Molecular Epidemiology of *Mycobacterium tuberculosis* in the Country of Georgia

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This thesis is dedicated to my children – Elene and Maxime – may you never stop learning, and without whom I would have completed two years earlier.

Summary

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (MTB), is one of the major contributors to human mortality globally. Being attributable to several socio-economic factors, national TB control programs still struggle to prevent transmission and treat active cases of the disease. In order to achieve sustainable development goal for ending TB in 2030, The World Health Organization (WHO) has endorsed the recommendations for prompt diagnosis and integrated molecular surveillance of TB. Molecular typing of MTB is progressively used for strengthening disease surveillance. However, in most limited-resources settings such as low/middle income countries, implementation of the typing methodologies is challenging.

In the first part of the thesis, we implemented a genotyping tool at the National Reference Laboratory (NRL) in Georgia, aiming to support disease surveillance at the molecular level. We took advantage of the isolate biobank, containing MTB isolates from 2009, and performed genotyping for the recurrent TB cases (chapter 3). The generated results gave the opportunity to customize and develop more practical application of MIRU-VNTR typing. The stepwise MIRU-VNTR typing approach described in chapter 4 represents a simplified and less time/cost/workload consuming way of the typing, which can be easily customized for different settings, thereby enhancing the usage of molecular typing for the TB control and surveillance.

Despite of being an ancient disease, treatment of TB remains to be complex, especially throughout evolution of the drug resistant variants, requiring treatment with toxic and expensive medications. Drug resistance is still hindering TB control strategies in Georgia. Moreover, resistance to the novel, promising medications, such as bedaquiline, bears utmost importance. Since 2019, bedaquiline has been used as the backbone for the treatment regimens for MDR, preXDR and XDR TB. However resistance has already been detected *in vivo*, translated into 22 (6.9%) of resistant cases in 2020-21 in Georgia. While transmission of resistant MTB strains is threatening TB control, the data for developing molecular test-systems for prompt detection of such cases is still lacking. This requires collated information of precise phenotypic information associated with corresponding sequencing data. In our second part of the thesis, we combined evidence of any influence between the genomic variants of bedaquiline resistance

associated genes and minimum inhibitory concentration values. Simultaneously, we looked for samples identified as phenotypically resistant using single, critical concentration (CC) provided by WHO, and correlated genomic information for those isolates. Herein, the chapter 5 provides valuable and essential data for the further developmental implications.

Abbreviations

Amk	Amikacin
BDQ	Bedaquiline
CC	Critical Concentration
DST	Drug susceptibility testing
ECOFF	Epidemiological cut-off
EMB	Ethambutol
EUCAST	European committee on antimicrobial susceptibility testing
FQ	Fluoroquinolones
INH	Isoniazid
HIV	Human immunodeficiency virus
Kan	Kanamycin
Lfx	Levofloxacin
LMICs	Low- and middle-income countries
Lzd	Linezolid
Mtb	*Mycobacterium tuberculosis*
MTBC	Mycobacterium tuberculosis complex
MDR	Multi-Drug Resistant
MGIT	Mycobacterial Growth Indicator Tube
Mfx	Moxifloxacin
MIC	Minimal Inhibitory Concentration
PXDR	Pre-Extensively Drug Resistant.
Pza	Pyrazinamide
Rif	Rifampicin
TB	Human Tuberculosis
tNGS	Targeted next generation sequencing
WGS	Whole genome sequencing
WHO	The World Health Organization
XDR	Extensively Drug Resistant

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1. Introduction

During the last 200 years, with more than a billion victims, tuberculosis (TB) has been a leader of human mortality caused by a single infectious agent (1). Ranking above HIV/AIDS, influenza and malaria, only the recent coronavirus (COVID-19) pandemic outnumbered the deaths caused with TB worldwide (2).

The World Health Organization`s (WHO) End TB Strategy and the United Nations (UN) Sustainable development Goals (SDGs), adopted in 2014 and 2015, respectively, aimed to achieve specific defined milestones by sharing the vision of a world free of TB. Within the milestone assessment of 2020, the major indicators such as percentage of reduction in TB incidence rate and percentage of reduction in the absolute number of TB deaths lagged behind the pre-defined targets (3). The incidence decline indicator compared to 2015 baseline was 11%, while the goal for 2020 was set on 20%. The deaths decline show bigger gap between the target and achieved percentage with 35% and 9.2%, respectively (2).

While having a slight but steady direction towards achievement of these targets, in 2020 the world fell significantly off track of the process due to the COVID-19 pandemic, restricting services for TB patients, dramatically reducing disease notification, limiting treatment and worsening the outcomes (4). The drop in TB notification with a fall of 18% between 2019-2020 years is translated into 5.8 million new case notifications, decreasing from 7.1 million in the previous year. The major consequence of this gap is increased deaths from TB worldwide (2). While this is the immediate outcome, the coming years will reveal the influence of the COVID-19 pandemic on TB transmission and the development of drug resistance, presumably worsening the epidemiological situation of TB.

1.1 From «White plague» to Nobel Prize

The history of TB covers several millennia. From ancient civilizations, clinical characteristics of this disease were described in archaic texts of India and China, plaguing

the world throughout history (5,6). A variety of names were devoted to this deadly disease, such as "phthisis" in Greece and Hippocrates aphorisms (7), "consumption" or "white plague" (8), and "tabes" in ancient Rome (6).

The TB prevalence in Europe and North America reached its peak during the 18th-19th centuries and this is the exact period when crucial discoveries about the etiology and pathogenesis of the disease were made (6). René-Théophile-Hyacinthe Laennec, a French physician, described clinical manifestation of TB in his book, in 1821. Along with inventing the stethoscope, he unraveled the concept of pulmonary and extra-pulmonary TB (9). However, the etiology of the disease was still unknown, until the experiment of French military surgeon Jean-Antoine Villemin, who infected rabbit with autopsy samples of patients with "consumption" and demonstrated the infectious characteristics of the disease (10).

In 1882, Robert Koch presented the infectious nature of TB and its causing agent "the tubercle bacillus" –*Mycobacterium tuberculosis*, leading to dramatic changes in the disease treatment and outcome (11). With his crucial discovery, he earned the Nobel Prize in Medicine or Physiology in 1905 (12).

With advances on the pathophysiology and etiology of TB, the wave of the disease decreased from mid-19th century, mostly owing to improved socio-economic conditions (6). Specific sanatoria were opened for the treatment of TB patients, offering rest at fresh air in the mountains and specific high-nutritional diet. These sanatoria are considered as one of the first facilities for TB treatment (13). While giving a patient piece and elementary sanitary activities, the effectiveness of "open-air" treatment was not enough, and the death rate of the disease at the end of the 19th century was 71% in average (14).

The decline of the TB incidence rate was facilitated with the introduction of the surgical interventions on the lung tissue. New practices in early 20th century, such as pulmonary collapse therapy, therapeutic pneumothorax and thoracoplasty resulted in cavity closure and better treatment outcomes (15). Nevertheless, the disease was persisting and preventive medicine was not able to reduce TB transmission.

Apart from Koch`s discovery, development of the so far one and only vaccine against TB, was one of the milestones of public health management. The Bacille Calmette Guèrin

(BCG) – vaccine, containing attenuated bacilli derived from *Mycobacterium bovis* – causing TB, introduced in 1921, presumably saved more than 100,000 children who were immunized in Europe (16). BCG is still used in middle and high incidence countries to prevent childhood TB meningitis and the miliary disease form of TB.

The steady decline of the "white plague" was challenged after World War I and II, leading to re-emergence of the disease into an epidemic level. In the mid and late 1900s, the World Health Organization (WHO) introduced the first disease control program pushing TB testing and BCG vaccination for European countries, providing guidelines for TB control (6).

In 1944, the era of the first anti TB drugs started with introduction of streptomycin (Sm) (17). Followed by isoniazid (Inh) in 1952 (18), pyrazinamide (Pza) in 1954 (19), ethambutol (Eth) in 1961 (20) and rifampicin (Rif) in 1963 (21), the numbers of new TB cases were rapidly decreasing. In addition to the new medications, exploring the usefulness of existing drugs towards other diseases, prompted inclusion of the fluoroqinolones (FQ), linezolid (Lzd) and clofazimine (Cfz) into the regimens against multi-drug resistant TB (22).

While proposed treatment regimens, policies and control programs attempted to keep the disease at least on a manageable level, the emergence of the human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) triggered huge increase in TB cases and death caused by the disease. In 1986, HIV was officially announced as a cause of rare lung infection, which was first described in 1981, nowadays marked as a first official reporting of AIDS (23). At the end of the 1990s, TB was one of the leading cause of death as an opportunistic infection in HIV positive patients (24), and the 21st century started with 1.84 million deaths from TB in 2000, with 226,000 of these attributable to HIV (25). Since then, until today, the "white plague" remains one of the major public challenges worldwide.

1.2 *Mycobacterium tuberculosis* complex

Ever since the first isolation of the tubercle bacillus – *Mycobacterium tuberculosis* (MTB) is still an object of the interest worldwide. In the meantime, years of technological

development and research partially revealed the nature of the bacterium and its main characteristics.

Containing more than 140 species, the genus *Myobacterium* comprises the *Mycobaterium tuberculosis* complex (MTBC), *Mycobacterium leprae* and a group of mycobacteria referred to as nontuberculous mycobacteria (NTM). The *Mycobacterium tuberculosis* complex (MTBC) comprises not only MTB, the main cause of the disease in humans, but also other bacterial species that cause TB in animals, such as *M. caprae, M. microti, M. orygis, M. pinnipedii M. mungi, M. suricattae,* the chimpanzee bacillus*, M. bovis* and several other sub-species (26–31). However, some of the animal-adapted species can cause disease in humans too, e.g. *M. bovis* – leading to bovine TB (32).

The uniqueness of the MTBC lies in its remarkably genetically homogenous nature (33,34). In 1998, Cole *et al* published the first complete genome sequence of MTB (35). Since then, the application of whole genome sequencing (WGS) and other genotyping techniques led to revealing high conservation in most of the regions of genome, where many other bacteria show high polymorphisms. In addition, there is no horizontal gene transfer observed in the MTB genome, supporting the theory of clonal evolution in the MTBC (36).

Despite of the low genetic diversity, there are nine main lineages within the human-adapted MTBC, distributed globally (37,38). Some of these lineages exhibit wide phylo-geographical distribution, such as Lineage 2 (East Asian) and Lineage 4 (Euro-American), considered as “generalists”, in contrast to Lineage 7 (mainly circulating in Ethiopia) and Lineages 5 and 6 (also known as *M. africanum* and prevalent in West Africa), which are referred to as “specialists” (37,39).

The phylo-geographical distribution and specific characteristics of each MTBC lineage are translated into their phenotypic features, which play a pivotal role in disease manifestation, development and outcome, by differing in complex interactions between the host and the pathogen (40,41). Specifically, Lineage 2, including its Beijing sub-lineage, is significantly associated with drug resistance, which is one of the major challenges for TB management (42). The strain genetic background plays an important role by contributing to the drug resistance prevalence, as it is not only influenced by resistance conferring mutations but also by additional compensatory mutations

increasing the fitness and transmissibility of the bacteria. While different lineage representatives exhibit various fitness cost and compensation abilities, Lineage 2 has shown the most profitable outcome with drug resistant bacteria with smaller, compensated fitness cost, leading to almost equal transmission ability as susceptible variant (43).

1.3 Molecular epidemiology – the world of research on *Mycobacterium tuberculosis*

After Koch's successful discovery of the one single infectious agent, causing all forms of TB, bacteriological methods became the main diagnostic tools for the disease. The methodology was limited to direct smears stained with the Ziehl-Nielsen method, colony morphology and growth rate comparisons (15). Chest radiography and clinical manifestation have been additional components of TB diagnosis. However, epidemiological research on TB, studying genetic and environmental risk factors, were limited until the late 1900s, when first molecular methods were introduced to discriminate between the clinical samples and evaluate TB transmission and outbreaks (15,44).

Starting from restriction enzyme analysis (REA) of MTB, where chromosomal DNA is digested with different restriction enzymes and the genetic fingerprint is interpreted as characteristics of each strain via gel electrophoresis, molecular typing of *Mycobacterium tuberculosis* had evolved to the various methods – using different genetic markers (45).

Pulsed-field gel electrophoresis (PFGE) was one of the pioneering tools successfully used for typing MTB as well as NTM species. Using large sections of DNA, it gives ability to compare different strain patterns using different duration and direction of electric pulses. Although the method is time consuming, with a long turn-around time, and does not give sufficient discrimination between the strains of MTB, the method is still used for typing other bacterial species (46).

IS*6110* typing was one of the most widely used method, based on a specific group of genetic elements called insertion sequences (IS). With a size of 1,355bp, it is found within all members of the MTBC, but positions and number of copies within each chromosome provides the ability to differentiate between strains. Restriction fragment length

polymorphism (RFLP), combining REA and southern blot technique, based on IS*6110* requires large amounts of DNA, and the process is quite cumbersome, not fully standardized, and therefore, the results comparability between the laboratories is complicated (33,45). Spoligotyping and mycobacterial interspersed repetitive units – variable number tandem repeat (MIRU-VNTR) have later gained more popularity over *IS6110* - restriction fragment length polymorphism (RFLP) (45).

Spoligotyping – spacer oligonucleotide typing - is still used for typing the MTBC, especially in low-resource countries. The process is simpler and the data is easily reproducible in a binary format (47). It is PCR-based technique based on identification of 43 interspersed spacer sequences – non-repetitive sequence of 35-41 bp, part of the direct variable repeats (DVR) (33). The international databases for spoligotypes SITVIT (formerly SpolDB4) contains more than 7,000 types, classified into 62 clades/lineages. The data generated differentiates main lineages and sub-lineages. However, its discriminatory power is lower, compared to IS6110-RFLP and MIRU-VNTR typing. The major drawback of this method is its lower resolution in discriminating between Beijing genotype representatives, which are mostly prevalent in Asia and former Soviet Union countries (48). Therefore, using spoligotyping alone for the molecular epidemiological studies is limited, especially in the geographical areas mentioned above (45).

MIRU-VNTR, originally described by Supply *et al*, uses the variable copy numbers of the tandem repeats as a genetic marker to discriminate between MTB strains (49). The process consists of PCR amplification of target regions and gel electrophoresis, followed by transferring the band length into the number of copies present in the sample. The outcome is a numerical code, which is compared between the isolates, and the global database miru-vntrplus.org offers the opportunity to browse the data worldwide (49,50). There are several panels proposed including different sets of 24 loci, with the advantage of detecting mixed infections and even discriminate between Beijing strains using additional 4 strain-specific loci (49,51). MIRU-VNTR is a reliable and effective methodology for molecular surveillance, and for a long time it was considered as the gold standard of TB molecular epidemiology studies. Recently, the conventional method has been replaced by automated capillary electrophoresis system, making the process simpler and more time-effective (52).

SNP typing. Several SNP typing methods are available for the MTBC phylogenetic classification, all of them using lineage/sub-lineage specific set of single nucleotide polymorphisms. Variation between cost, technical requirements and throughput of these methods are large. Additionally, there is no consensus set of SNPs proposed for the standard typing of MTBC (45). Stucki *et al* described two novel PCR-based methods: i. MOL-PCR, requiring Luminex flow cytometer platform as the method, uses fluorescent microspheres, and ii. TaqMan real-time PCR – commercially available system used for typing different species. In contrast to MOL-PCR, which is multiplexed and can assess simultaneously 50 SNPs, TaqMan real time PCR is performed per each SNP. Like most of the genotyping methods, SNP typing requires specific instruments – like real-time PCR machine or Luminex instrument, which might not be available or affordable in high incidence/low resource countries (53).

Whole genome sequencing (WGS). WGS is increasingly becoming the method of choice, not only for TB transmission and surveillance studies, but also for identifying novel markers for molecular diagnostic methodologies. Detecting resistance-conferring mutations in addition to phenotypic resistance data, gives the ability to produce the most complete information for genotypic-phenotypic correlations (54–56). Nowadays, several developed countries use WGS or targeted sequencing next-generation sequencing (tNGS), using only a number of gene targets for resistance detection and phylogenetic identification, as the primary diagnostic method for TB. Depending on the number of the samples to be processed, there are several platforms available for WGS and tNGS, varying from the largest available instrument NovaSeq6000 (illumina) generating output of 6,000Gb, to the smallest commercial benchtop instrument from Nanopore MinION with output of 50Gb per flow cell (57,58). However, the clinical implementation of these technologies is limited by several factors. Purified and precise DNA extraction, library preparation and bioinformatics are the main limitations associated with human resources, while instrument maintenance and cost of the sequencing kits are additional contributors hindering the wider usage of sequencing methodology, especially in the developing and low/middle resource countries (59).

While WGS is playing significant role in deciphering TB epidemiology and evolution, other genotyping methodologies are still used for particular molecular epidemiological studies. These methodologies still can be fast, reliable and relatively cost-effective

compared to sequencing, especially in countries where TB diagnostics and surveillance are limited.

1.4 "The captain of all these men of death"

When Alejandro Morales published one of his most successful books, which was based on real-life experience of TB patients, he artfully described the variety of disease treatment through hospital newsletters delivered to the fictional patients. From spiritual practices to the barbarism, aggravated medical experiments show how stigmatized TB patients were and how far the ideal "treatment" was deviated (13,22).

Indeed, TB treatment from rest in sanatorium and surgical procedures to the real chemotherapy took a long time, until 1944 when the first important drug in TB treatment – streptomycin was introduced (17). Later, streptomycin treatment was evaluated in the first randomized clinical trial in history of medicine, started by the British Medical Research Council, showing significant advantage of the streptomycin group over the controls (22). The development and industrialization of pharmacotherapy paved the way for the discovery of more drugs with effects on MTB, such as para-aminosalicylic acid (PAS) in 1946 and Isoniazid in 1952 (60,61). The combination of these three drugs – "triple therapy" reduced the number of TB cases, transmission and drug resistance, while increasing successful treatment outcomes (62). However, severe side effects, especially for PAS led to replacement of the latter with a more tolerable drug – ethambutol (1961) (20). Soon the arsenal of chemotherapeutical agents got bigger with the addition of pyrazinamide, and still later kanamycin, amikacin, capreomycin and thioamides, currently referred as second line medication (63–66).

The period of the late 1900s was called the golden era of antibiotics, when discovering new compounds was encouraging for pharmaceutical companies and scientists. This was the exact period when one of the major backbones of TB treatment – rifampicin was discovered and introduced. The compound was primarily derived from Streptomyces species, *S. mediterranei*, which produced the substance rifamycin, having various antimicrobial activity. Although, this compound showed very little effect in vivo, Piero Sensi and Maria Teresa Timbal, who isolated rifamycin, decided to modify the original

molecule in purpose to increase its activity (67,68). Several derivatives were under development and rifampicin showed the highest antimycobacterial properties. Implementing rifampicin in TB treatment revolutionized the chemotherapy and reduced the length of the treatment for susceptible patients to 9 months (22,69).

Since the introduction of rifampicin, the golden era has turned into a "golden fiasco". In 1998, the compound rifapentine was proposed but it did not earn much popularity, followed by 14 years of disruption in the development of new medications (70). The emergence of drug resistance due to disruption of the treatment supply, poor adherence, lack of medical service, in addition to bacterial mechanisms of drug resistance, significantly reduced the treatment arsenal (71).

Bedaquiline, also known as compound TMC207 or nowadays Sirturo, has been first described in 2005 by Andries *et al* (72). It has gained large success in clinical trials and was approved by the US Food and Drug Administration (FDA) in 2018. Along with bedaquiline, usage of pretomanid (approved by the FDA in 2019) and delamanid (approved by the FDA in 2014) showed increased capability to reduce the treatment duration for drug resistant TB cases (73–75). Similarly to delamanid, pretomanid also showed its effectiveness against both – replicating and non-replicating bacteria (75).

Several randomized trials with different combinations of these drugs as well as their independent use, showed significant increase in MDR and XDR TB successful outcome. Shortening the sputum and culture conversion rates, bedaquiline and delamanid gave the ability to shorten the drug-resistant TB regimens down to 9-12 months from up to 24 months (76,77). Moreover, nowadays treatment of MDR/XDR TB patients are moved on fully oral treatment regimens, recommended from 2019 by WHO, increasing patients adherence, reducing lost to follow-up rates and risk of further transmission of drug resistant strains (78).

Nowadays, TB treatment for drug-susceptible cases consists of isoniazid, rifampicin, ethambutol and pyrazinamide for 2 months, followed by isoniazid and rifampicin for 4 months. For the multi-drug resistant, pre-extensively drug resistant and extensively drug resistant cases (Table1.1), there are four treatment regimens proposed, depending on the resistance pattern and patient condition - i. fully oral long treatment regimen for 18-24 months using beadaquiline, linezolid, levofloxacin and clofazimine; ii. Fully oral modified

short regime - beadaquiline, linezolid, levofloxacin, clofazimine and cycloserin for 9 months; iii. Standard short regime with amikacin, moxifloxacin, prothionamide, clofazimin, pyrazinamide, high dose of isoniazid and ethambutol - 9-12 months; iv. Salvage regimen for MDR/XDR-TB – cyclsoserin, ethambutol, pirazynamide, amikacin, prothionamide/ethionamide, PAS - during 18-20 months (78,79).

During chemotherapy, follow-up smear microscopy, culture and drug susceptibility testing is performed on a bi-monthly basis to detect sputum/culture conversion and acquisition of any resistance towards any of the drugs included in the regimen. Phenotypic DST is the current gold standard, using only critical concentration (CC) proposed by WHO, as a cut-off for resistance identification. However, the data regarding genotype-phenotype correlations for novel medications is still lacking, leading to underestimated genotypic variants which might play a role in reduced susceptibility and misdiagnosis (80).

1.5 Drug resistance in *Mycobacterium tuberculosis*

Being naturally resistant to most antibiotics MTB holds a variety of mechanisms, which protects these bacteria from the effects of the vast majority of drugs.

The first shield contributing to “intrinsic” bacterial drug resistance is its unique, highly hydrophobic cell wall, serving as a permeability barrier. Several studies inducing mutants with defective cell wall lipids showed susceptibility to those drugs that they were resistant against beforehand (81,82). Additional mechanisms adopted by MTB to avoid drug exposure is enzymatic inactivation or modification of the compound using β-lactamases, aminoglycoside acetyl transferases, enhanced intracellular survival protein (Eis), that are encoded in the MTB genome, leading to resistance towards certain antibiotics (83,84).

Drug efflux systems, as a contributor to the drug resistance in MTB, is still controversial. However, it has been shown that the MTB genome encodes several efflux systems, such as 14 members of the major facilitator family and several transporters. In favor of the effect of efflux pumps on drug resistance development or increased minimum inhibitory concentration (MIC), is overexpression of these genes in drug resistant strains (85). Even

though, there is still a lack of data regarding the role of efflux systems, significant amount of data has been generated regarding upregulation of efflux pump and resistance or increased MIC towards bedaquiline and clofazimine. Specifically, increased MIC are associated with mutations in the transcriptional repressor MmpR, encoded by Rv0678 gene, influencing transcription of MmpL5 and MmpS5 proteins, leading to MmpL5-MmpS5 efflux pump composition (86).

Table 1.1 Old and new definitions of drug resistant TB

DEFINITIONS OF DRUG RESISTANT TUBERCULOSIS:	
MULTIDRUG-RESISTANT TUBERCULOSIS MDR	RESISTANCE TO RIFAMPICIN AND ISONIAZID (WHO, 2010)
EXTENSIVELY DRUG-RESISTANT TUBERCULOSIS XDR	RESISTANCE TO RIFAMPICIN, ISONIAZID, FLUOROQUINOLONE AND ANY SECOND-LINE INJECTABLE (WHO, 2006)
PRE-EXTENSIVELY DRUG-RESISTANT TUBERCULOSIS PXDR	RESISTANCE TO RIFAMPICIN, ISONIAZID AND ANY FLUOROQUINOLONE (UPDATED WHO 2021)
EXTENSIVELY DRUG-RESISTANT TUBERCULOSIS XDR	RESISTANCE TO RIFAMPICIN ISONIAZID, ANY FLUOROQUINOLONE AND AT LEAST ONE ADDITIONAL GROUP-A DRUG (BEDAQUILINE AND LINEZOLID) (UPDATED WHO 2021)

In addition to the transmission of drug resistant strains, which is still a major part of TB transmission globally, *de novo* drug resistance development is one of the challenges for TB management, especially in middle/low resource countries. Acquired drug resistance is mostly a consequence of the inadequate/interrupted treatment due to limited drug supply, poor adherence of the patients, due to long and complex treatment regimens or delays with drug susceptibility testing.

To define patient groups more precisely, WHO has recently updated the definitions for extensively drug-resistant TB and separated pre-extensively drug-resistant type of TB,

leading to the selection of specific and effective treatment regimens for each group of patients (87) (Table1.1).

1.6 Georgia and Tuberculosis

Georgia was one of the high burden MDR and XDR-TB countries until 2016. The last decade, with implementation of the new treatment regimens and introduction of the novel medications, helped reducing the numbers of drug resistant cases, ending with an incidence rate for all TB of 44 per 100,000 in 2021, compared to 131 per 100,000 in 2010. While having 12% of recurrent cases in 2021, with 31.4% of such cases being drug resistant, DR-TB remains a matter of concern. Additionally, the phylogenetic background of the bacteria (Lineage 2) plays a key role of having DR-TB transmission still on going in the country.

Gygli *et al* with a 6-year national wide collection of MTB isolates identified Georgian prisons as one of the main contributing factors for DR-TB transmission, with several spillovers of evolved DR strains from penitentiary system into the community. Moreover, the study identified the link between prisons and compensatory evolution among drug resistant strains, making TB transmission and control more challenging (43).

Despite of the decrease in numbers of cases from penitentiary system nowadays, the past mass amnesty in 2012, affected TB transmission for the following years, as most of the TB patients from prisons were not followed-up. The result was an increase of DR-TB incidence in 2014, from 96 per 100,000 in 2013 to 103 per 100,000 in 2014. From 2016, the country observed a significant decline in the number of cases from the penitentiary system, due to successful implementation of active and passive screening methods in prisons, ending up with 0.3 cases per 100 prisoners in 2021 (National TB surveillance program, unpublished data).

After the modification and implementation of new treatment regimens in 2019, including bedaquiline and delamanid as a backbone, the treatment success rate has been increasing in MDR/XDR treatment patients, from 56% in 2016 (calculated as an outcome of the second line treatment) to 72%/58% (MDR/XDR) in 2019 cohort of patients (National TB surveillance program, unpublished data). However, the resistance to these promising

new drugs has already emerged, narrowing the choice of the medications for the treatment of MDR and XDR-TB.

2. Objectives and outline

2.1 Thesis aims

This thesis aims to dive into the molecular epidemiology of MTB in Georgia, explore the diversity of MTB and implement molecular methodologies to address the epidemiological consequences of transmission of the disease. Additionally, the thesis aims to contribute new data regarding the emerging challenge of resistance detection in new medications, specifically bedaquiline.

2.2 Objectives

- Objective 1 - Chapter 3
  Implement new genotyping methodology, MIRU-VNTR, at the National Reference Laboratory (NRL) of National Center for Tuberculosis and Lung Diseases (NCTLD) in Georgia, for the purpose of molecular surveillance of MTB diversity and disease recurrence.

- Objective 2 – Chapter 4
  Develop and customize MIRU-VNTR genotyping in a cost/labor- effective manner based on MIRU-VNTR loci specificity relevant to MTB strain variation in Georgia.

- Objective 3 - Chapter 5
  Explore genetic variants and their correlation with phenotypic susceptibility to bedaquiline, one of the major challenges of current DR TB diagnostics and transmission.

2.3 Outline

This thesis covers aspects of MTB molecular epidemiology using different approaches. Starting with the implementation of a new genotyping technique at NRL, in the third chapter of this thesis, we used the opportunity to address one of the major problems of TB surveillance and management – recurrent TB. MIRU-VNTR genotyping gave the ability to differentiate between the main drivers of disease recurrence – relapse or reinfection.

In the fourth chapter, we customized and developed the previously implemented genotyping method in order to reduce cost, labor and processing time. For this purpose, we analyzed genotyping data and selected the minimal number of MIRU-VNTR loci with highest specificity to Georgian MTB strain diversity. Additionally, we adjusted the application of the remaining MIRU-VNTR loci in a step-wise manner for maximum resolution within the MIRU-VNTR typing capacity.

In the fifth chapter, we explored one of the emerging challenges of DR TB diagnostics and surveillance, which is the resistance detection to the new medications, specifically bedaquiline. As bedaquiline, along with additional new drugs, increased the rate of successful treatment outcomes globally, precise detection of bedaquiline resistance and identification of genetic variants contributing to the phenotypic resistance plays a key role for developing molecular diagnostic methods. In this chapter, we collated available data of sequenced MTB strains in Georgia and conducted minimum inhibitory concentration (MIC) testing to explore any genotype-phenotype associations leading to reduced susceptibility or resistance to bedaquiline.

The final chapter of this thesis is dedicated to the general discussion of the key findings of the thesis, where we focus on the general implications of the results and their impact on TB surveillance, in addition to the further possibilities of molecular epidemiological research in Georgia.

3. Classifying recurrent *Mycobacterium tuberculosis* cases in Georgia using MIRU-VNTR typing

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3.1 Abstract

Introduction

Recurrent tuberculosis (TB) is one of the main challenges in TB control. Genotyping based on Mycobacterial Interspersed Repetitive Units – Variable Tandem Repeats (MIRU-VNTR) has been widely used to differentiate between relapse and reinfection, which are the two main causes of recurrent TB. There is a lack of data regarding the causes of TB recurrence in Georgia, and while differentiating between relapse and reinfection plays a key role in defining appropriate interventions, the required genotyping methodologies have not been implemented. The objective of this study was to implement MIRU-VNTR genotyping at the National Center for Tuberculosis and Lung Diseases (NCTBLD) and differentiate between relapse and reinfection in multidrug resistant (MDR-) TB patients from Tbilisi, Georgia.

Methods

Recurrent MDR TB cases from 2014-2016 diagnosed at NCTLD were included in the study when bacterial samples from both episodes were available. Genotyping based on the MIRU-VNTR 24 loci was implemented and used for differentiating between relapse and reinfection. Paired samples showing the same MIRU-VNTR pattern or one locus difference were classified as relapse, while two and more loci differences were treated as reinfection. Exact logistic regression was used to identify predictors of recurrence.

Results

Thirty two MDR-TB patients (64 samples) were included and MIRU-VNTR 24 typing was performed on the corresponding paired samples. Of the 32 patients, 25 (83.3%) were identified as relapse while 5 (16.7%) were due to re-infection. Patients with a history of incarceration were significantly associated with TB reinfection ($p< 0.05$).

Conclusion

Recurrent TB in MDR patients in Georgia are mainly caused by relapse, raising concerns on the efficacy of the TB control program. An association between incarceration and reinfection likely reflects high levels of ongoing TB transmission in prisons, indicating the

need for better TB infection control measures in these settings. Our results add to the rationale for implementing genotypic surveillance of TB more broadly to support TB control in Georgia

3.2 Introduction

Tuberculosis (TB) remains a major global health problem (88). Recurrent TB, defined as a second episode of TB disease in patients previously declared as cured or with successful treatment completion, contributes to the global burden of TB, and thus needs to be properly addressed if TB is to be eliminated (88). Recurrent TB is caused by two fundamentally different mechanisms, i) relapse caused by the same strains of Mycobacterium tuberculosis and ii) exogenous reinfection with a different strain (89).

Relapse refers to the reactivation of a subset of bacteria that have not been successfully eliminated during patient treatment. The underlying causes of relapse are manifold, and include various bacterial and host factors (90). For example, phenotypic drug tolerance in bacterial persisters and differences in pharmaco-genetic characteristics of patients influence to likelihood of relapse (91,92). Relapse is thought to be the main contributor to recurrent TB in low incidence areas (93–95). In contrast, exogenous reinfection with a distinct M. tuberculosis strain is a particular problem in high incidence countries (96,97). In addition, several other risk factors such as HIV-infection have been associated with recurrent TB disease (97,98). Understanding the causes and risk factors driving recurrent TB in a specific epidemiological setting has important implications for defining adequate control strategies (89).

Georgia, located at the border of Eastern Europe and Western Asia, is a TB middle incidence country with an incidence rate of 84/100,000 per year. Multi-drug resistant / Rifampicin-resistant (MDR/RR) TB patients comprise 11% of all new TB cases and 30% of previously treated cases, translating into an incidence of MDR/RR TB of 19/100 000. The rate of recurrent TB cases has been increasing from 10.2 to 15.9 in 2014 and 2017, respectively, mainly due to lost to follow-up patients, especially for MDR and XDR TB (88). Despite of an overall decline in TB incidence from 228/100,000 in 2002 to 84/100,000 in 2018, Georgia remains one of the high MDR TB burden countries, nowadays showing 56% of successful outcome for 2nd line treatment (88). From 2013 until 2016, the proportion of lost to follow-up patients enrolled into second-line treatment in Georgia has almost halved, from 32% to 18%, but still remains high. Lost to follow-up patients often suffer from unfavorable outcomes, including a high rate of recurrent TB (95).

Currently, there is no data on the main causes of recurrent TB in Georgia. Moreover, most studies on recurrent TB to date in similar settings have only considered small numbers of drug-resistant patients (93,94,99). Here we implemented MIRU-VNTR typing in Georgia for the first time and used this technique to differentiate between the two major causes of recurrent disease in MDR TB cases in Georgia. We then tested for potential risk factors associated with either of these two causes of recurrent MDR-TB.

3.3 Materials and Methods

3.3.1 Data source and study design

National Tuberculosis Program (NTP), established in 1996 in Georgia, covers the whole country and includes 64 TB units, 11 microscopy laboratories, and the National Reference Laboratory (NRL) at the National Center for Tuberculosis and Lung Diseases (NCTBLD) in Tbilisi. The NRL is the only laboratory in the country with capacity to perform phenotypic M. tuberculosis drug susceptibility testing (DST). All TB cases diagnosed by smear microscopy and/or GeneXpert are sent to the NRL for DST. In addition, the active surveillance of the NTP in Georgia collects standard epidemiological and clinical information for every suspected TB case in the country, including from the civil sector and penitentiary system.

For the purpose of this study, we enrolled a retrospective cohort including bacteriologically confirmed recurrent MDR TB cases recruited between 2014 and 2016. Recurrent TB cases were identified through the National TB Surveillance electronic database and were classified as “successfully treated” (including treatment completion) and “lost to follow-up” depending on the outcome of the primary TB episode. Two cultures per patient and the related patient information were included in the study.

3.3.2 Ethics

The study was conducted at the National Reference Laboratory (NRL) in collaboration with the Swiss Tropical and Public Health Institute. Ethical approval was obtained by the relevant authorities in Georgia (the Institutional Review Board of the National Center for

Tuberculosis and Lung Diseases, Tbilisi, Georgia) and Switzerland (Ethikkommission Nordwest- und Zentralschweiz).

3.3.3 Definitions

A case of recurrent TB–was defined as subsequent occurrence of the disease due to relapse or reinfection, after declaring the patient as clinically cured based on WHO guidelines (88). **Cured**–bacteriologically confirmed TB patients were declared as "cured" in case of smear/culture negative result in at least once or in the last month of the treatment (100). **Lost to follow-up**–patients who did not initiate or interrupted the treatment process for at least two following months (100). **Relapse**–reactivation of endogenous infection that was not eliminated during treatment of a previous episode of TB (90). **Reinfection**–re-occurrence of TB disease caused by M. tuberculosis strain distinct from the strain that caused the previous episode of TB disease (90).

3.3.4 Case definition

Patients were considered eligible for the study in case of recurrent MDR TB episode during the period 2014–2016, while being declared cured/successfully treated or lost to follow-up for the first/second TB episode.

Epidemiological data from both episodes was collected, when available. For statistical analysis, epidemiological variables which are biologically plausible potential risk factors for reinfection or relapse were considered.

3.3.5 Bacteriology

The M. tuberculosis cultures from the primary and secondary episodes, stored at -80°C, were obtained from the Georgian NRL isolate collection. Frozen samples were thawed and sub-cultured on Lowenstein-Jensen medium. High quality DNA was extracted using the CTAB method as described previously (101). DNA was stored at -20°C before being used for genotyping.

3.3.6 MIRU-VNTR genotyping

Relapse and reinfection were differentiated using MIRU-VNTR genotyping; samples from both clinical episodes (pairs) were typed using the 24 loci panel as described (49). Positive and negative controls were included in each PCR reaction, as H37Rv and H2O, respectively. Double allele results were confirmed with two independent PCRs. The 24 loci results were converted into numerical codes based on an allelic table as published by Supply et al (49). The 24-digit profiles were compared using www.miru-vntrplus.org. Based on the results, each strain pair was defined as relapse or reinfection. Strain pairs with the same MIRU24 pattern or one locus of difference were considered as reflecting relapse. MIRU24 patterns differing by two or more loci between the two strains in a pair were defined as reinfection.

Double alleles in two or more loci were considered mixed infections and excluded from further analysis. Cases with double alleles in one locus were attributed to an event of intra-patient microevolution and considered a case of relapse (90).

3.3.7 Statistical analysis

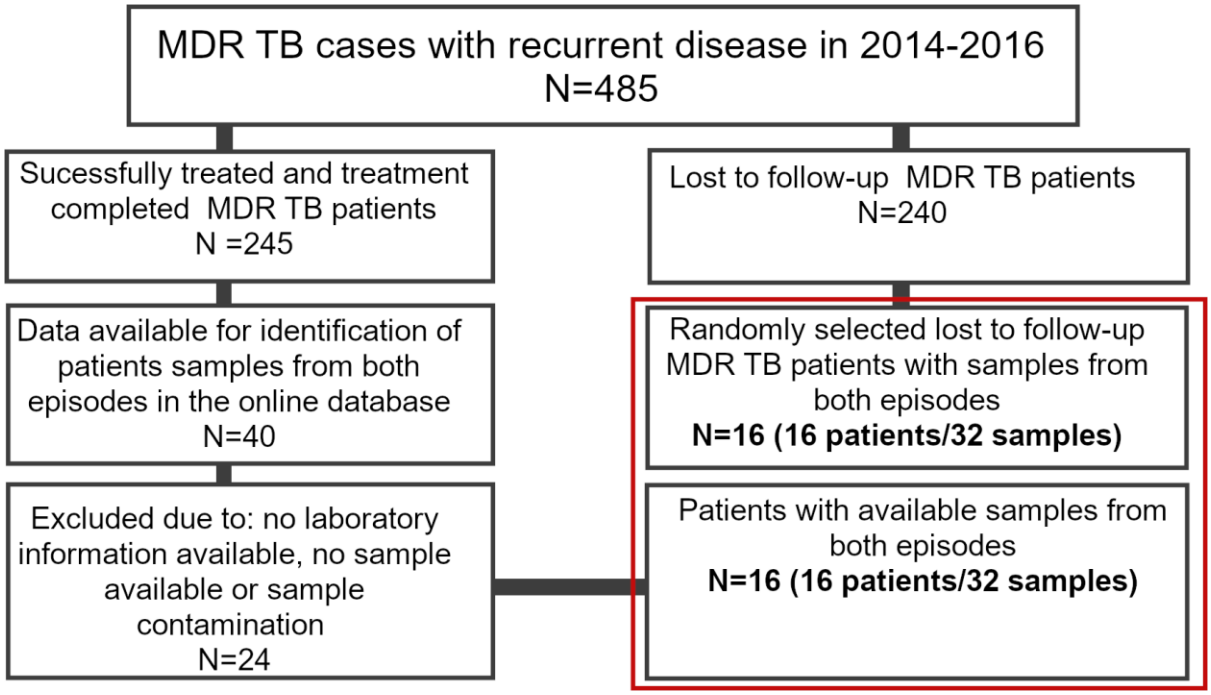
STATA v14.0 was used for statistical analysis. To compare categorical variables Fisher`s exact test was used and quantitative variables were compared using the Wilcoxon rank sum test. In all statistical comparisons, the significance level was set to 0.05. Logistic regression was used to assess associations between the odds of relapse / re-infection and the potential predictor variables gender, age, incarceration status during the primary or recurrent case, smear microscopy, HIV, number of people in household and smoking status were considered as independent variables. These predictor variables were considered one by one, in univariable models, and upon adjustment for age and imprisonment in multivariable models.

3.4 Results

3.4.1 Description of the study population

From a total of 1,361 MDR TB cases enrolled in 2nd-line treatment between 2014 and 2016 in Georgia, recurrent TB was detected in 485 (35.6%) patients, of whom 245 (50.5%) were successfully treated (treatment completed and cured) in the past, and 240 cases (49.5%) were reported as lost to follow-up. Epidemiological records included information for 40 (16.3%) of the successfully treated patients (Fig 1). From the total of 40, after exclusion due to missing laboratory data, missing specimen, or sample contamination, we ended up with a complete data set of 16 MDR-TB patients who were successfully treated (and 32 paired M. tuberculosis isolates). An additional 16 patients from the "lost to follow up" group were randomly selected and included in the study for comparison. Hence, a total of 32 patients (64 paired samples) were included in the study (Figure 3.1).

Figure 3.1. Flow diagram of selecting study participants.



3.4.2 MIRU-VNTR typing results: relapse vs. reinfection

Based on our MIRU24 typing results of the 64 paired M. tuberculosis isolates, 25/32 (78.1%) MDR TB patients had the same genotype in both paired isolates and were classified as relapse, while 5/32 (15.6%) showed different MIRU patterns and thus evidence of exogenous reinfection with a different strain. Two patients (6.3%) were classified as harboring a mixed infection.

3.4.3 Comparing patient characteristics across patient groups

After excluding the two patients with mixed infections, further analysis was performed using clinical and demographical data for the remaining 30 patients (Table 1). The median age of these patients was 37.5 (Inter Quartile Range (IQR) = 28–43 years) and 41.5 (IQR = 29–47 years), for the first and recurrent TB episodes, respectively. In the relapse group, the median age at the first episode was 41 years (IQR = 33–45 years) years and for the recurrent case 44 years (IQR = 35–49 years). In the reinfection group, patients' median age was 30 years (IQR = 26–42 years) at the first episode and 32 years (IQR = 28–43 years) at the second (i.e. reinfection) episode (Table 1). In the civil sector, relapse was observed in 20 (95.2%) patients out of 21. By contrast in the penitentiary system, 5 (55.6%) out of the 9 patients were due to relapse, while 4 (44.4%) were due to reinfection (Fisher's Exact Test, P = 0.004) (Table 3.1).

Table 3.1 Characteristics of the relapse/reinfection cases defined by MIRU-VNTR typing.

Characteristics		Total	Relapse *n* (%)	Reinfection *n* (%)	*P* value
Recurrence		30	25 (83.3)	5 (16.7)	
Sex	Female	5	5 (100)	0	0.37
	Male	25	20 (80)	5 (18.5)	
Age at first episode	<30	11	10 (90.9)	1 (9.1)	0.18
	31-40	6	6 (100)	0	
	41-50	11	8 (72.7)	3 (27.3)	
	>50	2	1 (50)	1 (50)	
Age at second episode	<30	9	9 (100)	0	0.28
	31-40	5	4 (80)	1 (20)	
	41-50	12	9 (75)	3 (25)	
	>50	4	3 (75)	1 (25)	
Group	Cured	15	11 (73.3)	4 (26.7)	0.33
	LFU*	15	14 (93.3)	1 (6.7)	
Origin	Civil	21	20 (95.2)	1 (4.8)	0.004
	Prison	9	5 (55.6)	4 (44.4)	
Smear [a]	Positive	14	12 (85.7)	2 (14.3)	0.75
	Discrepant	13	10 (76.9)	3 (23.1)	
	Negative	2	2 (100)	0	
	Unknown	1	1 (100)	0	
HIV	Positive	1	1 (100)	0	0.27

	Negative Unknown	24 5	21 (87.5) 3 (60)	3 (12.5) 2 (40)	
No. of people in household	0-2 3-5 ≥6 Unknown	6 6 12 6	5 (83.3) 10 (83.3) 5 (83.3) 2 (100)	1 (16.7) 2 (16.7) 1 (16.7) 0	0.57
Smoking	Yes No	16 10	13 (81.2) 9 (90)	3 (18.8) 1 (10)	0.65

For categorical variables Fisher`s exact test was used. Quantitative variables were analyzed with Wilcoxon rank sum test.

[a] Smear results from both episodes were compared, positive indicates Acid Fast Bacilli (AFB) (+) in both samples, discrepant refers to positive and negative results for either first or second sample, negative was defined with AFB(-) for both episodes. * LFU- denoted as lost to follow-up patients.

Finally, we were interested in the median time to relapse in both groups of patients—successfully treated (including treatment completion) and lost to follow-up. The median time after successful treatment and treatment completion was 49 months (26.7 months; IQR 22.6–89.4 months), while for the lost-to-follow-up patients, the median period until relapse was, as expected because of the interruption in treatment, much shorter—17.9 months (20.5 months, IQR 5.9–56.2 months).

3.4.4 Predictors for TB recurrence

Potential predictors for TB relapse and reinfection were analyzed using logistic regression (Table 3.2). Variables were adjusted for age and origin; odds ratios and CI were calculated. Odds ratios were obtained for lost to follow-up status, smear result, culture conversion, origin, smoking and number of people in the household, but no variable was statistically significant except imprisonment with high OR for reinfection (P-value = 0.03) (Table 3.2).

Table 3.2 Potential predictors of TB relapse and reinfection

Factors associated with relapse							
Characteristics	N(%)30	Un-adjusted OR (95%CI)	P-value	Adjusted OR (95%CI)[1]	P-value	Adjusted OR (95%CI)[2]	P-value
Lost to follow-up	15(50%)	5.1(52.3-0.5)	0.17	4.1 (44-0-37)	0.25	3.8 (47.9-0-29)	0.31
Smear positive	14(46.7%)	1.4 (9.8-0.2)	0.74	1.1 (8.74-0.15)	0.9	0.4 (5.7-0.03)	0.5
Culture conversion	18(60%)	1 (0.14-7.1)	1	1.21 (9.38-0.16)	0.86	2.86 (39.6-0.21)	0.43
Factors associated with reinfection							
Prison	18(60%)	16 (1.45-176.5)	0.02	51.93 (1.43-1891.5)	0.031	NA	NA
Smoker	16/26(61.5%)	2.08 (0.18-23.3)	0.55	3.3 (0.23-47.66)	0.38	1.64 (0.11-23.88)	0.72
Household members >2	22/28 (78.6%)	1.11 (0.1-12.31)	0.93	3.32 (0.16-70.11)	0.44	NA	NA

*CI= confidence interval; 1 - Adjusted OR (95%CI) for age; 2 - Adjusted OR (95%CI) for origin;

Lost to follow-up patients, patients with positive smear result and culture conversion were associated with relapse, but neither was statistically significant. While having been imprisoned, smoking habit and more than two cohabitants were positively associated with re-infection, but only the association with having been imprisoned was statistically significant.

3.5 Discussion

In this study, we used 24 loci based MIRU-VNTR typing to differentiate between relapse and reinfection in 32 patients with recurrent MDR-TB. Our results showed that the majority (>95%) of patients with recurrent MDR-TB from the civilian sector were due to relapse, whereas in patients with a history of incarceration, about half (44%) were due to reinfection.

The differentiation between relapse and reinfection in recurrent TB cases has major implications for the definition of national control measures. In the case of reinfection, prevention measures will need to be more inclusive at national level and require reducing TB transmission along with improving early detection of cases (89,102). On the other hand, preventing relapse, which mostly affects individual patients, requires strengthening treatment adherence in patient populations at high risk of relapse and more patient-oriented care (103).

Until now, all recurrent TB cases in Georgia were classified as “relapse” by the national surveillance program. This classification, as our data suggests, fails to describe the true nature of the infection in a substantial proportion of patients. Based on our data, 16.7% of recurrent cases were due to reinfection, and these were significantly associated with a specific high risk population, i.e. prisoners. Timely diagnosis of TB disease is a crucial part of controlling TB transmission in penitentiary system, while delay in diagnosis leads to an increased risk of TB transmission (104). Currently, the penitentiary system in Georgia provides an active TB screening program with questionnaires, followed by Xpert MTB/RIF test in case of disease suspicion. Regardless of the sharp decrease of TB cases in prison (National Surveillance Program, unpublished data) challenges still arise, in

addition to the lack of convenient tools for controlling the disease (105,106). Although our study was not directly focusing patients in prisons, our data shows that reinfection in TB is a significant problem in the penitentiary system that should be addressed.

Compared to drug susceptible TB, treatment for MDR-TB is long and more complex. Treatment for such patients includes second-line drugs that are known for their severe toxic side effects (107). Increased risk of recurrence due to relapse in MDR-TB patients suggests that the current MDR-TB treatment might not be enough to completely eliminate the bacteria (108–110). The percentage of MDR-TB cases in previously treated TB patients in Georgia has increased from 31% in 2010 to 38% in 2016, followed by a decrease to 22% in 2018 (National Surveillance Program, unpublished data), indicating the presence of a reservoir for drug-resistant bacteria in the country, partially due to incomplete treatment. Surprisingly, we did not find a statistically significant association between relapse and "lost to follow-up", but this could be due to the small sample set included in our study. We found no association between the number of people in households and risk of reinfection.

We managed to distinguish between relapse and reinfection in most of our cases. However, reinfection with the same strain of M. tuberculosis might suggest relapse, rendering the differentiation between relapse and reinfection more challenging. While MIRU-VNTR is still a widely used genotyping tool, whole genome sequencing (WGS) provides higher resolution to differentiate between closely related Mycobacterium tuberculosis strains. Due to the high costs and complex data analysis, using WGS is still limited, but gradually becoming more affordable, and thus likely to replace other genotyping methods in the near future (111).

One of the limitations of the study is the small sample size, leading to the large confidence intervals in our estimates. However, data from similar incidence settings show similar proportions of relapse and reinfection (96,112–114), and therefore we do not expect these proportions to change extensively, even if we included a larger sample size.

In conclusion, MIRU-VNTR typing base on 24 loci was implemented successfully in Georgia as a tool for differentiating between recurrent MDRTB caused by relapse versus reinfection. Our data revealed that relapse is a major contributor to problem of recurrent TB in Georgia. Despite the recent increases in resources made available to the National

Tuberculosis Program (NTP) to decrease TB incidence countrywide, our data highlights the need for improved treatment completion and a reduction in the number of patients who were lost to follow-up.

3.6 Acknowledgments

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4. Developing customized stepwise MIRU-VNTR typing for tuberculosis surveillance in Georgia

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4.1 Abstract

Introduction

Mycobacterial Interspersed Repetitive Units-Variable Tandem Repeats (MIRU-VNTR) typing has been widely used for molecular epidemiological studies of tuberculosis (TB). However, genotyping tools for Mycobacterium tuberculosis (Mtb) may be limiting in some settings due to high cost and workload. In this study developed a customized stepwise MIRU-VNTR typing that prioritizes high discriminatory loci and validated this method using penitentiary system cohort in the country of Georgia.

Methods

We used a previously generated MIRU-VNTR dataset from recurrent TB cases (32 cases) in Georgia and a new dataset of TB cases from the penitentiary system (102 cases) recruited from 2014 to 2015. A Hunter-Gaston Discriminatory Index (HGDI) was calculated utilizing a 24 standard loci panel, to select high discriminatory power loci, subsequently defined as the customized Georgia-specific set of loci for initial typing. The remaining loci were scored and hierarchically grouped for second and third step typing of the cohort. We then compared the processing time and costs of the customized stepwise method to the standard 24-loci method.

Results

For the customized Georgia-specific set that was used for initial typing, 10 loci were selected with a minimum value of 0.32 to the highest HGDI score locus. Customized 10 loci (step 1) typing of 102 Mtb patient isolates revealed 35.7% clustered cases. This proportion was reduced to 19.5% after hierarchical application of 2nd and 3rd step typing with the corresponding groups of loci. Our customized stepwise MIRU-VNTR genotyping approach reduced the quantity of samples to be typed and therefore overall processing time and costs by 42.6% each.

Conclusion

Our study shows that our customized stepwise MIRU-VNTR typing approach is a valid alternative of standard MIRI-VNTR typing panels for molecular epidemiological

investigation in Georgia that saves time, workload and costs. Similar approaches could be developed for other settings.

4.2 Introduction

Genotyping plays an important role in the surveillance and molecular epidemiology of tuberculosis (TB). However, the implementation of TB genotyping methods has been limited in many settings, especially in low- and middle-income countries (LMIC), given that they can be computationally demanding, time-consuming and costly (44,115). Mycobacterial Interspersed Repetitive Unit-Variable Number of Tandem Repeats (MIRU-VNTR) typing is widely used to evaluate the population structure, strain genetic diversity, and transmission of the Mycobacterium tuberculosis complex (MTBC) (116,117). Globally, the human-adapted MTBC can be classified into nine phylogenetic lineages that differ in their geographic distribution (37,38). This phylo-geographic genetic diversity also impacts the discriminatory power of MIRU-VNTR loci in a given population, which has led to the development of customized MIRU-VNTR loci sets for application in different geographical settings (50,115,118). Compared to the MIRU-VNTR typing using standard 12, 15 or 24 loci panels proposed by Supply et al (49), customized approaches have focused on the most sensitive and highly discriminatory loci (119,120), allowing for reduced workload, labor and consumable-dependent cost and sample processing time.

However, customization and reduction of the MIRU-VNTR loci set might be inefficient for reaching higher level of discrimination, especially where the variety of MTBC lineages is limited and the bacterial population is homogeneous. In such instances, various combinations of genotyping tools for increasing resolution have been proposed (48,120,121), but no systematic approach has yet been established. Considering the limited resources in LMIC, combining several distinct typing methods increases the complexity and costs, and the absence of standardization results in poor comparability between the laboratories. In the present study, we implemented customized stepwise MIRU-VNTR typing specifically for the epidemiological setting of Georgia (122). This approach is based on selecting and developing groups of MIRU-VNTR loci, to reduce the number of samples to be typed after each step.

Recently, standard 24-loci MIRU-VNTR typing has been established at the National Reference Laboratory (NRL) of National Center of Tuberculosis and Lung Diseases (NCTLD) in Tbilisi, Georgia, which has been used to differentiate between relapse and reinfection in recurrent TB cases (123). In this study, we modified the standard 24-loci

MIRU-VNTR panel into a customized stepwise typing approach for the Georgian Mtb population and applied it to a patient population from the penitentiary system.

While important progress in TB control and a substantial decrease in TB incidence in Georgia from 2002 (228 per 100,000) to 2019 (80 per 100,000) has occurred, many challenges to TB elimination remain, including high rates of drug-resistant TB cases. In particular, the penitentiary system has been identified as a hotspot of TB disease transmission including multidrug-resistant (MDR)-TB (43,104,106). Over the past six years, with the advent of new programmatic initiatives, cases of TB diagnosed in the penitentiary system have decreased, including MDR and extensively drug-resistant (XDR) TB, from 33% of all MDR cases in the country in 2011 to the 4.1% in 2020 (National Surveillance Program, unpublished data). However, the penitentiary system remains a persistent site for transmission of drug-resistant TB (43,104,105), bearing a risk of infection spillover into the community. Hence, rapid detection of TB transmission in prisons, along with infection surveillance in the country, is crucial, and resource-adapted molecular methods for surveillance are therefore needed.

4.3 Materials and Methods

4.3.1 Ethics

Ethical approval for the study was obtained from the relevant Institutional Review Boards (IRB) of the NCTLD in Georgia and in Switzerland (Ethikkommission Nordwest- und Zentralschweiz). Due to observational nature of the study which do not involve any physical intervention and was solely using the routinely collected samples from TB patient in Georgia, the need of informed consent was waived by the National Council on Bioethics of Ministry of Labor, Health and Social Affairs of Georgia, and therefore by local IRB. The data from archived isolates were fully anonymized to guarantee the confidentiality.

4.3.2 Customization and validation of country-specific minimal set of MIRU-VNTR loci

4.3.2.1 Genotyping dataset and parameters for customization

In order to develop a customized loci set for the Mtb strain population in Georgia, we first re-analyzed a dataset from a previous study of recurrent TB cases, with a retrospective cohort of 32 patients (64 samples) from 2014–2016, typed with the standard 24 loci panel (123). Inclusion criteria of the samples for the relapsed cases was availability of the isolates in the National Reference Laboratory biobank. From this dataset, we used allelic data for 37 isolates; 27 single Mtb isolates from relapsed patients and paired isolates from 5 reinfection cases. We defined genotypic clusters with a single locus variants (SLV), and calculated discriminatory indexes for each locus of the 24-loci panel using Hunter and Gaston discriminatory index (HGDI) (124). Based on the Simpson's index of diversity, HGD index was calculated using the online tool (http://insilico.ehu.es/mini_tools/discriminatory_power). Following the HGDI calculations and acknowledged cut-offs, the loci were classified into high (HGDI≥0.6), moderate (0.3≤ HGDI<0.6) and low (HGDI<0.3) discriminant categories (119). We ranked the 24 loci based on their HGDI score and selected the 10 loci with highest discriminatory indexes.

4.3.2.2 Genotyping dataset for customized set validation

For validating our customized set of loci, we focused on patients with pulmonary TB residing and diagnosed in the penitentiary system in 2014–2015. Cases were not overlapping the recurrent TB case isolates, were identified through the national TB surveillance database and were linked to the National Reference Laboratory (NRL) sample ID numbers to identify the NRL strain biobank and phenotypic drug susceptibility testing (pDST) results. We were able to link 136 TB cases in prisons from 2014.2015, with bio banked Mtb isolates for further use in the study, however, total of 102 Mtb isolates were recovered from the laboratory biobank and were typed using the customized 10 loci set. Specifically, the 10 customized MIRU-VNTR loci were multiplexed in four separate PCRs– 1) MIRU16, MIRU31 and ETRB; 2) MIRU40, MIRU26 and Mtub30; 3) Mtub 21, QUB11b and QUB26; 4) MIRU39. Negative (H_2O) and positive (H37Rv DNA) controls were included in each reaction. PCR amplification was performed under following conditions: the initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1.5 minutes with final extension at 72°C for

10 minutes. We used low-resolution electrophoresis for amplification check, followed by higher resolution electrophoresis with 1.8% agarose gel, using 1kb ladder as size marker. A numerical code was obtained by comparison of the allelic table published by Supply *et al* (49). Genotypic clusters were defined based on SLV.

4.3.3 Re-evaluation of the customized set and HGD indexes and stepwise approach development

For the stepwise typing approach development, we used the remaining 14 loci to type 35 Mtb isolates from the penitentiary system that were clustered after typing with the customized 10 loci set. Additionally, we re-assessed the discriminatory power of each locus based on the combined datasets of i) recurrent TB cases (37 isolates) and ii) prison cohort samples for which full 24 loci results were available (35 isolates). The HDGI was re-calculated and the loci were ranked following the cut-off scores described above. The criteria of locus hierarchy and inclusion into the two-step typing panel was defined by i) HGDIs, calculated within the recurrent TB cases and penitentiary system cohort; and ii) ability of the locus to discriminate and reduce proportion of the samples to be typed by the stepwise addition of each locus to the predefined 10 loci customized set (Figure 4.1).

Figure 4.1. Schematic representation of the MIRU-VNTR stepwise approach.

Schematic representation of the stepwise approach, with reduced amount of the samples to be typed after application of each step of locus group, resulting reduced time, cost and workload.

4.3.4 Cost and time assessment

Along with method modification, we assessed the processing time and approximate direct cost based on the technician per-hour labor cost, consumables and reagents pricing in Georgia for a batch of 54 samples processed with the customized stepwise MIRU- VNTR typing (excluding DNA extraction) and standard 24-locus panel, with 2 additional controls. The number of isolates in a batch was defined based on the number of wells available on our electrophoresis tray.

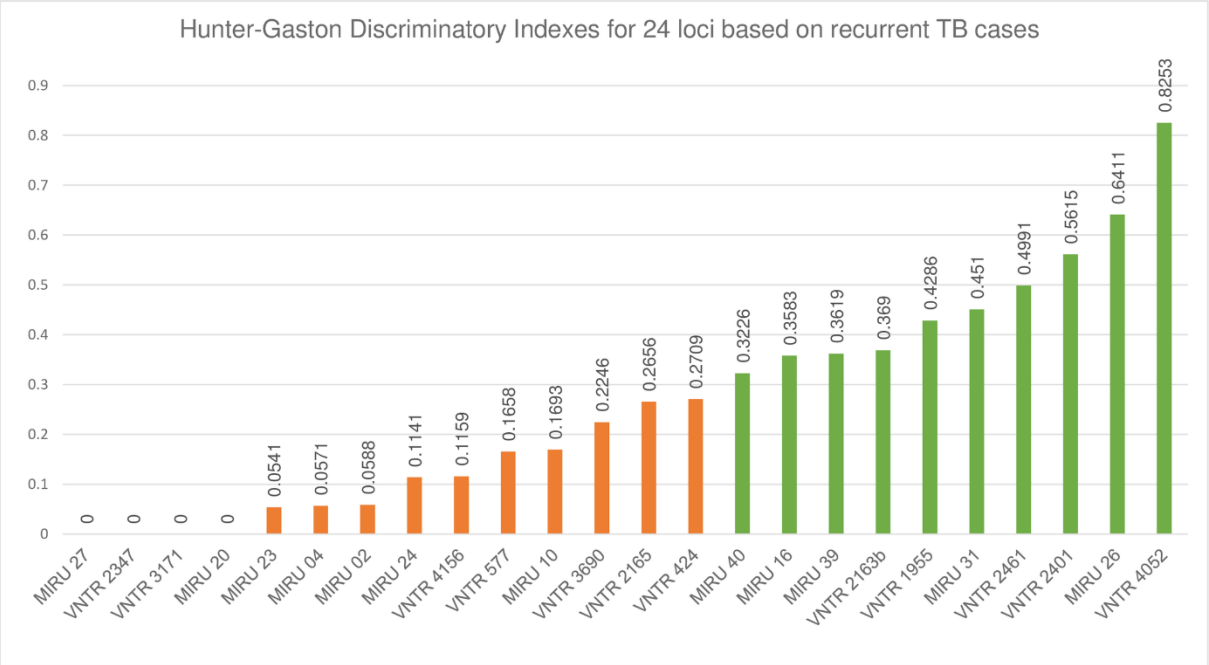
4.4 Results

4.4.1 Customization of the Georgia-specific MIRU-VNTR loci set using typing results from recurrent TB cases

Utilizing the standard 24-loci panel, MIRU-VNTR typing of 37 isolates from our previously published cohort of recurrent TB cases (123) revealed two highly discriminatory loci, namely - VNTR4052 (QUB-26) and MIRU26, with an HGDI of 0.83 and 0.64, respectively (Figure 4.2). Eight moderately discriminant loci were identified: VNTR2401/Mtub30 (HDGI=0.56), VNTR2461/ETR B (HGDI=0.50), MIRU31/ETR E (HGDI=0.45), VNTR1955/Mtub21 (HGDI=0.43), VNTR2163b/QUB-11b (HGDI=0.34), MIRU39 (HGDI=0.36), MIRU16 (HGDI=0.36), MIRU40 (HGDI=0.32). Another ten loci showed low discriminatory indices: VNTR424/Mtub04 (HGDI=0.27), VNTR2165/ETR A (HGDI=0.27), VNTR3690/Mtub39 (HGDI=0.22), MIRU10 (HGDI=0.17), VNTR577/ETR C (HGDI=0.17), VNTR4156/QUB-4156c (HGDI=0.12), MIRU24 (HGDI=0.11), MIRU02 (HGDI=0.06), MIRU04/ETR D (HGDI=0.06) and MIRU23 (0.05). Finally, four loci, MIRU20, VNTR2347/Mtub 29, MIRU27/QUB-5 and VNTR3171/Mtub34 showed no discriminatory power with an HGDI score of zero (Figure 4.2). A Georgia-specific panel of ten loci was defined, which included the two highly and the eight moderately discriminatory loci (Figure 4.2). The loci in the customized Georgia-specific set were compared to the standard 15- and 12-loci panels (2). Our customized panel shared four loci with both the 12- and 15-loci standard panels, while four and one loci were exclusively shared with the 15-loci set and the 12-loci set, respectively. VNTR2461 with

a moderate discriminatory power in the Georgia-specific panel has so far not been included in earlier proposed typing sets.

Figure 4.2. HGDI scores of 24-loci panel based on recurrent TB cases.



Graph represents individual discriminatory index for 24-loci panel generated from recurrent TB cohort typing results. Green bars indicate "the Georgian customized set" with high and moderate index loci included in the customized set.

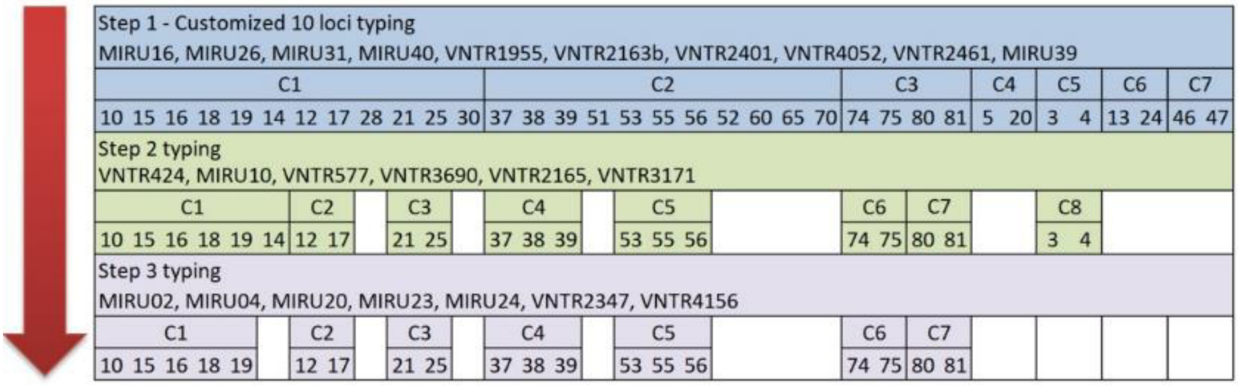
4.4.2 MIRU-VNTR typing of the Mtb samples from the penitentiary system

4.4.2.1 Genotyping with the customized 10-loci set

From total of unique 102 Mtb isolates from prison TB cases, full allelic data was obtained for 98 (96.1 %) isolates. MIRU-VNTR genotyping of 98 Mtb isolates with our customized 10-loci set revealed seven clusters by SLV comprising 35 clustered isolates and 63 singletons (non-clustered isolates). The 35 clustered isolates were distributed in the seven clusters as follows: cluster 1 – 12 isolates (34.4%), cluster 2 - 11 (31.4%), cluster 4

- 4 (11.4%), cluster 4 to 7 were represented with two (5.7%) isolates per cluster (Figure 4.3).

Figure 4.3. Stepwise application of customized and additional 14 loci and distribution of the clustered samples.



Selected loci for the stepwise approach and distribution of the clustered samples after typing with customized 10-loci set, step two typing with 6 loci and step 3 typing with remaining 8 loci.

For the stepwise typing approach development of the clustered isolates, we used the remaining 14 loci from the standard 24-loci panel not included in our customized 10-loci set. These resulted in an additional 16 singletons, while the number of clusters remained the same, with a sample distribution as follows: cluster 1 – five isolates (26.3%), cluster 2, 3, 6 and 7 each with two isolates (10.5%), while cluster 4 and 5 had three (15.8%) isolates each, resulting in a total of 19/98 (19.5%) clustered isolates (Figure 4.3).

4.4.2.2 Defining loci sets for the stepwise approach

In order to combine loci for the second typing step, we re-evaluated and compared rankings of HGDI scores (Figure 4.4 A-B) by assessing the number of discriminated isolates by each locus added separately to the customized 10-loci set within the combined dataset. VNTR424/Mtub04 showed an increased HGDI (within the additional 14 loci set) in the penitentiary system compared to recurrent TB typing data (Figure 4.4 B). Four loci – MIRU10, VNTR577/ETR C, VNTR2165/ETR A and VNTR3690/Mtub39 added most to the increase in HGDI. VNTR3171/Mtub34 showed an increased capacity to discriminate

between the isolates when added to these four loci, while the HGDI of VNTR3171/Mtub34 on its own was 0.05 and 0 in the penitentiary and recurrent TB cohort was, respectively. Application of the second step loci VNTR424/Mtub04, MIRU10, VNTR577/ETR C, VNTR2165/ETR A, VNTR3690/Mtub39, and VNTR3171/Mtub34 reduced the number of clustered isolates from 35 to 22, leading to the reduction of the samples to be genotyped by 37%.

The remaining eight loci (MIRU02, MIRU04/ETR D, MIRU20, MIRU23, MIRU24, MIRU27/QUB-5, VNTR2347/Mtub 29, and VNTR4156/QUB-4156c) were included in a third and last step of genotyping. The HGDI of MIRU27/QUB-5 remained zero in both cohorts, as it did not identify any discrete sample from the population. Application of the third step loci on 22 clustered samples identified 2 singletons, defining the final outcome of the customized stepwise MIRU-VNTR typing of penitentiary system cohort – 19 (19.4%) clustered cases from the total of 98 isolates.

Figure 4.4. Individual locus HGDI of 10 customized and additional 14 loci

(A) HGDI calculated for the customized 10 loci and (B) the additional 14 loci, based on typing of recurrent TB cases (civil cohort) in blue bars along with same indexes calculated within penitentiary system samples in orange. Grey bars indicate indexes calculated from combined datasets with 24 loci typing. Category definition: HGDI≥0.6 high discriminatory power, 0.3≤HGDI≤0.6 moderate discriminatory power, HGDI<0.3 low discriminatory power.

4.4.3 Processing time for the customized stepwise MIRU-VNTR typing

Considering a conventional method with two gel trays with 32-well gel comb, it is possible to run 54 isolates with 2 controls and 8 molecular size markers. To compare the total amount of time required for our customized stepwise genotyping approach and for the standard 24-loci method, we calculated the processing time for 56 isolates (including controls) for both methods. Our calculation included the following steps: master mix preparation (20 min.), adding sample DNA (20 min.), PCR (165 min.), and loading samples for gel electrophoresis (40 min.), running the gel (240 min), dying (20 min.) and imaging (8 min).

Applying the full 24-loci panel on the 102 patient isolates resulted in a total of 2,448 amplicons to run on gel electrophoresis, with a total processing time of 387.6 person-hours. By contrast, our customized stepwise typing approach resulted in a reduced number of amplicons to be processed from 2,448 to 1,020 (41.7%) after 1st step customized 10 loci typing. This was followed by 210 (8.6%) amplicons after applying the 2nd step typing with 6 loci, and to 176 (7.2%) amplicons to be typed for the 3rd step with the remaining 8 loci. Hence, our customized stepwise approach resulted in a total of 1,406 amplicons to run, with 41.7% and 8.6% reduction of typing with 1st step customized loci and 2nd step typing with an additional 6 loci, respectively. Compared to the 387.6 person-hours with 24-loci panel typing, the stepwise approach required 222.6 person-hours, which corresponds to a reduction in total processing time of 42.6%.

4.4.4 Cost comparison of the customized stepwise genotyping approach

The price of the 24-loci MIRU-VNTR per sample based on consumable prices in Georgia in addition to laboratory technician salary per-hour was approximately 1,124 Euros, while the customized stepwise approach using the 10-6-8 loci panels sequentially resulted in a decreased cost of approximately 648 Euros. This corresponds to a cost reduction of 42.6% compared to the standard method, indicating a major dependence of the total cost on the isolate quantity and processing time.

4.5 Discussion

We adapted the standard 24-loci MIRU-VNTR panel for the Georgian TB population by i) reducing the number of loci to a customized set of 10 loci for an initial step of genotyping; ii) developing a stepwise typing approach to retain adequate discriminatory power; iii) applying this approach on a retrospective TB patient cohort in a penitentiary system in Georgia. Our findings demonstrate that the customized stepwise MIRU-VNTR tying approach reduces the processing time, cost and workload, while achieving equal discriminatory power to 24 standard MIRU-VNTR loci panel. The modified MIRU-VNTR typing can be a benefit for the TB disease transmission control in the most effective way, especially for LMIC.

For the purpose of the MIRU-VNTR customization, using HGDI, we modified the 24-loci panel based on the discriminatory power of each loci. Initially, we chose the 10 highest HGDI score loci and compared them to the standardized 12- and 15-loci panel sets (Table 4.1). While four loci included in our customized set were shared with both the standard sets, one locus was shared only with the 12-loci panel, four loci only with the 15-loci panel, and one locus - VNTR2461 (ETRB) was not present either of the two standard sets. The bias of 15-loci and 24-loci panels towards MTBC lineage 4 has been well-documented (49). In Georgia, most MTBC belongs to lineage 4 and lineage 2/Beijing. Deviation from the standard loci panels is mostly influenced by HGDI sensitivity to local strain variation. Therefore, our results from assessing a customized set, based on the MTBC lineages circulating in Georgia, are consistent with previous studies showing the influence of strain diversity on the discriminatory power of genotyping methods (50,119,120). This variation thus should be considered when choosing appropriate methodologies for molecular epidemiological studies, whereby customizing based on local strain variation is one possible way, particularly in resource-limited settings (119,120,125).

Table 4.1. MIRU-VNTR 24 loci set with individual HGDI and inclusion in standardized and customized genotyping panel.

Locus (24 loci panel)	12 Loci Panel	15 Loci Panel	Customized 10–loci set
MIRU 02	X		
MIRU 04	X	X	
MIRU 40	X	X	X
MIRU 10	X	X	
MIRU 16	X	X	X
MIRU 20	X		
MIRU 23	X		
MIRU 24	X		
MIRU 26	X	X	X
MIRU 27	X		
MIRU 31	X	X	X
MIRU 39	X		X
VNTR 424		X	
VNTR 577		X	
VNTR 1955		X	X
VNTR 2163b		X	X
VNTR 2165		X	
VNTR 2347			
VNTR 2401		X	X
VNTR 2461			X

VNTR 3171			
VNTR 3690		X	
VNTR 4052		X	X
VNTR 4156		X	

For increased discrimination, we additionally typed isolates that were clustered based on the initial typing with the remaining loci from the 24-loci panel and re-evaluated the pre-defined customized set. The initial customized panel revealed 35.7% of clustered cases, which was subsequently reduced to 19.4% after applying an additional 14 loci. Re-evaluation of the HGDI indexes, along with assessment of the ability of each locus to identify the majority of the discrete isolates during the step-by step addition to the customized 10-loci set, allowed us to arrange the remaining 14 loci into the stepwise typing approach. While the 10-loci customized set might be sufficient as a standalone panel for particular epidemiological studies, it can be also considered as a primary typing set - step 1, for the hierarchical application of additional MIRU-VNTR loci. However, if a higher discriminatory power is required, our customized stepwise approach showed the capacity to reduce the number of samples to be typed by 41.7% and 8.6% using customized/step 1 and step two typing, respectively, leading to a reduced workload, processing time and cost.

We did observe the influence of the particular study population on the discriminatory power of specific loci, for example for locus VNTR424 and VNTR3171. Therefore, evaluation of the discriminatory power by HGDI needs to consider the homogeneity of the population to be typed.

Our customized stepwise approach advances classical 24 MIRU-VNTR panel by reduced workload, processing time and direct costs (technician per-hour labor cost, consumables and reagents) by 42.6%. Given that our findings are based on local pricing of the consumables and laboratory technician average salary, the result should thus be treated with caution, as they will vary based on the specific context concerning costs of technician salaries and consumables. Variation in cost reduction (absolute number) is attributable

to local pricing. In Georgia, a middle-income country, the consumable prices are much higher compared to Europe and United states due to procurement and shipping complexities, whereas estimated labor costs are lower. Therefore, the direct cost savings discussed in the paper are specific for the Georgian setting but this approach is likely to result in cost savings in other settings as well.

Based on our findings, we propose "The Georgian customized 10 MIRU-VNTR loci set", to be used for molecular epidemiological studies in the country of Georgia as a primary typing tool for identifying major clusters or defining relapse/reinfection. Moreover, the additional 14 loci could be used for further discrimination in clusters defined based on the initial typing. Such a stepwise approach using two typing sets will reduce time, cost and workload, while maintaining high discriminatory power and reproducibility in the most practical way.

Our study has several limitations. Firstly, our sample size was moderate. However, the study covered all the available isolates from the penitentiary system and the combination with previous dataset from the general population had increased the variation within the sample set. Secondly, even though MIRU-VNTR typing can produce sufficient data for specific questions, if higher resolution is needed, this method can be limiting, especially in settings with a large proportion of lineage2/Beijing strain (44). Ideally, whole genome sequencing (WGS) would be the method of choice; however, it requires the high-level facility with sequencing instruments, which currently are not available in Georgia. Even more importantly, the bioinformatics expertise is not widely available in Georgia, which increases complexity of using the WGS methodology. Tentatively, compared to WGS pricing in Europe and United States, the cost of the sequencing in Georgia would be much higher than MIRU-VNTR. Our study results combined with previous data showed that usage of the combination of a customized set of MIRU-VNTR loci and a stepwise approach gives useful information in the most feasible and cost-effective way.

Although conventional MIRU-VNTR is still a widely used tool, this method may still overestimate Mtb recent transmission events as confirmed by several studies (128–130). The next decade is likely to witness transition to sequencing technologies due to considerable decrease in sequencing cost (111). Additionally, decline of the TB burden within the country would give a significant advantage to these technologies to be used for

not only epidemiological investigations, but for diagnosis and drug resistance determination as well. However, until this transition occurs in developing countries, MIRU-VNTR typing based on a customized stepwise approach could be valuable addition to the surveillance tools used in Georgia to enhance the control and prevention of TB.

5. Linking genomic variants in bedaquiline resistance associated genes to phenotypical susceptibility

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This is a working chapter to be further developed and published subsequently.

5.1 Introduction

During the past decade, a number of novel anti-tuberculosis drugs have been under clinical trial for the drug resistant TB (DR-TB) treatment; the latter being still a major threat for public health and TB management (131). Rapid detection and effective treatment play a pivotal role in preventing disease transmission as well as drug-resistance development. The low effectiveness of DR-TB treatment is mainly due to long and complicated treatment. The most commonly used treatment regimens against DR-TB lasts for 18-24 including high-toxicity drug combinations. Due to severe side effects, non-adherence to the treatment contributes to selection of DR strains and relapses, complicating TB control (131).

Increasing effectiveness and adherence of the treatment can be achieved by shortening the treatment regimens with more tolerable medications, which also should be more efficient compared to the current alternatives. Long clinical trials, lacking knowledge regarding the phenomenon of tolerance (132), testing combinations of drugs took several decades of research for novel TB medications. A few candidates have been developed, with the highest effectiveness reached with the compound TMC207, originally described by Andries *et al* (72). TMC207, also known as bedaquiline (BDQ) that belongs to the group of diarylquinolines (DARQ), with mycobacterial ATP synthase inhibition as the main target of drug action.

In December 2012, the US Food and Drug Administration (FDA) approved BDQ as a novel chemical class of anti-mycobacterial medication with a high potency of bactericidal activity. Alongside with promising results for treatment of drug resistant cases and increased drug resistant TB treatment success globally (133–135), WHO recommended BDQ-based shorter treatment regimens for MDR and XDR TB treatment. Despite of huge advantages, much uncertainty still exists regarding the long-term effectiveness and resistance emergence.

5.1.1 Mechanism of action

As a member of the diarquinolones, BDQ has a particular effectiveness on a variety of mycobacteria, including *M. kansasii, M. fortuitum, M. abscessus*, MAC, with specific affinity to inhibit the ATP synthase function of these bacteria (72). Leading to ATP depletion, BDQ interferes with various ATP-dependent metabolic pathways (136).

Located in the inner membrane of mitochondria, mycobacterial ATP synthase is an enzyme crucial for survival of the bacteria. Consisting of two major sectors F1 and F0, each of which is comprised of several subunits – a3/b3/g/d/Ɛ and a/b2/c10-15, respectively. While F1 sector serves in the forming of ATP, after combining ADP and phosphate, the F0 sector is the main ion-conducting pathway. The proton force formed in F0 leads to a conformational change of the c subunit that is coupled with the b subunit of the F1 sector, triggering its enzymatic activity. BDQ has an affinity for the c subunit of the F0 sector, encoded by the *atpE* gene, and blocks the ATP synthesis by inhibiting the coupled rotation (137). Additionally, the affinity of BDQ towards the Ɛ subunit has also been described, even though it is much lower compared to c-subunit. *In vivo* experiment with *M. smegmatis* carrying a mutated Ɛ subunit showed that the modification of the Ɛ subunit reduced the susceptibility towards BDQ, with 2.5 fold increase in MIC (138) .

Resistance to BDQ has been detected in several clinical settings as well as in ex vivo studies, and is attracting widespread interest (139–143). In the population based surveillance study within several high burden DR-TB countries, Battaglia et al. explored genomic variants associated with BDQ resistance specifically in treatment-naïve MTB clinical strains, describing more than 100 mutations dispersed across the target genes (144). Along with other similar studies, these data show that these mutations occur frequently and are not necessarily associated with prior exposure to BDQ or clofazimine (145,146).

Although the major resistance mechanism to BDQ involves the *atpE* gene in laboratory strains, efflux-pump overexpression is a leading cause of reduced susceptibility in both, experimental and clinical MTB isolates. Mutations in the repressor gene *Rv0678*, leading to the overexpression of mmpS5-mmpL5 – encoding an efflux pump, is the main mechanism for observed increased MICs and cross resistance to clofazimine, along with mutations in the *pepQ* gene, encoding an aminopeptidase, presumably with a similar function to the creatinases (142). Interestingly, in a murine TB model, experimental *pepQ* mutants of MTB exhibited no fitness cost with less susceptibility to BDQ (147).

Cross-resistance between BDQ and clofazimine has been well documented and mainly associated with mutations in the Rv0678 gene, conferring “low level” resistance to both drugs. In addition, mutations in the *pepQ* gene lead to increased MIC to BDQ and

resistance to clofazimine. While clofazimine has been used for a several decades, cross-resistance to BDQ bears utmost importance considering resistance acquisition towards the latter (147,148).

In 2021, WHO published the first catalogue of the mutations in MTBC in correlation with phenotypic drug resistance, serving as a reference standard for mutations as genetic markers for all first line drugs and a number of second line medications. Although more than 17,000 mutations were included in this catalogue, a significant uncertainty regarding BDQ resistance-conferring variants remain, mainly due to the limited data available. Moreover, due to conservative and limited analysis framework, none of the mutations putatively associated with BDQ resistance met the criteria for final confidence grading, and were therefore not supported to be formally declared as resistance associated mutations (149). A recent study by the CryPTIC Consortium proposed a list of BDQ genotypic variants associated with increased MICs to BDQ, even though there is still a lack of data for these mutations to be regarded as high-confidence mutations (150).

The significant challenge for defining variants as high-confidence mutations is absence of high quality phenotypic DST data. Whereas it can provide results for variety of the drugs, the method produces susceptibility data for only single – critical concentration (CC), recommended by WHO. Although single concentration based phenotypic assays are still considered as a gold standard, there are several methods for multi concentration drug susceptibility testing, such as the agar proportion method (APM), the resazurin microtitre plate assay (REMA), the Sensititre MycoTB plate method; we used thelatter in our study to determinate MIC values of the BDQ.

Our study aimed to examine the emerging mechanisms of BDQ resistance by assessing three target genes – *atpE, pepQ* and *Rv0678*, and the influence of genomic variations on the MIC in addition to exploring the genomic basis of phenotypically resistant MTB strains.

5.2 Materials and methods

5.2.1 Ethics

The study was conducted at the National Reference Laboratory (NRL) in collaboration with the Swiss Tropical and Public Health Institute. Ethical approval was obtained by the relevant authorities in Georgia (the Institutional Review Board of the National Center for Tuberculosis and Lung Diseases, Tbilisi, Georgia) and Switzerland (Ethikkommission Nordwest- und Zentralschweiz).

5.2.2 Materials and datasets

5.2.2.1 Sample set with mutations in genes associated with bedaquiline resistance

From 2008 to 2019, up to 8,000 MTB genomes have been sequenced through the ERC project work. The sequencing data was analyzed using an in-house pipeline. Only high-quality genomes were considered: minimum mean coverage 15X, and no mixed infection. From the resulting genomes, we identified any nonsynonymous mutations, frameshift indels and nonsense mutations falling in BDQ-resistance associated genes - *atpE*, Rv0678, *pepQ*. Only variants that met the following thresholds were considered: minimum mapping quality of 20, minimum read depth 7X, minimum base quality score of 20 and maximum strand bias 90%. The lineage of each strain was determined by interrogating genomic positions from a well-defined catalog of lineage-defining SNPs.

5.2.2.2 Phenotypically bedaquiline resistant sample set

Phenotypically bedaquiline resistant isolates were identified through the NRL laboratory database. Selected isolates had pDST performed on liquid media using Mycobacteria Growth Indicator Tubes (MGIT) using standard protocol described elsewhere (151). The testing was done as a part of routine patient care. BDQ was included in the second line DST set along with moxiflocacin (0.25ug/ml and 1ug/ml), levofloxacin (1ug/ml), linezolid (1ug/ml), clofazimin (1ug/ml), delamanid (0.06ug/ml), amikacin (1ug/ml). The WHO interim critical concentration for BDQ of 1mg/L resistance was adopted to MGIT cultures.

5.2.3 Bacteriology and MIC evaluation

Isolates from both datasets, including isolates with mutations in any of the three key genes and phenotypically resistant cases, were retrieved from the biobank at NRL. Isolates were cultivated on Lowenstein Jensen media until sufficient growth was observed. Grown isolates were used to make suspension adjusted to McFarland standard 0.5 and 100ul of suspensions were cultivated on Sensititre MycoTB MIC plates with pre-prepared broth medium with a range of BDQ concentrations as follows: 0.03-0.06-0.12-0.25-0.5-1-2-4 mg/L. The Sensititre MycoTB MIC (MYCOTB; Trek Diagnostic Systems, Cleveland, OH) is a commercially available dry microdilution plate, which was used for MIC testing for all isolates. After inoculation, plates were covered with sealers and incubated at 37^0C. One growth control was included per isolate in 7H9 medium without BDQ. Each batch included the fully susceptible strain H37Rv as a control as well. For the purpose of the validation, isolate resistant to the BDQ, provided by supranational reference laboratory for quality control panel was also tested. Plates were checked for contamination after 24 and 48 hours. Cultures were monitored on 7-10-14-21 days by visual examination using mirrored viewer. For purpose of result validation, the tests were repeated for the isolates showing increased MIC.

5.2.4 DNA extraction and sequencing

Whole genome sequencing data, available through nationwide project funded by European Research Council (ERC) – ECOEVODRTB, was used for identifying mutations in BDQ resistance conferring genes. In addition, data was screened for clofazimine cross-resistance conferring mutations. DNAs were extracted with CTAB method and Nanodrop measurement was used for to check concentration and purity. Sequencing was performed at corefacility of ETHZ/University of Basel, Switzerland, on a Illumina Novaseq 6000 instrument.

5.2.5 Phylogenetic analysis

To construct the phylogenetic tree, we obtained a pseudo-alignment of polymorphic positions by concatenating all high-quality SNPs in the dataset, excluding resistance-associated positions. Non-fixed SNPs (<90% allele frequency) or positions covered with less than 7 reads were encoded as “X” in the alignment. Positions in the alignment where more than 10% of sequences had an X, were discarded. The SNP alignment was used to

infer a maximum-likelihood phylogeny using IQ-TREE 2 (REF: 11 Minh BQ, Schmidt HA, Chernomor O, et al. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Mol Biol Evol 2020; 37: 1530–4.) with the general time-reversible model of sequence evolution, indicating the invariant sites of each nucleotide. The tree and corresponding metadata was visualized and ploted using ggtree and ggtreextra.

5.3 Results

5.3.1 Sample set with mutation variants in BDQ resistance conferring putative genes

From 2009 to 2018, we identified 71 isolates with frameshift or non-synonymous mutations in the following genes: *atpE, pepQ* and *Rv0678*. 17 isolates were excluded due to missing primary culture in the biobank, or contamination during re-culturing. Among the 54 remaining isolates, mutations in the *atpE* gene were detected in 8 (14.8%) isolates, mutations in the *pepQ* gene in 34 (63%) isolates, and mutations in *Rv0678* in 30 (55.6%). Mutations were distributed into 36 variants as follows – 1 (2.8%) variant in the *atpE* gene, 17 (47.2%) variants in the *pepQ* gene and 18 (50%) variants in *Rv0678.* The distribution of unique mutations and additional mutations of isolates with MIC values ≥0.06, are described in Table 5.1 A-B-C.

As mentioned above, one variant was detected in the *atpE* gene. Among a total of 8 isolates with a mutation in *atpE,* variant – Phe76Leu occured in five isolates with no additional mutation, while two isolates had additional mutations in *Rv0678* or *pepQ*. The mutation Val39Ala in *atpE* with 5.3% of allele frequency was found together with additional mutations in both *pepQ* and *Rv0678* as well (Table 5.1). None of these mutations have been described so far.

Among 17 variants, twelve mutations (70.6%) were exclusive for *pepQ* gene; while 5 (29.4%) were in combination with *Rv0678* variants with low allele frequencies (Table 5.2 A-B-C). Additionally, insertion and frameshift mutations were observed in the *pepQ* gene in two isolates (variant frequency less than 20%), harboring mutations in both *atpE* and *Rv0678*. From 17 variants, 3 (12.5%) were declared as mutation with uncertain significance by WHO – Ser99Arg, Asp26Gly and Ala262Val.

There were 12, out of 18, (66.7%) unique variants in *Rv0678* gene. The variant - Phe93Leu was represented in 6 isolates, of which two had only a mutation in *Rv0678* and four isolates had additional mutations in *pepQ* at low allele frequency. Five variants (27.7%) harbored additional low frequency mutations in *pepQ*, while one variant (5.6%) had mutations in both *pepQ* and *atpE* genes (Table 5.1 A-B-C).

In our dataset, we observed 3 variants with a mutation in *Rv0678* of uncertain significance according to the WHO mutation catalogue – Asp47fs, Glu49fs and Arg96Trp. Whereas Gly11_pro14 deletion putatively is associated with BDQ resistance.

We considered all the isolates with mutations irrespective the allele frequencies. The minimum variant frequency was detected at 2.2%, while the maximum frequency was 99.6%. The median frequency was observed at 77.3% (IQR 6.1%-98.5%).

5.3.1.1 Mutations in clofazimine resistance conferring genes

Interestingly, 53 (98%) isolates out of 54, had additional mutations in both *Rv0676c* and *Rv1979c*, with 25 (47.2%) isolates having the same combination of amino acid substitution of Asp767Asn and Gly286Asp in corresponding genes (Table 5.1 A-B-C). One (1.9%) isolate with that combination had additionally Ala450Ser in *Rv1979c*. Seventeen (32.1%) had Ile794Thr and Gly286Asp in *Rv0676c* and *Rv1979c*, respectively, whilst 6 isolates (11.3%) had various additional mutations in *Rv1979c*. Four isolates were dispersed into the three variants of *Rv0676c*: Ile794Thr/Pro955Pro – one sample (1.9%); le794Thr/Val344Leu – two isolates (3.8%), Val55Met – one isolate (1.9%), all of these isolates also harbored the Gly286Asp substitution in *Rv1979c* (Table 5.1 A-B-C). Val55Met has been described as a mutation with uncertain significance, whereas Asp767Asp has not been associated with resistance. The Gly286Asp and Ille794Thr variants have not described before.

Table 5.1 Distribution of mutations in the *atpE, pepQ* and *Rv0678* genes and additional clofazimine resistance associated genes in MTB isolates with MIC values ≥0.06.

A	Mutation										MIC
	atpE	Variant frequency	***pepQ***	Variant frequency	***Rv0678***	Variant frequency	***Rv0676c***	Variant frequency	***Rv1979c***	Variant frequency	BDQ
	WT	NA	Arg77_Ala78del Ile75fs Glu72fs Asp70_Leu71insArgAla	2.6% 4.3% 4.3% 4.6%	Pro87fs Phe93Leu	3.2% 99.1%	Asp767Asn	99.2%	Gly286Asp	99.4%	0.12
	WT	NA	WT	NA	Asp47fs	99.1%	Asp767Asn	99.1%	Gly286Asp	99.2%	0.12
	WT	NA	Ala84fs Tyr82del	4.5% 4.5%	Phe93Leu	99.3%	Asp767Asn	99.4%	Gly286Asp	99.4%	0.12
	WT	NA	Gln67fs Gln67_Ala68insVal	4.4% 4.4%	Ala86Ser	98.9%	Asp767Asn	99.2%	Gly286Asp	99.2%	0.12
	WT	NA	WT	NA	Phe93Leu	99.2%	Asp767Asn	98.9%	Gly286Asp	99.2%	0.12
	WT	NA	WT	NA	Tyr92Cys	99.2%	Ile794Thr	99.1%	Gly286Asp	98.8%	0.25
	WT	NA	Arg77_Ala78del Ile75fs Glu72fs Asp70_Leu71insArgAla	9.7% 10.2% 9.4% 9.9%	Phe93Leu Ala682fs Phe680fs Leu634Gln	99.7% 14.3% 14% 5.4%	Asp767Asn	99%	Gly286Asp	97.7%	0.06
	WT	NA	WT	NA	Met1fs Phe680fs Ala682fs	99.1% 7.7% 8%	Asp767Asn	98.5%	Gly286Asp	98.9%	0.06
	WT	NA	WT	NA	Leu44Pro Glu49fs Ala682fs Phe680f Pro48Ala	81.1% 7.8% 4.6% 4.3% 3.5%	Asp767Asn	99.2%	Gly286Asp	98.9%	0.06
	WT	NA	WT	NA	Phe93Leu Asn428Asp Thr807Ser Leu634Gln	98.4% 8.7% 6% 4.7%	Asp767Asn	98.2%	Gly286Asp	98.1%	0.12
	WT	NA	WT	NA	Asn70Ile	95.6%	Asp767Asn	98.5%	Gly286Asp	98.5%	0.12

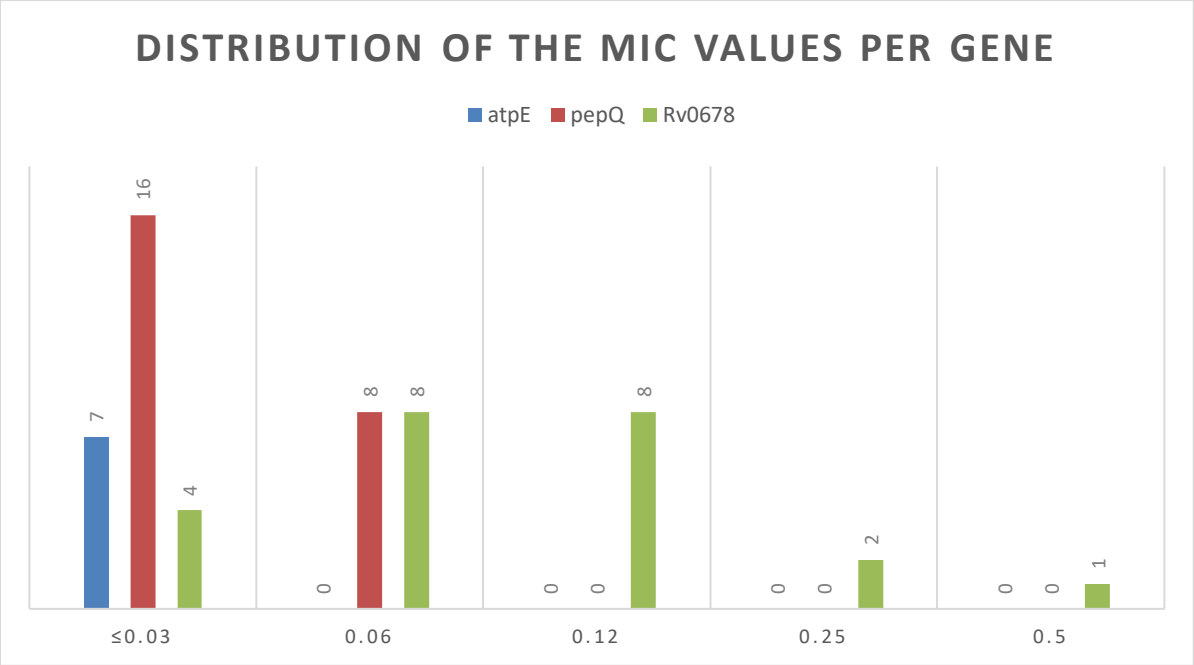
B	Mutation									MIC
	atpE	Variant frequency	***pepQ***	Variant frequency	***Rv0678***	Variant frequency	***Rv0676c***	Variant frequency	***Rv1979c***	Variant frequency	BDQ
	WT	NA	Asp70_Leu71insArgAlaGlu72fs Arg77_Ala78del	2.9% 2.7% 2.4%	Phe93Leu	98.9%	Asp767Asn	9.9%	Gly286Asp	98.8%	0.12
	WT	NA	Glu360*	3.9%	Leu95Ser Ala102Asp Glu28* Leu192_Val193insAspHisLeu	78.1% 15.1% 9.4% 4.4%	LysAsp766AsnAsn Asp767Asn	95.2% 3.8%	Gly286Asp Ala450Ser	99.3% 3.4%	0.25
	WT	NA	WT	NA	Arg96Trp Leu122Met	98.7% 98.7%	Asp767Asn	98.8%	Gly286Asp	98.8%	0.06
	WT	NA	Ser99Arg	97.9%	WT	NA	Ile794Thr Val344Leu	97.7% 97%	Gly286Asp	98.1%	0.06
	WT	NA	WT	NA	Tyr92Cys Glu451fs	98.9% 5.3%	Ile794Thr	98.7%	Gly286Asp Ala202delinsGlySerArgCysMetPro	98.6% 6.7%	0.5
	WT	NA	Gln262Arg	9.7%	WT	NA	Ile794Thr Pro955Pro	96.3% 9.6%	Gly286Asp	96.4%	0.06
	WT	NA	Gln262Arg	98.3%	WT	NA	Ile794Thr	98.3%	Gly286Asp	98.2%	0.06
	WT	NA	Gln262Arg	98.5%	WT	NA	Ile794Thr	96.3%	Gly286Asp	98.5%	0.06
	WT	NA	Thr315Arg	98.1%	Ser728Asn Val150Leu	5.4% 4.9%	Ile794Thr	98.2%	Gly286Asp Val402Leu	98.3% 5%	0.06
	WT	NA	Ala55Thr	98.4%	Arg175His	4.4%	Val55Met	98.6%	Gly286Asp	98.9%	0.06
	WT	NA	Ser99Arg	98.4%	WT	NA	Ile794Thr Val344Leu	98.3% 98.2%	Gly286Asp	98.1%	0.06
	WT	NA	Gln262Arg	98.4%	Ala45Gly Trp823Cys Glu163Asp	7.5% 5.8% 5%	Ile794Thr	97.9%	Gly286Asp	97.8%	0.06

C	Mutation										MIC
	atpE	Variant frequency	***pepQ***	Variant frequency	***Rv0678***	Variant frequency	***Rv0676c***	Variant frequency	***Rv1979c***	Variant frequency	BDQ
	Val39Ala	5.3%	Asn40Ile	3.4%	Gln9* Trp823Cys Gly677Arg	98.9% 5.2% 3.6%	Ile794Thr	99%	Gly286Asp	99%	0.06
	WT	NA	WT	NA	Met139Thr	52.5%	Asp767Asn	99%	Gly286Asp	99%	0.06
	WT	NA	WT	NA	Arg89Leu	99.1%	Asp767Asn	99.1%	Gly286Asp	99.1%	0.06

5.3.1.5 Distribution of MIC values within MTB isolates harboring genomic variants in BDQ resistance associated genes

A total of 54 MTB isolates were tested with the Sensititre MycoTB MIC plate for BDQ resistance. The median MIC was observed at 0.06 µg/ml, while the geometric mean was 0.07 µg/ml with a standard deviation of 0.08 µg/ml. The maximum MIC value was observed at 0.5 µg/ml, only in the one isolate with an amino acid change in *Rv0678* - Tyr92Cys. The distribution of the value ≤0.03 µg/ml between the three genes analyzed was as follows: 16 isolates (29.6%) with a mutation in *pepQ*, 7 (13%) isolates with a *atpE* mutation and 4 (7.4%) with a mutation in *Rv0678*. Isolates with MIC values of 0.06 µg/ml had mutations detected in two genes, represented by 8 (14.8%) isolates in each, *pepQ* and *Rv0678* (Figure 5.1). A transition of the isolates with mutations in *atpE* and *pepQ* having lower MIC values, to the isolates with substitutions in *Rv0678* is clearly visible in the figure 5.1. This trend is consistent with previous findings showing a higher reduction of susceptibility to BDQ with *Rv0678* mutations.

Figure 5.1 Distribution of MIC values per target gene.



The figure represents sample quantity and includes several isolates per variant based on availability.

Amino acid substitutions with 2 or more isolates per variant were observed in *pepQ* - Ala196Val, Val134Leu, Gln262Arg, Ser99Arg – distributed between ≤0.03 µg/mL - 0.06

ųg/mL; in *atpE* – Phe76Leu – with MIC values ≤0.03 ųg/mL; in *Rv0678* - Arg96Trp/Leu122Met, Phe93Leu and Tyr92Cys (Figure 5.1). None of the variants were shared between different MTBC lineages, indicating the likely absence of homoplastic mutations. Table 5.2 describes each variant with the corresponding MIC values with respect to the MTBC lineage distribution.

Table 5.2 BDQ MIC values for each variant of *pepQ, atpeE* and *Rv0678.*

	≤0.03 µg/mL	0.06 µg/mL	0.12 µg/mL	0.25 µg/mL	0.5 µg/mL	L2	L3	L4
Ala196Val[1]	3	-	-	-	-	3	-	-
Ser52Phe [3] *	1	-	-	-	-	1	-	-
Asp151Gly [1] *	1	-	-	-	-	1	-	-
Glu129Gln [1]	1	-	-	-	-	1	-	-
Gly285Asp [1]	1	-	-	-	-	1	-	-
His100Tyr [1]	1	-	-	-	-	1	-	-
Val336Ala [1]	1	-	-	-	-	1	-	-
Met73Val [3] *	1	-	-	-	-	1	-	-
Arg86Leu [1]	1	-	-	-	-	-	1	-
Ala263Val[1]; Asp26Gly[1] *	1	-	-	-	-	-	-	1
Phe76Leu [2] *	7	-	-	-	-	-	-	7
Gly11_Pro14del [3]; Asp15Tyr [3] *	1	-	-	-	-	-	-	1
Gly309Glu [1]	1	-	-	-	-	-	-	1
Ile202Met [1]	1	-	-	-	-	-	-	1
Ile28Met [1]	1	-	-	-	-	-	-	1
Val134Leu [1]	2	-	-	-	-	-	-	2
Ala71Val [3] *	1	-	-	-	-	-	-	1
Gln262Arg [1] *	1	4	-	-	-	-	-	5
Gln9* [3] *	-	1	-	-	-	-	-	1
Ser99Arg [1]	-	2	-	-	-	-	-	2
Thr315Arg [1] *	-	1	-	-	-	-	-	1
Ala55Thr [1] *	-	1	-	-	-	1	-	-
Met1fs [3] *	-	1	-	-	-	1	-	-
Arg89Leu [3] *	-	1	-	-	-	1	-	-
Arg96Trp [3]; Leu122Met; [3] *	-	2	-	-	-	2	-	-
Leu44Pro [3] *	-	1	-	-	-	1	-	-
Met139Thr [3] *	-	1	-	-	-	1	-	-
Phe93Leu [3] *	-	1	5	-	-	6	-	-
Ala86Ser [3] *	-	-	1	-	-	1	-	-
Asn70Ile [3]	-	-	1	-	-	1	-	-
Asp47fs [3]	-	-	1	-	-	1	-	-
Ala102Asp [3]; Leu95Ser [3] *	-	-	-	1	-	1	-	-
Tyr92Cys [3] *	-	-	-	1	1	-	-	2

[1] *pepQ*; [2] *atpE*; [3] *Rv0678;* * Isolates had and an additional mutations in same and/or *pepQ, atpE, Rv0678* genes below 20% of allele frequency. All isolates harbored amino acid change in CFZ resistance conferring genes - *Rv0676c* and/or *Rv1979c*.

5.3.2 Patient MTB isolates phenotypically resistant to BDQ

Thirty-seven phenotypically resistant TB cases were detected from June 2019 to January 2022 via MGIT phenotypic DST on Bactec 960 instrument. Genomic data was available for 16 (43%) of the corresponding patient isolates, out of which an *atpE* mutation was detected in 2 (12.5%), and 3 (18.7%) isolates exhibited mutations in Rv0678. Remaining eleven (68.8%) isolates had no mutation in the three target genes, however substitutions in Rv0676c and Rv1979c were present (Table 5.4).

The mutations in *atpE* detected in the phenotypically resistant isolates – Ile66Met – leading to increase of MIC value at 0.5 ųg/mL, has was not observed in cohort of genomic variants. However, the amino acid substitutions in Rv0678 – Ala102Asp – showed an MIC value of 0.25 ųg/mL in both cohorts, making latter noteworthy. Moreover, a patient isolate harboring the Ala102Asp mutation in Rv0678 had multiple previous active TB episodes with several different treatment regimens including Cfz and BDQ. During all episodes, the patient was declared lost to follow-up with a disrupted treatment process.

Low-level fluoroquinolone resistance was detected in 13 (81.2%) cases, whereas the remaining three (18.8%) patients exhibited high-level resistance to Mfx, resulting in death or treatment failure. Interestingly, Cfz resistance was detected only in one patient (6.2%), while we had no data for the other two cases (12.5%).

From 16 patients, two (12.5%) had treatment failure, eight (50%) had a successful treatment outcome, while one (6.25%) was lost to follow-up, and two (12.5%) patients were denoted as “other” – indicating death or that no information was available. The remaining three (18.75%) patients’ treatment is still going on therefore the final outcome has not been declared at this time point.

Validation of the MGIT pDST revealed seven (43.7%) susceptible results from those previously identified as resistant. None of these patients had a history of previous active TB disease.

Table 5.3 Phenotypic, genomic and epidemiological data for the patients with BDQ resistance defined by pDST.

Phenotypic data										Genomic data			Epidemiological data		
ID	**TB profile #**	**Phenotypic drug susceptibility ***							**MIC** µg/mL	***atpE***	***Rv0678****	**Additional mutation**	**TB history****	**Treatment regimen*****	**Treatment outcome**
		Mfx[1]	Mfx[2]	Lfx	BDQ	Lzd	Cfz	Amk							
1	preXDR	R	R	R	R	-	S	S	0.5	Ile66Met	WT	*Rv0676c* [Asp767Asn]	No	-	Death
2	preXDR	R	S	R	R/S	-	S	S	0.06	WT	WT	*Rv0676c* [Asp767Asn]]	-	-	-
3	preXDR	R	S	R	R/S	-	S	S	0.03	WT	WT	*Rv0676c* [Asp767Asn]	No	Lfx Cs Cfz Lzd BDQ	Cured
4	preXDR	S	S	R	R/S	-	S	S	0.03	WT	WT	*Rv0676c* [Asp767Asn]	No	Lfx Cs Cfz Lzd BDQ	Cured
5	preXDR	R	S	R	R	S	R	S	0.5	WT	Ala102Asp	*Rv0676c* [Asp767Asn]	No	Lfx Cfz Lzd BDQ	Cured
6	preXDR	R	R	R	R	-	S	S	0.5	Ile66Met	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	Yes	Lfx Cs Cfz Lzd BDQ	Failure
7	preXDR	R	S	-	R	-	-	-	0.25	WT	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	Yes	Cs Cfz Lzd BDQ Dld	-
8	preXDR	R	R	R	R	R	S	S	0.25	WT	Ala102Asp	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	Yes	Cm Pto Cfz BDQ Dld	Failure

9	XDR	R	S	R	R/S	-	S	R	0.12	WT	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	No	Cs Cfz Lzd BDQ Dld	Cured
10	MDR	S	S	S	R/S	-	S	S	0.12	WT	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	No	Lfx Cs Cfz Lzd BDQ	Cured
11	XDR	R	S	R	R/S	-	S	R	0.12	WT	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	-	-	-
12	MDR	S	S	S	R	-	S	S	0.25	WT	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	No	-	Cured
13	MDR	S	S	S	R	-	S	R	0.25	WT	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	-	-	-
14	MDR	S	S	S	R/S	-	S	S	0.25	WT	WT	*Rv1979c* [Gly286Asp] *Rv0676c* [Asp767Asn]	No	Lfx Cs Cfz Lzd BDQ	Cured
15	preXDR	R	S	-	R	-	-	-	0.5	WT	Ile794Thr; Leu32Se	*Rv1979c* [Gly286Asp]	Yes	Cs Cfz Lzd BDQ Dld	-
16	MDR	S	S	S	R	-	S	S	0.5	WT	WT	*Rv1979c* [Gly286Asp]	No	Lfx Cs Cfz Lzd BDQ Dld	Cured

 # Classification is based on updated WHO definitions.
** Phenotypic DST was performed on MGIT (Bactec 960): [1] Mfx concentration 0.25 μg/mL; [2]Mfx concentration 1.0 μg/mL; Lfx, BDQ, Lzd, Cfz and Amk concentrations on MGIT – 1.0 μg/mL.*
*** TB history denotes presence of active disease episode in past years.*
**** Treatment regimen indicated in the table is primary treatment medications started before pDST result availability.*
"-"denotes the missing information.
Repeated resistance result towards BDQ not concordant with primary results are indicated in red.
Low and borderline MIC values are indicated in purple.

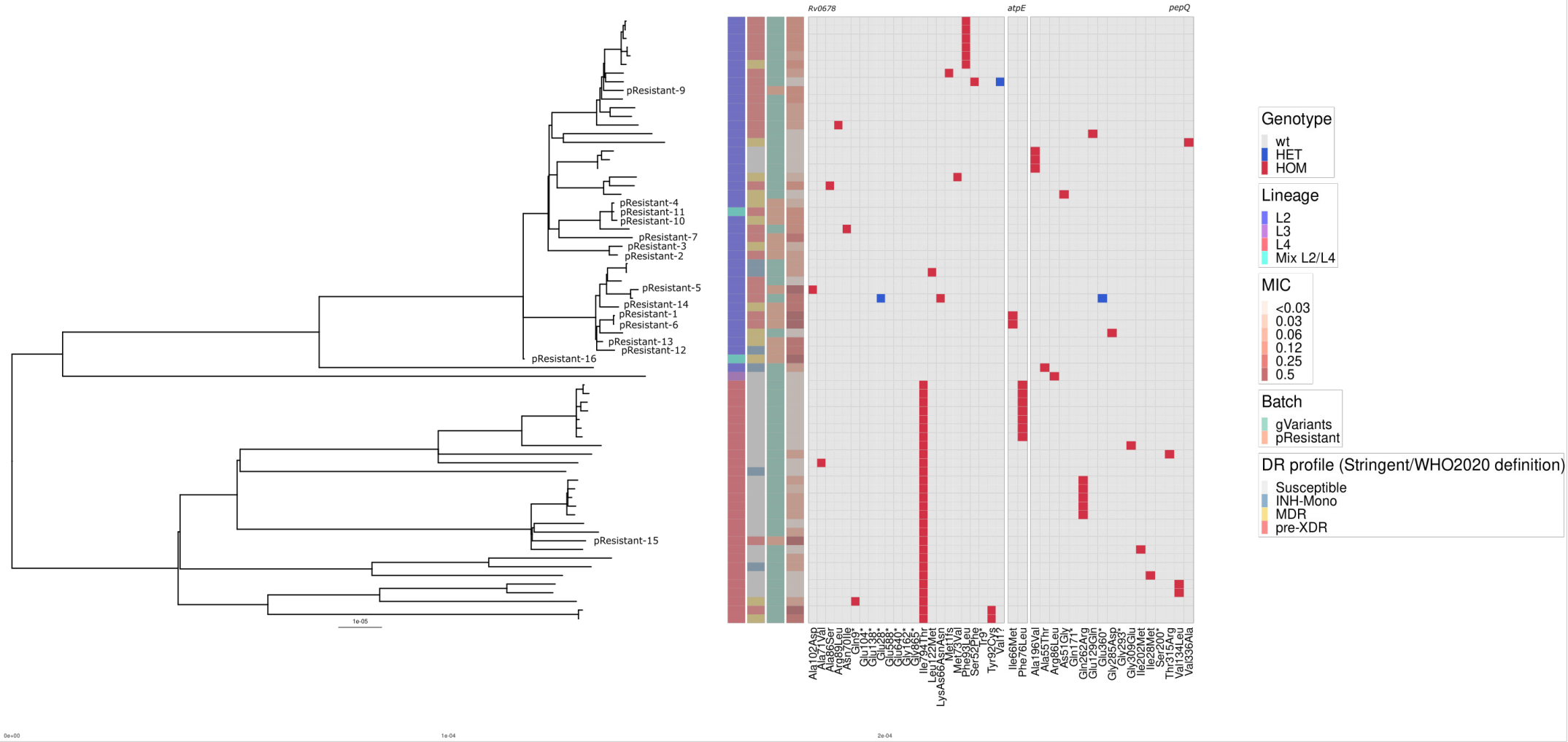
5.3.3 Phylogenetic analysis

We performed phylogenetic analysis of the *atpE, pepQ* and *Rv0678* mutations in our dataset, represented by the Lineages 2, 3 and 4. From the collated dataset (genomic variants and phenotypically susceptible) of 70 isolates, 39 (55.7%) were Lineage 2, while 28 (40%) represented Lineage 4, 2 (2.9%) - 1 (1.4%) isolates were identified as mixed Lineages (2&4) and Lineage 3, respectively.

Among Lineage 2 isolates, we identified three clades, within the clade distribution of respective amino acid substitutions – Phe93Leu in Rv0678, Ala196Val in *pepQ* gene and Ile66Met in *atpE*, likely indicating phylogenetic variants. The Ile794Thr mutation in Rv0678 was present in all isolates belonging to Lineage 4, whilst Phe76Leu in *atpE* and Gln262Arg, Val134Leu in *pepQ* were shared between the isolates from the clade.

Interestingly, increased MICs in nine phenotypically resistant isolates did not harbor any mutation in our defined target genes; on the other hand, these isolates shared specific mutation in mmpL-Rv0676c gene, influencing efflux pump expression similarly to Rv0678.

Figure 5.2 Phylogeny of study sample set



5.4 Discussion

This study explored variants in genes associated with BDQ resistance and their influence on minimum inhibitory concentration values. We also looked into the phenotypically resistant isolates and described mutations in BDQ resistance associated genes - *atpE, pepQ*, Rv0678 - in addition to the presence any further variants causing resistance towards clofazimine.

Currently, BDQ is one of the core drugs for the treatment of drug resistant TB as a part of the short all-oral treatment regimens (131). However, robust data for the specific resistance conferring mutations is still lacking and identification of resistance is limited due to uncertainty of critical concentration value to discriminate between resistance and susceptibility. EUCAST had provided rationale for the clinical breakpoints towards bedaquiline, aggregating the MIC values for the different medium and method used. Whilst 0.125 mg/L was given as presumptive epidemiological cut-off on 7H9 medium, frequently denoted as borderline resistance, there is still uncertainty and the cut-off should not be used to infer resistance (152). Defining variants, leading to the reduced susceptibility towards bedaquiline has fundamental value for optimizing culture-based drug susceptibility testing, for the development of molecular diagnostic methods (153), as nowadays, except for tNGS and WGS, there is no comparative molecular test available for bedaquiline.

5.4.1 Influence of genomic variants on BDQ MICs

In this study, we collated dataset of 54 genomic variants with mutations in *atpE, pepQ* and *Rv678* genes, in addition to 16 phenotypically resistant clinical isolates, herewith combined with MIC values defined by Sensititre MycoTB assay. We adopted 0.12 mg/L as the borderline susceptibility breakpoint for the 7H9 medium used in Sensititre 96-well plates defined by EUCAST. From the WGS dataset, genomic variants were distributed as follows – 1 (2.8%) variant in *atpE* gene, 17 (47.2%) variants in *pepQ* gene and 18 (50%) variants in *Rv0678.*

Mutations detected in *atpE* within the genomic variant dataset - Phe76Leu and within the phenotypically resistant dataset – Ile66Met have not been described elsewhere as

resistance-associated variants. However, the MIC value of 0.5 of two isolates with the latter amino acid change (Table 5.2) provides further evidence for presumptive resistance-conferring substitution. Furthermore, *atpE* mutations are not frequently found in clinical isolates, leading to the limited data, whereas our results substantiates with a few variants within the gene and might be useful for further studies.

We have observed 17 unique variants in *pepQ*; however, none of them induced any increase in MIC of more than 0.06 ug/mL, with only three substitutions resulting in the latter value. As mentioned in the Results section, three variants have been declared as mutations with uncertain significance by WHO – Ser99Arg, Asp26Gly and Ala262Val. Interestingly, no *pepQ* mutants were identified within the phenotypically resistant sample set. These results concurs well with previous findings, demonstrating that, despite of high number of *pepQ* variants, they are mostly causing reduced susceptibility but no resistance within clinical isolates.

In our sample set, we have identified several genomic variants and combinations leading associated with an increased MIC, exclusively driven by mutations in *Rv0678* gene, highlighting the complexity of bedaquiline-clofazimine cross-resistance (Table 5.2). Although mutations in efflux pump regulator are more frequently described in similar studies, consensus on resistance association cannot be determined, due to their uniqueness and not typically causing resistance (154). Similarly, out of two frameshift mutations described in studies, Asp47fs caused borderline resistance with an MIC value of 0.12 in our dataset, while it was associated with an increased MIC of up to 0.5 in previous studies (155). Although Glu49fs was represented in low allele frequency (7%) contributing to MIC of 0.06 (Table 5.1), homozygous variant identified in collated dataset resulted in bacterial growth at an MIC of 0.12 (155). Consequently, irregularities between variants and MIC values mediated by the MmpS5–MmpL5 efflux system mutations need considerably more data for a greater degree of accuracy on this matter.

5.4.2 Efflux system or essential mutations?

Sequencing data on phenotypically resistant patient isolates (resistance defined with MGIT, Bactec 960) in our cohort revealed a unique mutation in *atpE* – Ile66Met in two cases, not described elsewhere. Importantly, with MIC escalation to 0.5, these patients experienced unfavorable treatment outcomes. While patient #1 had a single mutation in

a BDQ resistance-conferring gene and one additional mutation in Rv0676c,, patient #6 harbored mutation in Rv1979c as well (Table 5.4). However, same patient isolates showed high-level Mfx resistance as well, and being associated with poor treatment prognosis, the influence of *atpE* mutations in such cases must be interpreted with caution.

We have observed relatively lower MIC values than expected in several cases, such as – patient #2, #3, #4, #9, #10 and #11 (Table 5.4). Comparatively to pDST on MGIT, the patient isolates mentioned should have resulted in MIC values ≥0.25. As mentioned in the Results section, validation of pDST results on 16 patients revealed susceptibility towards BDQ in 7 cases, providing additional support to the inconsistency of resistant result via current CC based methodologies and whereas the rate of BDQ resistance are discussed as an arising threat, the percentages of resistance should be debated cautiously. These results additionally offer evidence that mutations in the efflux regulator genes can cause borderline resistance with irreconcilable results. Moreover, borderline resistance points to the probability of the existence of low and high level resistance to BDQ.

It is interesting to note that, even though we observed increased MIC values dominated by Rv0678 gene mutations, which is known to be associated with cross-resistance to clofazimine, only one (6.2%) patient isolate (Table 5.4) exhibited resistance to Cfz, whereas 13 (81.2%) patients' isolates were susceptible. Studies confirm that prior Cfz intake does not predict BDQ resistance, and the cross-resistance in most cases is derived from BDQ resistant variants, while within Cfz resistant cases only one third would develop cross-resistance (146). Even though Cfz has been used in most of the treatment regimens in Georgia since 2005, our results, and previous literature findings, indicate that influence of CFZ use on BDQ susceptibility should not be overestimated.

Patients who experienced active TB episodes in our cohort did not receive BDQ within their past treatment regimens as long as it was not programmatically embedded in the treatment plan. However, they might have received Cfz, and exposure to the latter might have triggered activation of the MmpS5-MmpL5 efflux pump. Moreover, out of 4 previously treated patients, three (75%) had two mutations either in *Rv0678*, or *atpE*.

The phylogeny we constructed on our cohort revealed that majority of the described variants in this study are most likely to be phylogenetic markers. However, the Tyr92Cys substitution might be noteworthy considering the associated MIC values of 0.25 and 0.5.

Due to the prevalence of Lineage 2 strains in Georgia and across Post Soviet Union countries, with the ability to acquire resistance to BDQ quite rapidly (140), even heterozygous variants should be taken into consideration, as they might serve as a limitation due to unreliability of MIC testing. In our sample set, among 54 isolates, heterozygous variants were detected in 23 (43%), with the majority of these within *pepQ* and *Rv0678*. This might be pointing out the multiple emergence of resistance, which is quite common for fluoroquinolones.

We describe several variants associated with a significant increase of MICs, yet, limited sample size and homogenous population challenged identification of the multiple independent resistance emergence events. However, a greater focus on these variants could produce interesting findings that might support resistance prediction.

The need of additional data with genomic and phenotypic correlations for BDQ resistance determination is clearly supported by our findings. The collection of genomic data on variants is a key point for the further development of methodologies for rapid detection of resistance. Our findings add to the substantially growing body of literature on inconsistency of current culture-based drug susceptibility testing, indicating that both, molecular and pDST, ideally with two concentrations have to be used in parallel to detect BDQ resistance in MTB.

6. General Discussion

Integration of molecular epidemiology in TB control system has shown to increase effectiveness for the disease surveillance and management. While combination of genotyping data and epidemiological characteristics gives the ability to distinguish relapse from reinfection, or outbreak investigation, molecular typing demonstrates several possibilities for shaping TB control strategies. In this thesis, we attempted to cover several angles of the molecular epidemiology and surveillance of TB, starting from re-accessing and implementing a genotyping methodology such as MIRU-VNTR. We validated a novel approach of the well-known typing tool as a cost/time/labor effective method, and used it for exploring the causes of recurrent TB cases in Georgia. In chapter 5, we focused on drug-resistant TB surveillance, specially aiming for the novel medication, bedaquiline, for which data regarding the resistance conferring mutations is still limited. In this final chapter, I collate my main findings throughout the thesis and discuss the results in their wider context translating from single patient to the nation-wide public health issue.

6.1 MIRU-VNTR – golden oldie

The need for genotyping methodologies in the TB transmission and control program in Georgia was highlighted by several points, starting from the non-existence of any molecular typing tool for the disease surveillance so far, to the high rates of recurrent TB cases. Therefore, our primary aim was to choose and integrate the most appropriate typing method for a low/middle income country. A recent survey in 26 European countries revealed that 24 MIRU-VNTR was used by all countries, whereas seven performed TB surveillance solely by MIRU-VNTR, with the remaining countries combining that tool with spoligotyping, RFLP or/and WGS (156).

In chapters, three and four, we initially evaluated and implemented the conventional approach of MIRU-VNTR, as one of the widely used, comparatively cheaper and easy to interpret genotyping methods. Subsequently, we modified and developed a novel

approach aiming to reduce workload and cost, by stepwise typing of the patient isolates, leading to reduced quantity of isolates to be typed.

6.2 Relapse as a major driver of recurrent TB in Georgia

The distinction between relapse and reinfection in recurrent TB cases is important for the planning of precise interventions. As discussed in chapter 3, recurrence due to relapses linked to endogenous re-activation of the infection, indicates treatment inefficacy or poor adherence of the patient. Although we identified endogenous reactivation of the infection as the most common basis of the recurrent TB cases in Georgia, reinfection seems to be more common in the patients from penitentiary system. Considering the spillovers of highly transmissible strains from prisons into the general population, which had already been described by Gygli *et.al* (43), our results are consistent with these previous findings. However, primary and active TB screening in prisons has shown the positive impact on the number of new cases from penitentiary system, decreasing from 2.1% in 2017 to 1.6% in 2021 (National TB Surveillance, unpublished data).

We have also observed significant numbers of lost to follow-up patients contributing to the high number of the relapses, which might be explained by the complex and long-term treatment during the time period of the study, being conducted before the modification (2019) of the national guidelines for treatment of MDR/XDR TB patients. The influence of the shortened treatment regimens is apparent when comparing proportion of lost to follow up patients before and after implementing new regimens – from 24% om 2015 to 13.8% in 2020 (National TB Surveillance, unpublished data.

To monitor distinctions between relapse and reinfection proportions, TB control programs could use conventional genotyping method for differentiating between the two phenomena. Using typing methods, several studies in high incidence countries have detected reinfection as a major driver for the recurrence, mainly due to high risk of infection exposure (157). Despite of this logical explanation, a few studies have shown that the incidence rate does not always correlate with predominance of reinfection or relapse. E.g. a study in Finland revealed a high number of reinfection cases in close social

networks, while a study in the US detected a high number of recurrent cases in immigrant populations - both of the low-incidence countries with high rates of reinfection (93).

However, one should be cautious in geographical areas with predominant strain circulation, such as Beijing strains in Georgia, as the MIRU-VNTR will not provide the highest resolution discrimination and might be the limitation of using this typing tool exclusively (140,158). Meanwhile, the cost for whole genome sequencing has been rapidly decreasing over the past years, giving ability to produce robust data allowing full discrimination of the strains with detection of transmission clusters. Therefore, combination of MIRU-VNTR and WGS should be taken into consideration for standard TB surveillance in the Georgian and similar settings (159).

6.3 Stepwise MIRU-VNTR

The prevailing limitation for using genotyping for disease surveillance are financial constraints, as it was identified in the survey by Andres *et al. (156)* Additionally to that, human resources and data management/bioinformatics was also observed to be a barrier particularly for the WGS method (156). Therefore, we chose MIRU-VNTR as the most affordable tool for the implementation in the Georgian environment. For the best practice, we tried to modify and customize methods to reduce the cost, workload and time.

As MIRU-VNTR customization has been adopted by several countries, based on the highest discriminatory loci specifically for the circulating strains in the setting, step-wise approach has only been developed in Turkey (122). With such a modified approach, we proposed primarily combining the loci with the highest Hunter-Gaston Discriminatory Indexes to construct the customized panel of MIRU-VNTR for the first typing step. The customized panel consisted of the best combination of loci to differentiate between unique isolates. For the remaining clustered isolates, we proposed using next set of loci with medium discriminatory power. Finally, the third set of the remaining loci from the standard 24 panel was used for generating the full typing data for a reduced number of samples to be typed. The reduction of the number of isolates per step typing led to a proportionally reduced cost, processing time and workload. In chapter 4, we described

the proportional reduction with 42.6%, which is noteworthy and might enable to use the method in other resource-limited settings.

The stepwise approach can be used for other settings as well, as an innovative and more practical solution for the MIRU-VNTR typing. It can be easily implemented with combination of other typing methods such as spoligotyping and in case of availability, WGS.

The effective discrimination of MIRU-VNTR for the Beijing strains is relatively low, limiting usage of the tool in the Beijing predominant settings. In several studies, it was shown to have sufficient discriminatory power for the particular epidemiological research (158). However, this limitation is triggering a shift from conventional MIRU-VNTR to WGS, if the optimal conditions for performing sequencing and analysis are present.

6.4 Drug resistance surveillance: bedaquiline resistance – an emerging threat

Since 1994, WHO and International Union against Tuberculosis and Lung Disease (The Union) have started the global project for worldwide surveillance for anti-TB drug resistance (The Global Project for Anti-TB Drug Resistance Surveillance), aiming to collect and assess data on drug resistance in continuous manner (159). Collated datasets serve to evaluate intra and inter-population surveillance of drug resistance using routine DST methodologies with phenotypic and molecular tests. Specifically, the document aims to support national TB programs to provide the best possible mechanism of surveillance based on routine DST tests, taking into consideration that the data should be comparable between countries. However, the proposed system remains insufficient, due to

Group A - levofloxacin, moxifloxacin, bedaquiline, linezolid

Group B - clofazimine, cycloserine, terizidone

Group C – ethambuthol, delamanid, pyrazinamide, imipenem, meorepenem, amikacin, ethionamide, prothionamide, PAS

Consolidated guidelines on tuberculosis, WHO, 2019

inconsistency of the methodologies between the settings and need of in-depth measures for specific regions (159).

Current culture-based drug susceptibility testing provides results for a wide range of drugs starting from first-line medications to the extent of several drugs from Group A, B and C (see Table below). Phenotypic DST is often technically challenging, requires continuous quality control and for the most medications is tested at a single, critical concentration recommended by WHO. Nonetheless, due to the emerging resistance to the novel medications such as bedaquiline, the available drug arsenal is being more compromised, pointing to the need of defining levels of resistance drugs. Borderline or low-level resistance detection might allow using the medication with dose modifications as in case of moxifloxacin and isoniazid. The data presented in chapter 5, can be presumed as pre-formation of borderline resistance for bedaquiline, as phenotypically resistant patients harboring mutations, leading to up-regulation of efflux pump, can cause both – susceptible and resistant result on currently proposed phenotypic DST. Whether the borderline resistance has any clinical significance, needs to be further studied.

Apart from conventional phenotypic DST, several assays have been developed based on micro-dilution to assess multiple concentrations and define MICs of each drug on a single microtiter plate (153,160,161). Despite of several advantages of these methodologies, such as short turnaround time, no additional equipment requirement and low cost, plate-based test is not endorsed by WHO as it is less comparable between laboratories and there is still a lack of the data for the specificity and sensitivity (161). However, using multiple concentrations for the novel drugs on the validated pDST methodologies would generate crucial and valuable information for evaluating MIC values and their correlation with genomic data.

In order to facilitate the transition to genotypic DST, as a more rapid, cost-effective and automated assay for susceptibility testing, WHO with CRyPTIC Consortium published the WHO mutation catalogue, providing MTBC mutations associated with drug resistance (149). Among current medications used in TB treatment, data regarding bedaquiline resistance conferring genes are still insufficient, due to highly conservative criteria for providing confidence supporting each mutation.

Even though the majority of mutations described in our study can be interpreted as likely phylogenetic, we could argue that the limited number of isolates analyzed and the predominance of only two lineages in the Georgian population influence the full picture of the outcome. Notwithstanding these limitations, the data represented in chapter five aimed to extend current knowledge, and contributes to the global dataset of genomic variants of bedaquiline resistance associated genes along with MIC data of these isolates using Sensititre MycoTB plates. Additionally, we gathered phenotypically resistant clinical isolates and explored mutations in specific loci.

6.5 Rise of tNGS – is this the future?

Several sequencing based technologies for drug susceptibility testing are currently under development, aiming to reduce the diagnostic period and to provide the best treatment options for each patient. Compared to NGS, where culturing step is needed to provide enough bacterial biomass for DNA extraction and sequencing, targeted NGS (tNGS) is performed directly on clinical samples, making this tool relatively fast and feasible (162). Despite of being a comprehensive alternative to current pDST methods, for LMICs, the platform maintenance and lack of technicians are the main challenges in implementing tNGS tools, specifically in high incidence countries (163).

One of the limitations of sequence-based DST methods, especially for bedaquiline resistance detection, is associated with efflux-pump mechanisms, which is responsible for majority of increased MICs and are challenging to detect via tNGS (164). In our study, although, a number of isolates had additional mutations in resistance conferring genes, and the majority of high MIC values were driven by efflux pump upregulation. Therefore, for the future steps, in depth analysis is needed to make conclusion on resistance solely influenced by efflux mechanism.

While the data collection for phenotypic and genomic variations is still ongoing via global projects in addition to sources provided by various consortia for the new iteration of the WHO mutation catalogue, development of portable and affordable sequencing is advancing, potentially leading to a transition from phenotypic to genotypic susceptibility testing as a routine diagnostic.

6.6 Conclusions

TB surveillance is a multicomponent strategy, including molecular typing of bacteria to define patient-to-patient transmission and drug resistance control and surveillance at national level. Identification of the major challenges and implementing molecular techniques together reduce barriers against providing the best quality, patient-oriented treatment. Lacking a comprehensive understanding on current open issues such as the detection of the genetic basis of resistance towards novel, promising medications, restrains efficient drug resistance control leading to significant setback towards the goals of WHO's End TB Strategy.

In this thesis, we demonstrated the importance of embedding molecular epidemiological tools within TB surveillance, highlighting the fact that despite of increased availability of NGS platforms, conventional genotyping still stands as a valuable tool for epidemiological studies. Our customized MIRU-VNTR confirmed to be cost effective and easily adaptable between the different settings. The data generated while exploring BDQ resistance contributes to our understanding of the relationships between genomic variations and phenotype, particularly drawing attention to the role of the efflux systems and cross-resistance to clofazimine. While our findings add to the current literature, it also lays the groundwork for future research; the latter being the key for ensuring prompt diagnosis and appropriate treatment for TB.

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BIOGRAPHICAL SKETCH / CV

NAME: **Nino Maghradze, M.D., MBA, PhD**

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	COMPLETION DATE MM/YYYY	FIELD OF STUDY
Tbilisi State University, Georgia	MD	06/2012	Medicine
Ilia State University, Tbilisi, Georgia	MBA	06/2015	Business Administration in Public Health
University of Basel, Basel, Switzerland	PhD	03/2023	Epidemiology, Infection Biology

A. PERSONAL STATEMENT

I am a medical doctor who underwent doctoral studies in Epidemiology – Infection Biology at University of Basel, Switzerland, where I started my carrier as junior scientist and researcher with collaboration of Swiss Tropical and Public Health Institute (SwissTHP) and National Center for Tuberculosis and Lung Diseases (NCTLD) Georgia. Under supervision of Prof. Sebastien Gagneux our project was focused on studying compensatory evolution and epistasis of drug resistance conferring mutations using Mycobacterium tuberculosis (MTB) isolates from the country of Georgia. Under the scope of the project, my doctoral study was dedicated to i) explore molecular epidemiology of MTB in Georgia and implement genotyping methodology ii) extend the scope of drug resistance development and acquisition, specifically to the novel medications. In addition to my doctoral studies, since 2019, I have participated in the Emory-Georgia TB research-training program, which aims to improve TB research infrastructure in Georgia and help to develop experienced and qualified human resources for conducting independent research regarding TB treatment, prevention and control. Meanwhile, I have been taking part in multiple scientific projects and clinical trials carried out at NCTLD, with collaboration to Emory University, FIND, etc., leading to increasing interest in novel diagnostic methodologies, especially point of care tools and drug resistance detection. I had an opportunity to be part of the Seq&Treat project (FIND, Unitaid), generating evidence of targeted next generation sequencing (tNGS) as the feasible and rapid tool for drug resistance detection. As a laboratory team lead, I was working on MTB isolate sequencing on three platforms (MiSeq, iSeq, Minion), upgrading my experience in bioinformatics and sequencing technologies. Currently, being a part of basic and translational research projects, as well as clinical trials, enhances my expertise in field of TB molecular epidemiology, drug resistance and diagnostics.

B. POSITIONS AND HONORS

Positions and Employment

2024 – present	European University, Invited lecturer, Medical Faculty – Epidemiology
2021 –present	Laboratory team lead, scientific department, National Reference Laboratory, NCTLD Tbilisi, Georgia
2011-2012	Student assistant, Emergency Department, N. Kipshidze Tbilisi Central University Clinic
2013-present	Laboratory technician, junior scientist, National Reference Laboratory, NCTLD Tbilisi, Georgia
2011-2012	Student assistant, Emergency Department, N. Kipshidze Tbilisi Central University Clinic

Other Experience and Professional Memberships

2019	Fogarty Fellowship, NIH Funded Emory-Georgia TB Research Training Program (D43TWOO7124)
2010-2015	Co-founder, secretary general, European Medical Students Association (EMSA) at Tbilisi State University, member of EMSA international (EMSAi)
2017-2019	Member, Georgian International Medical and Public Health Association (GIMPHA)
2012	Clinicum Chemnitz gGmbH summer program, Department of Internal Medicine, Hematology and Oncology, Chemnitz, Germany
2012	Elective course in oncology, European Cancer Organization (ECCO), Antwerp, Belgium

C. CONTRIBUTIONS TO SCIENCE

1. **Implementing MIRU-VNTR typing and its application to clinically relevant questions:** Working with Prof. Sebastien Gagneux and collaborators at the Swiss TPH, we created and implemented a MIRU-VNTR typing tool to address one of the most important issues of TB management – recurrent TB disease. Before the study, differentiation of the basis of recurrent TB was unavailable. However, after customizing the methodology for Georgia and implementing it we were able to discriminate between reinfection and relapse in recurrent TB cases. The importance of the differentiation between these two phenomena is high, as it defines the level of intervention against TB recurrent cases, which contribute to an increased number of retreated cases in Georgia. **Maghradze N**, Jugheli L, Borrell S, Tukvadze N, Aspindzelashvili R, Avaliani Z, Reither K, Gagneux S. Classifying recurrent Mycobacterium tuberculosis cases in Georgia using MIRU-VNTR typing.
   PLoS ONE. 2019 Oct 18;14(10):e0223610. doi: 10.1371/journal.pone.0223610. PMID: 31626647. PMCID: PMC6799914.

2. **Utilizing new genotyping methodology (MIRU-VNTR) to describe TB transmission:** We adopted methods of customizing local loci set from countries, which had successfully used reduced panel of loci to customize the typing tool based on local MTB strain variation. We used this methodology to explore transmission characteristics in the penitentiary system, which is a major source of drug resistant MTB strains in Georgia. In addition, we tracked TB transmission among household contacts in a cohort study embedded in the "5% initiative". These studies allowed me to develop genotyping methodologies for further studies. **Maghradze N**, Jugheli L, Borrell S, Tukvadze N, Kempker RR, Blumberg HM, Gagneux S. Developing customized stepwise MIRU-VNTR typing for tuberculosis surveillance in Georgia.
   PLoS ONE. PMID: 35231041 PMCID: PMC8887741 DOI: 10.1371/journal.pone.0264472

3. **Prisons as ecological drivers of fitness-compensated multidrug-resistant Mycobacterium tuberculosis**
   Sebastian M Gygli, Chloé Loiseau, Levan Jugheli, Natia Adamia, Andrej Trauner, Miriam Reinhard, Amanda Ross, Sonia Borrell, Rusudan Aspindzelashvili, **Nino Maghradze**, Klaus Reither, Christian Beisel, Nestani Tukvadze, Zaza Avaliani, Sebastien Gagneux
   Nat Med. 2021 Jul;27(7):1171-1177. doi: 10.1038/s41591-021-01358-x. Epub 2021 May 24. PMID: 34031604

4. **The relative transmission fitness of multidrug-resistant Mycobacterium tuberculosis in a drug resistance hotspot**.
   Loiseau C, Windels EM, Gygli SM, Jugheli L, **Maghradze N**, Brites D, Ross A, Goig G, Reinhard M, Borrell S, Trauner A, Dötsch A, Aspindzelashvili R, Denes R, Reither K, Beisel C, Tukvadze N, Avaliani Z, Stadler T, Gagneux S. Nat Commun. 2023 Apr 8;14(1):1988. doi: 10.1038/s41467-023-37719-y. PMID: 37031225

D. RESEARCH SUPPORT

Ongoing Research Support

ERC-2019-AdG **Gagneux (PI)** **4/2020 – current**
Title: Linking Within-host and Between-host Evolution in Multidrug-resistant Mycobacterium tuberculosis.

The goal of this project is to explore the causes and consequences of within-host evolution in multidrug- resistant *Mtb* over time and across different body compartments, to look for potential trade-offs with between-host evolution during patient-to-patient transmission, and to define the role of suboptimal patient treatment and phenotypic drug tolerance on drug resistance evolution inside patients and across patient populations.
Role: Laboratory team lead

R2D2 **Cattamanchi (PI)** **05.2021-current**
Title: Rapid Research in Diagnostics Development for TB Network.
Role: Laboratory team lead

HDT **Wallis (PI)** **Site initiation**
Title: Stratified Host-Directed Therapy for Drug-Resistant Tuberculosis: A Randomized controlled Multi-Centre Trial.
Role: Laboratory team lead

Completed Research Support

NCT04239326 **Rodwell (PI)** **8/2021-11/2023**
Title: Multi-Centre Clinical Trial to Assess the Performance of Culture-free, End-to-end Targeted NGS (tNGS) Solutions for Diagnosis of Drug Resistant TB (DR-TB).
Culture-free, end-to-end targeted NGS (tNGS) Solutions for Diagnosis of Drug Resistant TB can offer higher throughput and greater accuracy across more TB drugs than current WHO endorsed molecular assays, and a significantly faster time to result than phenotypic drug susceptibility testing (DST). Evidence regarding the clinical diagnostic accuracy and operational characteristics of tNGS solutions is needed to comprehensively evaluate tNGS for diagnosis of drug-resistant TB among patients who have been diagnosed with TB, and will be critical to inform global and national policy.
Role: Laboratory team lead

D43 TW007124 **Blumberg (PI)** **9/27/2004–01/31/2021**
NIH/FIC Emory-Georgia TB Research Training Program

The goal of this program is focused on enhancing the tuberculosis (TB) related research and public health infrastructure in the country of Georgia (former Soviet republic) through providing didactic and mentored research training to Georgian investigators and trainees.

ERC-2012-StG_20111109 **Gagneux (PI)** **04/1/13 – 12/31/18**
Title: Compensatory Evolution and Epistasis in Multidrug-resistant Mycobacterium tuberculosis

This study aimed to combine experimental evolution and fitness assays in vitro and in human macrophages with comparative genome sequencing, RNAseq-based transcriptomics, and population-based molecular

epidemiology to 1) Identify and characterize compensatory mutations in M. tuberculosis resistant to rifampicin, streptomycin, and ofloxacin 2) Detect epistasis between drug resistance-conferring mutations in different strain genetic backgrounds 3) Investigate the effect of drug resistance-conferring mutations, compensatory mutations, and their epistatic interactions on the *M. tuberculosis* transcriptome.
Role: Researcher

SystemsX project TbX **Gagneux (PI)** **04/2014 – 3/2018**
Title: Systems Biology of Drug-resistant Tuberculosis in the Field

This study focused on mechanisms leading to the development and spread of *M. tuberculosis* resistance to develop an extensive model to simulate the involved processes.
Role: Researcher