

Genotype–Phenotype Characterization of Serial *Mycobacterium tuberculosis* Isolates in Bedaquiline-Resistant Tuberculosis

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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 mycobacteria growth indicator tube (MGIT)–based BDQ minimum inhibitory concentrations (MICs) 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 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 $\mu\text{g/mL}$) and 8 had BDQ-intermediate infections (MGIT MIC = 2 $\mu\text{g/mL}$). 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 single nucleotide polymorphisms, 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.

Keywords. tuberculosis; bedaquiline; drug resistance; whole-genome sequencing.

Bedaquiline (BDQ) has markedly improved clinical outcomes for individuals with drug-resistant tuberculosis (DRTB) [1–3]. Bedaquiline-containing regimens have been standard of care for all patients with rifampin-resistant tuberculosis (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.

Bedaquiline resistance in clinical TB infections is almost entirely attributable to mutations in *mmpR5* (*Rv0678*), which may also confer resistance to clofazimine. Bedaquiline resistance associated with mutations in 2 other genes, *pepQ* and *atpE*, are rare outside of in vitro experiments [6]. A diverse collection of *mmpR5* mutations, involving more than 200 different loci across the gene, have been reported in clinical isolates [7]. In addition, *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.

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Mycobacterium 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* subpopulations can 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 sequencing (WGS) data from serial *Mtb* isolates can differentiate infections with treatment-emergent resistance from infections involving strains with pre-existing (aka “primary”) resistance. Whole-genome sequencing also allows for the 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. Last, 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 receiving 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

Pharmacokinetics, Resistance, and Outcomes of Bedaquiline in MDR and XDR-TB (PROBeX) was an observational longitudinal cohort study conducted at 3 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 Measurements

We measured BDQ MIC values (0.125–8 µg/mL) of *Mtb* isolates using the BACTEC mycobacteria growth indicator tube (MGIT) 960 DST method [9] ([Supplementary Methods](#)). We performed repeat BDQ MIC measurements for any isolate with an initial MIC of 1 µg/mL or greater and, if repeat measurements were discrepant, we used the larger of the 2 MIC values. We defined BDQ-susceptible strains as those with BDQ MICs of 1 µg/mL or less (MGIT), intermediate strains as those with BDQ MICs = 2 µg/mL, and resistant strains as those with BDQ MICs of 4 µg/mL or greater [9]. (Note that both resistant and intermediate strains, as defined here, had MGIT-based MICs above the World Health Organization [WHO]–recommended critical concentration of 1 for BDQ in *Mtb* [18].)

Genetic Sequencing and Whole-Genome Sequencing–Based Strain Characterization

We conducted WGS for all available isolates from all participants who had at least 1 *Mtb* isolate with BDQ MIC greater than 1 µg/mL ($n = 98$). We used the Illumina NextSeq platform with read lengths of 150 bp and coverage at greater than 100× 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 of all reads for a given minority variant was greater than 0.2 or (2) the minority variant was identified in another sample from the same participant. [Supplementary Table 1](#) 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](#)). The WGS data identified 3 isolates with apparent admixture, involving multiple distinct strains within the same sample ([Supplementary Table 2](#) and [Supplementary Figure 1](#)), which were excluded from phylogenetic analyses.

Identification of Acquired Versus Pre-existing Bedaquiline 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—that is, those involving multiple, distinct *Mtb* strains.

We defined participants with pre-existing BDQ resistance as those who had any pretreatment isolate with BDQ MIC greater than 1 µg/mL. We defined participants as having treatment-emergent BDQ resistance if all pretreatment isolates had MICs less than 2 and any subsequent isolate (after starting treatment) had MICs of 2 or greater. 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 2 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 more than 12 SNP differences. Last, we defined acquired BDQ resistance as *mmpR5*-mediated if pretreatment, 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 2 thresholds to classify isolates as genomically linked: fewer than 5 SNP differences [22] and a more conservative threshold of fewer than 2 SNP differences.

RESULTS

Pre-existing and Acquired Bedaquiline Resistance

We obtained *Mtb* BDQ MIC data for 147 of 195 participants in the PROBeX study, of whom 24 had at least 1 isolate with a BDQ MIC greater than 1. Sixteen of these participants (10.9% of 147) had at least 1 *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 with those without any BDQ-resistant isolates ([Supplementary Table 3](#)). The remaining 8 participants had at least 1 isolate with intermediate BDQ susceptibility (MIC = 2). Participants with BDQ-resistant or -intermediate strains were less likely to achieve clinical cure compared with participants with only BDQ-susceptible isolates, but this difference was not statistically significant (odds ratio: 0.87; 95% confidence interval: .36–2.11) ([Supplementary Table 4](#)).

[Figure 2](#) summarizes BDQ DST results for the 24 participants who had any isolate with a BDQ MIC greater than 1 µg/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 was *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 (ie, those separated by >12 SNP differences). One participant with polyclonal treatment-emergent resistance had isolates with a genetic admixture of multiple strains ([Supplementary Table 2](#)). Five participants did not have pretreatment 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 time spans (<56 days apart) and had no interval changes in *mmpR5* genotypes ([Supplementary Figure 2](#)).

Genomically Linked Isolate Pairs

[Supplementary Figure 3](#) 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 less than 5 SNPs ([Figure 3](#)); we identified 4 participant pairs sharing genomically linked isolates when the less-than-2 SNP threshold was applied.

Using the less-than-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 3 other strains (strains B, C, and D) were each involved in a single pair of linked participants. Using the most stringent threshold of less than 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](#)).

Tuberculosis Infections With Elevated Bedaquiline 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

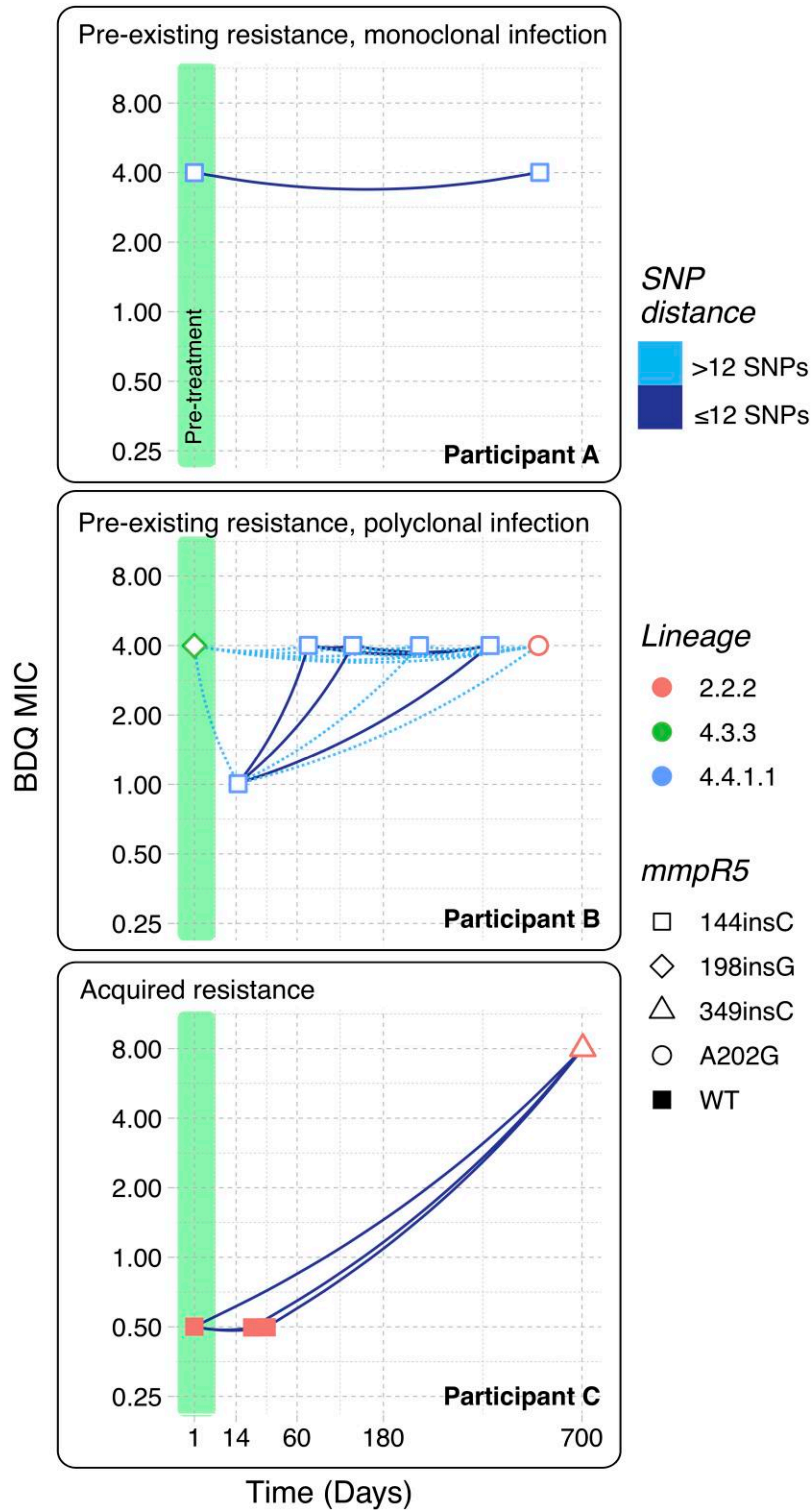


Figure 1. Pre-existing resistance, polyclonal infection, and acquired resistance identified using WGS characterization of serial *Mtb* isolates. Each panel shows BDQ MICs over time for an individual participant (participants A–C). Each timepoint represents a single isolate, colored by phylogeographic 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. Abbreviations: BDQ, bedaquiline; MIC, minimum inhibitory concentration; *Mtb*, *Mycobacterium tuberculosis*; SNP, single nucleotide polymorphism; WGS, whole-genome sequencing; WT, wild-type.

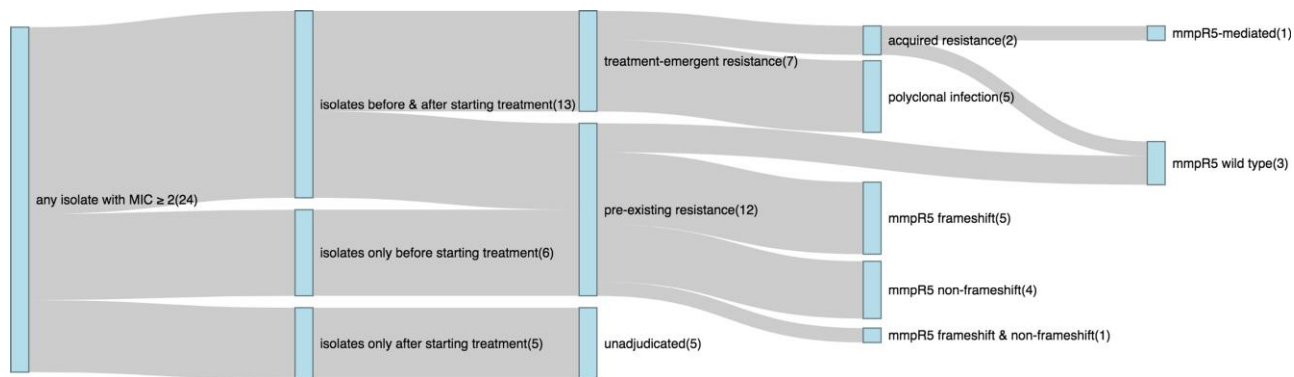


Figure 2. *Mtb* isolate collection and BDQ MIC results for 24 participants who had any isolate with a BDQ MIC ≥ 2 . Counts for the number of participants at each node are shown in parentheses. Plot generated using the *networkD3* package in R [23, 24]. Abbreviations: BDQ, bedaquiline; MIC, minimum inhibitory concentration; *Mtb*, *Mycobacterium tuberculosis*.

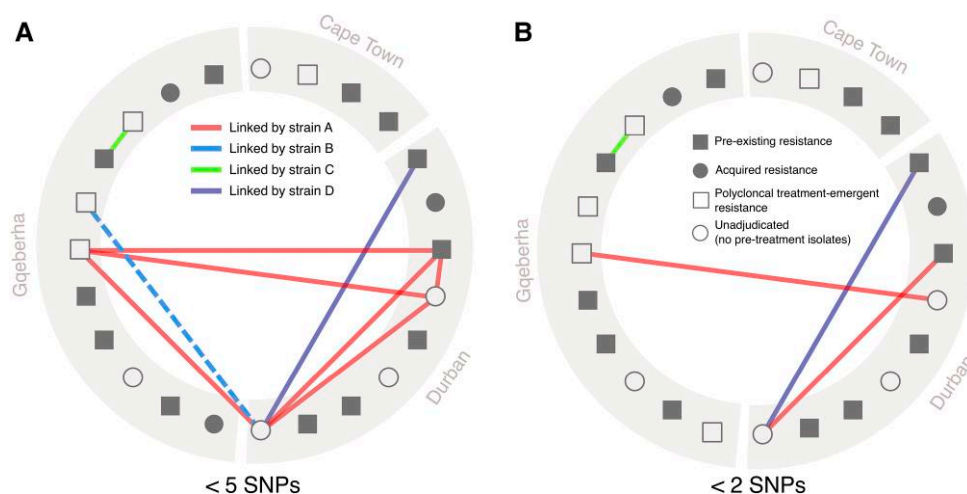


Figure 3. Genomically linked *Mtb* isolate pairs shared between participants. A, Participant pairs linked by highly related *Mtb* isolates differing by < 5 SNPs. Symbols denote whether each participant had pre-existing or treatment-emergent resistance, no categorically intermediate or resistant strains (all with MIC ≤ 1), or no pretreatment isolates available (such that pre-existing vs 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 1 of 4 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 [25]. Abbreviations: BDQ, bedaquiline; MIC, minimum inhibitory concentration; *Mtb*, *Mycobacterium tuberculosis*; SNP, single nucleotide polymorphism; WT, wild-type.

above), 4.3.2, and 4.8 (Supplementary Figure 4). 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 Bedaquiline 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. Forty-two 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). The *mmpR5* mutations in participants with pre-existing resistance (139insG [D47fs], 139insGATC [P48fs], 144insC [E49fs], 198delG [I67fs], 198insG [I67fs], A202C

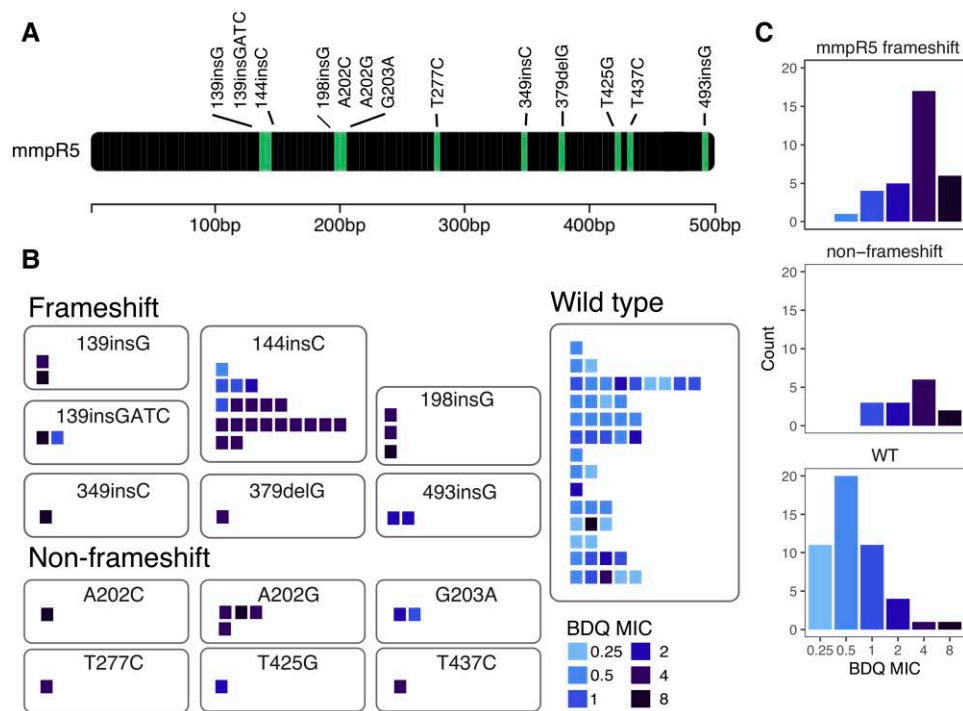


Figure 4. *mmpR5* Mutations identified among study participants. The gene map (A) shows locations of *mmpR5* mutations identified among participants in this study. Panels B and C show isolates grouped by *mmpR5* mutation type (frameshift, non-frameshift, and WT). In panel B, each square represents a single isolate, each row of squares represents a single participant, and squares are colored by BDQ MIC. Abbreviations: BDQ, bedaquiline; MIC, minimum inhibitory concentration; WT, wild-type.

[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 the importance of pretreatment BDQ susceptibility testing, which can have important implications for therapeutic decision making and preventing selection for resistance to other TB drugs (including pretomanid and delamanid, which have relatively lower barriers to resistance [26]). 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, 27]. Emerging strategies for sequencing directly from sputum samples, without re-culturing [28], 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 (eg, DST on a single sample per participant, without WGS) may fail to capture the complexity and dynamic nature of drug-resistant 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 (eg, treatment failure) may be incorrectly attributed to just 1 of multiple strains involved in their

infection. In our study, 8 BDQ-resistant infections would have been misclassified as susceptible if only their pretreatment 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. Whole-genome sequencing 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 2). 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 [29] and variable sampling from different disease sites and/or bacterial subpopulations [30–32]) 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 [33]). Last, 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 2 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 was 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 predate the clinical use of BDQ [34]. 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 initiation, which is likely exceedingly rare. The evolutionary processes behind the initial emergence of currently circulating BDQ-resistant strains remain uncertain [35]. Expanded surveillance for BDQ-resistant *Mtb* strains, including studies specifically designed to track potential transmission of these strains, is urgently needed [36].

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 an SNP distance threshold and bias toward either over- or under-calling linkages based on which threshold value is used [37]. The SNP distance thresholds we used are comparable to or more stringent than other studies [22] (including those in South Africa [38]) 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 a restricted number of strains (compared with drug-susceptible TB), making it more likely that isolates identified in this cohort would be genetically similar. Last, the small sample size in this study and nonrandomized selection of participants for serial genotype–phenotype characterization likely limit 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.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author Contributions. Study conception and design: B. M., J. C. M. B., T. S. B., and L. T. Data acquisition: S. V. O., L. J., G. Meintjes, G. Maartens, and S. W. Data analysis: L. T., T. S. B., S. V. O., L. J., and M. R. F. Funding and supervision: J. C. M. B., N. R. G., N. S. S., N. I., G. Meintjes, G. Maartens, and B. M. Drafting of the manuscript: T. S. B., L. T., J. C. M. B., and B. M.

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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