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Drug resistance mechanisms and drug susceptibility testing for tuberculosis

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ABSTRACT

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is the deadliest infectious disease and the associated global threat has worsened with the emergence of drug resistance, in particular multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). Although the World Health Organization (WHO) End-TB Strategy advocates for universal access to antimicrobial susceptibility testing, this is not widely available and/or it is still underused.

The majority of drug resistance in clinical MTB strains is attributed to chromosomal mutations. Resistance-related mutations could also exert certain fitness cost to the drug-resistant MTB strains and growth fitness could be restored by the presence of compensatory mutations. Understanding these underlying mechanisms could provide an important insight into TB pathogenesis and predict the future trend of MDR-TB global pandemic. This review covers the mechanisms of resistance in MTB and provides a comprehensive overview of current phenotypic and molecular approaches for drug susceptibility testing, with particular attention to the methods endorsed and recommended by the WHO.

Key words: antimicrobial susceptibility testing, drug resistance, tuberculosis, molecular mechanisms.

Abbreviations: AMK, amikacin; BDQ, bedaquiline; CAP, capreomycin; CB, clinical breakpoint; CC, critical concentration; CFZ, clofazimine; CS, cycloserine; DLM, delamanid; DST, drug susceptibility testing; ECOFF, epidemiological cut-off value; EMB, ethambutol; ETH, ethionamide; FQ, fluoroquinolone; INH, isoniazid; IQR, interquartile range; KAN, kanamycin; LiPA, line probe assay; LZD, linezolid; MDR-TB, multidrug-resistant TB; MIC, minimum inhibitory concentration; MGIT, Mycobacteria Growth Indicator Tube; MODS, microscopic observation dr susceptibility; MOX, moxifloxacin; MTB, *Mycobacteriu tuberculosis*; MUT, mutation; NGS, next generation sequencir NRA, nitrate reductase assay; NTM, nontuberculo mycobacteria; OFX, ofloxacin; PAS, para-aminosalicylic ac pDST, phenotypic DST; POA, pyrazinoic acid; PT prothionamide; PZA, pyrazinamide; PZase, pyrazinamida QRDR, quinolone resistance-determining region; RIF, rifampic RRDR, rifampicin resistance-determining region; rRN ribosomal RNA; SLID, second-line injectable drugs; ST streptomycin; TB, tuberculosis; WGS, whole-genor sequencing; WHO, World Health Organization; WT, wild-ty XDR-TB, extensively drug-resistant TB.

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculo* (MTB) remains a major public health concern. Despi having an effective treatment regimen, 10.4 million ne cases and 1.7 million TB-related deaths were reported 2016.[1,2] Although the rate of infection has declined glo ally, the threat of TB on public health has further wo ened with the emergence of drug-resistant TB, particular multidrug-resistant TB (MDR-TB) and exte sively drug-resistant TB (XDR-TB). It is paramount understand the molecular mechanisms of drug-resista TB in order to limit the spread of drug-resistant strai reduce treatment duration, minimize adverse drug effe and improve treatment outcomes of patients.

This review focuses on the understanding of resi tance mechanisms of the common anti-TB drugs, a the complexity related to proper drug susceptibil testing (DST). According to the most recent Wo Health Organization (WHO) treatment guidelin drugs for MDR treatment have now been classified the following groups: A (fluoroquinolones (FQ)—lev floxacin, moxifloxacin and gatifloxacin), B (second-li injectable agents—amikacin (AMK), capreomyc (CAP), kanamycin (KAN) and streptomycin (STR)), (other core second-line agents—ethionamide (ETH prothionamide (PTH), cycloserine (CS)/terizidor linezolid (LZD) and clofazimine (CFZ)) and D (add- agents—pyrazinamide (PZA), ethambutol (EMB), hig dose isoniazid (INH), bedaquiline (BDQ), delaman

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(DLM), para-aminosalicylic acid (PAS), imipenem-cilastatin, meropenem, amoxicillin-clavulanate and thioacetazone)[3]; in this review, we follow this classification, even though further updates might occur.[4]

MECHANISMS OF DRUG RESISTANCE IN MTB

The majority of drug resistance in clinical MTB strains is attributed to chromosomal mutations. The main mechanisms of resistance leading to drug resistance include drug target alteration, overexpression of drug target, disruption of prodrug activation and the activation of efflux pump.[5] This section focuses on the main mechanisms of resistance in MTB. Table 1 summarizes the main genetic regions involved in drug resistance, whereas Table 2 provides an overview on the studies focusing on fitness of drug resistance-associated mutations.

First-line drugs

First-line drugs include rifampicin (RIF) and INH as core compounds, and plus PZA and EMB as add-on agents (group D1).[2,3]

RIF was first introduced in 1972 as an anti-TB drug, and it is highly bactericidal for both growing and non-growing MTB. RIF inhibits the elongation of messenger RNA,[43] thus the majority of RIF-resistant MTB strains harbour resistance-associated mutations in the structural region of *rpoB* encoding for β-subunit of RNA polymerase, particularly at the rifampicin resistance-determining region (RRDR) from codons 426 to 452 of *rpoB* (please refer Andre *et al.*[42] for MTB reference codon numbering system). Mutations at codons 450, 445 and 435 are the most common associated with RIF resistance.[6,7] Recent studies have also reported a number of mutations outside RRDR such as V170F and I491F.[44] Mutations in *rpoB* may also lead to cross-resistance with other rifamycin derivatives, such as rifabutin.[7,45] The occurrence of monoresistance to RIF varies in different settings, and most of the RIF-resistant strains are also resistant to other anti-TB drugs. Although RIF resistance is in general a good surrogate marker for MDR-TB, in some settings up to 10% of cases can be monoresistant to RIF.[46,47]

INH, a prodrug that is activated by the catalase/peroxidase enzyme (KatG) encoded by *katG*[8] to generate nicotinoyl-NAD adduct, was introduced in 1952. The activated complex tightly binds with enoyl-acyl carrier protein reductase (InhA) and inhibits mycolic acid synthesis.[8] INH is only active against metabolically active tubercle bacilli.[5] The majority of INH-resistant strains are associated with missense mutations at codon 315 of KatG, where the S315T mutation is the most prevalent mutation accounting for 60–95% of INH resistance occurrence. The nucleotide change c-15t in the promoter region of *fabG1-inhA* leads to an overexpression of InhA, and is the second most common INH resistance associated mutation, causing resistance by drug titration mechanism. Although less frequent, mutations at the active sites such as S94A and I194T were also reported. In contrast to *katG* mutations, mutations in *inhA* are more commonly associated with low-level INH resistance (minimum inhibitory concentration, MIC: 0.2–1 mg/L). Approximately 10% of INH-resistant strains do not harbour any mutation in *katG* or *inhA*, suggesting alternative resistance mechanisms to be identified.[5] Cross-resistance between INH and ETH/PTH is discussed in Section on *Other core second-line agents (group C)*.

PZA was widely used as an anti-TB drug since 1972. PZA is a prodrug in which the antimycobacterial activity requires hydrolysis by MTB pyrazinamidase (PZase)/nicotinamidase encoded by *pncA* to convert into its active form pyrazinoic acid (POA) causing cytoplasmic acidification and depletion of membrane potential.[21,48] The activity of PZA is generally thought to be dependent on acidic pH[49,50]; however, it has recently been reported that the PZA activity can be independent of acidic pH and intrabacterial acidification.[51,52] The latter finding can be confusing as it does not mean that PZA works at neutral pH in general, irrespective of the metabolic status of the TB bacteria. Rather, it indicates that the bacterial metabolic status is important in determining the activity of PZA where in dormant persister bacteria with low metabolisms PZA could even show activity at near neutral pH, even then PZA would be expected to show more activity under acidic pH. PZA has a critical role in the current first-line anti-TB regime for its sterilizing properties allowing to eliminate a population of persister bacilli not killed by other TB drugs.[21,53] Mutations in *pncA*, resulting in a loss of function of PZase, represent the primary molecular mechanism for PZA resistance in clinical strains.[48] *pncA* mutations are diverse in nature and scattered along the entire gene (561 base pairs in length), plus additional mutations found in the promoter regions. Furthermore, not all mutations in the *pncA* gene confer PZA resistance in MTB.[54,55] Additional studies are still in need to delineate the connection of PZA mechanism and its targets. PZA was found to inhibit and trans-translation in MTB by targeting the ribosomal protein I (RpsA),[56] however a recent study challenged the proposed model.[57] More recently, it has been suggested that PZA inhibits aspartate decarboxylase (PanD) as a drug target, an enzyme critical for pantothenate and coenzyme A synthesis and in turn important for energy production.[58,59] Although there was initial confusion and doubt about PanD as a target of PZA,[60] a more recent study by Gopal *et al.* confirms the earlier finding that PanD is indeed a target of PZA.[59,61] In addition, recent studies also identified another possible target of PZA as ClpC1,[62,63] part of a protease complex involved in protein degradation, which is presumably important for persister survival. Furthermore, a recent study identified novel mutations in LprG (rv1411c), rv0521, rv3630, rv0010c, ppsC and cyp128 associated with POA/PZA resistance in MTB,[64] which sheds new light on mode of action and resistance of this intriguing persister drug.

Whereas all the first-line drugs considered above show bactericidal activity, EMB is a bacteriostatic agent. EMB primarily targets at arabinosyltransferase encoded by *embCAB* operon, which inhibits the arabinogalactan biosynthesis in MTB cell wall.[65] Resistance towards EMB is generally caused by missense mutations at *embCAB* operon, in particularly at codons 306, 406 and 497 of *embB*.[66] Among all the EMB resistance-related mutations, the most common one is *embB* M306V. In addition, missense mutation in *Rv3806c* (*ubiA*) V188A, A237V, R240C and A249G as well as overexpression of the gene were confirmed to cause increased EMB resistance.[13,67] Whereas the occurrence of

Table 1 List of drug resistance-related genes against anti-TB drugs

Drug	Resistance-related genes	Occurrence(%)	Gene function	Referen
Rifampicin	*rpoB*	95–99	RNA polymerase subunit B	6,7
Isoniazid	*katG*	60–95	Catalase-peroxidase	8,9
	inhA	8–43	Promoter region for 2-trans-enoyl-acyl carrier protein reductase	10,11
Ethambutol	*embB*	40–68	Arabinosyltransferase	12
	ubiA	9.5–45.5	5-Phospho-α-d-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyltransferase	13
Streptomycin	*rpsL*	70–85	Ribosomal protein S12	14,15
	rrs	70–85	16S rRNA	14
	gidB	N/A	Putative 16S rRNA methyltransferase	16
Quinolones	*gyrA*	97–98	DNA gyrase subunit A	17
	gyrB	N/A	DNA gyrase subunit B	18
Aminoglycosides	*rrs*	86–97	16S rRNA	19
	eis	N/A	Aminoglycoside acetyltransferase	20
Pyrazinamide	*pncA*	Up to 99	Amide conversion	21
	rpsA		S1 ribosomal protein	
	panD		Aspartate decarboxylase	
	clpC1		Protease	
Cycloserine	*ald*	N/A	L-alanine dehydrogenase	22
	alr	N/A	Alanine racemase	22,23
Para-aminosalicylic acid	*folC*	N/A	Dihydrofolate synthase	24
	dfrA	N/A	Dihydrofolate reductase	25
	thyA	37	Thymidylate synthase	24
Linezolid	*rplC*		50S ribosomal protein L3	26
	rrl		23S rRNA gene	27
Clofazimine	*rv0678*	N/A (based on laboratory-derived strains; one clinical isolate)	Transcriptional regulator to repress the expression of multisubstrate efflux pump MmpL5	28,29
	rv1979c		Possible permease	
	rv2535c		PepQ putative aminopeptidase	
Bedaquiline	*rv0678*	N/A (based on one laboratory-derived strain; one clinical isolate)	Transcriptional regulator to repress the expression of multisubstrate efflux pump MmpL5	28,30
Bedaquiline	*atpE*	N/A (laboratory-derived strain)	F0 subunit, ATP synthase	31
Delamanid	*ddn*	N/A	Deazaflavin-dependent nitroreductase	32
	fgd1	N/A	Glucose-6-phosphate dehydrogenase	33
	fbiA	N/A (based on one clinical isolate)	Protein FbiA for flavin cofactor F_{420} biosynthesis	34
	fbiB	N/A (laboratory-derived strains)	Protein FbiB for flavin cofactor F_{420} biosynthesis	35
	fbiC	N/A (laboratory-derived strains)	Protein FbiC for flavin cofactor F_{420} biosynthesis	35

rRNA, ribosomal RNA; TB, tuberculosis.

embB mutations among EMB-resistant clinical isolates was consistent throughout the globe, occurrence of *ubiA* mutations in EMB-resistant strains can vary according to the geographical region considered.[68] Although the causal relationship between *embB* and *ubiA* missense mutations with EMB resistance has been established, missense mutations at *embB* can only explain 40–68% of the global EMB resistance occurrence.[12,69] Moreover, the presence of EMB-susceptible clinical isolates with *embB* M306V or M306I missense mutations has been reported in multi studies.[70–72] Such discrepancies might be due to inappr priate critical concentrations (CC; i.e. higher than the e demiological cut-off value, ECOFF).

Fluoroquinolones (group A)

Group A includes FQ. These compounds are effecti against both growing and non-growing tubercle baci

Table 2 Overview of fitness cost conferred by drug resistance-related mutations in RIF, INH and EMB

Drug	Mutation[†]	Experiment conditions[‡]	Relative fitness[§]	Reference
RIF	*rpoA* T187A	N/A	~1.00	36
	rpoA T187P	N/A	~1.20	36
	rpoB S531L (S450L)	Competition	>1.00	37
	rpoB S531W (S450W)	Competition	0.67–0.88	37,38
		Independent	0.71	38
		In macrophage	0.28	38
	rpoB H526Y (H445Y)	Competition	0.81–0.89	37,38
		Independent	0.86	38
		In macrophage	0.63	38
	rpoB S522L (S441L)	Competition	0.54–0.88	38
		Independent	0.95	38
		In macrophage	0.50	38
	rpoB S531L (S450L)	Competition	0.91, 0.96,	37,38
	rpoB H526D (H445D)	Competition	0.78–0.81	37,38
	rpoB H526R (H445R)	Competition	0.82	37,38
	rpoB Q513L (Q432L)	Competition	0.83	37,38
	rpoB H526P (H445P)	Competition	0.84	37,38
	rpoB R529Q (R448Q)	Competition	0.58	38
	rpoC D485N	N/A	~1.00	36
INH	*ahpC* downregulation	Animal model[¶]	Reduced[¶]	39,40
	inhA C-15T	Independent	0.82–1.01	41
	katG S315T	Independent	0.82–0.96	41
EMB	*embB* M306V	Competition	0.80–0.90	13
	ubiA A237V	Competition	1.00	13
	Rv3792 L198L	Competition	0.95–1.00	13
	embB M306V + *ubiA* A237V	Competition	0.80–0.90	13
	embB M306 V + *Rv3792* L198L	Competition	0.95–1.00	13

[†]Refer Andre *et al.*[42] for *rpoB* MTB numbering system reported in parenthesis.

[‡]Experimental conditions were referred as competition, pairwise competition assay; independent, independent mtb growth assay; in macrophage, macrophage challenge experiment and N/A, not available.

[§]Relative fitness was calculated by (growth rate of mutated strain)/(growth rate of reference strains).

[¶]For study conducted to understand the effect of *ahpC* downregulations, the study was conducted to evaluate the virulence of *ahpC* knockdown MTB in immunocompromised mice.

EMB, ethambutol; INH, isoniazid; MTB, *Mycobacterium tuberculosis*; RIF, rifampicin.

Their molecular mechanism of action is by inhibiting the activity of topoisomerase II (DNA gyrase), thus inhibits subsequent DNA transcription and bacterial replication in MTB.[73] DNA gyrases are tetramers composed of two α, and two β subunits encoded by *gyrA* and *gyrB*, respectively.[74,75] FQ resistance in MTB is mainly caused by mutations in the highly conserved quinolone resistance-determining region (QRDR) of *gyrA*. The most common FQ resistance-associated mutations are at codons 90, 91 and 94, but missense mutations at codons 74 and 88 have also been reported.[76–78] Missense mutations at both codons 80 (T/A) and 95 (T/S) are regarded as natural polymorphisms unrelated to FQ resistance.[79] Mutations in *gyrB* such as A508S and G512R have been reported to confer high-level resistance to FQ.[18] Cross-resistance between FQ is common. In addition, the involvement of high-level expression of efflux pumps, such as *pstB*, has been suggested as the possible cause for the early state of FQ resistance in MTB.[80]

Second-line injectable agents (group B)

Among the group B, there are injectable anti-TB agents such as aminoglycosides (STR, KAN and AMK) and cyclic peptides (CAP) that inhibit protein synthesis by binding to the mycobacterial ribosome.

STR was first isolated from the soil microorganism *Streptomyces griseus* and became the first antibiotic used for TB treatment. Mutations in *rpsL* (30S ribosomal protein) are the major mechanism of resistance and accounts for around 50% of the resistance. Mutations in *rrs* also account for approximately 15% of STR resistance. Recently, mutations in *gidB*, a gene encoding 7-methylguanosine methyltransferase, have been suggested to reduce 16S ribosomal methylation. The mutation can lead to the decrease in the affinity between STR and 16S ribosomal RNA (rRNA)-binding site, thus causing low-level STR resistance.[81,82]

KAN and AMK inhibit protein synthesis by alteration at the level of 16S rRNA. The major aminoglycoside resistance-related mutations are located at nucleotide positions 1401 and 1402 of *rrs* gene, leading to high-level KAN and AMK resistance. Meanwhile, uncommon mutations at codon 1484 were also reported to confer resistance in KAN and AMK.[83,84] Mutations at positons −10 and −35 of the promoter region of *eis* have been also been shown to confer resistance to KAN.[19,85]

Mutation at nucleotide position 1401 of *rrs* is the major molecular mechanism of CAP resistance.

