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The Performance of Genotypic Profiling and Distribution of Minimum Inhibitory Concentrations in Bedaquiline-Resistant Tuberculosis

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Abstract

Background: Bedaquiline (BDQ) is critical for multidrug-resistant tuberculosis (MDR-TB) treatment, but emerging resistance threatens its efficacy. Detection of BDQ resistance relies on slow phenotypic drug-susceptibility testing (pDST), delaying treatment decisions. We determined the diagnostic accuracy of whole-genome sequencing (WGS) for predicting BDQ resistance against culture-based pDST. **Methods:** *Mycobacterium tuberculosis* isolates underwent BDQ pDST (1.0 μg/mL in MGIT) in a prospective study of patients with MDR-TB with additional resistance to fluoroquinolones or aminoglycosides in KwaZulu-Natal, South Africa (2018–2022). Isolates with single nucleotide polymorphisms showing a read depth of ≥10 and allele frequency of ≥0.7 were included. Isolates exhibiting phenotypic resistance underwent quantitative minimum inhibitory concentration (MIC) testing (ranging from 0.25 to 8.0 μg/mL) using the BACTEC MGIT 960 system. Diagnostic performance of WGS compared to MGIT-based pDST was assessed by calculating sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV). **Results:** Analysis included 428 isolates: 88 phenotypically resistant isolates (87 with potential BDQ-resistance-associated variants (RAVs) and one wild-type) and 340 susceptible isolates (7 with potential RAVs and 333 wild-type). Presence of potential RAVs accurately predicted phenotypic resistance: sensitivity 98.9% (95% CI: 93.8–99.8), specificity 97.9% (95% CI: 95.8–99.0), PPV 92.6% (95% CI: 85.4–96.4), and NPV 99.7% (95% CI: 98.3–99.6). Among the 94 isolates with potential RAVs, we observed gene mutations in *Rv0678* (88/94, 93.6%), *pepQ* (2/94, 2.1%), and *Rv1979c* (4/94, 4.3%). The median BDQ MIC for resistant isolates was 4.0 μg/mL [IQR:2.0–4.0 μg/mL]. **Conclusions:** Potential BDQ RAVs, predominantly in *Rv0678*, are

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strong predictors of BDQ phenotypic resistance, highlighting the potential use of genotypic catalogues to inform timely treatment decisions.

Keywords: *Mycobacterium tuberculosis*; *Rv0678*; bedaquiline; minimum inhibitory concentrations; drug susceptibility testing; phenotypic resistance

1. Introduction

Tuberculosis (TB) remains a profound global health crisis, with 10.7 million incident cases and 1.2 million deaths in 2024 [1]. The persistence of drug-resistant TB (DR-TB) presents a severe threat, fundamentally undermining standard DR-TB short-course chemotherapy. In 2024, an estimated 390,000 individuals developed multidrug-resistant or rifampicin-resistant TB (MDR/RR-TB), yet only 164,545 (42%) were diagnosed and received appropriate second-line treatment [1].

The introduction of bedaquiline (BDQ), a first-in-class diarylquinoline, prompted a recent shift from prolonged, toxic regimens to all-oral short-course therapies [2–5]. However, expansion of BDQ utilization has been accompanied by the emergence and dissemination of BDQ-resistant *Mycobacterium tuberculosis* (*Mtb*) strains [6,7]. Global meta-analyses indicate a BDQ resistance prevalence of 5.7% among people with RR-TB at baseline, before initiating treatment [8]. South African RR-TB national surveillance data identified BDQ resistance in 3.8% of patients at baseline and 2.3% among on-treatment samples [6]. A recent study conducted between 2023 and 2024 in the Western Cape, South Africa, showed BDQ resistance in 12% of baseline isolates and 41% of on-treatment isolates [9].

The molecular architecture of BDQ resistance is complex and distributed across multiple genetic loci [10,11] that span at least four primary candidate genes: *atpE*, *mmpR5* (*Rv0678*), *pepQ*, and *Rv1979c* [11,12]. Mutations within *atpE*, the direct biochemical target of BDQ, definitively confer high-level resistance [13]; however, they account for less than 5% of clinical resistance [14]. Instead, evolutionary pressure selects overwhelmingly for loss-of-function mutations in *Rv0678*, present in >96% of phenotypically resistant isolates [15]. The genetic profile of *Rv0678*-mediated resistance is diverse, with over 200 mutations identified across its entire coding sequence and promoter regions [16–18]. The resulting phenotypic impact is highly variable, frequently generating intermediate phenotypes and clofazimine (CFZ) cross-resistance [16,19]. Mutations in accessory genes *pepQ* and *Rv1979c* are implicated in resistance, though infrequent, occurring in <5% of phenotypic resistant isolates [20,21].

Historically, DR-TB management relied heavily on empirical treatment, often resulting in the use of drugs to which the patient was already resistant. This approach suppresses susceptible *Mtb* subpopulations while simultaneously providing a competitive advantage to resistant *Mtb* mutants [22]. Thus, there is a need for diagnostic tools to rapidly detect BDQ resistance to guide treatment and prevent further emergence and spread of resistant strains. The diffuse mutational distribution across the 498-base-pair *Rv0678* gene renders targeted, cartridge-based PCR probe technologies currently unfeasible. In addition, sequencing known candidate genes yields poor predictive values for many variants: an "any mutation" diagnostic approach for BDQ resistance candidate genes (*Rv0678*, *atpE*, *atpB* and *pepQ*) yields a modest sensitivity of 69% and a positive predictive value (PPV) of only 14% [23]. The modest sensitivity observed was due to the majority of isolates demonstrating an intermediate phenotype (minimum inhibitory concentration (MIC) at the critical concentration), likely resulting from heteroresistance. Operating a molecular assay with such low positive predictive value (PPV) would lead to over-diagnosis and the

inappropriate withholding of a highly efficacious drug. The World Health Organization (WHO) has developed a comprehensive mutation catalogue for genotypic and phenotypic resistance calls for antitubercular drugs, including BDQ [17]. Even employing the stringent statistical criteria of the WHO mutation catalogue, the maximum achievable sensitivity for predicting phenotypic resistance using high-confidence mutations remains severely limited at approximately 49.4% [12,17].

Given the current difficulties in interpreting diverse non-target mutations, genomic sequencing cannot yet serve as a standalone proxy for phenotypic testing [24]. Consequently, the diagnosis of BDQ resistance remains heavily reliant on culture-based pDST, which is labour-intensive and can take up to eight weeks to provide results [25]. Developing rapid molecular diagnostics requires closing the gap in understanding the genotypic and phenotypic relationship. This involves expanding comprehensive predictive catalogues to accurately capture noncanonical resistance mutations, paired with standardized, quantitative MIC testing to resolve intermediate or borderline phenotypes. Although quantitative MIC testing is time-consuming and currently unsuitable for widespread programmatic use, it provides critical epidemiological and clinical insights into the spectrum of drug resistance, particularly for efflux-mediated mechanisms that often result in modest MIC elevations [26].

Current methods for detecting BDQ resistance are largely culture-based, and rapid resistance detection of BDQ will likely depend on granular understanding of genotypic-phenotypic relationships that underpin the genesis of phenotypic resistance. Global efforts aim to achieve this through a cumulative catalogue of mutations, curated under the WHO to inform the field implementation of sequencing-based drug susceptibility strategies. This strategy is limited by poor sensitivity and accuracy; however, there is limited genotypic-phenotypic data emanating from large, well-characterized clinical cohorts in TB endemic settings. We sought to determine the diagnostic accuracy of mutations occurring in BDQ resistance candidate genes for detecting MGIT-based phenotypic BDQ resistance in clinical *Mtb* isolates from a large cohort of patients with DR-TB in a high TB burden setting to strengthen the clinical implementation and utility of sequencing methods. In addition, we aimed to characterize the genotypic profiles underlying phenotypic BDQ resistance in this setting and to describe the spectrum of MICs of these isolates.

2. Results

A total of 428 *Mtb* isolates from people with MDR-TB were included in this study. BDQ pDST identified 88 BDQ-resistant isolates and 340 BDQ-susceptible isolates. WGS revealed 94 isolates with potential BDQ resistance-associated variants (RAVs) (variants identified in genes previously implicated in BDQ resistance: *atpE*, *Rv0678*, *pepQ*, and *Rv1979c*), while the remaining 334 isolates possessed a wild-type genotype. Among the 88 phenotypically resistant isolates, the vast majority (87 isolates, 98.9%) possessed potential BDQ RAVs, and only 1 (1.1%) isolate was wild-type. Conversely, among the phenotypically susceptible isolates, 333 (97.9%) were wild-type, while 7 (2.1%) harboured potential BDQ RAVs. Bedaquiline MICs were performed for 95 isolates: all 88 isolates with BDQ phenotypic resistance and the 7 BDQ-susceptible isolates with potential BDQ RAVs.

In our primary analysis using all isolates included in this study (N = 428), the presence of a potential BDQ RAV was a strong predictor of phenotypic BDQ resistance (Table 1). The overall accuracy of a potential BDQ RAV for detecting phenotypic resistance was 98.1% (95% CI: 96.4–99.1). The markers demonstrated a sensitivity of 98.9% (95% CI: 93.8–99.8) and a specificity of 97.9% (95% CI: 95.8–99.0). Furthermore, the PPV was 92.6% (95% CI: 85.4–96.4), and the negative predictive value (NPV) was 99.7% (95% CI: 98.3–99.6) (Table 1). When we performed analysis using one isolate per participant (N = 418), the results were

comparable to those of the primary analysis with a sensitivity, specificity, PPV and NPV of 98.7% (95% CI: 93.2–99.8), 98.2% (95% CI: 96.2–99.2), 92.9% (95% CI: 85.3–96.7) and 99.7% (95% CI: 98.3–99.9), respectively (Supplementary Table S1).

Table 1. Diagnostic Performance of Potential BDQ RAVs for Predicting Phenotypic Bedaquiline Resistance.

Genotype	BDQ Phenotype at 1.0 μg/mL CC		Total	Predictive Values & Accuracy, % (95% CI)
	BDQ Resistant	BDQ Susceptible		
Potential BDQ RAV	87	7	94	PPV, 92.6 (85.4–96.4)
Wild-type	1	333	334	NPV, 99.7 (98.3–99.6)
Total	88	340	428	Accuracy, 98.1% (96.4–99.1)
Sensitivity & Specificity, % (95% CI)	Sensitivity, 98.9 (93.8–99.8)	Specificity, 97.9 (95.8–99.0)		

Abbreviations: BDQ, bedaquiline; CC, critical concentration; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; RAV, resistance-associated variant.

Based on the WHO catalogue of mutations (Version 2), the 94 isolates with potential BDQ RAVs were stratified into WHO-classified and noncanonical/unclassified variants (Table 2 and Supplementary Table S2). WHO-classified variants were identified in 87 isolates. Of these, 32 isolates had variants in the *Rv0678* Asp47fs (n = 10), Glu49fs (n = 12), Ile67fs (n = 9), and Met146Thr (n = 1) associated with BDQ resistance (Table 2). Meanwhile, 38 isolates had variants with an interim determination of association with BDQ resistance. Of these 38 isolates, 3 [Pro48fs (n = 1), Leu95fs (n = 1) and Leu83fs (n = 1)] had a susceptible phenotype with MICs of ≤0.25, 1.0 and 1.0 μg/mL, respectively. Even though Glu138fs and Leu95fs are currently not listed in the WHO catalogue, they were categorized as associated with BDQ resistance of interim significance based on an expert rule that all frameshifts in *Rv0678* be considered a marker for phenotypic BDQ resistance. Seventeen isolates contained *Rv0678* and *Rv1979c* variants of uncertain significance, yet these isolates had MICs of 4.0–8.0 μg/mL, except for Met139Ile, where 1/4 isolates had an MIC at the CC (1.0 μg/mL) and were classified as phenotypically susceptible (Table 2). Therefore, of the 87 isolates with a WHO-classified variant, 83 (95.4%) were phenotypically resistant, and 4 (4.6%) were susceptible (Supplementary Table S2). Noncanonical variants were found in 7 isolates. Among these, 4 isolates were phenotypically resistant [*Rv0678* upstream insertion (n = 1), Gly25Ser (n = 1), Val1Leu (n = 1), and Gly66Trp (n = 1)] (Table 2 and Figure 1), and 3 isolates were susceptible [*Rv0678*, c.-11C>A (n = 2), and Pro48Ser (n = 1).

In a parallel analysis, we evaluated the performance of only WHO-classified potential BDQ RAVs and observed results that were similar to those obtained when using an "any potential RAV" approach (Supplementary Table S2). The overall accuracy of WHO-classified BDQ potential BDQ RAVs in predicting phenotypic resistance was 97.9% (95% CI: 96.1–99.0). The demonstrated sensitivity was 94.2% (95% CI: 87.4–97.6) and a specificity of 98.8% (95% CI: 97.0–99.5). Furthermore, the PPV was 95.4% (95% CI: 88.8–98.2), and the NPV was 98.5% (95% CI: 96.6–99.4).

Genotypic profiling using the WGS detailed in Table 2 highlights that 94 variants spanned several targets, including *Rv0678* in 88 (93.6%) isolates, solely *pepQ* in 2 (2.1%), and *Rv1979c* in 4 (4.3%). Most *Rv0678* variants were located within the coding region, spanning both DNA-binding and dimerisation domains, with a minority occurring at the promoter region (Supplementary Figure S1). Among the 88 isolates with *Rv0678* variants, three had additional *pepQ* variants (3.4%), and two had *atpE* variants (2.3%). The most frequently observed WHO-classified variants, with an MIC range of 2–8 µg/mL, included *Rv0678* Asp47fs (10/88, 11.4%), Glu49fs (12/88, 13.6%), Ile67fs (9/88, 10.2%), Leu32Ser (6/88, 6.8%), and Cys46fs (4/88, 4.5%). We observed two isolates with a *pepQ* variant (Val273fs) occurring alone, exhibiting MICs of 2 and 4 µg/mL, respectively. While *Rv1979c* variants are not a common mechanism of BDQ phenotypic resistance, four isolates with BDQ MICs of 2–4 µg/mL had *Rv1979c* variants with no mutations in the *Rv0678*, *pepQ*, or *atpE* genes (Glu38Asp and Arg409Gln, graded as of uncertain significance).

Table 2. Genotypic Profiling of Isolates by Bedaquiline Minimum Inhibitory Concentrations (MICs) and WHO Mutation Classification.

	Bedaquiline Concentration in MGIT (µg/mL)						
	≤0.25	**0.5**	**1.0 (CC)**	**2.0**	**4.0**	**8.0**	**WHO Classification**
Phenotypically resistant isolates, *n* = 88							
Wild type [a]	-	-		-	1	-	
Rv0678 variants							
Asp47fs	-	-	-	3	4	2	Associated with BDQ-R
Glu49fs	-	-	-	4	4	1	Associated with BDQ-R
Ile67fs	-	-	-	1	6	2	Associated with BDQ-R
Met146Thr	-	-	-	1	-	-	Associated with BDQ-R
Gln51fs, Glu49fs, Glu104Lys	-	-	-	1	-	-	Glu49fs—Associated with BDQ-R
Leu32Ser	-	-	-	2	4	-	Associated with BDQ-R interim
Gly121Arg, C.-11C>A	-	-	-	1	-	-	Gly121Arg—Associated with BDQ-R interim
Pro48fs	-	-	-	1	1	-	Associated with BDQ-R interim
Glu55fs	-	-	-	2	-	-	Associated with BDQ-R interim
Arg30fs	-	-	-	-	1	-	Associated with BDQ-R interim
Asp165fs	-	-	-	1	-	-	Associated with BDQ-R interim
Leu142fs	-	-	-	1	6	2	Associated with BDQ-R interim
Gln159fs	-	-	-	-	3	-	Associated with BDQ-R interim
Arg72fs	-	-	-	-	1	-	Associated with BDQ-R interim
Cys46fs, Asp47Glu	-	-	-	-	3	1	Cys46fs—Associated with BDQ-R interim Asp47Glu—Uncertain significance
Glu138fs	-	-	-	1	-	-	Associated with BDQ-R interim [b]
Tyr92Cys	-	-	-	-	1	-	Uncertain significance
Arg89Leu	-	-	-	-	2	-	Uncertain significance
Gly65Arg	-	-	-	1	-	-	Uncertain significance
Thr58Pro	-	-	-	-	1	2	Uncertain significance

Table 2. *Cont.*

	Bedaquiline Concentration in MGIT (μg/mL)						
	≤0.25	**0.5**	**1.0 (CC)**	**2.0**	**4.0**	**8.0**	**WHO Classification**
Leu60Pro	-	-	-	-	1	-	Uncertain significance
Met139Ile	-	-	-	-	2	1	Uncertain significance
Phe93Leu	-	-	-	-	1	-	Uncertain significance
Upstream insertion	-	-	-	-	1	-	Not classified
Gly25Ser	-	-	-	-	1	-	Not classified
Val1Leu	-	-	-	-	-	1	Not classified
Gly66Trp	-	-	-	-	1	-	Not classified
pepQ variants							
Val273fs	-	-	-	1	1	-	Associated with BDQ-R interim
Rv0678 & pepQ variants							
Asp47fs, Phe79Leu & Ser200Leu	-	-	-	-	-	1	Asp47fs—Associated with BDQ-R Phe79Leu—Uncertain significance Ser200Leu—Uncertain significance
Leu142fs & Val79_Gly80ins	-	-	-	-	1	-	Leu142fs—Associated with BDQ-R interim
Arg156 * & Ala87fs	-	-	-	-	1	-	Arg156 *—Associated with BDQ-R interim
Rv0678 & atpE variants							
Glu49fs & Asp28Asn	-	-	-	-	-	2	Glu49fs—Associated with BDQ-R
Rv1979c variants							
Glu38Asp	-	-	-	2	1	-	Uncertain significance
Arg409Gln	-	-	-	-	1	-	Uncertain significance
Phenotypically susceptible isolates (n = 7) [c]							
Rv0678 variants							
Met139Ile			1				Uncertain significance
Pro48fs	1						Associated with BDQ-R interim
Leu95fs	-	-	1	-	-	-	Associated with BDQ-R interim [b]
Leu83fs	-	-	1	-	-	-	Associated with BDQ-R interim
c.-11C>A	2	-	-	-	-	-	Not classified
Pro48Ser	-	1	-	-	-	-	Not classified

Abbreviations: BDQ, bedaquiline; BDQ-R, bedaquiline resistance; MGIT, mycobacteria growth indicator tube. [a] Wild-type for candidate genes associated with BDQ resistance, i.e., *Rv0678*, *pepQ*, *Rv1979c*, and *atpE*. [b] Not listed in the WHO catalogue, but categorized as associated with BDQ resistance interim based on the WHO guidelines that all frameshifts in *Rv0678* be considered as a marker for BDQ resistance. [c] Additional 333 phenotypically susceptible isolates were all wild-type by WGS and did not undergo MIC testing. * premature stop codon.

The distribution of BDQ MICs among the 88 phenotypically resistant isolates was mapped across concentrations ranging from 2.0 to 8.0 μg/mL (Figures 1 and 2). Specifically, 23 (26.1%) isolates had an MIC of 2.0 μg/mL, 50 (56.8%) isolates (including the wild-type resistant isolate) had an MIC of 4.0 μg/mL, and 15 (17.1%) isolates had an MIC of 8.0 μg/mL.

Distribution of BDQ MICs amongst phenotypically resistant isolates by genotypic profile (WHO-classified and noncanonical variants) in BDQ resistance-associated candidate genes is presented in Figure 1. Isolates with noncanonical potential RAVs had an MIC distribution of 4.0–8.0 µg/mL [interquartile range (IQR): 4.0–8.0 µg/mL], while WHO-classified potential RAVs had an MIC distribution of 2.0–8.0 µg/mL [IQR: 2.0–4.0 µg/mL]; both groups had a median of 4.0 µg/mL [IQR: 2.0–4.0 µg/mL]. No statistically significant difference was observed in the MIC distribution between groups (Mann–Whitney U, $p = 0.9$). Frameshift mutations in *Rv0678* were the most common variants, occurring in 47/88 (53.4%) isolates, followed by point mutations, which occurred in 22 (25.0%) isolates (Figure 2).

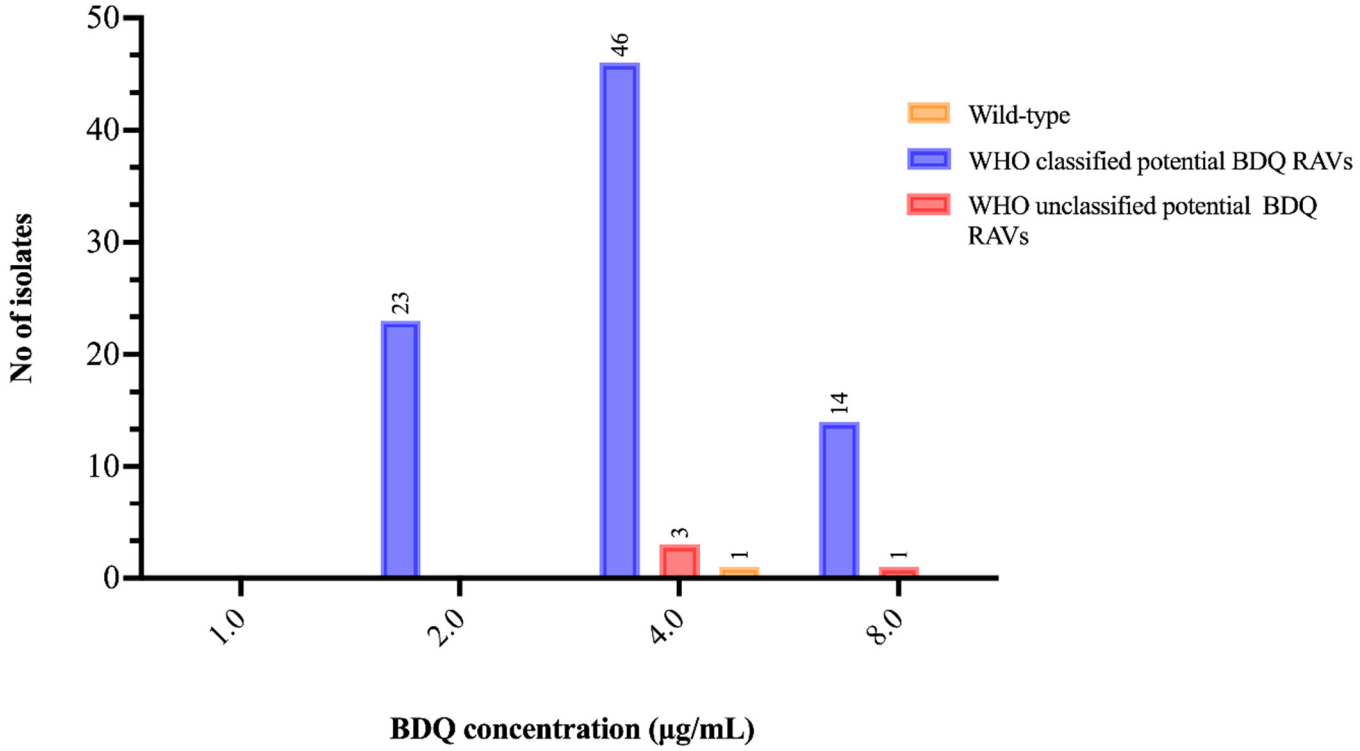


Figure 1. Distribution of BDQ MICs amongst phenotypically resistant isolates, by genotypic profile; wild-type, WHO-classified potential RAVs and unclassified variants in BDQ resistance candidate genes ($n = 88$). There were no differences in MIC distribution between classified and unclassified variants; both had a median MIC of 4.0 µg/mL. No statistical significance was observed between groups (Mann–Whitney U test, $p = 0.9$). Abbreviations: BDQ, bedaquiline; MIC, minimum inhibitory concentration; RAV, resistance-associated variant; WHO, World Health Organization.

Of the 88 isolates with BDQ phenotypic resistance, 86 (97.7%) were CFZ cross-resistant, and 2 (2.3%) remained susceptible to CFZ. Among the two isolates with BDQ phenotypic resistance and CFZ susceptibility, one had a variant in *Rv0678* (Gly66Trp), and the other had dual variants in *Rv0678* (Cys46fs and Asp47Glu). While there was no other isolate with the Gly66Trp variant, 3 other isolates with Cys46fs and Asp47Glu variants were resistant to CFZ.

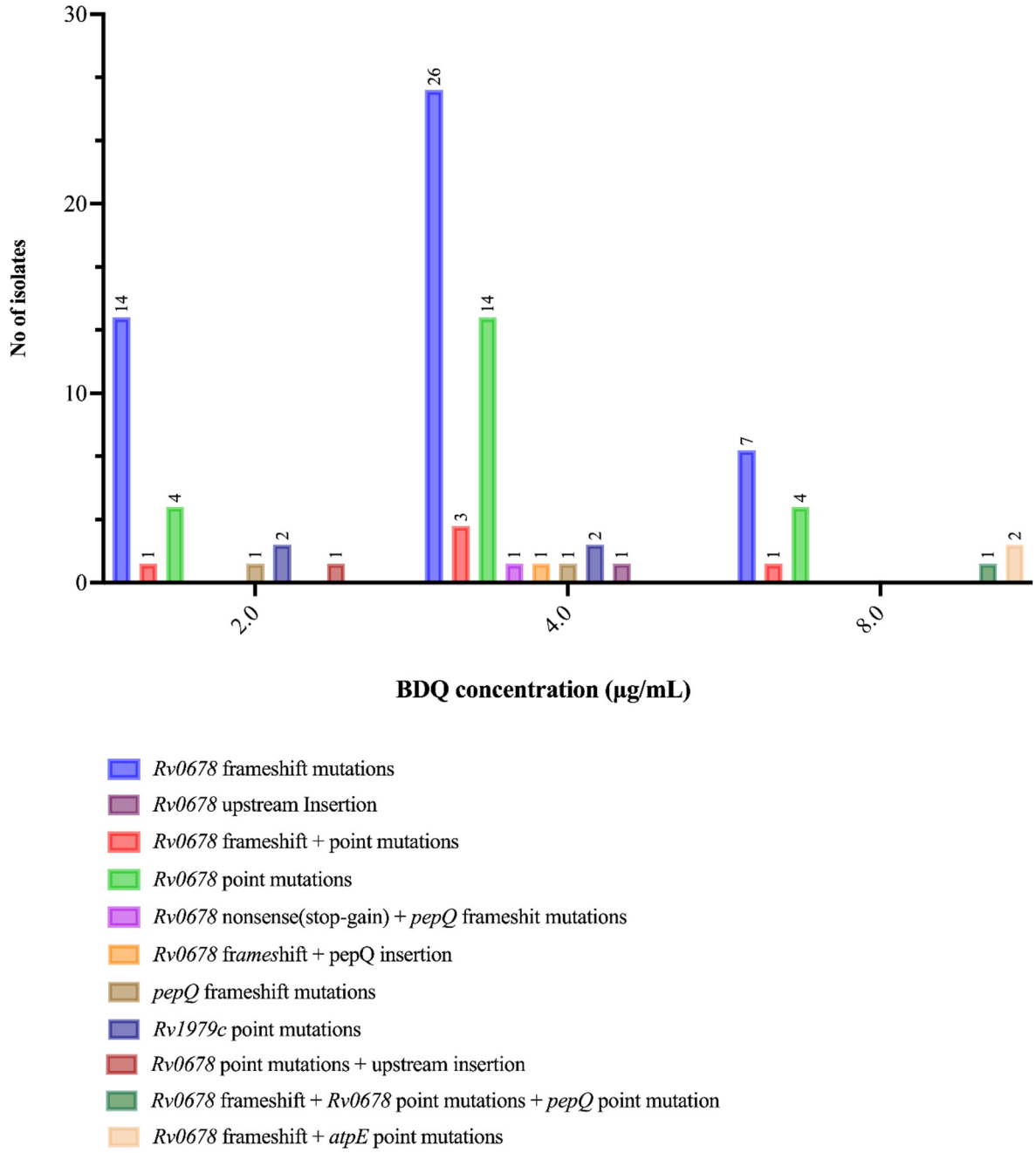


Figure 2. Bedaquiline phenotypically resistant isolates (n = 87) MIC distribution shown by type of mutation: frameshifts, point mutations, insertions, and nonsense.

3. Discussion

We found that potential BDQ RAVs are strong predictors of phenotypic resistance, achieving a sensitivity of 98.9% (95% CI: 93.8–99.8) and a PPV of 92.6% (95% CI: 85.4–96.4). This performance is exceptionally high compared to previous comprehensive analyses that have shown genomic sequencing of candidate genes yields a maximum sensitivity of 69% and a PPV of 14–26% for diagnosing clinical BDQ resistance [23]. Genomic methods have historically been considered insufficiently sensitive to serve as standalone diagnostic tools [23,27]. In contrast, our findings support the potential utility of genomic data in guiding the treatment of DR-TB in specific cohorts dominated by potentially highly fixed RAVs obtained using WGS. It has recently been shown that using WGS to select regimens achieves similar bacteriological and superior clinical efficacy compared to the standard

of care [28]. Our findings provide locally contextualised genotypic-phenotypic mapping to support a change in practice in our setting where confirmatory pDST may no longer be required in the presence of variants restricted to *Rv0678*. This analysis demonstrates that, for novel drugs where genomic diagnostic sensitivity remains moderate, improving the understanding of the genetic basis underlying phenotypic resistance mechanisms is critical for future development of molecular methods and strengthening the clinical implementation and utility of sequencing methods [29].

Our genotypic profiling revealed that BDQ phenotypic resistance in 93% of isolates was driven by variants that were WHO-classified, with only 7% not classified. The second edition of the WHO mutation catalogue relies on highly conservative and rigorous statistical grading, achieving an overall sensitivity of only 49.4%. This conservative approach provides high confidence for the identified variants. The high proportion of phenotypically resistant isolates harbouring classified variants in our setting supports the idea that the current WHO catalogue may be sufficient in predicting phenotypic resistance in our clinical setting.

Our findings demonstrate that over 90% of phenotypic resistance to BDQ is attributable to mutations in the *Rv0678* gene, with a smaller proportion exclusively linked to variants in the *pepQ* and *Rv1979c* genes. We identified a minor set of *Rv0678* variants not currently included in the WHO catalogue of mutations, observed among isolates exhibiting phenotypic resistance. These variants showed a potential contribution to BDQ phenotypic resistance, though these finding still needs to be validated in other settings. Nonetheless, our findings are consistent with work by Steyn et al., where uncharacterised *Rv0678* variants had a similar likelihood of being phenotypically resistant to BDQ regardless of inclusion in the catalogue [9]. The analysis of the unclassified variants was exploratory and should not be interpreted as a clinically validated diagnostic rule. One of the unclassified variants, Val1Leu, may contribute to BDQ phenotypic resistance since it disrupts the start codon, preventing transcription. However, since this mutation changes from GTG to TTG, a rare alternative start codon, transcription may still occur. Therefore, the functional impact of this mutation remains unclear, with no precise classification or categorization. We report unclassified BDQ RAVs in a minority of phenotypically susceptible isolates. Among these RAVs, c.-11C>A, an insertion upstream of *Rv0678,* has been reported to be associated with hypersensitivity to BDQ [6,13]. Furthermore, *Rv0678* Met139Ile, which carries an uncertain significance classification, occurred in one of the susceptible isolates with an MIC of 1.0 μg/mL, while three other isolates with this mutation had MICs of >1.0 μg/mL.

Interestingly, we observed *pepQ* variants in five isolates. Three isolates harboured both a *pepQ* variant and an *Rv0678* variant, with a 4-8-fold increase in BDQ MIC, while the remaining two isolates carried only a *pepQ* frameshift mutation (Val273fs) with a 2-4-fold increase in BDQ MIC. Mutations in the *pepQ* gene have been previously detected in phenotypically resistant isolates, suggesting a role in clinical resistance [20,30]. However, *pepQ* mutations are uncommon in clinical isolates. We also identified two isolates carrying both *Rv0678* (Glu49fs) and *atpE* (Asp28Asn) variants conferring an 8-fold increase in BDQ MIC. Although the *atpE* variant is not yet classified in the WHO catalogue, it has previously been reported as being associated with BDQ susceptibility [31]. We also observed *Rv1979c* variants in four isolates, without concomitant mutations in other BDQ resistance candidate genes. These variants were associated with a 2-4-fold increase in BDQ MIC. Mutations in *Rv1979c* have been mostly associated with CFZ resistance; however, emerging evidence also implicates *Rv1979c* variants in BDQ resistance [11,18,32]. The variants observed in this study (Glu38Asp and Arg409Gln) have previously been documented with MICs below the CC for BDQ and CFZ resistance [16]. However, our data suggest that these *Rv1979c* variants may contribute to BDQ phenotypic resistance in clinical strains. Separate studies have also demonstrated an association between *Rv1979c*

variants and BDQ phenotypic resistance [18,21]. Furthermore, one phenotypically resistant isolate exhibited an MIC of 4.0 μg/mL despite lacking mutation/s in any of the canonical candidate genes. A similar observation, where wild-type isolates were phenotypically resistant with MGIT MIC > 1.0 μg/mL, has been reported by Ismail and colleagues [18]. This genotype–phenotype discordance highlights the possibility that there may be uncharacterised or undetected mechanisms contributing to BDQ resistance [33]. Further investigation of phenotypically resistant, genotypically wild-type isolates is required, as they may provide an opportunity to identify novel resistance mechanisms. Although the PPV of the potential BDQ RAVs evaluated in this study was high, these findings should be carefully interpreted, as prevalence, distribution, and transmission of BDQ genetic variants, and their associated genetic backgrounds across geographic settings may result in nuanced genotypic-phenotypic relationships and therefore epidemiologically linked diagnostic performance.

A high proportion of isolates demonstrated cross-resistance between BDQ and CFZ, highlighting the widely described phenomenon and known shared mechanism of resistance [16,34,35]. The presence of rare cases in which discordances occur, specifically isolates that remain susceptible to CFZ despite BDQ resistance, suggests that the relationship between genotype and phenotype may be more complex than a single-mutation effect, requiring additional contextual factors to meaningfully elevate MICs.

To the best of our knowledge, this represents the first large-scale quantitative profiling of phenotypic BDQ resistance among *Rv0678*-mutated isolates within a single, contemporary, and highly characterized clinical cohort. Among this collection of phenotypically resistant isolates, we observed a range of BDQ MICs from 2.0 to 8.0 μg/mL, with 26% of isolates having a 2-fold increase in MICs (2.0 μg/mL) above the WHO-defined CC, 57% demonstrating a 4-fold increase in MICs (4.0 μg/mL), and 17% demonstrating an 8-fold increase in MICs (8.0 μg/mL), arising exclusively from the frameshift and substitution mutations in this collection of isolates. The BDQ MIC distribution in this cohort was characterized predominantly by modest 2- to 4-fold increases, notably lacking the high-level resistance (>8-fold) typically driven by target-based mutations [11,13,36–38]. Dose escalation has successfully overcome low- and intermediate-level resistance to drugs such as isoniazid and moxifloxacin [39] and may present a compelling clinical strategy for overcoming low-level BDQ resistance mediated by *Rv0678*. Modest elevation in MICs observed in this study does not imply that higher BDQ exposure would reliably overcome resistance; however, they do support efforts to explore BDQ dose optimization as a potential therapeutic strategy. Any consideration of optimised BDQ dosing in the presence of elevated BDQ MICs would require appropriate pharmacokinetic-pharmacodynamic evidence, and thorough safety and clinical evaluation. Currently, clinical evidence is limited, but modelling studies do suggest that higher BDQ exposure could improve bacterial clearance [13,40–43], highlighting the need for clinical trials evaluating the safety and efficacy of high-dose BDQ to establish whether this approach can overcome *Rv0678*-mediated efflux resistance and whether pipeline agents, such as DprE1 inhibitors, which share this exact cross-resistance vulnerability, can still be used effectively.

While we report one of the largest contemporary clinical datasets of BDQ-resistant isolates [7], our study has several limitations: the parent study focused on the transmission of MDR-TB and may have been enriched for additional resistance to FQs and/or aminoglycosides, potentially contributing to a narrow representation of a restricted number of circulating strains. However, we evaluated all clinical isolates from this cohort, which had systematic coverage of all second-line drug-resistant cases across a wide geographic region spanning over 90,000 km^2 with a population of ~12 million people. The diagnostic performance of genotypic data reported in this study should be interpreted within the

context of the parent study population, which selected *Mtb* isolates based on predefined criteria (RR/MDR-TB with additional resistance to fluoroquinolones or aminoglycosides). Consequently, these findings may not be readily generalizable to all populations or routine diagnostic settings, and further evaluation across diverse populations, resistance profiles, and laboratory settings is warranted. Treatment outcome data of participants that contributed isolates to the study are unknown, limiting our ability to correlate resistance amplification and treatment outcomes with phenotypic and genotypic data. We did not perform MIC measurements in replicates or in isolates with BDQ susceptibility and genotypic wild-type due to the labour intensity, costs, and infrastructure requirements associated with MIC experiments. However, we used standardised and validated methods under strict standard operating procedures within a well-established biosafety level 3 laboratory. Moreover, in all isolates, our MIC estimates were concordant with the overall phenotype of the isolate as determined by the national reference laboratory. Unlike previously published papers that showed a predominance of BDQ RAVs (up to 63%) driven by insertion or deletion mutants [18], only 3 of our isolates showed insertion mutations solely. It is important to note that insertion or deletion mutants often occur upstream of *Rv0678* in susceptible isolates [18]. Additionally, traditional sequencing approaches can struggle to detect minority bacterial subpopulations (heteroresistance), which are common in *Rv0678* and can lead to misclassification of the resistance phenotype. These limitations are mitigated in our study by the exceptionally high genotypic-phenotypic concordance observed, suggesting that the clinical isolates in our cohort possessed largely fixed, highly penetrant resistance mechanisms.

Even though our study is enriched for classified variants, the minority of unclassified variants suggest that novel resistance-associated variants are continuously emerging in clinical settings. Global scale-up of genotypic DST, alongside, not to the exclusion of, pDST testing, is required to continuously update the genotype–phenotype catalogues. Additionally, reference laboratories need to supplement the WHO mutation catalogue with newly identified variants of significance that are frequently observed within their specific epidemiologic context. While we support the use of intensified local genomic surveillance data to periodically update the WHO mutation catalogue, we highlight the need for concurrent phenotypic characterisation of newly identified variants to support understanding of their clinical and public relevance.

4. Methods and Materials

4.1. Study Design and Mtb Clinical Isolates Selection

This study was performed on stored *Mtb* clinical isolates obtained from participants enrolled in the CONTEXT study (BE059/18 & MOD002-IRB00098594), published elsewhere [7]. Briefly, CONTEXT was a population-based, prospective study of people with MDR-TB and second-line drug resistance [fluoroquinolones (FQs) and/or aminoglycosides] in KwaZulu-Natal, South Africa, from 2018 to 2022. In 2021, phenotypic testing was initiated for BDQ and CFZ at a critical concentration (CC) of 1.0 µg/mL for each drug, as per the WHO recommendation in MGIT [BACTEC™ MGIT 960™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). All *Mtb* isolates enrolled in the CONTEXT study were shipped to the South African National Institute of Communicable Diseases for WGS.

This nested sub-study identified 651 *Mtb* isolates that had at least BDQ phenotypic testing at 1.0 µg/ml in MGIT and a valid genotypic profile (mutation read depth of ≥10), of which 442 had both phenotypic DST and WGS. Of the 442, 102 were BDQ phenotypically resistant, and 340 were phenotypically susceptible (Figure 3). Among 102 BDQ phenotypically resistant isolates, 14 were excluded: 6 were not found in the biorepository despite being in the database, 6 were contaminated, and 3 were mixed with mycobacteria other than

tuberculosis. We finally included 428 *Mtb* isolates: 88 BDQ phenotypically resistant isolates contributed by 79 patients (i.e., 9 serial isolates: 7 individuals each contributed one serial isolate and one individual contributed 2 serial isolates). Among 340 BDQ phenotypically susceptible isolates, 338 patients contributed one isolate each, while one patient contributed a baseline and a serial isolate. All isolates with either (1) BDQ phenotypic resistance or (2) BDQ susceptibility but having variants in BDQ resistance candidate genes underwent BDQ MIC testing.

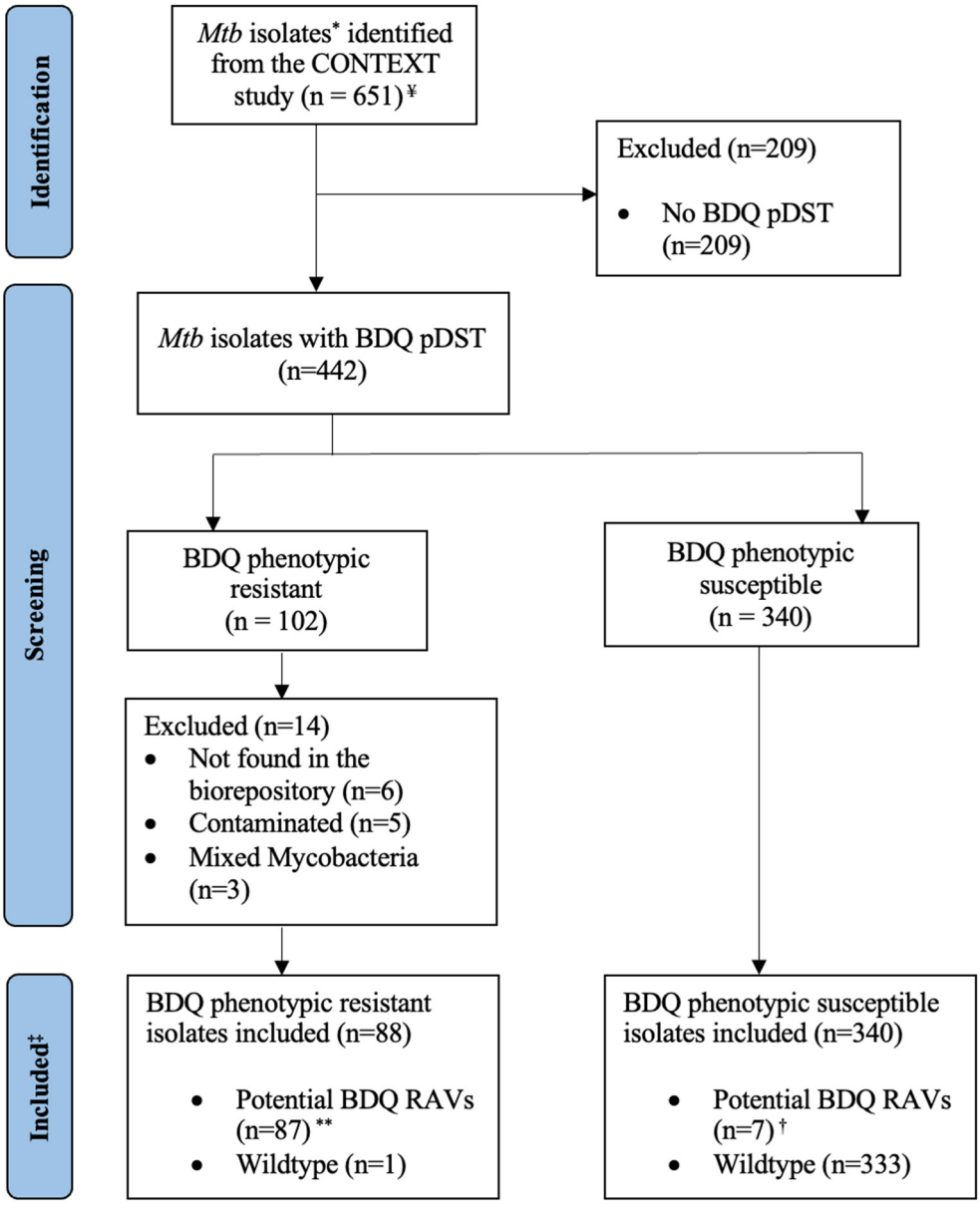


Figure 3. The clinical *Mtb* isolates identified from the CONTEXT study database, screened and excluded, and the final number of isolates included in the final analysis and tested for BDQ MICs. Abbreviations: BDQ, bedaquiline; MIC, minimum inhibitory concentration; pDST, phenotypic drug susceptibility testing; RAV, resistance-associated variant. * Resistant to rifampicin and isoniazid with additional resistance to second-line drugs (fluoroquinolone or aminoglycoside); ¥ All isolates with at least one of BDQ phenotypic DST at a CC of 1.0 μg/mL or valid WGS (read depth of ≥10); ** Included 7 individuals who each contributed one serial isolate and one individual who contributed 2 serial isolates; † Included one individual who contributed 1 serial isolate; ‡ BDQ MIC testing was performed for all BDQ phenotypic resistant isolates and only 7 phenotypically susceptible isolates with potential BDQ RAVs.

4.2. Minimum Inhibitory Concentrations Testing

Bedaquiline powder was purchased from Merck Life Science (Pty) Ltd., Modderfontein, Johannesburg, South Africa, and was stored at −20 °C upon receipt. Stock concentrations to achieve final concentrations of 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 µg/mL in MGIT were made using dimethyl sulfoxide (Merck Life Science (Pty) Ltd., Modderfontein, Johannesburg, South Africa) and stored at −20 °C until use (not more than 6 months). Minimum inhibitory concentration testing and determination were performed using BACTEC™ MGIT 960™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) within epicentre/TB Exist (Version 5.02A) as described previously [44–47]. Detailed methodology can be found in the Supplementary Material.

4.3. Whole Genome Sequencing Analysis

In the CONTEXT study, WGS for *Mtb* clinical isolates was conducted using the Illumina NextSeq platform (Illumina, Inc, San Diego, CA, USA) and Nextera DNA Flex Library Preparation kit (Illumina, Inc, San Diego, CA, USA). Raw sequencing reads were trimmed using Prinseq version 0.20.04, and non-*Mtb* (contaminant) reads were removed with Kraken [48,49]. The remaining reads were aligned to the H37Rv reference genome using BWA-MEM version 0.7.18 [50]. Single nucleotide polymorphisms (SNPs) with a read depth of ≥10 and an allele frequency of ≥0.7 were used to determine potential drug resistance-conferring mutations. Pilon version 1.24 was used to generate high-confidence variant calls from the resulting alignments, and variants within 50 base pairs of hypervariable PPE/PE gene families, repeat regions, and mobile elements [51,52]. We defined any mutation identified in the candidate genes (*Rv0678*, *atpE*, *Rv1979c*, *and pepQ*) as a potential BDQ RAV. We classified potential BDQ RAVs by referencing the WHO *Mtb* catalogue of mutations version 2: (1) associated with BDQ resistance, (2) associated with BDQ resistance interim, (3) uncertain significance, or (4) noncanonical potential RAVs (i.e., not classified).

5. Data Analysis

Diagnostic concordance between WGS–derived genotypic profiles and reference pDST for BDQ was assessed by cross-classifying isolates in a standard 2 × 2 contingency table. We reported sensitivity, specificity, PPV, NPV, and accuracy values. Sensitivity was defined as the proportion of phenotypically resistant isolates correctly identified by the presence of a potential BDQ RAV, while specificity was defined as the proportion of phenotypically susceptible isolates correctly identified as wild type. Overall diagnostic accuracy was calculated as the proportion of isolates correctly classified (true resistant and true susceptible) across the total analytic sample (N = 428). The estimated proportions for all the diagnostic performance metrics were accompanied by two-sided 95% confidence intervals (CIs), calculated using the Wilson score method. The distribution of BDQ MIC values was assessed for normality using the Shapiro–Wilk test. Because MIC values deviated significantly from normality, summary statistics are reported as medians with interquartile ranges (IQRs). The Mann–Whitney U test was then used to compare MIC distributions between isolates harbouring WHO-classified versus noncanonical (unclassified) potential BDQ RAVs. Statistical significance for all comparisons was defined as a two-sided p-value <0.05. All diagnostic accuracy metrics and confidence interval estimation were performed using R version 4.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 10.6.0 (GraphPad Software, Boston, MA, USA).

6. Conclusions

In conclusion, our findings demonstrate a strong association between potential BDQ RAVs driven overwhelmingly by a diverse array of mutations throughout the *Rv0678* gene

and BDQ phenotypic resistance. These findings support the potential utility of genomic sequencing in predicting phenotypic BDQ resistance. We identified a range of WHO-classified and unclassified variants in the *Rv0678*, *pepQ*, and *Rv1979c* genes associated with elevated BDQ MICs. The minority of unclassified potential BDQ RAVs require further investigation in independent prospective cohorts in different geographical settings for global clinical translation evidence. These findings narrow the gap in our understanding of the genotypic–phenotypic correlations of BDQ resistance, a critical step toward the development of molecular diagnostic assays, the optimization of treatment strategies, and the prevention of further emergence of BDQ resistance. Integrating routine genomic surveillance with rapid phenotypic testing is imperative to guide the design of effective, personalized treatment regimens and to promptly interrupt the ongoing transmission of highly drug-resistant *Mtb* strains.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics15111044/s1, Figure S1: Potential bedaquiline resistance-associated variants (BDQ RAVs) in the Rv0678 and associated minimum inhibitory concentrations (MICs). Dashed lines represent susceptible MICs, while solid lines represent resistant MICs, and variants associated with susceptible and resistant MICs are presented by dotted lines; Table S1: Diagnostic Performance of Potential BDQ RAV for Predicting Phenotypic Bedaquiline Resistance Using One Isolate Per Patient; Table S2: Classification of Potential BDQ RAV According to the WHO Mtb Catalogue of Mutations.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original sequences data presented in this study are openly available at NCBI under accession number PRJNA1257273.

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