Evolution of drug resistance in *Mycobacterium tuberculosis*: a review on the molecular determinants of resistance and implications for personalized care

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Drug-resistant TB (DR-TB) remains a significant challenge in TB treatment and control programmes worldwide. Advances in sequencing technology have significantly increased our understanding of the mechanisms of resistance to anti-TB drugs. This review provides an update on advances in our understanding of drug resistance mechanisms to new, existing drugs and repurposed agents. Recent advances in WGS technology hold promise as a tool for rapid diagnosis and clinical management of TB. Although the standard approach to WGS of *Mycobacterium tuberculosis* is slow due to the requirement for organism culture, recent attempts to sequence directly from clinical specimens have improved the potential to diagnose and detect resistance within days. The introduction of new databases may be helpful, such as the Relational Sequencing TB Data Platform, which contains a collection of whole-genome sequences highlighting key drug resistance mutations and clinical outcomes. Taken together, these advances will help devise better molecular diagnostics for more effective DR-TB management enabling personalized treatment, and will facilitate the development of new drugs aimed at improving outcomes of patients with this disease.

Introduction

Resistance to anti-TB drugs is an escalating global health crisis. The global burden of TB remains alarmingly high, with ~10.4 million incident cases and ~1.5 million deaths reported by the WHO in 2015.¹ *Mycobacterium tuberculosis* (MTB) strains displaying *in vitro* resistance to isoniazid and rifampicin accounted for ~480000 incident cases and 250000 deaths in 2015.³ XDR-TB strains display additional resistance to both the fluoroquinolones and second-line injectable agents, and have been reported to cause disease in 106 countries to date.¹² With high mortality rates, XDR-TB poses a dire threat to public health, exacerbated by its deadly interaction with the HIV/AIDS epidemic.

Additional resistance beyond XDR has been described as totally drug-resistant TB, which displays further resistance to drugs used to treat XDR-TB, resulting in programmatically untreatable forms of TB.³ This, coupled with estimates from published studies that suggest that current treatment options for XDR-TB fail to cure 30%–75% of patients with XDR-TB, contributes to an emerging public health crisis.⁴⁻⁶ New drugs such as bedaquiline and delamanid, and repurposed drugs such as linezolid, have been introduced into drug-resistant TB (DR-TB) treatment regimens. Despite the availability of new drugs, limited access to these agents and/or the inability to construct an effective regimen containing at least four active drugs, contribute to ongoing poor outcomes in DR-TB, including treatment failure and mortality. The management of DR-TB is further complicated by the high cost, long duration and debilitating toxicity of currently available second-line drugs.³ Current treatment guidelines indicate standardized fixed dose 6 month regimens for new treatment and re-treatment of drug-susceptible TB (DS-TB). In the case of DR-TB, the conventional 18–24 month treatment regimen has been redesigned and now ranges between 9 and 24 months based on individual patient eligibility such as previous TB history and drug exposure. In addition to duration, complex multidrug regimens and optimal medication adherence are required for effective treatment of TB infection.⁵,¹⁰ Challenges of adherence are linked to complex dosing strategies, serious and often life-threatening drug side effects, and drug–drug interactions.

This review provides an update on scientific advances in understanding drug resistance mechanisms in MTB, to new, existing and repurposed drugs. We also highlight developments in sequencing technology and bioinformatics that enable personalized therapy for DR-TB.

Implications for personalized therapy for DR-TB

The diagnosis of DR-TB remains a challenge. Currently, the frontline molecular diagnostic assay for the detection of drug resistance...
is the Cepheid Xpert MTB/RIF (GeneXpert), which detects the presence of MTB bacilli and simultaneously detects rifampicin resistance.11 Whilst rapidly identifying patients eligible for MDR-TB treatment, the test is limited to the detection of rifampicin resistance. In addition to the GeneXpert, the WHO endorsed the use of the Hain line probe assay.12 Current versions include the Genotype MTBDRplus and Genotype MTBDRslv2.0, which collectively detect resistance to isoniazid, rifampicin, fluoroquinolones and second-line injectable agents.13,14 However, analysis of published performance data of the tests shows suboptimal sensitivities.15,16 Thus, WGS remains the most desirable platform to perform follow-on testing on rifampicin-resistant TB, which can accelerate the initiation of effective treatment.

The current gold standard for MTB drug susceptibility testing (DST) is culture on solid media, which takes several weeks to months owing to the slow growth rate of TB in vitro. Treatment is therefore often empirical, based on factors such as past medical or social history, or local prevalence of resistance. This results in delays in the initiation of appropriate treatment.1,7 The use of empirical treatment regimens often leads to overprescribing of drugs with adverse effects including irreversible hearing loss, renal toxicity and hepatotoxicity. In contrast, suboptimal treatment increases the potential for the development of drug resistance.18,19

An additional challenge is the misdiagnosis of infection by nontuberculous mycobacteria (NTM) as TB. Pulmonary infections caused by NTM are gaining recognition for increasing isolation in clinical settings worldwide.20 The presence of NTM as commensals in pulmonary samples confounds MTB diagnosis, particularly in patients with a previous TB history and other chronic pulmonary conditions. Furthermore, the clinical signs and symptoms of NTM infection are clinically and radiologically indistinguishable from MTB infection, underscoring the need for a reliable molecular-based diagnostic.21 These factors clearly underscore the urgent need to detect rapidly the drug resistance and initiate personalized treatment for every patient presenting with DR-TB to prevent ongoing DR-TB transmission and effectively control TB globally.

Drug resistance in M. tuberculosis

The primary vehicle driving drug resistance in MTB is the acquisition of mutations in genes that code for drug targets or drug-activating enzymes. These are mainly in the form of SNPs, insertions or deletions (indels) and to a lesser extent, large deletions. Unlike other bacteria, resistance is not acquired via horizontal gene transfer by mobile genetic elements.22

Drug resistance in TB occurs through two main mechanisms: (i) primary or transmitted drug resistance, occurs when resistant strains are transmitted to a new host, and (ii) secondary or acquired drug resistance, which occurs through the acquisition of drug resistance mutations to one or more drugs.23-25

Studies that have examined the progressive development of drug resistance using WGS have shown the initial acquisition of isoniazid resistance, followed by resistance to rifampicin or ethambutol, then resistance to pyrazinamide and finally, resistance to second- and third-line drugs. These studies have provided valuable insights into the evolution of the organism.26-30 Estimated probabilities for the acquisition of resistance by spontaneous mutation are ~1 in 10^8 bacilli for rifampicin, to ~1 in 10^6 bacilli for isoniazid, streptomycin and ethambutol.31 However, recent studies report that the rate of mutations causing drug resistance varies according to the lineage to which the strain belongs. The Beijing strain family, which is strongly associated with DR-TB in many settings, has demonstrated increased mutation rates in vitro.32-34

The following section details the mutations mediating resistance to each of the anti-TB drugs. Table 1 summarizes the genes associated with resistance, MICs and mutation frequencies to existing anti-TB agents and new/repurposed agents, respectively.

Mechanisms of resistance to first-line drugs

Isoniazid

Isoniazid is a prodrug activated by the catalase/peroxidase enzyme encoded by the katG gene. Once activated, isoniazid inhibits mycolic acid synthesis via the NADH-dependent enoyl-acyl carrier protein reductase, encoded by the inhA gene.23,24 The molecular basis of isoniazid resistance is mediated by mutations in the katG, inhA gene or within the promoter region of the inhA gene. The most common resistance mechanism has been identified as the katG S315T mutation, which leads to an inefficient isoniazid–NAD product inhibiting the antimicrobial action of isoniazid. This mechanism is associated with low-level isoniazid resistance in MDR isolates.35-38 Mutations of the inhA promoter region, the most common being found at position −15, result in an overexpression of inhA. This mechanism is associated with low-level resistance in isoniazid monoresistant isolates and has been implicated in cross-resistance to a structural analogue, ethionamide. Mutations in the active region of the inhA gene result in a decreased affinity of the isoniazid–NAD product. Such mutations are less frequent.36,39 A recent study reported that mutations occurring in the inhA regulatory region and coding region resulted in high-level isoniazid resistance and cross-resistance to ethionamide.40 Mutations in the dfrA gene have recently been implicated in resistance to isoniazid. The 4R isomer of the isoniazid–NAD product inhibits dihydrofolate reductase, encoded by dfrA. However, studies have failed to demonstrate a correlation between mutations in dfrA and isoniazid resistance.41 Mutations in the promoter region of the ahpC gene were proposed as proxy markers for isoniazid resistance. The ahpC gene in MTB codes for an alkyl hydroperoxidase reductase enzyme responsible for resistance to reactive oxygen and nitrogen derivatives. Further analysis of such mutations revealed that this is a compensatory mechanism for the reduction or loss of activity of the catalase–peroxidase system and does not confer isoniazid resistance.42 Studies have also reported mutations in the kasA, oxyR-ahpC and furA-katG in isoniazid-resistant isolates of MTB. However, their exact role in mediating isoniazid resistance is yet to be demonstrated.43,44 More recently, a silent mutation in the inhA promoter gene resulting in the upregulation of inhA resulted in isoniazid resistance.45 A recent systematic review found that mutations in katG and inhA accounted for 64.2% and 19.2% of isoniazid resistance, respectively. These two mutations, in combination with commonly occurring mutations in the inhA promoter and the ahpC-oxyR, account for ~84% of global phenotypic isoniazid resistance.46 Recent WGS analysis showed overwhelming evidence that isoniazid resistance precedes rifampicin resistance, associated with the katG S315T mutation. This makes this mutation an ideal marker of the pre-MDR phenotype.28 Globally, the case rate of isoniazid mono-resistance is estimated to be 2%–15%, and is
Table 1. Common genes involved in resistance in *Mycobacterium tuberculosis* to classical, new and repurposed anti-TB drugs

| Drug                                      | MIC range (mg/L) | Mutation frequency (%) among resistant isolates | Compensatory mechanisms       |
|-------------------------------------------|------------------|-------------------------------------------------|-------------------------------|
| Isoniazid: inhibition of cell wall mycolic acid synthesis | 0.02–0.2          | 70                                              | *oxyR* and *ahpC*              |
| katG                                      |                  | ~10                                             |                               |
| inhA                                      |                  | ~10                                             |                               |
| kasA                                      |                  |                                                 |                               |
| Rifampicin: inhibition of RNA synthesis    | 0.05–1           | 95                                              | *rpoA* and *rpoC*              |
| rpoB                                      |                  |                                                 |                               |
| Ethambutol: inhibition of cell wall arabinogalactan biosynthesis | 1–5              | ~70                                             | unknown                       |
| embB                                      |                  | ~45, occurs with embB mutations                  |                               |
| ubiA                                      |                  |                                                 |                               |
| Pyrazinamide: reduction of membrane energy; inhibition of trans-translation; inhibition of pantothenate and coenzyme A synthesis | 16–100           | ~99                                             | no clinical evidence          |
| pncA                                      |                  |                                                 |                               |
| rpsA                                      |                  |                                                 |                               |
| panD                                      |                  |                                                 |                               |
| Streptomycin: inhibition of protein synthesis | 2–8              | ~6                                              | unknown                       |
| rpsL                                      |                  | <~10                                            |                               |
| rrs                                       |                  |                                                |                               |
| gidB                                      |                  |                                                |                               |
| Fluoroquinolones: inhibition of DNA synthesis | 0.5–2.5         | ~90                                             | *gyrA* (T80A and A90G) putative *gyrB* |
| gyrA                                      |                  |                                                 |                               |
| gyrB                                      |                  |                                                 |                               |
| Capreomycin, amikacin and kanamycin: inhibition of protein synthesis | 2–4              | 60–70                                           | *rrs* (C1409A and G1491T)      |
| rrs                                       |                  | ~80 (low-level kanamycin)                        |                               |
| eis                                       |                  | ~3 (capreomycin)                                |                               |
| tlyA                                      |                  |                                                 |                               |
| Ethionamide: inhibition of cell wall mycolic acid synthesis | 2.5–25           | mutations occurring in various combinations in these genes account for ~96% of ethionamide resistance | unknown                       |
| ethA                                      |                  |                                                 |                               |
| mshA                                      |                  |                                                 |                               |
| ndh                                       |                  |                                                 |                               |
| inhA                                      |                  |                                                 |                               |
| inhA promoter                             |                  |                                                 |                               |
| Para-aminosalicylic acid: inhibition of folic acid and thymine nucleotide metabolism | 1–8              | ~40                                             | to be determined               |
| thyA                                      |                  |                                                 | ~90                           |
| folC                                      |                  |                                                 |                               |
| ribD                                      |                  |                                                 |                               |
| Bedaquiline: inhibition of mycobacterial ATP synthase | 0.06–1           | clinical relevance of mutations to new drugs is to be determined. *atpE* described in two clinical isolates to date. *rv0678* occurs intrinsically, without prior exposure to drug. *PepQ* not detected in clinical isolates | unknown                       |
| *rv0678*                                  |                  |                                                 |                               |
| *atpE*                                    |                  |                                                 |                               |
| *pepQ*                                    |                  |                                                 |                               |
associated with worse outcomes.47–54 This underscores the need to detect resistance in its earliest form. A study by the same authors describes the novel katG A290P, katG L427P, fadE24 R454S and fabD A159T mutations in lineage 1 and 3 isolates from southern India. The role of these mutations in mediating resistance to isoniazid requires further investigation.55-free acid.44

### Rifampicin

Rifampicin is one of the most effective anti-TB drugs because it is effective against actively metabolizing and slow-metabolizing bacilli, making the drug a key component of the current first-line treatment regimen applied to the treatment of DS-TB.23,24,56,57 In MTB, rifampicin binds to the β subunit of the RNA polymerase, resulting in the inhibition of elongation of mRNA. Resistance to rifampicin is mediated by mutations clustered in codons 507–533 of the gene coding for the RNA polymerase β subunit, rpoB. This region is known as the rifampicin resistance-determining region, which is the target of modern molecular-based assays and accounts for 96% of rifampicin resistance. Codons 526 and 531 harbour the most common mutations associated with rifampicin resistance.23,24,58-61 Mutations outside the rifampicin resistance-determining region have been reported in rifampicin-resistant isolates.62 Studies have also demonstrated a lack of alteration in the rpoB gene in a fraction of rifampicin-resistant isolates, suggesting other mechanisms of rifampicin resistance.62 Rifampicin monoresistance is rare as rifampicin resistance occurs in conjunction with resistance to other drugs, most commonly isoniazid, making rifampicin targets a surrogate marker of the MDR phenotype.63 Mutations in rpoB have been associated with cross-resistance to all rifamycin antibiotics. Significantly, cross-resistance between rifampicin and rifabutin has been reported and attributed to mutations within the hotspot region, early regions of the rpoB gene and double mutations in codons 516 and 529. WGS analysis demonstrated mutations in the rpoA and rpoC genes, which encode the α and β\′ subunits of the RNA\(\beta\) polymerase as compensatory mechanisms in isolates that bear mutations in the rpoB gene. These mutations are associated with increased fitness and transmissibility of resistant strains. However, recently the phenomenon of rifampicin-dependent/–enhanced strains has emerged. Such strains have been reported to grow poorly in normal culture media that lacks rifampicin. Zhong et al. described the phenomenon in 39% of the MDR-TB strains analysed in a study conducted in China. Whilst the mechanism under which such strains arise is yet to be elucidated, it is believed that they occur as MDR-TB, and are selected by repeated treatment with the drug.65,66

### Pyrazinamide

Pyrazinamide is a nicotinamide analogue that has significantly reduced the duration of DS-TB treatment to 6 months. A vital characteristic of pyrazinamide is its ability to inhibit semi-dormant bacilli located in acidic environments such as that of TB lesions.57 Pyrazinamide not only constitutes a part of the standard first-line regimen to treat TB but is also a key component of all current regimens undergoing evaluation in Phase II and III clinical drug trials for the treatment of DS- and DR-TB.57 Pyrazinamide is a prodrug that is activated by the pyrazinamidase/nicotinamidase (PZase) enzyme, encoded by the pncA gene.68,69 Once activated, pyrazinoic acid disrupts the bacterial membrane energetics thereby inhibiting membrane transport. Pyrazinamide enters the bacterial cell by passive diffusion and is then converted into pyrazinoic acid.
The pyrazinoic acid is then pumped out of the bacterial cell by a weak efflux mechanism. In an acidic environment, the pyrazinoic acid is protonated allowing for reabsorption into the cell, resulting in cellular damage. Pyrazinamide acid and its n-propyl ester have also been implicated in inhibition of fatty acid synthase I in MTB. It has been recently proposed that pyrazinoic acid is involved in inhibiting trans-translation in MTB. Mutations in the pncA gene and its promoter region remains the most common mechanism mediating pyrazinamide resistance. The mutations identified within this gene are diverse, with 600 unique mutations in 400 positions reported to date, accounting for 72%–99% of pyrazinamide resistance. However, pyrazinamide-resistant strains with diverse mutations in the pncA gene do not display any loss of fitness or virulence. Isolates lacking alteration in the pncA gene are reported to have mutations in the rpsA (ribosomal protein L) gene. Overexpression of rpsA has also been implicated in increased resistance to pyrazinamide. However, there was no clear demonstration that mutations in rpsA were linked to pyrazinamide resistance. A small proportion of resistant isolates lack mutations in the pncA gene, suggesting another mechanism of resistance exists. DST for pyrazinamide is technically challenging and unreliable owing to the acidic pH required for the culture. Thus, based on the reliability of pncA gene mutations described in the literature, it is proposed that prediction of pyrazinamide susceptibility based on these mutations should be applied to achieve improved treatment outcomes. A recent multi-country study reported that pyrazinamide resistance is strongly associated with rifampicin resistance, confirming that the burden of pyrazinamide resistance is in patients with rifampicin resistance. More recently, panD mutations have been associated with pyrazinamide resistance. WGS analysis revealed the presence of panD mutations in pyrazinamide-resistant isolates and the inclusion of these in screening has been recommended to enhance the detection of pyrazinamide resistance. In a separate study aimed at understanding the molecular basis of pyrazinamide resistance it was demonstrated that H21R and I49V double mutations occurring in panD decrease the binding affinity for pyrazinamide.

**Ethambutol**

Ethambutol was first introduced as an anti-TB drug in 1966 and remains a part of the current first-line regimen. Ethambutol is active against actively multiplying bacilli, disrupting the biosynthesis of the arabinogalactan in the cell wall. The embCAB operon encodes the mycobacterial arabinosyl transferase enzyme. Resistance to ethambutol is mediated via mutations in the embB gene. Alteration in codon 306 of the embB gene is the most common resistance mechanism reported to date. It was further reported that this mutation predisposes the isolate to develop resistance to other drugs and is not necessarily involved in ethambutol resistance. Allelic exchange experiments have demonstrated that only certain amino acid substitutions led to ethambutol resistance. Studies have shown that mutations in the decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes (Rv3806c and Rv379), which occur simultaneously with mutations in embB and embC, result in a variable MIC range for ethambutol. This depends on the type of mutation present. Furthermore, this implies that the embB306 mutation results in varying degrees of ethambutol resistance but does not cause high-level ethambutol resistance on its own. Approximately 30% of ethambutol-resistant isolates lack alteration in embB, suggesting a different mechanism of resistance. Additive mutations occurring in ubiA have been reported to cause high-level ethambutol resistance when they occur with embB mutations. The ubiA gene encodes decaprenyl-phosphate 5-phosphoribosyltransferase synthase, which is involved in cell wall synthesis. Alteration in ubiA is reported to be lineage specific, and is predominant in the African isolates.

**Streptomycin**

Streptomycin, an aminoglycoside antibiotic, was the first drug to be applied to the treatment of TB in 1942. Owing to the initial application of the drug as TB monotherapy, resistance rapidly emerged. Streptomycin is active against slow-growing bacilli and acts by irreversibly binding to the ribosomal protein S12 and 16S rRNA, which are the components of the 30S subunit of the bacterial ribosome. Through this interaction, streptomycin blocks translation thereby inhibiting protein synthesis. The main mechanism of resistance to streptomycin is believed to be mediated via mutations in the rpsL and rrs genes, encoding the ribosomal protein S12 and the 16S rRNA, respectively, accounting for ~60%–70% of streptomycin resistance. Recently, mutations in the gidB gene, encoding a 7-methylene-guanosine methyltransferase specific for methylation of the G527 in loop of the 16S rRNA, have been implicated in low-level streptomycin resistance. Whole-genome analysis has also demonstrated a 130 bp deletion within the gidB gene possibly mediating streptomycin resistance.

**Mechanisms of resistance to second-line drugs**

**Second-line injectable agents**

The aminoglycosides kanamycin and amikacin and the cyclic polypeptide capreomycin are second-line injectable agents currently applied to the treatment of drug-resistant TB. Although these belong to different classes of antibiotics, they all exert their effect via the same target. All three drugs are protein synthesis inhibitors that act by binding to the bacterial ribosome resulting in a modification of the 16S rRNA structure. High-level resistance has been associated with mutations in the 1400 bp region of the rrs gene and additional resistance to capreomycin has been associated with polymorphisms of the ftyA gene. This gene codes for rRNA methyltransferase required for 2′-O-methylation of ribose in rRNA. The A–G polymorphism at position 1401 of the rrs gene and additional resistance to capreomycin has been associated with polymorphisms of the ftyA gene. This gene codes for rRNA methyltransferase required for 2′-O-methylation of ribose in rRNA. The A–G polymorphism at position 1401 of the rrs gene and additional resistance to capreomycin has been associated with polymorphisms of the ftyA gene. This gene codes for rRNA methyltransferase required for 2′-O-methylation of ribose in rRNA. The A–G polymorphism at position 1401 of the rrs gene and additional resistance to capreomycin has been associated with polymorphisms of the ftyA gene. This gene codes for rRNA methyltransferase required for 2′-O-methylation of ribose in rRNA. The A–G polymorphism at position 1401 of the rrs gene and additional resistance to capreomycin has been associated with polymorphisms of the ftyA gene. This gene codes for rRNA methyltransferase required for 2′-O-methylation of ribose in rRNA. The A–G polymorphism at position 1401 of the rrs gene and additional resistance to capreomycin has been associated with polymorphisms of the ftyA gene. This gene codes for rRNA methyltransferase required for 2′-O-methylation of ribose in rRNA.
between kanamycin and amikacin was initially assumed; however, other studies have demonstrated discordant resistance patterns between these two agents.\textsuperscript{100} It has also been reported that capreomycin resistance varies according to the level of resistance to kanamycin, and high-level resistance to kanamycin was associated with cross-resistance to capreomycin.\textsuperscript{101} More recently, mutations in the promoter region of the \textit{eis} gene have been reported to result in low-level resistance to kanamycin. The \textit{eis} gene encodes an aminoglycoside acetyltransferase enzyme. Polymorphisms at positions $-10$ and $-35$ of this gene resulted in an overexpression of its protein product and low-level kanamycin resistance. A study reported that 80\% of the clinical isolates with low-level resistance to kanamycin had genetic alterations in the promoter region of this gene.\textsuperscript{102,103}

**Fluoroquinolones**

Fluoroquinolones are potent bactericidal antibiotics currently used as second-line treatment for DR-TB. Ciprofloxacin and ofloxacin represent an older generation of antibiotics that are derivatives of nalidixic acid.\textsuperscript{77} New generation fluoroquinolones, such as moxifloxacin and gatifloxacin, are currently being considered for use in regimens for DR-TB.\textsuperscript{3} This class of antibiotics targets the DNA gyrase enzyme, thereby preventing transcription during cell replication. DNA gyrase enzymes are encoded by the \textit{gyrA} and \textit{gyrB} genes. Resistance to the fluoroquinolones has been linked to mutations occurring in a conserved region known as the quinolone resistance-determining region in the \textit{gyrA} and \textit{gyrB} genes.\textsuperscript{104–106} Fluoroquinolone-resistant strains of MTB most frequently display mutations in codons 90, 91 and 94 of the \textit{gyrA} gene. Mutations in codons 74, 88 and 91 have also been associated with fluoroquinolone resistance.\textsuperscript{107–109} It has been reported that clinically significant resistance to ciprofloxacin and ofloxacin (MIC of 2 mg/L) is conferred by a single gyrase mutation, whereas double mutations in the \textit{gyrA} or concomitant \textit{gyrA} and \textit{gyrB} mutations result in high MICs.\textsuperscript{109} A mutation detected in codon 95 of \textit{gyrA} is a natural polymorphism that has no role in mediating fluoroquinolone resistance.\textsuperscript{110} The complexity of fluoroquinolone resistance in MTB has been demonstrated by the hypersusceptibility induced by the presence of mutations in codon 80 of the \textit{gyrA} gene, particularly when occurring with other resistance-conferring mutations.\textsuperscript{111} Efflux mechanisms have also been reported to mediate fluoroquinolone resistance.\textsuperscript{112} Mutations in the \textit{gyrB} gene are rare.\textsuperscript{26} A recent multi-country analysis revealed low-level resistance to new generation fluoroquinolones. This may be accounted for by the widespread use of this drug class. Furthermore, the proposed breakpoint of 2.0 mg/L for this drug class has been reported to be too high, thus, representing an underestimation of the burden of resistance to new generation fluoroquinolones.\textsuperscript{78}

**Para-aminosalicylic acid**

Para-aminosalicylic acid, an analogue of para-aminobenzoic acid, was one of the first antibiotics used in the treatment of TB together with isoniazid and streptomycin. Para-aminosalicylic acid now forms a part of second-line treatment regimens applied to the treatment of drug-resistant TB. The mechanism of para-aminosalicylic acid resistance has only very recently been elucidated. It is suggested that para-aminosalicylic acid competes with p-aminobenzoic acid for the enzyme dihydropteroate synthase, inhibiting folate synthesis. The main mechanism mediating para-aminosalicylic acid resistance has been identified as mutations occurring in the \textit{thyA} gene, accounting for 40\% of para-aminosalicylic acid resistance.\textsuperscript{113,114} The T202A \textit{thyA} mutation, initially associated with para-aminosalicylic acid resistance was found to be a phylogenetic marker associated with the Latin American strain families rather than resistance to para-aminosalicylic acid. A recent study demonstrated that mutations in \textit{folC}, which encodes dihydrofolate synthase, conferred resistance in clinical isolates.\textsuperscript{115} \textit{ribD}, an additional enzyme of the folate pathway has been associated with para-aminosalicylic acid resistance. The A11G mutation in \textit{ribD}, resulting in overexpression of the gene, was detected in 91.7\% of clinical isolates by Zhang et al.\textsuperscript{116} Further studies are required to elucidate fully the mechanisms of para-aminosalicylic acid resistance.\textsuperscript{117}

**Ethionamide**

Ethionamide, a derivative of isonicotinic acid, is a structural analogue of isoniazid. Ethionamide is a prodrug that is activated by the mono-oxygenase enzyme, encoded by the \textit{ethA} gene. Once activated, ethionamide inhibits mycolic acid synthesis during cell wall biosynthesis by inhibiting the enoyl-acyl carrier protein reductase enzyme. Regulatory control of the \textit{ethA} gene occurs via the transcriptional repressor, EthR.\textsuperscript{118} Resistance to ethionamide is mediated by mutations in the \textit{etaA}/\textit{ethA}, \textit{ethR} and \textit{inhA} genes. Mutations in the \textit{inhA} gene mediate co-resistance to both isoniazid and ethionamide.\textsuperscript{119,120} A study has recently demonstrated the role of the mshA gene, encoding an enzyme essential to mycothiol biosynthesis as a target for ethionamide resistance using spontaneous isoniazid- and ethionamide-resistant mutants.\textsuperscript{121} Rueda et al. reported on 30 clinical isolates with high- and low-level ethionamide resistance. High-level resistance (21 of 30), defined at an MIC of $\geq 25$ mg/L, had various mutations, occurring in varying combinations in one or more of the ethionamide resistance genes. In contrast, low-level resistance (9 of 30), defined at an MIC range of 2.5–10 mg/L, was associated mainly with mutations occurring in the \textit{ethA} gene only. The role of these combinations requires further investigation.\textsuperscript{122}

**Mechanisms of resistance to new and repurposed drugs**

**Bedaquiline**

Bedaquiline is the first drug in a new class of agents, the diarylquinolines, to be used for TB treatment. Bedaquiline acts by targeting mycobacterial ATP synthase, inhibiting bacterial respiration. The drug is therefore active against dormant bacilli, an invaluable characteristic for MTB infection. \textit{In vitro} studies show MIC values of 0.03 mg/L, approximately equivalent to those of rifampicin and isoniazid in DS-MTB.\textsuperscript{123,124} Bedaquiline in combination with pyrazinamide has demonstrated remarkable sterilizing activity in a mouse model.\textsuperscript{123} Target-based mutations in the \textit{atpE} gene described in strains selected \textit{in vitro} have been associated with high-level resistance to bedaquiline, with up to 4-fold increase in MIC.\textsuperscript{125,126} The gene encodes the mycobacterial $F_{1}F_{0}$ proton ATP synthase, a key enzyme in ATP synthesis and membrane potential generation.\textsuperscript{125,126} Zimenkov et al.\textsuperscript{127} recently described the first
occurrence of \textit{atpE} D28N and A63V mutations in two clinical isolates of MTB associated with an MIC of 0.12 and 1.00 mg/L, respectively. Prior to this report, the mutations observed in the \textit{atpE} gene were described for lab-generated strains. Non-target-based mutations, such as the presence of mutations in \textit{rv0678}, result in the upregulation of the \textit{MmpL5} efflux pump, resulting in low-level bedaquiline resistance and cross-resistance to clofazimine.\cite{126,128} These mutations have been associated with at least a 4-fold increase in MIC.\cite{126} Veziris et al.\cite{129} reported the M139T \textit{rv0678} mutation that resulted in a 16-fold increase in MIC after treatment including bedaquiline. The same study reported a double nucleotide deletion at positions 18–19 and an insertion at position 140 of \textit{rv0678}, corresponding to MICS of 0.5 and 0.25 mg/L, respectively. Zimenkov et al. reported the most common mechanism associated with increased bedaquiline MICS was the presence of mutations in \textit{rv0678}. Paired isolates representing bedaquiline pre- and post-treatment for 17 patients, revealed elevated bedaquiline MICS prior to treatment. Four of these patients had mutations in the \textit{rv0678} gene associated with an MIC range of 0.06–0.25 mg/L.\cite{127} This is in keeping with a report of the high frequency of \textit{rv0678} mutations detected in MDR-TB and DS patients with no prior exposure to bedaquiline or clofazimine.\cite{130} Mutations in the second non-target mechanism, \textit{pepQ}, were reported with the association of low-level bedaquiline resistance and cross-resistance to clofazimine. Similar to \textit{rv0678}, mutations in \textit{pepQ} result in modest increases of bedaquiline and clofazimine MICS.\cite{131} However, none of the studies reported above documented \textit{pepQ} mutations in clinical isolates with confirmed resistance to bedaquiline or clofazimine.\cite{127,129,132}

\textbf{Delamanid and pretomanid}

Delamanid and pretomanid belong to the nitroimidazole class of antibiotics. Pretomanid, formerly PA-824 is a prodrug that requires activation by deazaflavin-dependent nitro-reductase, which is encoded by \textit{fdg1}. \textit{fdg1} converts the prodrug into three metabolites, which include des-nitro-imidazole and two unstable by-products. Des-nitro-imidazole compounds promote the anaerobic activity of these compounds by generating reactive nitrogen species, including nitric oxide, which may then boost the host-macrophage killing of MTB.\cite{133,134} Pretomanid has been reported to be highly active against MTB with an MIC range of 0.015–0.25 mg/L.\cite{135} Resistance to pretomanid has been linked to mutations occurring in the genes associated with prodrug activation (\textit{fdn} and \textit{fdg1}), or in genes associated with the F420 biosynthetic pathway (\textit{fdIA}, \textit{fdIB} and \textit{fdIC}).\cite{136} However, analysis of 65 strains of the MTB complex, representing the various lineages, revealed no significant impact on pretomanid MICS.\cite{136} Delamanid, formerly OPC-67683, inhibits the synthesis of methoxy-mycolic and keto-mycolic acid, components of the mycobacterial cell wall.\cite{137} Delamanid displayed potent \textit{in vitro} activity against lab strains and clinical isolates of MTB, with a reported MIC range of 0.006–0.24 mg/L.\cite{138} Bloemberg et al.\cite{139} recently reported D49Y in the \textit{fdIA} gene and a frameshift mutation in codon 49 of the \textit{fadG} gene that corresponded with increasing phenotypic delamanid resistance. Similar to pretomanid, it is a prodrug that requires activation via the same pathway, and thus, resistance to delamanid is associated with mutations in one of the five genes described above.\cite{133} The exclusive role of the drug in TB treatment regimens makes it a desirable agent for treatment regimens as this diminishes the likelihood for the development of resistance.\cite{137}

\textbf{Linezolid}

Linezolid, an oxazolidinone, is the first antibiotic in its class to be approved for the treatment of TB. Recent studies have found that treatment outcomes with regimens containing linezolid for complicated cases of MDR-TB are equal to or better than those reported for uncomplicated MDR-TB and better than those reported among patients treated for XDR-TB.\cite{108} Linezolid acts by binding to the V domain of the 5OS ribosomal subunit, thereby inhibiting an early step in protein synthesis.\cite{139} Resistance to linezolid has been associated with mutations in the 23S rRNA (\textit{rrl}) gene. Richter et al.\cite{139} reported the occurrence of four linezolid-resistant isolates in a cohort of 210 MDR-TB isolates. The MICS of the resistant isolates ranged between 4 and 8 mg/L, with no mutations present in the target genes.\cite{139} Subsequent studies conducted on \textit{in vitro} selected mutants reported the G2061T and G2572T mutations in the \textit{rrl} gene associated with high-level resistance in the range of 16–32 mg/L. Mutants bearing low-level resistance of 4–8 mg/L, had no alteration in the \textit{rrl} gene, thus supporting the former report.\cite{140} Bloemberg \textit{et al.} detected the \textit{rrl} G2576T and A2572C mutations in a patient with corresponding phenotypic linezolid resistance. They sequenced sequential isolates from the patient using WGS and confirmed that subsequent isolates represented a single Beijing strain that evolved over time. A striking observation was the loss of the \textit{rrl} mutations over time.\cite{141} More recently, a mutation in the \textit{rplC} gene, encoding the 5OS ribosomal L3 protein, in \textit{in vitro} selected mutants and clinical isolates has been described as a mechanism of linezolid resistance.\cite{142} Zimenkov \textit{et al.} reported on acquired linezolid resistance in 10 patients in a cohort of 27. The most frequent resistance mechanism is the C154R mutation in the \textit{rplC} gene. Seven of the 10 patients had alterations in the \textit{rplC} gene only. The study also reported the presence of the G2294A and C1921T \textit{rrl} mutations in two resistant isolates, together with mutations in the \textit{rplC} gene. One isolate has only the G2814T mutation. The study did not conduct MIC evaluation, resistance to linezolid was defined at a concentration of 1 mg/L in the MIGIT960 system.\cite{127} The mechanisms resulting in resistance to linezolid are yet to be elucidated.\cite{116}

\textbf{Clofazimine}

Clofazimine is conventionally used for the treatment of leprosy. Owing to the increasing prevalence of drug resistance in MTB, the drug emerged as a candidate for the treatment of DR-TB. Clofazimine is now a part of the new standardized short-course regimens proposed by WHO for the treatment of DR-TB.\cite{25} The precise mechanism of action is unknown. However, studies conducted in \textit{Mycobacterium smegmatis} revealed that it is probably a prodrug, which is reduced by NADH dehydrogenase, to release reactive oxygen species upon reoxidation by O$_2$.\cite{144} Resistance to clofazimine has been attributed to non-target mutations in \textit{rv0678}, leading to efflux of the drug. Resistance to clofazimine has been linked to cross-resistance with bedaquiline.\cite{126,128} The \textit{pepQ} and the \textit{rv1979c} genes have been recently described as additional mechanisms associated with clofazimine resistance.\cite{145} The implications of the \textit{pepQ} gene have been described above. Xu \textit{et al.} recently identified five clofazimine-resistant isolates from a cohort of patients with...
previous treatment for pre-XDR and XDR-TB, with MICs of 1.2 mg/L for bedaquiline. Four of the five patients with cross-resistance to bedaquiline had mutations in the rv0678 gene. The remaining isolate with no cross-resistance to bedaquiline had a mutation in the rv1979c gene.64

**Other mechanisms of drug resistance in M. tuberculosis**

**Compensatory evolution**

It has been postulated that resistance mutations bear a fitness cost to the bacterium. This concept emanates from the observation that isoniazid-resistant isolates displayed decreased virulence in the guinea pig model.144-146 However, studies have since demonstrated the presence of co-occurrence of secondary mutations that act as compensatory mechanisms for the impaired fitness of the pathogen. These compensatory mutations are believed to occur in genes encoding the same protein or genes involved in similar metabolic pathways.64 Sherman and Mduli demonstrated this phenomenon in isoniazid-resistant isolates of MTB with an inactivated katG gene.145 The absence of katG catalase–peroxidase activity resulted in mutations in the regulatory region of the ahpC (alkyl hydroperoxidase reductase) gene, leading to overexpression of this gene. Mutations of the ahpC gene are believed to be compensatory for the loss of katG activity.145 More recently, whole-genome analysis demonstrated that mutations occurring in RNA polymerases rpoA and rpoC were compensatory for the loss of fitness mediated by mutations in the rpoB gene in rifampicin-resistant isolates.146-148 Reports on the varying levels of capreomycin resistance amongst A1401G laboratory mutants and clinical isolates bearing the same mutation, imply a possible interplay of a compensatory mechanism.99,149 Similarly, mutations in gyrB may account for resistance-conferring mutations found in the gyrA gene.132,150

**Efflux-mediated resistance**

Efflux pump systems are involved in expelling drugs from the bacterial cell, enabling acquisition of resistance mutations in the bacterial genome. MTB presents with one of the largest number of putative efflux pumps with 148 genes coding for membrane transport proteins within its 4.4 Mb genome. The contribution of these efflux systems in acquiring multidrug resistance in MTB has been demonstrated by a number of studies.151,152 The overexpression of efflux pumps is believed to mediate the build-up of resistance mutations, which confers high-level drug resistance allowing MTB to survive and persist at clinically relevant drug concentrations. The ability of the efflux pumps to extrude a diversity of compounds allows them to expel multiple drugs leading to the MDR phenotype.151,152 Efflux pump inhibitors are compounds capable of restoring the activity of antibiotics independent of the level of resistance. The inhibitor–antibiotic combination decreases the MIC of the antibiotic. The use of efflux pump inhibitors has been considered as an adjuvant in TB treatment and has the potential to reduce the duration of TB treatment.64,126,152-154

**Deficient DNA repair mechanisms**

Mutations occurring in DNA repair systems alter the ability of such systems to repair efficiently the damaged DNA, thereby increasing mutation rates. This provides a selective advantage to bacteria that bear resistance-conferring mutations.155,156 Missense mutations occurring in putative antimutator (mut) genes have been identified in the Beijing strain family, and have been associated with increased mutation rates.157,158 Whilst further studies are required to elucidate this mechanism fully, WGS studies have indicated increased variability in the genes encoding DNA repair proteins in Beijing strains.29,34,159

Another mechanism associated with an increase in mutation rates is the exposure to suboptimal drug concentrations. Suboptimal fluoroquinolone concentrations have demonstrated the ability to induce transcriptional changes in genes responsible for DNA repair mechanisms, such as the SOS mechanism.160 Further studies addressing DNA repair mechanisms are warranted and will improve our understanding of the adaptive evolution in the organism.64

**Developments in genomics**

WGS remains the most desirable resistance-testing platform with the potential to identify resistance to all drugs in a single analysis and simultaneously track outbreaks with high resolution.161,162 However, the clinical utility of WGS is dependent on its capacity to test specimens directly from sputum samples. However, WGS requires a higher concentration of MTB DNA than is available in sputum specimens and is therefore performed on cultured isolates. An additional challenge is the contamination by host DNA in clinical specimens.3 Recent approaches, such as the selective removal of host DNA and enrichment of cultures to increase the proportion of MTB to host DNA, circumvent the need for culture-based DNA extraction. Votintseva et al.163 developed a modified Nextera XT protocol to extract and purify mycobacterial DNA within hours to 3 days from an early positive Bactec 960 Mycobacteria Growth Indicator Tube culture (median culture age of 4 days). Using this technique, they successfully sequenced 98% of the clinical samples and mapped them to the reference MTB H37Rv genome with >90% of sequence coverage.163 Recently, the same group reported antibiotic susceptibility and surveillance data in 8 h using the Oxford Nanopore Technologies MinION sequencer. Results were reported to be fully concordant with genotypic data. They report this technology to be advantageous in combating the problem associated with low quantities of DNA in samples as the sequencer has the ability to continue sequencing until sufficient coverage of the genome is obtained.164 Brown et al.165 sequenced the whole genome of MTB directly from uncultured sputa using biotinylated RNA baits designed specifically for MTB DNA. With a depth of the coverage achieved of 20x, the read alignment with the reference genome was >90% and the concordance between predicted genotypic resistance and phenotypic resistance profiles was 100% in a sample of 20 of 24 strains analysed. The whole process could be accomplished within 96 h, even from low-grade smear-positive sputa.165

Expertise in bioinformatics and computational analysis is integral to the interpretation of large-scale WGS data. Parallel to advancements in sequencing technologies, novel bioinformatics algorithms have also been developed for rapid analysis and clinical
interpretation of MTB sequence data. The introduction of large-scale data-sharing platforms, such as the Relational Sequencing TB Data Platform, has allowed the collection of whole-genome sequences from various studies. This allows users to attain validated, clinically relevant genetic data linked to associated MTB metadata. Currently, the TB profiling tool is the most accurate in predicting the relationship between detected mutation and its association with resistance.

Conclusions and future prospects

DR-TB remains a key public health challenge of modern times. In light of recent reports of the dissemination of drug resistance beyond XDR-TB to programatically untreatable TB, there is an urgent need for personalized management for DR-TB through drug resistance screening. This review highlights the complexity of drug resistance in MTB, captured by the advances of sequencing technologies.

The underlying drug resistance mechanisms in M. tuberculosis and their implications for potential clinical outcomes are increasingly well understood. Current diagnostic techniques for monitoring resistance mutations are, however, largely limited to our current knowledge of mutation patterns. Large-scale analyses conducted on WGS sequences have assisted in cataloguing various causative to compensatory or adaptive mutations and their varying roles in mediating drug resistance in the organism. Of significance, such studies have also revealed harbinger mutations, capable of predicting resistance at the earliest possibility. In contrast, they have also highlighted the lack of genetic variation in some phenotypically resistant isolates, as well as the complexities of lineage-specific variation. The recent launch of the Relational Sequencing TB Data Platform database, holds significant promise for a rapid DST approach and will aid in addressing the problem of phenotypic and genotypic discordance. In addition to integrated culture-based DSTs and whole-genome sequences, this platform allows for the addition of clinical outcome data, which can facilitate rapid, personalized patient care.

In addition, host-specific factors such as pharmacogenomics and the nutritional status of the host further impact on the response to anti-TB treatment. The pathophysiological profile associated with malnutrition impacts on pharmacokinetic processes, drug responses and toxicity. Reports on the effect of malnutrition on drug treatment indicate delayed or decreased absorption, reduced protein binding of several drugs, fluctuations in volume of distribution, altered hepatic oxidative drug biotransformations and conjugations, and reduced elimination of conjugates and renally excreted drugs. Poor nutritional status has also been reported to impact adversely on treatment outcomes and increase the risk of relapse. Optimal nutritional care is essential for successful health promotion and TB prevention.

Current anti-TB treatment dosing is based on the patient’s body weight. Drug concentrations and pharmacokinetics vary among patients, resulting in adverse reactions due to toxicity as well as suboptimal drug concentrations that impact on the development of drug resistance. In the context of personalized care for TB, the introduction of therapeutic drug monitoring will allow individualized dosing based on serum drug concentrations. This will reduce the likelihood of adverse drug reactions and improve treatment outcomes. Plasma concentrations of HIV and anti-TB drugs have been reported to display wide inter-individual variability associated with genetic mutations in the respective drug-metabolizing enzymes or transporter proteins. Indications for therapeutic drug monitoring should be prioritized for patients who fail to show sputum culture conversion 2 months after treatment initiation, those with adverse drug reactions and those with comorbidities such as HIV coinfection, type 2 diabetes, gastrointestinal abnormalities and severely ill patients.

The dynamics of developing resistance and the factors that facilitate resistance development within a patient are still poorly understood and require further elucidation. Resistance-conferring mutations in bacteria can evolve dynamically over time under antibiotic pressure in patients. The relationship between drug resistance, fitness and virulence of the organism requires further study. Current rapid diagnostics, such the Xpert MTB/rifampicin and Hain line probe assays, although rapid, are limited in their ability to produce a comprehensive resistance profile. Rapid WGS is the most promising utility for the personalized care of patients with DR-TB. Personalized treatment offers the potential to improve treatment outcomes, through limiting the therapeutic regimen to effective drugs only, thereby reducing the unnecessary pill burden and significantly reducing harm resulting from the debilitating side effects of current treatment. This is the only remaining strategy for managing the drug resistance crisis as our existing antimicrobial repertoire is quickly diminishing.

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