In vitro approaches for generation of *Mycobacterium tuberculosis* mutants resistant to bedaquiline, clofazimine or linezolid and identification of associated genetic variants

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Highlights

- Two *in vitro* approaches capable of generating drug-resistant mutants to three drugs.
- Reference strains with varying resistance profiles used to successfully create mutants.
- Novel *rv0678* mutations identified for clofazimine and bedaquiline.
- A novel *atpE* mutation identified for bedaquiline.

ABSTRACT

Bedaquiline, clofazimine and linezolid are pertinent drugs for drug-resistant tuberculosis. Drug-resistant mutants provide insight into important resistance acquisition mechanisms. Methods for *in vitro Mycobacterium tuberculosis* mutant generation are poorly described.

Induction (serial passaging) and spontaneous (adapted Luria-Delbrück assay) approaches using *M. tuberculosis* ATCC reference strains (one fully-susceptible, four unique mono-resistant) were performed. Mutant MIC values were confirmed (MGIT960) and resultant RAVs compared between approaches and to a catalog of previously published RAVs.

Mutant MIC values showed a 3-4-fold (induced) and a 1-4-fold (spontaneous) increase compared to baseline. The pyrazinamide-resistant strain had higher baseline MIC values and acquired resistance (≥ 4-fold) in fewer passages than other strains (induction approach) for bedaquiline. Previously described and novel RAVs in *atpE* (8 *vs.* 1) and *rv0678* (4 *vs.* 12) genes were identified in bedaquiline- and clofazimine-resistant mutants. No *rv1979c* and *rv2535c* RAVs were identified. Previously described RAVs were identified in *rplC* and *rrl* genes for linezolid-resistant mutants.

Both approaches successfully led to *in vitro* mutants with novel RAVs being described in *atpE* and *rv0678* genes. It was observed that pre-existing resistance may influence mutant phenotypic and genotypic characteristics and warrants further attention.

Keywords: *M. tuberculosis*, mutants, bedaquiline, clofazimine, *in vitro*

1. INTRODUCTION

The emergence of drug resistance in bacterial populations has widely been studied through the generation of *in vitro* mutants (Zhou, 2000). However, in the field of tuberculosis (TB), the majority of TB drugs were discovered almost 60 years ago and treatment in TB patients often followed demonstration of efficacy in guinea pig models (Murray, 2015). This left much to be desired for identification of RAVs and resistance acquisition mechanisms. *In vitro* mutants resistant to (candidate) drugs, could be seen as an early step to prevent drug failure during later stages of drug development and deployment. Generated mutants can be used to identify variants or mutations in genes associated with resistance (i.e. resistance associated variants; RAVs), thus providing insight into modification of bacterial drug targets due to drug exposure (Koser, 2015, Zhou, 2000). *In vitro* studies may also provide information around rates or frequencies associated with genetic resistance accumulation (Martinez, 2000). In addition to pre-clinical drug development these *in vitro* studies could provide valuable information for post-clinical management and surveillance of drug resistance. This can be achieved by using *in vitro* identified RAVs to facilitate and inform diagnostic tests and molecular screening strategies for evaluation of drug susceptibility. Obtained mutants, which are phenotypically and genotypically confirmed resistant could also serve as reference strains for resistance.

Mycobacterium tuberculosis accumulates drug resistance through two mechanisms; induction of responsive RAVs following drug exposure and spontaneous mutations through *de novo* evolution (Gygli, 2017). Both mechanisms can be mimicked *in vitro*. Induction of mutations can be mimicked through the use of serial passaging, while spontaneous mutation can be mimicked through fluctuation assays (such as the Luria-Delbrück assay). It is possible that RAVs, arising spontaneously, differ from induced RAVs (Gillespie, 2002, McGrath, 2014) and that both of these could differ from RAVs occurring *in vivo* (Bergval et al., 2009).

The principle of the induction approach through the use of serial passage is universal, has been applied to both Gram-positive and Gram-negative bacteria, and a variety of media types, inoculum sizes and drug concentrations have been used (Gullberg, 2011, Martinez, 2011). The induction approach begins with inoculation of a strain (susceptible to the drug of interest) onto media containing a low drug concentration

(~0.5× minimal inhibitory concentration (MIC) of drug). Colonies grown on this initial drug-containing plate are then selected and re-exposed to higher drug concentrations (1× MIC). The process is repeated till the desired resistance arises (2× MIC and higher).

The experimental design to study spontaneous RAVs was first described by Luria and Delbrück in 1943 and has since been used extensively (Luria, 1943). The principle of this process is based on the assumption that within an actively growing bacterial population multiple replication cycles have taken place, resulting in naturally occurring mutagenesis (Rosche, 2000). Thus, the starting point of this approach is a parent culture (susceptible to the drug of interest) with a low number of cells to prevent pre-existing mutants from being selected. This culture is then divided into parallel cultures and grown to log phase. This actively growing culture is then plated onto drug-containing agar and only mutants that have occurred spontaneously, with resulting resistance to the drug tested, are capable of growth. Putative resistant colonies can then be selected and sub-cultured for further characterization.

Characterization of mutants as resistant can be conducted using phenotypic as well as genotypic approaches. Phenotypic resistance is assigned when a mutant possesses a minimal inhibitory concentration (MIC) value higher than the critical concentration (CC). Directed Sanger sequencing to identify variants within defined genetic targets is often used to genotypically characterize obtained mutants. However, the importance of analyzing multiple targets (global genome picture) through whole genome sequencing was demonstrated with studies performed on *in vitro* spontaneous bedaquiline-resistant mutants where “off-target” mutations of the *rv0678* gene were only identified four years later (Hartkoorn, 2014, Huitric, 2010). That delayed discovery was due to the sole focus on *atpE* targeted sequencing of the original bedaquiline-resistant mutants (Huitric, 2010). The spontaneous approach has been applied previously for bedaquiline (Andries et al., 2005, Hartkoorn, 2014, Huitric, 2010), clofazimine (Zhang, 2015) and linezolid (Balasubramanian et al., 2014, Beckert, 2012, Hillemann, 2008, Zhang, 2016); and for the latter drug the induction approach has also been applied to investigate RAVs. However, mutants derived from the same strains using both approaches have not been compared for their resultant RAVs. The differences that exist between the two methods is detailed in Table 1.

Table 1: Theoretical comparison of spontaneous and induction approaches.

Approach	Hands on time	Labor Intensity	Time To result	Features observed or associated with approach	RAVs generated	MIC values generated	Type of growth
Induction (5 passages)	Plate preparation- 15 minutes	Low	~5 months (1 month/ passage)	-Time to resistance -Sequencing after each passage provides information around genetic variants	RAVs associated with high MIC values	High	Confluent
	Isolate preparation and inoculation- 1 hour	Medium					
Spontaneous	Plate preparation- 1 hour	Medium	2 months	-Mutation frequency -Mutation rate	Distinct RAVs correlating with low and high MIC values	Range of above borderline to high	Single colonies
	Isolate preparation and inoculation- 2-3 hours	High					

We aimed to compare *in vitro* mutants resistant to either bedaquiline, clofazimine or linezolid obtained through the induction approach with those isolated using the spontaneous approach. As both approaches are based on the unique principles described above (Section 1), inoculum preparation as well as selective concentrations for each drug differ. However, the reference strains, solid media for selection, as well as the platforms to confirm resistance were standardized. A summary of the approximate cost, labor intensity and phenotypic and genotypic data generated from each approach is also provided in Table 1. We further described the RAVs in pre-selected genes (alluded to in literature), from mutants obtained between the two approaches, and compared these to a compiled catalog of previously published RAVs from *in vitro*, *in vivo* and clinically resistant strains (Ismail N., 2018).

2. MATERIALS AND METHODS

All experimental work was performed in a BSL3 facility at the Centre for Tuberculosis (WHO TB Supranational Reference Laboratory), National Institute for Communicable Diseases, National Health Laboratory services. Ethical approval (REF: 309/2016) was obtained from The Research Ethics Committee (University of Pretoria, Faculty of Health Sciences). An experimental workflow for *in vitro* mutant generation and phenotypic and genotypic confirmation of derived mutants is provided in Figure 1.

2.1. Antimicrobial preparation

Bedaquiline (Janssen Therapeutics, Titusville, NJ, USA), clofazimine (REF: C8895, Sigma-Aldrich Co., St Louis, USA) and linezolid (REF: PZ0014, Sigma-Aldrich Co.) were formulated in DMSO (REF: 41639, Sigma-Aldrich Co.) to stock concentrations of 1 mg/ml and maintained at -20^{o}C (for a maximum of 3 months). The stock solutions were further diluted using DMSO to obtain the required concentrations for testing.

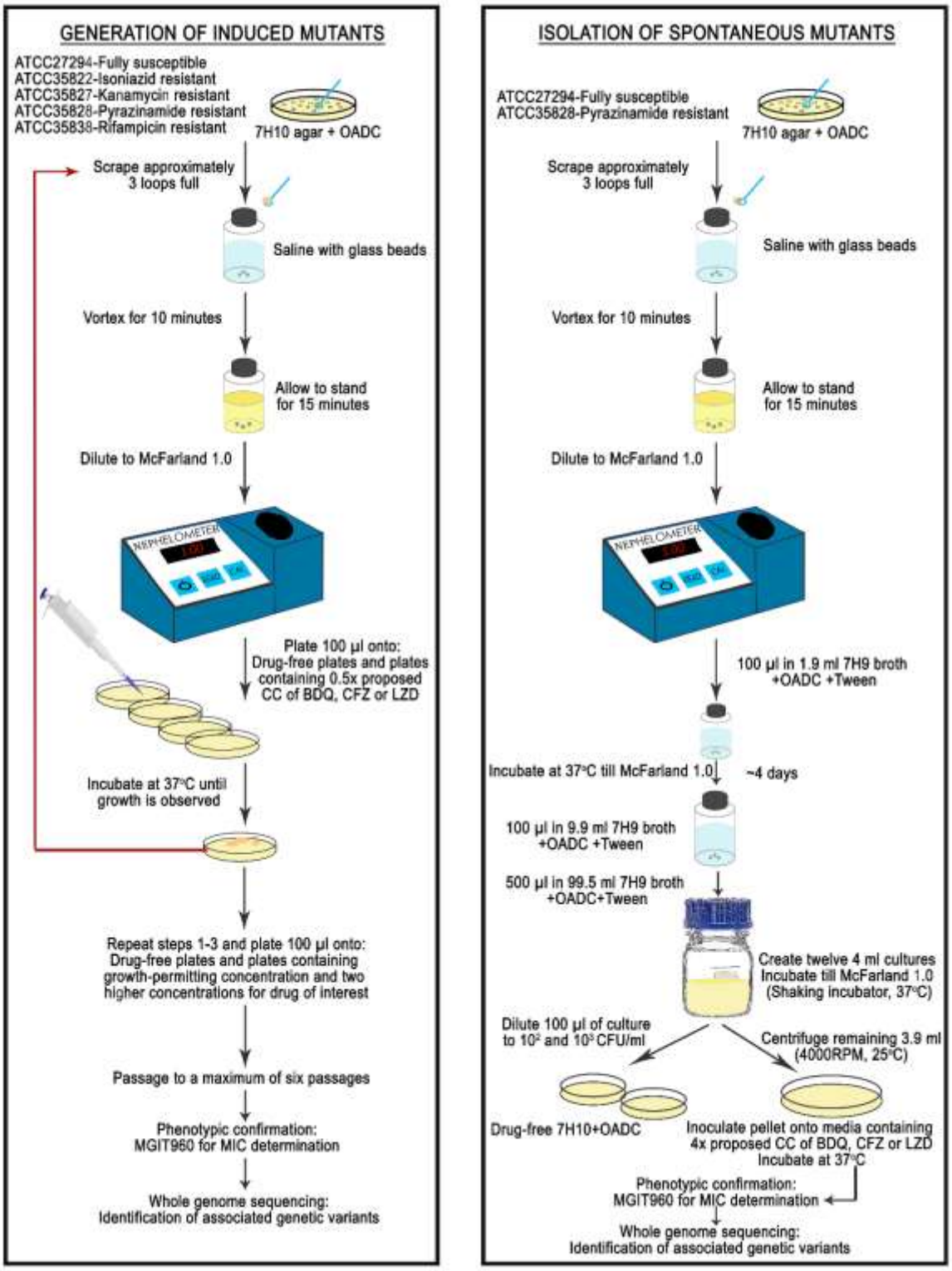

Figure 1: Experimental overview for generation of induced mutants and selection of spontaneous mutants and resultant mutant confirmation.

2.2. Bacterial strains

Five *M. tuberculosis* American Type Culture Collection (ATCC) reference strains with varying susceptibility profiles were used to determine if inherent resistance plays a role in the type of RAVs obtained. All resistant reference strains were described by the ATCC to be derived from an H37Rv strain. The strains used were ATCC27294: wild type [WT]/ fully susceptible; ATCC35822: isoniazid-resistant [INH^R] (*katG*: complete gene deletion); ATCC35827: kanamycin-resistant [KAN^R] strain (*rrs*: A1401G mutation); ATCC35828: pyrazinamide-resistant [PZA^R] (pncA: G394A (Gly132Ser) mutation) and ATCC35838: rifampicin-resistant [RIF^R] (*rpoB*: C1349T (Ser450Leu) mutation).

2.3. Generation of induced mutants (Figure 1)

All five ATCC strains were grown on Middlebrook 7H10 agar with 10% v/v OADC (oleic acid, albumin, dextrose, catalase) supplement (BD Biosciences) and then used to prepare cell suspensions with the turbidity adjusted to that of a McFarland 1.0 standard (~3× 10^8 CFU/ml (NCCLS, 2003)) as described (GLI). In brief, 2 to 3 loops full of an actively growing culture were added to Phoenix ID Broth (BD Biosciences) containing three 5 mm glass beads. The suspension was then vortexed for 10 minutes (Heidolph™ Multi Reax Vortex Mixer, Schwabach, Germany) and allowed to settle for 15 minutes. The McFarland turbidity of the suspension was measured using a PhoenixSpec™ Nephelometer (BD Biosciences) and adjusted using normal saline. One hundred microliters of each cell suspension was inoculated onto agar plates containing 0.5×, 1× and 2× the proposed CC for solid agar of bedaquiline (0.25 µg/ml), clofazimine (0.25 µg/ml) or linezolid (0.5 µg/ml) as well as onto a drug-free control plate (WHO, 2018). A sub-inhibitory concentration (0.5× proposed CC), as opposed to a standardized concentration for all three drugs, provides a growth starting point with increasing accumulation of resistance through each passage.

Plates were incubated at 37°C until sufficient growth (from the highest drug concentration plate) appeared to prepare a suspension (McFarland 1.0) as described above (Section 2.3). The suspension was then used to inoculate four plates; a drug-free control; a plate with the growth permitting drug concentration (same as which growth was scraped from) as well as on plates with 2- and 4-fold higher drug. This process was repeated until 6 passages were completed or until concentrations of at

least 4× proposed CC were reached for each drug, i.e. bedaquiline (1 µg/ml), clofazimine (1 µg/ml) or linezolid (4 µg/ml).

2.4. Isolation of spontaneous mutants (Figure 1)

The Luria-Delbrück protocol further adapted by Ford *et al.* (Ford, 2011) was used to generate spontaneous mutants for two reference strains of *M. tuberculosis*. The *M. tuberculosis* strains ATCC27294 (WT), which served as a control and ATCC35828 (PZA^R) were used. A McFarland 1.0 cell suspension was diluted 1:20 with 7H9 broth. This was left to grow for 4 days at 37ºC in an orbital shaking incubator (Labcon, Model Number: FSIM-SP024) or until the culture reached the turbidity of a McFarland 1.0 standard. Two sequential dilutions of 1:100 followed by a 1:200 were then prepared and used to create twelve 4 ml cultures in Middlebrook 7H9 broth (OADC, Tween-20). These parallel cultures were then incubated at 37ºC as above (Section 2.3) till the turbidity reached that of a McFarland 1.0 standard. For each culture, a log dilution was prepared and the 10^3 and 10^4 cells/ml dilutions were plated on drug-free plates. The remaining culture was then spun down and the pellet re-suspended in 200 µl of Middlebrook 7H9 broth and spread onto a 120 mm plate (Gosselin™, Borre, France) containing 4× proposed CC of bedaquiline, clofazimine or linezolid corresponding to 1, 1 and 2 µg/ml. After 21 days of incubation at 37ºC, the total number of cells was determined on growth control plates. After a further 11 days of incubation, the mutant colonies on drug-containing plates were counted. Mutation frequencies were calculated using the equation $m = \frac{Nm}{Nt}$ where *m* is the mutation frequency, *Nm* is the number of mutants (on drug-containing plates) and *Nt* is the total number of cells (on growth control plates).

2.5. Phenotypic and genotypic characterization of obtained mutants

Induced or spontaneous mutant's MIC values for each drug were determined using the MGIT 960 platform (Becton Dickinson Diagnostic Systems (BD Biosciences), Sparks, Maryland, USA). Confluent growth of the entire induced mutant population obtained at the ceiling concentration (or after the last passage) was scraped from across drug-containing plates and used to create a suspension. Ten microliters of this suspension was sub-cultured for a drug-free passage. Following this passage, MIC determination was performed and genomic DNA was extracted from MGIT tubes

containing growth at the highest drug concentration. For spontaneous mutants, single colonies were picked and sub-cultured for a drug-free passage. When the number of spontaneous colonies was too high, i.e. >15; 3 colonies were randomly selected from each of the 12 plates. Following the drug-free passage, cultures obtained from the 36 spontaneous mutant colonies are (Ismail et al., 2018) subjected to MIC determination, followed by randomly selecting three for Whole Genome Sequencing (WGS). Thus, induced mutants are representative of an entire population while spontaneous mutants represent a single colony.

2.5.1. *Determination of MIC values*

Using the stock solutions described above (Section 2.1), two-fold dilutions with a final concentration ranging from 8 to 0.125 µg/ml (bedaquiline and linezolid) and 4 to 0.06 µg/ml (clofazimine) were prepared. The clofazimine tested range could not be extended as the color of the drug solution at 8 µg/ml interfered with the florescent detection of this instrument. Determination of MIC values was performed using the methodology described in the MGIT manual (Siddiqi and Rüsch-Gerdes, 2006). Both baseline and mutant MIC values were determined using the MGIT960 platform. Briefly, within 3-5 days of flagging positive, 500 µl of a 1:5 dilution of the culture was used to inoculate MGIT tubes containing a serial dilution of bedaquiline, clofazimine or linezolid. A further 1:100 dilution was then used to inoculate a drug-free growth control. The tubes were incubated in the BACTEC MGIT960 system until the growth units of the growth control reached 400 or for a maximum of 28 days. The MIC was determined as the first concentration within the series which had a growth unit value of <100.

2.5.2. *Identification of resistance associated variants*

Genomic DNA extraction was performed using the NucliSENS easyMAG (BioMérieux, Marcy-l'Étoile, France). In brief, a 200 µl aliquot of the liquid culture was used as the input volume for DNA extraction using the on-board generic protocol, with a final elution volume of 25 µl. DNA concentration was measured using a Qubit® 2.0 fluorometer (Life technologies, Carlsbad, CA, USA) with the Qubit dsDNA High Sensitivity (HS) Assay kit (Life technologies). Preparation of paired-end libraries was performed using the Nextera XT DNA library kit (Illumina, San Diego, CA, USA), following manufacturer's protocol for tagmentation, size selection and a modified library normalization step (Omar, under review). WGS was carried out using the

Illumina MiSeq at the National Institute for Communicable Diseases Sequencing Core Facility, using an Illumina MiSeq 2× 300bp V3 cartridge.

Identification of RAVs was performed using the CLC Genomics workbench, version 10 and by mapping to an edited, annotated, reference genome of *M. tuberculosis* H37Rv (Genebank NC000962.3). The following parameters were set in order to identify single nucleotide polymorphisms (SNPs) or insertions/deletions (indels): minimum paired coverage depth of 5×, frequency of ≥30% (Black et al., 2015), length and similarity fractions of 0.8 each and a Phred score of ≥Q20 (≥99% accuracy) at both variant positions and nucleotides within a radius of 5bp.

For linezolid resistance, all spontaneous mutants were confirmed using Sanger sequencing, replacing WGS due to the uniformity of mutants detected, targeting *rplC* (for mutants with MIC values of 8 or >8 µg/ml) and *rrl* regions (for mutants with MIC values <8 µg/ml) as described previously (Beckert, 2012, Zimenkov, 2017).

2.6. Comparison of mutant genetic variants to previously published RAVs

For bedaquiline-resistant mutants, RAVs were searched for within *atpE, rv0678*, *rv1979c* and *rv2535c*; clofazimine-resistant mutants within *rv0678*, *rv1979c* and *rv2535c* and linezolid-resistant mutants the *rplC* and *rrl* genetic targets. These were compared to a catalog of previously published RAVs (Ismail N., 2018), which were assigned as references for comparison with the RAVs identified in this study. The latter were considered novel if they were not described in literature.

3. RESULTS

3.1. Induction approach

3.1.1 *Number of passages to generate induced mutants*

Four bedaquiline-resistant mutants were generated within 5 passages and one was generated within 4 passages (PZA^R strain, Table 2). Clofazimine-resistant mutants from all five reference strains were generated within five passages (Table 2). Linezolid-resistant mutants were generated within six passages. According to the ATCC: “A passage is defined as a subculture involving growth of the viable microorganism with

fresh medium" (ATCC, 2013). We utilized fresh drug-containing 7H10 medium and ensured that a culture with fresh growth was used (21-28 days) (Pfyffer, 2012). Cultures were used at this point or when sufficient growth appeared for the creation of a suspension with the turbidity of a McFarland 1.0 standard.

3.1.2. *MIC values obtained*

Although it could be sufficient to phenotypically confirm on the basis of their growth on solid media containing high drug-concentrations (at their final passage), we established MGIT960 MIC values as well to establish rigor. All bedaquiline-resistant mutants possessed MIC values of >8 µg/ml (Table 2). The MIC values for the clofazimine-resistant mutants were 4 µg/ml (from WT and KAN^R strains) and >4 µg/ml (from PZA^R, INH^R and RIF^R strains). Linezolid-resistant mutants possessed MIC values of 8 µg/ml (KAN^R and RIF^R strains) and >8 µg/ml (INH^R and PZA^R). The characterization of all strains from the induction approach as mutants was confirmed through the observed 4-fold increase in MIC values from baseline.

3.1.3. *Genotypic characterization (RAVs generated)*

Four out of five of the bedaquiline-resistant induced mutants exhibited *atpE* RAVs, with a substitution at the amino acid Asp28 as the favored location (4 of 6 *atpE* RAVs, Table 2). Three bedaquiline-resistant mutants displayed two distinct RAVs in the *atpE* and *rv0678* genes (INH^R, KAN^R and RIF^R). The bedaquiline-resistant mutant population from the WT strain was dominated by a single *atpE* RAV. The resultant bedaquiline-resistant induced mutant population from the PZA^R strain was unique in that two distinct *atpE* RAVs (Glu61Asp and Asp28Gly) were found. All clofazimine-resistant mutant populations possessed *rv0678* RAVs, with no overlap in the type of RAVs identified. By contrast, linezolid-resistant mutants exhibited no genotypic differences as all mutants possessed the Cys154Arg amino acid change in the *rplC* gene (Table 2).

Table 2: Mutants obtained through induction approach for five ATCC strains. Mutant MIC values determined using MGIT960. Resistance associated variants identified with whole genome sequencing. Novel mutations (not previously identified in literature) are highlighted as bold text. One mutant population was generated per strain; with some populations exhibiting more than one mutation.

Drug	ATCC strain	Baseline MGIT MIC (µg/ml)	No. of passages	Mutant MGIT MIC (µg/ml)	Gene	ΔNT (DNA)	ΔAA (Protein)	Frequency (%)
BDQ	WT	0.5	5	>8	*atpE*	G187C	Ala63Pro	100
	INH^R	0.25	5	>8	*atpE*	A83G	Asp28Gly	98
					rv0678	**T461C**	**Leu154Pro**	100
	KAN^R	0.5	5	>8	*atpE*	A83**C**	Asp28**Ala**	62
					rv0678	**201_206del**	**Ser68_Thr69del**	88
	PZA^R	1	4	>8	*atpE*	G183T	Glu61Asp	100
					atpE	A83G	Asp28Gly	31
	RIF^R	0.25	5	>8	*atpE*	A83G	Asp28Gly	100
					rv0678	**A63T**	**Glu21Asp**	86
CFZ	WT	0.125	5	4	*rv0678*	**G74A**	**Gly25Asp**	100
	INH^R	0.25	5	>4	*rv0678*	**T131C**	**Leu44Pro**	100
	KAN^R	0.125	5	4	*rv0678*	**T407C**	**Leu136Pro**	86
	PZA^R	0.5	5	>4	*rv0678*	**C204A**	**Ser68Arg**	52
	RIF^R	0.5	5	4	*rv0678*	**T131C**	**Leu44Pro**	55
LZD	WT							Not available
	INH^R	1	6	>8	*rplC*	T460C	Cys154Arg	96
	KAN^R	1	6	8	*rplC*	T460C	Cys154Arg	100
	PZA^R	2	6	>8	*rplC*	T460C	Cys154Arg	100
	RIF^R	0.5	6	8	*rplC*	T460C	Cys154Arg	100

Footnote for Table 2:

ATCC reference strains (ATCC27294- Wild type/Fully susceptible [WT], ATCC35822- Isoniazid resistant [INH^R], ATCC35827- Kanamycin resistant [KAN^R], ATCC35828-Pyrazinamide resistant [PZA^R] and ATCC35838- Rifampicin resistant [RIF^R])

BDQ-bedaquiline, CFZ-clofazimine, LZD-linezolid

MIC-Minimum Inhibitory Concentration

Gene-Resistance associated gene, ∆ NT- Nucleotide change, ∆ AA-Amino acid change

3.2. Spontaneous approach

3.2.1. *MIC values obtained*

For the bedaquiline-resistant spontaneous mutants only a single mutant was derived from the WT strain, which exhibited a high MIC value (>8 µg/ml). From the 619 mutants derived from the PZA^R strain, three selected mutants had MIC values of 4 to >8 µg/ml (Table 3). From the clofazimine-containing plates for both strains, large numbers of mutants were found, but only 3 from each strain were selected. All 6 clofazimine-resistant spontaneous mutants displayed MIC values within the range of 1 to 4 µg/ml (Table 3). Two linezolid-resistant spontaneous mutants isolated from the WT strain and 11 from the PZA^R had MIC values of ≥8 µg/ml, while the remaining two mutants from the PZA^R strain had MIC values of 4 µg/ml (Table 3).

3.2.2. *Genotypic characterization (RAVs generated)*

For the single bedaquiline-resistant spontaneous mutant isolated from the WT strain, an *atpE* RAV (Asp28Val) was found. Bedaquiline-resistant mutants from the PZA^R strain either possessed *atpE* or *rv0678* RAVs (Table 3). All three clofazimine-resistant spontaneous mutants selected from the WT strain possessed RAVs in the *rv0678* gene (Table 3). Two of these exhibited identical *rv0678* RAVs with MIC values only a dilution apart (Table 3, 2 and 4 µg/ml respectively). The third clofazimine-resistant mutant had two *rv0678* RAVs (frequencies of 23 and 34%). All three clofazimine-resistant mutants from the PZA^R strain had *rv0678* RAVs (Table 2). No selection process was required for the linezolid-resistant spontaneous mutants as all of these were analyzed at the *rplC* gene using targeted Sanger sequencing. Based on the observed uniformity of RAVs associated with linezolid-resistant induced mutants (100% possessed *rplC* Cys154Arg RAV) analyzed with WGS, we used Sanger

sequencing for the associated regions of *rplC* and *rrl* genes for spontaneous mutants. Both linezolid-resistant spontaneous mutants from the WT strain and 11/13 mutants from the PZA^R strain, possessed the *rplC* RAV, Cys154Arg. The remaining 2/13 linezolid-resistant spontaneous mutants, had RAVs in the *rrl* gene at nucleotide positions G2270C and A2810C (Table 2).

3.2.3. *Mutation frequencies*

Mutation frequencies were calculated as described in Section 2.4. For the WT strain, the mutation frequencies for bedaquiline and clofazimine at a 1 µg/ml concentration were ~6×10^{-9} and ~5×10^{-5} and for linezolid at a 2 µg/ml concentration was ~1×10^{-8}. For the PZA^R strain, the mutation frequencies for bedaquiline, clofazimine and linezolid are ~4×10^{-7}, ~7×10^{-7} and ~1×10^{-7} respectively.

Table 3: Mutants obtained through the spontaneous approach for fully susceptible (WT) and pyrazinamide-resistant (PZA^R) ATCC strains. Mutant MIC values determined using MGIT960. Resistance associated variants identified with whole genome sequencing for bedaquiline- and clofazimine-resistant mutants and with Sanger sequencing for linezolid-resistant mutants. Novel mutations (not previously identified in literature) are highlighted as bold text.

Drug mutant	ATCC Strain	Total no. of mutants	Mutation frequency	Mutant MGIT960 MIC (µg/ml)	Gene	∆NT	∆AA	Frequency (%)
BDQ	WT	1	~6x 10^{-9}	>8	*atpE*	A83T	Asp28Val	93
	PZA^R	619	~4x 10^{-7}	4	*rv0678*	**C403G**	**Arg135Gly**	81
				8	*atpE*	A83G	Asp28Gly	100
				>8	*atpE*	G187C	Ala63Pro	100
CFZ	WT	6937	~5x 10^{-5}	2	*rv0678*	193delG	Ile67fs	38
				4	*rv0678*	193delG	Ile67fs	69
				4	*rv0678*	**A65T**	**Gln22Leu**	33
	PZA^R	12371	~7x 10^{-7}	1	*rv0678*	**T407C**	**Leu136Pro**	97
				2	*rv0678*	C214**T**	Arg72**Trp**	93
				4	*rv0678*	G137A	Cys46Tyr	37
					rv0678	A97G	Thr33Ala	32
LZD	WT	2	~1x 10^{-8}	>8	*rplC*	T460C	Cys154Arg	-
				>8	*rplC*	T460C	Cys154Arg	-
	PZA^R	13	~1x 10^{-7}	4	*rrl*	G2270C	-	-
				4	*rrl*	A2810C	-	-
				8[a]	*rplC*[a]	T460C	Cys154Arg	-
				>8[b]	*rplC*[b]	T460C	Cys154Arg	-

Footnote for Table 3:

a) Representative of 3/11 of the linezolid spontaneous mutants with *rplC* mutations

b) Representative of 8/11 of the linezolid spontaneous mutants with *rplC* mutations

BDQ-bedaquiline, CFZ-clofazimine, LZD-linezolid

MIC-Minimum Inhibitory Concentration

Gene-Resistance associated gene, ∆ NT- Nucleotide change, ∆ AA-Amino acid change

ATCC reference strains (ATCC27294- Wild type/Fully susceptible [WT] and ATCC35828- Pyrazinamide resistant [PZA^R]

3.3. Comparison of RAVs between approaches and to previously published RAVs

In Tables 2 and 3, RAVs identified in induced and spontaneous mutants are compared to the catalog of mutations compiled from literature (Ismail N., 2018). Novel mutations are highlighted in bold text. Amongst the 9 bedaquiline-resistant mutants (5 induced and 4 spontaneous), 13 RAVs are identified. Of these, 9 are *atpE* RAVs and 4 are *rv0678* RAVs. Eight of the 9 *atpE* RAVs correspond with literature with Asp28Gly/Val being favored. An additional novel *atpE* mutation, Asp28Ala (WT strain), was also found. Thus, RAVs at position 28 occurred as 6/9 *atpE* mutations. Mutations within the *atpE* gene at Ala63Pro were identified in 2 of the 9 bedaquiline-resistant mutants. A single Glu61Asp mutation was associated with one of the induced bedaquiline-resistant mutants. All four *rv0678* RAVs associated with bedaquiline-resistant mutants (3 induced and 1 spontaneous) are novel.

Within the group of clofazimine-resistant mutants (5 induced and 6 spontaneous), 11 *rv0678* RAVs are identified. Of these, 8 are novel. Previously described RAVs were associated with spontaneous clofazimine-resistant mutants, i.e. Thr33Ala and Ile67fs (identified in 2 distinct spontaneous mutant colonies). Additionally, we observed identical RAVs in clofazimine-resistant mutants derived from different strains, i.e. the novel RAVs within the *rv0678* gene Leu44Pro and Leu136Pro. The Leu44Pro RAV was observed in two induced mutants derived from INH^R and RIF^R strains, with MIC values only one dilution apart. However, the Leu136Pro RAV occurred in spontaneous (PZA^R) and induced (KAN^R) mutants with mutant MIC values of 1 and 4 µg/ml respectively.

Linezolid-resistant mutants from both approaches exhibit the well-characterized and dominant *rplC* RAV, Cys154Arg. The two *rrl* mutations have also been previously described and were found in linezolid-resistant spontaneous mutants with MIC values of 4 µg/ml.

3.3.1 *Inter-approach mutant comparison*

A comparison of the two strains (WT and PZA^R) used in both the induction and spontaneous approaches was performed to determine if any similarities in the RAVs obtained exist (Figure 2). Looking at the bedaquiline-resistant mutants, the *atpE* Ala63Pro RAV (G187C) can be found in an induced and a spontaneous mutant, though derived from WT and PZA^R strains respectively (Figure 2). Similarly, the *atpE* RAV, Asp28Gly (A83G), is found in an induced and a spontaneous mutant; here, both mutants are derived from the PZA^R strain exclusively. An overlap observed amongst clofazimine-resistant mutants was the RAV, Ile67fs (193delG) identified in two distinct spontaneous mutants derived from the WT strain (Figure 2). From the 16 linezolid-resistant mutants (from WT and PZA^R strains using both approaches), 14 harbored the *rplC* Cys154Arg RAV and two mutants (PZA^R strain) possessed *rrl* mutations (Figure 2).

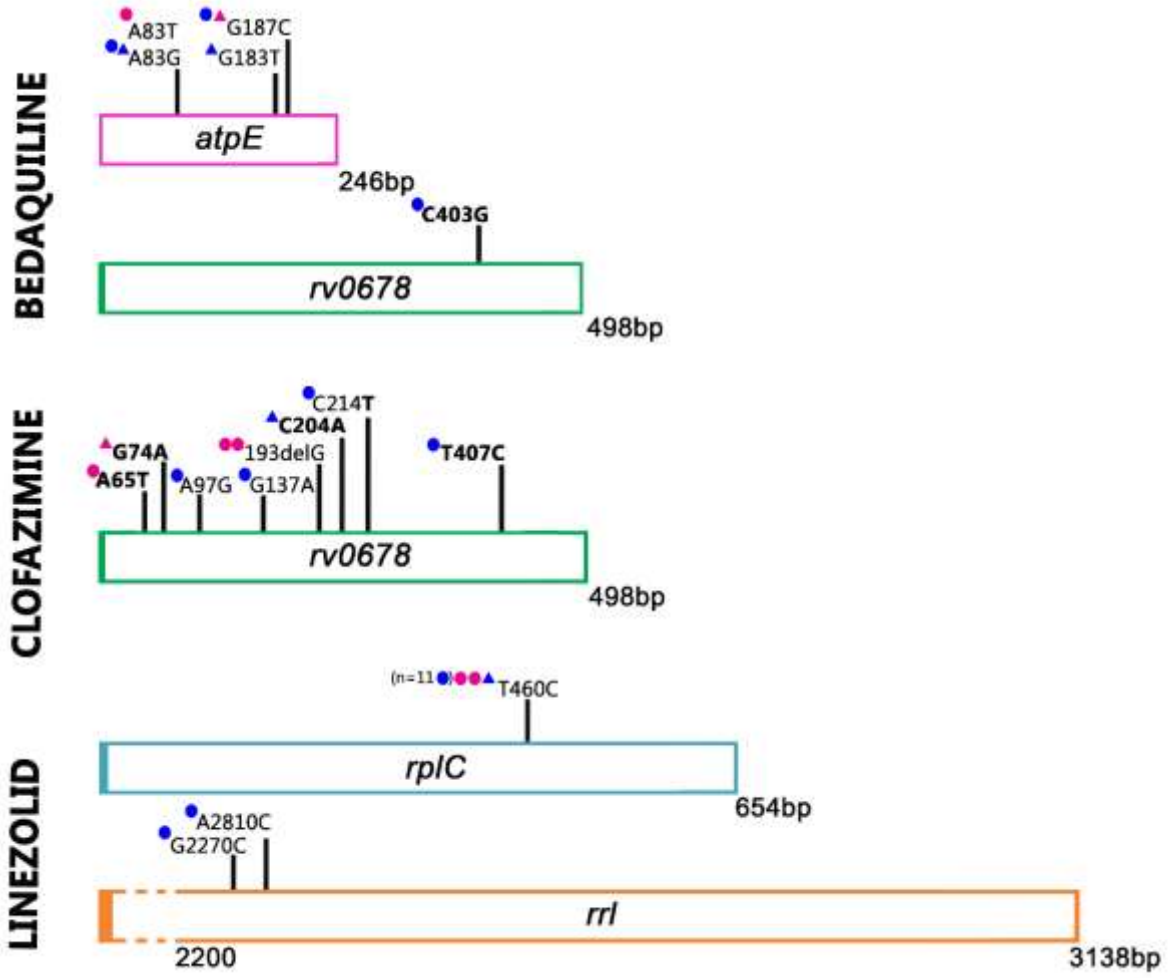

Figure 2: Resistance associated variants observed for the fully susceptible (WT) and pyrazinamide-resistant (PZA^R) strains from the spontaneous and induction approaches. Novel mutations (not previously described in literature) are depicted in bolded text. RAVs are allocated to mutants from different approaches derived from two strains using colored shapes. The induced (triangle) or spontaneous (circle) mutants derived from either the WT (pink) or PZA^R (blue) strain

4. DISCUSSION

The global burden of drug-resistant TB has led to increased use of WHO category 5 drugs including bedaquiline, clofazimine and linezolid (WHO, 2017), for which a paucity of information is available about their resistance acquisition mechanisms. This study is the first to comprehensively investigate both the generation of induced mutants and isolation of spontaneous mutants for three key TB drugs. Mutants were

successfully obtained using an induction and spontaneous approach, however, with varying MIC levels and genetic changes.

We observed the following procedural differences for mutant generation between the two approaches (Table 1). The time to result is significantly different- an induced mutant takes 5 months to obtain, while a spontaneous mutant can be selected within 2 months. By way of design, the isolation of spontaneous mutants for a single strain requires a larger number of plates for the parallel cultures used. As a result, the spontaneous approach is more costly and plate preparation and inoculation for this approach is more labor intensive than for the induction approach. However, the spontaneous approach can be used for the calculation of mutation frequencies (shown in this study) as well as mutation rates. Key information that can be obtained from the induction approach is the rate of resistance development, by observing the number of passages in which mutants are obtained. The mutants that arise using each approach are representative of either a population or a colony for the induction and spontaneous approach respectively. A sample of a suspension of the induced mutant population was used for culturing. The culture derived from this was subjected to a selection step by extracting DNA from cultures in the highest drug containing tube (following MIC determination). Both the creation of a suspension and the selective DNA extraction enabled enrichment of bacteria possessing the resistance-causing determinants.

We ensured that the platform used to evaluate phenotypic resistance (i.e. MGIT960) was different from the platform used to create putative mutants (i.e. solid agar) to establish rigor. Mutants obtained using the induction approach possessed high MIC values (≥4-fold proposed CC) and the RAVs identified have previously been associated with this high level of resistance (Nguyen, 2017, Zhang, 2016). Spontaneous mutants isolated have either high or above borderline MIC values associated with distinct RAVs, e.g. *atpE* or *rv0678* and *rplC* or *rrl* associated with bedaquiline and linezolid resistance, respectively. This could possibly be explained by the principle of the spontaneous approach, where all mutations that could result in resistance are given an opportunity to arise on selection, whereas for the induction approach the organism is driven towards high-level resistance. To evaluate this further, a higher selection concentration for the spontaneous method could be used to investigate whether mutants possessing MIC values associated with low-level resistance could still be selected for.

For bedaquiline-resistant mutants, the observed *atpE* RAVs correspond well with published literature. These RAVs can be found within the *c*-subunit of ATP synthase enzyme (at amino acid positions 28, 61 and 63) and influence drug binding (Matteelli, 2010, Segala, 2012). Interestingly, in our study we were unable to isolate a mutant harboring a mutation at amino acid position 66, a hotspot region previously described in a *M. tuberculosis* H37Rv strain displaying phenotypic bedaquiline resistance (Petrella, 2006). In addition to *atpE* RAVs, certain bedaquiline-resistant induced mutant populations and a single bedaquiline-resistant spontaneous mutant colony exhibited concomitant *rv0678* RAVs. Since *rv0678* RAVs are associated with an efflux response they could appear first as described previously (Gygli, 2017). To further understand this, bacteria could be analyzed after each passage (*versus* only after they achieve high MIC values) to uncover RAV accumulation patterns. No *rv1979c* and *rv2535c* mutations could be identified amongst bedaquiline-resistant mutants.

The bedaquiline-resistant induced mutant population (PZA^R strain) exhibited two *atpE* RAVs (occurring at frequencies of 31 and 100% respectively). Thus, a critical feature to note regarding *M. tuberculosis* resistance acquisition mechanisms, is the ability for this single strain to use two different gene positions for mutation under the same conditions. Further to this, the use of five strains from a common progenitor with varying susceptibility profiles to obtain mutants with a variety of RAVs leads us to believe that pre-existing resistance may play a role on the genetic pathway chosen for resistance generation.

For clofazimine-resistant mutants, we found the majority of RAVs occurring within the *rv0678* gene. This correlates well with previously published research. No *rv1979c* and *rv2535* gene mutations were found. However, in comparison to the Zhang *et al.* study first describing these RAVs sequence data of *in vitro* clofazimine-resistant mutants showed *rv0678* RAVs in 93/96 mutants and only 3/96 mutants possessed *rv1979c* and *rv2535* mutations (Zhang, 2015). The cost of WGS prohibited us from sequencing such a large number of mutants. Additionally, our study used a different mutant selection concentration compared to this study (1 *vs.* 0.25 µg/ml).

We identified 13 novel *rv0678* RAVs in this study and found that, unlike the *atpE* and *rplC* mutations, *rv0678* mutations appear to occur randomly along the gene. This corresponds well with the functionality of *mmpR5.* Various mutations within the *rv0678*

gene possibly influences *mmpR5* protein translation and subsequent repression of *mmpL5* and *mmpS5* efflux proteins expression (Andries et al., 2014, Zhang, 2015). The identification of novel RAVs (Leu44Pro and Leu136Pro) in mutants obtained from strains with varied pre-existing resistance and resulting in different MIC values demonstrate the importance of inherent susceptibility profiles in resistance acquisition mechanisms.

Linezolid-resistant mutants displayed uniformity within the *rplC* gene through WGS (induced mutants) and targeted Sanger sequencing (spontaneous mutants). The majority of observed mutants possessed the *rplC* mutation Cys154Arg, corresponding with literature (Beckert, 2012). Ribosomal protein L3 is encoded for by *rplC* and the Cys154Arg mutation has been previously proposed as a marker for linezolid resistance in *M. tuberculosis* (Makafe, 2016). Only two of the linezolid-resistant spontaneous mutants exhibit previously described *rrl* mutations, which were associated with lower MIC values (Zhang, 2016).

When using the PZA^R strain for our induction approach we observed its ability to acquire resistance faster than other mono-resistant and WT strains used for bedaquiline and we also observed that this strain possessed higher baseline MIC values for all three drugs compared to the other strains used. This could indicate an intrinsic resistance acquisition mechanism associated with the PZA^R strain. PZA resistance is highly prevalent amongst multi-drug resistant (MDR) TB strains compared to other mono-resistance TB profiles (NICD). Key research has also linked the majority of PZA resistance to RIF-resistant cases (Zignol), and rifampicin resistance has been loosely associated with *rv0678* mutations (Villellas, 2017). Clearly, the entire picture around resistance acquisition mechanisms is incomplete, particularly in the context of MDR-TB regimens and the new drugs bedaquiline and clofazimine. Cure rates of DR-TB could possibly be at risk as PZA is a key drug within the DR-TB regimen (Alame-Emane et al., 2015, Njire, 2016). This observation opens avenues for investigation of the role that pre-existing PZA resistance could play as a predictor for XDR-TB. Future studies to understand the role of pre-existing resistance on the acquisition of mutations need to be conducted.

Both approaches successfully lead to mutants resistant to each drug. The RAVs identified by both methods correspond well with previously published data as

summarized in our catalog (Ismail N., 2018). However, for bedaquiline, clinically resistant isolates have been associated primarily with *rv0678*, with limited *atpE* RAVs (Bloemberg, 2015, Zimenkov, 2017). In terms of RAVs identified, for both approaches, we find an overlap for bedaquiline in specific *atpE* regions, for clofazimine resistance we find associated *rv0678* mutations and in the *rplC* RAVs associated with linezolid resistance. It is ascertained that the RAVs identified mimic *in vivo* resistance profiles confirming rigor of the approaches used.

The spontaneous approach is based on *de novo* mutagenesis occurring within replication cycles-with *in vitro* replication occurring under different conditions from those *in vivo*. Additionally, recent work by Sarathy *et al.* shows that drug penetration of bedaquiline and clofazimine into the caseum may be poor and thus the selective drug concentrations of 2× the CC or higher may be unlikely to occur *in vivo* (Sarathy, 2016). Thus, the induction approach with continual exposure of bacteria to low concentrations could be a more accurate representation of the *in vivo* situation for these hydrophobic drugs. With this approach, investigation of induced mutants after three to four passages could provide more clinically relevant information than driving for mutants with high MIC values. This could also be used to study the accumulation of mutations when different pathways are involved (e.g. efflux and targeted; *rv0678* and *atpE*). However, a major constraint of performing the induction approach on *M. tuberculosis* isolates is the growth rate and the time taken for each passage.

Limitations of our study include the use of only two strains for the spontaneous approach; however, we assume from the type of mutants obtained from both WT and PZA^R strains (spontaneous approach), that mutants from the INH^R, RIF^R and KAN^R strains would not differ significantly. Due to the principle of the spontaneous approach, the number of cells inoculated cannot be adjusted, thus the inoculum size may differ between drugs and strains. However, mutation frequencies are calculated using both the number of mutants and the total number of cells and are therefore adjusted for this. Linezolid-resistant spontaneous mutants isolated were analyzed using Sanger sequencing and not WGS as for other mutants. The uniformity of mutations observed in the former mutants warranted the sequencing approach chosen. Finally, novel *v0678* mutations were assigned as the resistance associated variants on the basis of phenotypic confirmation; further cloning is required to confirm the role of these mutations. In addition to this, *rv0678* mutations were assigned as resistance conferring

as these mutations were found to appear on comparison of mutant to baseline genomes for two of the strains (data not shown). All limitations were as a result of the cost involved.

In conclusion, we demonstrate two *in vitro* approaches for the generation and isolation of resistant mutants. These approaches resulted in mutants that can provide key information due to the close resemblance to *in vivo* resistant isolates, but with some important methodological differences between the two approaches. Novel RAVs detected in clofazimine- and bedaquiline-resistant mutants from this study require further research. A larger sample set comprising strains with varied resistance profiles to create mutants would provide essential data regarding *M. tuberculosis* resistance acquisition mechanisms.

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest for this study.

8. REFERENCES

Alame-Emane, A.K., Xu, P., Pierre-Audigier, C., Cadet-Daniel, V., Shen, X., Sraouia, M., Siawaya, J.F., Takiff, H., Gao, Q., Gicquel, B., 2015. Pyrazinamide resistance in *Mycobacterium tuberculosis* arises after rifampicin and fluoroquinolone resistance. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 19, 679-684.

Andries, K., Verhasselt, P., Guillemont, J., Gohlmann, H.W., Neefs, J.M., Winkler, H., Van Gestel, J., Timmerman, P., Zhu, M., Lee, E., Williams, P., de Chaffoy, D., Huitric, E., Hoffner, S., Cambau, E., Truffot-Pernot, C., Lounis, N., Jarlier, V., 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science. 307, 223-227.

Andries, K., Villellas, C., Coeck, N., Thys, K., Gevers, T., Vranckx, L., Lounis, N., de Jong, B.C., Koul, A., 2014. Acquired resistance of Mycobacterium tuberculosis to bedaquiline. PLoS One. 9, e102135.

ATCC, 2013. Reference Strains: How many passages are too many?, Technical bulletin No. 6, American Type Culture Collection.

Balasubramanian, V., Solapure, S., Iyer, H., Ghosh, A., Sharma, S., Kaur, P., Deepthi, R., Subbulakshmi, V., Ramya, V., Ramachandran, V., Balganesh, M., Wright, L., Melnick, D., Butler, S.L., Sambandamurthy, V.K., 2014. Bactericidal activity and mechanism of action of AZD5847, a novel oxazolidinone for treatment of tuberculosis. Antimicrobial Agents and Chemotherapy. 58, 495-502.

Beckert, P., Hillemann, D., Kohl, T. A., Kalinowski, J., Richter, E., Niemann, S. and Feuerriegel, S., 2012. rplC T460C identified as a dominant mutation in linezolid-resistant *Mycobacterium tuberculosis* strains. Antimicrobial Agents and Chemotherapy. 56, 2743-2745.

Bergval, I.L., Schuitema, A.R., Klatser, P.R., Anthony, R.M., 2009. Resistant mutants of *Mycobacterium tuberculosis* selected *in vitro* do not reflect the *in vivo* mechanism of isoniazid resistance. Journal of Antimicrobial Chemotherapy. 64, 515-523.

Black, P.A., de Vos, M., Louw, G.E., van der Merwe, R.G., Dippenaar, A., Streicher, E.M., Abdallah, A.M., Sampson, S.L., Victor, T.C., Dolby, T., Simpson, J.A., van Helden, P.D., Warren, R.M., Pain, A., 2015. Whole genome sequencing reveals genomic heterogeneity and antibiotic purification in *Mycobacterium tuberculosis* isolates. BMC Genomics. 16, 857.

Bloemberg, G.V., Keller, P. M., Stucki, D., Trauner, A., Borrell, S., Latshang, T., Coscolla, M., Rothe, T., Hömke, R., Ritter, C., Feldmann, J., Schulthess, B., Gagneux, S. and Böttger, E. C., 2015. Acquired Resistance to Bedaquiline and Delamanid in Therapy for Tuberculosis. New England Journal of Medicine. 373, 1986-1988.

Ford, C.B., Lin, P. L., Chase, M. R., Shah, R. R., Iartchouk, O., Galagan, J., Mohaideen, N., Ioerger, T. R., Sacchettini, J. C., Lipsitch, M., Flynn, J. L. and Fortune, S. M., 2011. Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. Nature Genetics. 43, 482-486.

Gillespie, S.H., 2002. Evolution of Drug Resistance in Mycobacterium tuberculosis: Clinical and Molecular Perspective. Antimicrobial Agents and Chemotherapy. 46, 267-274.

GLI, Global Laboratory Initiative, 2014, Mycobacteriology Laboratory Manual, First Edition, Drug Susceptibility Testing: The MGIT system, 51-66.

Gullberg, E., Cao, S., Berg, O. G., Ilback, C., Sandegren, L., Hughes, D. and Andersson, D. I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathogens. 7, e1002158.

Gygli, S.M., Borrell, S., Trauner, A. and Gagneux, S., 2017. Antimicrobial resistance in *Mycobacterium tuberculosis*: mechanistic and evolutionary perspectives. FEMS Microbiology Reviews. 41, 354-373.

Hartkoorn, R.C., Uplekar, S. and Cole, S. T., 2014. Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 58, 2979-2981.

Hillemann, D., Rusch-Gerdes, S. and Richter, E., 2008. *In vitro*-selected linezolid-resistant *Mycobacterium tuberculosis* mutants. Antimicrob Agents Chemother. 52, 800-801.

Huitric, E., Verhasselt, P., Koul, A., Andries, K., Hoffner, S. and Andersson, D. I., 2010. Rates and mechanisms of resistance development in *Mycobacterium tuberculosis* to a novel diarylquinoline ATP synthase inhibitor. Antimicrobial Agents and Chemotherapy. 54, 1022-1028.

Ismail, N., Omar, S.V., Ismail, N.A., Peters, R.P.H., 2018. In vitro approaches for generation of Mycobacterium tuberculosis mutants resistant to bedaquiline, clofazimine or linezolid and identification of associated genetic variants. J Microbiol Methods. 153, 1-9.

Ismail N., O.S.V., Ismail N.A. and Peters R.P.H, 2018. Catalog of previously published mutations and mutation frequencies associated with bedaquiline, clofazimine and linezolid resistance for *Mycobacterium tuberculosis*. Data in brief. Submitted.

Koser, C.U., Javid, B., Liddell, K., Ellington, M. J., Feuerriegel, S., Niemann, S., Brown, N. M., Burman, W. J., Abubakar, I., Ismail, N. A., Moore, D., Peacock, S. J. and Torok, M. E., 2015. Drug-resistance mechanisms and tuberculosis drugs. Lancet (London, England). 385, 305-307.

Luria, S.E.a.D., M., 1943. Mutations of Bacteria from Virus Sensitivity to Virus Resistance. Genetics. 28, 491-511.

Makafe, G.G., Cao, Y., Tan, Y., Julius, M., Liu, Z., Wang, C., Njire, M. M., Cai, X., Liu, T., Wang, B., Pang, W., Tan, S., Zhang, B., Yew, W. W., Lamichhane, G., Guo, J. and Zhang, T., 2016. Role of the Cys154Arg Substitution in Ribosomal Protein L3 in Oxazolidinone Resistance in *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. 60, 3202-3206.

Martinez, J.L., Baquero, F. and Andersson, D. I., 2011. Beyond serial passages: new methods for predicting the emergence of resistance to novel antibiotics. Current opinion in pharmacology. 11, 439-445.

Martinez, J.L.a.B., F., 2000. Mutation frequencies and antibiotic resistance. Antimicrobial Agents and Chemotherapy. 44, 1771-1777.

Matteelli, A., Carvalho, A. C., Dooley, K. E. and Kritski, A., 2010. TMC207: the first compound of a new class of potent anti-tuberculosis drugs. Future Microbiology. 5, 849-858.

McGrath, M., Gey van Pittius, N. C., van Helden, P. D., Warren, R. M. and Warner, D. F., 2014. Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*. Journal of Antimicrobial Chemotherapy. 69, 292-302.

Murray, J.F., Schraufnagel, Dean E. and Hopewell, Philip C., 2015. Treatment of Tuberculosis. A Historical Perspective. Annals of the American Thoracic Society. 12, 1749-1759.

NCCLS, 2003. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard NCCLS Document M24-A. 23.

Nguyen, T.V.A., Anthony, R. M., Banuls, A. L., Vu, D. H. and Alffenaar, J. C., 2017. Bedaquiline resistance: Its emergence, mechanism and prevention. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.

NICD, National Institute for Communicable Diseases. South African Tuberculosis Drug Resistance Survey. 2012-2014.

Njire, M., Tan, Y., Mugweru, J.,Wang, C.,Guo, J.,Yew, W.,Tan, S. and Zhang, T., 2016. Pyrazinamide resistance in *Mycobacterium tuberculosis*: Review and update. Advances in medical sciences. 61, 63-71.

Omar, S.V., Joseph, L., Said, H.M., Ismail, F., Ismail, N., Gwala, T.L. and Ismail, N.A. , under review Feasibility of Next-Generation Whole Genome Sequencing for drug resistance determination of *Mycobacterium tuberculosis* in a South African Tuberculosis Reference Laboratory. Diagnostic Microbiology & Infectious Diseases (manuscript under review February 2018).

Petrella, S., Cambau, E., Chauffour, A., Andries, K., Jarlier, V. and Sougakoff, W., 2006. Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. Antimicrobial Agents and Chemotherapy. 50, 2853-2856.

Pfyffer, G.E.a.W., F., 2012. Incubation time of mycobacterial cultures: how long is long enough to issue a final negative report to the clinician? J Clin Microbiol. 50, 4188-4189.

Rosche, W.A.a.F., P. L., 2000. Determining Mutation Rates in Bacterial Populations. Methods (San Diego, Calif.). 20, 4-17.

Sarathy, J.P., Zuccotto, F., Hsinpin, H., Sandberg, L., Via, L. E., Marriner, G. A., Masquelin, T., Wyatt, P., Ray, P. and Dartois, V., 2016. Prediction of Drug Penetration in Tuberculosis Lesions. ACS infectious diseases. 2, 552-563.

Segala, E., Sougakoff, W., Nevejans-Chauffour, A., Jarlier, V. and Petrella, S., 2012. New Mutations in the Mycobacterial ATP Synthase: New Insights into the Binding of the Diarylquinoline TMC207 to the ATP Synthase C-Ring Structure. Antimicrobial Agents and Chemotherapy. 56, 2326-2334.

Siddiqi, S.H., Rüsch-Gerdes, S., 2006. MGIT ™ Procedure Manual for BACTEC ™ MGIT 960 ™ TB System. Foundation for Innovative New Diagnostics.

Villellas, C., Coeck, N., Meehan, C. J.,Lounis, N., de Jong, B.,Rigouts, L. and Andries, K., 2017. Unexpected high prevalence of resistance-associated Rv0678 variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline. J Antimicrob Chemother. 72, 684-690.

WHO, 2017. Global Tuberculosis Report. World Health Organisation.

WHO, 2018. Technical report on critical concentrations for TB drug susceptibility testing of medicines used in the treatment of drug-resistant TB. World Health Organisation.

Zhang, S., Chen, J., Cui, P., Shi, W., Shi, X., Niu, H., Chan, D., Yew, W. W., Zhang, W. and Zhang, Y., 2016. *Mycobacterium tuberculosis* Mutations Associated with

Reduced Susceptibility to Linezolid. Antimicrobial Agents and Chemotherapy. 60, 2542-2544.

Zhang, S., Chen, J., Cui, P., Shi, W., Zhang, W. and Zhang, Y., 2015. Identification of novel mutations associated with clofazimine resistance in *Mycobacterium tuberculosis*. Journal of Antimicrobial Chemotherapy. 70, 2507-2510.

Zhou, J., Dong, Y., Zhao, X., Lee, S., Amin, A., Ramaswamy, S., Domagala, J., Musser, J. M. and Drlica, K., 2000. Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations. The Journal of infectious diseases. 182, 517-525.

Zignol, M., Dean, A.S., Alikhanova, N., Andres, S., Cabibbe, A. M., Cirillo, D.M., Dadu, A. Dreyer, A., Driesen, M., Gilpin, C., Hasan, R., Hasan, Z., Hoffner, S., Husain, A., Hussain, A., Ismail, N., Kamal, M., Mansjö, M., Mvusi, L, Niemann, S., Omar, S.V., Qadeer, E., Rigouts, L., Ruesch-Gerdes, S., Schito, M., Seyfaddinova, M., Skrahina, A., Tahseen, S., Wells, W.A., Mukadi, Y.D., Kimerling, M., Floyd, K. Weyer, K. and Raviglione, M.C., Population-based resistance of Mycobacterium tuberculosis isolates to pyrazinamide and fluoroquinolones: results from a multicountry surveillance project. The Lancet Infectious Diseases. 16, 1185-1192.

Zimenkov, D.V., Nosova, E. Y., Kulagina, E. V., Antonova, O. V., Arslanbaeva, L. R., Isakova, A. I., Krylova, L. Y., Peretokina, I. V., Makarova, M. V., Safonova, S. G., Borisov, S. E. and Gryadunov, D. A., 2017. Examination of bedaquiline- and linezolid-resistant *Mycobacterium tuberculosis* isolates from the Moscow region. Journal of Antimicrobial Chemotherapy. 72, 1901-1906.