoroquinolones (FQs) are the most promising antituberculous therapeutic agents to be developed in 40 years (Salah Eldin, 2012). Mutations in a small region of gyrA, called Quinolone Resistance-Determining Region (QRDR) are the primary mechanism of FQ resistance in M. tuberculosis (Chang, 2010).

The second mechanism which causes resistance to the fluoroquinolones is through the efflux mechanism, which pumps the drug back to the cell (Abraham, 2003).

Mutation in the gyrA gene and the two single nucleotide polymorphisms Asn538Asp and Asp500His in the gyrB gene are associated with cross-resistance to FQs in M. tuberculosis (Nosova, 2013).

Specifically, the D94G (GAC→GGC) gyrA mutation, out of five different variants, caused resistance to fourth-generation moxifloxacin (Gillespie, 2016). Using a PCR-sequencing method, the direct identification of mutations in the gyrase A (gyrA) gene's fluoroquinolone resistance determining region (QRDR) is evaluated (Lau, 2011).

Among 25 gyrA mutants, it was found that 18 (72%) resistant isolates carried mutations at codon 94, with five different amino acid changes- D94A, D94N, D94Y, D94G and D94H. The remaining 7 (28%) had mutations in codon A90V (GCG to GTG) and S91P (TCG to CCG).

Among 7 gyrB mutants, it was found that 5 (71.4%) resistant isolates carried mutation at codon 538 (Asparagine to Aspartic acid) and remaining 2 (28.5%) had mutation in codon 540 (Glutamic acid to Valine).

There is already concern about high levels of pre-existing FQ-resistant TB in regions with a high prevalence of drug resistance (Sharma, 2019). Since these medications are commonly sold over the counter and additionally prescribed as broad-spectrum antibiotics for the treatment of undiagnosed respiratory infections. To make this situation severe, few individuals are developing Bedaquiline resistance, which is extremely dangerous.

Fluoroquinolone-resistant M. tuberculosis incidence was apparently high, in the selected group of clinical isolates. Due to the improper use of second line medication, as a result of a poorly adhered to regimen in the treatment of multidrug-resistant TB, is one significant

mechanism for the acquisition of fluoroquinolone-resistant TB.

This study provides the standardization of atpE gene amplification. And the resultant product will be subjected to DNA sequencing and Mutational analysis for further molecular characterization in the next level of study among M. tuberculosis isolates in North-zone of Tamilnadu and Puducherry. So that it could help in the population-based surveillance study.

The inhibition of targeted and main enzyme is caused by knowing the binding affinity and bond formation (hydrogen and hydrophobic) of the most potent molecule. From docking analysis, BDQ utilizes slightest binding energy of –5.23k calorie/mole with atpE. The BDQ form two hydrogen bond interactions with all the target gene atpE. Hydrophobic amino acid residues ALA34 and LEU49 were found to be key residues that aid in the development of major hydrophobic interactions with BDQ. The molecular docking study in this paper showed the inhibition capability of Bedaquiline towards the target genes. So this drug may work as a potent inhibitor of ATP biosynthesis in M. tuberculosis. Our study further focuses on amplifying the target sequences of the gene of interest in the 1st line drug-resistant clinical isolates for the prevalence study of the pre-existence of Bedaquiline resistance in the selected population of North-zone of Tamilnadu and Puducherry.

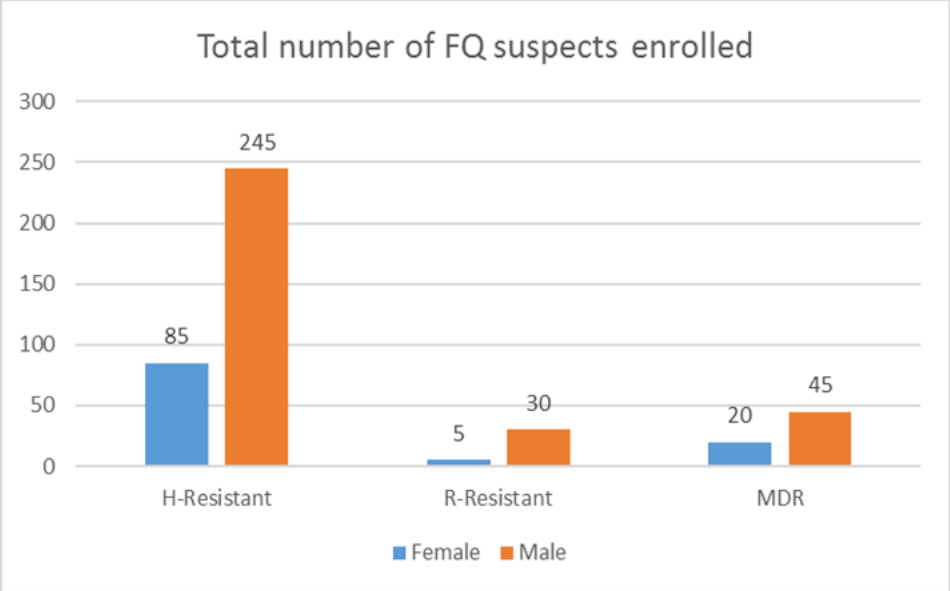


Figure 1: Total number of FQ suspects enrolled for the study from first line drug-resistant on gender wise (H-Isoniazid, R-Rifampicin and MDR-Multi drug resistant)

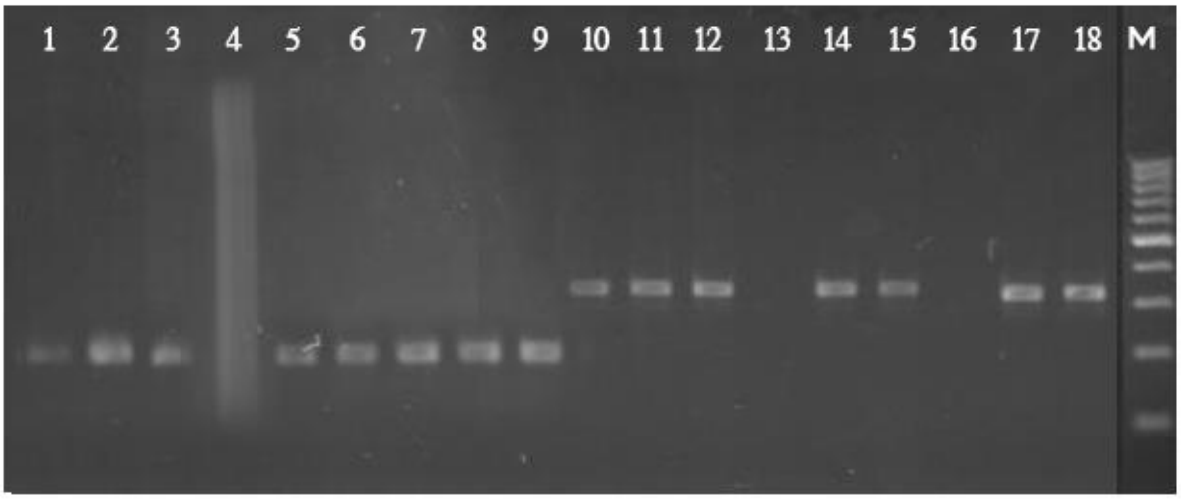
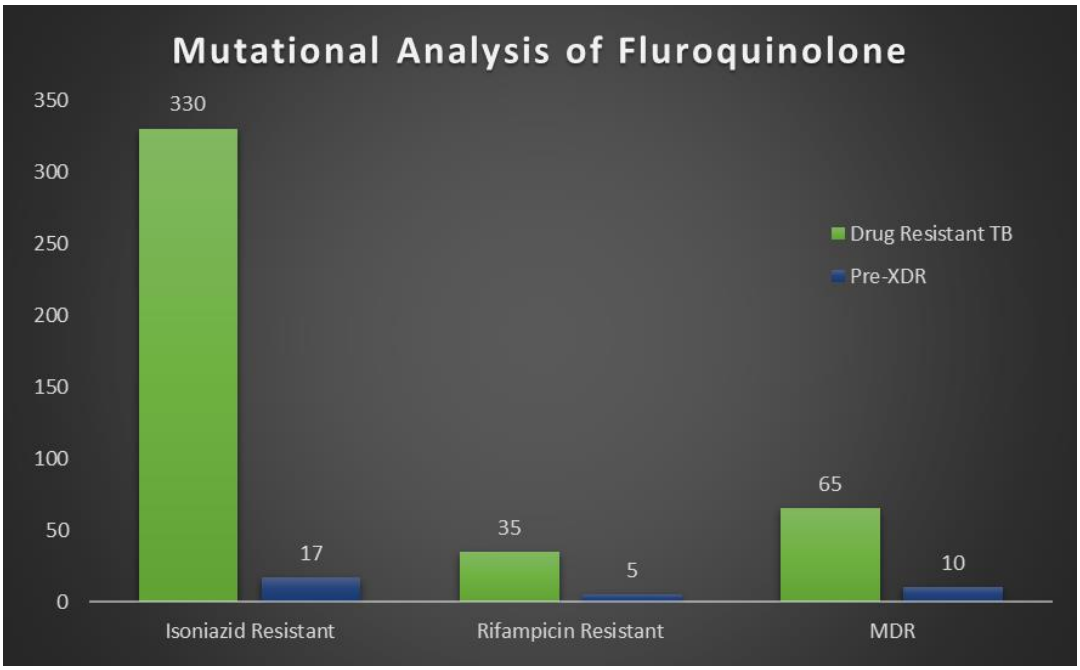


Figure 2: The above gel is for gyrA the (ladder) used is from 100,200, 300, 400, 500, 600, 700, 800, 900, 1000 and gyrA is at 216 bp, LAN M from the right side is for the marker (ladder), LAN 1–9, are positive for gyrA except LAN 4. gyrB is at 322 bp, LAN 10–16, are positive for gyra B, except LAN13 &16. atpE is at 317bp LAN 17 & 18. (This photo is taken by gel documentation system).



TYPE OF DRUG RESISTANT TB		FQ-RESISTANT
Isoniazid Resistant	330	17
Rifampicin Resistant	35	5
MDR	65	10
TOTAL NO OF DR-TB CASES	430	32

Figure3: Mutational analysis of fluoroquinolone-resistant among the monoresistant and MDR-TB isolates

Table.1 Frequency of mutations on codons of gyrA and gyrB genes
WT-Wild type, WTΔ-Mutant type, MUT-Mutation

SECOND LINE TB DRUG RESISTANCE	GENE	BAND	MUTATION OR REGION INTERROGATED	MUTATION PATTERN AND FREQUENCY OF FQ RESISTANT	
				FQ-RESISTANT (n=32)	NO.(%) DETECTED
FQ RESISTANCE PATTERN	gyrA	WT1		30	93.75%
		WTΔ1	Codon(s) 85–89	2	6.25%
		WT2		27	84.34%
		WTΔ2	Codon(s) 89–93	5	15.63%
		WT3		25	78.13%
		WTΔ3	Codon(s) 92–96	7	21.88%
		MUT1	A90V	6	18.75%
		MUT2	S91P	1	3.13%
		MUT3A	D94A	1	3.13%
		MUT3B	D94N	3	9.38%
			D94Y		
		MUT3C	D94G	11	34.38%
		MUT3D	D94H1	3	9.38%
	gyrB	WT1		25	78.13%
		WTΔ1	Codon(s) 536–541 (codon 497-502)	7	21.88%
		MUT1	N538D	5	15.6%
		MUT2	E540V	2	6.25%

Table.2 Frequency of mutated codon and Amino acid change in gyrA and gyrB genes

gyrA Mutation probe	Codon Position	Substitution	Amino acid change	Frequency n=32
MUT1	90	GCG→GTG	Ala > Val	6
MUT 2	91	TCG→CCG	Ser > Pro	1
MUT 3A	94	GAC→GCC	Asp > Ala	1
MUT 3B	94	GAC→AAC	Asp > Asn	3
MUT 3B	94	GAC→TAC	Asp > Tyr	
MUT 3C	94	GAC→GGC	Asp > Gly	11
MUT 3D	94	GAC→CAC	Asp > His	8

gyrB Mutation probe	Codon Position	Amino acid change	Frequency n=83
MUT1	538	Asn > Asp	5
MUT 2	540	Glu > Val	2

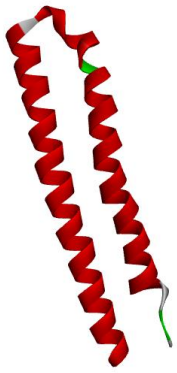


Figure4: 3D Structure of Target gene-atpE

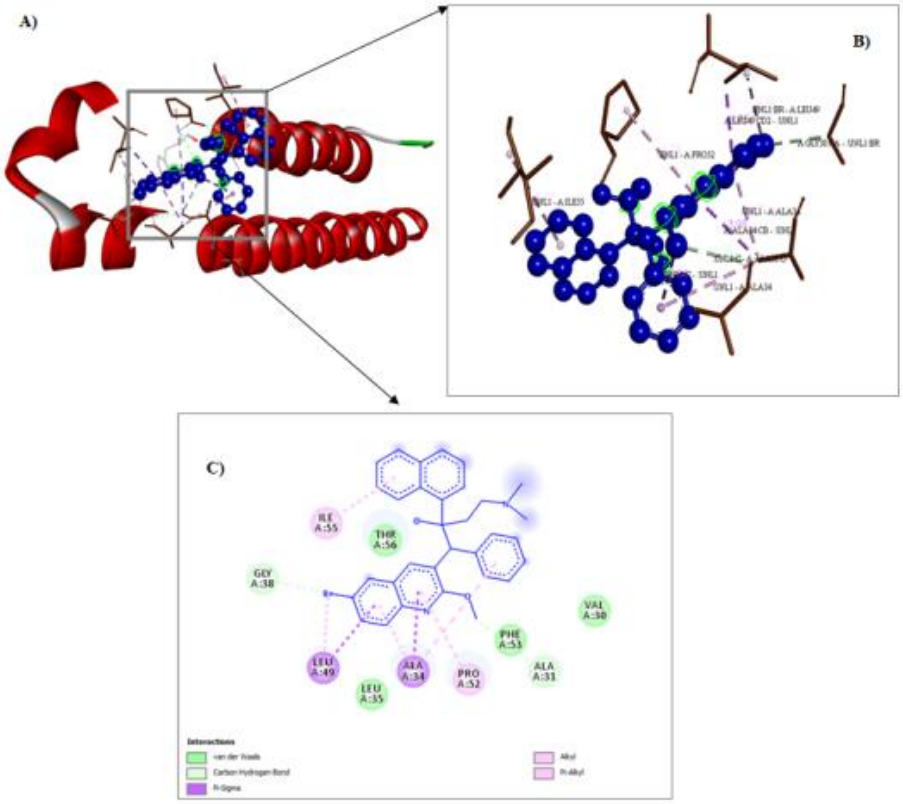


Figure5: Bedaquiline (blue colour ball and stick model) interactions with atpE represented in (A) solid ribbon model with; (B) active site amino acid residues amino acid residues represented in red colour line model; (C) Alkyl and Pi-Alkyl are shown in pink dotted lines and Pi-sigma are shown in purple dotted lines), Carbon hydrogen bonds are shown in (light blue colour circles). van der Waals interactions (light green colour circles)

Table.4 Inhibition constant, energy values of docking simulation of atpE with Bedaquiline (All energy values are given in Kcal/mol)

Conformation	Binding Energy	Ligand efficiency	Inhibitory constant, K_i (μm)	Intermolecular energy	vdW + H bond + desolv Energy	Electrostatic energy	Torsional energy	Total internal Unbound
Bedaquiline + atpE	-5.23	-0.14	147.1	7.91	-7.98	0.07	2.68	-2.81

Table.5 Bedaquiline interacting residues of the atpE genes of Mycobacterium tuberculosis are summarized with the number of hydrogen bonds, hydrophobic interactions.

Conformation	Hydrogen-bonding Donor-Acceptor linkage of Amino acid		Hydrophobic interactions	
	(Atom···Ligand atom)	Distance (Å)	(Atom···Ligand atom)	Distance (Å)
PepQ + Bedaquiline atpE + Bedaquiline	CA- BR O- C	3.39608 3.35511	ALA34 (CB)	3.98454
			LEU49 (CD2)	3.7457
			LEU49 (BR)	4.09817
			ILE55	5.27285
			ALA34	5.05325
			PRO52	4.79309
			ALA34	4.07882

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Conflict of Interest:

There is no conflict of interest between authors.

Author's Contribution:

The research was performed with equal contributions and teamwork from all authors.

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