**TITLE**

Genotype-phenotype characterization of serial *Mycobacterium* *tuberculosis* isolates in bedaquiline-resistant tuberculosis

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**KEYWORD**

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**RUNNING TITLE**

Bedaquiline-resistant tuberculosis

**SUMMARY**

Bedaquiline (BDQ) is a critical component in new regimens for drug-resistant (DR) TB. Characterizing serial *M. tuberculosis* isolates collected during BDQ-based treatment identified diverse and dynamic subgroups of BDQ-resistant TB, including those with treatment-associated and pre-existing resistance.

**ABSTRACT**

*Background*: Emerging resistance to bedaquiline (BDQ) threatens to undermine advances in the treatment of drug-resistant tuberculosis (DRTB). Characterizing serial *Mycobacterium tuberculosis* (*Mtb*) isolates collected during BDQ-based treatment can provide insights into the etiologies of BDQ resistance in this important group of DRTB patients.

*Methods*: We measured mycobacterium growth indicator tube (MGIT)-based BDQ minimum inhibitory concentrations (MIC) of *Mtb* isolates collected from 195 individuals with no prior BDQ exposure who were receiving BDQ-based treatment for DRTB. We conducted whole genome sequencing (WGS) on serial *Mtb* isolates from all participants who had any isolate with a BDQ MIC > 1 collected before or after starting treatment (95 total *Mtb* isolates from 24 participants).

*Results*: Sixteen of 24 participants had BDQ-resistant TB (MGIT MIC ≥ 4 mcg/mL) and 8 had BDQ-intermediate infections (MGIT MIC = 2 mcg/mL). Participants with pre-existing resistance outnumbered those with resistance acquired during treatment, and 8 of 24 participants had polyclonal infections. BDQ resistance was observed across multiple *Mtb* strain types and involved a diverse catalog of *mmpR5 (Rv0678)* mutations, but no mutations in *atpE* or *pepQ*. Nine pairs of participants shared genetically similar isolates separated by < 5 SNPs, concerning for potential transmitted BDQ resistance.

*Conclusions*: BDQ-resistant TB can arise via multiple, overlapping processes, including transmission of strains with pre-existing resistance. Capturing the within-host diversity of these infections could potentially improve clinical diagnosis, population-level surveillance, and molecular diagnostic test development.

**MAIN TEXT**

**Introduction**

Bedaquiline (BDQ) has markedly improved clinical outcomes for individuals with drug-resistant tuberculosis (DRTB) [1-3]. BDQ-containing regimens have been standard of care for all patients with rifampin-resistant TB in South Africa since 2018 [4], where approximately 50,000 patients have received BDQ to date. Recent national-level surveillance data found baseline BDQ resistance in 3.8% of individuals with rifampin-resistant TB in South Africa, and emerging resistance to this critical new drug could jeopardize future progress in TB care and prevention [5]. Expanding population-level surveillance for BDQ-resistant TB and providing early, accurate diagnosis of BDQ-resistant infections are urgent public health priorities.

BDQ resistance in clinical TB infections is almost entirely attributable to mutations in *mmpR5* (*Rv0678*), which may also confer resistance to clofazimine. BDQ resistance associated with mutations in two other genes, *pepQ­* and *atpE*, are rare outside of *in vitro* experiments [6]. A diverse collection of *mmpR5* mutations, involving >200 different loci across the gene, have been reported in clinical isolates [7]. *mmpR5* mutations are associated with a wide range of BDQ resistance phenotypes, and some *mmpR5* mutations do not appear to confer BDQ resistance at all [7-9]. Continued uncertainty about how these mutations influence phenotypic BDQ susceptibility poses an important problem for the development of rapid genotypic tests for BDQ resistance.

*M. tuberculosis (Mtb*) bacterial populations in individual hosts are more diverse and dynamic than previously understood [10, 11], posing challenges for the scientific and public health response to DRTB. Difficulty capturing diverse *Mtb* subpopulations [12, 13]*,* including those with divergent drug-resistance phenotypes, is an important and enduring problem in the clinical management of DRTB [14]. Failure to detect these cryptic *Mtb* subpopulationscan also result in misclassification of patients or research participants by resistance phenotype, with important implications for public health surveillance, clinical research, and molecular diagnostics for DRTB.

More comprehensive characterization of individual *Mtb* infections, using serial *Mtb* isolates collected before and during DRTB treatment, has yielded important insights into the diversity and within-host evolution of *Mtb* [15, 16]. Joint analysis of phenotypic drug susceptibility testing (DST) and whole genome sequence (WGS) data from serial *Mtb* isolates can differentiate infections with *treatment-emergent* resistance from infections involving strains with *pre-existing* (aka “*primary”*)resistance. WGS also allows for identification of *polyclonal infections*, which can involve different *Mtb* strains with divergent susceptibility phenotypes; without WGS, it is impossible to clearly differentiate this subset of polyclonal infections from monoclonal infections with treatment-emergent resistance. Lastly, the number of single nucleotide polymorphism (SNP) differences between isolates, derived from WGS data, provides an important quantitative measure of *Mtb* within-host genetic diversity and between-host genetic similarity.

This study used WGS and BDQ minimum inhibitory concentration (MIC) testing to characterize serial *Mtb* isolates from a sample of participants with DRTB who had evidence of decreased BDQ susceptibility while on BDQ-based treatment. Using this approach, we describe how infection with pre-existing resistant strains, within-host evolution, and polyclonal infection shape the dynamics of BDQ-resistant TB at the individual level.

**Methods**

*Study population and design*

PROBeX was an observational longitudinal cohort study conducted at three TB referral hospitals in Gqeberha, Cape Town, and Durban, South Africa between 2016 and 2018 [3]. PROBeX recruited 195 adult participants with culture-confirmed extensively drug-resistant (XDR) or pre-XDR TB, as defined at the time of the study [17], who received treatment with a standardized BDQ-containing regimen. The study was approved by the institutional review boards at Albert Einstein College of Medicine, the University of Cape Town, Columbia University and Emory University. Additional details on the PROBeX parent study [3] are included in the **Supplementary Methods**.

*Bedaquiline Minimum Inhibitory Concentration (MIC) measurements*

We measured BDQ MIC values (0.125–8 mcg/mL) of *Mtb* isolates using the BACTEC™ MGIT™ 960 DST method [9] **(Supplementary Methods)**. We performed repeat BDQ MIC measurements for any isolate with an intial MIC ≥ 1 mcg/mL and, if repeat measurements were discrepant, we used the larger of the two MIC values. We defined BDQ-susceptible strains as those with BDQ MICs ≤1 mcg/mL (MGIT), intermediate strains as those with BDQ MICs = 2 mcg/mL, and resistant strains as those with BDQ MICs ≥ 4 mcg/mL [9]. (Note that both resistant and intermediate strains, as defined here, had MGIT-based MICs above the WHO-recommended critical concentration of 1 for BDQ in *Mtb*. [18])

*Genetic sequencing and WGS-based strain characterization*

We conducted WGS for all available isolates from all participants who had at least 1 *Mtb* isolate with BDQ MIC > 1 mcg/mL (n=98). We used the Illumina NextSeq platform with read lengths of 150bp and coverage at >100x using the Nextera DNA Flex Library Preparation kit. Additional information on WGS data processing, sequence quality metrics, and WGS data availability is provided in the **Supplementary Methods**.

We conducted targeted Sanger sequencing to confirm *mmpR5* genotypes obtained via WGS, as described in the **Supplementary Methods**. We accepted *mmpR5* minority variants identified via WGS if they met the following criteria: (1) the proportion all reads for a given minority variant was > 0.2; or (2) the minority variant was identified in another sample from the same participant. **Table S1** compares the WGS-based and Sanger-based *mmpR5* genotypes for isolates in this study.

We determined lineages for each isolate using fast-lineage-caller v1.0 (<https://github.com/farhat-lab/fast-lineage-caller>) and the SNP-based lineage classification scheme developed by Coll et al [19]. We used Mykrobe [20] and the WHO TB drug resistance mutations catalog [21] to generate genotypic resistance profiles to other non-BDQ TB drugs. We calculated the number of SNP differences for all between-participant and within-participant isolate pairs and constructed a phylogenetic tree using maximum-likelihood methods (**Supplementary Methods**). WGS data identified 3 isolates with apparent admixture, involving multiple distinct strains within the same sample (**Table S2 and Figure S1**), which were excluded from phylogenetic analyses.

*Identification of acquired versus pre-existing BDQ resistance*

We used clinical information on each participant, combined with BDQ MIC values and WGS-based strain characterization to delineate whether participants had pre-existing versus treatment-emergent resistance; we also determined whether these infections were polyclonal, i.e. those involving multiple, distinct *Mtb* strains.

We defined participants with pre-existing BDQ resistance as those who had any pre-treatment isolate with BDQ MIC > 1 mcg/mL. We defined participants as having *treatment-emergent* BDQ resistance if all pre-treatment isolates had MIC < 2 and any subsequent isolate (after starting treatment) had MIC ≥ 2. Treatment-emergent resistance, as defined, can be due to either monoclonal infections that acquire resistance *de novo* or polyclonal infections involving multiple strains with different BDQ MICs. To differentiate between these two situations, we further defined *acquired* resistance as the subset of cases with treatment-emergent resistance that were not polyclonal. Polyclonal infections were defined as participants who had isolates from multiple phylogeographic lineages or different isolates from the same lineage that were separated by >12 SNP differences `. Lastly, we defined acquired BDQ resistance as *mmpR5­-mediated* if pre-treatment, susceptible isolates were *mmpR5­* wild type and resistant isolates collected later were *mmpR5­* mutants. **Figure 1** provides individual participant examples to illustrate these definitions.

*Genomically linked isolates*

We used SNP differences, calculated from high quality WGS-derived SNPs, to identify genomically linked isolate pairs. We applied two thresholds to classify isolates as genomically linked: <5 SNPs differences [22] and a more conservative threshold of <2 SNP differences.

**Results**

*Pre-existing and acquired BDQ resistance*

We obtained *Mtb* BDQ MIC data for 147 of 195 participants in the PROBeX study, of which 24 had at least one isolate with BDQ MIC > 1. Sixteen of these participants (10.9% of 147) had at least one *Mtb* isolate with BDQ resistance (MICs ≥ 4) collected at any time during treatment. There were no significant differences in the demographic characteristics of these 24 participants compared to those without any BDQ-resistant isolates **(Table S3)**. The remaining 8 participants had at least one isolate with intermediate BDQ susceptibility (MIC = 2). Participants with BDQ-resistant or -intermediate strains were less likely to achieve clinical cure compared to participants with only BDQ-susceptible isolates, but this difference was not statistically significant (Odds ratio: 0.87, 95% confidence interval: 0.36-2.11, **Table S4**).

**Figure** **2** summarizes BDQ DST results for the 24 participants who had any isolate with BDQ MIC > 1 mcg/mL. Nineteen of these participants had multiple *Mtb* isolates collected during the study; 5 had only a single isolate. Thirteen participants had isolates collected both before and after treatment initiation.

Taken together, 12 of 147 participants with available BDQ MIC data (8.2%) in the PROBeX study had pre-existing BDQ resistance. Only 1 participant with pre-existing resistance had prior clofazimine exposure. Seven of 147 participants (4.8%) had evidence of treatment-emergent BDQ resistance, of which 5 were polyclonal infections and 2 met criteria for acquired resistance. One case of acquired BDQ resistance were *mmpR5­*-mediated; the second had no evidence of any mutations in *mmpR5, atpE*, or *pepQ*. Eight of 24 participants had polyclonal infections involving strains from multiple *Mtb* lineages or genetically distinct strains from the same lineage (i.e., those separated by > 12 SNP differences). One participant with polyclonal treatment-emergent resistance had isolates with genetic admixture of multiple strains (**Table S2**). Five participants did not have pre-treatment isolates collected, such that we were unable to adjudicate whether these participants had pre-existing or treatment-emergent BDQ resistance. We identified 4 participants with widely different BDQ MICs (≥ 4-fold difference) for highly-related isolates from the same lineage that were collected within relatively short timespans (<56 days apart) and had no interval changes in *mmpR5* genotypes (**Supplementary Figure S2)**.

*Genomically linked isolate pairs*

**Supplementary Figure S3** shows the distributions of pairwise SNP distances for all within-participant and between-participant *Mtb* isolate pairs in our study sample. Using between-participant pairwise SNP distances, we identified 9 participant pairs that shared genomically linked isolates differing by <5 SNPs (**Figure 3**); we identified 4 participant pairs sharing genomically linked isolates when the <2 SNP threshold was applied.

Using the <5 SNP threshold, we found 4 specific strains (Strains A, B, C, and D in **Figure 3**) involved in genetically similar between-participant isolate pairs. Strain A, a BDQ-resistant lineage 4.4.1 strain with a frameshift *mmpR5­* mutation (144insC [E49fs]), was involved in a genomically linked cluster of 4 participants recruited at both the Durban and Gqeberha study sites (**Figure 3A**). The three other strains (Strains B, C, and D) were each involved in a single pair of linked participants. Using the most stringent threshold of <2 SNPs, we identified 4 participant pairs, involving 7 unique participants, that shared highly related isolates from strains A, B, or D, but not C (**Figure 3B**).

*TB infections with elevated BDQ MICs involve diverse* Mtb *populations*

Phylogenetic analysis of 95 total sequenced isolates from 24 participants identified BDQ resistance across multiple lineages, including lineages 2.2.2, 4.4.1 (including strain A described above), 4.3.2, and 4.8 (**Figure S4**). Isolates from certain phylogeographic lineages shared similar resistance profiles for other, non-BDQ TB drugs. For example, the majority of isolates from lineages 2.2.2 and 2.2.1 were XDR by genotyping and the majority of isolates from lineage 4.4.1 were pre-XDR (2006 definition).

*Multiple mmpr5 mutations are associated with elevated BDQ MICs*

**Figure 4** shows the *mmpR5* mutations identified among isolates in our cohort and the BDQ MIC values of isolates carrying each mutation. Considering all non-admixed isolates from each participant (including highly similar serial isolates collected from the same patient), 23 of 33 (69.7%) isolates with *mmpr5* frameshift mutations were BDQ resistant, 5 had intermediate susceptibility (15.1%), and 5 were susceptible (15.1%). Eight of 14 (57.1%) isolates with *mmpR5* non-frameshift mutations were BDQ resistant, 3 (21.4%) had intermediate susceptibility, and 3 (21.4%) were susceptible. 42 of 48 (87.5%) *mmpR5* wild-type isolates were BDQ susceptible, 4 (8.3%) were intermediate, and 2 (4.2%) isolates were BDQ resistant **(Figure 4C)**.  *mmpR5* mutations in participants with pre-existing resistance (139insG [D47fs], 139insGATC [P48fs], 144insC [E49fs], 198delG [I67fs], 198insG [I67fs], A202C [S68R], G203A [S68N], T277C [F93L], and T437C[M146T]) differed from those with acquired resistance (349insC [L117fs] and 379delG [D127fs]). There were no isolates with polymorphisms detected in *atpE* or *pepQ*.

**Discussion**

This study characterized serial *Mtb* isolates from individuals who had increased BDQ MICs during BDQ-based treatment for DRTB. This cohort included participants with pre-existing BDQ resistance, polyclonal infections with divergent BDQ resistance phenotypes, and a small number of infections with acquired BDQ resistance associated with *de novo* mutations in *mmpR5*. These infections were diverse across different participants, involving multiple *Mtb* strain types harboring a relatively large set of different *mmpR5* mutations, and also within individuals, with diverse isolates sampled at different timepoints during TB diagnosis and treatment.

Two of our findings have implications for clinical management of BDQ-resistant TB. First, the high proportion of participants with pre-existing BDQ resistance underscores importance of pre-treatment BDQ susceptibility testing, which can have important implications for therapeutic decision-making and preventing selection for resistance to other TB drugs (including pretomanid and delaminid, which have relatively lower barriers to resistance [23]). Second, our results indicate that mixed *Mtb* infections, involving polyclonal infections with different strain types, should be considered in patients who experience treatment failure or have unexpected changes in BDQ susceptibility during treatment. Clinical sampling often does not capture the full diversity of TB strains in a single host [10] and failure to identify *Mtb* populations with more extensive resistance profiles can have important consequences for TB treatment decisions and clinical outcomes [14, 24]. Emerging strategies for sequencing directly from sputum samples, without re-culturing [25], and microbiological methods for increasing the yield of differentially culturable *Mtb* populations [12] may be important for capturing the full genetic diversity of *Mtb* isolates. Repeating phenotypic DST in individuals with poor clinical or microbiological response to treatment can help identify treatment-emergent resistance and inform therapeutic decision-making.

Our findings also highlight important issues relevant to the classification of TB infections by resistance phenotype. Clinical studies that rely on more limited microbiological data (for example, DST on a single sample per participant, without WGS) may fail to capture the complexity and dynamic nature of DR and BDQ-resistant TB infections, resulting in multiple kinds of potential misclassification. For example, if clinical trial participants have unrecognized polyclonal infection, their clinical outcomes (e.g., treatment failure) may be incorrectly attributed to just one of multiple strains involved in their infection. In our study, 8 BDQ-resistant infections would have been misclassified as susceptible if only their pre-treatment samples were used for classification. Misclassification of this kind can also confound genotype-phenotype correlations, obfuscating attempts to identify molecular markers of resistance to BDQ. WGS is thus an important asset for clinical studies where avoiding potential misclassification, and disambiguating strains involved in polyclonal infections, are important priorities.

Four participants in our study had wide differences in BDQ MIC values on nearly clonal samples that had no identifiable changes in their *mmpr5* genotypes **(Supplementary Figure S2)**. Variability is a well-documented feature of MIC measurements in *Mtb*, and many of the known factors underlying this variability (including inherent laboratory measurement variance [26] and variable sampling from different disease sites and/or bacterial subpopulations [27-29]) likely apply here. Additional work is needed to understand other determinants of BDQ resistance, including genetic variants that are difficult to identify with conventional sequencing approaches (including large-scale genomic re-arrangements [30]). Lastly, variability in BDQ MIC measurements poses an additional, important challenge for characterizing and classifying *Mtb* strains by resistance phenotype.

We found genomically linked infections with a specific BDQ-resistant strain (Strain A in Figure 2) involving 4 different participants recruited at two different study sites (Durban and Gqeberha). Strain A is a highly drug-resistant lineage 4.4.1 strain that carries the *mmpR5* frameshift mutation *144insC* [E49fs]. This same mutation has been observed in previous studies of BDQ-resistant TB in South Africa, where a lineage 2 strain with *mmpR5 144insC* [E49fs] was identified in a cluster of linked infections [5]. A local genomically linked cluster of BDQ-resistant strains from lineage 4.4.1 were recently observed in eSwatini, but in this case, BDQ resistance was associated with a distinct and more diverse set of *mmpR5* mutations (G121R, N98D, and M146T) that pre-date the clinical use of BDQ [31]. Importantly, our study was not designed to infer transmission of specific strains between individual participants, and population-level estimates of transmission-associated BDQ-resistant TB cannot be extrapolated from our data. Infections with pre-existing resistance are arguably all attributable to transmission, except for potential *de novo* acquisition of resistance in the interval between initial infection and treatment inititation, which is likely exceedingly rare. The evolutionary processes behind the initial emergence of currently circulating BDQ-resistant strains remain uncertain [32, 33]. Expanded surveillance for BDQ-resistant *Mtb* strains, including studies specifically designed to track potential transmission of these strains , is urgently needed [34].

Our study has important limitations. First, conventional WGS can have difficulty resolving minority bacterial subpopulations without high-depth read coverage, and the standard read depths used in our WGS may fail to capture the full *Mtb* genetic diversity in each sample, including heteroresistant infections and polyclonal infections with very small minority strain populations. Second, SNP distance-based methods for inferring genomic linkages between isolates have several drawbacks, including ambiguity about how to choose a SNP distance threshold and bias toward either over- or under-calling linkages based on which threshold value is used [35]. The SNP distance thresholds we used are comparable to or more stringent than other studies [22] (including those in South Africa [36]) and our analysis is likely biased toward under-calling the number of genetically linked infections in our cohort. Third, potential sampling bias may have enriched our study cohort for individuals with genomically linked isolates. Participants all had highly drug-resistant TB, relatively rare infections that are caused by restricted number of strains (compared to drug-susceptible TB), making it more likely that isolates identified in this cohort would be genetically similar. Lastly, the small sample size in this study and non-randomized selection of participants for serial genotype-phenotype characterization likely limits the generalizability of our findings.

Our findings highlight the complex, dynamic nature of *Mtb* strains involved in BDQ-resistant TB, with important implications for patient care and scientific and clinical research. Increased investment is needed to enhance public health surveillance for BDQ-resistant TB and mitigate its potential spread.

**Potential conflicts of interest:**

All authors: No reported conflicts.

**Author contributions:**

Study conception and design: BM, JCMB, TSB, LT

Data acquisition: SVO, LJ, GrM, GM, SW

Data analysis: LT, TSB, SVO, LJ, MRF

Funding and supervision: JCMB, NRG, SS, NI, GrM, GM, BM

Drafting of manuscript: TSB, LT, JCMB, BM

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**FIGURES**

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**Figure 1: Pre-existing resistance, polyclonal infection, and acquired resistance identified using WGS characterization of serial *Mtb* isolates**. Each panel shows BDQ MICs over time for an individual participant (Participants A-C). Each timepoint represents a single isolate, colored by phylogeographic lineage. The shape of each point shows the *mmpR5* genotype of each isolate. Lines between each point are colored by the SNP difference between each isolate pair. Isolates collected prior to treatment initiation are highlighted in green. The x-axis (time in days) is square root-transformed to better display isolates collected early during treatment.

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**Figure 2: *Mtb* isolate collection and BDQ MIC results for 24 participants who had any isolate with BDQ MIC ≥** **2.** Counts for the number of participants at each node are shown in parentheses. Plot generated using the *networkD3* package in R [37, 38].

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**Figure 3: Genomically-linked *Mtb* isolate pairs shared between participants.** (A) Participant pairs linked by highly related *Mtb* isolates differing by < 5 SNPs. Symbols denote whether each participant had pre-existing or treatment-emergent resistance, no categorically intermediate or resistant strains (all with MIC ≤ 1), or no pre-treatment isolates available (such that pre-existing versus treatment associated resistance could not be adjudicated). Lines between symbols denote participants linked by closely related isolates (differentiated by < 5 SNPs or < 2 SNPs in panel B) from one of four strains. Strain A: BDQ-resistant, lineage 4.4.1, *mmpR5* 144insC [E49fs]; Strain B: BDQ-resistant, lineage 2.2.2, *mmpR5* A202G; Strain C: BDQ-susceptible, lineage 2.2.2, *mmpR5* WT; Strain D: BDQ-resistant, lineage 4.3.3, *mmpR5* 198insC. Figure generated using the *igraph* package in R [39].



**Figure 4: *mmpR5* mutations identified among study participants**. Gene map (top) shows locations of *mmpR5* mutations identified amongst participants in this study. Bottom panels show isolates grouped by*mmpR5* mutation type (frameshift, non-frameshift, and wild type): each square represents a single isolate, each row of squares represents a single participant, and squares are colored by BDQ MIC.

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