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Translating basic science insight into public health action for multidrug- and extensively drug resistant-tuberculosis

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Abstract

Multidrug (MDR)- and extensively drug resistant (XDR) -tuberculosis (TB) impose a heavy toll of human suffering and social costs. Controlling drug-resistant-TB is a complex global public health challenge. Basic science advances including elucidation of the genetic basis of resistance have enabled development of new assays which are transforming the diagnosis of MDR-TB. Molecular epidemiologic approaches have provided new insights into the natural history of TB with important implications for drug resistance. In the future, progress in understanding *M. tuberculosis* strain-specific human immune responses, integration of systems biology approaches with traditional epidemiology and insight into the biology of mycobacterial persistence have potential to be translated into new tools for diagnosis and treatment of MDR- and XDR-TB. We review recent basic sciences developments which have contributed or may contribute to improved public health response.

Keywords

Tuberculosis, Multidrug-Resistant/diagnosis; Extensively Drug-Resistant Tuberculosis/diagnosis; Tuberculosis, Multidrug-Resistant/epidemiology; Extensively Drug-Resistant Tuberculosis/epidemiology*; Extensively Drug-Resistant Tuberculosis/prevention & control

Introduction

Multi-drug resistant (MDR) tuberculosis (TB) poses an immense global public health challenge, with an estimated 650,000 prevalent cases in 2010.¹ MDR-TB (defined as resistance to both isoniazid and rifampicin) is the consequence of injudicious antibiotic use over the course of decades and inadequate TB control programs.^{2,3} Further acquisition of

resistance among MDR-TB strains has generated extensively drug resistant TB (XDR-TB) (defined as MDR-TB with additional resistance to a fluoroquinolone and a second-line injectable agent). “Completely” or “totally” drug resistant (TDR-TB) strains for which there is no effective treatment are increasingly reported⁴⁻⁶ although criteria for TDR-TB have not yet been formally defined.⁷ Confronting the spread of resistant *Mycobacterium tuberculosis* poses an extraordinarily complex programmatic, clinical and scientific challenge.

This review focuses on how insights from basic science are contributing or could contribute in the future to better clinical care and to improved public health response to MDR- and XDR-TB. We survey developments in microbiology, immunology and systems biology to identify findings with the greatest potential impact. Successful translation from basic science to real-world tools demands a thorough understanding of the operational challenges posed by MDR- and XDR-TB. Therefore, our discussion is grounded in the concrete and immediate needs of clinicians, TB program managers and policymakers. We will address both the benefits as well as unintended negative consequences of novel technologies. The challenge of controlling MDR- and XDR-TB is inseparable from the broader effort to contain non-MDR-TB. For example, measures that would reduce the burden of all TB such as interruption of transmission or vaccines indirectly reduce opportunities for amplification of drug resistance.⁸ However this review will be limited to translational topics that most directly relate to control of MDR- and XDR-TB.

1. Scope of the Global MDR/XDR Challenge

1.1. Epidemiology of MDR- and XDR-TB

After the discovery of anti-TB agents in the 1940 and 1950s, the benefits of single drug therapy were short-lived as resistance developed quickly and predictably with deleterious clinical consequences. Subsequently, the core principle that TB must be treated with multiple effective agents was developed.⁹⁻¹² At the time antibiotics became available, however, there was no model for coordinated global TB control and the principles that now guide TB control programs such as directly observed therapy (DOT), infection control and monitoring until cure had yet to evolve.¹³ During the ensuing decades, insufficient governmental funding and social and political instability led to weak TB control programs in certain countries which were unable to effectively identify cases, monitor therapy and treat to documented cure. The consequence was progressive amplification of resistance culminating in the spread of MDR in the 1990s³ and, more recently, the global expansion of XDR-TB.¹⁴ Globally, the World Health Organization (WHO) estimates that 3.4% of new TB cases (defined as TB in persons with less than 1 month of prior TB treatment) and 20% of retreatment cases (defined as one or more months of prior TB treatment) have MDR-TB.¹⁵

The distribution of MDR-TB varies geographically. The two most populous countries, India and China, contribute more than 50% of the world's MDR-TB burden. However, the proportion of MDR-TB cases vary within high burden countries; including rates as high as 62% for retreatment cases in parts of Tajikistan. Seven countries report areas with MDR-TB rates of greater than 50% among retreatment cases.¹⁵ While improvements in TB care and control have begun to lower overall TB incidence and mortality during the past decade, the

WHO concludes that there is no definitive epidemiologic evidence that rates of MDR-TB are changing globally.^{15,16} The distribution and burden of XDR-TB is harder to estimate since diagnosis depends on susceptibility testing for second-line agents which is not widely available. By the end of 2010, 68 countries had reported at least one case of XDR-TB.¹

1.2. The Current Landscape: Diagnostic , Therapeutic and Programatic Challenges Posed by MDR- and XDR-TB

Controlling MDR- and XDR-TB presents staggering operational, clinical and financial challenges and, in general, the countries with the greatest burden are the least equipped to respond.

1.2.1. Poor Laboratory Capacity Limits Diagnosis of MDR- and XDR-TB—

Capacity to perform culture and drug susceptibility testing (DST) is fundamental since MDR- and XDR-TB are defined on the basis of susceptibility results. Conventional laboratory capacity is woefully inadequate in the 27 countries classified by WHO as high-MDR-TB burden (defined by at least 4,000 new MDR-TB cases annually or >10% MDR among newly registered cases). Of these, 14 (52%) had less than one AFB smear microscopy center per 100,000 persons and less than one laboratory capable of performing DST per 5 million persons in 2010.¹⁵ Globally, less than 2% of new cases and less than 6% of retreatment cases were tested for MDR-TB.¹ Consequently, just over 50,000 cases of MDR-TB were reported to WHO in 2010, representing just 18% of MDR-TB cases estimated to exist among the 5.7 million cases of all active TB reported.¹⁵ Additional MDR-TB cases go unrecognized among the estimated additional 3.1 million projected cases of TB that are never diagnosed or reported to WHO.

1.2.2. Existing Molecular Diagnostics for MDR-TB—

Resurgent TB rates in the 1990s and the menace of MDR- and XDR-TB prompted investment in both laboratory capacity-building and in novel molecular diagnostics.¹⁷ After decades with minimal innovation in diagnostics, a private-public partnership fostered development of a number of novel assays, presenting a range of new options for rapid molecular detection of drug resistance. The relative advantages and disadvantages of these assays for TB control programs are thoroughly reviewed in two recent publications.^{18,19}

The two assays with the greatest potential impact on diagnosis of MDR-TB and the focus of accelerated implementation are the line probe assays (LPA) (Figure 1A) and the Xpert MTB/RIF (Figure 1B and Box 1). LPAs are based on reverse-hybridization of DNA using DNA strip technology to identify *M. tuberculosis* and new generations of these tests assay mutations in genes associated with rifampicin, isoniazid, fluoroquinolone and aminoglycoside resistance.¹⁸⁻²⁰ These assays are recommended for testing pure *M. tuberculosis* culture or acid-fast positive sputum and can provide results within hours. Recent reports suggest LPAs may also be accurate in smear-negative TB.²⁰⁻²²

1.2.3 Scaling-up MDR-TB treatment: A balance between saving lives and amplifying resistance—

Treatment of MDR-TB generally requires two years of therapy with second-line agents that are substantially more costly, more toxic and less effective than

standard drugs used for drug susceptible-TB.²³ The long course of treatment, need for continual monitoring for both adherence and adverse events, need for laboratory support, and the risk of amplification from MDR-TB to XDR-TB demands a coordinated program with substantial logistical and clinical capacity.²⁴ As a result of these challenges, as of 2010, only 9% of TB basic management units worldwide provide treatment services for MDR-TB patients.¹⁵

Since MDR-TB or XDR-TB treatment regimens have rarely been evaluated in randomized clinical trials,²⁵ MDR-TB treatment regimens vary widely, depending on individual clinicians, institutions and countries. Unavailability of traditional DST frequently precludes individualized treatment regimens so most high-burden settings use standardized MDR-TB or XDR-TB treatment regimens based on epidemiologic data from periodic drug-resistance surveys.⁴ Rates of treatment success are generally around 60%²³ and amplification from MDR-TB to XDR-TB occurs even in well-functioning MDR-TB treatment programs following current WHO guidelines.²⁶

There is broad agreement that universal access to MDR-TB services is a moral, social and economic imperative.^{34,35} However, the inevitable consequence of poorly managed use of second-line agents will be further amplification of XDR- and TDR-TB.³³ For scaling up treatment of MDR-TB, there needs to be a rapid expansion of laboratory services combined with access to quality-assured second-line drugs delivered through effective treatment programs.

2. Translation of basic science for public health impact

2.1. Molecular mechanisms of drug resistance

2.1.1 Connecting genotypic resistance, phenotypic resistance and clinical outcome—Unlike many other bacteria, in which there is an ongoing exchange of mobile elements, drug resistance in *M. tuberculosis* appears to occur exclusively as a consequence of sequential, spontaneous chromosomal mutations, including point mutations, deletions, and insertions.²⁷ These resistance mutations arise as a consequence of selective pressure of anti-TB drug treatment regimens, often initially resulting from either inadequate treatment or patient non-adherence. To date, there is no clear evidence that horizontal gene transfer of resistance-encoding elements occurs in *M. tuberculosis*.

Gene targeted sequencing²⁸ and whole genome sequencing^{29,30} have revolutionized our understanding of the molecular mechanisms of resistance. Key mutations which confer resistance to first and second-line drugs are shown in Figure 2 and catalogued in a comprehensive online TB Drug Resistance Database.^{31,32} Genetic insights have enabled the development of a range of genotypic resistance tests such as LPAs, Xpert MTB/RIF and other molecular diagnostics.³³ However, resistance mechanisms are incompletely understood and questions remain about how to translate new developments for maximal clinical and programmatic impact.

Critical challenges remain in translating new insights into the genetic basis of resistance into practice. The proportion of phenotypically resistant (i.e., *in vitro* resistance as measured by

DST) *M. tuberculosis* isolates for which known mutations can be identified varies for different drugs. At one end of the spectrum, mutations underlying resistance to isoniazid and rifampicin are well-characterized. Isoniazid is a prodrug that must be activated by the katG catalase-peroxidase to isonicotinyl-NAD. The activated form interacts and binds to its target protein, the enoyl-acyl carrier protein InhA, inhibiting synthesis of mycolic acid, resulting in mycobacterial cell death. In a large sequencing study, 90% of phenotypically isoniazid resistant isolates had either mutations in the *katG* or *inhA* gene. All isolates with these mutations were isoniazid resistant (100% specificity).³⁴ Rifampin blocks bacterial RNA transcription by binding to the beta-subunit of DNA-dependent RNA polymerase encoded by the *rpoB* gene. In the same series, 97% of isolates with phenotypic rifampicin resistance had mutations in the *rpoB* gene, and the specificity of *rpoB* mutations was 94%. This comprehensive understanding of isoniazid and rifampicin mutations, at the gene level, has facilitated the success of molecular diagnostics for resistance to these two important drugs.

By contrast, for ethambutol and second-line drugs such as capreomycin and fluoroquinolones, the sensitivity of sequencing, relative to phenotypic resistance was lower, ranging from 61 to 81%.³⁴ These results indicate that additional, currently unidentified, resistance-conferring mutations exist. An important goal therefore is to identify these other mechanisms of drug resistance at the genomic level, enabling us to not only design more robust molecular diagnostics, but also to use this knowledge in strategizing therapeutic regimens.

The relationship between genotypic and phenotypic resistance is not always straightforward. The best characterized and most common resistance mutations predictably result in phenotypic resistance but sequencing and molecular beacon assays (which identify deviations from wild type) identify other mutations in resistance-determining regions, that can be either silent (not resulting in resistance) or result in only low-level resistance.³⁴ In order to resolve these issues, further research correlating mutations discovered through sequencing with phenotypic DST in clinically relevant resistant strains will be necessary. This will allow us not only to better document and catalogue resistance mutations, but also to develop more robust platforms for accurate diagnostics.

Finally, for several second-line drugs, phenotypic resistance measured via DST does not necessarily indicate that the drug will have no efficacy in the treatment of human infection. Numerous factors can contribute to this, including differing activity *in vivo* and *in vitro*, infection in any one individual with a mixture of *M. tuberculosis* strains, and transcriptional resistance mechanisms that may be upregulated *in vitro* but not *in vivo*. The synergistic and antagonistic effects of combination therapy also come into play *in vivo*. For instance, rifampicin appears to induce efflux pumps which reduce ofloxacin sensitivity.³⁵

2.1.2 Why do some resistance mutations “succeed?”—Although chromosomal mutations occur randomly in the *M. tuberculosis* genome, through the process of natural selection a relatively small number of mutations cause most observed phenotypic resistance. In some cases the mutations occur in a small subset of potential positions in the protein. For example, >95% of mutations in *rpoB* occur in a small 100 bp region, and of these, only three

specific mutations result in 70-80% of phenotypic resistance to rifampicin in clinical strains.³⁶

Why these few mutations dominate in strains that have succeeded in causing human MDR-TB is an important question. One explanation is that “successful” mutations enable survival in the presence of drug while minimizing the fitness cost of resistance. Fitness cost is based on the premise that clinical *M. tuberculosis* strains are optimally adapted for transmission and causing disease, and that mutations in highly conserved genes such as *rpoB* come with a fitness cost, or impaired ability to be transmitted or cause disease. The myriad of other possible *rpoB* mutations may not be seen clinically because they may impose too great a fitness cost.

The degree to which drug-conferring mutations impose a fitness cost has important implications for predicting the spread of MDR-TB³⁷ and has been the subject of intense laboratory and epidemiologic investigation.^{38,39} *In vitro* data confirm a fitness cost with resistance and indicate that different mutations affect fitness to variable degrees. Gagneux *et. al.* generated a panel of nine different *rpoB* mutants by exposing a rifampicin-sensitive clinical strain to rifampicin and then compared the fitness of the *rpoB* mutants to their non-mutated parent through competition assays. In this study, all *rpoB* mutants were less fit than the parent strain and the fitness of different *rpoB* mutants differed significantly.³⁹

Gagneux *et. al.* additionally evaluated whether the fitness cost of resistance differed depending on the *M. tuberculosis* genotype. Repeating the experiment with a Beijing strain, they found that the fitness cost of the same *rpoB* mutations was different than in the initial experiment, indicating that certain genotypes may be more capable of tolerating particular mutations. Other investigators have also observed that fitness cost varies with genotype.^{38,40}

Finally, Gagneux *et. al.* observed an epidemiologic association between fitness cost of *rpoB* mutations and the frequency with which those mutations cause human disease. The *rpoB* mutation with the lowest *in vitro* fitness cost was the most commonly observed, accounting for over 50% of rifampicin resistance in clinical strains from different parts of the world. Conversely, the *rpoB* mutation with the greatest *in vitro* fitness cost was not found among clinical isolates. They concluded that the fitness cost of resistance is not only heterogeneous, depending on the particular mutation and strain, but is inversely related to the ability of *M. tuberculosis* to successfully establish itself in human disease.

Complicating the assessment of fitness cost on the epidemiology of MDR- and XDR-TB is *M. tuberculosis*' capacity to adapt to initially deleterious mutations via compensatory mutations. With repeated passage *in vitro*, the fitness cost of resistance appears to diminish.³⁶ Compensatory mutations in the same gene or other genes may arise to compensate for a decrease in fitness due to the original drug resistance mutation. Compensatory mutations in the RNA polymerase genes *rpoA* and *rpoC*, for instance, are thought to compensate for rifampicin resistance mutations in the *rpoB* gene.⁴¹ One intriguing idea, that has some experimental backing, is that in some cases, mutations that result in decreased fitness may revert, if a drug is withheld, as has been shown with the reversion of an isoniazid resistant strain in the absence of isoniazid.⁴²

2.1.3 Generation of resistance during human infection—The generation of resistance during human infection can be attributed to many factors, including the misuse or mismanagement of drugs, patient non-adherence, and inadequate drug resistance screening. Acquired resistance occurs as a result of new mutations in the infecting strains, evolving under the selective pressures of drugs, while primary resistance occurs as a result of transmission and infection with a previously resistant strain. Genome sequencing efforts have helped elucidate the mutations responsible for clinical anti-TB resistance on a genome-wide basis,^{29,30} and genome sequencing used in conjunction with social network analysis has provided new tools to understand the patterns of disease transmission,⁴³ complementing more traditional epidemiological approaches.

The rate at which mutations result in phenotypic resistance is an area of considerable discussion, and many of the rates that are commonly cited were determined over 40 years ago, before specific resistance-conferring mutations were identified (Table 1).⁴⁴ Rates differ substantially between drugs. For example, as shown in Table 1, phenotypic resistance to ethambutol emerges at a rate that is more than 2,000-fold greater than for rifampicin. These studies have been complemented by recent genome sequencing efforts to estimate mutation rates *in vivo*.⁴⁵

Drug concentrations and microenvironmental conditions influence both the rate of mutation and specifically which mutations occur.³⁶ For example, Jenkins *et al.* found a wider spectrum of *rpoB* mutations in *M. tuberculosis* cultivated at low pH than at higher pH.⁴⁰ During human infection, *M. tuberculosis* may be exposed to considerable heterogeneity in both drug concentrations and local tissue conditions. For example, in the center of a caseous mass or in empyema, *M. tuberculosis* may experience low pH, low oxygen tension, and low drug concentrations due to limited penetration.⁴⁶ *M. tuberculosis* also has distinct metabolic adaptations to different *in vivo* conditions as demonstrated by different transcriptional profiles among bacilli isolated from the caseous cavity, pericavity wall and architecturally normal distant lung tissue.⁴⁷ It is postulated that these factors may increase rates of *M. tuberculosis* mutation.

Non-genetic mechanisms of resistance can also occur, and may play an important role in the progression from low-level resistance to high-level resistance. Transcriptional responses of drug efflux pumps, for instance have been suggested to play a role in resistance,^{48,49} and in at least one case it has been shown that rifampicin treatment of rifampicin-resistant strains contributes to ofloxacin resistance through the transcriptional upregulation of efflux pumps.³⁵ Together this suggests that genetic-based diagnostics might be complemented with the co-development of expression-based assays in some instances.

2.1.4 Future biomarkers for MDR- and XDR-TB—Xpert MTB/RIF and LPAs represent an enormous stride forward in our quest for rapid molecular diagnostics, but have several crucial limitations: (1) they are laboratory-based assays, as opposed to point-of-care tests, (2) they are ultimately screening tests that do not supplant the need for DST to formulate drug regimens and (3) they cannot, for the most part, be used in peripheral settings without stable electricity, where much of the world's TB is diagnosed.

Optimally, development of future molecular assays would meet two critical niches: a point-of-care test to screen for MDR-TB in peripheral settings and a molecular platform for use in reference laboratories that could serve as an alternative to DST. The Foundation for Innovative New Diagnostics (FIND) spearheads international collaboration to improve TB diagnostics. The current FIND assay development pipeline includes point-of-care tests for detection of *M. tuberculosis*, but these assays are not specifically oriented towards MDR-TB.⁵⁰

The second ultimate, long-term, goal will be a rapid genomic-based test, sufficient to direct therapy in the absence of culture and conventional phenotypic DST. Despite substantial international efforts to build laboratory capacity across the globe, the infrastructure and expertise required for phenotypic DST continues to make it difficult to establish and maintain in many parts of the world. Supplanting culture and phenotypic DST would require a comprehensive cataloguing of resistance mutations underlying clinical resistance and would require development of robust platforms capable of either sequencing or applying a large number of probes to sputum specimens. To date, the most comprehensive approach to molecular detection of drug resistance use in clinical practice is direct sequencing of resistance-determining loci by the U.S. Centers for Disease Control and Prevention's Molecular Detection of Drug Resistance service. In the U.S., this service provides sequencing-based assessment of drug resistance faster than is available via traditional DST. However, because of our understanding of the genetic basis of phenotypic resistance remains incomplete, such testing cannot substitute for the gold-standard DST.

In summary, our knowledge of the molecular basis for drug resistance is growing rapidly, particularly with the implementation of faster and less expensive sequencing technologies. These discoveries are being translated to molecular diagnostics, which hold the potential to revolutionize our ability to rapidly identify drug resistance, even in low-resource peripheral settings. The ultimate goals of a point-of-care test for MDR-TB and molecular assays that can replace phenotypic DST seem distant today, but it is hoped that the sustained funding and international collaboration that enabled development of LPAs and Xpert MTB/RIF will someday bring these goals to fruition as well.

2.2. Implications of reinfection and mixed infections

The application of molecular typing methods for *M. tuberculosis* during the past two decades has shaken key assumptions about the natural history and microbiology of TB. Specifically, molecular epidemiologic studies have provided new evidence regarding the occurrence of exogenous reinfection, mixed infections and the immune protection conferred by infection and disease. These findings have important implications for drug resistance. For several of these phenomena, the science is far from settled so we will review existing data and highlight key unanswered questions.

2.2.1. Classic TB natural history paradigm—The classical paradigm, termed the Unitary Theory of Tuberculosis, suggested that the natural history of TB followed a consistent sequence (Figure 3A).⁵¹ In high-incidence settings, primary infection was believed to occur once with a single strain of *M. tuberculosis*. Infection could either cause

primary progressive disease or, more commonly, result in latent TB infection (LTBI). LTBI was believed to provide protective immunity from reinfection with other *M. tuberculosis* strains despite continuing exposures. For individuals who developed reactivation TB, disease was caused by the single infecting strain. If a recurrence of TB occurred after completion of therapy, the second episode was due to relapse rather than reinfection.

2.2.2 Reinfection during latency—The premise that the immune response associated with a positive tuberculosis skin test (TST) prevents reinfection with additional *M. tuberculosis* strains has been contested for decades.⁵² However, since there is no way to assay *M. tuberculosis* directly during latency, it has been difficult to demonstrate sequential infections with multiple strains before a patient presents with TB.

Evidence that adaptive immunity associated with LTBI prevents reinfection includes studies in which individuals exposed to a case of active TB were less likely to develop acute TB if they had a prior positive TST than those with a prior negative TST.^{53,54} For example, medical and nursing students entering training in the U.S. in the 1930-50s with a positive TST had about half the risk of developing TB as those entering with a negative TST.⁵³ Experiments in guinea pigs also suggested that an initial *M. tuberculosis* infection protects from subsequent infection.⁵⁵

Conversely, a body of circumstantial epidemiologic evidence (thoroughly reviewed by Chiang *et al.*⁵⁶) suggests that sequential recurrent infections do occur among asymptomatic persons (Figure 3B). Mathematical models indicate that LTBI is minimally, if at all, protective against reinfection.⁵⁷⁻⁵⁹ The fact that multiple *M. tuberculosis* strains are sometimes isolated during initial episodes of TB lends further credence to the hypothesis that reinfection occurs during latency.

Recently, Houben *et al.* linked data on TST testing from before 1990 with a molecular epidemiologic analysis of TB cases occurring during 1996 to 2008 in a population-based study in Malawi.⁶⁰ Of 17 HIV-uninfected TB patients documented to have a positive TST over a decade earlier, 5 (29%) had a clustered isolate suggesting recent reinfection. Among 25 HIV-infected TB patients with prior positive TST, 22 (88%) had clustered isolates. Assuming the previous TST truly indicated previous infection with *M. tuberculosis*, this study strongly supports the occurrence of reinfection during latency.

Reinfection during latency has implications for MDR- and XDR-TB. The degree to which initial infection prevents subsequent infection with other strains is an essential input in models forecasting the future course of the MDR- and XDR-TB epidemics. For example, if latency due to non-MDR-strains prevents superinfection with resistant strains, high rates of LTBI in many developing countries would provide a bulwark against the spread of MDR- and XDR-TB. Based on this assumption, some models come to the conclusion that successful control of non-MDR-TB in low-incidence industrialized countries has created greater vulnerability to the spread of MDR-TB.⁶¹ Based on the observation that different *M. tuberculosis* lineages may elicit different host responses, investigators have hypothesized that lineage-specific immunity following initial infection may slow expansion of MDR-TB epidemics in regions with few circulating lineages (e.g., Europe) more than in regions with a

greater diversity of circulating lineages (e.g., sub-Saharan Africa).⁶² It is critical to note that these models hinge on assumptions about the protective immunity associated with LTBI but the evidence supporting these assumptions is limited.

In summary, the existence and importance of protective immunity, like many aspects of the natural history of LTBI, remains controversial and in need of continued research.⁶³ The key scientific breakthrough needed is an assay capable of clarifying the spectrum of host and pathogen interaction during latency.⁶⁴

2.2.3 Reinfection causing recurrence after an episode of TB—The existence and importance of exogenous reinfection leading to recurrent TB had been debated for decades.^{51,65-67} Over the past 25 years, the development of accurate molecular typing techniques enabled comparison of molecular fingerprints of *M. tuberculosis* isolated during successive TB episodes in individual patients. Recurrence is attributed to reinfection rather than relapse when different strains are isolated during a patient's first and second episode of TB (Figure 3B).

Whether two isolates are classified as different strains can depend on the typing method used. Early methods such as phage typing were insensitive for detecting strain differences since many strains share a single phage type. Typing methods have become increasingly sensitive with progression from spoligotyping to IS6110-based restriction fragment length polymorphism (RFLP) to PCR-based methods such as mycobacterial interspersed repetitive unit–variable-number tandem repeats (MIRU-VNTR) and finally to whole genome sequencing.⁶⁸ Strains that appear identical using one typing method sometimes are identified as different with the use of higher-resolution methods. For example, during a recent TB outbreak in British Columbia, isolates from 37 patients were identical by RFLP and MIRU-VNTR, suggesting transmission of a single strain. However, sequencing of the same isolates revealed that two distinct *M. tuberculosis* lineages were causing simultaneous outbreaks.⁴³

Reinfection causing recurrent TB with a new *M. tuberculosis* strain has now been repeatedly documented using different molecular techniques.⁶⁹⁻⁸³ In general, the proportion of recurrent TB attributable to reinfection is associated with the local TB incidence, presumably reflecting risk of re-exposure to *M. tuberculosis*.⁸⁴⁻⁸⁶ In high-incidence settings, 40-100% of recurrent TB may be attributable to reinfection.^{79,81,82,87} In low TB incidence settings, data on the proportion of recurrent TB attributable to reinfection is mixed. Studies in Italy,⁷⁵ Spain,^{71,72,88} the Netherlands⁷⁸ and Australia⁷⁷ indicate that 16-33% of recurrent TB is due to reinfection while a study in the U.S. and Canada⁷⁴ found that only 4% of recurrent TB represents reinfection. Although reinfection has most frequently been documented in TB which recurs *after* the completion of therapy, reinfection with a new strain has also been reported *during* initial anti-TB therapy.^{70,83}

Host immune status is also an important predictor of exogenous reinfection after an episode of TB.⁸⁶ In South India, Narayanan *et al.* found that 22 (88%) of 25 HIV-infected patients with recurrent TB were reinfected with a new strain. Among HIV-uninfected patients only 2 (9%) of 23 were reinfected.⁷⁶ Similarly, in a population-based study in Malawi, risk of reinfection was >6-fold higher among HIV-infected individuals than among HIV-uninfected

individuals.⁸⁰ The degree to which these differences occur because HIV-infected patients are more likely to have nosocomial exposures or are more likely to become reinfected if exposed or simply are more likely to rapidly progress to active disease once reinfected is unclear.⁸⁰

Reinfection with MDR- or XDR-strains during or after anti-TB therapy can confound surveillance. MDR-TB in a patient previously treated with more than one month of anti-TB therapy is traditionally interpreted as amplification of drug resistance due to inappropriate drug exposure (Figure 4A).¹ However, MDR-TB can also emerge during or after treatment as a consequence of reinfection (Figure 4B) and high rates of reinfection may cause either under- and over-estimation of on-going amplification of resistance. In an example of under-estimation of amplification, Caminero *et. al.* observed that in Gran Canaria Island, Spain, a site with moderate TB incidence, 3 (16%) of 18 patients with recurrent TB had MDR-TB at the second episode. However, for 8 of these patients, recurrent TB was the result of reinfection with a non-MDR strain. For the remaining 10 patients, recurrent TB occurred due to relapse with the original strain and three (30%) of these had amplification of resistance to MDR.⁷²

Conversely, in an example of over-estimation of amplified resistance, Andrews *et. al.* identified 17 patients in KwaZulu-Natal, South Africa successfully treated for non-MDR TB who subsequently recurred with MDR- or XDR-TB. All 17 recurrences occurred due to different TB strains, indicating ongoing transmission and reinfection rather than amplification of resistance. As the authors note, enhancing TB treatment to prevent amplification of resistance will do little to reduce MDR-TB rates if most MDR- and XDR-TB occur due to primary transmission.⁸⁷

The implications of recurrent TB due to reinfection for clinical trials in which recurrent TB is a primary outcome⁸⁹ depend on the setting. In trials in the U.S. and Canada, only 4% of recurrence was due to reinfection.⁷⁴ By contrast, in the high-incidence settings where drug trials are increasingly conducted, >50% of recurrent TB may be secondary to reinfection and failure to distinguish between relapse and reinfection could critically confound assessment of the efficacy of new treatment regimens.⁷⁶

2.2.4 Mixed infection—Prior to the advent of molecular typing, simultaneous infection with a mixture of strains was suspected based on the fact that different colonies from the same sample sometimes gave different DST results.⁹⁰ In the 1970s, phage typing confirmed mixed infection.^{91,92} For example, Mankiewicz *et. al.* analyzed three randomly-selected colonies per patient from 233 indigenous Alaskan TB patients and identified 33 (14.2%) patients with multiple strains.⁹² Mixed infection was subsequently detected using RFLP^{90,93} and individual patients have been observed to have different strains simultaneously in pulmonary and extra-pulmonary sites.^{91,94}

How commonly mixed infections occur and how significantly they impact interpretation of DST and amplification of resistance is contested. A variety of technical and conceptual challenges make interpretation and comparison between studies difficult. One challenge introduced by highly sensitive modern typing methods is distinguishing between the subtle

genetic variability that may develop within a population of *M. tuberculosis* through spontaneous mutations following infection with a single strain (clonal heterogeneity due to *in vivo* microevolution) and genetic distance which indicates that isolates arose from two separate strains of *M. tuberculosis* (mixed infection). Clonal heterogeneity has been defined by MIRU-VNTR as allelic variation at a single locus while allelic variation at two or more loci indicates mixed infection.⁹⁵⁻⁹⁷ The conceptual distinction between clonal heterogeneity and mixed infection with two or more strains is important because in mixed infection the minority population may occur at a much higher frequency ($\sim 10^{-2}$) than spontaneous mutations ($\sim 10^{-6}$ to 10^{-8}).⁹⁸

Using MIRU-VNTR, clonal heterogeneity has been defined as detection of two or more alleles at a single locus. Samples with no allelic differences are classified as clonally homogeneous and samples with two or more alleles at two or more loci are classified as mixed infection with more than one strain.^{95,99} As noted above, the fact that genotyping has recently demonstrated distinct lineages with identical MIRU-VNTR types will require re-evaluation of these definitions.⁴³

Additionally, the proportion of mixed infections that are detected depends critically on sampling and specific laboratory techniques used.^{100,101} A single isolate may not be representative of the entire bacillary population and multiple isolates may improve sensitivity.^{94,101} Most analyses of mixed infection are conducted using DNA extracted from culture isolates rather than directly from sputum and the culture step appears to reduce the sensitivity for detection of clonal complexity.¹⁰² A variety of different strain typing methods have been used, some of which have low sensitivity for detecting multiple strains.^{98,103,104} In light of the challenges and technical variability discussed above, ascertaining the true incidence of mixed infection and systematically comparing among sites is challenging. In general, most studies in high-incidence settings have observed mixed infection rates of 5-18%^{83,95,101,103-105} although lower rates were documented in Malawi¹⁰⁰ and South India.¹⁰⁶

When a patient presents with mixed infection, it is difficult to ascertain the time sequence through which the patient was infected with multiple strains. Investigators have outlined several possible pathways including: (1) simultaneous exposure to and infection by multiple strains, (2) sequential reinfection during latency with reactivation of one strain impairing immunity and prompting reactivation of the second strain (Figure 3B).^{56,99,105}

Mixed infections have the potential to confound the diagnosis and management of MDR- and XDR-TB, particularly if a minority resistant strain is not detected by DST (Figure 4C). For example, in a high-incidence South African setting, van Rie *et. al.* detected mixed infection in 5 (10%) of 48 patients with MDR-TB.⁸³ For four of these patients, phenotypic DST on the initial specimen was entirely susceptible but repeat DST (after months of first-line therapy) demonstrated MDR-TB. In the absence of molecular testing, this would have been interpreted as amplification of resistance. PCR indicated that an undetected MDR-TB strain was present initially and was “unmasked” by first-line drug therapy.

Mixed infections certainly occur but the question remains how much they affect the epidemiology of MDR- and XDR-TB. Investigators have argued that mixed infection may affect the population dynamics of *M. tuberculosis* in high-prevalence settings by enabling less fit drug-resistant strains to persist in a co-infected host, thereby providing the opportunity to develop fitness-enhancing mutations and positioning the strain for emergence when first-line therapy is provided.¹⁰⁷

In summary, molecular epidemiologic analyses based on *M. tuberculosis* genetic markers have provided new insight into the complex dynamics of reinfection during latency, reinfection causing recurrence after an episode of TB and mixed infection. These more nuanced natural history paradigms have important implications for diagnosis, control and prediction of MDR- and XDR-TB.

The next frontier in epidemiology will be integrating the tools of systems biology with traditional epidemiologic approaches.^{108,109} Systems biology has been defined as the study of complex interactions in biological systems through quantification and modeling of molecular constituents.¹¹⁰ Specifically, development of high-throughput biological assays capable of simultaneous quantification of thousands or tens of thousands of biological compounds has enabled the study of patterns of proteins (proteomics), RNA (transcriptomics), metabolites (metabolomics) and lipids (lipidomics). “Omics” approaches are enhancing our understanding of host-*M. tuberculosis* interactions and these insights may lead to improved biomarkers that can be used to better understand the natural history and epidemiology of TB. For example, a whole blood transcriptional signature thought to represent host response to TB was recently identified in TB patients from South Africa and the U.K., and this immune signature resolved with successful TB therapy.¹¹¹ Will there be a “signature” proteomic or transcriptomic pattern to predict whom among the approximately 2 billion persons with LTBI will progress to TB? The systems biology revolution has thus far not resulted in concrete tools for TB but prospects for significant practical applications from this field are good.

2.3. Implications of strain-specific characteristics

In 2007, a NIH panel prioritizing research on MDR- and XDR-TB raised concern regarding the impact of *M. tuberculosis* strain differences on host immunity and the implications for vaccines and drug regimens against emerging strains, especially MDR- and XDR-strains.¹¹² Unfortunately, the majority of our knowledge of virulence and host immunity has been based on research using laboratory adapted strains H37Rv and Erdman.¹¹³⁻¹¹⁵ However, continued growth and repeated passage of laboratory strains reduces virulence, and alters cell wall properties. This section will consider the implications of strain differences for MDR- and XDR-TB with particular attention to emerging Beijing strains (Box 2).

Anti-TB immunity results from a balance of protective T_H1 immune responses which are essential to containing infection but may result in tissue-damage as well as counter-regulatory immune-dampening regulatory T cell (Treg) responses (Figure 5A). With the onset of adaptive immunity, T cells capable of expressing IFN γ accumulate in the lung. These T cells secrete other pro-inflammatory chemokines and cytokines which in turn drive continuous recruitment and activation of lymphocytes, macrophages, dendritic cells and

granulocytes. During this process, expression of the type 1 inflammatory mediators IL-1 β , IFN γ and TNF α are crucial for phagocyte activation, intracellular killing, organized granuloma formation and apoptosis of *M. tuberculosis*-infected macrophages. However, these initial responses can also drive tissue damage, which leads to the emergence of Foxp3+CD4+ Tregs. While Treg response dampens tissue-damaging inflammation, it may also down-regulate the host IFN γ -mediated protective immune response.

M. tuberculosis genotypes with variable virulence may interact differently with host immunity.^{113,114,116-122} In particular, strains in the Beijing genotype family appear to induce distinctly different host immune responses compared to other clades of *M. tuberculosis* strains (Figure 5B).^{123,124} The hyper-virulent W-Beijing strain HN878 was originally described by Kaplan and her colleagues,¹²⁵⁻¹²⁷ who demonstrated that this strain grew extremely rapidly in the mouse lung, and resulted in reduced survival (mortality at 60-100 days) compared to laboratory strains (mortality around 400 days). They found no evidence for any T_H1 response, using RT-PCR, and instead noted an increase in type I interferon levels in these mice. They concluded that the hyper-virulence of HN878 was due to its avoidance of needed T_H1 immunity, mediated by an increased IFN γ response and the production of T_H2-related monokines/cytokines. They then provided evidence for the molecular basis of these events by showing that these monokines were directly induced by an unusual cell wall polyketide synthase-derived phenolic glycolipid which is not produced by members of the other principal genetic groups.¹²⁷

M. tuberculosis lineage has also been shown to influence innate immune response, and virulence was associated with distinct cell envelope lipid profiles.¹²⁸ Wang *et al.* confirmed that low production of TNF α and IL-6 by infected human macrophages and dendritic cells *in vitro* was a signature of the Beijing family as a whole impairing innate immunity.¹²⁹ Ordway *et al.* found that in mice W-Beijing HN878 can induce a very robust and early T_H1 response (peaking ~30 days after aerosol infection), followed by a rapid decline due to strong induction of CD4+Foxp3+IL-10+ Tregs.¹¹⁴ The unique and greater ability of W-Beijing strains to induce immunosuppressive Tregs compared to laboratory strains of *M. tuberculosis* such as H37Rv was further corroborated in guinea pigs using W-Beijing strains TN14149 and SA161.¹¹³

A population-based molecular epidemiological study tested representative isolates from different sublineages of the East Asian *M. tuberculosis* lineage. Ten strains from four of the five sublineages that have the characteristic Beijing spoligotype were tested in guinea pigs infected by aerosolization.¹³⁰ The results of this study demonstrated that all strains were capable of growing and causing lung pathology in guinea pigs exposed by low-dose aerosol infection. Differences among the four sublineages were not overt, but members of the RD207 lineage consisting of strains more likely to cause secondary cases, grew more quickly and caused more severe pathology and enhanced influx of Tregs. All strains tested induced T_H1 immunity, and in addition, all strains induced some degree of regulatory host molecules (Foxp3, TGF β , and IL-17) indicating the influx of Tregs and T_H17 cells. This study supports that the current molecular-based sublineage classification appears to be associated biologically with clinical and pathological consequences, and differences

between sublineages may favor or influence the capacity of these isolates to be more readily transmitted within communities.

Mixed evidence suggests that the BCG vaccine may be less protective against Beijing strains than other *M. tuberculosis* strains and this has led to the hypothesis that favorable selection in BCG-vaccinated populations is contributing to the on-going expansion of Beijing strains (Box 2). In a 2003 study, mice were vaccinated intravenously with BCG and then infected with a high dose of different *M. tuberculosis* clades. Protection induced by BCG against the W-Beijing strain was only about half that seen against the laboratory strain H37Rv.¹²² In contrast, a 2008 study in mice tested BCG-induced protective immunity against nine different *M. tuberculosis* strains (including Beijing strains) and found no strain-specific differences.¹³¹ A third study showed that BCG was ineffective in protecting mice against a W-Beijing strain while a more potent rBCG vaccine was highly protective.¹³² Finally, a recent study demonstrated that BCG-vaccinated mice challenged with W-Beijing strains were protected at day 30, but by day 60 this protection was diminished,¹¹⁸ an event not seen in mice challenged with the laboratory strain H37Rv. Together the studies support the hypothesis that some clinical strains are able to modulate host immunity and evade vaccine-induced protection better than others, most likely by induction of early inflammation causing an indirect expansion of Tregs which interfere with protective immunity. In summary, while the full implications of strain-specific differences in virulence and proclivity to acquired resistance remain to be delineated, there is now compelling evidence that traditional experimental strains like H37Rv and Erdman may fail to recapitulate the full range of pathogenesis seen during human infection with clinical strains.

2.4. Drug Targets and Development

Shorter, less toxic and more efficacious drug regimens for MDR- and XDR-TB are needed urgently to improve outcomes and expand access to treatment.¹³³ Following the discovery of rifamycins in 1963, the last *M. tuberculosis*-specific drug class developed, TB elimination was thought to be within reach. Consequently, by the 1980s, TB drug development research had nearly ceased.¹³⁴ In the 1990s, the emergence of HIV and global spread of MDR-TB inspired a sustained and concerted effort by a public, private, academic and philanthropic collaboration which has yielded a number of new compounds that are in pre-clinical and clinical testing.¹³⁵ The process of developing, testing and implementing new drugs is an extraordinarily complex pharmacologic, financial, regulatory and programmatic endeavor. A number of recent reviews have detailed the current state of the TB drug development pipeline and we will not discuss specific drug candidates here.^{134,136,137} Instead, we will highlight how recent advances in understanding the biology of *M. tuberculosis* may contribute to drug development, and how translational developments could address challenges in the drug development pipeline.

2.4.1 Persisters, resuscitation and the need for sterilizing drugs—Actively replicating *M. tuberculosis* are readily killed by antibiotics and the burden of viable bacteria declines exponentially within days of starting therapy.¹³⁸ Nonetheless, human TB must be treated for months or even years to prevent relapse.¹³⁹ In the 1970s, D. A. Mitchison articulated a conceptual model that remains central to current drug therapy: during human

disease, a population of rapidly growing *M. tuberculosis* is accompanied with sub-populations of metabolically-quiescent, antibiotic-impervious bacteria.¹⁴⁰ Rapidly growing bacteria are killed by antibiotics which inhibit growth-related processes such as cell wall synthesis (isoniazid, ethambutol, ethionamide, cycloserine), nucleic acid synthesis (rifampicin, fluoroquinolones) or protein synthesis (aminoglycosides). The non-replicating population frequently referred to as persisters are affected by only a limited number of “sterilizing” agents such as pyrazinamide and rifampicin (which has both bactericidal and sterilizing properties). Persisters have transient functional antibiotic insensitivity rather than fixed genotypic resistance. If, under the right conditions, persisters begin to replicate, sensitivity to antibiotics is restored.¹⁴¹

Until recently, however, the existence and characteristics of the persister population *in vivo* was based primarily on inference because a persistent phenotype could not be directly identified during human disease.¹⁴² Recent findings have shed some light on key questions regarding the persister phenotype that are relevant to both active TB and LTBI. Understanding the biology of persistence and developing novel sterilizing drugs that can shorten therapy is a leading priority for all forms of TB but is particularly important for MDR- and XDR-TB which currently require years of treatment.¹⁴³

What is the metabolic state of *M. tuberculosis* during persistence?: Non-replicating phenotypes can be generated *in vitro* by exposing *M. tuberculosis* to stresses encountered during human infection such as hypoxia,^{144,145} nitric oxide,¹⁴⁶ nutrient deprivation,¹⁴⁷ drug exposure,^{141,148} or combinations of these stresses.¹⁴⁹ Analysis of RNA and protein expression in these experiments has identified bacterial adaptations that are likely key components of persistence. Exposure to low oxygen tension, nitric oxide, and carbon monoxide result in increased expression of the DosR regulon, a 48 gene program that enables prolonged survival under anaerobic conditions by inhibiting aerobic respiration, limiting bacterial replication, and maintaining redox balance.¹⁵⁰ However, in both murine and *in vitro* models of chronic TB, mutants unable to express the DosR regulon are not necessarily more susceptible to antibiotics than wild-type *M. tuberculosis*,¹⁵¹ indicating that a drug-tolerant, non-replicating persister phenotype is achievable through other pathways. As a mechanism for rapid adaptation to and recovery from anaerobic conditions, DosR may be a component of an as-of-yet poorly defined viable but not culturable state.^{152,153} Non-replication induced by stress is associated with accumulation of triacylglycerol in lipid bodies through induction of *Tgs1*, a DosR regulon gene coding for triacylglycerol synthetase^{154,155}, and by incorporation of host lipids.¹⁵⁶ These lipid bodies are postulated to serve as an energy source for the bacilli during resuscitation from dormancy.^{149,156}

How accurately do *in vitro* models represent the persister population present during active disease?: Increasing evidence suggests that the adaptations observed *in vitro* are also present during human infection. A sub-population of bacilli with lipid-bodies was universally noted in the sputum of Gambian patients with smear-positive TB and lipid body accumulation is now considered a putative phenotypic marker for persistence.^{142,152} *M. tuberculosis* RNA expression profiles in sputum were suggestive of a mixed population of replicating and non-replicating bacteria.¹⁴² The triggers for persistence during human

infection are not clear. Two non-exclusive hypotheses are that (1) a population of metabolically quiescent persisters is always stochastically present, although the proportion may vary with growth phase, and (2) micro-environmental conditions such as hypoxia, low pH, nutrient deprivation, DNA damage, exposure to antimicrobials and biofilm formation induce persistence.^{157,158} *M. tuberculosis* encounters a wide range of conditions during human infection and transcriptionally adapts to existence in the acidic and hypoxic caseous mass as opposed to the peri-cavity wall or residence in inflamed but structurally intact lung parenchyma.⁴⁷ Given these diverse environments and adaptations, a binary model of replicating versus persistent *M. tuberculosis* is likely over simplistic, and human TB may involve many metabolically heterogeneous sub-populations in different metabolic states over time.¹⁵²

What prompts persisters to “wake up” or be resuscitated?: *M. tuberculosis* secretes resuscitation-promoting factors (Rpfs) which appear to modify bacterial cell wall by cleaving glycosidic bonds in peptidoglycan.¹⁵² The five *Rpf* genes are expressed differentially and dynamically in the course of resuscitation and appear to be influenced by stressors such as low pH, nutrient deprivation, and hypoxia. Rpfs revive viable but non-culturable *M. tuberculosis in vitro*, in mouse models and in human sputum. Among patients with pulmonary TB, the number of *M. tuberculosis* cultured from sputum increased >5-fold with the addition of Rpfs, suggesting the population may be dominated by viable but non-culturable bacteria that are dependent on Rpfs for resuscitation.¹⁵⁹ Key aspects of the regulation of Rpfs and mechanisms of resuscitation remain obscure but these molecules appear to be an important part of the puzzle in the biology of persistence.

2.4.2 Targeted drug development versus screening—Increasing insight into mechanisms of persistence provides the opportunity for designing pharmaceutical compounds that target processes essential for *M. tuberculosis* survival such as the triacylglycerol synthetase enzyme that enables lipid body formation. Unfortunately, while rational drug design is intellectually appealing, the practical challenge of identifying compounds that specifically inhibit target processes while being “drugable” (*i.e.*, bioavailable, nontoxic to the host, able to penetrate poorly vascularized areas of necrotic lung and the *M. tuberculosis* cell wall) has thus far not proven feasible.¹⁶⁰ The leading compounds currently under investigation were identified through the sometimes slow and laborious screening of libraries of chemical compounds.^{136,137} The development of increasingly sophisticated computational models which leverage pre-existing knowledge to focus high-throughput screening on classes of compounds with the highest likelihood of drug activity and drugability may improve the yield of screening.¹⁶¹ Among computational methods being employed are methods of virtual screening to identify new inhibitors¹⁶² and network based approaches to identify novel drug targets.¹⁶³

2.4.3 Which drugs to test? Finding the right pre-clinical models—Selecting only the most promising drug candidates is essential since capacity to perform randomized controlled trials (RCT) in MDR- and XDR-TB patients is extremely limited. Which compounds are tested in humans depends on results of *in vitro* and animal models, but unfortunately, there are inherent differences in these experimental models from human TB

infections. For example, the primary *in vitro* screen for drug activity, minimum inhibitory concentration (MIC), quantifies drug concentration required to reduce colony-forming units by 90 or 99% in *M. tuberculosis* cultures during an unrestricted growth phase.¹⁶⁴ MIC thus reflects activity against actively growing bacteria and appears to be a reasonable surrogate for bactericidal or bacteriostatic activity but do not reflect sterilizing activity.¹⁶⁵ Pyrazinamide, the potent sterilizing agent that enabled modern short-course chemotherapy, has no activity against actively growing *M. tuberculosis* and would not be identified via MIC screening.¹⁶⁶ Indeed, Zhang notes that this essential drug was discovered serendipitously.¹⁶⁶ Thus, to develop new drugs with sterilizing activity, the aforementioned *in vitro* models of persistence can be adapted for drug screening,^{144,147,167,168} but how well activity in these models is correlated with *in vivo* sterilization is unknown.

Animal models are also problematic in some instances. For example, the granuloma-like lesions formed by mice during *M. tuberculosis* infection (the standard model for assessment of *in vivo* drug efficacy) differ substantially from human granulomas.^{169,170} Furthermore, new drug candidates have historically been tested against the *laboratory* strains of *M. tuberculosis* (e.g. H37Rv and Erdman) discussed in Section 3.3, but a recent head-to-head comparison in mice showed that bactericidal drug activity differed substantially between these lab strains and clinical strains.¹²⁰ In summary, the current methods of pre-clinical drug evaluation have a somewhat limited ability to predict *in vivo* efficacy. Research to improve our understanding of host-pathogen interactions during human infection and disease holds promise to help us better optimize the predictive accuracy of preclinical models.

2.4.4 Accelerating drug trials: The need for biomarkers for treatment response

—Current guidelines for MDR- and XDR-TB treatment are based primarily on expert opinion and observational cohorts.²⁵ It is imperative that new anti-TB drugs be evaluated in RCTs but the challenge of conducting such studies in MDR- and XDR-TB has proven a critical bottleneck to drug development.^{25,133} A major impediment to RCTs in MDR- and XDR-TB is the lack of a robust surrogate endpoint that correlates with relapse, the gold-standard endpoint in TB drug trials.¹⁷¹ Since MDR- and XDR-TB may require years of drug treatment, the completion of a sufficiently large RCT with relapse as the endpoint could take a decade or more for each drug being evaluated. The presence of *M. tuberculosis* in sputum cultures collected after two months of treatment is currently used as a surrogate marker for treatment failure but the predictive accuracy is questionable.¹⁷¹ Therefore, a biomarker that can accurately predict a durable treatment response is therefore urgently needed.

4. Conclusions

MDR- and XDR-TB impose a staggering burden of human suffering and social costs. Global implementation of key steps in control such as early accurate diagnosis of drug resistance, interruption of primary transmission of resistance, and intensively monitored treatment in quality-assured programs present enormous programmatic and financial challenges. While there is no quick fix, basic science insights may be translated into new tools that will improve clinical care and public health response.

Understanding of the genetic basis of drug resistance has already led to novel diagnostics such as Xpert MTB/RIF and LPAs that are transforming the diagnosis of MDR-TB. Optimally, future assays will lead to both true point-of-care assays for MDR-TB and to molecular assays that would replace traditional DST. Enhanced molecular subtyping enabled the field of molecular epidemiology which has challenged traditional paradigms of TB natural history with important implications for MDR- and XDR-TB. In the future, integration of systems biology with traditional epidemiologic approaches should lead to increased understanding of the pathobiology of TB and to novel biomarkers.

Improved insight into host-pathogen interaction and the different immune responses induced by different *M. tuberculosis* strains may identify the causes of global emergence of highly virulent Beijing strains that appear to have greater propensity towards developing resistance. Similarly, increasing understanding of the biologic basis of persistence may lead to development of new, more effective sterilizing drugs to shorten the course of MDR- and XDR-TB treatment.

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Box 1 Xpert MTB/RIF: a case study in translation from bench to global public health impact

Sequencing the *Mycobacterium tuberculosis* genome set the stage for Xpert MTB/RIF, a robust and user-friendly rapid assay that is changing the landscape of multidrug-resistant (MDR) tuberculosis (TB). Translation of scientific insight into public health practice was the result of a unique collaboration between public and private funders, private industry and the World Health Organization.

Described as ‘game changing’,²⁰ Xpert MTB/RIF is a real-time polymerase chain reaction assay performed directly on sputum that simultaneously detects the presence of *M. tuberculosis* and the 23 commonly occurring mutations in the *rpoB* gene that confer rifampin resistance.²¹ Rifampin resistance was chosen as a marker for MDR-TB because many rifampin-resistant strains are also resistant to isoniazid.

The sensitivity of Xpert MTB/RIF for detection of *M. tuberculosis* under operational conditions is greater than 98% among smear-positive and greater than 70% among smear-negative patients with TB.^{22,23} The sensitivity of Xpert MTB/RIF is also high among human immunodeficiency virus (HIV)-infected patients and children, populations in which TB is difficult to diagnosis because smears are frequently negative.^{24,25} Specificity for TB diagnosis is greater than 98% in all populations. Under operational conditions, the sensitivity and specificity for rifampin resistance is greater than 94% and 98%, respectively.^{6,22,23}

Xpert MTB/RIF provides results within 2 h of specimen collection and uses disposable single-use cartridges that reduce biosafety and cross-contamination risks. Operation is straightforward and the platform may be adapted for other high-priority pathogens.²⁶ Disadvantages are that stable electricity and air conditioning are often required to maintain the platform within the 15–30°C range, and annual recalibration is necessary.²⁶ The subsidized cost is currently a challenging \$16.86 US dollars per assay.

The positive predictive value of positive rifampin resistance on Xpert MDR/RIF depends critically on the local prevalence of MDR-TB. The positive predictive value of MDR-TB is greater than 90% if the prevalence of rifampin resistance is >15% but only 49% if the prevalence is 2%.²⁶ Additionally, a recent report indicates that in low MDR-TB incidence settings, more than 40% of rifampin-resistant isolates were not isoniazid-resistant.²⁷ In these settings, a positive Xpert MDR/RIF result will not reliably indicate MDR-TB.

In 2010, the WHO recommended the use of Xpert MTB/RIF as the initial diagnostic test among individuals suspected of having MDR-TB, among HIV-infected persons, and as a secondary test for smear-negative TB suspects.²⁸ A concerted international effort to support worldwide implementation has facilitated orders of >500 instruments at concessional prices as of the third quarter of 2011.²⁹ The strategy is based on decentralizing testing to make Xpert MTB/RIF available at the district level.

How will use of Xpert MTB/RIF change clinical and public health practice for MDR-TB? Drug susceptibility testing (DST) will continue to be essential to choose a treatment

regimen, but DST is not widely available. MDR-TB treatment is currently available for less than 10% of existing MDR-TB cases. Xpert MTB/RIF will substantially widen the gap between MDR-TB cases diagnosed and available treatment. Diagnosis of rifampin resistance via Xpert MTB/RIF is already posing ethical challenges in settings without MDR-TB treatment. Clinicians face the choice of giving no therapy or improvising second-line treatment based on incomplete information with agents such as fluoroquinolones that may be locally available. If clinicians chose to improvise, the perverse consequence of Xpert MTB/RIF could be amplification of extensively drug-resistant TB.

Xpert MTB/RIF illustrates that translation to public health impact for TB requires more than simply scientific advances—it requires understanding of the public health needs, appropriate technology and keen attention to potential unintended consequences.

Box 2 Epidemiology of Beijing strains: emergence and association with drug resistance

The spread of Beijing strains of *Mycobacterium tuberculosis* is of great concern because these strains appear to be both more virulent and more commonly drug-resistant than non-Beijing strains. The ‘success’ of Beijing strains raises compelling, if still unanswered, questions about how these strains are different and what the ramifications will be for multidrug-resistant and extensively drug-resistant tuberculosis (TB).

EPIDEMIOLOGICAL EXPANSION

Beijing strains form a distinct genotype family with unusual genetic homogeneity despite their worldwide spread. Phylogenetic analyses suggest Beijing strains emerged relatively recently (~1000 years ago) in China and disseminated along migration and trade routes.¹²⁶ A systematic review indicated that these strains continue to expand in some sites and are stable in others.¹²⁷ In much of East and Southeast Asia, Beijing strains are the dominant endemic strains causing up to 90% of TB. The proportion of disease caused by Beijing strains is expanding in Western Europe, South Africa and countries of the former Soviet Union, with explosive growth in some sites. For example, among children in South Africa's Western Cape Region, Beijing strains were absent before 1965 and rare before 1995, but grew from 13% to 33% of TB cases between 2000 and 2003.¹²⁸ Analysis of both children and adults in the same region demonstrated exponential increase in Beijing strain TB with a doubling time of 4.6 years.¹²⁹ Conversely, in other sites, such as the US and Sweden, rates of TB due to Beijing strains do not appear to be increasing.^{127,130}

What factors enabled the remarkable expansion of Beijing strains? This is the subject of intensive ongoing scientific inquiry, but hypotheses can be broadly categorized. First, the increased pathogenicity of the Beijing strains means that they are more likely to cause disease and be transmitted. Second, the bacillus Calmette Guérin vaccine may be less protective against Beijing strains thereby favouring their spread. Finally, because drug resistance may be more common among Beijing strains (see discussion later), empirical TB treatment may have selected for and permitted continuing transmission of these strains.

ASSOCIATION WITH DRUG RESISTANCE

Many epidemiological analyses (renewed by Hanekom *et al.*¹³¹ and Parwati *et al.*¹³²) have demonstrated strong association between Beijing strains and higher rates of drug resistance. However, for unclear reasons, this association is notably stronger in sites where the incidence of Beijing is rising as compared with sites where it is endemic.¹²⁷ What would account for a higher frequency of drug resistance among Beijing strains? An early, appealing explanation was that Beijing strains are ‘hypermutators’ due to unique alterations in DNA repair mechanisms.¹³³ However, *in vitro* experiments suggested that the rate of mutations leading to rifampin resistance was no higher among Beijing strains than non-Beijing strains,¹³⁴ and no evidence for hypermutation has been detected among clinical isolates.¹³⁵ An alternative explanation is that even if Beijing strains develop resistance mutations at the same rate as non-Beijing strains, the more pathogenic Beijing

strains may bear less of a fitness cost from resistance mutations and therefore may be more likely to propagate.^{131,132-135}

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A) Line-probe assay **B) GeneXpert MTB/RIF** **Figure 1.**

Technology underlying line probe assays (LPA) and Xpert MTB/RIF. With LPAs (**A**), DNA is first extracted from *M. tuberculosis* culture or sputum and the resistance-determining regions of rifampicin, isoniazid, fluoroquinolones and aminoglycosides are amplified via PCR. The labeled PCR products are then exposed to a strip impregnated with oligonucleotide primers specific to both resistance-conferring mutations and wild-type sequence. Color change on the strip indicates the presence of either resistance-conferring mutations or wild-type sequence for each of the target drugs. Xpert MTB/RIF (**B**) is an automated nucleic acid amplification test in which the only manual processing required is sputum liquifaction and inactivation. The automated platform purifies, concentrates, and amplifies DNA to identify target sequence indicating the presence of *M. tuberculosis* and *rpoB* mutations.

Drug	Mechanism of Action	Key Resistance-conferring Mutations
Isoniazid (INH)	INH is converted to isonicotinic acid by the catalase peroxidase protein KatG. The activated form, INH-NAD, then binds to the NADH-dependent enoyl ACP reductase, inhA, inhibiting fatty acid elongation and mycolic acid synthesis.	i) Point mutations in katG, inhA or NADH dehydrogenase gene (ndh) resulting in loss of enzymatic activity; ii) Mutations in the promoter region of the mabA / inhA operon, leading to an increase inhA expression; iii) Overexpression of the antioxidant enzyme AhpC (alkyl hydroperoxide reductase)
Rifampin (RIF)	RIF binds to and inhibits the β -subunit of the mycobacterial DNA-dependent RNA polymerase (rpoB)	Point mutation in β -subunit of RNA polymerase protein (rpoB)
Ethambutol (EMB)	EMB inhibits the arabinosyl transferase (embB), involved in the synthesis/polymerization of arabinan component of arabinogalactan and LAM in the cell wall	Mutations of the embB gene
Pyrazinamide (PZA)	PZA is activated to pyrazinoic acid (POA) by the mycobacterial pncA protein, leading to inhibition of mycobacterial fatty acid synthesis	Mutation of the mycobacterial pyrazinamidase (pncA) protein, involved in converting PZA to POA
Fluoroquinolones (FQN)	Fluoroquinolones bind to and inhibit the mycobacterial DNA-gyrase complex, preventing negative supercoiling of DNA during replication	Mutations in the DNA gyrase A (gyrA) and gyrase B (gyrB) genes. Non-gyrase mediated resistance mechanisms also occur among mycobacteria challenged with low FQN concentrations.
Aminoglycosides	Aminoglycosides bind to the mycobacterial 16S ribosomal RNA, leading to misreading of mRNA and inhibition of mycobacterial protein synthesis	i) Amikacin and kanamycin resistance results from point mutations of the 16S ribosomal RNA gene (rpsL); ii) Resistance to streptomycin is due to both rpsL mutations and mutations in the ribosomal protein rpsL, or the methyltransferase gidB.
Ethionamide (ETH)	Activated form of ETH inhibits mycolic acid biosynthesis by binding to inhA	Point mutations in ethA or inhA

Figure 2.
Mechanisms of action and common resistance-conferring mutations for key anti-TB drug classes.

A) Classical paradigm of the natural history of TB **B) Reinfection-mixed infection paradigm** **Figure 3.**

The classical paradigm of TB natural history (**A**) suggested that primary infection occurred due to a single strain (Strain A) and that LTBI due to Strain A was protective against infection with additional strains. If the individual developed active disease, was treated but developed recurrent TB, recurrence would be the result of relapse with the original Strain A. Molecular epidemiology studies indicate a more dynamic paradigm (**B**) in which reinfection and mixed infections occur. Individuals may be simultaneously or sequentially infected with multiple strains of *M. tuberculosis* (Strains A, B, C). LTBI may be incompletely or non-protective. Reactivation TB may occur due to a single strain or multiple strains. Individuals who develop recurrent TB following treatment may have relapse due to their original strains (A, B or C) or be reinfected due to exposure to a new strain (Strain D – in this case MDR).

Figure 4.

MDR-TB can develop during the course of anti-TB drug therapy via several mechanisms. Resistance may be amplified (A) as a result of sequential drug exposure. A wild-type population of susceptible *M. tuberculosis* includes a low frequency subpopulation of bacilli with spontaneously occurring INH-resistance (estimated 2.5 mutants per 108 bacilli). Exposure to INH alone selects for survival and replication of the INH-resistant mutants. The resulting INH-resistant population includes a low frequency subpopulation of RIF-resistant bacilli. Now addition of RIF selects for survival of the INH- and RIF-resistant (MDR) bacilli. MDR-TB may also occur due to reinfection. (B) Drug-susceptible is appropriately treated but the patient may have exogenous reinfection with a new MDR-strain either during or following drug treatment. MDR-TB may occur as a result of mixed infection with drug susceptible and MDR-TB strains. (C) MDR-*M. tuberculosis* may be present as a minority population that is not detected on initial DST. Anti-TB drug therapy will select for survival of resistant subpopulations.

Figure 5.

Infection with virulent Beijing strains of *M. tuberculosis* can result in a distinctive host immunophenotype over time as compared to infection with laboratory strains of *M. tuberculosis*. During the early stages of an infection, virulent strains may induce a greater TH1 immune response, resulting in tissue damage and leading to excessive counter-regulatory regulatory T cell (Treg) responses that ultimately impairs host immunity against the tubercle bacillus.

Table 1Mutation rate *in vitro* for essential anti-TB drugs.

Drug	Rate of mutations causing phenotypic resistance
Isoniazid	2.56 mutations per 10^8 cell divisions
Rifampicin	2.25 mutations per 10^{10} divisions
Ethambutol	1 mutation per 10^7 divisions
Streptomycin	2.95 mutations per 10^8 divisions