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Comparison of Mycobacterium tuberculosis Strains with H37Rv Using Whole Genome Sequence Analysis to Identify Virulence Factors and Phylogenetic Relationships

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ABSTRACT: *Mycobacterium tuberculosis (Mtb) poses a significant disease burden in developing countries, largely due to the rising issue of drug resistance. In Pakistan, the genetic and molecular characteristics of resistant strains have not been thoroughly analyzed or compared with the reference Mtb strain, H37Rv, to understand the molecular and genetic epidemiology. Mtb employs various strategies to resist anti-tuberculosis drugs, and antimicrobial resistance, including single and multidrug resistance, has increased extensively in developing nations like Pakistan. In this study, the whole genomes of three Mtb strains (335, 335-2, and 311) were uploaded to the Rapid Annotation Subsystems Technology (RAST) server to retrieve coding DNA sequences (CDS). The RAST server identified 4,454, 4,445, and 4,396 CDS for Mtb strains 335, 335-2, and 311, respectively. Mutation analysis was performed using NCBI nucleotide BLAST and CLUSTAL Omega, comparing the sequences with the H37Rv reference strain. The analysis revealed 909, 832, and 1,135 mutations in the 335, 335-2, and 311 strains, respectively. Phylogenetic analysis based on the most virulent protein (EccC3) indicated a close resemblance between all three strains and the H37Rv strain. Furthermore, Mycobacterium canettii (accession number WP_203037448.1) was identified as the closest related bacterium to the studied strains and H37Rv.*

Keywords: Mutations, XDR-TB, Drug-resistant TB, Tuberculosis

INTRODUCTION

There are more than 1.5 million cases of tuberculosis (TB) in Pakistan which is caused by *Mycobacterium tuberculosis* (*Mtb*). The molecular existence of this pathogen has been evidenced back to 17,000 years The prevalence of *Mtb* infections is currently very high in Pakistan which is mainly due to poverty, undiagnosed cases, failure to define the accurate case definitions at the clinics, unawareness, misreporting, and lack of intensive active surveillance (Siddiqui et al., 2019). The developed countries are very successful in controlling these kinds of challenges and efficiently reducing the incidence of *Mtb* (Aftab et al., 2021), but even then, despite newer methods for diagnosis and treatment, TB is still among the top 10 deadly infectious diseases (Sandhu, 2011). This is because *Mtb* uses several strategies to resist the action of anti-TB drugs (Yasmin et al., 2014).

Additionally, antimicrobial resistance prevalence in terms of single and multiple drug resistance has already been extensively increased in developing countries including Pakistan (Gao et al., 2018; Wang et al., 2018; Desai and Joshi 2019; Kabir et al., 2020). Furthermore, these drug-resistant strains also pose higher susceptibility to cause various types of mutations in the *Mtb* strains which is also becoming a serious public health concern for the medical sciences (Yar et al., 2018; Mukati et al., 2019; Sharma et al., 2020). Therefore, it is imperative to explore all the possible resistance strains of *Mtb* so that counteractions at molecular levels can be established to prevent the occurrence of *Mtb* resistance genes (Jabbar et al., 2019; Kabir et al., 2021).

The emergence of drug resistance in tuberculosis is due to the collective contribution of the various mechanisms. Mainly, two factors i.e., internal and external are concerned with enhancing resistance to MTB. The genetic mutation acquisition, presence of drug efflux pumps, modification of drug target enzymes, low drug permeability through cell envelope, drug inactivation, and the fitness cost are the internal factors connected with resistance strategies in bacteria (da Silva et al., 2011; Gygli et al., 2017; Swain et al., 2020; Allue Guardia et al., 2021). On the other hand, factors concerning social determinants in a population of tuberculosis patients are called external factors (Nguyen et al., 2018).

The genetic studies and analysis of molecular patterns of identified strains of *Mtb* are essential to monitor the prevalence of specific strains of *Mtb* in Pakistan (Ali et al., 2011; Jabbar et al., 2019). The extent of genetic variability, mutations, diversity, and measured resistance level can be studied through comparative genomic studies to identify drug-resistant genes in different isolated strains of

Mtb in Pakistan (Jabbar et al., 2019; Kabir et al., 2023). Bioinformatics tools such as rapid annotation subsystems technology (RAST) utilize the genetic sequences to predict genes for elaborative protein sequencing and inform virulence factor databases through genetic comparison with the reference strains of *Mtb* (Ilina et al., 2013; Jia et al., 2017). These and similar other tools also predict the possible reasons behind the genetic mutations and drug resistance in *Mtb* strains (Khan et al., 2019). Given this context, the present study has been designed to compare the whole genome of 3 strains of *Mtb i.e.* 335, 335-2, and 311 with reference strain of *Mtb* H37Rv.

MATERIALS AND METHODS

Uploading of Genome Sequences at RAST

The genomes of the strains i.e. 335, 335-2, and 311 used in the current study were obtained from a recently published work (Kabir et al., 2019). The genomes of studied strains i.e. 335, 335-2, and 311 were uploaded one by one on the Rapid Annotation using Subsystems Technology (RAST) for getting complementary DNA sequences (CDS) of DNA and protein. The RAST server supports the data and procedures established within the SEED framework (http://pubseed.theseed.org/) to provide high-quality functional annotation. The RAST supports both the automated annotation and the analysis of the genome. Annotated genomic information was obtained through the SEED viewer which offers a wide set of information to browse, compare, and download for genome. The result consists of domain, genus, species, strains, genome size, and number of contigs. The procedure of finding gene location and coding regions in the genome was performed by aligning the submitted genomes against the reference genomes. Hence, the filtering and trimming were performed by the server based on reference genomes. After the genome sequence, annotation is necessary to make it useful. The SEED viewer gave genomic information in graphically and tabulated form.

Analysis of Gene Prediction

In computational biology, gene finding refers to the procedure of identifying
the regional genomic DNA that encodes genes. It comprises RNA genes (non-coding), protein-coding genes, and other functional elements (regulatory regions). Gene prediction of *Mtb* was done through the RAST server in the presence of reference genome H37Rv.

DNA Sequence Alignment analysis

Sequence alignment is a process of arranging the sequence of DNA, RNA, or protein to know similar regions having functional, structural, or evolutionary relationships. CLUSTAL Omega

was used for multiple sequence alignment among 3 *Mtb* strains and the reference strain H37Rv.

Analysis through Virulent Factor Database

The mutated sequences were obtained from CLUSTAL Omega and BLAST alignment and uploaded on the virulence factor database (http://www.mgc.ac.cn/VFs/) through which we get the bit score and E values of the most virulent genes. The list of all virulent genes was arranged in descending order to get the highest bit scored gene at the top. Finally, the most virulent gene was BLAST against other organisms excluding *Mtb* to check its evolutionary relationship.

Construction of phylogenetic tree

The phylogenetic tree provides an evolutionary relationship of the specific gene of one organism to the other organisms. For this purpose, molecule evolutionary genomic analysis (MEGA X**)** software was employed. FASTA files of drug resistance genes in 3 *Mtb* strains and reference genome (H37Rv) were aligned and converted to a mega format to build the tree. The files were opened in Mega X one by one and then alignment was made by a CLUSTAL Omega with falling parameter gap penalty 10, gap extension penalty 0.1, multiple alignments having gap opening penalty 1.0, and gap extension penalty. 0.2, protein weight matrix Gonnet, Residue specific penalties on, hydrophilic penalties on, gap separation distance 4, end gap separation off, use negative metrics of and delay divergence. Cut off 30% and the gaps contacts were removed for the aligned sequences and then the data were exported to mega format. The mega format file was opened, and phylogenetic trees were constructed by having an analysis of phylogenetic reconstruction, and maximum likelihood.

RESULTS

Genome annotation and features

High-quality functional annotation of *Mtb* was obtained from the RAST server. Results showed information about sequence size, shortest contig size, number of contigs, % GC content, longest contig size, median sequence size, mean sequence size, L50 value, and N50 value. This information is illustrated in Table 1.

Table 1: Genome annotation and analysis in 3 different Mtb strains

Statistic	As uploaded			As splitting into scaffolds		
	***Mtb* 335**	***Mtb* 335-2**	***Mtb* 311**	***Mtb* 335**	***Mtb* 335-2**	***Mtb* 311**
Sequence size	4406929	4392079	4399363	4406929.	4392079	4399363
Number of contigs	237	224	194	237	224	194
GC content (%)	65.7	65.7	65.7	65.7	65.7	65.7
Shortest contig size	92	92	104	92	92	104
Median sequence size	2245	6594	6594	2245	6594	6594
Mean sequence size.	18594.6	19607.5	22677.1	18594.6	19607.5	22677.1
Longest contigs size	199215	174386	178690	199215	174386	178690
N50 value	62939	61172	64257	62939	61172	64257
L50 value	-	26	24	-	26	24

RAST server provided 4454, 4445, and 4396 CDS of DNA/protein for *Mtb* 335, 335-2, and 311 strains, respectively. DNA sequences were then compared for mutation analysis using NCBI nucleotide BLAST and CLUSTAL omega against H37Rv reference strain and obtained 909, 832, and 1135 mutated regions in *Mtb* 335, 335-2, and 311 strains, respectively. Most of the mutations were due to single nucleotide polymorphism (SNPs). Moreover, some mutations were related to the resistance genes of the bacteria including mutations in gyrA and EthA in *Mtb* 335 strain, mutations in katG, gyrA, embB, embC, and EthA in *Mtb* 335-2 strain, and mutations in katG, embA, embB, embC and EthA in *Mtb* 311 strain.

Predicted virulence factors in *Mtb* 335 strain

When the DNA sequences of *Mtb* were compared with reference genome H37Rv we get the most mutated sequences. After getting the mutated sequences, we checked out the most virulent sequences in those mutated sequences through the virulence factor database (VFDB) which shows the bit score and e-values of sequences. The bit score and e-values define how much the sequence is virulent. Based on bit score and e-values 20, 19, and 20 virulence-related factors for *Mtb* 335, 335-2, and 311 strains, respectively. The VFDB analysis showed that the most virulent protein was EccC3 which is a component of the Type VII secretion system as mentioned in Table 2.

Table 2: Results of virulence factor database in *Mtb* strain. The most virulent protein found was FtsK/SpoIIIE family protein EccC3, the component of the Type VII secretion system ESX-3

Strain	Sequence no.	length	bit score
335	fig|6666666.737179.peg.1587	3993	7908
335-2	fig|6666666.737181.peg.2161	3993	7908
311	fig|6666666.736742.peg.2371	3993	7908

Phylogenetics analysis

The construction of a phylogenetic tree takes place to check the evolutionary relationship of *Mtb* 335, *Mtb* 335-2, and *Mtb* 311 strains with other species based on the most virulent protein i.e. EccC3 which is a component of the Type VII secretion pathway. This has provided us the opportunity to observe which proteins have the same mutated protein sequence, are interlinked to each other, and belong to the same family. Phylogenetics analysis of Mtb strains and the reference strain H37rV was performed using a maximum likelihood algorithm. Furthermore, the closest related bacteria with studied strains and H37Rv found was *Mycobacterium canettii* with accession number WP_203037448.1. From a phylogenetic point of view, strains that are closely related to each other have more similarities. The results of the phylogenetic analyses are shown in Fig. 1.

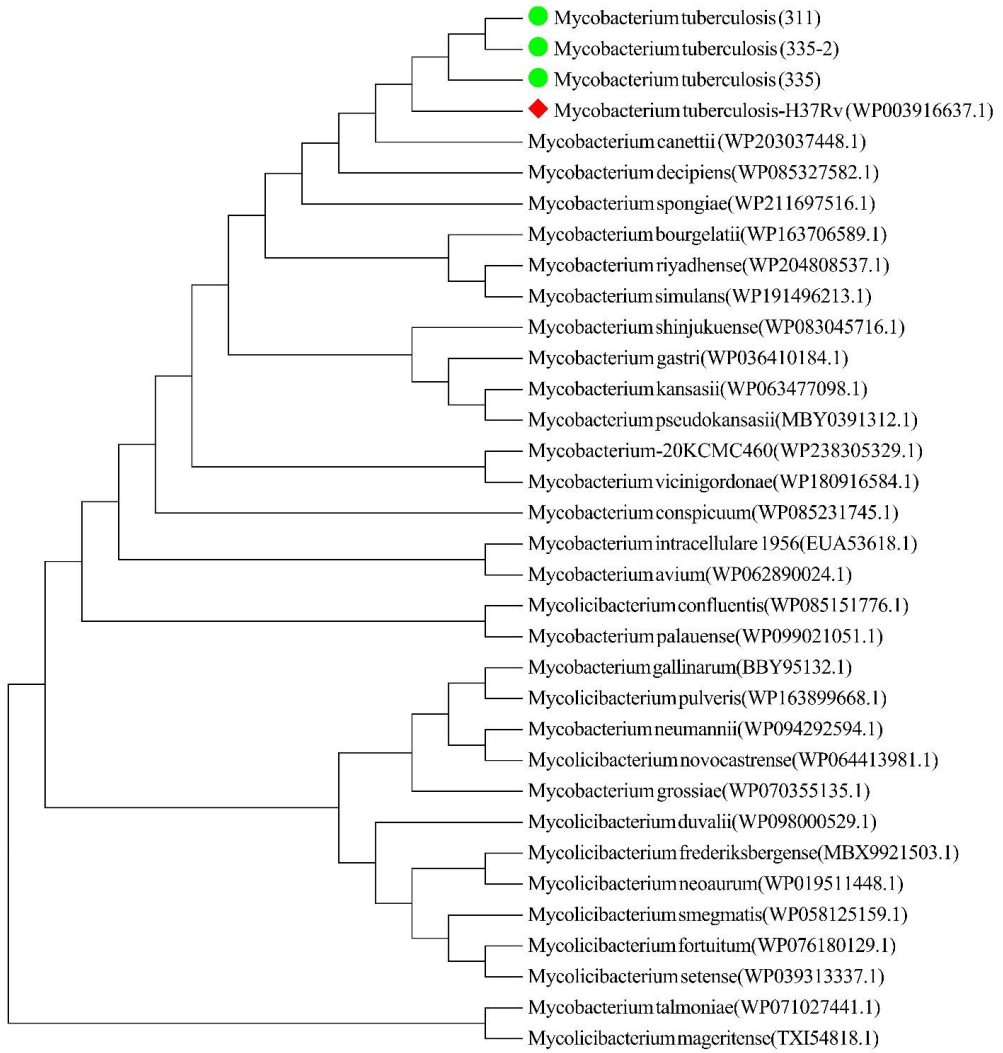

Fig. 1: Phylogenetics analysis of Mtb strains and the reference strain H37rV. The analysis is based on the most virulent protein i.e. EccC3 which is a component of Type VII secretion system. The analysis was performed using maximum likelihood algorithm. The closely related bacteria found was *Mycobacterium canettii* with accession number WP_203037448.1

DISCUSSION

In the current investigation, genomes of 3 *Mtb* strains (335, 335-2, and 311) were individually compared with the reference genome H37Rv of *Mtb*. All strains not only showed similarity to the reference genome but also shared several characteristics with the pathogenic reference genome. The CLUSTAL Omega tool generated multiple sequence alignments among 3 or more sequences. The alignment was carried out in genes that had 99% identity to the reference strain of *Mtb*. Mutation

analysis by CLUSTAL Omega provided 909, 832, and 1135 mutated regions in *Mtb* 335, 335-2, and 311 strains, respectively. Comparison of virulence factors encoded in *Mtb* 335, 335-2, and 311 strains reported multiple clades which showed phylogenetic lineage between these species' clades. These clades were based on structural or functional similarity. The prime objectives of the study were to identify the mutation and compare it with the reference genome, phylogenetic analysis, evolutionary relationships among *Mtb* strains, and evolutionary comparison among other bacteria and *Mtb* 335-2 strain.

Bloom and Cadarette (2019) described that bacterial pathogens pose major threats to public health worldwide in the 21st century. Current work on bacterial pathogenesis has increased considerably our information about the pathways of the disease occurrence at the molecular level over (Chen et al., 2005). To enhance future work, it is essential to create a database collectively providing the virulence factors (VFs) of different medically important bacterial pathogens (Chen et al., 2005). The virulence factor database (VFDB) provides current information on virulence factors from different pathogenic bacterial species. VFDB can be used by browsing each genus or by using keywords. VFDB gives a unified gateway for VFs from various bacterial pathogens (Chen et al., 2005). Forrellad et al. (2013) reported that a variety of virulence factors have been produced in MTBC members in response to the host's immune reaction

Pule et al. (2016) reported that bacterial EPs are responsible for providing resistance against various anti-TB drugs by pressing out the drug molecules that enter the cell. Isoniazid, the most potent first-line anti-TB drug, is resisted due to overexpression of the MmpL7 protein (Pasca et al., 2005). The efflux-mediated resistance can be decreased using efflux inhibitors. Drug efflux pumps i.e., reserpine (Shaheen et al., 2019), verapamil (de Souza et al., 2020), chlorpromazine, and thioridazine (Rodrigues et al., 2012) show reduced activity against various inhibitors.

As mentioned in Table 2, the most virulent protein found in the bacteria was EccC3 (a component of the Type VII secretion system ESX-3) based on the highest bit score in VFDB. The 4 ESX conserved protein constituents (EccB3, EccC3, EccD3, and EccE3) are involved in making secretion machinery of the ESX-3 system of *Mycobacterium tuberculosis* (Famelis et al., 2019). Out of these 4 constituents, EccC3 (Uniprot ID: P9WNA9) is a malleable arrangement of 4 ATPase domains and has been crosslinked with its neighbouring (EccD3) protein's small hydrophilic domain. Its 4 ATPase domains are connected to the

bacterial cell membrane via a stem domain. Next to the stem domain another unknown function domain (DUF) is considered an ATPase domain that is necessary for ESX-3 secretory pair release (Famelis et al., 2019). The conformational alterations in the stem domain which is stimulated by the binding of substrate/stimulant at the posterior end of EccC3 lead to hydrolysis of ATP in the DUF and hence, secretory pair discharge through the periplasm (Famelis et al., 2019). Moreover, Costa et al., declared that the structural design of type VII secretion pathways (T7SS) varies significantly from the other known secretory apparatuses (Costa et al., 2015). This study furnishes the comprehension of secretory pathways conformation that will be expedient for the novel antimicrobial tactics designed to hit bacterial virulence (Famelis et al., 2019).

CONCLUSION

This investigation reveals that the genomes of three Mtb strains (335, 335-2, and 311) were uploaded to the RAST server, which identified 4,454, 4,445, and 4,396 coding DNA sequences (CDS) for each strain, respectively. Phylogenetic analysis based on the most virulent protein (EccC3) showed a close relationship between all three strains and the H37Rv reference strain. Additionally, *Mycobacterium canettii* (accession number WP_203037448.1) was identified as the closest related bacterium to the studied strains and H37Rv. These findings can support researchers in utilizing the identified virulent genes for developing vaccines and new treatment strategies against these resistant Mtb strains.

Conflict of Interest

The authors have no competing interests.

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Ethical Approval Statement

Not applicable

REFERENCES

1. Aftab A, Afzal S, Qamar Z, Idrees M (2021). Early detection of MDR *Mycobacterium tuberculosis* mutations in Pakistan. Sci Rep. 11(1): p. 16736.

2. Ali, A., Hasan, R., Jabeen, K., Jabeen, N., Qadeer, E., Hasan, Z (2011). Characterization of mutations conferring extensive drug resistance to *Mycobacterium tuberculosis* isolates in Pakistan. Antimicrob. Agents Chemother. 55(12): 5654-5659.

3. Allue Guardia A, Garcia JI, Torrelles JB (2021). Evolution of drug-resistant Mycobacterium tuberculosis strains and their adaptation to the human lung environment. Front. Microbiol. 12: 137.

4. Bloom DE, Cadarette D (2019). Infectious Disease Threats in the Twenty-First Century: Strengthening the Global Response. Front Immunol. 10: p. 549.

5. Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, Jin Q (2005). VFDB: a reference database for bacterial virulence factors. Nucleic Acids Res. 33(Database issue): p. D325-8.

6. Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat. Rev. Microbiol. 13(6): p. 343-59.

7. da Silva PEA, Von Groll A, Martin A, Palomino JC (2011). Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*. FEMS Immunol. Med. Microbiol. 63(1): 1-9.

8. de Souza JVP, Murase LS, Caleffi-Ferracioli KR, Palomo CT, de Lima Scodro RB, Siqueira VLD, Cardoso RF (2020). Isoniazid and verapamil modulatory activity and efflux pump gene expression in *Mycobacterium tuberculosis*. Int. J. Tuberc. Lung Dis. 24(6): 591-596.

9. Desai U, Joshi JM (2019). Extrapulmonary drug-resistant tuberculosis at a drug-resistant tuberculosis center, Mumbai: Our experience–Hope in the midst of despair. Lung India: Official Organ of Indian Chest Society 36(1): 3.

10. Famelis N, Rivera-Calzada A, Degliesposti G, Wingender M, Mietrach N, Skehel JM, Fernandez-Leiro R, Böttcher B, Schlosser A, Llorca O, Geibel S (2019). Architecture of the mycobacterial type VII secretion system. Nature. 576(7786): p. 321-325.

11. Forrellad MA, Klepp LI, Gioffré A, Sabio y García J, Morbidoni HR, de la Paz Santangelo M, Cataldi AA, Bigi F (2013). Virulence factors of the *Mycobacterium tuberculosis* complex. Virulence. 4(1): 3-66.

12. Gao Y, Zhang Z, Deng J, Mansjö M, Ning Z, Li Y, Li X, Hu Y, Hoffner S, Xu B (2018). Multi-center evaluation of GenoType MTBDR*sl* line probe assay for rapid detection of pre-XDR and XDR Mycobacterium tuberculosis in China. J. Infect. 77(4): 328-334.

13. Gygli SM, Borrell S, Trauner A, Gagneux S (2017). Antimicrobial resistance in *Mycobacterium tuberculosis*: mechanistic and evolutionary perspectives. FEMS Microbiol. Rev. 41(3): 354-373.

14. Ilina EN, Shitikov EA, Ikryannikova LN, Alekseev DG, Kamashev DE, Malakhova MV, Parfenova TV, Afanas'ev MV, Ischenko DS, Bazaleev NA, Smirnova TG, Larionova EE, Chernousova LN, Beletsky AV, Mardanov AV, Ravin NV, Skryabin KG, Govorun VM (2013). Comparative genomic analysis of *Mycobacterium tuberculosis* drug-resistant strains from Russia. PLoS One. 8(2): p. e56577.

15. Jabbar A, Phelan JE, de Sessions PF, Khan TA, Rahman H, Khan SN, Cantillon DM, Wildner LM, Ali S, Campino S, Waddell SJ, Clark TG (2019). Whole

genome sequencing of drug resistant *Mycobacterium tuberculosis* isolates from a high burden tuberculosis region of North West Pakistan. Sci Rep. 9(1): p. 14996.

16. Jia X, Yang L, Dong M, Chen S, Lv L, Cao D, Fu J, Yang T, Zhang J, Zhang X, Shang Y, Wang G, Sheng Y, Huang H, Chen F (2017). The Bioinformatics analysis of comparative genomics of *Mycobacterium tuberculosis* complex (MTBC) provides insight into dissimilarities between intraspecific groups differing in host association, virulence, and epitope diversity. Front. Cell Infect. Microbiol. 7: p. 88.

17. Kabir S, Ejaz H, Hussain SZ, Rasheed MA, Junaid K, Rehman, A (2023). Effect of tellurite on growth of extensively drug resistant (XDR) *Mycobacterium tuberculosis* and action on mycobacterial drug efflux pump. J. King Saud Uni. Sci. 35(4): 102629

18. Kabir S, Junaid K, Rehman A (2021). Variations in rifampicin and isoniazid resistance associated genetic mutations among drug naïve and recurrence cases of pulmonary tuberculosis. Int. J. Infect. Dis. 103: 56-61.

19. Kabir S, Tahir Z, Mukhtar N, Sohail M, Saqalein M, Rehman A (2020). Fluoroquinolone resistance and mutational profile of gyrA in pulmonary MDR tuberculosis patients. BMC Pulm. Med. 20: 1-6.

20. Khan MT, Ali S, Khan AS, Ali A, Khan A, Kaushik AC, Irfan M, Chinnasamy S, Zhang S, Zhang YJ, Cui Z, Wei AJ, Wang Y, Zhao M, Liu K, Wang H, Zeb MT, Wei DQ (2019). Insight into the drug resistance whole genome of *Mycobacterium tuberculosis* isolates from Khyber Pakhtunkhwa, Pakistan. Infect Genet Evol. 92: p. 104861.

21. Mukati S, Julka A, Varudkar HG, Singapurwala M, Agrawat JC, Bhandari D, Jain A (2019). A study of clinical profile of cases of MDR-TB and evaluation of challenges faced in initiation of second line Anti tuberculosis treatment for MDR-TB cases admitted in drug resistance tuberculosis center. Indian J. Tuberc. 66(3): 358-363.

22. Nguyen QH, Contamin L, Nguyen TVA, Bañuls AL (2018). Insights into the processes that drive the evolution of drug resistance in *Mycobacterium tuberculosis.* Evol. Appl. 11(9): 1498-1511.

23. Pasca MR, Guglierame P, De Ross, E, Zara F, Riccardi G (2005). *mmpL7* gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium smegmatis*. Antimicrob. agents Chemother. 49(11): 4775-4777.

24. Pule CM, Sampson SL, Warren RM, Black PA, van Helden PD, Victor TC, Louw GE (2016). Efflux pump inhibitors: targeting mycobacterial efflux systems to

enhance TB therapy. J. Antimicrob. Chemother. 71(1): 17-26.

25. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M (2012). Contribution of efflux activity to isoniazid resistance in the Mycobacterium tuberculosis complex. Infect. Genet. Evol. 12(4): 695-700.

26. Sandhu GK (2011). Tuberculosis: current situation, challenges and overview of its control programs in India. J Glob Infect Dis. 3(2): p. 143-50.

27. Shaheen A, Afridi WA, Mahboob S, Sana M, Zeeshan N, Ismat F, Rahman M (2019). Reserpine is the new addition into the repertoire of AcrB efflux pump inhibitors. Mol. Biol. 53**(**4): 596-605.

28. Siddiqui S, Brooks MB, Malik AA, Fuad J, Nazish A, Bano S, Becerra MC, Hussain H (2019). Evaluation of GenoType MTBDRplus for the detection of drug-resistant *Mycobacterium tuberculosis* on isolates from Karachi, Pakistan. PLoS One. 14(8): e0221485.

29. Swain SS, Sharma D, Hussain T, Pati S (2020). Molecular mechanisms of underlying genetic factors and associated mutations for drug resistance in *Mycobacterium tuberculosis*. Emerg. Microbes Infect. 9(1): 1651-1663

30. Wang Z, Xie T, Mu C, Sun R, Wang C, Zhao H, Ju H (2018). Performance of sequencing in predicting ofloxacin resistance in *Mycobacterium tuberculosis* from positive Bactec MGIT 960 cultures. Ann. Clin. Lab. Sci. 48(1): 69-74.

31. Yar AM, Zaman G, Hussain A, Changhui Y, Rasul A, Hussain A, Bo Z, Bokhari H, Ibrahim M (2018). Comparative genome analysis of 2 *Mycobacterium tuberculosis* strains from Pakistan: insights globally into drug resistance, virulence, and niche adaptation. Evol. Bioinform. Online, 14: p. 1176934318790252.

32. Yasmin M, Gomgnimbou MK, Siddiqui RT, Refrégier G, Sola C (2014). Multi-drug resistant Mycobacterium tuberculosis complex genetic diversity and clues on recent transmission in Punjab, Pakistan. Infect Genet Evol. 27: p. 6-14.