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Research Paper

Genetics and roadblocks of drug resistant tuberculosis

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ABSTRACT

Considering the extensive evolutionary history of *Mycobacterium tuberculosis*, anti-Tuberculosis (TB) drug therapy exerts a recent selective pressure. However, in a microorganism devoid of horizontal gene transfer and with a strictly clonal populational structure such as *M. tuberculosis* the usual, but not sole, path to overcome drug susceptibility is through *de novo* mutations on a relatively strict set of genes. The possible allelic diversity that can be associated with drug resistance through several mechanisms such as target alteration or target over-expression, will dictate how these genes can become associated with drug resistance. The success demonstrated by this pathogenic microbe in this latter process and its ability to spread is currently one of the major obstacles to an effective TB elimination.

This article reviews the action mechanism of the more important anti-TB drugs, including bedaquiline and delamanid, along with new findings on specific resistance mechanisms. With the development, validation and endorsement of new *in vitro* molecular tests for drug resistance, knowledge on these resistance mechanisms and microevolutionary dynamics leading to the emergence and fixation of drug resistance mutations within the host is highly important.

Additionally, the fitness toll imposed by resistance development is also herein discussed together with known compensatory mechanisms. By elucidating the possible mechanisms that enable one strain to reacquire the original fitness levels, it will be theoretically possible to make more informed decisions and develop novel strategies that can force *M. tuberculosis* microevolutionary trajectory down through a path of decreasing fitness levels.

1. Introduction

The introduction of streptomycin (STR) as the first active drug against tuberculosis (TB) is not only a major milestone in TB treatment history but, has also comprised a new selective pressure leading to the selection, emergence and amplification of drug resistance in a disease with an already broad intrinsic resistance spectrum (Emmart, 1945; Jones et al., 1944). In fact, it did not take long for STR-resistant TB cases to be detected and described (Waksman et al., 1945; Youmans et al., 1946). Over the following decades, new drugs showing activity against TB were gradually developed leading to a generalized belief that victory in the war against TB was now within reach and, that it would be only a matter of time until TB was eradicated. Eventually, much driven by multidrug resistant (MDR) TB outbreaks, TB was declared as a global emergency by the World Health Organization (WHO) in 1993 and, presently, more than two decades after, TB still accounts for approximately 1.7 million deaths each year, making it the ninth leading cause of death worldwide (World Health Organization, 1994, 2017).

There are major obstacles lying in the way for an effective TB worldwide elimination and, in this regard, TB is no different from other diseases of bacterial aetiology: antimicrobial resistance is among such obstacles (Tacconelli et al., 2018). The new WHO's End TB framework is aiming towards TB elimination by 2035 but, to be successful, this new strategy must effectively address increasingly different challenges such as the roll-out of new drug resistant TB diagnostic technologies that need to be locally adequate and should also translate into an effective link to care and early treatment initiation (Stop TB Partnership, 2015). However, the development and adequate implementation of such methods must draw information from sequence data and known mechanisms of drug resistance in TB. The latter usually show some degree of geographical variability, which must be considered. Herein, we review the basic genetic mechanisms underlying resistance to all first line drugs, injectable second line drugs, fluoroquinolones and the new drugs bedaquiline (BDQ) and delamanid (DLM). Moreover, we also address some evolutionary aspects associated with the microevolutionary

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trajectory of *Mycobacterium tuberculosis* towards drug resistance.

2. A Brief epidemiological perspective on drug resistant tuberculosis

The specter of drug resistant TB poses an increasingly recognized public health problem around the globe. According to the World Health Organization, 490,000 new multidrug resistant (MDR) cases are estimated to have occurred in 2016 (World Health Organization, 2017). MDR-TB is defined as resistance to the two most important anti-TB drugs: isoniazid (INH) and rifampicin (RIF) and, in fact, concomitant resistance to both drugs will likely result in the death of approximately 78,400 of these 490,000 MDR-TB patients. This, assuming the latest MDR-TB mortality rate estimated for the 2014 cohort by the WHO: 16%. Also, the lower treatment success rate observed in this same cohort was only 54%, significantly lower than the overall treatment success rate, 83%. Yet, to make matters worse another type of drug resistance, extensively drug resistance (XDR), defined in 2006 as MDR-TB with simultaneous resistance to one second line injectable drug (amikacin [AMK], kanamycin [KAN] or capreomycin [CAP]) and one fluoroquinolone (FQ), has now been reported by 123 countries (World Health Organization, 2007, 2017). XDR-TB associated mortality and treatment success are even worse than those for MDR-TB: 28% and 30%, respectively (World Health Organization, 2017).

Across the WHO European region alone, 15,363 (17.7%) MDR-TB cases among new cases have been reported in 2016. But to this number, 24015 (51.9%) MDR-TB cases reported in the same year should be added from previously treated cases (European Centre for Disease Prevention and Control, 2018). The latter comprises the main risk factor for MDR-TB (Desissa et al., 2018; Guglielmetti et al., 2018). Moreover, only approximately 40% of all RIF-resistant and MDR-TB cases are currently being detected in Europe, owing to the lack of universal DST coverage or rapid testing which potentiates the increase of primary MDR-TB transmission (European Centre for Disease Prevention and Control, 2018). This detection rate is still very far from the WHO established target of 85%, rendering the deployment of locally adequate molecular testing an urgent matter and, the knowledge of the resistance determinants a pressing need.

3. Mycobacterium tuberculosis Complex: an unexpected pathogen and the path to drug resistance

Mycobacterium tuberculosis sensu stricto, *Mycobacterium africanum* or *Mycobacterium bovis* are the most prominent members of a group known as *Mycobacterium tuberculosis* Complex (MTC) which is composed of an increasing number of highly related members sharing 99.9% nucleotide sequence similarity (Euzéby, 2014). The MTC shows a strict clonal population structure and its evolutionary trajectory to obligate pathogenicity has mostly been marked by genomic downsizing occurring over an extensive process of adaptive radiation towards more restricted ecological niches (Brosch et al., 2002; Gagneux et al., 2006a). Unlike other well-known bacterial pathogens, or even for that matter, many mycobacterial species, it is assumed that the MTC is devoid of Horizontal Gene Transfer, thereby exhibiting a closed genome, coupled with a low mutation rate (Eldholm and Balloux, 2016). As a consequence, *M. tuberculosis* is regarded as a paradigm of a monomorphic bacteria, but, still, a successful pathogen that has subsisted as such since the dawn of mankind (Comas et al., 2013; Dos Vultos et al., 2008). *M. tuberculosis* may therefore come as an unlikely pathogen or, one with a much higher genetic barrier to drug resistance development, but the true and devastating reality is illustrated by the numbers already mentioned above which show us otherwise.

Concerning the process of resistance development, *M. tuberculosis* mode of evolution carries along severe limitations. The most notorious being the impossibility to acquire resistance mediating genes that could eventually be mobilized by diverse genetic platforms. As a result, the usual course towards resistance development is through the selection of *de novo* mutations, either Single Nucleotide Polymorphisms (SNPs) or indels, at loci usually termed as resistance associated genes (Table 1). The nature of the associations that are established between these genetic markers and specific phenotypic resistance may have distinct origins and four main classical mechanisms can be outlined (Fig. 1): i) drug target modification, as a result of non-synonymous mutations that can alter, or completely avert, the pharmacokinetic binding of the drug to its target; ii) abrogated prodrug activation, due to mutations resulting in a partial or total loss of the activity of prodrug activation enzymes that, in turn, prevents the prodrug of reaching its active form; iii) target overexpression, usually resulting from hypermorphic mutations occurring at the promoter region that controls the expression of the drug target and, thereby, allowing it to outcompete the drug; and, iv) overexpression of drug modifying enzymes, through similar mutational mechanisms as the former but, leading to higher abundance of drug modifying enzyme that renders the drug inactive. A fifth increasingly recognized resistance mechanism consists in drug efflux and will also be discussed in more detail below as it can play a key role in the emergence of resistance driven by any of the above mechanisms. Also, drug modification coupled with reduced permeability may also underlie the intrinsic drug resistance exhibited by the MTC to several antimicrobial drugs (Buriankova et al., 2004; Warrier et al., 2016).

Table 1
– Drugs, mechanisms of action and genes associated with resistance.

Drug	Action mechanism	Main genes associated with resistance
INH	Inhibition of mycolate synthesis	*katG, inhA, ndh*
RIF	Transcription inhibitor	*rpoB*
EMB	Inhibition of AG and LAM arabinosylation (cell wall biosynthesis)	*embB, ubiA* (Rv3806c)
STR	Translation inhibitor	*rpsL, rrs, gidB, whiB7*
PZA	Inhibition of stress-response mechanisms (not completely elucidated); inhibitor of trans-translation	*pncA*
FQ	Inhibition of DNA replication (DNA gyrase)	*gyrA, gyrB*
SLID	Translation inhibitor	*rrs, tlyA, eis, whiB7*
DLM	Inhibition of mycolate synthesis	*ddn, fbiA, fbiB, fbiC, fgd1*
BDQ	ATP synthase inhibitor	*atpE, Rv0678, pepQ*

4. Anti-TB Drugs: Mode of Action and Resistance Mechanisms

4.1. Isoniazid: a potent bactericidal drug targeting the mycobacterial cell wall

INH or isonicotinic acid hydrazide is a synthetic prodrug that requires activation by the bacterial catalase peroxidase encoded by the *katG* gene (Heym et al., 1993). Upon activation, an isonicotinic acyl radical that binds NAD^+ is produced, forming an INH-NAD adduct that targets the InhA enoyl-ACP reductase, an essential enzyme for mycolic acid elongation by the type II fatty acid synthase system (FASII) (Rozwarski et al., 1998; Vilcheze et al., 2006; Vilcheze et al., 2005). The inhibition of mycolic acid biosynthesis leads to cell lysis and death. INH enters the cell by passive diffusion but its activity is restricted to dividing bacilli under aerobic conditions (Bardou et al., 1998).

INH notorious efficacy is in part due to its low minimum inhibitory concentration (MIC): 0.02 mg/L for *M. tuberculosis* H37Rv and 0.02–0.05 mg/L in susceptible clinical isolates (Rastogi et al., 1996). INH has a bactericidal activity against rapidly growing mycobacteria and bacteriostatic against slow-growers, although bactericidal activity is observed against *M. tuberculosis* (Heifets et al., 1991; Yamori et al., 1992)..

INH resistance usually develops because of *katG* mutations that decrease the ability of the catalase-peroxidase to convert INH to its

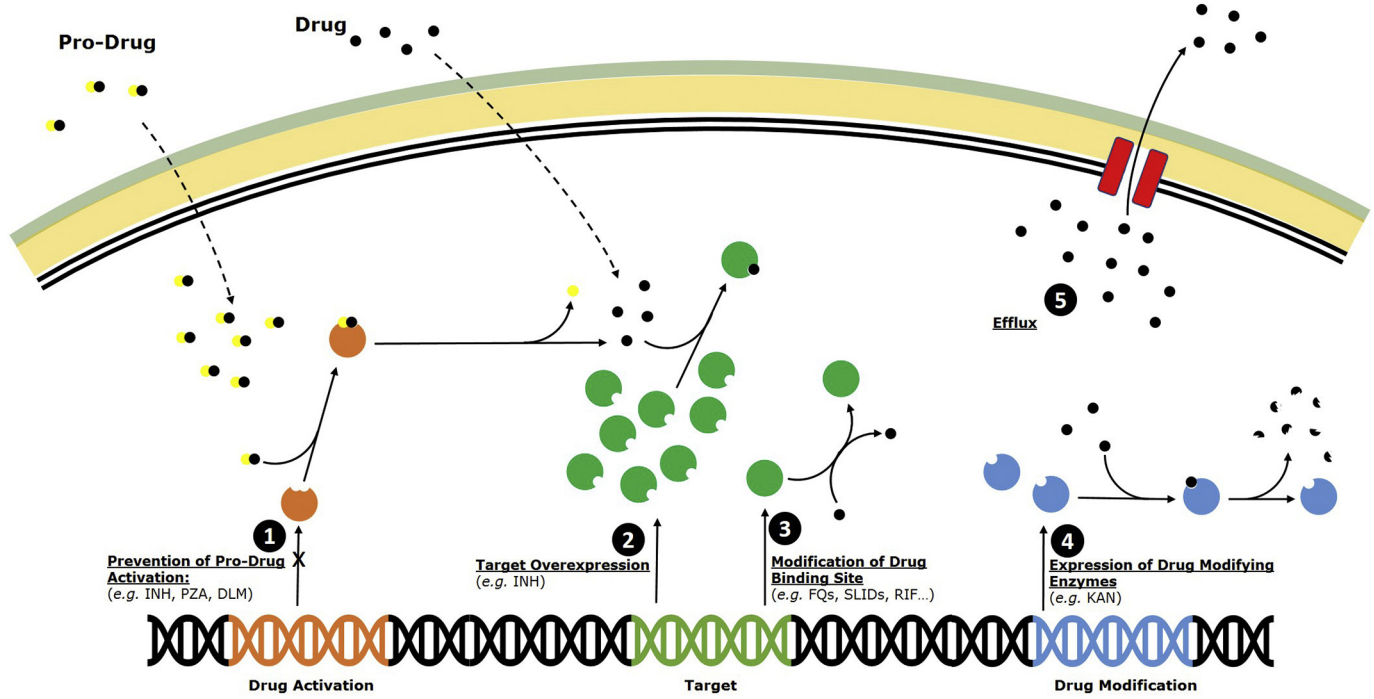

Fig. 1. – Overview of basic resistance mechanisms in M. tuberculosis. Four main classical resistance mechanisms are depicted: 1) abrogation of drug activation by loss of function or structural alteration of drug activating enzymes driven by mutations on such genes or corresponding promoter regions; 2) overexpression of the drug target, which outcompetes the drug; 3) target structural modification that can result, e.g., on the alteration of the geometry of the drug binding pocket or modification of drug binding sites, rendering the drug inactive by preventing its binding to its target enzyme; and, 4) overexpression of drug modification enzymes that alter or degrade the drug chemical structure. Efflux (5) is depicted as an alternative drug resistance pathway that acts by reducing the intracellular drug concentration and can thus provide a gateway to the emergence of stable and high-level drug resistance by one of the classical mechanisms.

Table 2
– Main resistance associated mutations along with estimated prevalence among drug resistant strains, cross-resistance and resistance levels if available.

Drug	Gene	Mutation	Cross-resistance[b]	Prevalence[c]	MIC[c]
INH	*katG*	S315 T		7.0-93.0	4.0- ≥ 32.0
INH	*inhA*	inhA Promoter (e.g. C-15 T)	ETH	0.75–91.4	0.25–1.0
RIF	*rpoB*	D435V[a]	RFB	3.4-28.6	20.0–50.0
RIF	*rpoB*	H445D[a]	RFB	7.7-43.0	160.0
RIF	*rpoB*	S450L[a]	RFB	31.0-76.9	100.0- ≥ 160.0
EMB	*embB*	M306 V		8.7–48.3	8.0–16.0
EMB	*embB*	M306 L		8.0–28.2	8.0–16.0
EMB	*embB*	M306I		0–8.7	4.0–16.0
STR	*rpsL*	K43R		12.1–89.0	> 256.0
STR	*rpsL*	K88R		1.7–6.1	8.0–64.0
SLIDs	*rrs*	A1401G	KAN/AMK/CAP	49.3–88.6	30.0–80.0 (AMK); 24.0–96.0 (KAN); 5–25 (CAP)
SLIDs	*rrs*	G1484 T	KAN/AMK/CAP/VIO	0–16.6	7.5 (AMK); 40 (KAN); 20- ≥ 40 (CAP)
FQ	*gyrA*	A90V		4.0–38.5	4.0–16.0 (OFX); 0.25–2.5 (MFX)
FQ	*gyrA*	D94G		46.2–71.9	4.0–50.0 (OFX); 0.5–8.0 (MOX)
FQ	*gyrA*	D94A		4–38.5	4.0–10.0 (OFX); 0.5–2.5 (MOX)

[a] *M. tuberculosis* H37Rv RpoB numbering.
[b] Abbreviations: ETH, etionamide; RFB, rifabutin.
[c] Sources for prevalence and resistance levels are cited throughout the text.

active form. Depending on the mutations found, *katG* mutations may result in low to high-level resistance which is negatively correlated with the remaining KatG INH oxidase activity (Ando et al., 2010). The most common mutation found in *katG* is a serine to threonine substitution at codon 315 (S315T), found in up to 93% of INH resistant isolates and associated with an INH intermediate to high-level resistance (Table 2) (Afanas'ev et al., 2007; Ali et al., 2011; Alves et al., 2011; Bakonyte et al., 2003; Brossier et al., 2009; Cambau et al., 2015; Campbell et al., 2011; Chan et al., 2007; Chaoui et al., 2009; Cho et al., 2009; Dalla Costa et al., 2009; Feuerriegel et al., 2012a; Hausner et al., 1988; Heym et al., 1993; Homolka et al., 2010; Imperiale et al., 2013; Minh et al., 2012; Muller et al., 2011; Nikolayevsky et al., 2004; Rindi et al., 2005; Sajduda et al., 2004; Silva et al., 2003; Valvatne et al., 2009; Yao et al., 2010; Yuan et al., 2012; Zhang et al., 2005; Zhang et al., 1993; Zhang et al., 1992). S315T has been shown to decrease KatG catalytic activity and binding affinity towards INH (Saint-Joanis et al., 1999; Wengenack et al., 1998; Yu et al., 2003).

Another important mechanism of INH resistance, usually second to *katG* mutations, relies on the acquisition of hypermorphic mutations in the promoter region of *mabA(fabG1)-inhA* operon, usually in up to 32% of INH resistant isolates (Afanas'ev et al., 2007; Ali et al., 2011; Alves et al., 2011; Bakonyte et al., 2003; Campbell et al., 2011; Chaoui et al., 2009; Cho et al., 2009; Homolka et al., 2010; Larsen et al., 2002; Minh et al., 2012; Nikolayevsky et al., 2004; Rindi et al., 2005; Sajduda et al.,

2004; Silva et al., 2003; Valvatne et al., 2009; Yao et al., 2010; Yuan et al., 2012; Zhang et al., 2005). Although such mutations usually lead to INH low-level resistance, an unusual high-prevalence of *inhA* promoter mutations, up to 91%, have been detected in Lisbon, Portugal, and in *M. africanum* West-Africa 1 lineage (Brossier et al., 2009; Cambau et al., 2015; Homolka et al., 2010; Imperiale et al., 2013; Muller et al., 2011; Perdigao et al., 2008). Furthermore *inhA* ORF/promoter double mutations can bring INH resistance towards intermediate levels (Machado et al., 2013).

Also, NAD^+/NADH altered ratios, mediated by *ndh* mutations, can also result in INH and ethionamide (ETH) resistance (Vilcheze et al., 2005). Mutations found in other genes, namely *kasA* and *ahpC*, have an unknown or questionable role in INH resistance and failed to meet statistical significance by genome-wide association studies (GWAS) (Ali et al., 2011; Coll et al., 2018; Homolka et al., 2010; Rindi et al., 2005; Valvatne et al., 2009; Zhang et al., 2005). Some studies have however reported up to 35% of INH resistant clinical isolates without mutations in any of the above genes, suggesting alternate mechanisms for INH resistance, such as drug efflux (Cardoso et al., 2007; Ho et al., 2009; Machado et al., 2012; Rindi et al., 2005).

4.2. Rifampicin: loosing transcription

RIF or rifampin is a semi-synthetic antibacillary drug derived from rifamycin and introduced in TB chemotherapy in 1967 (Vall-Spinosa et al., 1970). RIF binds to the β-subunit of the DNA-dependent RNA polymerase, encoded by the *rpoB* gene (Wehrli et al., 1968). Binding to the RNA polymerase β-subunit is thought to physically block transcription of growing RNA chains when these become 2–3 nucleotides in length (Campbell et al., 2001; McClure and Cech, 1978). RIF has a bactericidal activity against most Gram-positive, some Gram-negative and mycobacteria (Stottmeier et al., 1969). Active against metabolically active bacteria, RIF also possesses some degree of sterilizing activity as it is active against latent bacilli with spurts of activity (Gillespie, 2002; Mitchison, 1979).

RIF MIC ranges between 0.2 and 0.4 mg/L for susceptible clinical isolates (0.4 mg/L for *M. tuberculosis* H37Rv) (Rastogi et al., 1996).

Acquisition of RIF resistance is usually the result of aminoacid substitutions in a 81-bp region of the *rpoB* gene named RIF resistance determining region (RRDR) or cluster I (Heep et al., 2001; Kapur et al., 1994; Miller et al., 1994). Besides aminoacid substitutions, deletions or insertions in *rpoB* have been reported in some studies (Dalla Costa et al., 2015; Herrera et al., 2003; Suresh et al., 2006). The most common substitutions occur in codons 450 (prevalence of 31.0–76.9% in RIF-resistant isolates), 445 (7.7–43.0%) and 435 (3.4–28.6%), according to *M. tuberculosis* RpoB numbering (Table 2) (Ahmad and Mokaddas, 2005; Aristimuno et al., 2006; Bakonyte et al., 2005; Campbell et al., 2001; Feuerriegel et al., 2012a; Heep et al., 2001; Herrera et al., 2003; Homolka et al., 2010; Kapur et al., 1994; Lee et al., 2005; O'Sullivan et al., 2005; Rahim et al., 2012; Shemyakin et al., 2004; Siu et al., 2011; Suresh et al., 2006; Valvatne et al., 2009; Yao et al., 2010; Yuan et al., 2012). Mutations in these three codons are thought to have the least impact on fitness, particularly S450L mutations (Gagneux et al., 2006b; Mariam et al., 2004).

RIF resistance level depends on the substituted residue, or combination of mutations, and the type of substitution, *e.g.*, S450L and H445D result in high-level resistance whereas D435V mostly results in an intermediate-level resistance (Table 2) (Cambau et al., 2015; Jamieson et al., 2014; Van Deun et al., 2009; van Ingen et al., 2011; Zaczek et al., 2009).

Up to 11% of RIF resistant isolates do not show any RRDR mutations, with some isolates displaying N-terminal mutations that may affect protein-drug interaction or, mutations in *rpoB* cluster II (codons 490–491) (Heep et al., 2001; Siu et al., 2011).

A different resistance mechanism involving inactivation of RIF by ribosylation in mycobacteria has been identified as partially responsible for the low susceptibility of some species to RIF, but no mechanism of this type has been described for *M. tuberculosis* (Dabbs et al., 1995; Quan et al., 1997; Rominski et al., 2017).

Noteworthy, resistance to RIF rarely emerges before resistance to other drugs and, for this reason, is usually considered as a surrogate marker for MDR-TB as it is often associated with INH resistance, albeit through independent underlying mechanisms (Manson et al., 2017). The latter, coupled with the strong association between the RRDR mutations and phenotypic RIF resistance has been exploited by several molecular diagnosis methods for the early detection of MDR-TB (e.g. GeneXpert MTB/RIF) (Dorman et al., 2018).

4.3. Ethambutol: undermining the mycobacterial cell wall

EMB is an antimycobacterial drug synthesized from ethylenediamine and used in TB treatment since 1968. EMB targets the cell wall biosynthesis, more specifically by inhibiting the arabinosylation of the cell wall arabinogalactan (AG) and lipoarabinomannan (LAM) (Takayama and Kilburn, 1989). EMB targets the arabinosyltransferases encoded by the *embCAB* operon but, shows a higher affinity towards EmbB. Since e*mbAB* gene products are responsible for the arabinosylation of AG, whereas arabinosylation of LAM is catalyzed by the *embC* gene product, EMB leads to a more rapid inhibition of AG biosynthesis than LAM biosynthesis (Goude et al., 2009; Mikusova et al., 1995; Shi et al., 2006; Zhang et al., 2003). This EMB mediated inhibition of AG biosynthesis leads to further mycolic acid accumulation due to the depletion of mycolate attachment sites (Mikusova et al., 1995).

EMB enters the cell in a passive manner and, has a bacteriostatic activity against metabolically active bacilli rather than bactericidal (Bakker-Woudenberg et al., 2005; Beggs and Auran, 1972; Forbes et al., 1962). EMB MICs range between 0.5 and 2 mg/L for susceptible isolates (0.5 mg/L for *M. tuberculosis* H37Rv) (Rastogi et al., 1996).

EMB resistance has been traditionally associated with *embB* mutations, of special incidence on a EmbB 142 aa extracytoplasmatic loop (Alcaide et al., 1997; Lety et al., 1997; Ramaswamy et al., 2000; Telenti et al., 1997). The most common mutations occur in codon 306 in up to 68% of EMB resistant isolates and usually involve the substitution of a methionine by a valine, leucine or isoleucine (Table 2) (Ahmad et al., 2007; Campbell et al., 2011; Giri et al., 2018; Jadaun et al., 2009; Lee et al., 2002; Mokrousov et al., 2002; Parsons et al., 2005; Plinke et al., 2006; Sreevatsan et al., 1997b; Srivastava et al., 2006; Tracevska et al., 2004b). Strains bearing *embB*306 mutations have been associated with a higher level of EMB resistance than other *embB* mutations (Park et al., 2012; Srivastava et al., 2009; Srivastava et al., 2006). On the other hand, M306V/L mutations appear to confer a higher level of EMB resistance than M306I (Campbell et al., 2011; Giri et al., 2018; Ramaswamy et al., 2000; Sreevatsan et al., 1997b; Sun et al., 2018). Yet, the molecular basis of EMB resistance is not straightforwardly determined as, over a decade ago, it has been reported that *embB*306 mutations occasionally fail to deliver a phenotypic resistance level that overcomes the critical concentration used in standardized drug susceptibility testing, with several authors reporting EMB susceptible isolates bearing *embB*306 mutations whereas others only detected *embB*306 mutations among EMB resistant isolates (Cambau et al., 2015; Campbell et al., 2011; Johnson et al., 2006; Mokrousov et al., 2002; Plinke et al., 2009; Plinke et al., 2006; Shen et al., 2007). As a result, a high degree of uncertainty fell upon *embB*306 mutations as a molecular determinant of EMB resistance and its eventual utility for rapid molecular testing. Subsequently, allelic exchange experiments have shown that *embB*306 mutations only moderately increase EMB MIC to levels below the ones observed in clinical isolates but, that *embB*306 mutations appear to be required for high-level resistance, highlighting that this level of resistance is the result of multigenic mutational events (Plinke et al., 2011; Safi et al., 2010; Safi et al., 2008). At that point, other genes such as the *iniA* gene, essential to EMB efflux, were proposed to be involved in EMB resistance, although the paucity of data

prevented a robust association (Colangeli et al., 2005; Jaber et al., 2009; Ramaswamy et al., 2000).

The advent of Next Generation Sequencing (NGS) played a pivotal role in the identification of mutations in Rv3806c/*ubiA*, whose gene product is involved in the decaprenylphosphoryl-β-D-arabinose (DPA) biosynthetic pathway and, are thought to lead to increased intracellular levels of DPA that may compete with EMB for the Emb enzymes' active site (Safi et al., 2013). Moreover, Rv3806c mutations had multiplicative effects over *embB* mutations leading to EMB high-level resistance. Moreover, the association of Rv3806c with EMB resistance was recently corroborated by GWAS (Coll et al., 2018). Also, in the latter study, Rv2820c and Rv3300c, encoding two hypothetical proteins of unknown function were found to be associated with EMB resistance. Truncation of Rv2820c mediated by RD207 in Beijing/W strains has been shown to enhance mycobacterial virulence *ex vivo* and *in vivo* and evokes higher levels of anti-inflammatory cytokine IL-10 (Lam et al., 2011; Zhai et al., 2018). However, the molecular mechanism by which these two proteins may interfere with EMB resistance is not yet understood. Additionally, *embC-embA* intergenic mutations are also strongly associated with EMB resistance (Coll et al., 2018).

4.4. Streptomycin: the first drug for TB treatment

STR was the first antibiotic used in TB treatment, in 1944, and is an aminoglycoside isolated from *Streptomyces griseus* (Smith and Waksman, 1947; Waksman et al., 1946). STR interferes with protein synthesis by inhibiting genetic translation with a more pronounced effect on elongation rather than initiation (Hausner et al., 1988). Crystallography of *Thermus thermophilus* 30S ribosomal subunit complexed with STR, shows that STR tightly binds 16S rRNA (*rrs* gene) through both salt bridges and hydrogen bonds to four different parts of the molecule and across ribosomal protein S12 (*rpsL* gene) (Carter et al., 2000). The binding of STR appears to stabilize the ribosomal ambiguity state (ram state) and have a negative effect on the ribosomal proof-reading activity (Carter et al., 2000; Ruusala and Kurland, 1984). This appears to be due to the STR-mediated stabilization of the near-cognate anticodon stem-loop analogue complex and destabilization of the cognate anticodon stem-loop analogue complex (Demirci et al., 2013). STR-induced mistranslation leads to an increased proteolysis rate and abnormal misread proteins appear to create protein channels causing loss of permeability control, accelerating STR irreversible uptake and block of translation initiation (Bryan and Kwan, 1983; Busse et al., 1992; Davis et al., 1986; Hewitt and Kogut, 1977). The stabilization of the peptidyl-tRNA in the A-site has also been demonstrated to reduce the translocation rate (Karimi and Ehrenberg, 1994).

The initial use of STR in monotherapeutic regimens has led to the emergence of high number of resistant cases, and with subsequent development of more efficacious antibacillary drugs, STR gradually lost its role as a first-line drug, mainly in developed countries (Ruiz et al., 2003).

STR MICs range between 1.0 and 2.0 mg/L (1.0 mg/L for *M. tuberculosis* H37Rv) and has a moderate bactericidal activity against susceptible isolates (Rastogi et al., 1996; Smith and Waksman, 1947). On comparison with the two other aminoglycosides used in TB treatment, KAN and AMK, STR is the least toxic (Peloquin et al., 2004).

Resistance towards STR is usually mediated by mutations in the *rpsL* gene, generally a substitution of a lysine by an arginine at codon 43, although mutations on codon 88 are often reported and associated with STR resistance (Meier et al., 1994). Mutations in *rpsL* gene are associated with high-level resistance, particularly K43R mutations (Table 2) (Cooksey et al., 1996; Meier et al., 1996; Nhu et al., 2012; Springer et al., 2001; Tudo et al., 2010). Another mechanism of STR resistance occurs through *rrs* gene mutations that generally occur in the 530 and 910 loops, which interact with STR and are in close proximity due to the 16S rRNA secondary structure (Meier et al., 1994). Mutations in the *rrs* gene usually yield a resistance level below the resistance level of isolates bearing *rpsL* mutations, but high enough to be considered an intermediate resistance level, although MICs can vary significantly depending on the mutation (Cambau et al., 2015; Meier et al., 1996; Springer et al., 2001).

The prevalence of each of these mutations displays a wide variability, depending on strain lineage or geography. Mutations in *rpsL* gene are usually more prevalent (up to 89%) than *rrs* mutations (0–28.6%) (Brzostek et al., 2004; Chaoui et al., 2009; Cooksey et al., 1996; Cuevas-Cordoba et al., 2012; Feuerriegel et al., 2012b; Lipin et al., 2007; Ramaswamy et al., 2004; Sekiguchi et al., 2007; Shi et al., 2007; Sreevatsan et al., 1996; Sun et al., 2010; Tracevska et al., 2004b; Tudo et al., 2010). Nevertheless, in a study by Cuevas-Cordoba *et al, rrs* mutations were found to be more prevalent than *rpsL* mutations among STR-resistant isolates from southeast Mexico: 28.6% *vs* 19.8%, respectively (Cuevas-Cordoba et al., 2012).

The loss of the *gidB*-encoded rRNA methyltransferase function, found to be responsible for the methylation of G527 of the 16S rRNA, leads to a reduced affinity of STR towards the ribosome and STR low-level resistance (Okamoto et al., 2007; Spies et al., 2008). GidB mutations have been detected in clinical isolates, although the precise quantitative role in STR resistance is still unclear as some of the mutations detected in resistant isolates are also present in susceptible ones (Feuerriegel et al., 2012a; Spies et al., 2011). Other polymorphisms detected in *gidB* have been associated with strain lineage rather than STR resistance (Ballif et al., 2012; Spies et al., 2011). This is the case for the endemic MDR/XDR-TB Q1 clade in Portugal which has been defined based on the A80P mutation on GidB, which is simultaneously associated with an intermediate-level resistance to STR (Perdigao et al., 2013).

An alternative STR resistance mechanism has been initially discovered by selecting kanamycin-resistant mutants at low kanamycin concentrations and, by mapping the underlying mutations to the 5′ untranslated region of the *whib7* transcript (Reeves et al., 2013). The latter codes for a transcriptional activator and mutations leading to the overexpression of WhiB7 have also been demonstrated to result in the overexpression of Eis acetyl transferase and Tap efflux pump (Morris et al., 2005; Reeves et al., 2013). While Eis overexpression is linked with KAN low-level resistance, Tap overexpression was shown to be concomitantly associated with low-level STR resistance and to the emergence of STR high-level resistance at an increased frequency driven mostly by *gidB* mutations (Okamoto et al., 2007; Reeves et al., 2013). This situation parallels with the increased mutational frequency for high-level STR spontaneous mutants by *gidB* mutants except for the fact this phenomenon was always driven by *rpsL* secondary mutations, which were not observed in *whiB7* mutants (Okamoto et al., 2007).

4.5. Pyrazinamide: a stress-targeting drug?

PZA is a nicotinamide synthetic prodrug that has been introduced in TB treatment in 1952 and has allowed the shortening of TB treatment to the current, and widely adopted, 6-month short-course regimen (Anthony et al., 2018; den Hertog et al., 2015; Yeager et al., 1952). The action mechanism of PZA has recently been thoroughly reviewed by Anthony et al. (Anthony et al., 2018). Briefly, the classical Zhang model is based on PZA entering the cell by passive diffusion where it is converted by the bacterial pyrazinamidase (PZase)/nicotinamidase into pyrazinoic acid (POA), which *per se* would behave as an ionophore and could accumulate in the cell due to a deficient efflux system, only leaving the cell by passive diffusion in an acidic pH environment. Extracellular protonation of POA would allow re-entrance by passive diffusion where deprotonation would lead to cytoplasm acidification (Zhang and Mitchison, 2003; Zhang et al., 2002; Zhang et al., 1999). Moreover, the acidification of the cytoplasm would also contribute to the depletion of the membrane energy potential (Wade and Zhang, 2004). Yet, this early proposed mechanism dating back to 2003 does not fully explain phenotypic resistance to PZA in clinical isolates, nor is

it compatible with recent evidence showing that PZA can act on neutral pH conditions and that other environmental stress factors can trigger a susceptible phenotype to PZA, independently of the extracellular pH (den Hertog et al., 2016; Peterson et al., 2015).

Despite numerous attempts, the exact target(s) for PZA remained elusive for several years since the isolation of POA resistant mutants were unsuccessful (Scorpio et al., 1997; Zimhony et al., 2000). This was also true for POA structural analogues nicotinic acid and benzoic acid (Zhang and Mitchison, 2003). These failed attempts strongly supported the view in which the target(s) of PZA/POA would be essential to cell viability and to circumvent this, binding assays enabled the identification of *rpsA*-encoded ribosomal protein S1 as a major POA binding protein, subsequently leading to the proposal that PZA inhibits trans-translation, the primary rescue system for ribosomal stalling (Shi et al., 2011). More recent efforts aimed at selecting POA-resistant mutants *in vitro* have been successfully carried out on nearly neutral agar instead of acidic agar (Gopal et al., 2016). WGS analysis of these *in vitro* spontaneous mutants initially enabled the identification of mutations on: *panD*, coding for an aspartate decarboxylase from the coenzyme A (CoA) biosynthetic pathway; mycocerosic acid synthase *mas* gene, involved in the synthesis of the cell wall lipid pthiocerol dimycoserosate (PDIM), a cell-envelope-associated virulence factor; and, *ppsA-E* genes, coding for phenolpthiocerol synthesis type-1 poliketide synthases, also from the PDIM biosynthetic pathway (Gopal et al., 2016). Using the same methodology, unfoldase CplC1 mutants, part of the Caseinolytic Protease Complex, have also been associated with PZA resistance in *in vitro* POA-selected mutants and, therefore, as a new candidate target of PZA (Yee et al., 2017). From these putative drug targets, POA but not PZA has been experimentally demonstrated to bind PanD and this binding to be abrogated by *panD* missense mutations. Conflicting evidences regarding RpsA as a putative target still remain with PZA becoming increasingly recognized as an antivirulence agent that becomes active upon stress conditions (Anthony et al., 2018; Dillon et al., 2017; Gopal et al., 2016).

Concerning resistance in clinical isolates, the main mechanism underpinning PZA resistance is the acquisition of mutations in the *pncA* gene (Scorpio and Zhang, 1996). Mutations in the *pncA* gene have been identified in numerous studies in about 72.0–99.9% of the PZA resistant isolates studied (Alexander et al., 2012; Barco et al., 2006; Bishop et al., 2001; Campbell et al., 2011; Cheng et al., 2000; Chiu et al., 2011; Feuerriegel et al., 2012a; Hou et al., 2000; Jureen et al., 2008; Kim et al., 2012; Lee et al., 2001; Lemaitre et al., 1999; Mestdagh et al., 1999; Morlock et al., 2000; Muthaiah et al., 2010; Rodrigues Vde et al., 2005; Scorpio et al., 1997; Somoskovi et al., 2007; Sreevatsan et al., 1997a; Stoffels et al., 2012; Tracevska et al., 2004a). Most of the mutations identified are predicted to affect the enzymatic activity of PncA due to the alteration of the active site or destabilization of the protein structure leading to the loss of the PZase activity (Stoffels et al., 2012). As the *pncA* gene is not essential to bacterial viability, the gene is under a less stringent mutational constraint leading to a high mutational diversity and independent emergence of PZA resistance at a lower rate (10^{-5}) (Cheng et al., 2000; David, 1970; Hou et al., 2000; Stoffels et al., 2012). The resistance level to PZA depends on the mutation although mutational hotspots have been identified with a higher proportion in the loop between the β2 and β3 strands which comprise the metal coordination site of the enzyme's catalytic centre (Lemaitre et al., 1999; Scorpio et al., 1997; Zimic et al., 2010). In fact, docking simulations support the association between specific mutations and resistance levels (Unissa et al., 2010).

PZA resistant isolates with a wild-type *pncA* gene fall in two groups: PZase-negative and PZAase-positive isolates (Barco et al., 2006; Bishop et al., 2001; Mestdagh et al., 1999). While PZA resistance in PZase-positive isolates may be explained by mutations at other *loci*, namely on the putative targets of POA, resistance in PZase-negative isolates with a wild-type *pncA* gene suggest a differential regulation of *pncA* gene expression.

Mutations in these putative targets are not, however, robust markers for drug resistance (Alexander et al., 2012; Werngren et al., 2017). This has been corroborated by global GWAS analysis in which only *pncA* or its promoter region were found to meet statistical significance and independent association with PZA resistance (Coll et al., 2018). The absence of non-*pncA* mutations in clinical isolates might be related with *in vivo* gene essentiality as *clpC1* and *mas/ppsA-E* mutants have been shown to exhibit an attenuated phenotype on a mouse model of infection (Gopal et al., 2017).

4.6. The Second-Line Injectable Trio: Amikacin, Kanamycin and Capreomycin

The second-line injectable drugs (SLIDs) for TB treatment are KAN, AMK and CAP. Although KAN and AMK are, such as STR, aminoglycosides and CAP is a macrocyclic peptide from the tuberactinomycin family, these drugs share the same basic action mechanism and incomplete cross-resistance between the three has been well documented (Felnagle et al., 2011; Maus et al., 2005a). KAN, AMK and CAP have shown *in vitro* bactericidal activities but CAP has a bactericidal effect against non-replicating *M. tuberculosis* (Heifets et al., 2005).

These three drugs act through the inhibition of the genetic translation due to ribosomal binding. Nevertheless, the exact mechanism through which these drugs inhibit the ribosomal activity during translation is not fully understood or studied to the extent that has been with STR. AMK and KAN have been shown to bind to the A-site of the ribosome and inhibit translation (Kondo et al., 2006). CAP mode of action has been initially deduced from early studies of ribosomal translation inhibition with its analogue viomycin (VIO) and confirmed by the crystal structure of CAP and VIO in complex with the 70S ribosomes (Modolell and Vazquez, 1977; Stanley et al., 2010; Yamada and Bierhaus, 1978). These studies have shown that both VIO and CAP affect the dissociation of the 70S ribosome of *M. smegmatis* by stabilization of the 70S couples and, inhibit the translocation by arresting the peptidyl-tRNA in the ribosomal A-site (Modolell and Vazquez, 1977; Stanley et al., 2010; Yamada and Bierhaus, 1978). CAP binds to the interface between helix 44 and helix 69 of the small (16S) and large (23S) ribosomal subunits, which is dependent on the methylation, by the TlyA 2'-O-methyltransferase, of C1409 in helix 44 and C1920 in helix 69 of the 16S and 23S rRNA, respectively (Johansen et al., 2006; Stanley et al., 2010).

Mutations in the 16S rRNA-encoding *rrs* gene can mediate cross-resistance between CAP, KAN and AMK. The most common mutation found in the *rrs* gene is the A1401G mutation, present in about 49.3–88.6% of the resistant isolates and, associated with resistance to KAN and AMK, and CAP low-level resistance (Table 2) (Ali et al., 2011; Bauskenieks et al., 2015; Cambau et al., 2015; Campbell et al., 2011; Du et al., 2013; Engstrom et al., 2012; Feuerriegel et al., 2009; Georghiou et al., 2012; Jugheli et al., 2009; Maus et al., 2005a; Via et al., 2010; World Health Organization, 2018; Yuan et al., 2012). The C1402T mutation does, on the other hand, mediate resistance to KAN, CAP, VIO but not AMK whereas the G1484T mutation mediates resistance to all four drugs (KAN, AMK, CAP and VIO) (Bauskenieks et al., 2015; Du et al., 2013; Maus et al., 2005a).

KAN resistance has also been linked with *eis* overexpression, a gene encoding an acetyl transferase that can multi acetylate several aminoglycosides, rendering the drugs ineffective (Chen et al., 2011). Since Eis acetylates KAN more efficiently than AMK, *eis* overexpression has been associated with KAN low-level resistance but not AMK resistance (Zaunbrecher et al., 2009). In this regard, several *eis* promoter mutations have been identified in clinical isolates (Engstrom et al., 2011; Gikalo et al., 2012). Alternatively, *eis* overexpression can be driven by mutations on the 5′ untranslated region of *whiB7* causing cross-resistance with STR as described above (Reeves et al., 2013).

CAP resistance can also be mediated by *tlyA* mutations (Engstrom et al., 2011; Maus et al., 2005b). Maus *et al* have shown that loss of TlyA

2′-o-methyltransferase function causes CAP resistance and, Manshupanee *et al* have shown that TlyA N-terminal aminoacid substitutions reduce its ability to methylate C1409 and C1920 in the 16S and 23S rRNA and that subtle changes in the level of rRNA methylation lead to significant differences in the CAP sub-inhibitory concentrations (Maus et al., 2005b; Monshupanee et al., 2012). Nevertheless, *tlyA* mutations in CAP resistant isolates are only seldom observed in CAP resistant isolates as it is more likely that CAP resistance develops because of KAN/AMK cross-resistance (Engstrom et al., 2011; Jugheli et al., 2009).

4.7. The Fluoroquinolones

FQs are quinolones fluorinated at the central ring system. These are broad-spectrum antibacterial drugs that target the bacterial DNA gyrase and topoisomerase IV, therefore inhibiting DNA replication (Badet et al., 1982). *M. tuberculosis* lacks the topoisomerase IV *parE* and *parC* subunit homologues, limiting the inhibitory activity of FQs to the DNA gyrase through interaction with both GyrA and GyrB subunits (Cole et al., 1998; Malik et al., 2012b). Quinolones bind to the DNA Gyrase-DNA binary complex producing a ternary complex that halts DNA replication leading to cell death (Piton et al., 2010). The binding site of quinolones - the Quinolone Binding Pocket (QBP), constituted by protein and DNA, is located in the enzyme's catalytic core where the drug interacts with both the GyrA and GyrB subunits and is intercalated between the dinucleotide step (Piton et al., 2010).

FQ resistance has been reported to be increasing in several settings and several studies have described an association between previous exposure to FQ prior to TB diagnosis and treatment, and an increased risk of having FQ-resistant TB (Devasia et al., 2012; Migliori et al., 2012). Devasia *et al* also associated a previous FQ treatment for > 10 days with FQ resistance (Devasia et al., 2009). This data seem to correlate with the fact that quinolones induce the bacterial SOS repair system, which is error-prone, and can induce the development of FQ resistance in *M. smegmatis* and *M. tuberculosis* (Malik et al., 2012a). Gatifloxacin (GAT) and moxifloxacin (MXF) appear to induce FQ resistant mutants at a lower rate than ciprofloxacin (CIP) and levofloxacin (LVX) (Malik et al., 2012a). Furthermore GAT and MXF are more effective (lower MICs) than CIP and ofloxacin (OFX) and can be used to treat FQ low-level resistant isolates (Chen et al., 2012). Von Groll *et al* has also observed that almost complete cross-resistance between OFX, MXF and GAT existed (Von Groll et al., 2009).

The molecular basis of FQ resistance has been associated with *gyrA* and *gyrB* mutations, particularly in mutational hotspots denominated Quinolone Resistance Determining Regions (QRDR) (Takiff et al., 1994). GyrA QRDR is located between codons 74–95, whereas GyrB QRDR is proposed to be comprehended between codons 500–540 (Pantel et al., 2012; Shi et al., 2006). Both QRDRs are located at the QBP according to the DNA gyrase crystal structure (Pantel et al., 2012; Piton et al., 2010). The most common mutations associated with FQ resistance occur in *gyrA* in codons 94 and 90, in 46.2–71.9% and 4.0–43.0% isolates, respectively (Table 2) (Campbell et al., 2011; Chen et al., 2012; Cheng et al., 2004; Cui et al., 2011; Devasia et al., 2012; Feuerriegel et al., 2009; Long et al., 2012; Singh et al., 2015; Suzuki et al., 2012; Von Groll et al., 2009). Mutations occurring in the *gyrB* gene have also been described, although at a lesser frequency and some with questionable role in FQ resistance (Feuerriegel et al., 2009; Malik et al., 2012b; Pantel et al., 2011). Double mutations have also been described to act synergistically to confer FQ-resistance or decreased FQ susceptibility (Long et al., 2012; Malik et al., 2012b; Suzuki et al., 2012).

Data from structural analysis show that *gyrA* and *gyrB* mutations can cause FQ resistance through the modification of the QBP geometry or, lead to the modification of the DNA structure in the QBP when mutations in aminoacid residues that interact with the DNA occur (Piton et al., 2010).

Regarding resistance levels, several studies convey the notion that mutations at the *gyrA*94 codon are usually associated with a slightly higher resistance level, particularly the D94G mutation (Table 2) (Chien et al., 2017; Kambli et al., 2015; World Health Organization, 2018). Differences in MIC-mutation correlations between studies may also be related with the observation that FQ MIC can exhibit some degree of variability according with the strain's genetic background and efflux activity (Chien et al., 2017; Malik et al., 2012b; Singh et al., 2011).

4.8. The new kids on the block: Bedaquiline and Delamanid

BDQ and DLM are the first new anti-TB drugs to become available in 40 years and the approval of both drugs by the Food and Drug Administration and European Medicine Agency led to a new hope for improved and more effective treatment regimens for MDR and XDR-TB (European_Medicines_Agency, 2013; Master and Furin, 2016).

DLM is a dihydro-nitroimidazooxazole derivative with a highly potent activity against either actively growing or dormant *M. tuberculosis* (Matsumoto et al., 2006; Stinson et al., 2016). As PZA or INH, DLM is a pro-drug that requires activation to its reactive form: an intermediate metabolite produced between DLM and desnitro-imidazooxazole derivative (Chen et al., 2017; Fujiwara et al., 2018). This activation step from the pro-drug DLM is catalysed by the deazaflavin (F_{420})-dependent nitroreductase Ddn and leads to the synthesis inhibition of methoxy-mycolic acids and keto-mycolic acids by its active intermediate metabolite (Matsumoto et al., 2006).

Loss of Ddn function by mutations on *ddn* gene is one of the main genetic mechanisms leading to DLM resistance by disruption of the F_{420}-dependent nitroreductase pathway. Other resistance mechanisms include non-synonymous mutations in *fbiA*, *fbiB* and *fbiC*, coding for enzymes involved in the synthesis of F_{420} from its precursor molecule (5-amino-6-ribtylamino-2,4(1H,3H)-pyrimidinedione), that produce non-functional forms of F_{420} thereby impairing the F_{420}-dependent DLM bioactivation. Also, *fgd1* mutants lead to the accumulation of an oxidized form of F_{420} which has also been associated with DLM resistance (Fujiwara et al., 2018; Purwantini and Mukhopadhyay, 2009). Mutations in any of these five genes described above are thought to mediate DLM resistance but have been mostly studied in *in vitro* selected mutants and *fbiB* mutations have not yet been detected in the few clinical isolates resistant to DLM that, despite its recent introduction, have already been reported (Bloemberg et al., 2015; Fujiwara et al., 2018; Hoffmann et al., 2016; Stinson et al., 2016). As such, further studies are warranted to fully elucidate alternative resistance pathways that underlie the genetic basis of resistance in phenotypically resistant isolates that do not harbor any mutation in these genes (Haver et al., 2015).

Regarding BDQ, it is a diarylquinoline with potent activity against *M. tuberculosis*, including *in vitro* induced dormant cells, that targets the subunit c of the ATP synthase (Andries et al., 2005; Koul et al., 2007; Koul et al., 2008). Recent experimental data also shows that BDQ acts as a H^+/K^+ ionophore that can lead to the dissipation of proton motive force and K^+ gradient and, that this activity is enhanced by binding to the ATPase thereby leading to the uncoupling of proton motive force and ATP synthesis (Hards et al., 2018). Although BDQ has been more recently introduced, some evidence points out that resistance to this drug appears to emerge rapidly (Veziris et al., 2017).

Three genes are so far known to be involved in the emergence of resistance to BDQ: *atpE*, *Rv0678* and *pepQ*. *atpE* codes for the bedaquiline target and mutations in this gene are thought to abrogate the binding of this drug to its target, resulting in high-level resistance (Koul et al., 2007). Specifically, mutations in codon A63 and I66, located on the protein membrane-spanning domain, were first detected among *in vitro* selected spontaneous mutants but since then other mutations have been found (Petrella et al., 2006; Segala et al., 2012). However, only two mutations (D28N and A63V) have been so far reported in clinical isolates which may imply an *in vivo* impaired fitness regarding other *atpE* mutations (Zimenkov et al., 2017). On the other hand, Rv0678 association with BDQ resistance concerns efflux regulation: BDQ is a

suitable substrate for MmpS5-MmpL5 efflux pump and the expression of its coding genes is kept under the control of Rv0678, that acts as a repressor to downregulate the transcription of *mmpS5* and *mmpL5* genes (Hartkoorn et al., 2014). Mutations in Rv0678 are thought to derepress both *mmpS5* and *mmpL5* and lead to BDQ resistance mediated by an increased efflux activity (Andries et al., 2014; Somoskovi et al., 2015). Also, Rv0678 mutations can mediate resistance to clofazimine (CFZ), a second-line drug, and thus result in cross-resistance between CFZ and BDQ (Gupta et al., 2014; Hartkoorn et al., 2014). This forms the genetic basis underlying several clinical cases and isolates that have never been exposed or treated with BDQ but that, nevertheless, do exhibit phenotypic resistance to BDQ (Villellas et al., 2017). *pepQ* mutations have also been linked with BDQ and CFZ cross-resistance, albeit low-level, but only in a mouse model (Almeida et al., 2016). The precise resistance mechanism or function of the *pepQ* gene is unclear.

5. Efflux: showing drugs the way out as a pathway to drug resistance

Efflux of specific molecules is mediated by a highly diverse set of efflux pumps (EPs), that can be found across both Gram-negative and positive bacteria, as well as eukaryotes. The description of the full diversity of EPs in *M. tuberculosis* is beyond the scope of this review as it has been previously addressed elsewhere (da Silva et al., 2011; Rodrigues et al., 2017). But, essentially, EPs are protein transporters that can extrude a diverse array of compounds, that can be structurally or functionally distinct, working against the influx of these molecules (da Silva et al., 2011). These transporter proteins therefore ensure a concentration gradient across the cell membrane and thereby maintain lower concentrations of these molecules on the intracellular compartment. In *M. tuberculosis*, the number of putative efflux pump coding genes is surprisingly high when compared with the efflux gene content of other bacterial species (da Silva et al., 2011). This is particularly surprising considering the restricted ecological niche that *M. tuberculosis* faces in comparison with species that have a wider distribution.

Presently, five EP superfamilies are described and classified according to its energy source and structural homology: ATP-binding cassette (ABC), major facilitator superfamily (MFS), resistance nodulation division (RND), small multidrug resistance (SMR) and, multidrug and toxic-compound extrusion (MATE). All superfamilies but MATE have been described so far in *M. tuberculosis* (Rodrigues et al., 2017). EPs are primarily designed to provide protection against toxic compounds and *M. tuberculosis* EPs such as Mmr are known to contribute to intrinsic resistance to a broad range of toxic molecules such as ethidium bromide or cetyltrimethylammonium bromide with knock-out mutants showing an increased susceptibility to such compounds (Rodrigues et al., 2013). From a clinical standpoint, *M. tuberculosis* has found a new use for EPs and has repurposed these transporter molecules to withstand anti-TB or other known antibiotics, albeit with different degrees according to the compound (Pasca et al., 2005).

In fact, efflux plays a major role in intrinsic resistance to several drugs such as vancomycin and ceftriaxone (Dinesh et al., 2013). But, probably, the most important clinical aspect pertains the phenomenon of induced drug resistance where overexpression of EPs have been demonstrated to occur upon drug exposure and can lead to reversible high-level resistance (Viveiros et al., 2002). This induced resistance phenomenon gains special importance when inadequate treatment regimens brings drug serum levels to sub-inhibitory ranges and induces the overexpression of EPs while maintaining a viable and actively replicating population. This provides a window of opportunity for the accumulation of drug resistance mutations that can lead to stable high-level resistance (Machado et al., 2012). Induced resistance has also been observed with *Mycobacterium avium* where exposure to sub-inhibitory levels of azithromycin leads to the overexpression of at least one ABC transporter and a putative MFS EP, followed by acquisition of high-level resistance to azithromycin that could not be reversed by efflux pump inhibitors such as thioridazine (Schmalstieg et al., 2012).

Not only direct exposure to drugs can elicit the upregulation of EP coding genes but it has been described that EPs are also induced upon phagocytosis by the macrophage (Szumowski et al., 2013). This upregulation of EP coding genes can lead to an antimicrobial tolerant state and it is yet to be elucidated if this induced tolerant state within the macrophage can also initiate a similar window of opportunity for the accumulation and selection of drug resistance mutations when adequate selective pressure is exerted. Consistent with these findings, EPs are also upregulated when *M. tuberculosis* is exposed to the lysosomal soluble fraction (Lin et al., 2016). Additionally, strain-specific variability may be linked with differences on the gene expression patterns involving general EP regulators, such as the MarA or WhiB7, and promote resistance development in such strains (Szumowski et al., 2013).

Another highly relevant aspect concerns the findings in which EP overexpression resulting from drug exposure can become an inheritable trait over a strain's evolutionary trajectory towards MDR or XDR. In uninduced drug resistant isolates the basal expression level of 11 genes coding for EPs were found to be significantly higher when comparing with pansusceptible isolates (Li et al., 2015). The role of efflux in the adaptive process of resistance development and amplification was demonstrated also within a single host over the evolution from pansusceptibility to XDR-TB (Eldholm et al., 2014). In this latter study, basal expression levels of the *iniBAC* operon in uninduced isolates were increased in comparison with the parental susceptible isolate, stressing the importance of efflux in the resistance acquisition process. There is an apparent evidence that upregulation of EPs is evolutionary selected during resistance development and upon drug exposure, ensuring high expression levels even when grown in the absence of anti-TB drugs. In line with these hypothesis, the recent global GWAS study by Coll et al. identified a specific mutation in *drrA*, a gene thought to code for an antibiotic transporter across the membrane, therein showing a strong association with XDR-TB when compared to susceptible isolates (Coll et al., 2018). In this same study, a promoter mutation potentially leading to the overexpression of the *mmpL13a/b* operon, encoding efflux transport proteins, was also highly associated with XDR-TB.

Altogether, efflux contributes to the resistance level shown by many clinical strains to several anti-TB drugs (Coelho et al., 2015; Machado et al., 2018). Reducing efflux activity through EP inhibitors such as thioridazine or verapamil has been exploited as an adjuvant therapeutical option that can potentiate the activity of anti-TB drugs as these inhibitors are able to reduce resistance levels and show synergism with several of these drugs (Machado et al., 2017; Yadav et al., 2016). Moreover, inhibition of efflux in intracellular *M. tuberculosis* bacilli has also been demonstrated to enhance macrophage killing by increasing phagosome acidification and activating lysosomal hydrolases and, in a mouse model, to accelerate bacterial clearance and reduce relapse (Gupta et al., 2013; Machado et al., 2018). Efflux inhibition therefore poses as an area of great interest for innovative product design with potential to prevent the emergence of resistance while simultaneously boosting the activity of current drugs. In this regard, novel EP inhibitors active within the macrophage and with intrinsic antimycobacterial activity constitute research avenues of enormous potential for the development of new lead compounds (Kumar et al., 2016).

6. Fitness and Epistasis: evolutionary consequences and workarounds for drug resistance

6.1. Tipping the Fitness Balance: how much is resistance costing?

The MTC is thought to have emerged in Africa, 70,000 years ago, from where it radiated into distinct lineages that are now distributed around the globe (Comas et al., 2013). Over its evolutionary history the MTC has been subjected to several populational bottlenecks and, as a result, its evolutive process has been mainly driven by genetic drift (Hershberg et al., 2008). However, over the last 80 years that extend

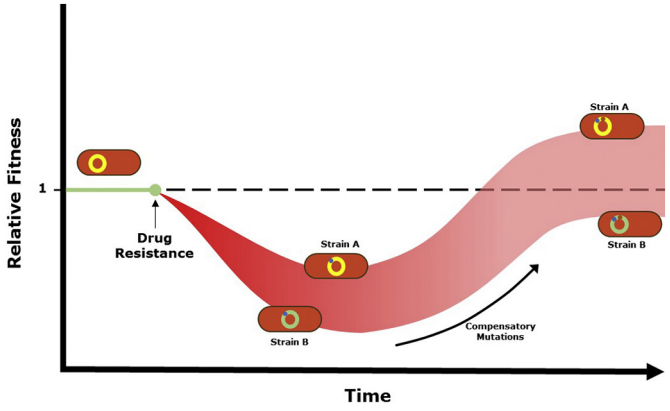

Fig. 2. - General perspective on the fitness reduction and compensation upon resistance development. Considering a given strain, a period of lower fitness can follow the acquisition of drug resistance when measured on a drug-free environment. This reduction in fitness is driven by a sub-optimal configuration of enzymes or gene expression that is required to ensure a resistance phenotype. Yet, compensatory mutations can ameliorate this reduction in fitness and reset the strain's relative fitness to levels close to the original fitness level or to levels that can even surpass the initial relative fitness. An additional layer of complexity is driven by genetic epistasis that translate into variable fitness levels according to the genetic background which, can lead to different fitness costs or to different compensated phenotypes due to different allelic configurations present in each strain.

since the dawn of the antibiotic era, new selective pressures have been imposed on the *M. tuberculosis* natural cycle of infection leading to the selection of drug resistance mutations. Often, as detailed above, most of these mutations occur at genes that are either essential or may play an important physiological part which, means that some mutations can be accommodated whilst others are partially detrimental and are not observed in isolates other than *in vitro* selected mutants. Alternatively, some mutations can be fully deleterious and are never observed. Even so, these mutations that do not result in an *in vivo* impaired phenotype may result in modified enzymes that show a sub-optimal activity when compared with the parental wild-type enzyme. As a result, resistance may come with a fitness cost in which drug resistant strains exhibit a decreased relative fitness when compared with the susceptible parental strain (Fig. 2).

One of the early methods used to assess the fitness cost associated with resistance was through competition assays, originally developed for *Escherichia coli*, in which the two clinical isolates, a drug resistant and a susceptible strain, are co-cultured and left to compete in the same environment for the same nutrients. Using this design, the number of generations is determined for each isolate within a specified timeframe and the relative fitness calculated (Wiser and Lenski, 2015). Using this approach with *M. tuberculosis* initially led to the identification and ranking of specific *rpoB* mutations associated with RIF resistance but simultaneously encompassing increasing fitness costs (Billington et al., 1999). The authors proposed that the most frequently observed mutations underlying RIF resistance in clinical isolates are the ones that are generally associated with lower fitness costs.

Similar results were obtained for INH, where KatG S315T high prevalence among INH-resistant clinical isolates is thought to be associated with a non-significant fitness cost since KatG S315T retains catalase and peroxidase activity, albeit with a 6 to 2-fold reduction, respectively, but simultaneously bearing an impaired ability to convert INH to its active form (Pym et al., 2002; Wengenack et al., 1997).

Also, mutations in *rpsL* conferring resistance to STM in clinical isolates were shown to be, more usually, of the non-restrictive type regarding translation fidelity (Bottger et al., 1998). However, *in vitro* spontaneous mutants bearing restrictive mutations arise at comparable frequencies implying that in an *in vivo* scenario a decreased translation fidelity is likely selected against.

Similar results were also obtained when comparing MDR-TB and susceptible strains sharing the same RFLP-IS6110 profile which to some degree provided a control for the genetic background (Davies et al., 2000).

This early, and more simplistic, conceptual notion that resistant strains were equal to less fit strains carried along significant implications because, when incorporated in epidemiological models, it conveyed a scenario in which MDR-TB would remain mostly a localized problem and would not pose an obstacle to TB control (Dye and Espinal, 2001). Nevertheless, this was assuming that the average fitness of drug resistant strains is significantly reduced and that it would remain unaltered.

6.2. So, is the development of drug resistance a dead-end for *M. tuberculosis*?

From an evolutionary standpoint S. Gillespie put it quite clearly in a review article back in 2001 by stating that “resistance will not disappear because there is no evolutionary disadvantage in being resistant once the adaptation has taken place” (Gillespie, 2001). This important insight came from the observation that strains belonging to the same outbreak can display a variable relative fitness over the transmission chain (Gillespie et al., 2002). This variable fitness cost suggests that strains can eventually adapt and overcome the initial decrease in fitness through a process aimed at ameliorating this fitness toll. This process was further elucidated by Gagneux et al. through the analysis of paired isolates recovered from the same patient before and after development of RIF resistance (Gagneux et al., 2006b). In the host, and contrarily to *in vitro* obtained mutants, the fitness deficit of RIF resistant strains not only was dependent on the mutation but the fitness deficit itself was reduced. In this study the authors showed that RpoB S450L mutation always showed a relative fitness equal to that of the parental susceptible isolate, if not greater, which was not verified in isolates with an underlying mutational basis for resistance other than the S450L mutation. These findings and others lent further support to strain adaptation within the host and to the amelioration of fitness costs associated with RIF resistance (Bhatter and Mistry, 2013; Shorten et al., 2013).

This process of resistance compensation was later found to be mainly driven by compensatory mutations that may or not occur at distinct *loci* (Fig. 2). The concept of compensatory mutations was not entirely novel since Sherman et al., 1996 had already reported *ahpC* overexpression as a compensatory mechanism to the loss or decreased activity of KatG catalase-peroxidase activity (Sherman et al., 1996). Secondary mutations at the A-site of the 16S rRNA were also found to mediate resistance compensation by restoring base pairing with primary resistance site (Shcherbakov et al., 2010). Yet, the development of NGS platforms, which allowed not only variant discovery at the genome-wide scale but also provided an unprecedented resolution for modelling phylogenetic scenarios, led to the identification of an increasing number of homoplasic mutations occurring after the emergence of resistance (Casali et al., 2012). Compensatory mutations for RIF resistance are thought to be usually distributed across the *rpoA*, *rpoB* and *rpoC*, coding genes for RNA polymerase subunits, and this is transversal to other microorganisms (Brandis and Hughes, 2013; Brandis et al., 2012). In fact, in *M. tuberculosis*, *rpoC* has also been identified as a target of independent mutation highlighting its putative role in RIF resistance compensation (de Vos et al., 2013; Farhat et al., 2013). A high prevalence of mutations in this latter gene have been found in RIF resistance isolates usually mapping to the RpoA-RpoC interaction region, although some putative compensatory mutations in RpoC do not fall into these regions (Comas et al., 2012; de Vos et al., 2013; Perdigao et al., 2014).

Whilst compensatory mutations usually emerge either as secondary mutations to the same target (e.g. *rrs*) or as hypermorphic mutations that drive the overexpression of genes that can compensate for the

decreased activity of an altered target gene (e.g. *ahpC/katG*), another compensatory mechanism has been recently reported and based on a non-mutational compensation of A1401 mutations in *rrs* (Freihofer et al., 2016). According to this study, through an unknown regulatory mechanism, the overexpression of methyltransferase TlyA, which methylates the adjacent position in the 16S rRNA (C1402), reduces the fitness cost of A1401 mutations at the expense of a reduced resistance level (Freihofer et al., 2016; Gygli et al., 2017). Corroborating this finding is the fact that the Lisboa3 strains in Portugal acquired CAP resistance by TlyA loss of function mediated by a frameshift mutation (Perdigao et al., 2013). In these strains, resistance to KAN was concomitantly acquired through a G-10A hypermorphic mutation at the *eis* gene instead of an A1041G mutation as has happened with the Q1 strains also in Portugal. According to the mechanism proposed by Freihofer et al. for rrs A1401G, resistance compensation in these strains would be impossible due to TlyA loss of function and, may explain the reason why these strains never evolved high level resistance to KAN and AMK (Freihofer et al., 2016; Perdigao et al., 2013).

6.3. In the end, resistant strains do bypass fitness costs and achieve epidemiological success!

If a strain can ameliorate the fitness toll imposed by drug resistance, the consequences of being less fit may not be, from an epidemiological viewpoint, as dramatic for drug resistant strains as initially thought and the current incidence of M/XDR-TB already discussed herein attests for it. In fact, other epidemiological models showed that a relatively fit MDR strain can eventually outcompete the drug sensitive strain and other less fit MDR strains (Cohen and Murray, 2004). This is the case for many MDR-TB clones displaying less costly mutations coupled with compensatory mechanisms and is also concordant with the report that, e.g., some W-Beijing strains did not show reduced growth rates and were therefore presumed to maintain their fitness even after resistance acquisition (Toungoussova et al., 2004). Furthermore, even atypical Beijing strains (“ancient” branch, without an IS*6110* at the NTF locus), which are regarded as less virulent, have been reported to acquire resistance and disseminate in vulnerable populations (Strauss et al., 2008).

In line with these evidences the way in which we measure *M. tuberculosis* fitness also suffers from a conceptual problem as competition assays and *in vitro* growth rates do not necessarily predict the species reproductive fitness over its host population, which also depends on multiple factors. In this regard, molecular epidemiological data plays a tremendous part in providing data on the transmission of a given strain at the populational level. The incorporation of molecular epidemiological data from distinct settings in epidemiological disease models showed that the fitness of drug resistant TB can be as high as the fitness of susceptible TB (Luciani et al., 2009). This latter study also addresses a key parameter: treatment cure rates and effective programmatic management. The authors suggest that settings having a high incidence of drug resistant TB are also more likely of having strains with a higher fitness as inefficacy in controlling the transmission of these strains have allowed these to adapt and compensate for the fitness deficits associated with resistance (Luciani et al., 2009). The implications of this rationale do in fact corroborate the notion that some M/XDR-TB strains such as the Lisboa3, Q1 or KZN do not appear to be severely impaired despite the number of drugs to which these strains are resistant (Cohen et al., 2015; Perdigao et al., 2014). The ineffective containment of these strains, and others around the globe, has likely ensured gradually decreasing fitness costs. A recent report from Golla et al., using a community-level approach, showed that a higher risk for TB infection exist in childs in a MDR-TB household when compared with a drug susceptible TB household, but a lower risk of TB disease was observed in children exposed to MDR-TB (Golla et al., 2017). This study on one hand supports an impaired virulence by MDR-TB in its setting by suggesting a lower rate of progression to active disease but, on the other hand, it suggests that MDR-TB can spread faster, which probably owes to a prolonged infectious period associated with MDR-TB.

Summarising, when transposing the notion of fitness cost to public health, a more holistic perspective on drug resistance associated costs in fitness must necessarily encompass, beyond the biological fitness, setting-associated limitations to TB programmatic management and local epidemiological constraints, such as the prevalence and incidence of other potential co-morbidities, which by itself can ensure the successful spread of less fit strains. In line with this, more recent studies, including one from Trauer et al., defines the detection rate and treatment commencement as very important factors that can significantly affect MDR-TB transmission and incidence (Trauer et al., 2014). Also, Knight et al. demonstrates that variable fitness costs due to adaptive processes and selection can also lead to a high prevalence of drug resistance (Knight et al., 2015).

6.4. A role for genetic epistasis

Another important point that is necessary to take into consideration is that fitness costs and/or drug resistance levels can and do show variability according to the genetic background. For example, Gagneux et al. in a study already mentioned above observed that the genetic background of the parental strain from which spontaneous mutants selected *in vitro* were studied did affect the fitness cost of the H450D mutation but not of S450 L or even H445Y (Gagneux et al., 2006b). These differences regarding the phenotypic outcome of the same mutation when placed in a distinct genetic environment is often referred to as epistasis and results from the interaction between multiple allelic variants (Fig. 2) (Wong, 2017). In *M. tuberculosis* there is some degree of evidence supporting the importance of epistatic interactions between drug resistance mutations and transmissibility. In North China, for example, the association between *rpoB* S450L and *katG* S315T mutations are proposed to facilitate transmission of the “modern” Beijing sublineage and therefore to comprise a positive epistatic interaction which does not take place among “ancient” Beijing strains (Li et al., 2017). These positive epistatic interactions underpin the effect of compensatory mutations but the outcome might not be the most advantageous in distinct genetic backgrounds. The Lisboa3, Q1 and KZN strains have all successfully spread throughout its endemic settings but show a different association between INH and RIF conferring mutations (Cohen et al., 2015; Perdigao et al., 2014). Also, the epistatic interaction between different mutations can have a negative effect: the association between KatG S315T mutations and RpsL R43K was less likely found in households with multiple cases which is suggestive of a decreased transmissibility even though these mutations both confer high-level resistance to INH and STR, respectively (Salvatore et al., 2016).

Positive epistasis thereby appears to play an important role in the evolutionary trajectory and selection of MDR and XDR-TB strains as multiple combinations of drug resistant mutations can result in resistance to multiple anti-TB drugs without fitness costs. In fact, Borrell et al. showed, using *M. smegmatis* as a model, that both negative and positive epistatic interactions could be established between specific combinations of *rpoB* and *gyrA* mutations (Borrell et al., 2013). In this latter study, double-mutants that bore the D94G mutation on gyrA consistently displayed a relative fitness above 1 which is consistent with its high prevalence among MDR and XDR-TB. The opposite was found for gyrA mutation G88C, which was always associated with decreased fitness levels (Borrell et al., 2013). Recently, the GWAS analysis carried out by Coll et al. identified several new putative epistatic interactions based on non-random co-occurrence of mutations in pairs of *loci* (Coll et al., 2018). Nonetheless, experimental evidence that attests for improved fitness regarding these strains is lacking but, will be of the utmost importance to generate a more comprehensive picture concerning the more likely evolutionary paths that lead to MDR/XDR in *M. tuberculosis* and, which are costlier.

7. Concluding remarks

The body of knowledge concerning the genetics of drug resistance has met a tremendous increase over the past two decades. NGS platforms have brought us the ability to characterize the full genome of drug resistant bacilli in search for otherwise cryptic mutations and further epistatic interactions underpinning compensatory mechanisms (Coll et al., 2018). Our present knowledge already allows us to predict, with a fair specificity and sensitivity, drug resistance and susceptibility from sequence data alone and WGS is becoming an important tool for resistance surveillance (Coll et al., 2015; Zignol et al., 2018). However, and although several initiatives are in pursuit, the need to bridge the gap between genotype and phenotype is still a pressing need since obtaining sequence data is becoming increasingly cheaper while obtaining quantitative susceptibility data is lagging behind (Heyckendorf et al., 2018; Starks et al., 2015; World Health Organization, 2018).

It is not only important to know the full spectrum of resistance associated genes and variants but, it is equally important to have a better understanding of how this genetic variation is geographically structured and its setting-specific prevalences. This knowledge has a broad impact on surveillance, molecular diagnosis, patient-centred approaches and, design of newer drugs. However, many countries are apparently far from benefiting from such a comprehensive knowledge since new technologies and even gold standard methods face enormous obstacles for implementation in settings lacking adequate laboratory support. Paradoxically, many of these contribute heavily to the TB global burden and its full contribution to the drug-resistant TB burden is yet to be fully seen.

Moreover, integrating molecular drug resistance data with evolutionary biology is already providing a deeper understanding on how we can reshape TB treatment regimens, and impose microevolutionary trajectories that maximize the fitness costs to *M. tuberculosis*, so that we can preserve the effectiveness of current anti-TB drugs and ensure that they will still be available for future generations (Rochford et al., 2018). Presently, the impact of MDR-TB and XDR-TB is a major roadblock to the WHO End TB strategy that aims to eliminate TB by 2035 and, in this context, it must be addressed. It is important to bear in mind that even before XDR-TB, MDR-TB was once described as "*Ebola with wings*", a still valid description and, in itself, a call to action.

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Additional information

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