International Journal of Infectious Diseases 32 (2015) 94–100

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Review

The complex evolution of antibiotic resistance in *Mycobacterium tuberculosis*

J.D. Fonseca [a,*], G.M. Knight [b], T.D. McHugh [a]

[a] Centre for Clinical Microbiology, University College London, London, NW3 2PF, UK
[b] TB Modelling Group, TB Centre, Centre for the Mathematical Modelling of Infectious Diseases, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK

ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form 12 January 2015
Accepted 14 January 2015

Corresponding Editor: Eskild Petersen, Aarhus, Denmark.

Keywords:
Tuberculosis
Drug resistance
Molecular mechanisms of resistance

SUMMARY

Multidrug-resistant and extensively drug-resistant tuberculosis (TB) represent a major threat to the control of the disease worldwide. The mechanisms and pathways that result in the emergence and subsequent fixation of resistant strains of *Mycobacterium tuberculosis* are not fully understood and recent studies suggest that they are much more complex than initially thought. In this review, we highlight the exciting new areas of research within TB resistance that are beginning to fill these gaps in our understanding, whilst also raising new questions and providing future directions.

1. Introduction

Tuberculosis (TB) remains one of the leading causes of death worldwide. In 2013, an estimated 9 million people developed TB and 1.5 million died from the disease.[1] Despite evidence that TB is slowly declining, the emergence and spread of multidrug-resistant strains of *Mycobacterium tuberculosis* (MDR-TB) represents a major challenge to the global control of the disease.[1,2]

High cure rates for susceptible TB can be achieved with treatment regimens consisting of a 2-month 'intensive' phase, where isoniazid (INH), rifampicin (RIF), pyrazinamide, and ethambutol are administered, followed by a 4-month 'continuation' phase with only INH and RIF.[3] However, the effectiveness of such regimens is threatened by rising resistance to these first-line drugs. In its 2014 report on TB, the World Health Organization (WHO) estimated that 3.5% of new and 20.5% of previously treated cases have MDR-TB.[1] MDR-TB is caused by *M. tuberculosis* (M.tb) isolates resistant to the two most powerful anti-TB drugs, RIF and INH, and is notoriously difficult to treat, with global success rates of around 48%.[1] The treatment of MDR-TB patients commonly lasts for 2 years or longer and relies on the use of second-line drugs (such as fluoroquinolones and injectable aminoglycosides) that are less effective, more toxic, and far more costly.[2] The additional acquisition of resistance to these second-line drugs defines extensively drug-resistant (XDR) cases of TB. The prognosis of patients infected with XDR-TB is extremely poor,[4] and the spread of these strains raises the possibility of the return to a pre-antibiotic era.[5]

The *de novo* emergence of drug resistance in an individual patient can occur as a result of low adherence to treatment, inadequacy of the drug regimen (e.g., wrong antibiotic choices or dosages, poor drug quality), and patient-dependent pharmacodynamic and pharmacokinetic properties of the drugs administered.[6] In recent years, several determinants of acquired resistance to the drugs commonly used in the treatment of TB have been elucidated. These are associated with spontaneous mutations that interfere with drug–target binding (e.g., for RIF in the *rpo*B gene, for fluoroquinolones in the *gyr*A/B genes), compromise prodrug activation (e.g., for INH in the *kat*G gene, for PA-824 in the *fgd* gene), or cause overexpression of the target (e.g., for INH/ethionamide in the promoter region of *inh*A).[7] However, the resistance phenotypes of a significant proportion of clinical isolates of M.tb cannot solely be explained by these mutations: up to 30% of isolates resistant to INH and approximately 5% of those resistant to RIF do not harbour mutations in the known resistance genes.[8] On the other hand, there is growing evidence that bacteria are not merely spectators of their own evolution; instead, they are able to develop a series of mechanisms that facilitate a rapid adaptation to changes in their environment (such as exposure to drugs) and modulate the effects of drug resistance.[9–12] These observations illustrate the complexity of drug resistance in M.tb and highlight

* Corresponding author. Tel.: +020 7472 6402x33540; fax: +020 7317 7710x31151.
E-mail address: rebmjco@ucl.ac.uk (J.D. Fonseca).

http://dx.doi.org/10.1016/j.ijid.2015.01.014

the need for deeper exploration into the repertoire of strategies that lead to the emergence and subsequent fixation of drug-resistant strains of M.tb.

This review outlines recent findings relating to the evolution and spread of drug resistance. The aim is not to provide an exhaustive description of all the mechanisms known to date, but to illustrate the most important concepts brought to light by recent studies.

2. Identification of additional mechanisms of drug resistance in *Mycobacterium tuberculosis*

Several approaches have been used to elucidate the molecular mechanisms employed by bacteria to survive antibiotic treatment. They include transcriptional studies to profile responses to the exposure to different drugs, comparative studies of whole genome sequences of susceptible and resistant strains, and the use of mutagenesis to study gene function. The application of these approaches to M.tb has provided us with insights into the modes of action of current anti-TB drugs and new compounds in the TB drug discovery pipeline. It has also enabled the identification of new genes and intergenic regions (particularly genes involved in lipid metabolism, cell wall homeostasis, purine metabolism, and transcriptional regulation) under positive evolutionary selection by drug pressure.[13–19] However, the role of most of these genes in drug resistance remains unclear and warrants further study.

Recent efforts have, for example, led to the description of new mechanisms of resistance to ethambutol. Resistance to this drug was firstly attributed to mutations in the *emb*CAB operon (particularly mutations in codons 306, 406, and 497 of *emb*B), which encodes for mycobacterial arabinofuranosyltransferases thought to be the targets of ethambutol.[20–22] Subsequently, Rv3806c and Rv3792 have also been shown to play a role in ethambutol resistance by increasing the synthesis of decaprenyl-phosphoryl-β-D-arabinose (a substrate of the EmbCAB enzymes) and the expression of *emb*C, respectively.[23] Some of the mutations identified in these genes only conferred low-level resistance to ethambutol.[23] Low-level resistance is defined as an increase in the minimum inhibitory concentration (MIC) of a particular drug above that of the average susceptible bacterial population but below the threshold for clinically relevant resistance.[24] The low-level resistance to ethambutol associated with these mutations might be a stepping-stone to higher levels of resistance, a view that is supported by the observation that several ethambutol resistance mutations can be present in the same resistant isolate.[22,23] These findings point to a complex pathway to clinical resistance towards ethambutol: it involves mutations in several genes that are acquired in a stepwise manner and that interact to determine the overall level of resistance to this drug.[20,21,25] Mutations within the *gid*B gene (encoding a putative 16S rRNA methyltransferase) have similarly been found to produce a low-level streptomycin resistance phenotype.[26] Further work is needed to investigate the existence of low-level resistance mechanisms against other anti-TB drugs and determine their clinical significance.

3. Contributing factors to the emergence of drug resistance in *Mycobacterium tuberculosis*

Exposure to drugs induces a complex stress response in M.tb and produces changes in metabolic state and activity that functionally contribute to resistance. The study of such responses, some of which are discussed below, provides a better understanding of how M.tb can enhance its ability to survive antibiotic treatment.

3.1. DNA repair system, mutation rates, and antibiotic resistance

There has been increasing interest in the study of the role of DNA repair systems in the emergence of antibiotic resistance, as they directly influence the type and frequency of mutations that occur in bacteria. Alterations within these systems can result in a reduced ability to repair DNA damage and, as a consequence, increase the rates of mutation.[27,28] Such mutator phenotypes can represent a selective advantage under stressful conditions, as bacteria can more readily develop mutations that will enhance their survivability (e.g., resistance mutations).[27,28]

In M.tb, the interest in the study of the link between mutations within DNA repair genes, hypermutator phenotypes, and an increased ability to acquire antibiotic resistance was fuelled by the observation that polymorphisms in three putative antimutator genes (*mut*) appeared to be unique to the Beijing lineage.[29] Beijing strains have been associated, in some settings, with a higher risk of drug resistance (including MDR and XDR) when compared with that of other lineages.[30] Ebrahimi-Rad et al.[29] hypothesized that this higher propensity to acquire resistance could be attributed to increased mutation rates produced by missense mutations in the *mut* genes. Subsequent studies were unable to establish a definitive link between particular mutations in such genes and antibiotic resistance.[31] Nonetheless, there is some indication that the Beijing lineage might be associated with higher mutation rates.[32,33] This seems to be in line with results from whole genome sequencing that suggest that Beijing isolates are more variable than non-Beijing ones.[34-36] Some of this variability involves genes encoding DNA repair proteins.[37] The implications of these findings in the emergence of antibiotic resistance in Beijing strains warrant further study.

Mutation rates may be raised during exposure to sub-inhibitory concentrations of certain antibiotics, particularly those whose primary mode of action is DNA damage.[38] For example, fluoroquinolones target DNA gyrase, generating lethal double-stranded DNA (dsDNA) breaks that induce transcriptional changes in the genes responsible for DNA repair and preservation of the genome integrity, such as those involved in the SOS response.[39] Genome-wide expression studies in M.tb have shown the up-regulation of DNA repair clusters in response to the exposure to fluoroquinolones.[40,41] For example, sub-inhibitory concentrations of ciprofloxacin induce the expression of LexA and RecA, the two key regulatory proteins of the SOS regulon, and of the error-prone DNA polymerase DnaE2 (even though this response is significantly delayed after exposure to the drug).[40] In patients with TB infection, the previous use of quinolones has not only been associated with the development of resistance to this antibiotic class, but also to first-line anti-TB drugs.[42,43] Interestingly, exposure to INH (a drug that does not directly act on DNA metabolic processes) might also result in the induction of the SOS response and in higher mutation rates.[44,45]

SOS-induced DNA polymerases, such as DnaE2, lack intrinsic proofreading activity, which leads to mutations when DNA replication bypasses lesions or errors.[46] DnaE2 is thought to be an important mediator of induced mutagenesis (damage-induced expression of DnaE2 can increase the mutation rate up to 20–50 times) and to play a role in the emergence of drug resistance in M.tb.[47,48] In an *in vitro* study, resistance to RIF was found to emerge more frequently in the wild-type than in the *dna*E2 knockout strain.[48] Furthermore, specific RIF resistance-conferring mutations within the *rpo*B gene have been associated with the overexpression of *dna*E2 in fitness-impaired strains, suggesting a role for DnaE2 in the adaptation of these strains.[47] A better understanding of this and other mechanisms of DNA repair in M.tb will allow us to identify strains with an enhanced ability to adapt and resist antibiotic treatment.

3.2. Efflux pumps—a gateway to high-level resistance?

Many efflux pumps of M.tb (belonging to the major facilitator superfamily, the ATP-binding cassette superfamily, the resistance

nodulation division family and to the small multidrug resistance family) have been characterized and their role in antibiotic resistance investigated. Drug efflux pumps are constitutively expressed in wild-type cells but can be induced by mutations within their regulatory genes or by the presence of antibiotics (due to interactions with such regulatory systems).[49]

The overexpression of several efflux systems has been shown in clinical isolates of MDR-TB upon exposure to common anti-TB drugs.[50,51] In contrast with the high-level resistance caused by mutations in genes that encode the primary targets of the main TB drugs, the reduction in intracellular levels of antibiotics caused by increased activity of efflux systems is generally responsible for only conferring low-level resistance.[8,52] The same efflux pump can induce cross-tolerance to structurally and mechanistically diverse compounds,[53,54] and there is a vast overlap in substrate specificity among the pumps present in M.tb.[55] These findings, together with the observation that genes encoding for efflux pumps are overexpressed soon after exposure to drugs, provide evidence to support the notion that the extrusion of antibiotics mediated by efflux pumps represents a rapid, non-specific response to highly noxious agents.[52,56] The level of tolerance conferred by these mechanisms might confer a selective advantage in the presence of suboptimal, low antibiotic concentrations, enabling the survival of certain bacterial subpopulations until a classical high-level mutation emerges and a population with clinically significant antibiotic resistance is established.[28,56-58] On the other hand, individual mechanisms may not be sufficient to confer clinical resistance but may interact (e.g., additively or synergistically) with other resistance determinants resulting in high-level resistance. The differential expression of efflux pumps might, at least in part, explain the observation that some clinical isolates harbouring the same classical resistance-conferring mutations can have different antibiotic susceptibility patterns.[59]

Efflux pump inhibitors have been shown to reduce the MICs of a wide range of drugs (including RIF, INH, ciprofloxacin, ofloxacin, streptomycin, and linezolid) in resistant strains of M.tb.[52,60-62] Although *in vivo* data are limited, there is some indication that the efflux pump inhibitor verapamil is able to restore the activity of RIF, INH, and pyrazinamide against M.tb in mice.[53] These findings have stimulated an interest in the introduction of inhibitors of efflux pumps into the treatment regimen for TB as an adjunctive therapy that could increase the intracellular concentration of (and therefore the susceptibility to) certain drugs, which is particularly relevant in the treatment of MDR-TB cases. It should be noted that the exposure of MDR-TB isolates to efflux pump inhibitors does not generally produce a phenotype of full susceptibility, as resistance-conferring mutations have often accumulated in other genes that are not inhibited by these agents.[63]

Efflux systems are responsible for fundamental physiological processes: for example, some of those that have been linked to antibiotic resistance in M.tb are also involved in virulence, oxidative stress responses, and growth.[64,65] Thus physiological regulatory systems may determine the levels of drug resistance.[63,64] The regulation of these efflux systems is still poorly understood, but several of them have been shown to be induced during macrophage infection.[65,66] In the future, a better understanding of the efflux substrate specificities and of the mechanisms of efflux pump regulation will help the development of strategies to inhibit them.

4. The evolutionary trajectory of drug-resistant *Mycobacterium tuberculosis*

Antibiotic resistance mutations represent a selective advantage during antibiotic treatment, but *in vitro* studies have shown that they can significantly impair bacterial fitness in the absence of antibiotics.[10,67,68] Interestingly, clinical isolates harbouring such mutations can be more fit than isogenic laboratory-constructed strains.[69] This observation suggests the existence of other determinants of fitness that could mitigate the deficits incurred by resistance mutations. These could be secondary compensatory mutations or factors related to the genetic background of the strain.[10]

Compensatory evolution, by reducing or eliminating the fitness costs associated with antibiotic resistance, might play a pivotal role in the spread of drug-resistant M.tb. However, only a few compensatory mechanisms have been described so far. Sherman et al.[70] showed that INH-resistant strains of M.tb with an inactivated *kat*G gene can acquire mutations in the regulatory region of the alkyl hydroperoxide reductase *ahp*C gene conducive to its overexpression. The authors concluded that these mutations might represent a compensatory mechanism for the loss of KatG catalase–peroxidase activity. In another study, *Mycobacterium smegmatis* was used to model the evolution of aminoglycoside resistance in M.tb.[71] It was observed that the fitness cost of an aminoglycoside resistance-conferring mutation (G1491U) in the 16S rRNA gene, *rrs*, could be ameliorated by a secondary mutation within the same gene (C1409A). These dual mutations were identified in clinical isolates of M.tb but at low frequencies, suggesting that their epidemiological relevance might be minor.[71]

More recently, a set of non-synonymous mutations in the genes that encode RNA polymerase subunits RpoA and RpoC were implicated in the compensatory evolution of RIF resistance.[72-74] These gene sequences are generally highly conserved in M.tb, so non-synonymous mutations are likely to represent recently acquired adaptive mutations and not natural polymorphisms.[72] These putative compensatory mutations, which appear to be preferably located in the *rpo*A–*rpo*C interaction region of the *rpo*C gene, have been associated with increased *in vitro* fitness[73,75,76] and are often present in MDR-TB strains.[72-74] The acquisition of *rpo*A-*rpo*C mutations generally occurs in strains that are already RIF-resistant due to alterations in the *rpo*B gene[77], in a process that seems to be facilitated by the continued exposure to drugs.[76] This compensatory evolution leads to improved transmissibility as evidenced by the clonal expansion of M.tb strains harbouring such combinations of resistance and compensatory mutations.[72,73] Interestingly, these changes are frequently present in strains that harbour *rpo*B resistance mutations associated with the lowest *in vitro* fitness deficits.[72,74] For example, they are often co-present with *rpo*B mutation S531L,[72] simultaneously one of the most frequent mutations in RIF-resistant clinical isolates and one of the least costly.[10,68,72,77]

In an *in vitro* study, double drug-resistant mutants of *M. smegmatis* containing certain combinations of *rpo*B and *gyr*A mutations (conferring resistance to RIF and ofloxacin, respectively) were found to have higher fitness than at least one of the corresponding single drug-resistant mutants.[12] This means that, in some cases, the overall cost of carrying multiple resistance mutations can be less than what would be expected if those mutations had independent (multiplicative) effects on fitness. Further work is needed to confirm these findings (as the authors were unable to exclude the acquisition of compensatory mutations as a possible explanation for the observed phenotypes) and elucidate the mechanisms behind them. Nonetheless, the identification of the same combinations of *rpo*B and *gyr*A mutations in clinical isolates of M.tb suggests that this might represent another form of compensatory evolution with a potential significant impact on the emergence of transmissible MDR-TB strains.[78] The acquisition of additional resistance determinants, instead of further impairing fitness, can ameliorate the deficits produced by resistance to other drugs. The resulting strains will have both a selective advantage in the presence of

antibiotics and increased competitiveness in the absence of such selective pressure.

The strain genetic background can also modulate *in vitro* fitness and drug susceptibility, meaning that the same resistance mutation can incur different fitness costs and produce different resistance patterns depending on the host strain. Mutations in the *rpo*B, *kat*G, and *inh*A genes were found to convey different levels of antibiotic resistance in strains from different phylogenetic lineages of M.tb.[79,80] Furthermore, some resistance mutations appear to be lineage-specific,[81-83] or more likely to be present in certain M.tb lineages.[69,80,84] There is also an indication that some lineages are particularly associated with multi-drug resistance[85-87] and that strain background can affect transmissibility.[69,80,88,89] On the other hand, evidence that the genetic background can influence the fitness costs of resistance came from the observation that the same *rpo*B mutation (H526D) could confer different fitness deficits in M.tb strains CDC1551 (which belongs to lineage 4) and T85 (lineage 2).[69] Further work is needed to elucidate the mechanisms behind these findings.

The *in vitro* studies described above have shed some light on the interplay between resistance determinants, strain background, bacterial fitness, and compensatory mutations in the evolution of antibiotic resistance in M.tb. Less is known about the emergence of resistance *in vivo* and the relative importance of each of these factors. Data from available studies point to the complex evolution of drug resistance in TB patients undergoing treatment. Within-host M.tb populations appear to be heterogeneous and highly dynamic: there are successive changes in antibiotic resistance and fitness profiles of isolates obtained in serial samples from the same patient.[35,36,90,91] The co-existence of subpopulations with different levels of resistance in the same sample, mixed clonal infections, and exogenous re-infection, have also been observed.[35,36,90-93] MDR is the result of the stepwise accumulation of resistance-conferring mutations, and highly resistant mutations that do not impair fitness or transmissibility are favoured in the long-term.[19,35,36,90,91]

5. Mathematical modelling—what it can tell us about the evolution of drug resistance in *Mycobacterium tuberculosis*

Many of the above outlined mechanisms and aspects of drug resistance within M.tb are considered in *in vitro* conditions and in isolation. Determining how important they are to clinical populations can be achieved, in part, using mathematical models. These allow predictions to be made about the impact of such mechanisms, for example relative fitness,[94] on levels of drug resistance in the future, but can also explore the experimental data and help in the guidance of new experimental work.[9] This becomes especially important as the pipeline of new drugs for TB treatment fills.[95] However, it is also useful for understanding the dynamics of evolution of resistance to the existing spectrum of anti-TB drugs.

The quality of the output from mathematical modelling is highly dependent on the quality of the data used to construct the model structure and to inform parameters. Within drug resistance epidemiology, the data on transmission is relatively poor due to the current low prevalence and availability of drug susceptibility tests. These tests are also usually phenotypic and hence may not capture detailed data on the varying mechanisms or levels of resistance. Other parameters may be extremely difficult to measure, such as the relative fitness, or ability to transmit, of different resistance strains.[94] Fortunately, it is likely that commonly used fitness measures in vitro are matched to clinical success.[10,68] Moreover, mathematical models are hampered by a desire to accurately capture biological complexity, such as the many facets of drug resistance mechanisms, without becoming overly complicated and too difficult to parameterize.

The power of mathematical models to generalize conclusions from experimental and genetic work, however, means that there is great scope for collaboration and interaction. Through an interdisciplinary approach, experimental results can be broadened to applications in the clinical population suffering from TB disease. Modellers can help to guide experimentalists to collect needed data, whilst aiding experimentalists to understand the full impact of their explorations. This is especially true for the mechanisms outlined in this review – models could be used in the future to explore both their complex interplay but also to highlight their potential importance in the spread of drug resistance within M.tb.

6. Impact for drug development, resistance diagnosis, and treatment

In light of recent research, the mechanisms and rates of resistance, as well as the associated fitness costs and compensatory mutations, should be investigated in the early stages of drug development, as they provide relevant information about the potential for the emergence, spread, and fixation of resistance towards a particular drug. The ideal anti-TB drug would be one for which resistance mutations are not only rare but also associated with high fitness costs.[96,97] In addition, it is important to identify targets for which the rate of fitness-restoring mutations is low. Restoration of fitness will be less likely when the fitness defect is pleiotropic and affects more than one cellular process.[97] The study of the interactions between mutations conferring resistance to different antibiotics (namely their combined effect on bacterial fitness) could help optimize treatment regimens by identifying those less likely to be associated with the emergence of multiple drug resistance.

Drug resistance in M.tb is commonly believed to be caused by single-step mutations, but there is now evidence to suggest that, at least for certain anti-TB drugs, it is the result of a stepwise acquisition of mutations leading to a gradual decrease in susceptibility. The first step in this process may involve a mutation that does not increase the MIC of a drug above the breakpoint for clinical resistance. These findings highlight the importance of unveiling the full spectrum of resistance mechanisms towards a particular drug. However, low-level resistance is often overlooked because it is not generally thought to be associated with a higher likelihood of therapeutic failure. Low-level mutants can, nonetheless, be selected in the presence of suboptimal antibiotic concentrations and serve as a gateway for the amplification of resistance.[24,98] TB patients undergoing treatment can be exposed to such concentrations of anti-TB drugs either as a result of inadequate dosing (e.g., low patient compliance or poor drug quality) or gradients of drug concentration created in the body by the differential penetration of drugs into tissues and lesions.[24,99] The identification of patients infected with these 'pre-resistant' M.tb mutants may suggest the use of increased antibiotic dosages or an alteration of treatment strategies before the establishment of full-scale drug resistance.[23] However, low-level mutants (such as those recently described for ethambutol and streptomycin) cannot be detected by standard culture-based susceptibility testing. Current methods rely on breakpoint concentrations to qualitatively define resistance and are, therefore, insensitive to changes in drug susceptibility below those thresholds. Further studies are needed to clarify the relationship between the presence of these low-level mutations, the emergence of clinical resistance, and patient outcomes to inform potential changes in current diagnostic tests (e.g., broaden molecular tests for drug resistance to include screening for such mutations). Given the putative pivotal role of low-level mutations in the spread of resistance, surveillance systems based on resistance produced by high-level mutations

Table 1
Potential future directions in anti-TB drug resistance research

Mechanisms of acquired resistance	-Identify additional mechanisms of drug resistance to anti-TB drugs, including those that produce low-level resistance -Study the role of low-level resistance determinants in the emergence of high-level resistance and their clinical relevance -Clarify the observed association between certain resistance mutations and the development of MDR-TB -Investigate how strain genetic background can influence the level of resistance conveyed by certain mutations
Fitness costs of resistance	-Further characterize the fitness effects of resistance determinants (including those responsible for low-level resistance) and identify additional mechanisms of compensatory evolution -Study the interactions between the fitness effects of different resistance mutations -Investigate how strain genetic background can modulate the fitness impact of drug resistance-conferring mutations -Better characterize the evolution of drug resistance, bacterial fitness, and acquisition of compensatory mutations in M.tb populations within the host during antibiotic treatment
Mutation rates to antibiotic resistance	-Gain a better understanding of the factors and mechanisms that affect mutation rates in M.tb and their role in the emergence of drug resistance -Investigate the role of DnaE2 in the emergence of drug resistance in the host and its potential as a target for therapeutic intervention -Investigate the potential association of certain sub-lineages of M.tb with drug resistance and increased mutation rates -Clarify observations of an association between certain drug resistance-conferring mutations and elevated mutation rates -Determine whether anti-TB drugs, other than fluoroquinolones, can increase the *in vitro* mutation rates of M.tb -Determine whether particular drug combinations are associated with a higher probability of the development of resistance -Investigate the occurrence of stable mutator strains within M.tb populations during human infection

TB, tuberculosis; MDR, multidrug-resistant; M.tb, *Mycobacterium tuberculosis*.

alone may be insufficient. Indeed, high-level resistance mutations might be just the tip of the iceberg.

Finally, the heterogeneity of within-host M.tb populations can also have implications for resistance diagnostics. Culture-based drug susceptibility testing usually involves the examination of a single isolate from a single sample from a given disease episode, with the assumption that it is representative of a homogeneous bacterial population, which might not be the case. Knowing the existence and prevalence of the different subpopulations and their corresponding drug susceptibilities could be useful for the management of the patient. To obtain a measure of the heterogeneity of the TB infection, several independent bacterial isolates should to be examined from each patient sample. Similarly, the heterogeneity of M.tb infections might also confound molecular antibiotic resistance tests. For example, the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) (which is becoming a leading screening tool for TB) has an increased false-negative rate for detecting RIF resistance in mixed M.tb complex infections, failing to identify it when <90% of the organisms present in the sample are RIF-resistant. In order to avoid poor clinical outcomes, GeneXpert results might warrant further confirmation in settings where mixed infections are common.[100]

7. Concluding remarks

The mechanisms by which drug resistance emerges and is fixated in M.tb populations are not wholly understood (Table 1). *In vitro* studies have identified several determinants of resistance to the main anti-TB drugs, but whole-genome analyses suggest that the response to drug exposure might be much more complex than initially thought and involve a set of strategies developed by mycobacteria to enhance their ability to adapt and evolve. Furthermore, there are indications that the acquisition of clinically significant resistance to certain drugs might be a stepwise process that often involves an initial low-level mutation that acts as a gateway for high-level resistance. The clinical implications of these findings should be investigated.

Recent research suggests that bacterial fitness might play a pivotal role in the spread of antibiotic-resistant M.tb. *In vitro*, bacterial fitness is determined by the interplay of numerous factors including the growth deficits incurred by resistance mutations, strain genetic background, and compensatory evolution. Future studies should elucidate the interactions and relative importance of these and other factors in the transmissibility of drug-resistant TB. The development of new tools, such as mathematical modelling, will facilitate this process and provide important information for the control of drug-resistant TB.

The growing threat of MDR- and XDR-TB highlights the need for a better understanding of the complexity of drug resistance in M.tb. This will allow the development of enhanced diagnostic tests to identify resistant strains and strategies to curb their spread, and also help the design of more powerful anti-TB drugs.

Conflict of interest: None to declare.

Acknowledgments

JDF was supported by grant SFRH/BD/70037/2010 from FCT, Portugal.

References

1. World Health Organization. *Global tuberculosis report 2014* 2014.
2. Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, Van Soolingen D, et al. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. *Lancet* 2010;**375**:1830–43.
3. Espinal MA, Kim SJ, Suarez PG, Kam KM, Khomenko AG, Migliori GB, et al. Standard short-course chemotherapy for drug-resistant tuberculosis: treatment outcomes in 6 countries. *JAMA* 2000;**283**:2537–45.
4. Martinson NA, Chaisson RM. Survival in XDR TB: Shifting the Curve and Shifting the Paradigm. *J Acquir Immune Defic Syndr* 2011;**57**:89.
5. Raviglione M. XDR-TB: entering the post-antibiotic era? *Int J Tuberc Lung Dis* 2006;**10**:1185–7.
6. Pasipanodya JG, Gumbo T. A new evolutionary and pharmacokinetic–pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs. *Curr Opin Pharmacol* 2011;**11**:457–63.
7. Da Silva PEA, Palomino JC. Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: classical and new drugs. *J Antimicrob Chemother* 2011;**66**:1417–30.
8. Louw G, Warren R, Van Pittius NG, McEvoy C, Van Helden P, Victor T. A balancing act: efflux/influx in mycobacterial drug resistance. *Antimicrob Agents Chemother* 2009;**53**:3181–9.
9. MacLean RC, Hall AR, Perron GG, Buckling A. The population genetics of antibiotic resistance: integrating molecular mechanisms and treatment contexts. *Nat Rev Genet* 2010;**11**:405–14.
10. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJ. The competitive cost of antibiotic resistance in Mycobacterium tuberculosis. *Science* 2006;**312**:1944–6.
11. Borrell S, Gagneux S. Strain diversity, epistasis and the evolution of drug resistance in Mycobacterium tuberculosis. *Clin Microbiol Infect* 2011;**17**:815–20.
12. Borrell S, Teo Y, Giardina F, Streicher EM, Klopper M, Feldmann J, et al. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol Med Publich Health* 2013;**2013**:65–74.
13. Chatterjee A, Saranath D, Bhatter P, Mistry N. Global transcriptional profiling of longitudinal clinical isolates of Mycobacterium tuberculosis exhibiting rapid accumulation of drug resistance. *PLoS One* 2013;**8**:e54717.
14. Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Victor TC, et al. Genomic analysis identifies targets of convergent positive selection in drug-resistant Mycobacterium tuberculosis. *Nat Genet* 2013;**45**:1183–9.

15. Manjunatha UH, Boshoff H, Dowd CS, Zhang L, Albert TJ, Norton JE, et al. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in Mycobacterium tuberculosis. *Proc Natl Acad Sci USA* 2006;**103**:431–6.
16. de Knegt GJ, Bruning O, ten Kate MT, de Jong M, van Belkum A, Endtz HP, et al. Rifampicin-induced transcriptome response in rifampicin-resistant Mycobacterium tuberculosis. *Tuberculosis (Edinb)* 2013;**93**:96–101.
17. Jiang X, Zhang W, Gao F, Huang Y, Lv C, Wang H. Comparison of the proteome of isoniazid-resistant and -susceptible strains of Mycobacterium tuberculosis. *Microb Drug Resist* 2006;**12**:231–8.
18. Maus CE, Plikaytis BB, Shinnick TM. Mutation of tlyA confers capreomycin resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 2005;**49**:571–7.
19. Zhang H, Li D, Zhao L, Fleming J, Lin N, Wang T, et al. Genome sequencing of 161 Mycobacterium tuberculosis isolates from China identifies genes and intergenic regions associated with drug resistance. *Nat Genet* 2013;**45**:1255–60.
20. Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B, et al. The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. *Nat Med* 1997;**3**:567–70.
21. Safi H, Sayers B, Hazbon MH, Alland D. Transfer of embB codon 306 mutations into clinical Mycobacterium tuberculosis strains alters susceptibility to ethambutol, isoniazid, and rifampin. *Antimicrob Agents Chemother* 2008;**52**:2027–34.
22. Plinke C, Cox HS, Zarkua N, Karimovich HA, Braker K, Diel R, et al. embCAB sequence variation among ethambutol-resistant Mycobacterium tuberculosis isolates without embB306 mutation. *J Antimicrob Chemother* 2010;**65**:1359–67.
23. Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, et al. Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-beta-D-arabinose biosynthetic and utilization pathway genes. *Nat Genet* 2013;**45**:1190–7.
24. Baquero F:. Low-level antibacterial resistance: a gateway to clinical resistance. *Drug Resist Updat* 2001;**4**:93–105.
25. Safi H, Fleischmann RD, Peterson SN, Jones MB, Jarrahi B, Alland D. Allelic exchange and mutant selection demonstrate that common clinical embCAB gene mutations only modestly increase resistance to ethambutol in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 2010;**54**:103–8.
26. Wong SY, Lee JS, Kwak HK, Via LE, Boshoff HI, Barry 3rd CE. Mutations in gidB confer low-level streptomycin resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 2011;**55**:2515–22.
27. Chopra I, O'Neill AJ, Miller K. The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Resist Updat* 2003;**6**:137–45.
28. Martinez J, Baquero F. Mutation frequencies and antibiotic resistance. *Antimicrob Agents Chemother* 2000;**44**:1771–7.
29. Ebrahimi-Rad M, Bifani P, Martin C, Kremer K, Samper S, Rauzier J, et al. Mutations in putative mutator genes of Mycobacterium tuberculosis strains of the W-Beijing family. *Emerg Infect Dis* 2003;**9**:838–45.
30. Stagg HR, Cohen T, Becerra MC, Anderson LF, Abubakar I. A tale of two settings: the role of the Beijing genotype in the epidemiology of multidrug-resistant tuberculosis. *Eur Respir J* 2014;**43**:632–5.
31. Lari N, Rindi L, Bonanni D, Tortoli E, Garzelli C. Mutations in mutT genes of Mycobacterium tuberculosis isolates of Beijing genotype. *J Med Microbiol* 2006;**55**:599–603.
32. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, et al. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet* 2013;**45**:784–90.
33. de Steenwinkel JE, ten Kate MT, de Knegt GJ, Kremer K, Aarnoutse RE, Boeree MJ, et al. Drug susceptibility of Mycobacterium tuberculosis Beijing genotype and association with MDR TB. *Emerg Infect Dis* 2012;**18**:660–3.
34. Saunders NJ, Trivedi UH, Thomson ML, Doig C, Laurenson IF, Blaxter ML. Deep resequencing of serial sputum isolates of Mycobacterium tuberculosis during therapeutic failure due to poor compliance reveals stepwise mutation of key resistance genes on an otherwise stable genetic background. *J Infect* 2011;**62**:212–7.
35. Sun G, Luo T, Yang C, Dong X, Li J, Zhu Y, et al. Dynamic population changes in Mycobacterium tuberculosis during acquisition and fixation of drug resistance in patients. *J Infect Dis* 2012;**206**:1724–33.
36. Merker M, Kohl TA, Roetzer A, Truebe L, Richter E, Rüsch-Gerdes S, et al. Whole genome sequencing reveals complex evolution patterns of multidrug-resistant Mycobacterium tuberculosis Beijing strains in patients. *PloS One* 2013;**8**:e82551.
37. Dos Vultos T, Mestre O, Rauzier J, Golec M, Rastogi N, Rasolofo V, et al. Evolution and diversity of clonal bacteria: the paradigm of Mycobacterium tuberculosis. *PloS one* 2008;**3**:e1538.
38. Gillespie SH, Basu S, Dickens AL, O'Sullivan DM, McHugh TD. Effect of subinhibitory concentrations of ciprofloxacin on Mycobacterium fortuitum mutation rates. *J Antimicrob Chemother* 2005;**56**:344–8.
39. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* 1997;**61**:377–92.
40. O'Sullivan DM, Hinds J, Butcher PD, Gillespie SH, McHugh TD. Mycobacterium tuberculosis DNA repair in response to subinhibitory concentrations of ciprofloxacin. *J Antimicrob Chemother* 2008;**62**:1199–202.
41. Boshoff HI, Myers TG, Copp BR, McNeil MR, Wilson MA, Barry CE. *The transcriptional responses of Mycobacterium tuberculosis to inhibitors of metabolism:novel insights into drug mechanisms of action J Biol Chem* 2004;**279**:40174–8.
42. Long R, Chong H, Hoeppner V, Shanmuganathan H, Kowalewska-Grochowska K, Shandro C, et al. Empirical treatment of community-acquired pneumonia and the development of fluoroquinolone-resistant tuberculosis. *Clin Infect Dis* 2009;**48**:1354–60.
43. Deutschendorf C, Goldani LZ, Santos RP. Previous use of quinolones: a surrogate marker for first line anti-tuberculosis drugs resistance in HIV-infected patients? *Braz J Infect Dis* 2012;**16**:142–5.
44. Chen L-C, Yeh H-Y, Yeh C-Y, Arias CR, Soo V-W. Identifying co-targets to fight drug resistance based on a random walk model. *BMC Syst Biol* 2012;**6**:5.
45. Waddell SJ, Stabler RA, Laing K, Kremer L, Reynolds RC, Besra GS. The use of microarray analysis to determine the gene expression profiles of Mycobacterium tuberculosis in response to anti-bacterial compounds. *Tuberculosis* 2004;**84**:263–74.
46. Nohmi T. Environmental stress and lesion-bypass DNA polymerases. *Annu Rev Microbiol* 2006;**60**:231–53.
47. Bergval IL, Klatser PR, Schuitema AR, Oskam L, Anthony RM. Specific mutations in the Mycobacterium tuberculosis rpoB gene are associated with increased dnaE2 expression. *FEMS Microbiol Lett* 2007;**275**:338–43.
48. Boshoff HI, Reed MB, Barry 3rd CE, Mizrahi V. DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. *Cell* 2003;**113**:183–93.
49. Rossi ED, Aínsa JA, Riccardi G. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiol Rev* 2006;**30**:36–52.
50. Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, Sharma VD. Microarray analysis of efflux pump genes in multidrug-resistant Mycobacterium tuberculosis during stress induced by common anti-tuberculous drugs. *Microb Drug Resist* 2010;**16**:21–8.
51. Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, Wang H. Assessment of efflux pump gene expression in a clinical isolate Mycobacterium tuberculosis by real-time reverse transcription PCR. *Microb Drug Resist* 2008;**14**:7–11.
52. Machado D, Couto I, Perdigao J, Rodrigues L, Portugal I, Baptista P, et al. Contribution of efflux to the emergence of isoniazid and multidrug resistance in Mycobacterium tuberculosis. *PLoS One* 2012;**7**:e34538.
53. Louw GE, Warren RM, Gey van Pittius NC, Leon R, Jimenez A, Hernandez-Pando R, et al. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant Mycobacterium tuberculosis through efflux. *Am J Resp Crit Care Med* 2011;**184**:269–76.
54. Aínsa JA, Blokpoel MC, Otal I, Young DB, De Smet KA, Martín C. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in Mycobacterium fortuitum and Mycobacterium tuberculosis. *J Bacteriol* 1998;**180**:5836–43.
55. Balganesh M, Kuruppath S, Marcel N, Sharma S, Nair A, Sharma U. Rv1218c, an ABC transporter of Mycobacterium tuberculosis with implications in drug discovery. *Antimicrob Agents Chemother* 2010;**54**:5167–72.
56. Srivastava S, Musuka S, Sherman C, Meek C, Leff R, Gumbo T. Efflux-pump-derived multiple drug resistance to ethambutol monotherapy in Mycobacterium tuberculosis and the pharmacokinetics and pharmacodynamics of ethambutol. *J Infect Dis* 2010;**201**:1225–31.
57. Webber M, Piddock L. The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* 2003;**51**:9–11.
58. Viveiros M, Portugal I, Bettencourt R, Victor TC, Jordaan AM, Leandro C, et al. Isoniazid-induced transient high-level resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 2002;**46**:2804–10.
59. Huitric E, Werngren J, Juréen P, Hoffner S. Resistance levels and rpoB gene mutations among in vitro-selected rifampin-resistant Mycobacterium tuberculosis mutants. *Antimicrob Agents Chemother* 2006;**50**:2860–2.
60. Escribano I, Rodriguez JC, Llorca B, Garcia-Pachon E, Ruiz M, Royo G. Importance of the efflux pump systems in the resistance of Mycobacterium tuberculosis to fluoroquinolones and linezolid. *Chemotherapy* 2007;**53**:397–401.
61. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M. Contribution of efflux activity to isoniazid resistance in the Mycobacterium tuberculosis complex. *Infect Genet Evol* 2012;**12**:695–700.
62. Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, Zaha A. Identification of mutations related to streptomycin resistance in clinical isolates of Mycobacterium tuberculosis and possible involvement of efflux mechanism. *Antimicrob Agents Chemother* 2008;**52**:2947–9.
63. Martins A, Iversen C, Rodrigues L, Spengler G, Ramos J, Kern W, et al. An AcrAB-mediated multidrug-resistant phenotype is maintained following restoration of wild-type activities by efflux pump genes and their regulators. *Int J Antimicrob Agents* 2009;**34**:602–4.
64. Ramón-García S, Martín C, Thompson CJ, Aínsa JA. Role of the Mycobacterium tuberculosis P55 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. *Antimicrob Agents Chemother* 2009;**53**:3675–82.
65. Adams KN, Takaki K, Connolly LE, Wiedenhoft H, Winglee K, Humbert O, et al. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 2011;**145**:39–53.
66. Rohde KH, Abramovitch RB, Russell DG. Mycobacterium tuberculosis invasion of macrophages: linking bacterial gene expression to environmental cues. *Cell Host Microbe* 2007;**2**:352–64.
67. Mariam DH, Mengistu Y, Hoffner SE, Andersson DI. Effect of rpoB mutations conferring rifampin resistance on fitness of Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 2004;**48**:1289–94.

68. Billington O, McHugh T, Gillespie S. Physiological cost of rifampin resistance induced in vitro in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 1999;**43**:1866–9.
69. Gagneux S, Burgos MV, DeRiemer K, Enciso A, Muñoz S, Hopewell PC, et al. Impact of bacterial genetics on the transmission of isoniazid-resistant Mycobacterium tuberculosis. *PLoS pathogens* 2006;**2**:e61.
70. Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE, et al. Compensatory ahpC gene expression in isoniazid-resistant Mycobacterium tuberculosis. *Science* 1996;**272**:1641–3.
71. Shcherbakov D, Akbergenov R, Matt T, Sander P, Andersson DI, Böttger EC. Directed mutagenesis of Mycobacterium smegmatis 16S rRNA to reconstruct the in vivo evolution of aminoglycoside resistance in Mycobacterium tuberculosis. *Mol Microbiol* 2010;**77**:830–40.
72. De Vos M, Müller B, Borrell S, Black P, van Helden P, Warren R, et al. Putative compensatory mutations in the rpoC gene of rifampin-resistant Mycobacterium tuberculosis are associated with ongoing transmission. *Antimicrob Agents Chemother* 2013;**57**:827–32.
73. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, et al. Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. *Nat Genet* 2012;**44**:106–10.
74. Casali N, Nikolayevskyy V, Balabanova Y, Ignatyeva O, Kontsevaya I, Harris SR, et al. Microevolution of extensively drug-resistant tuberculosis in Russia. *Genome Res* 2012;**22**:735–45.
75. Brandis G, Wrande M, Liljas L, Hughes D. Fitness-compensatory mutations in rifampicin-resistant RNA polymerase. *Mol Microbiol* 2012;**85**:142–51.
76. Brandis G, Hughes D. Genetic characterization of compensatory evolution in strains carrying rpoB Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates. *J Antimicrob Chemother* 2013;**68**:2493–7.
77. Lanzas F, Karakousis PC, Sacchettini JC, Ioerger TR. Multidrug-resistant tuberculosis in Panama is driven by clonal expansion of a multidrug-resistant Mycobacterium tuberculosis strain related to the KZN extensively drug-resistant M. tuberculosis strain from South Africa. *J Clin Microbiol* 2013;**51**:3277–85.
78. Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I. Positive epistasis drives the acquisition of multidrug resistance. *PLoS Genet* 2009;**5**:e1000578.
79. Zaczek A, Brzostek A, Augustynowicz-Kopec E, Zwolska Z, Dziadek J. Genetic evaluation of relationship between mutations in rpoB and resistance of Mycobacterium tuberculosis to rifampin. *BMC Microbiol* 2009;**9**:10.
80. Fenner L, Egger M, Bodmer T, Altpeter E, Zwahlen M, Jaton K, et al. Effect of mutation and genetic background on drug resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 2012;**56**:3047–53.
81. Spies FS, Ribeiro AW, Ramos DF, Ribeiro MO, Martin A, Palomino JC, et al. Streptomycin resistance and lineage-specific polymorphisms in Mycobacterium tuberculosis gidB gene. *J Clin Microbiol* 2011;**49**:2625–30.
82. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. *PLoS medicine* 2009;**6**:e1000002.
83. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. *Nat Genet* 2010;**42**:498–503.
84. Baker L, Brown T, Maiden MC, Drobniewski F. Silent nucleotide polymorphisms and a phylogeny for Mycobacterium tuberculosis. *Emerg Infect Dis* 2004;**10**:1568–77.
85. Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN. Global dissemination of the Mycobacterium tuberculosis W-Beijing family strains. *Trends Microbiol* 2002;**10**:45–52.
86. Drobniewski F, Balabanova Y, Nikolayevsky V, Ruddy M, Kuznetzov S, Zakharova S, et al. Drug-resistant tuberculosis, clinical virulence, and the dominance of the Beijing strain family in Russia. *JAMA* 2005;**293**:2726–31.
87. Borrell S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant Mycobacterium tuberculosis State of the art. *Int J Tuberc Lung Dis* 2009;**13**:1456–66.
88. Baker LV, Brown TJ, Maxwell O, Gibson AL, Fang Z, Yates MD, Drobniewski FA. Molecular analysis of isoniazid-resistant Mycobacterium tuberculosis isolates from England and Wales reveals the phylogenetic significance of the ahpC -46A polymorphism. *Antimicrob Agents Chemother* 2005;**49**:1455–64.
89. van Doorn HR, de Haas PE, Kremer K, Vandenbroucke-Grauls CM, Borgdorff MW, van Soolingen D. Public health impact of isoniazid-resistant Mycobacterium tuberculosis strains with a mutation at amino-acid position 315 of katG: a decade of experience in the Netherlands. *Clin Microbiol Infect* 2006;**12**:769–75.
90. Meacci F, Orrù G, Iona E, Giannoni F, Piersimoni C, Pozzi G, et al. Drug resistance evolution of a Mycobacterium tuberculosis strain from a noncompliant patient. *J Clin Microbiol* 2005;**43**:3114–20.
91. Mariam SH, Werngren J, Aronsson J, Hoffner S, Andersson DI. Dynamics of antibiotic resistant *Mycobacterium tuberculosis* during long-term infection and antibiotic treatment. *PLoS One* 2011;**6**:e21147.
92. van Rie A, Victor TC, Richardson M, Johnson R, van der Spuy GD, Murray EJ, et al. Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. *Am J Respir Crit Care Med* 2005;**172**:636–42.
93. Shamputa IC, Jugheli L, Sadradze N, Willery E, Portaels F, Supply P, Rigouts L. Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. *Respir Res* 2006;**7**:99.
94. Cohen T, Sommers B, Murray M. The effect of drug resistance on the fitness of *Mycobacterium tuberculosis*. *Lancet Infect Dis* 2003;**3**:13–21.
95. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. *Nat Rev Drug Discov* 2013;**12**:388–404.
96. Bjorkman J, Andersson DI. The cost of antibiotic resistance from a bacterial perspective. *Drug Resist Updat* 2000;**3**:237–45.
97. Andersson DI. The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr Opin Microbiol* 2006;**9:**:461–5.
98. Goldstein F. The potential clinical impact of low-level antibiotic resistance in Staphylococcus aureus.. *J Antimicrob Chemother* 2007;**59**:1–4.
99. Dartois V. The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat Rev Microbiol* 2014;**12**:159–67.
100. Zetola NM, Shin SS, Tumedi KA, Moeti K, Ncube R, Nicol M, et al. Mixed Mycobacterium tuberculosis complex infections and false-negative results for rifampin resistance by GeneXpert MTB/RIF are associated with poor clinical outcomes. *J Clin Microbiol* 2014;**52**:2422–30.