Chapter 10

First– and Second–Line Drugs and Drug Resistance

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Additional information is available at the end of the chapter

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1. Introduction

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis*, which is transmitted through inhalation of aerosolized droplets. TB mainly attacks the lungs, but can also affect other parts of the body. TB is highly contagious during the active stage of the disease and can infect an individual through inhalation of as few as 10 *Mycobacterium tuberculosis* (MTB) bacteria. After inhalation, these bacteria are mainly captured by the alveolar macrophages, but they can evade the host immune system and remain in the dormant stage for a long period of time, at which point they can reactivate to a virulent form under immune-compromised conditions of the host. This is possible because *M. tuberculosis* can persist in slow growing as well as in fast growing stages which makes treatment challenging. Almost all of the antibiotics that can be used to treat TB work when the bacteria are actively dividing. In the intensive phase of TB treatment, the antibiotics mainly kill rapidly growing bacteria, which causes rapid sputum conversion, and the eradication of clinical symptoms. However, in order to kill the persistent or slow growing strains of MTB, the continuation phase of the treatment is essential. TB can be treated effectively by using first line drugs (FLD) isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (SM). However, this first line therapy often fails to cure TB for several reasons. Relapse and the spread of the disease contribute to the emergence of drug resistant bacteria. The emergence of multidrug resistant TB (MDR-TB), i.e. which is resistant to at least isoniazid (INH) and rifampicin (RIF), is of great concern, because it requires the use of second-line drugs that are difficult to procure and are much more toxic and expensive than FLDs [1]. Therefore, the detection and treatment of drug susceptible or single drug resistant TB is an important strategy for preventing the emergence of MDR-TB [2]. *M. tuberculosis* strains with extensively drug resistant-TB (XDR-TB), that is resistant to either isoniazid or rifampicin (like MDR tuberculosis), any fluoroquinolone, and at least one of three second-line antituberculosis injectable drugs—*i.e.*, capreomycin, kanamycin, and amikacin have also been reported [3].

First- and second-line drugs, minimum inhibitory concentrations (MICs) and mechanisms of drug resistance are presented in Table 1 [4]. Antituberculosis drugs are mainly divided into two parts.

1. First-line antituberculosis drugs- Isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA) and streptomycin (SM).
2. Second-line antituberculosis drugs- Sub divided into two
   - i. Fluoroquinolones- Ofloxacin (OFX), levofloxacin (LEV), moxifloxacin (MOX) and ciprofloxacin (CIP).
   - ii. Injectable antituberculosis drugs- Kanamycin (KAN), amikacin (AMK) and capreomycin (CAP).
   - iii. Less-effective second-line antituberculosis drugs- Ethionamide (ETH)/Prothionamide (PTH), Cycloserine (CS)/Terizidone, P-aminosalicylic acid (PAS).

Drug	MIC (mg/L)	Gene	Role of gene product
Isoniazid	0.02–0.2 (7H9/7H10)	*katG*	catalase/peroxidase
		inhA	enoyl reductase
		ahpC	alkyl hydroperoxide reductase
Rifampicin	0.05–0.1 (7H9/7H10)	*rpoB*	β-subunit of RNA polymerase
Pyrazinimide	16–50 (LJ)	*pncA*	PZase
Streptomycin	2–8 (7H9/7H10)	*rpsL*	S12 ribosomal protein
		rrs	16S rRNA
		gidB	7-methylguanosine methyltransferase
Ethambutol	1–5 (7H9/7H10)	*embB*	arabinosyl transferase
Fluoroquinolones	0.5–2.0 (7H9/7H10)	*gyrA/gyrB*	DNA gyrase
Kanamycin	2–4 (7H9/7H10)	*rrs*	16S rRNA
		eis	aminoglycoside acetyltransferase
Amikacin	2–4 (7H9/7H10)	*rrs*	16S rRNA
Capreomycin	2-4 (7H9/7H10)	*rrs*	16S rRNA
		tylA	rRNA methyltransferase
Ethionamide	2.5–10 (7H11)	*inhA*	enoyl reductase
p-aminosalicylic acid	0.5 (LJ)	*thyA*	thymidylate synthase A

Table 1. First- and second-line drugs, MICs and mechanisms of drug resistance

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2. First-line antituberculosis drugs

2.1. Isoniazid

Isoniazid (INH) is one of the most effective and specific antituberculosis drugs, which has been a key to treatment since its introduction in 1952 [5]. *M. tuberculosis* is highly susceptible to INH (MIC 0.02–0.2 μg/ml). INH is only active against growing tubercle bacilli, and is not active against non-replicating bacilli or under anaerobic conditions. INH enters the mycobacterial cell by passive diffusion [6]. The most significant adverse reactions associated with isoniazid administration are hepatotoxicity and neurotoxicity.

Resistance to isoniazid is a complex process. Mutations in several genes, including *katG*, *ahpC*, and *inhA*, have all been associated with isoniazid resistance. INH is a prodrug that is activated by the mycobacterial enzyme KatG [7]. INH-resistant clinical isolates of *M. tuberculosis* often lose catalase and peroxidase enzyme encoded by *kat*G [8], especially in high-level resistant strains (MIC > 5 μg/ml) [9]. Low-level resistant strains (MIC < 1 μg/ml) often still possess catalase activity [9]. Although mutations in *katG* have been shown to be responsible for INH resistance [10], it is not clear whether the regulation of *katG* expression plays a role in INH resistance. The *katG* gene encodes a bifunctional catalase-peroxidase that converts INH to its active form [7]. Activated INH inhibits the synthesis of essential mycolic acids by inactivating the NADH-dependent enoyl-acyl carrier protein reductase encoded by *inhA* [11].

A study by Hazbo´n et al. [12] analysed 240 alleles and found that mutations in *katG*, *inhA* and *ahpC* were most strongly associated with isoniazid resistance. A decrease in or total loss of catalase/peroxidase activity as a result of *katG* mutations are the most common genetic alterations associated with isoniazid resistance [7]. Ser315Thr is the most widespread *katG* mutation in clinical isolates, but there are many mutations that result in inactivation of catalase-peroxidase, with MICs ranging from 0.2 to 256 mg/L.

Mutations in *inh*A or its promoter region are usually associated with low-level resistance (MICs = 0.2 −1 μg/ml) and are less frequent than *kat*G mutations [10, 12]. INH-resistant *M. tuberculosis* harboring *inh*A mutations could have additional mutations in *kat*G, conferring higher levels of INH resistance [13]. The most common *inhA* mutation occurs in its promoter region (-15C → T) and it has been found more frequently associated with mono-resistant strains [14].

In *M. tuberculosis*, *ahpC* codes for an alkyl hydroperoxidase reductase that is implicated in resistance to reactive oxygen and reactive nitrogen intermediates. It was initially proposed that mutations in the promoter of *ahpC* could be used as surrogate markers for the detection of isoniazid resistance [15]. However, several other studies have found that an increase in the expression of *ahpC* seems to be more a compensatory mutation for the loss of catalase/peroxidase activity rather than the basis for isoniazid resistance [4, 16].

2.2. Rifampicin

Rifampicin (RIF) was introduced in 1972 as an antituberculosis drug and has excellent sterilizing activity. Rifampicin acts by binding to the β-subunit of RNA polymerase (*rpoB*) [17], the en-

zyme responsible for transcription and expression of mycobacterial genes, resulting in inhibition of the bacterial transcription activity and thereby killing the organism. An important characteristic of rifampicin is that it is active against actively growing and slowly metabolizing (non-growing) bacilli [18]. RIF produces relatively few adverse reactions. It may cause gastrointestinal upset. Hepatotoxicity occurs less frequently than with isoniazid administration.

Rifampicin MICs ranging from 0.05 to 1 μg/ml on solid or liquid media, but the MIC is higher in egg media (MIC = 2.5–10 μg/ml). Strains with MICs < 1 μg/ml in liquid or agar medium or MICs < 40 μg/ml in Lowenstein-Jensen (LJ) medium are considered RIF-susceptible. The great majority of *M. tuberculosis* clinical isolates resistant to rifampicin show mutations in the gene *rpoB* that encodes the β-subunit of RNA polymerase. This results in conformational changes that determine a low affinity for the drug and consequently the development of resistance [19]. Mutations in a 'hot-spot' region of 81 bp of *rpoB* have been found in about 96% of rifampicin-resistant *M. tuberculosis* isolates. This region, spanning codons 507–533 (numbering according to the *Escherichia coli rpoB* sequence), is also known as the rifampicin resistance-determining region (RRDR) [17]. Mutations in codons 531, 526 and 516 (Ser531Leu, His526Tyr, and Asp516Val) are the most frequently reported mutations in most of the studies [20, 21]. Some studies have also reported mutations outside of the hot-spot region of *rpoB* in rifampicin-resistant *M. tuberculosis* isolates [22].

2.3. Pyrazinamide

Pyrazinamide (PZA) is an important first-line antituberculosis (anti-TB) drug that is used in short-course chemotherapy and is one of the cornerstone drugs in the treatment of MDR-TB [23]. One key characteristic of pyrazinamide is its ability to inhibit semidormant bacilli residing in acidic environments [23]. Pyrazinamide is a structural analogue of nicotinamide and is a pro-drug that needs to be converted into its active form, pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase (PZase) [24]. PZA is only active against *M. tuberculosis* at acid pH (e.g., 5.5) [25]. Even at acid pH (5.5), PZA activity is quite poor, with MICs in the range of 6.25–50 μg/ml [26]. Hypersensitivity reactions and gastrointestinal upset may occur with PZA administration.

PZase is encoded in *M. tuberculosis* by the gene *pncA* [27]. Mutations in the *pncA* gene may cause a reduction in PZase activity which may be the major mechanism of PZA resistance in MTB [28, 29]. The mutations of the *pncA* gene in PZA-resistant MTB isolates has been well characterized, however the correlation varies between different geographical areas including missense mutations, one or more base insertions or deletions, and complete deletion [28-32]. Despite the highly diverse and scattered distribution of *pnc*A mutations, there is some degree of clustering of mutations within different regions of the *pncA* gene such as at amino acid residues 3–17, 61–85 and 132–142 has been reported [33, 34]. The highly diverse mutation profile in the *pncA* gene observed in PZA-resistant strains is unique among drug-resistance genes in *M. tuberculosis* [28]. While the reason behind this diversity is still unclear, it is thought that this could be due to adaptive mutagenesis or due to deficiency in DNA mismatch repair mechanisms [23]. Most PZA-resistant *M. tuberculosis* strains (72–97%) have mutations in *pnc*A; [28, 29, 34, 35] however; some resistant strains do not have *pnc*A mutations.

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2.4. Ethambutol

Ethambutol (EMB) [dextro-2,2′-(ethylenediimino)di-1-butanol], which is an essential first-line drug in tuberculosis treatment, plays an important role in the chemotherapy of drug-resistant TB [36]. EMB is also an important antimycobacterial drug as it enhances the effect of other companion drugs including aminoglycosides, rifamycins and quinolones. The most common side effects observed with ethambutol are dizziness, blurred vision, color blindness, nausea, vomiting, stomach pain, loss of appetite, headache, rash, itching, breathlessness, swelling of the face, lips or eyes, numbness or tingling in the fingers or toes. Patients taking ethambutol should have their visual acuity and color vision checked at least monthly.

The MICs of EMB for *M. tuberculosis* are in the range of 0.5–2 μg/ml. EMB is a bacteriostatic agent that is active for growing bacilli and has no effect on non-replicating bacilli. EMB interferes with the biosynthesis of cell wall arabinogalactan [37]. It inhibits the polymerization of cell-wall arabinan of arabinogalactan and of lipoarabinomannan, and induces the accumulation of D-arabinofuranosyl-P-decaprenol, an intermediate in arabinan biosynthesis [38, 39].

Arabinosyl transferase, encoded *by embB*, an enzyme involved in the synthesis of arabinogalactan, has been proposed as the target of EMB in *M. tuberculosis* [40] and *M. avium* [41]. In *M. tuberculosis*, *embB* is organized into an operon with *embC* and *embA* in the order *embCAB*. *embC, embB* and *embA* share over 65% amino acid identity with each other and are predicted to encode transmembrane proteins [40]. Mutations in the *embCAB* operon, in particular *embB*, and occasionally *embC*, are responsible for resistance to EMB [40]. Point mutations of the *embABC* gene commonly occurred in *embB* codon 306 [40, 42, 43], and mutations in the *embB306* codon have been proposed as a marker for EMB resistance in diagnostic tests [44]. However, point mutations in the *embB306* locus occur in only 50 to 60% of all EMB-resistant clinical isolates [42, 45-47], and *embB306* mutations can also occur in EMB-susceptible clinical isolates [46, 47]. Five different mutations were uncovered in this codon (ATG→ GTG/CTG/ATA, ATC and ATT), resulting in three different amino acid shifts (Met→ Val, Leu, or Ile) [43]. Although the association between *embB306* mutation and ethambutol resistance or broad drug resistance has been observed in several groups' studies with either clinical or laboratorial isolates [48, 49], the exact role of *embB306* mutations play in the development of ethambutol resistance and multidrug resistance in *M. tuberculosis* is not fully understood. About 35% of EMB-resistant strains (MIC <10 μg/ml) do not have *embB* mutations [39, 45], suggesting that there may be other mechanisms of EMB resistance. Further studies are necessary to identify the potential new mechanisms of EMB resistance.

2.5. Streptomycin

Streptomycin (SM), an aminocyclitol glycoside antibiotic, was the first drug to be used in the treatment of TB, in 1948 [50]. SM kills actively growing tubercle bacilli with MICs of 2–4 μg/ml, but it is inactive against non-growing or intracellular bacilli [23]. The drug binds to the 16S rRNA, interferes with translation proofreading, and thereby inhibits protein synthesis [51, 52]. Ototoxicity and nephotoxicity are associated with SM administration. Vestibular dysfunction is more common than auditory damage. Renal toxicity occurs less frequently than with

kanamycin or capreomycin. Hearing and renal function should be monitored in patients getting SM.

Mutations associated with streptomycin resistance have been identified in the genes encoding 16S rRNA (*rrs*) [53] and ribosomal protein S12 (*rpsL*) [54-57]. Ribosomal protein S12 stabilizes the highly conserved pseudoknot structure formed by 16S rRNA [58]. Amino acid substitutions in RpsL affect the higher-order structure of 16S rRNA [51] and confer streptomycin resistance. Alterations in the 16S rRNA structure disrupt interactions between 16S rRNA and streptomycin, a process that results in resistance [59]. Mutations in *rps*L and *rrs* are the major mechanism of SM resistance [54, 56, 57], accounting for respectively about 50% and 20% of SM-resistant strains [54, 56, 57]. The most common mutation in *rps*L is a substitution in codon 43 from lysine to arginine [54, 56, 57], causing high-level resistance to SM. Mutation in codon 88 is also common [54, 56, 57]. Mutations of the *rrs* gene occur in the loops of the 16S rRNA and are clustered in two regions around nucleotides 530 and 915 [39, 54, 56, 57]. However, about 20–30% of SM-resistant strains with a low level of resistance (MIC < 32 μg/ml) do not have mutations in *rps*L or *rrs* [60], which indicates other mechanism(s) of resistance. A mutation in *gid*B, encoding a conserved 7-methylguanosine (m(7)G) methyltransferase specific for 16S rRNA, has been found to cause low-level SM resistance in 33% of resistant *M. tuberculosis* isolates [61]. A subsequent study showed that while Leu16Arg change is a polymorphism not involved in SM resistance, other mutations in *gid*B appear to be involved in low-level SM resistance [62]. In addition, some low-level SM resistance seems to be caused by increased efflux as efflux pump inhibitors caused increased sensitivity to SM, although the exact mechanism remains to be identified [62].

3. Second-line antituberculosis drugs

3.1. Fluoroquinolones

The fluoroquinolones (FQs) have broad-spectrum antimicrobial activity and so are widely used for the treatment of bacterial infections of the respiratory, gastrointestinal and urinary tracts, as well as sexually transmitted diseases and chronic osteomyelitis [63]. In contrast to many other antibiotics used to treat bacterial infections, the FQs have excellent in vitro and in vivo activity against *M. tuberculosis* [64, 65]. FQs include ciprofloxacin, ofloxacin, levofloxacin, and moxifloxacin. So, FQs are currently in use as second-line drugs in the treatment of TB. Adverse effects are relatively infrequent (0.5–10% of patients) and include gastrointestinal intolerance, rashes, dizziness, and headache. Most studies of fluoroquinolone side effects have been based on relatively short-term administration for bacterial infections, but trials have now shown the relative safety and tolerability of fluoroquinolones administered for months during TB treatment in adults.

The cellular target of FQs in *M. tuberculosis* is DNA gyrase, a type II topoisomerase consisting of two A and two B subunits encoded by *gyrA* and *gyrB* genes, respectively [66]. Mutations in a small region of *gyrA*, called quinolone resistance-determining region (QRDR) and,

less frequently, in *gyrB* are the primary mechanism of FQ resistance in *M. tuberculosis* [66, 67]. Analysis of QRDR alone by genotypic tests has been suggested as sufficient for rapid identification of vast majority of FQ-resistant *M. tuberculosis* strains as additional targeting of *gyrB* did not enhance the sensitivity significantly [67, 68].

Mutations within the QRDR of *gyr*A have been identified in clinical and laboratory-selected isolates of *M. tuberculosis*, largely clustered at codons 90, 91 and 94 [69-73], with Asp94 being relatively frequent [71, 74]. Codon 95 (Ser95Thr) contains a naturally occurring polymorphism that is not related to fluoroquinolone resistance, as it occurs in both fluoroquinolone-susceptible and fluoroquinolone-resistant strains [75]. A less common involvement is codon 88 [76]. For clinical isolates, *gyr*B mutations appear to be of much rarer occurrence [72, 73]. Generally, two mutations in *gyr*A or concomitant mutations in *gyr*A plus *gyr*B are required for the development of higher levels of resistance [69, 77].

3.2. Aminoglycosides (kanamycin, amikacin and capreomycin)

The aminoglycosides amikacin (AMK)/kanamycin (KAN) and the cyclic polypeptide capreomycin (CAP) are important injectable drugs in the treatment of multidrug-resistant tuberculosis. Although belonging to two different antibiotic families, all exert their activity at the level of protein translation. Renal toxicity occurs from these drugs. Regular monitoring of hearing and renal function is recommended.

AMK and KAN are aminoglycosides that have a high level of cross-resistance between them [78-80]. The cyclic polypeptide CAP is structurally unrelated to the aminoglycosides and thus is a potential candidate to replace AMK or KAN if resistance to either of them is suspected [81, 82]. It has been demonstrated that the risk of treatment failure and mortality increase when CAP resistance emerges among MDR-TB cases [83]. However, cross-resistance in *M. tuberculosis* between AMK/KAN and CAP has been observed in both clinical isolates and laboratory-generated mutants [79, 80, 84, 85].

AMK/KAN and CAP primarily affect protein synthesis in *M. tuberculosis* and resistance to these drugs is associated with changes in the 16S rRNA (*rrs*) [78, 80, 81, 85, 86]. The *rrs* mutation A1401G causes high-level AMK/KAN and low-level CAP resistance. C1402T is associated with CAP resistance and low-level KAN resistance. G1484T is linked to high-level AMK/KAN and CAP resistance [79, 80, 84, 86]. Low-level resistance to kanamycin has been correlated to mutations in the promoter region of the *eis* gene encoding aminoglycoside acetyltransferase, the enhanced intracellular survival protein, Eis [87].

Resistance to the cyclic peptide capreomycin has also been associated with mutations in *tlyA* [86]. The gene *tlyA* encodes a putative 2′-O-methyltransferase (TlyA) that has been suggested to methylate nucleotide C1402 in helix 44 of 16S rRNA and nucleotide C2158 in helix 69 of 23S rRNA in *M. tuberculosis* [81, 88]. Capreomycin binds to the 70S ribosome and inhibits mRNA–tRNA translocation [89]. It is believed that TlyA methylation enhances the antimicrobial activity of capreomycin [81] and that disruption of *tlyA* leads to cap-

reomycin resistance because the unmethylated ribosome is insensitive to the drug [81, 86, 88]. The identified mechanism of capreomycin resistance on the basis of in vitro selected mutants has found that *tlyA* mutations were common [80, 86] whereas infrequent in clinical isolates of *M. tuberculosis* [79, 80].

3.3. Ethionamide/prothionamide

Ethionamide (ETH, 2-ethylisonicotinamide) is a derivative of isonicotinic acid and has been used as an antituberculosis agent since 1956. The MICs of ETH for *M. tuberculosis* are 0.5–2 μg/ml in liquid medium, 2.5–10 μg/ml in 7H11 agar, and 5–20 μg/ml in LJ medium. Ethionamide and the similar drug prothionamide (PTH, 2-ethyl-4-pyridinecarbothioamide) act as prodrugs, like isoniazid. Which is activated by EtaA/EthA (a mono-oxygenase) [90, 91] and inhibits the same target as INH, the InhA of the mycolic acid synthesis pathway [92]. Once delivered into the bacterial cell, ethionamide undergoes several changes. Its sulfo group is oxidized by flavin monooxygenase, and the drug is then converted to 2-ethyl-4-aminopyridine. The intermediate products formed before 2-ethyl-4-aminopyridine seem to be toxic to mycobacteria, but their structures are unknown (may be highly unstable compounds). Mutants resistant to ethionamide are cross-resistant to prothionamide. ETH frequently causes gastrointestinal side effects, such as abdominal pain, nausea, vomiting and anorexia. It can cause hypothyroidism, particularly if it is used with *para*-aminosalicyclic acid.

3.4. *p*-Amino salicylic acid

p-Amino salicylic acid (PAS) was one of the first antibiotics to show anti-TB activity and was used to treat TB in combination with isoniazid and streptomycin [93]. Later, with the discovery of other more potent drugs including rifampicin, its use in first line regimens was discontinued. PAS is still useful as part of a treatment regimen for XDR TB although its benefit is limited and it is extremely toxic. Thymidylate synthase A, encoded by *thyA*, an enzyme involved in the biosynthesis of thymine, has been proposed recently as the target of PAS in *M. bovis* BCG [94]. Most common mutation in *thyA* was Thr202Ala, though few susceptible isolates also showed the same mutation [95]. However, its mechanism of action was never clearly elucidated. The most common adverse reactions associated with PAS are gastrointestinal disturbances.

3.5. Cycloserine

Cycloserine (CS) is an antibiotic that is used to treat TB. The exact mechanism of action of cycloserine is unknown, but it is thought to prevent the tuberculosis bacteria from making substances called peptidoglycans, which are needed to form the bacterial cell wall. This results in the weakening of bacteria's cell wall, which then kills the bacteria. Cycloserine possesses high gastric tolerance (compared with the other drugs) and lacks cross-resistance to other compounds. But it causes adverse psychiatric effects; [96, 97] which is its main drawback. So, psychiatric interrogation is necessary before prescribing cycloserine drug. Cycloserine is one of the cornerstones of treatment for MDR and XDR tuberculosis [96, 97, 98]. Terizidone (a combination of two molecules of cycloserine) might be less toxic [96, 97], although studies of this drug are scarce.

4. Conclusions

Despite all the advances made in the treatment and management, TB still remains as one of the main public health problems that have plagued mankind for millennia. The challenges posed by *M. tuberculosis* infection, through its interaction with the immune system and its mechanisms for evasion, require many more breakthroughs to make a significant impact on the worldwide tuberculosis problem. The introduction of MDR and XDR strains of *M. tuberculosis* poses several problems in mycobacterial genetics and phthisiotherapy. Among the response priorities, rapid detection of anti-tuberculosis drug resistance, use of appropriate regimens for treatment, and new drug development are of paramount importance. However, regarding the dynamics of TB transmission, and also in view of rational development of new anti-TB drugs, it is extremely important to extend our knowledge on the molecular basis of drug resistance and all its complexity. It is necessary to clarify the association between specific mutations and the development of MDR-TB or the association between drug resistance and fitness. This would allow better evaluation of the transmission dynamics of resistant strains and more accurate prediction of a future disease scenario. Adequate monitoring of drug resistance, especially MDR/XDR-TB in new patients and its transmission, molecular characterization of the drug-resistant strains, and analysis of patients' immune status and genetic susceptibility are also needed to address the problem of the fitness, virulence and transmissibility of drug-resistant *M. tuberculosis* strains.

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