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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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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.

1. Introduction

The emergence of drug resistance in bacterial populations has widely been studied through the generation of *in vitro* mutants (Zhou et al., 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 et al., 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 et al., 2015, Zhou et al., 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 et al., 2017). Both mechanisms can be mimicked *in vitro*. Induction of

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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 et al., 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 et al., 2011, Martinez et al., 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 et al., 2014, Huitric et al., 2010). That delayed discovery was due to the sole focus on *atpE* targeted sequencing of the original bedaquiline-resistant mutants (Huitric et al., 2010). The spontaneous approach has been applied previously for bedaquiline (Andries et al., 2005; Hartkoorn et al., 2014; Huitric et al., 2010), clofazimine (Zhang et al., 2015) and linezolid (Balasubramanian et al., 2014, Beckert et al., 2012, Hillemann et al., 2008, Zhang et al., 2016); and for the latter drug the induction approach has also been applied to investigate RAVs (Ismail et al., 2018). 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.

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 et al., 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 Fig. 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 °C (for a maximum of 3 months). The stock solutions were further diluted using DMSO to obtain the required concentrations for testing.

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 (Fig. 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, 2014). 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 min (Heidolph™ Multi Reax Vortex Mixer, Schwabach, Germany) and allowed to settle for 15 min. 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).