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Authors

Rivière, Emmanuel
Verboven, Lennert
Dippenaar, Anzaan
et al.

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Variants in Bedaquiline-Candidate-Resistance Genes: Prevalence in Bedaquiline-Naive Patients, Effect on MIC, and Association with *Mycobacterium tuberculosis* Lineage

Emmanuel Rivière,^{a,b} Lennert Verboven,^{a,b} Anzaan Dippenaar,^a Sander Goossens,^{a,b} Elise De Vos,^a Elizabeth Streicher,^c Bart Cuypers,^{b,d} Kris Laukens,^b Fathia Ben-Rached,^e Timothy C. Rodwell,^{f,g} Arnab Pain,^{a,h} Robin M. Warren,^c Tim H. Heupink,^a Annelies Van Rie^a

^aTuberculosis Omics Research Consortium, Family Medicine and Population Health, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium

^bAdrem Data Lab, Department of Computer Science, Faculty of Sciences, University of Antwerp, Antwerp, Belgium

^cDivision of Molecular Biology and Human Genetics, South African Medical Research Council Centre for Tuberculosis Research, DSI-NRF Centre of Excellence for Biomedical Tuberculosis Research, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

^dMolecular Parasitology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

^eComputational Bioscience Research Centre, Biological Environmental Sciences and Engineering Division, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia

^fFIND, Geneva, Switzerland

^gDivision of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, University of California San Diego, La Jolla, California, USA

^hInternational Institute for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido, Japan

ABSTRACT Studies have shown that variants in bedaquiline-resistance genes can occur in isolates from bedaquiline-naive patients. We assessed the prevalence of variants in all bedaquiline-candidate-resistance genes in bedaquiline-naive patients, investigated the association between these variants and lineage, and the effect on phenotype. We used whole-genome sequencing to identify variants in bedaquiline-resistance genes in isolates from 509 bedaquiline treatment naive South African tuberculosis patients. A phylogenetic tree was constructed to investigate the association with the isolate lineage background. Bedaquiline MIC was determined using the UKMYC6 microtiter assay. Variants were identified in 502 of 509 isolates (98.6%), with the highest (85%) prevalence of variants in the *Rv0676c* (*mmpl5*) gene. We identified 36 unique variants, including 19 variants not reported previously. Only four isolates had a bedaquiline MIC equal to or above the epidemiological cut-off value of 0.25 $\mu\text{g}/\text{mL}$. Phylogenetic analysis showed that 14 of the 15 variants observed more than once occurred monophyletically in one *Mycobacterium tuberculosis* (sub)lineage. The bedaquiline MIC differed between isolates belonging to lineage 2 and 4 (Fisher's exact test, $P = 0.0004$). The prevalence of variants in bedaquiline-resistance genes in isolates from bedaquiline-naive patients is high, but very few (<2%) isolates were phenotypically resistant. We found an association between variants in bedaquiline resistance genes and *Mycobacterium tuberculosis* (sub)lineage, resulting in a lineage-dependent difference in bedaquiline phenotype. Future studies should investigate the impact of the presence of variants on bedaquiline-resistance acquisition and treatment outcome.

KEYWORDS antibiotic resistance, antimicrobial resistance, bedaquiline, drug-resistant tuberculosis, epidemiology, phenotypic drug susceptibility testing, phylogeny, tuberculosis, whole-genome sequencing

Bedaquiline (BDQ) was the first drug approved for drug-resistant tuberculosis (DR-TB) treatment in 40 years. In 2018, the World Health Organization (WHO) included BDQ as one of three core drugs for treating rifampicin-resistant TB (RR-TB). Unfortunately, cases of BDQ treatment failure were reported soon after its introduction (1–4).

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Address correspondence to Emmanuel Rivière, emmanuel.riviere@uantwerpen.be.

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The WHO recently listed five tier 1 (*atpE*, *Rv0678*, *Rv0676c*, *Rv0677c*, and *pepQ*) and one tier 2 (*Rv1979c*) genes as BDQ candidate resistance genes (5). Clinical BDQ resistance has been mainly attributed to variants in genes involved in the MmpL5/MmpS5 *Mycobacterium tuberculosis* (*Mtb*) efflux pathway (6). Most reported variants occur in the *Rv0678* gene, encoding a transcriptional repressor protein MmpR5. The *Rv0676c* and *Rv0677c* genes, encoding the MmpL5/MmpS5 transport proteins, respectively, have been implicated in BDQ resistance (7). Although rarely observed in clinical isolates, variants in the *atpE* gene, encoding the BDQ drug target ATP synthase, have been reported repeatedly in *in vitro* isolates (1, 8). While variants in the *Rv2535c* (*pepQ*) gene, encoding a cytoplasmatic peptidase, reduce efficacy of BDQ and clofazimine (CFZ) in mice, it remains unclear if loss of function *pepQ* variants lead to a reduced BDQ susceptibility in clinical setting (8–10). Lastly, evidence for the role of *Rv1979c* in BDQ resistance remains limited (5, 8).

It has been reported that variants in BDQ resistance-associated genes can occur at low prevalence (6% to 12%) in clinical *Mtb* isolates from BDQ-naive patients, with varying rate of phenotypic BDQ resistance (2.3% to 66.7%) (11–15). This is worrisome with regard to treatment outcomes, even if they do not confer phenotypic resistance at the current WHO endorsed critical concentration as they could serve as a steppingstone to higher MIC BDQ resistance (16).

In this study, we analyzed whole-genome sequencing (WGS) data of 509 isolates from South African BDQ-naive patients and performed BDQ MIC analyses on a subset to investigate the prevalence of variants in all tier 1 and tier 2 BDQ candidate resistance genes, assess their association with *Mtb* (sub)lineages, and evaluate the effect of these variants on BDQ MIC.

RESULTS

Prevalence of genomic variants. Almost all (98.6%, 502/509) isolates had a variant in a BDQ candidate resistance gene. Most (305 or 59.9%) isolates had a single, 188 (36.9%) had two, and nine samples (1.8%) had three variants in the genes analyzed. A total of 36 unique variants were identified in 32 combinations: one in the upstream (*atpB*) region of *atpE*, one in *Rv0677c* (*mmpS5*), six in *Rv0678* (*mmpR*), eight in *Rv2535c* (*pepQ*), nine in *Rv1979c*, and 11 in *Rv0676c* (*mmpL5*) (Table 1 and Fig. 1).

The highest prevalence of genomic variants was observed in the *Rv0676c* (*mmpL5*) gene, with two of the 11 variants observed occurring at p.Ile794Thr (48.1%, 245/509) and p.Asp767Asn (36.0%, 183/509) (Table 1). Only one variant (c.306C>T) was observed in the *Rv0677c* (*mmpS5*) gene at low prevalence (0.2%, 1/509). Six variants were observed in the *Rv0678* gene, of which the promoter variant n.-11C>A occurred frequently (20.0%, 102/509). The other five *Rv0678* variants were observed once. All eight variants observed in the *Rv2535c* (*pepQ*) gene were rare (prevalence <0.5%). The nine variants observed in the *Rv1979c* gene occurred at low prevalence (<5%), with p.Arg409Gln being the most prevalent (10.4%, 53/509). One variant in the *atpB* gene, p.His250Pro, 53 nucleotides upstream of the *atpE* coding region, was observed in a single isolate. In the sensitivity analysis excluding the 58 isolates from unconfirmed BDQ naive patients, the same variants were observed, with the exception of the *Rv0676c* p.Thr495Ile variant (Table S2).

Prevalence of variants by *Mtb* lineage. Most isolates belonged to lineage 2 (46.4%, 236/509) or 4 (48.1%, 245/509); few isolates to lineage 1 (4.1%, 21/509) or 3 (1.4%, 7/509). The prevalence of variants in the BDQ resistance genes differed by lineage (Table 2). While none of the seven lineage 3 isolates contained any variant in the BDQ candidate resistance genes, lineages 1, 2, and 4 isolates contained at least one variant (Table 2). For lineage 1 isolates, variants occurred frequently in the *Rv1979c* (100%, 21/21), and *Rv0676c* (14.3%, 3/21). For lineage 2, variants occurred at high prevalence in the *Rv0676c* (78.0%, 184/236), *Rv0678* (44.1%, 104/236), and *Rv1979c* (33.5%, 79/236) genes. For lineage 4, variants were common in the *Rv0676c* (100%, 245/245) and *Rv1979c* (7.8%, 19/245) genes. Similar differences between lineages could be observed when excluding the 58 isolates from unconfirmed BDQ naive patients (Table S3).

TABLE 1 All observed variants in the data set

Observed variants in clinical isolates						
Gene	DNA level	Protein level	Frequency	Oldest sampling yr	WHO classification (5)	BDQ ^a MIC range (μg/mL)
<i>Rv1304 (atpB)</i>	749A>C	His250Pro	1	2012	Uncertain	0.015
<i>Rv0678 (mmpR)</i>	310G>T	Glu104*	1	2011	NA	0.06
	198delG	Ile67fs	1	2011	NA	0.015
	-11C>A	NA	102	2003	NA	≤0.008 to 0.06
	214delC	Arg72fs	1	2010	NA	0.03
	413A>G	Glu138Gly	1	2013	NA	0.03
	418C>T	Arg140Trp	1	2013	NA	≤0.008
<i>Rv2535c (pepQ)</i>	150_151dupCG	Asp51fs	2	2012	NA	0.03 to 0.06
	573G>C	Glu191Asp	2	2010	NA	≤0.008
	347G>T	Gly116Val	1	2013	NA	NA
	-118A>G	NA	1	2012	NA	NA
	190G>A	Ala64Thr	1	2013	NA	0.03
	196T>C	Ser66Pro	1	2005	NA	0.015
	892G>A	Val298Ile	1	2011	NA	≤0.008
	1011C>T	Arg337Arg	1	2015	NA	≤0.008
<i>Rv1979c</i>	-129G>A	NA	4	2012	Not assoc w R ^c	0.015 to 0.06
	114G>C	Glu38Asp	14	2006	Uncertain	≤0.008 to 0.12
	857A>G	Asp286Gly	21	1998	Uncertain	≤0.008 to 0.12
	151T>A	Tyr51Asn	24	2007	Uncertain	≤0.008 to 0.5
	1226G>A	Arg409Gln	53	2010	Uncertain	≤0.008 to 0.25
	1216G>A	Ala406Thr	1	2010	NA	0.03
	-147G>A	NA	1	2012	NA	NA
	520C>T	Leu174Phe	1	2013	Uncertain	≤0.008
	1181T>C	Ile394Thr	1	2012	Uncertain	≤0.008
<i>Rv0676c (mmpL5)</i>	172>T	Gln58*	2	2000	NA	≤0.008
	1792T>A	Trp598Arg	3	2012	Uncertain	≤0.008 to 0.03
	740T>C	Val247Ala	3	2012	Uncertain	NA
	2842G>A ^b	Val948Ile ^b	5	NA	Not assoc w R ^c	0.015 to 0.06
	1065G>T	Pro355Pro	24	2007	NA	≤0.008 to 0.12
	2299G>A	Asp767Asn	183	2003	Not assoc w R	≤0.008 to 0.5
	2381T>C	Ile794Thr	245	2000	Not assoc w R ^c	≤0.008 to 0.5
	1161C>G	Val387Val	1	2012	NA	≤0.008
	1783C>T	Pro595Ser	1	2013	NA	0.015
	1484C>T	Thr495Ile	1	2015	NA	≤0.008
	2661G>T	Val887Val	1	2012	NA	0.015
<i>Rv0677c (mmpS5)</i>	306C>T	Asn102Asn	1	2012	NA	≤0.008

^aBDQ, bedaquiline.^bVariants were observed independently in the phylogenetic tree.^cClassification based on variant identified through calling compared to H37Rv and likely observed due to acquired variant in the reference genome.

Of 36 unique variants identified, 15 were observed more than once. Of these, 14 were classified as monophyletic and one as homoplastic (Fig. 2; Data Set S1, S2, S3, and S4). The *Rv0676c* p.Ile794Thr variant was present in all lineage 4 ($n = 245$) isolates but absent in all other lineages. The *Rv0676c* p.Asp767Asn variant was observed in all sublineage 2.2.1 isolates ($n = 183$), but absent in all other (sub)lineages. Likewise, the *Rv0678* n.-11C>A variant was exclusively observed in sublineage 2.2.1 isolates ($n = 102$), but absent in all other (sub)lineages. The *Rv1979c* p.Asp286Gly variant was present in all lineage 1 isolates ($n = 21$) and absent from all other lineages. The *Rv1979c* p.Arg409Gln variant was observed in all sublineage 2.2.2 ($n = 50$) and sublineage 2.2 ($n = 3$) isolates, but absent in all other (sub)lineages. The *Rv1979c* p.Tyr51Asn variant was observed in all 2.2.1.1 sublineage isolates ($n = 24$), but absent in all other (sub)lineages. The other monophyletic variants (*Rv2535c* p.Glu191Asp, *Rv2535c* c.150_151dupCG, *Rv1979c* n.-129G>A, *Rv1979c* p.Glu38Asp, *Rv0676c* p.Gln58*, *Rv0676c* p.Trp598Arg, *Rv0676c* p.Val247Ala, and *Rv0676c* c.1065G>T) were reported in subclades of a sublineage. The oldest sampling year of all monophyletic variants was prior to the introduction of BDQ, except for *Rv0676c* p.Thr495Ile, which was observed in an isolate collected in 2015. Only the *Rv0676c* p.Val948Ile variant was homoplastic as it was observed in both sublineage 4.9 and sublineage 2.2.1 isolates (Fig. 2; Table 1; Data Set S1, S2, S3, and S4).

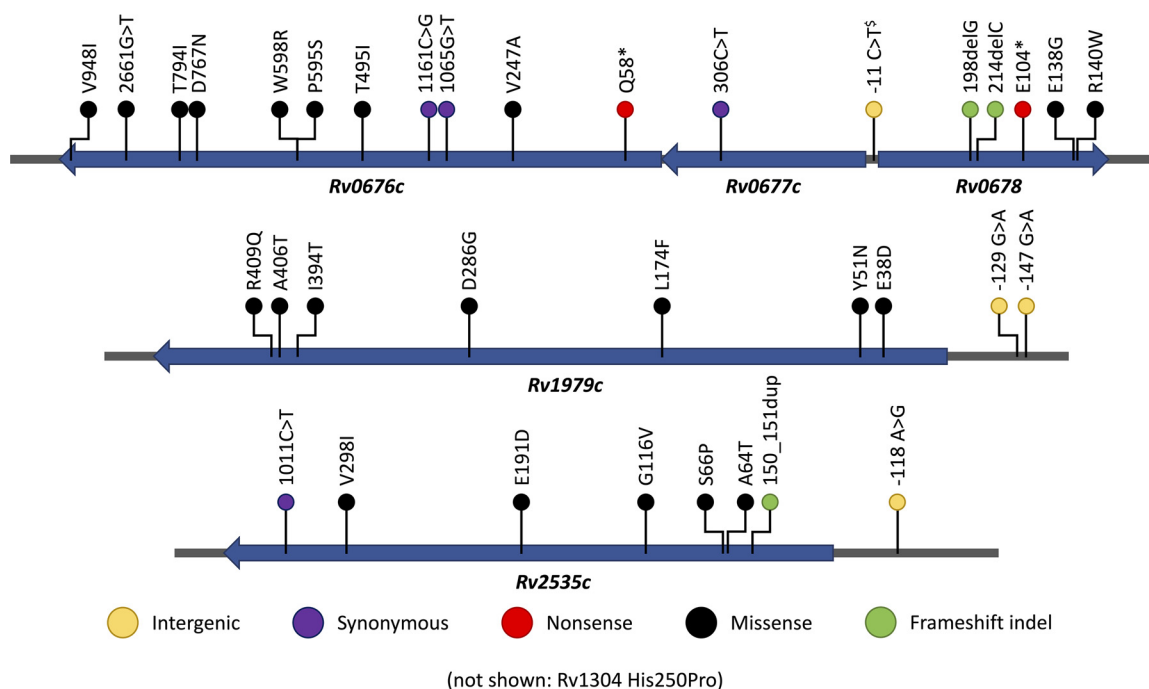


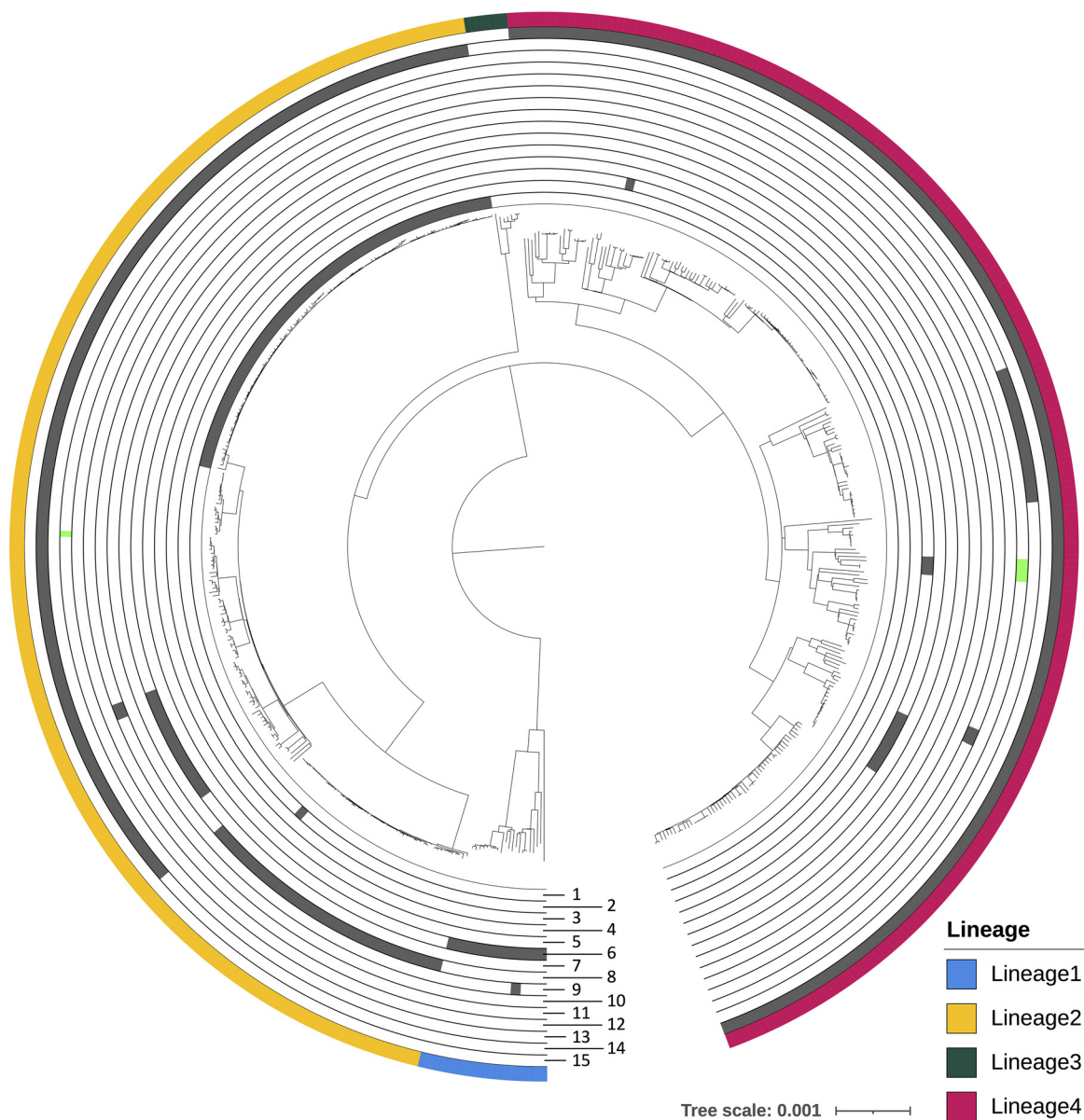
FIG 1 Variants identified in bedaquiline candidate resistance genes *Rv0676c*, *Rv0677c*, *Rv0678*, *Rv1979c*, and *Rv2535c*, and flanking promoter regions. The single variant in *Rv1304* (upstream of *atpE*) is not shown. Nucleotide positions are used for frameshift indels and intergenic variants, codon positions are used for synonymous, nonsense and missense variants. ⁵-11 position is upstream relative to the *Rv0678* gene.

BDQ MIC by variant and lineage. Phenotypic data could be obtained for 32 of the 36 observed variants (isolates containing *Rv0676c* p.Val247Ala, *Rv1979c* n.-147G>A, *Rv2535c* p. Gly116Val, or *Rv2535c* n.-118A>G lost viability). The BDQ MIC was below the epidemiological cut-off value (ECOFF) in 287 (98.6%) of 291 isolates tested (Fig. S1) with a wide distribution for almost all variants (Fig. 3; Fig. S2). The BDQ MIC was above the ECOFF for one isolate solely containing the *Rv0676c* p.Ile794Thr variant and one isolate containing the *Rv1979c* p.Tyr51Asn and *Rv0676c* p.Asp767Asn variants. The latter also contained one low frequency variant (*Rv2535c* c.306C>G) with allele frequency of 12% (Table 3). The BDQ MIC was equal to the ECOFF for one isolate solely containing the *Rv0676c* p.Ile794Thr variant and one isolate containing the *Rv1979c* p.Arg409Gln variant. The latter also contained six low frequency *Rv0678* variants with varying frequencies (Table 3). All four samples with BDQ MIC above or equal to the ECOFF, originated from confirmed BDQ naive patients.

The MIC distribution of lineage 2 isolates differed from that of lineage 4 isolates as lineage 4 isolates had a median MIC of 0.03 μ g/mL and a uniform distribution from

TABLE 2 Proportion of samples with mutated bedaquiline (BDQ) resistance gene, overall and stratified by lineage

Lineage	Proportion of samples with mutated BDQ resistance gene					
	Rv1304	Rv0678	Rv2535c	Rv1979c	Rv0676c	Rv0677c
1	0% (0/21)	4.8% (1/21)	0% (0/21)	100% (21/21)	14.3% (3/21)	0% (0/21)
2	0% (0/236)	44.1% (104/236)	0.8% (2/236)	33.5% (79/236)	78.0% (184/236)	0% (0/236)
3	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)
4	0.4% (1/245)	0.4% (1/245)	3.3% (8/245)	7.8% (19/245)	100% (245/245)	0.4% (1/245)
All	0.2% (1/509)	20.8% (106/509)	2.0% (10/509)	23.4% (119/509)	84.9% (432/509)	0.2% (1/509)



<i>Rv0678</i>	<i>Rv2535c</i>	<i>Rv1979c</i>	<i>Rv0676c</i>
1 n.-11C>A	2 c.150_151dupCG	4 n.-129G>A	9 p.Gln58*
	3 p.Glu191Asp	5 p.Glu38Asp	10 p.Val247Ala
		6 p.Asp286Gly	11 p.Trp598Arg
		7 p.Tyr51Asn	12 p.Val948Ile ^S
		8 p.Arg409Gln	13 c.1065G>T [#]
			14 p.Asp767Asn
			15 p.Ile794Thr

FIG 2 Maximum likelihood phylogenetic tree of the 509 *Mycobacterium tuberculosis* samples collected from South African patients. Each concentric circle represents a unique variant (corresponding number in legend table) that was observed more than once (variants observed only once are listed in Table 1). Variants occurring exclusively in one clade are shown in gray. Homoplastic variants (occurring in independent samples) are shown in vibrant green (number 12). ^SVariants were observed independently in the phylogenetic tree; [#] Synonymous SNPs.

BDQ MIC per Combination of Mutations

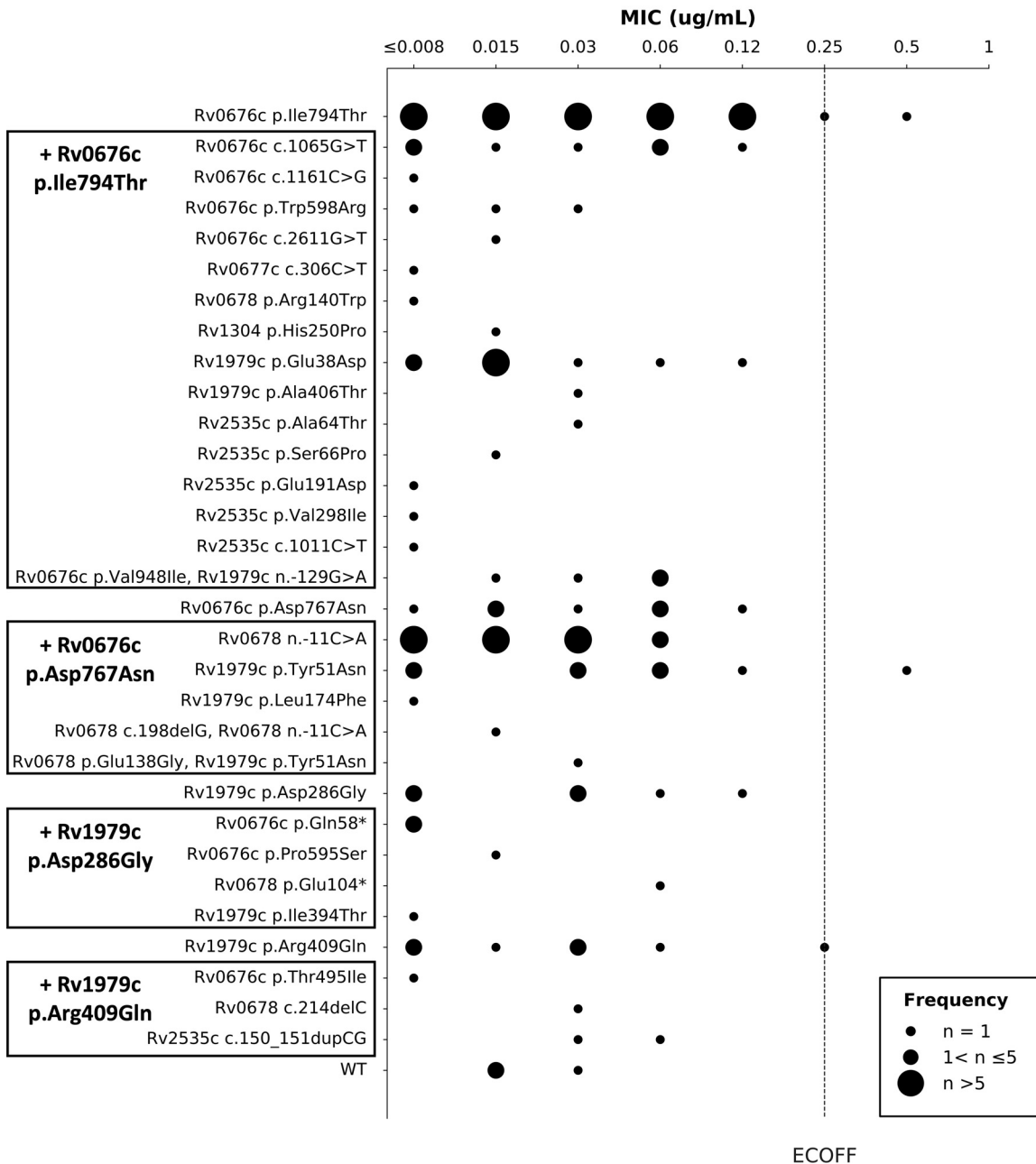


FIG 3 Observed bedaquiline (BDQ) MIC values for each combination of variants in the data set for which phenotypic data were available. MIC reading was done after 2 weeks of incubation.

≤0.008 to 0.06 μg/mL, whereas the MIC distribution of lineage 2 isolates was skewed with a lower median (0.015 μg/mL) and a mode of ≤0.008 μg/mL (Fisher’s exact test $P = 0.0004$) (Fig. 4). No differences were observed between lineage 1 and lineage 2 ($P = 0.65$) or 4 ($P = 0.68$), likely due to the low number of lineage 1 isolates with phenotypic data ($n = 13$). Comparisons with lineage 3 were not performed due to scarce data ($n = 3$) (Fig. S5, panel C).

In the sensitivity analysis excluding unconfirmed BDQ naive patients, similar phenotypic effects were observed (Fig. S3, S4, and S5) and the MIC difference between lineages 2 and 4 remained significant ($P = 0.003$).

TABLE 3 Low frequency variants identified in samples with high (≥ 0.25 $\mu\text{g/mL}$) bedaquiline MIC

LoFreq variants in samples with high BDQ MIC						
Sample ID	BDQ ^a MIC ($\mu\text{g/mL}$)	Sublineage	Gene	DNA level	Protein level	Allele frequency (%) ^b
R4330	0.5	2.2.1.1	Rv0676c	c.2299G>A	p.Asp767Asn	100
			Rv1979c	c.151T>A	p.Tyr51Asn	100
			Rv2535c	c.306C>G	p.Val102Val	12
R7726	0.5	4.7	Rv0676c	c.2381T>C	p.Ile794Thr ^c	NA ^c
R8361	0.25	2.2.2	Rv0678	c.38delA	NA	3
			Rv0678	c.198dupG	NA	33
			Rv0678	c.198delG	NA	4
			Rv0678	c.226C>A	p.Gln76Lys	10
			Rv0678	c.275dupA	NA	6
			Rv0678	c.466dupC	NA	10
			Rv1979c	c.1226G>A	p.Arg409Gln	100
R2835	0.25	4.1.2.1	Rv0676c	c.2381T>C	p.Ile794Thr ^c	NA ^c

^aBDQ, bedaquiline.^bAllele frequencies <50% are highlighted in gray.^cVariants were identified by comparing with the ancestral genome; no allele frequencies can therefore be reported.

DISCUSSION

We detected 36 unique variants in the six BDQ candidate resistance genes in isolates of 509 South African RR-TB patients, of which 89% were confirmed BDQ naive. While virtually all (98.6%, 502/509) isolates contained at least one variant, few (1.4%, 4/291 tested) had a MIC at or above the ECOFF. Interestingly, a variant-lineage dependency observed, which resulted in MIC distribution differences between lineage 2 and 4.

The prevalence of observed variants was higher than those reported by other studies of BDQ naive patients (11–14). In a clinical MDR-TB trial, 23 of 347 (6.6%) BDQ-naive participants harbored variants in *Rv0678* (13). In a study from China, five of 90 (5.6%) isolates from BDQ naive patients harbored variants (four in *Rv0678*, one in *Rv1979c*) (14). In a larger study, 106 of 4795 (2.2%) isolates from patients likely not exposed to BDQ contained a variant (11). The lower prevalence of BDQ variants in these three studies may be explained by their exclusion of the *Rv0676c* (*mmpL5*) gene, which had the highest (84.9%) prevalence of variants in our study. Only one small Iranian study ($n = 24$ isolates) included all six genes in their analysis. Contrary to our findings, only three of the 24 (12.4%) isolates contained variants (two in *Rv0676c* and one in *Rv1979c*) even though the lineage distribution was similar to our study (10).

Of the 36 variants, 23 (63.9%) are not included in the WHO catalogue and 19 (52.8%) have not been reported previously (8). Of the 13 variants in the WHO catalogue, nine could not be classified and four were classified as “not associated with resistance.” Interestingly, three of these four variants (*Rv0676c* p.Thr794Ile, *Rv1979c* c.-129A>G, and *Rv0676c* p.Ile948Val) are likely acquired variants in the H37Rv reference genome and should be listed preferably as their ancestral equivalent (*Rv0676c* p.Ile794Thr, *Rv1979c* c.-129G>A, and *Rv0676c* p.Val948Ile) (Table 1).

The prevalence of phenotypic BDQ resistance (MIC > ECOFF) was only 0.69% (two of 291), much lower than what was observed in prior studies of BDQ-naive patients. In the MDR-TB clinical trial, 34.8% (eight of 23) of isolates with a variant in *Rv0678* had a BDQ MIC above the critical concentration (13). In a study from China, 80.0% (four of five) of isolates with a *Rv0678* variant had an increased BDQ MIC (14). In a study from Iran, 66.7% (two of three) samples with a variant in *Rv0676c* and *Rv1979c* had a BDQ MIC above the ECOFF (12). In a study with isolates originating from surveillance activities across six different high TB countries, 7.8% (four of 51 tested) of isolates with a *Rv0678* variant had a BDQ MIC above the critical concentration (11). In the largest study to date conducted in South Africa, 3.6% (72 of 2004) of baseline isolates from patients

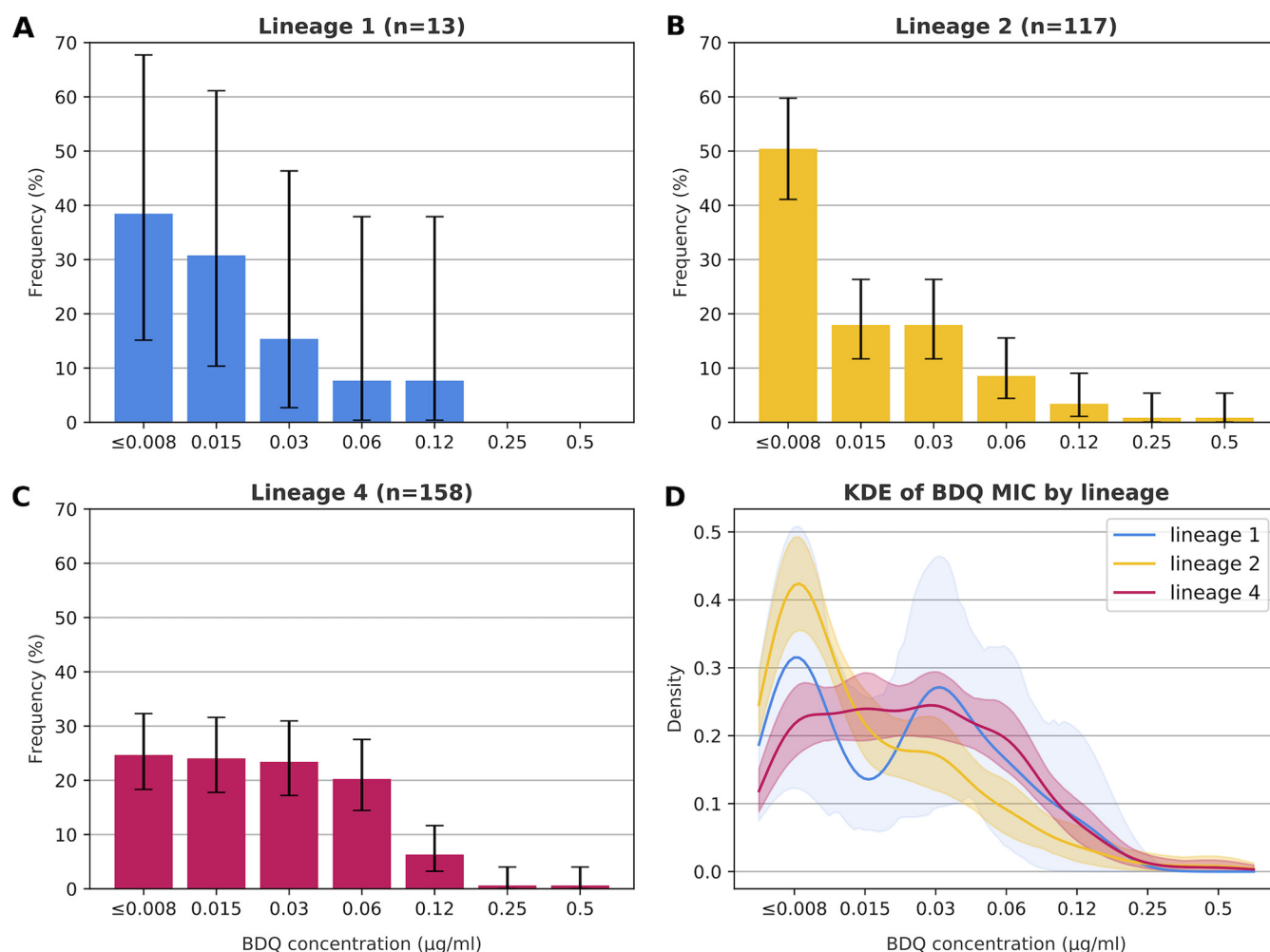


FIG 4 Bedaquiline (BDQ) MIC stratified by lineage. (A) BDQ MIC using the CRyPTIC (UKMYC6) plates for lineage 1 isolates. (B) BDQ MIC using the CRyPTIC (UKMYC6) plates for lineage 2 isolates. (C) BDQ MIC using the CRyPTIC (UKMYC6) plates for lineage 4 isolates. (D) Kernel density estimation (KDE) of BDQ MIC of lineage 1, 2, and 4 isolates. KDE and corresponding confidence intervals were calculated using the `prop.test` function in the R *stats* package (version 4.0.0). MIC reading was done after 2 weeks of incubation.

with no previous exposure to BDQ (1987 patients) or unknown exposure status (17 patients) were phenotypically resistant to BDQ (15).

The variants observed in the four isolates with a MIC at or above the ECOFF in our study have been reported before. In contrast to our observation, the *Rv0676c* p.Asp767Asn was graded as not associated with BDQ resistance in the WHO Variant Catalog (5) and the *Rv1979c* p.Tyr51Asn variant was only reported in susceptible isolates ($n = 152$). The *Rv1979c* p.Arg409Gln variant has been reported in 146 susceptible and five resistant isolates (5). The *Rv0676c* p.Ile794Thr (or p.The794Ile variant in H37Rv) is graded as not associated with BDQ resistance (5). Of the low frequency variants observed in two of these four isolates, the *pepQ* c.306C>G variant and *Rv0678* c.38delA and p.Gln76Lys variants have not been reported before. The *Rv0678* c.198dupG variant has been reported in one susceptible and two resistant clinical isolates (13); the c.198delG variant in three susceptible clinical isolates from exposed patients and two resistant *in vitro* samples (1, 17); the c.275dupA variant in two resistant BDQ naive clinical isolates (11, 13) and three susceptible BDQ exposed clinical isolates (18); and the c.466dupC variant in two susceptible clinical isolates (1, 18). These results highlight the importance of, including low frequency variants analysis in studies investigating BDQ genotype-phenotype associations (19).

In contrast to most studies (11, 13), we found an association between variants in BDQ-resistance genes and lineages. The only prior report of lineage dependency is for

the n.-11C>A *Rv0678* promoter variant which was found exclusively in lineage 2 isolates (13), similar to our findings. Furthermore, our results suggest that genetic differences between (sub)lineages may result in phenotype differences between lineages. While the clinical relevance of this finding remains to be investigated, this is interesting given three recent observations: (i) diverging evolutionary trajectories between *Mtb* lineages, particularly lineages 2 and 4, in response to antibiotic pressure (20); (ii) different levels of resistance for variants in the *rpoB*, *katG*, and *inhA* genes depending on *Mtb* lineage (21); and (iii) patients with an increased MIC for rifampicin or isoniazid below the breakpoint are at increased risk of developing antibiotic resistance and relapse (22).

Several limitations should be considered. First, exposure to BDQ could not be excluded in 58 (11.4%) patients. BDQ exposure was unlikely as all 58 patients were diagnosed before the BDQ roll-out and homoplastic variants were rare. Furthermore, our sensitivity analysis showed similar findings when excluding these 58 isolates. Second, we could not assess the BDQ MIC for four of the 36 unique variants as some samples failed to grow. Third, reflecting the epidemiology of *Mtb* in South Africa (23), isolates of lineage 5, 6 or 7 were absent and isolates of lineages 1 and 3 were rare. Lastly, CFZ exposure, which can result in BDQ cross-resistance through mutations in the *Rv0678* gene, was not considered because data on CFZ exposure status was not available (24).

In conclusion, while 98.6% of BDQ naive patients harbored *Mtb* with one or more variants in one of the six candidate genes for BDQ resistance, the proportion of isolates with a BDQ MIC at or above the ECOFF was low (1.4%). The identification of variants in these genes might not warrant exclusion of BDQ from the treatment regimen until further data have accumulated. Future studies should investigate which variants increase the risk of acquisition of phenotypic BDQ resistance and impact treatment outcomes. The observation that the prevalence of variants is (sub)lineage dependent may result in differential impact of these variants by geographic region.

MATERIALS AND METHODS

Clinical *Mtb* isolates. WGS data from 509 isolates collected from 509 RR-TB patients before BDQ treatment initiation were obtained from two sources. Data from 263 confirmed BDQ-naive patients enrolled in a prospective cohort study (“EXIT-RIF”) from 2012 and 2013 in three South African provinces were included in this analysis. Data from 246 patients diagnosed between 2001 and 2018 in South Africa with an isolate in the *Mtb* biobank at Stellenbosch University were added. Of these, 133 were classified as BDQ-naive as they were diagnosed before the introduction of BDQ in South Africa in January 2013 (25), and 55 were assumed BDQ-naive as they were diagnosed with RR-TB during the BDQ clinical access program but were not eligible for BDQ given their resistance profile. BDQ exposure could not be excluded for the remaining 58 patients who were eligible for the Bedaquiline Clinical Access Program.

Whole-genome sequencing. Isolates were cultured on 7H10 media and DNA was extracted using the phenol chloroform method (26). Genomic libraries were prepared using either TruSeq DNA Sample Preparation Kits V2 (Illumina Inc., San Diego, CA, USA) or NEBNext Ultra II DNA library prep kit for Illumina (New England BioLabs). Samples were sequenced on an Illumina HiSeq 2000/4000 or NextSeq 550.

DNA sequence analysis. Sequencing data were analyzed using the XBS pipeline (27). All FASTQ sequence data were mapped to the H37Rv reference genome using the Burrows-Wheeler alignment tool BWA-MEM (28). After marking duplicate reads with Genome Analysis Tool Kit (GATK [29]) MarkDuplicates, single nucleotide polymorphisms (SNP), insertions, and deletions were called using GATK Haplotypecaller. Samples with a coverage depth of $\geq 10\times$ and breadth of $\geq 90\%$ were identified for inclusion in joint variant calling. QuantB was used to filter out samples with $\geq 20\%$ nontuberculous Mycobacteria (30). Genomic variant call format (GVCF) files were merged and genotypes were jointly called using GATK GenotypeGVCFs (31). Next, GATK VQSR was used to identify true variants (32) using a target sensitivity of 99.9%. Variants in the merged GVCF file were annotated and isolate lineage and sublineage were determined using TB-Profiler (33).

The *Rv0676c*, *Rv0677c*, *Rv0678*, *Rv1305* (*atpE*), *Rv1979c*, and *Rv2535c* (*pepQ*) genes were investigated (Table S1). All variants were compared to their matching position in the *Mtb* ancestral genome to confirm whether they are true variants or due to acquired variants in H37Rv (sublineage 4.9) (34). The latter were translated to their ancestral equivalent (*Rv1979c* c.-129G>A, *Rv0676c* p.Val948Ile, and *Rv0676c* p.Ile794Thr). Variants were described using HGVS notation (35); nonsynonymous substitutions were described on protein level.

IQtree (version 1.6.12) was used to infer the best substitution model (36) (K3Pu+F+ASC+R5) and construct a maximum likelihood tree (37, 38). The phylogenetic tree was visualized using iTOL v6, rooted at midpoint, and annotated with variants occurring more than once in the data set and isolate lineage (39). For each variant, the oldest sampling year was identified based on the sampling dates of all isolates displaying that variant. A variant was classified as monophyletic when observed in a single clade which

could be explained by common ancestry or as homoplastic when observed in two or more independent samples that could not be explained parsimoniously by common ancestry.

Bedaquiline MIC. Bedaquiline MIC determined using the microtiter plate assay (UKMYC6) (40) was available for 291 isolates: 260 of the 509 (51.1%) isolates purposefully selected to represent all combinations of lineages and variants in the six BDQ candidate resistance genes and 31 isolates with incomplete variant calling. Plates containing standard inoculum aliquots of selected isolates and BDQ at concentrations 0.008, 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, and 1 $\mu\text{g}/\text{mL}$ were incubated at 35°C to 37°C and read after 14 and 21 days of incubation if growth was observed in both positive-control wells. For isolates with a BDQ MIC equal to or higher than the ECOFF of 0.25 $\mu\text{g}/\text{mL}$ (41), LoFreq (version 2.1.5) was used to investigate low-frequency variants (42).

Statistical analysis. For each *Mtb* lineage, BDQ MIC proportions and corresponding 95% confidence intervals were determined using the proportion test (R *stats* package, R version 4.0.0). Pairwise comparisons of the distribution of MICs between lineages were made using Fisher's exact test with Monte Carlo simulated *P*-values. Differences between lineages were considered significant when *P*-value < 0.05. To investigate whether the inclusion of 58 isolates from unconfirmed BDQ-naïve patients biased our results, we performed a sensitivity analysis in which these isolates were excluded.

Ethics. All isolates and sequences originating from the SAMRC-CTR biobank were collected, stored, and analyzed under the ethical clearance obtained from Stellenbosch University Health Research Ethics Committee (N09/11/269). The EXIT-RIF study was approved by the institutional review board of the University of North Carolina, Chapel Hill, NC, USA (11-2273) and the Human Ethics Research Committee of the University of the Witwatersrand, Johannesburg, South Africa (M111139).

Data availability. Sequencing reads have been deposited at the European Nucleotide Archive (project accession number: [PRJEB50385](https://www.ebi.ac.uk/ena/record/PRJEB50385)). Accession numbers and manuscript links of previously published data are listed supplemental materials.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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