

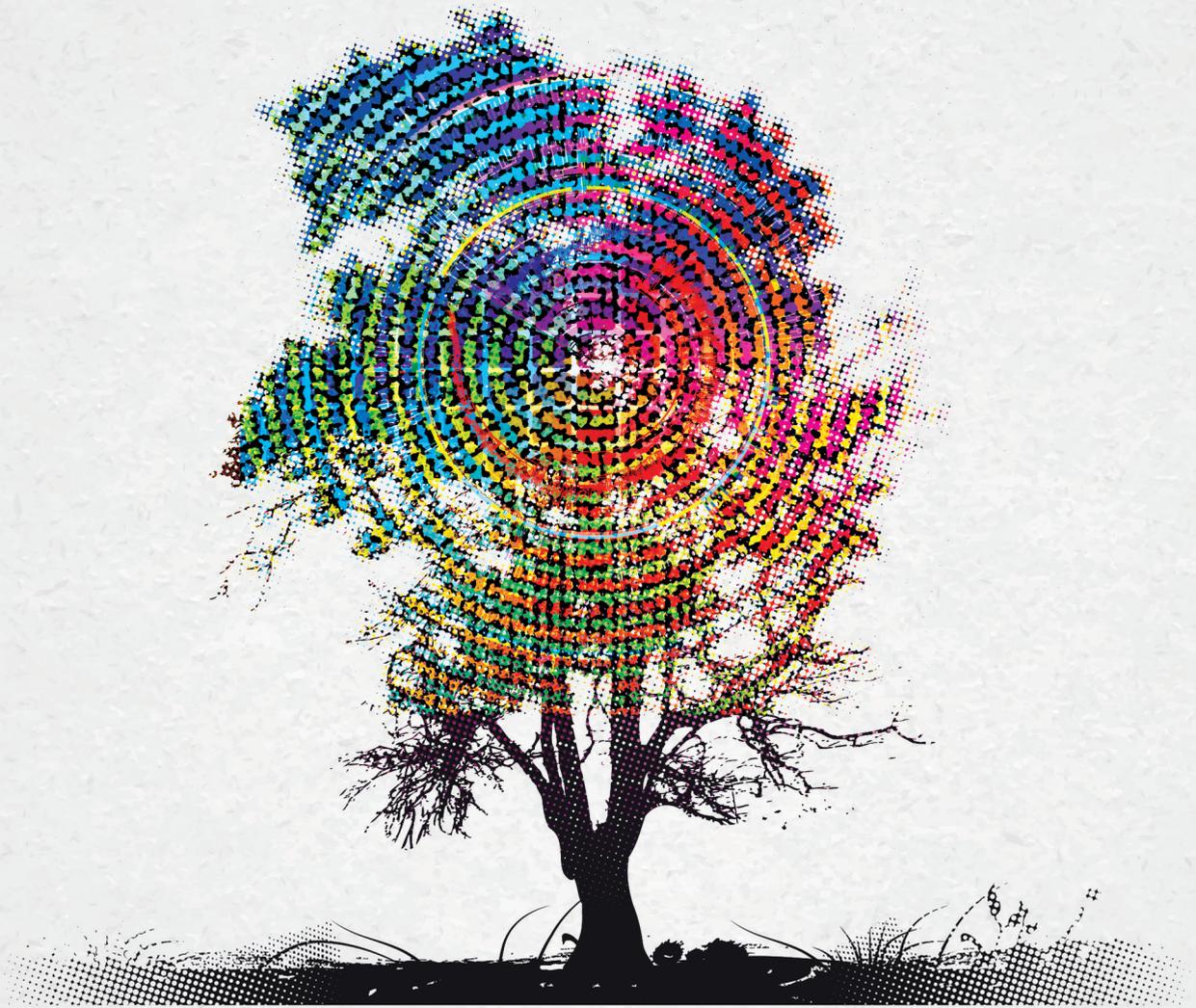
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Understanding tuberculosis drug resistance, disease phenotype
and transmission by mycobacterial genome analysis

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Understanding
tuberculosis drug resistance,
disease phenotype and transmission
by mycobacterial genome analysis

Carolien Ruesen

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1

General introduction

Tuberculosis

Tuberculosis is the number one cause of death in humans due to a single infectious agent¹, with an estimated 10 million new cases and 1.6 million deaths in 2017². Tuberculosis is caused by infection with *Mycobacterium tuberculosis*, which is spread from a person with the respiratory form of the disease to another person via air droplets generated by coughing and sneezing. After exposure to *M. tuberculosis*, the outcome can vary (**Figure 1.1**). Following infection, the bacteria can be cleared through the action of the innate immune system (early clearance); the infection can rapidly progress to active tuberculosis disease, or it can be contained in a latent form with no clinical signs of disease². One fourth of the world's population has this latent form of tuberculosis infection, which may or may not reactivate up to several decades after initial exposure³. In addition, not only the outcome of *M. tuberculosis* infection is variable, the course of active disease can also vary. Pulmonary tuberculosis and various forms of extrapulmonary disease such as tuberculous meningitis and miliary tuberculosis are all presentations of active tuberculosis disease.

Various factors contribute to the heterogeneity of *M. tuberculosis* infection and tuberculosis disease. For example, weakened immunity, such as in human immunodeficiency virus (HIV)-positive individuals, is a strong risk factor for rapid progression from *M. tuberculosis* infection to active disease⁴. To date, most of the research efforts on tuberculosis have focused on host and environmental factors influencing transmission, drug resistance, and susceptibility to infection and disease⁵. However, it is becoming increasingly clear that bacterial factors are also important. Not only is the genetic diversity of *M. tuberculosis* higher than previously thought⁶, knowledge is also expanding that this genomic diversity translates into relevant disease phenotypic variation. Over the past decades, studies have shown that *M. tuberculosis* genotype families show differences in terms of their geographical spread^{7,8}, immune responses in the patient⁹, and virulence^{6,10}. Virulence in tuberculosis relates to the ability of the bacteria to survive in the face of the host immune responses, their capacity to cause lung damage, to survive in aerosols outside the host, and lastly to successfully transmit and infect a new host^{11,12}. Altogether, this suggests that *M. tuberculosis* genetic variation may play an important role in the success of the pathogen, which has been coexisting with mankind since hunter-gatherer times for an estimated 70,000 years¹³.

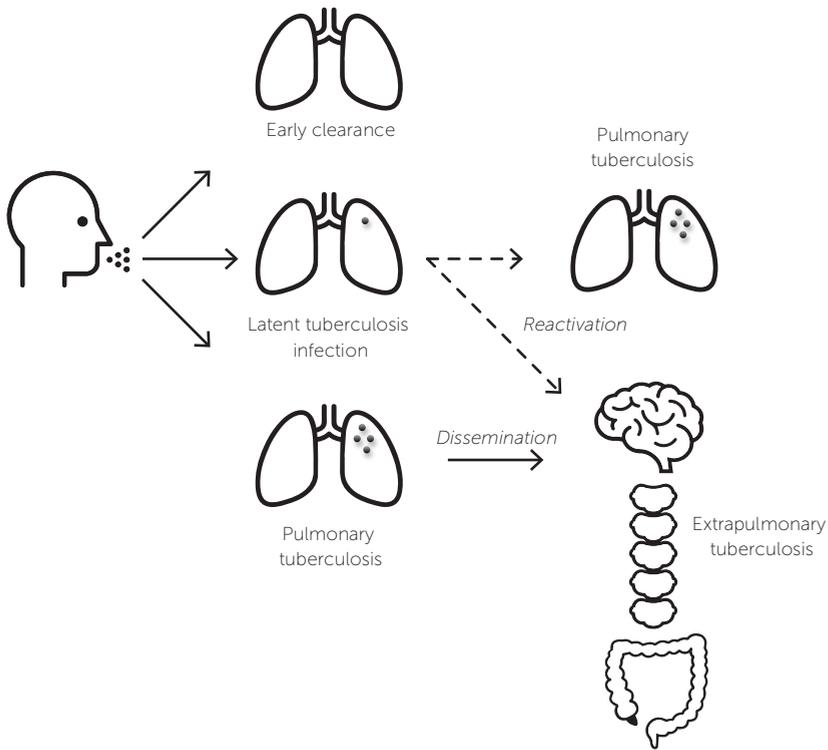


Figure 1.1. The different outcomes and disease manifestations after exposure to *M. tuberculosis*

Human tuberculosis is mainly caused by *M. tuberculosis* and *M. africanum*. Both are human-adapted members of a bigger *Mycobacterium tuberculosis* complex (MTBC), which emerged as a professional pathogen from an environmental *Mycobacterium* through stepwise adaptation to an intracellular milieu. MTBC has little sequence variation compared with other bacteria and some of the genotyping tools applied to other bacterial pathogens are uninformative in MTBC¹⁴. Hence, classifying MTBC clinical strains requires analytical methods that capture genomic diversity in a more comprehensive manner. Whole genome sequencing, in contrast with other typing techniques, allows indexing of MTBC diversity across the whole genome, capturing as much variation as possible and enabling detailed analyses of the evolutionary forces driving this diversity.

Today, based on whole genome sequence analyses, we know that the human-adapted members of MTBC can be further divided into seven phylogenetic lineages: Lineage 1 to 4 and Lineage 7 belong to *M. tuberculosis sensu stricto*, whereas Lineage 5 and 6 are traditionally known as *M. africanum* West Africa 1 and 2, respectively¹⁵. These lineages can be grouped into evolutionarily “ancient” and “modern” lineages, with a deletion in the TbD1 genomic region found exclusively in the evolutionarily “modern” lineages, serving as a genetic marker separating the two groups. The geographical spread of these lineages differs markedly, with the modern lineages (Lineage 2, 3 and 4) exhibiting a global distribution and the ancient lineages (Lineage 1, 5, 6 and 7) a strong geographical restriction⁶. Lineage 2 (also known as the East-Asian lineage) and Lineage 4 (Euro-American lineage) are the most widespread globally. Comparisons with *M. canetti* and other smooth tubercle bacilli, which are phylogenetically the closest relatives of MTBC¹⁶, have shown that many individual genomic features including point mutations in key genes, as well as epistatic interactions between these features might have contributed to the evolution of *M. tuberculosis* towards an obligate human pathogen¹⁶. This in contrast to many other bacterial pathogens in which pathogenicity results from the presence or absence of specific “virulence factors”.

Strains of MTBC differ genetically in their content of single nucleotide polymorphisms (SNPs), small insertions and deletions, mobile and repetitive elements, large genomic deletions, and large duplications. Whole genome sequencing can reveal all sources of genomic variation. The complete genome sequence of the best-characterized strain of *M. tuberculosis*, H37Rv (belonging to Lineage 4), has been determined and published for the first time by Cole and colleagues in 1998¹⁷. The *M. tuberculosis* H37Rv genome comprises approximately 4.4 million base pairs and contains around 4,000 genes. *M. tuberculosis* differs from other bacteria in that a very large proportion of its coding potential accounts for the production of enzymes involved in lipogenesis and lipolysis, and for two families of glycine-rich proteins with characteristic Pro-Glu (PE) or Pro-Pro-Glu (PPE) motifs that may represent a source of antigenic variation^{17,18}. Based on whole genome sequencing analyses of all MTBC lineages, we know that on average, two human-adapted MTBC strains differ by about 1,200 SNPs (0.03% of the genome when excluding repetitive elements)⁶. Strains from the same lineage show less genetic diversity than strains from different lineages, with the least diversity for strains belonging to Lineage 7 (on average 230 SNPs difference between any two strains belonging to this lineage), and largest for strains belonging to Lineage 1 (730 SNPs on average)⁶.

In addition to exploring and quantifying the genomic diversity in MTBC to get a better understanding of the evolution and molecular epidemiology, whole genome sequencing data can be used together with computational methods to predict the impact of this

variation on a range of clinical or immunological phenotypes. Ultimately, differences among MTBC strains are determined by their genomic differences. In this thesis I have used whole genome sequencing data to link *M. tuberculosis* genetic variation to three relevant phenotypes: drug resistance (**Chapter 2, 3 and 4**), disease localisation (**Chapter 5**) and transmission (**Chapter 6**), which I will discuss in the following sections.

Antituberculosis drug resistance

Drug-resistant *M. tuberculosis* was first documented in the late 1940s, shortly after the introduction of the first anti tuberculosis drug, streptomycin¹⁹. However, drug-resistant tuberculosis did not receive global attention until the 1990s when large outbreaks of multidrug-resistant tuberculosis were identified in the United States and Europe²⁰⁻²². It has since become clear that drug-resistant tuberculosis, caused by rifampicin-resistant, multidrug-resistant or extensively drug-resistant strains (**Box 1.1**), is a growing concern and threatens global tuberculosis control. Treatment for patients with multidrug-resistant or extensively drug-resistant tuberculosis is prolonged and expensive, and associated with morbidity and mortality^{23,24}. The drugs used are toxic and poorly tolerated, and adverse events are common and may be severe and irreversible^{25,26}.

Patients can carry a drug-resistant *M. tuberculosis* strain as a result of two processes: acquisition or transmission of resistance. Noncompliance or misuse of antituberculosis drugs, such as monotherapy or the addition of single drugs to failing regimens, can result in the emergence of resistant mutants (acquired resistance). Transmission of such resistant strains to another person may result in infection and eventually disease (primary or transmitted resistance)²⁴. Outbreaks of highly fatal drug-resistant tuberculosis have been documented in several settings, especially those in which the prevalence of HIV infection is high³²⁻³⁴.

Phenotypic testing is the standard for drug susceptibility testing despite its challenges, which are felt most acutely in resource-limited settings. These challenges include the slow growth rate of *M. tuberculosis*, the high-level biosafety infrastructure required and uncertainties around the proposed clinical breakpoints for some drugs³⁵. The mechanism underlying drug resistance in *M. tuberculosis* differs from other bacterial species that often acquire resistance through horizontal gene transfer systems such as plasmid exchange. In *M. tuberculosis*, drug resistance is solely conferred by de novo mutations in genes coding for drug targets or for proteins involved in drug metabolic pathways, possibly preceded by efflux pump up-regulation. Changes in the DNA of *M. tuberculosis* usually result from point mutations, indels, or, more rarely, large deletions³⁶. Together with *M. tuberculosis*' low mutation rate of an estimated 0.3-0.5

Box 1.1. Definitions of drug-resistant tuberculosis**Drug-susceptible tuberculosis**

Tuberculosis caused by *M. tuberculosis* strains that are susceptible to all first-line anti-tuberculosis drugs. The first-line standard regimen that is currently recommended for the treatment of drug-susceptible tuberculosis is based on a 2-month intensive phase with four drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) followed by a 4-month consolidation phase with two drugs (isoniazid and rifampicin)²⁷.

Rifampicin-resistant tuberculosis

Tuberculosis caused by *M. tuberculosis* strains that are resistant to rifampicin.

Multidrug-resistant tuberculosis

Tuberculosis caused by *M. tuberculosis* strains that are resistant to at least isoniazid and rifampicin, the two most effective first-line antituberculosis drugs. Treatment is more costly and toxic and has lower cure rates than drug-susceptible tuberculosis^{28,29}, although this has improved since the introduction of bedaquiline³⁰.

Extensively drug-resistant tuberculosis

Tuberculosis caused by multidrug-resistant *M. tuberculosis* strains that are additionally resistant to any fluoroquinolone and any second-line injectable drug (i.e., kanamycin, amikacin, or capreomycin). Cure and survival rates are worse for extensively drug-resistant tuberculosis than for multidrug-resistant tuberculosis³¹.

substitutions per genome per year³⁶, this makes whole genome sequencing a well-suited technique to study *M. tuberculosis*, creating a unique opportunity for genotypic drug susceptibility testing. Early results from an extensive study linking whole genome sequencing data to phenotypic drug susceptibility testing have provided information for a pilot of drug-susceptibility testing based on whole genome sequencing in the United Kingdom³⁷.

Tuberculosis disease phenotype

The result of exposure to *M. tuberculosis* depends on the interplay between the innate and adaptive immune mechanisms of the human host on the one hand, and the bacterial mechanisms to evade or antagonize these immune responses on the other hand. Early clearance can be defined as the eradication of infecting *M. tuberculosis* by the innate immune system before an adaptive immune response develops, and was

recently found to occur in 25% of exposed individuals³⁸. A persistent infection (latent tuberculosis) develops in those who fail to clear the infection, with an approximately 10% lifetime risk of progression to active disease, highest in the first 18 months after infection³⁹. Although active tuberculosis predominantly affects the lungs (pulmonary tuberculosis), it can cause disease in any other organ (extrapulmonary disease). The classic clinical features of pulmonary tuberculosis include cough, sputum production, haemoptysis, loss of appetite, weight loss, fever, and night sweats⁴⁰. The wide variation in clinical manifestations, disease severity and outcome is one of the most intriguing aspects of tuberculosis and has been mainly attributed to host factors, among others HIV infection, diabetes, smoking, age, and malnutrition. However, host factors do not fully explain the observed variation and evidence is compiling that *M. tuberculosis* strain diversity is also important^{5,41}.

M. tuberculosis strain differences have been characterized *in vitro* in macrophage infection models and *in vivo* in animals⁴² and differences have been defined by bacterial growth in cells or organs, the death of infected cells or animals, and differences in the histopathology of infected animal tissues⁴³. Highly virulent *M. tuberculosis* isolates appeared to grow faster⁴⁴, to cause more lung damage and mortality^{43,45}, and were more capable to transmit⁴⁶ than attenuated or low virulence strains. Differences between strains in clinical settings have been demonstrated with respect to their inflammatory response, the severity of the disease, transmission rate and disease presentation⁸. In addition, results from clinical and epidemiological studies suggested that strains from Lineage 5 and Lineage 6 are metabolically different, grow slower, and are less virulent than the other human-adapted MTBC lineages. Similarly, at least certain groups of Lineage 2 and Lineage 4 strains have been shown to be more virulent in terms of disease severity and human-to-human transmission, and strains belonging to the East-Asian/Beijing lineage were more likely to progress to active tuberculosis disease and were associated with extra-pulmonary tuberculosis, multidrug resistance, treatment failure, and relapse⁴⁷⁻⁴⁹.

These findings support that, next to human and environmental factors, *M. tuberculosis* strain diversity contributes to the variable outcome of infection and disease in human tuberculosis. However, the specific *M. tuberculosis* genetic factors determining the variation in disease phenotypes remain largely unknown, and differences between *M. tuberculosis* lineages do not explain the full spectrum of phenotypic variation observed. Whole genome sequencing offers the possibility to study *M. tuberculosis* genomic diversity at the highest possible resolution, at the level of individual nucleotide changes, therewith also capturing differences within *M. tuberculosis* lineages⁵⁰. A better understanding of the genetic determinants of disease phenotypes, what I aim for in this thesis, would in turn aid in the understanding of tuberculosis' pathogenesis.

Mycobacterium tuberculosis transmission

Transmission from one person to another is crucial for *M. tuberculosis*' survival. In order to be able to transmit, the bacterium needs to cause disease, leading to destruction of lung tissue and consequent expulsion of aerosolized *M. tuberculosis* bacilli. Disease development is a function of the host's immunocompetence; individuals with HIV, for example, are at increased risk of progression to active disease. However, disease development is also a reflection of the evolutionary strategy of *M. tuberculosis* as a pathogen, which during human existence has needed to ensure transmission to the next host. *M. tuberculosis* has to undertake a delicate balancing act: cause enough disease to ensure transmission but not so much that patients rapidly die, taking the pathogen's progeny with them²⁷.

Preventing tuberculosis transmission is one of the spear points of tuberculosis control, but has proven to be extremely difficult. One of the reasons is the absence of an effective vaccine against tuberculosis. The bacillus Calmette-Guérin (BCG) vaccine has been widely used and is effective at preventing severe childhood forms of tuberculosis, but protection wanes by adolescence and the protective efficacy in adults is highly variable. The elimination of tuberculosis cannot reasonably be achieved by treatment of individual patients; hence an improved vaccine is required. The main challenge in developing an improved vaccine against tuberculosis has been the lack of understanding of correlates of a protective immune response. So far, vaccine development in tuberculosis has focused on inducing protective adaptive immunity to prevent progression to active tuberculosis disease, but the observation that some individuals do not develop *M. tuberculosis* infection even after heavy exposure to tuberculosis, suggests that the innate immune system is important for preventing initial infection with *M. tuberculosis*. Recent evidence suggests that BCG vaccination also plays a role in the natural protection or early clearance of *M. tuberculosis*. Alternative approaches aimed at boosting innate immunity could improve vaccine development to protect against *M. tuberculosis* infection, so contributing to tuberculosis prevention.

Transmission of tuberculosis is currently measured by genotyping of the isolates, epidemiological contact investigation, or a combination of the two⁵¹. However, epidemiological data are often challenging to obtain, and genotyping data are difficult to interpret without them. Whole genome sequencing facilitates identifying likely transmission events among patients without the requirement of epidemiological data; based on the principle that patients infected with genetically similar strains are considered to be part of the same transmission cluster^{52,53}. Using this 'DNA fingerprinting' technique, many studies to date have investigated the association between *M. tuberculosis* genetic variation and transmission. However, two processes are involved in transmission;

initial infection of a contact, followed by progression of latent tuberculosis infection to active disease, and these cannot be distinguished by studying genetic clustering. This distinction is important when examining early clearance, which relates to direct transmission: passing infection from index case to contact persons⁵⁴. *M. tuberculosis* could promote transmission by evading early clearance in the contacts, and the extent to which this evasion is successful is possibly genetically determined. Understanding which *M. tuberculosis* strain variation is associated with a more, or less successful innate immune response in the host will aid the identification of new vaccine targets and is topic of one of the studies in this thesis.

Aim and scope of the thesis

This thesis aims at better understanding of the *M. tuberculosis* genetic factors determining drug resistance, disease presentation and transmission of tuberculosis. We examined the genomes of *M. tuberculosis* strains isolated from more than 1,000 patients from Indonesia, Peru and Romania, and linked genomic data with clinical metadata. The thesis consists of two parts. The first part focuses on antituberculosis drug resistance, combining *M. tuberculosis* genetic data with phenotypic drug susceptibility testing results, as well as information on patient-related risk factors for drug resistance. The second part addresses the effect of *M. tuberculosis* genetic variation on tuberculosis disease presentation and on transmission within households (**Figure 1.2**).

Drug resistance

The first part of this thesis describes the use of *M. tuberculosis* whole genome sequencing data to predict antituberculosis drug resistance in high-burden, low-resource countries. In Indonesia, which has the third-highest tuberculosis burden in the world, diagnosis of drug-resistant tuberculosis is difficult as *M. tuberculosis* culture is not routinely performed and phenotypic drug susceptibility testing is only available in reference laboratories. In **Chapter 2**, we examined for the first time the resistance-conferring mutations to first- and second-line drugs in Indonesia using whole genome sequencing, and assessed the level of agreement between genotypic and phenotypic drug susceptibility testing.

Although whole genome sequencing can identify mutations associated with antituberculosis drug resistance, the impact of the many resistance mutations on the minimum inhibitory concentration remains unclear⁵⁵. The level of resistance that a single nucleotide polymorphism causes, reflected by the minimum inhibitory concentration, is important for clinicians treating patients in order to determine whether to increase the dosage or change the regimen. Therefore, in **Chapter 3** we examined the genomes

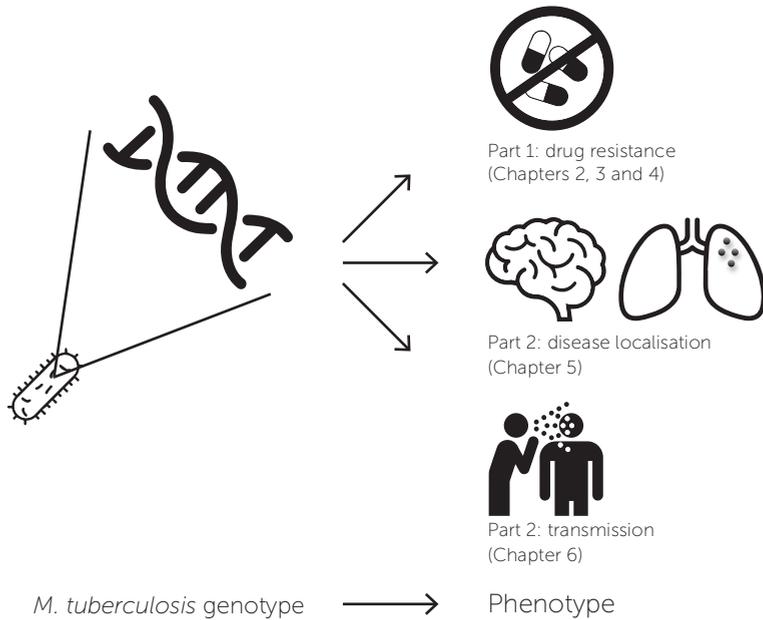


Figure 1.2. The *M. tuberculosis* genotype was linked to multiple tuberculosis phenotypes in my thesis

of phenotypically drug-resistant *M. tuberculosis* isolates from Romanian patients for drug resistance mutations, and linked these to the measured minimum inhibitory concentrations.

Phenotypic antituberculosis drug resistance has been associated with diabetes mellitus comorbidity⁵⁶ and this may contribute to the poor treatment outcomes among tuberculosis patients with diabetes, but a basic understanding of the molecular mechanism behind this association is lacking. As opposed to phenotypic drug susceptibility testing, whole genome sequencing has the potential to unlock valuable information on the specific mutations underlying resistance, transmission clusters and on the phylogenetic background of drug-resistant *M. tuberculosis* strains. In **Chapter 4**, we used whole genome sequencing to assess genotypic drug resistance in *M. tuberculosis* isolates from Indonesian and Peruvian patients with and without diabetes. We examined if drug resistance mutations were more frequently found in isolates from patients with diabetes, compared to those without, after adjusting for other risk factors for drug resistance.

Tuberculosis disease phenotype and transmission

Many studies have attempted to link host or environmental factors to various tuberculosis disease phenotypes, but together they do not fully explain the observed spectrum. In this part of the thesis we studied the effect of bacterial factors, using whole genome sequencing. Animal studies have shown that *M. tuberculosis* strains differ in their ability to disseminate to and survive in the central nervous system^{57,58}, and *in vitro* studies have identified *M. tuberculosis* genes that were crucial for invading an artificial blood-brain barrier⁵⁹. Much less is known about the role of *M. tuberculosis* genotype on the disease phenotype in humans. In **Chapter 5**, we therefore examined the effect of the infecting *M. tuberculosis* genotype on the susceptibility to tuberculous meningitis, compared to pulmonary tuberculosis. We used a novel approach of counting homoplasmy events to detect genetic variants associated with either the pulmonary or meningeal tuberculosis presentation.

In addition to disease phenotype, *M. tuberculosis* genetic variation could also influence the risk of *M. tuberculosis* transmission. *M. tuberculosis* factors can increase the risk of transmission in two ways: first; through the index case, by inducing a disease phenotype that favours transmission. A higher bacillary load with more immunopathology and worse cavitory disease results in increased spread of *M. tuberculosis*⁴¹. Second; through the contact, by resistance to the contact's innate immune system⁶⁰. Understanding these pathogen factors is essential to understanding on the one hand the virulence of *M. tuberculosis* in the index case, related to outgrowth and cavities, and on the other hand the virulence of *M. tuberculosis* in the contact, related to resistance to innate immunity. However, epidemiological evidence for *M. tuberculosis* genotype influencing risk of infection is scarce. Therefore, in **Chapter 6**, we investigated whether *M. tuberculosis* genotype influences the disease phenotype in the contact, or the success with which it can escape the innate immune defence of the contact, using a household-based case-contact study in Bandung, Indonesia.

Lastly, in **Chapter 7**, the findings of this thesis are summarized and discussed, and future challenges and opportunities of whole genome sequencing of *M. tuberculosis* are delineated.

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PART ONE

Antituberculosis drug resistance

2

Use of whole genome sequencing to predict *Mycobacterium tuberculosis* drug resistance in Indonesia

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Abstract

Objectives

Whole-genome sequencing (WGS) is rarely used for drug resistance testing of *Mycobacterium tuberculosis* in high-endemic settings. Here we present the first study from Indonesia, which has the third highest tuberculosis (TB) burden worldwide, with <50% of drug-resistant cases currently detected.

Methods

WGS was applied for strains from 322 human immunodeficiency virus (HIV)-negative adult TB patients. Phenotypic drug susceptibility testing (DST) was performed for a proportion of the patients.

Results

Using WGS, mutations associated with drug resistance to any TB drug were identified in 51 (15.8%) of the 322 patients, including 42 patients (13.0%) with no prior TB treatment (primary resistance). Eight isolates (2.5%) were multidrug-resistant (MDR) and one was extensively drug-resistant (XDR). Most mutations were found in *katG* (n = 18), *pncA* (n = 18), *rpoB* (n = 10), *fabG1* (n = 9) and *embB* (n = 9). Agreement of WGS-based resistance and phenotypic DST to first-line drugs was high for isoniazid and rifampicin but was lower for ethambutol and streptomycin. Drug resistance was more common in Indo-Oceanic lineage strains (37.5%) compared with Euro-American (18.2%) and East-Asian lineage strains (10.3%) ($P = 0.044$), but combinations of multiple mutations were most common among East-Asian lineage strains ($P = 0.054$).

Conclusions

These data support the potential use of WGS for more rapid and comprehensive prediction of drug-resistant TB in Indonesia. Future studies should address potential barriers to implementing WGS, the distribution of specific resistance mutations, and the association of particular mutations with endemic *M. tuberculosis* lineages in Indonesia.

Introduction

Drug-resistant tuberculosis (TB) threatens the global control of TB in many parts of the world. There were an estimated 580 000 cases of multidrug-resistant TB (MDR-TB) in 2015, with <50% being detected and even fewer receiving appropriate treatment^{1,2}. Culture-based drug susceptibility testing (DST), the gold standard to diagnose drug-resistant TB, is technically difficult and takes about 4-6 weeks after the isolation of *Mycobacterium tuberculosis*. Furthermore, inconsistencies in DST results are widely reported, especially for ethambutol (EMB) and second-line drugs^{3,4}, whilst phenotypic DST for pyrazinamide (PZA) requires different protocols⁵. Molecular-based DST testing using the Xpert MTB/ RIF assay can rapidly detect most rifampicin (RIF) resistance⁶, but this assay has incomplete sensitivity for RIF, does not examine resistance for other TB drugs and may fail to detect heteroresistance^{7,8}.

Whole-genome sequencing (WGS) has been shown to be a potential tool for reliable prediction of the drug susceptibility phenotype of *M. tuberculosis* isolates within a clinically relevant timeframe⁹. However, so far WGS is mostly applied in well-resourced, low TB burden settings. Because of rapid advances in WGS technology and its decreasing cost and turnaround time, WGS is now becoming accessible in limited-resourced, high TB burden countries¹⁰. In the current study, WGS was applied to predict drug resistance in a selection of *M. tuberculosis* isolates from Indonesia, which has the third-highest TB burden in the world. In Indonesia, diagnosis of drug-resistant TB is challenging as *M. tuberculosis* culture is not routinely performed and DST is only available in certain reference laboratories. To date, the contribution and concordance of phenotypic and genotypic DST in Indonesia is unknown. This study aimed to describe resistance-conferring mutations to first- and second-line TB drugs in Indonesia using WGS and to examine their concordance with phenotypic DST and their distribution among different *M. tuberculosis* lineages.

Materials and Methods

Selection of *M. tuberculosis* isolates and drug susceptibility testing

WGS was performed on a random sample of archived *M. tuberculosis* isolates from 322 human immunodeficiency virus (HIV)-negative adult patients (216 with pulmonary TB and 106 with meningeal TB) with complete medical information. Pulmonary TB patients had been diagnosed in Hasan Sadikin Hospital (Bandung, Indonesia) between 2012–2013 and in the TB-HIV Research Centre of Universitas Padjadjaran (Bandung, Indonesia) between 2013–2015. TB meningitis patients had been diagnosed at Hasan Sadikin Hospital between 2006–2013¹¹. Specimens from each patient were processed

accordingly and were inoculated on solid Ogawa medium or in MODS (Microscopic Observation of Drug Susceptibility) liquid medium¹². Positive cultures from each method were subcultured and aliquots were archived at -80 °C prior to DNA extraction.

Xpert MTB/RIF was available in the study setting only after 2012 and was accessible only for patients with suspected MDR-TB according to Indonesian national guidelines. Phenotypic DST was not performed routinely for all patients but only when requested by treating physicians. DST was performed in the provincial referral laboratory using the proportion method on Löwenstein–Jensen (LJ) medium at concentrations of 40.0 µg/mL for RIF, 0.2 µg / mL for isoniazid (INH), 2.0 µg /mL for EMB and 4.0 µg /mL for streptomycin (STR). Briefly, a 1.0 McFarland standard isolate suspension was serially diluted 10-fold (from 10⁻¹ to 10⁻⁵) in sterile distilled water. Dilutions 10⁻³ and 10⁻⁵ were inoculated, respectively, onto LJ slants with and without drugs, and were incubated at 37 °C. The results were read at 28 days and up to 42 days, depending on the control growth. An isolate was considered resistant to a given drug when growth of ≥1% above the control was observed in drug-containing medium. DST for second-line drugs was not available during patient inclusion for this study. Phenotypic DST was repeated for several isolates that had tested INH-susceptible but that harboured mutations conferring resistance to INH in the *katG315* gene. In these cases, *M. tuberculosis* was subcultured from frozen isolates onto Ogawa slopes prior to DST. The study protocols for the inclusion of patients and for bioanalysis were approved by the Ethical Committee of the Faculty of Medicine of Universitas Padjadjaran.

Whole genome sequencing and analysis

Frozen isolates were subcultured on Ogawa slopes and mycobacterial DNA was extracted for sequencing using Ultra- Clean¹ Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's protocol, or using the cetyltrimethylammonium bromide (CTAB) method of DNA purification. The concentration and purity of extracted DNA were measured using a NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the intactness of DNA was checked by agarose gel electrophoresis.

M. tuberculosis DNA was sequenced on an Illumina HiSeq 2000 instrument (Illumina Inc., San Diego, CA) using 2 x 100-bp paired-end reads. Sequencing was performed at BGI (Hong Kong). After sequencing, the raw FASTQ sequence reads were filtered, including removal of adapter sequences, contamination, and low-quality reads that had more than 10% N base calls or had a quality score ≤4 in more than 40% of the bases. Five TB meningitis strains and four pulmonary TB strains were contaminated, based on a low GC content and were excluded from further analyses. Sequencing coverage was determined using the FastQC v.0.10.1 (<http://www.bioinformatics>).

babraham.ac.uk/projects/fastqc/) quality control tool. The proportion of bases sequenced with a sequencing error rate of $\leq 1\%$ per base ranged from 93% to 97% per genome. The average depth of coverage for the remaining 322 sequenced strains was 121.1, and the average percentage of bases covered by at least one read was 98.9%. The sequence reads were aligned to reference strain *M. tuberculosis* H37Rv (GenBank accession no. NC_000962.3) and variants were called using Breseq software v.0.27.1¹³. Mutations with low-quality evidence (i.e. possible mixed read alignment) were not included.

In silico determination of drug resistance

Raw FASTQ sequencing files were uploaded to TB Profiler, an online tool to determine drug resistance *in silico*¹⁰. It uses raw sequence data as input and compares identified single nucleotide polymorphisms (SNPs) and indels to a curated list of 1,325 drug resistance mutations and displays related output. The precision of TB Profiler's curated mutation catalogue for predicting resistance had been assessed using six geographically distinct data sets from China, Pakistan, Malawi, Portugal, Russia and Canada¹⁰, and its accuracy compared with other *in silico* drug resistance prediction tools has been proven recently¹⁴.

Phylogeny construction

A phylogenetic tree was constructed to determine the evolutionary relationship of the isolates. All 29,199 variable positions identified by breseq across the 322 *M. tuberculosis* sequences were extracted and concatenated into a single alignment. Solely for the purpose of creating the phylogenetic tree, SNPs occurring in PE/PPE genes and genes related to mobile elements (listed in Table S2.1) were excluded to avoid any concern about inaccuracies in the read alignment in these parts of the genome. In addition, SNPs in an additional 40 genes previously associated with drug resistance¹⁰ were also removed to exclude the possibility that homoplasmy of drug resistance mutations would significantly affect the phylogeny¹⁵. After applying these filters to the initial set of 29,199 SNPs, the 28,544 remaining SNPs were used to construct the phylogenetic tree using PhyML v.3.0¹⁶ using the HKY85 model with four categories for the gamma model of rate distributions and 100 bootstraps.

To examine possible associations between *M. tuberculosis* lineage and drug susceptibility, the lineage was determined for each of the 322 strains using a 62-SNP barcode¹⁷. The resulting classification in the main *M. tuberculosis* lineages also served as a quality check for the generated maximum-likelihood phylogenetic tree, as it enabled us to validate that isolates belonging to the same lineage clustered together in the tree.

Statistical analysis

The χ^2 test was used to statistically test the association between *M. tuberculosis* lineage and drug susceptibility. Cohen's κ was used to determine the level of agreement between WGS and phenotypic DST for first-line drugs. In addition, enrichment values were calculated for drug resistance per lineage based on the ratio of lineage-specific observed and expected occurrence of drug resistance. The ratios were visualized in a heat map as a measure of association between *M. tuberculosis* lineage and drug susceptibility.

Results

Patient characteristics

Patients were mostly young (median age 33 years, interquartile range 23.5–45.0 years), 51.9% were male and 16.1% reported a history of TB treatment. Using WGS, mutations associated with drug resistance to any TB drug were identified in 51 (15.8%) of the 322 patients; 42 (13.0%) were identified to have primary resistance, MDR-TB was present in 8 patients (2.5%), including primary MDR-TB in 5 patients (1.6%) and primary extensively drug-resistant TB (XDR-TB) in 1 patient (0.3%) (Table 2.1, Table S2.2).

Mutations associated with drug resistance

Genetic variants in multiple genes associated with drug resistance in *M. tuberculosis* were identified by WGS (Table 2.2). A total of 29 isolates (9.0%) had mutations in genes associated with resistance to INH, including *katG* ($n = 18$), *kasA* ($n = 3$) and the promoter region of *fabG1* C-15T ($n = 9$). Nine isolates with mutations at the *fabG1* promoter site were predicted to have co-resistance to INH and ethionamide. The most common mutation in *katG* was Ser315Thr ($n = 14$), but the uncommon mutation *katG* Ser315Met was also found in two isolates, and *katG* Trp191Arg and *katG* Ala106Val were found in one isolate each. The *katG* Ser315Thr mutation was found in seven of eight MDR-TB isolates (Table 2.2). RIF resistance-conferring mutations were identified in ten isolates (3.1%), all in the *rpoB* gene. Six common mutations distributed over codons 435, 445 and 450 were found, mostly as single mutations at one of these positions. Rare mutations at codon 432 and 441 were found together in one isolate. One MDR-TB isolate with *rpoB* Ser450Leu also harboured the well-known compensatory mutation at *rpoC* Leu527Val.

For EMB, nine isolates (2.8%) showed mutations in the *embB* gene, with the mutation Met 306Val being most common ($n = 4$), and rare mutations at codon 406 and codon 497 in three MDR isolates. PZA resistance-conferring mutations were identified in 18 isolates (5.6%). All mutations occurred as a single mutation in five codons in the *pnxA*

gene. For fluoroquinolones (FQs), mutations in *gyrA* codon 90 and 94 were identified in 4 isolates (1.2%), three with FQ monoresistance and one XDR-TB isolate. With regard to resistance to STR and injectable agents, mutations were found in the *rpsL* and *rrs* loci in nine isolates (2.8%). Mutations at *rrs* codon 492 were only found in STR-monoresistant strains. The XDR-TB isolate harboured a mutation at the *eis* promoter linked to kanamycin resistance.

Table 2.1. Presence of drug resistance mutations in 322 clinical *M. tuberculosis* isolates detected by whole genome sequencing

| Resistance pattern | Number (%) of strains |
|-----------------------------------|-----------------------|
| Susceptible to all drugs | 271 (84.3) |
| Resistant to any drug | 51 (15.8) |
| Resistant to first-line drugs | |
| Any first-line drug | 48 (14.9) |
| Isoniazid | 29 (9.0) |
| Rifampicin | 10 (3.1) |
| Ethambutol | 8 (2.5) |
| Pyrazinamide | 18 (5.6) |
| Streptomycin | 9 (2.8) |
| Resistant to second-line drugs | |
| Ethionamide | 9 (2.8) |
| Fluoroquinolones | 4 (1.2) |
| Amikacin | 1 (0.3) |
| Kanamycin | 1 (0.3) |
| Monoresistance | |
| Isoniazid | 10 (3.1) |
| Rifampicin | 1 (0.3) |
| Pyrazinamide | 15 (4.7) |
| Streptomycin | 3 (0.9) |
| Fluoroquinolones | 3 (0.9) |
| Resistance to multiple drugs | |
| Multidrug resistance (MDR) | 8 (2.5) |
| Extensively drug resistance (XDR) | 1 (0.3) |
| Polyresistance* | 10 (3.1) |

*Defined as resistance to multiple drugs but not M/XDR

Table 2.2. Distribution of drug resistance-associated mutations in 51 *M. tuberculosis* isolates with any drug resistance identified by whole genome sequencing

| Drug | Gene | Amino acid change | No. of isolates | No. of MDR-TB isolates |
|---------------------------------------|--|----------------------------|-----------------|------------------------|
| INH | <i>katG</i> | Ser315Thr | 14 | 7 ^a |
| | | Ser315Met | 2 | 0 |
| | | Trp191Arg | 1 | 0 |
| | <i>fabG1</i> | C-15T promoter | 8 | 1 |
| | <i>kasA</i> | Gly312Ser | 3 | 0 |
| | Double loci <i>fabG1</i> + <i>katG</i> | C-15T promoter + Ala106Val | 1 | 1 |
| RIF | <i>rpoB</i> | His445Tyr | 1 | 1 |
| | | His445Asp | 1 | 1 |
| | | Asp435Val | 1 | 1 |
| | | Asp435Tyr | 2 | 2 |
| | | Ser450Leu | 2 | 2 ^a |
| | | His445Cys | 1 | 1 |
| | | Gln432Lys + Ser441Leu | 1 | 0 |
| Double loci <i>rpoB</i> + <i>rpoC</i> | Ser450Leu + Leu527Val | 1 | 1 | |
| EMB | <i>embB</i> | Met306Ile | 2 | 1 ^a |
| | | Met306Val | 4 | 1 |
| | | Gly406Asp | 1 | 1 |
| | | Gln497Lys | 1 | 1 |
| | | Met306Val + Gly406Asp | 1 | 1 |
| STR | <i>rrs</i> | C492T | 3 | 0 |
| | | A514C | 1 | 1 |
| | <i>rpsL</i> | Lys43Arg | 5 | 3 ^a |
| PZA | <i>pncA</i> | His82Arg | 6 | 0 |
| | | Thr87Met | 9 | 1 |
| | | Ser66Pro | 1 | 1 |
| | | Pro62Leu | 1 | 0 |
| | | Ala171Val | 1 | 1 ^a |
| ETO | <i>fabG1</i> | C-15T promoter | 9 | 2 |
| FLQ | <i>gyrA</i> | Asp94Gly | 1 | 0 |
| | | Ala90Val | 1 | 0 |
| | | Asp94Asn | 1 | 0 |
| | | Asp94Ala | 1 | 1 ^a |
| AMK | <i>rrs</i> | A514C | 1 | 1 |
| KAN | <i>eis</i> | G-14A promoter | 1 | 1 ^a |

*Abbreviations: INH: isoniazid; RIF: rifampicin; EMB: ethambutol; STR: streptomycin; PZA: pyrazinamide; ETO: ethionamide; FLQ: fluoroquinolones; AMK: amikacin; KAN: kanamycin; MDR-TB: multidrug-resistant.

^aMutation occurred in the extensively drug-resistant (XDR) isolate.

Agreement of phenotypic and genotypic drug susceptibility testing to first-line drugs

Overall, there was considerable agreement between genotypic and phenotypic DST. Phenotypic DST for the first-line drugs RIF, EMB and STR was available for 103 isolates with WGS data and for INH it was available for 102 isolates, with resistance found to INH (n = 17), RIF (n = 7), EMB (n = 6) and STR (n = 7). Concordance between WGS and phenotypic DST was high for RIF (k = 0.865; P < 0.001) and INH (k = 0.814; P < 0.001) but was low for EMB (k = 0.712; P < 0.001) and STR (k = 0.136; P = 0.148). Agreement of genotypic and phenotypic DST for each drug is shown in Figure 2.1.

The common mutations *katG* Ser315Thr and *fabG1* C-15T were highly predictive of phenotypic INH resistance in this setting (89.5% of the isolates with either one of these mutations were phenotypically resistant). Similarly, mutations in the RIF resistance-determining region (RRDR) of *rpoB* were highly predictive of resistance to RIF (100% of the isolates with a mutation were phenotypically resistant). Nine isolates showed drug resistance-associated mutations but were susceptible by phenotypic DST. In those isolates, mutations were found for STR at *rrs* C492T (n = 3) and A514C (n = 1) and *rpsL* Lys43Arg (n = 1); for INH at *katG* Ser315Thr (n = 1), *katG* Trp191Arg (n = 1), *fabG1* C-15T (n = 1) and *kasA* Gly312Ser (n = 3); and for EMB at *embB* Met306Val (n = 1) and Met306Ile (n = 1). Conversely, nine other isolates that were drug-resistant according to phenotypic DST (n = 7 for STR, n = 2 for RIF and n = 1 for EMB) showed no known drug resistance mutations using WGS. However, one isolate with phenotypic RIF resistance but without well-known drug resistance mutations harboured other mutations in *rpoB* (*rpoB* Cys681Gly and *rpoB* Pro1014Ser).

Phylogenetic distribution of drug resistance

Drug resistance rates and patterns differed significantly between different *M. tuberculosis* lineages (Figure 2.2; Table S2.3). Among 116 isolates belonging to the East-Asian lineage, 12 (10.3%) were genotypically drug-resistant compared with 36 (18.2%) of 198 Euro-American strains and 3 (37.5%) of 8 Indo-Oceanic strains ($\chi^2 = 6.258$; P = 0.044). Although fewer strains belonging to the East-Asian lineage had drug resistance mutations, they more often had multiple drug resistance mutations ($\chi^2 = 5.844$; P = 0.054). From the phylogeny, it was observed that most of the *pncA* mutations in isolates harbouring PZA resistance clustered together. The nine isolates with a *pncA* Thr87Met mutation were adjacent in the tree, and the same goes for the six isolates carrying the *pncA* His82Arg mutation, suggesting transmitted resistance. However, the isolates differed by more than 12 SNPs, the commonly used threshold for (recent) transmission [18]. The other three mutations occurred only once and in genetically distant strains.

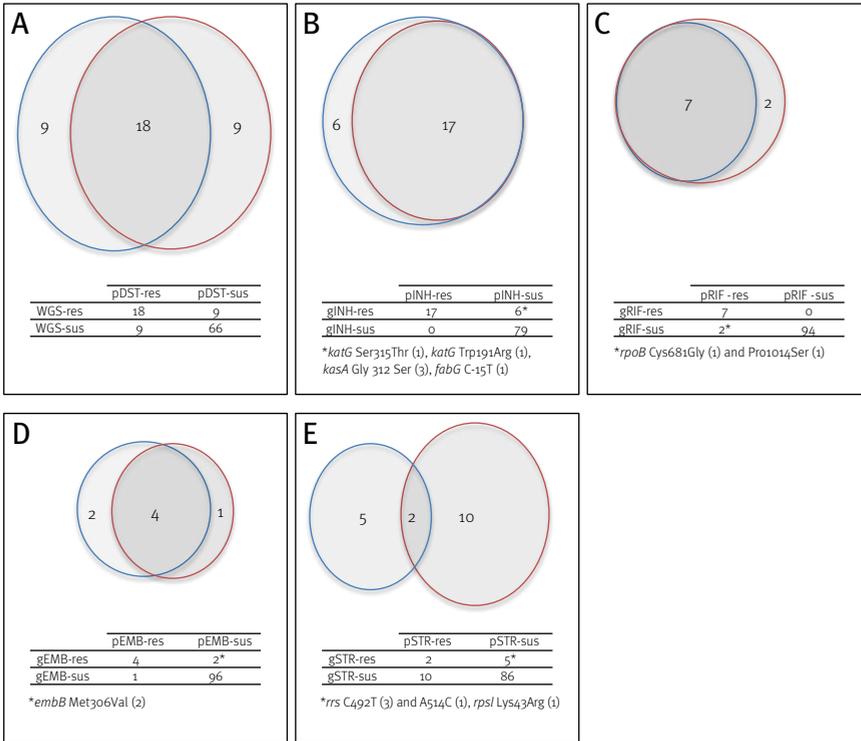


Figure 2.1. Comparison of phenotypic drug susceptibility testing (DST) and DST by whole-genome sequencing (WGS) for *Mycobacterium tuberculosis* isolates to (A) any first-line drug, (B) isoniazid (INH), (C) rifampicin (RIF), (D) ethambutol (EMB) and (E) streptomycin (STR). Light grey, drug resistance determined by WGS; dark grey, drug resistance determined by phenotypic DST. Data are presented as number of strains. N = 103 for RIF, EMB and STR and N = 102 for INH.

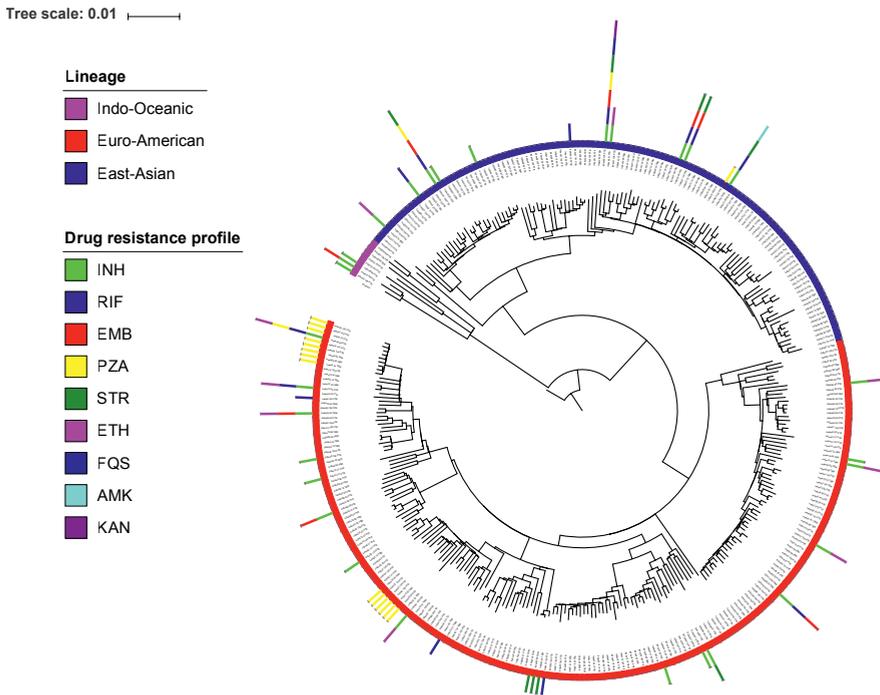


Figure 2.2. Phylogenetic tree of 322 *Mycobacterium tuberculosis* isolates indicating drug resistance profiles and main lineages based on single nucleotide polymorphism (SNP) barcoding. INH, isoniazid; RIF rifampicin; EMB, ethambutol; PZA, pyrazinamide; STR, streptomycin; ETH, ethionamide; FQS, fluoroquinolones; AMK, amikacin; KAN, kanamycin.

Discussion

This is the first study to report the use of WGS on clinical *M. tuberculosis* isolates from Indonesia, showing its potential for clinical management and TB control in Indonesia. Indonesia has a huge gap between MDR-TB incidence and MDR-TB treatment. Together with China, India, Nigeria and the Russian Federation, it accounted for >60% of this gap on a global scale in 2015². Of the 10,000 estimated MDR- or RIF-resistant notified pulmonary TB cases in Indonesia in 2015, only an estimated 15% were started on treatment². A large part of this gap can be explained by poor detection of drug resistance. Phenotypic DST is poorly accessible in remote parts of the country. WGS could offer a rapid and comprehensive diagnostic solution, especially with the introduction of portable platforms and the decreasing price and turnaround time of WGS¹⁸, leading to quicker and more appropriate treatment.

In a random collection of patient isolates, it was found that 15.8% carried mutations associated with drug resistance to any TB drug, often in patients without a history of TB treatment. This is much lower than reported in an earlier study in Indonesia where resistance to first-line drugs was reported in 38.2% of 262 culture-positive samples, although these samples were collected over the whole country and resistance was determined by phenotypic DST¹⁹. In the current study, resistance mutations to all first-line drugs, FQs and second-line injectable drugs were found. MDR-TB was present in eight isolates (2.5%), which is lower than expected based on recent survey data², and XDR-TB was present in one isolate (0.3%).

In contrast to the findings from previous studies^{19–21}, in the current study drug resistance was observed to occur more often in strains belonging to the Indo-Oceanic lineage compared with the Euro-American and East-Asian lineages. On the other hand, strains belonging to the East-Asian lineage more often harboured multiple mutations. One possible explanation is that the East-Asian lineage, which includes Beijing strains, appears to have a higher mutation rate that could lead to accelerated acquisition of drug resistance mutations. It may be one of the reasons why this lineage has been repeatedly associated with drug resistance^{22,23}. However, this would also lead to more resistance against a single drug.

Published studies from Indonesia have clearly shown disparities in the relative distribution of *M. tuberculosis* genotypes across the archipelago. In this article, we reported resistance mutations in a urban setting in Java where modern lineage 2 (East-Asian) and lineage 4 (Euro-American) are highly prevalent. These findings should be confirmed in other regions, especially eastern Indonesia, where lineage 1 (Indo-Oceanic) occurs at a substantially higher frequency^{24–26}. Current catalogues of drug resistance mutations rely predominantly on data from modern lineages 2 and 4^{10,27}. A recent study from India, where lineage 1 and 3 predominate, reported putative novel resistance-conferring mutations in lineage 1 that had not previously been implicated in resistance²⁷, suggesting that the mutations underlying genotypic drug resistance differ by lineage.

WGS offers the possibility to learn more about the influence of the genetic background of strains on all aspects of drug resistance evolution in *M. tuberculosis*. WGS could provide insight into secondary compensatory mutations, not conferring resistance but reducing the fitness cost of the resistance mutation by interacting epistatically with it. The rate of acquiring new drug resistance mutations and the fitness costs of these mutations may vary as a function of the strain genetic background^{27,28}.

Most mutations associated with INH resistance were found in *katG* (62.1%) and the promoter region of *fabG1* (31.0%). Testing only for *katG* and *fabG1* promoter mutations would detect INH resistance in 89.6% of the INH-resistant isolates. All mutations associated with RIF resistance were found only in the RRDR of *rpoB*. This therefore confirms the usefulness of these three specific regions for prediction of INH and RIF resistance²⁹. Conversely, *kasA* Gly312Ser and *katG* Trp191Arg mutations were found in phenotypically INH-susceptible isolates. *KasA* (β -ketoacyl ACP synthase), coded by the *kasA* gene, is an enzyme involved in mycolic acid synthesis. Mutations in the *kasA* gene have been associated with low-level INH resistance^{30,31} but the role of these mutations in INH resistance remains unclear. Therefore, the significance of this gene in conferring drug resistance in Indonesia should be carefully assessed. Two isolates were phenotypically RIF-resistant but harboured mutations in *rpoB* that have not been associated with drug resistance before (Cys681Gly and Pro1014Ser), therefore their possible role in RIF resistance should be confirmed in other studies.

Regarding EMB, three loci of the *embB* gene (loci 306, 406 and 497) were identified, with mutations at locus 306 being the most frequent. Mutations in *embB* were present only in combination with other drug resistance mutations, in line with several studies that have demonstrated a strong association between *embB*₃₀₆ mutations and INH-, RIF- or multidrug-resistant TB³². This finding suggests that *embB*₃₀₆ mutations may have a selective advantage upon treatment with multiple drugs³³. Regarding STR, isolates carried mutations in *rpsL* ($n = 5$) and *rrs* ($n = 4$). A common variant in *rpsL* (Lys43Arg) has been associated with high-level STR resistance³² and this variant was indeed the most common in this study. Phenotypic STR resistance was only confirmed in two of seven genotypically STR-resistant isolates with DST results available. Three isolates had a single mutation in *rrs* codon 492; their relevance should be carefully interpreted since this mutation has been reported as a marker for the LAM3 genetic lineage of *M. tuberculosis* rather than for STR resistance³⁴.

In line with previous studies, concordance between WGS and phenotypic DST was good for INH and RIF²⁹ but had low agreement for EMB and STR^{35,36}. There are several possible explanations for this finding. First, discordance was mainly found with uncommon genotypic mutations, which may be associated with low-level resistance that can be missed by conventional phenotypic DST. Second, the difference between the epidemiological breakpoint and the minimum inhibitory concentration (MIC) for EMB and STR is relatively small^{35,36}, which complicates phenotypic DST. Third, detection of phenotypic STR resistance in the absence of known mutations for STR resistance suggests the existence of other resistance mechanisms such as efflux pumps³⁶. Finally, we cannot exclude errors in phenotypic DST despite good quality control in the laboratory. In this regard, the fact that one isolate was phenotypically

drug-susceptible with a high confidence katG Ser315Thr mutation for INH in WGS is a matter of concern since this mutation is associated with high-level resistance^{29,37}. Given this high specificity, mutations at katG315 might even be used to assess laboratory quality for DST.

Second-line drug resistance was observed in a number of isolates. At present, there is no standard phenotypic DST for PZA and the second-line drugs in Indonesia, and evidence is limited on the performance of DST for these drugs³⁸, indicating once more the potential added value of WGS in this setting. Also, reported prior treatment for TB poorly corresponded with genotypic drug resistance, and primary drug resistance was common in this study population of Indonesian patients. Of concern, the only XDR-TB strain was found in a patient who had reported no prior TB treatment. This suggests that selective use of genotypic or phenotypic DST, targeting only those with previous treatment or other factors presumed to be associated with drug resistance, will result in many undetected resistant cases and ongoing transmission of drug-resistant strains³⁹. This is further supported by the observation that 10 of 51 isolates with mutations associated with resistance to any drug were not phenotypically tested for drug susceptibility because they did not have these risk factors. WGS could help in early identification of resistance in these patients, limiting the spread of drug-resistant TB.

Molecular drug resistance for PZA mostly involves mutations in the *pncA* gene. There is no predominant drug resistance mutation in *pncA* but a range of diverse mutations across the gene, each associated with a different MIC₅. Five different mutations in *pncA* were found in the isolates in this study, almost all in PZA-mono-resistant strains. Most previous studies reported that PZA resistance is common in MDR-TB strains⁴⁰ although significant rates of PZA monoresistance have been reported in some settings^{40–42}. A study in China showed that PZA monoresistance contributes to delay in the resolution of lung cavitation without affecting the sputum conversion and lesion elimination rates⁴¹. The non-essential nature of the *pncA* gene, such that it can accumulate various mutations without affecting the viability of the organism, might explain the spread of PZA-mono-resistant strains. Two *pncA* mutations exclusively occurred in strains adjacent in the phylogenetic tree, suggesting possible transmission. However, the genetic difference between these strains was too large to conclude that this was indeed the case.

This study has several limitations. First, WGS was performed on a random sample of archived *M. tuberculosis* isolates so we cannot conclude that the proportions of drug resistance shown in this study are representative. This was a convenience sample collected from patients with pulmonary and meningeal TB. We estimate that several thousands of patients are treated for TB each year in Bandung. However, culture is not

routinely performed and isolates are not archived. Therefore, our sampling fraction is likely to be <10%. Second, phenotypic DST was available for most genotypically resistant strains but only for a fraction of isolates without resistance mutations. As a consequence, specificity estimation for genotypic resistance was not possible. Third, sequencing was performed retrospectively on archived isolates; therefore it was not possible to evaluate time to diagnosis of drug resistance using WGS. Nevertheless, we for the first time highlight the potential benefit of using WGS to generate an *in silico* drug susceptibility profile in Indonesia and show that mutations associated with drug resistance are highly predictive for phenotypic resistance to RIF and INH in the region. Larger studies are needed to confirm the clinical relevance of several uncommon mutations found in this strain collection. Given the fact that phenotypic DST is complex and slow and is poorly accessible in large parts of Indonesia, WGS could offer a rapid and comprehensive diagnostic solution. This technology is now more accessible with the introduction of portable platforms and an automatic bioinformatic pipeline as well as the decreasing price and turnaround time of WGS⁴³. It could lead to quicker and more appropriate treatment in low-income settings where many still rely on empirical treatment regimens. This pilot study offers a good starting point to further evaluate the impact of WGS in the diagnosis, treatment, surveillance and control of drug-resistant TB in Indonesia.

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Competing interests

None declared.

Ethical approval

Health Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran (Bandung, Indonesia).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jgar.2018.08.018>.

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Supplementary tables

Table S2.1. PE/PPE genes and drug resistance genes excluded for the phylogeny construction

| PE/PPE genes and genes in repetitive regions | | | | | | Known drug resistance genes |
|--|---------|---------|---------|---------|---------|-----------------------------|
| Rv0031 | Rv0922 | Rv1575 | Rv2107 | Rv2741 | Rv3381c | |
| Rv0096 | Rv0977 | Rv1576c | Rv2108 | Rv2768c | Rv3386 | |
| Rv0109 | Rv0978c | Rv1577c | Rv2123 | Rv2769c | Rv3387 | accD6 |
| Rv0124 | Rv0980c | Rv1578c | Rv2126c | Rv2770c | Rv3388 | ahpC |
| Rv0151c | Rv1034c | Rv1579c | Rv2162c | Rv2791c | Rv3425 | efpA |
| Rv0152c | Rv1035c | Rv1580c | Rv2167c | Rv2810c | Rv3426 | embA |
| Rv0159c | Rv1036c | Rv1581c | Rv2168c | Rv2812 | Rv3427c | embB |
| Rv0160c | Rv1039c | Rv1582c | Rv2177c | Rv2814c | Rv3428c | embC |
| Rv0256c | Rv1040c | Rv1583c | Rv2278 | Rv2815c | Rv3429 | embR |
| Rv0278c | Rv1041c | Rv1584c | Rv2279 | Rv2853 | Rv3430c | ethA |
| Rv0279c | Rv1042c | Rv1585c | Rv2328 | Rv2885c | Rv3474 | fabD |
| Rv0280 | Rv1047 | Rv1586c | Rv2340c | Rv2892c | Rv3475 | fadE24 |
| Rv0285 | Rv1054 | Rv1646 | Rv2352c | Rv2943 | Rv3477 | fbpC |
| Rv0286 | Rv1067c | Rv1651c | Rv2353c | Rv2943A | Rv3478 | furA |
| Rv0297 | Rv1068c | Rv1705c | Rv2354 | Rv2944 | Rv3507 | gid |
| Rv0304c | Rv1087 | Rv1706c | Rv2355 | Rv2961 | Rv3508 | gyrA |
| Rv0305c | Rv1088 | Rv1753c | Rv2356c | Rv2978c | Rv3511 | gyrB |
| Rv0335c | Rv1089 | Rv1756c | Rv2371 | Rv3018A | Rv3512 | inhA |
| Rv0354c | Rv1091 | Rv1757c | Rv2396 | Rv3018c | Rv3514 | iniA |
| Rv0355c | Rv1135c | Rv1763 | Rv2408 | Rv3021c | Rv3532 | iniB |
| Rv0387c | Rv1149 | Rv1764 | Rv2424c | Rv3022A | Rv3533c | iniC |
| Rv0388c | Rv1168c | Rv1765A | Rv2430c | Rv3022c | Rv3539 | kasA |
| Rv0442c | Rv1169c | Rv1768 | Rv2431c | Rv3023c | Rv3558 | katG |
| Rv0453 | Rv1172c | Rv1787 | Rv2479c | Rv3115 | Rv3590c | fabG1 |
| Rv0532 | Rv1195 | Rv1788 | Rv2480c | Rv3125c | Rv3595c | manB |
| Rv0578c | Rv1196 | Rv1789 | Rv2487c | Rv3135 | Rv3621c | ndh |
| Rv0741 | Rv1199c | Rv1790 | Rv2490c | Rv3136 | Rv3622c | nat |
| Rv0742 | Rv1214c | Rv1791 | Rv2512c | Rv3144c | Rv3636 | oxyR |
| Rv0746 | Rv1243c | Rv1800 | Rv2519 | Rv3159c | Rv3637 | pncA |
| Rv0747 | Rv1313c | Rv1801 | Rv2591 | Rv3184 | Rv3638 | rmlD |
| Rv0754 | Rv1325c | Rv1802 | Rv2608 | Rv3185 | Rv3640c | rpoB |

Table S2.1. Continued

| PE/PPE genes and genes in repetitive regions | | | | | | Known drug resistance genes |
|--|---------|---------|---------|---------|---------|-----------------------------|
| Rv0755A | Rv1361c | Rv1803c | Rv2615c | Rv3186 | Rv3650 | |
| Rv0755c | Rv1369c | Rv1806 | Rv2634c | Rv3187 | Rv3652 | |
| Rv0795 | Rv1370c | Rv1807 | Rv2646 | Rv3191c | Rv3653 | rpsL |
| Rv0796 | Rv1386 | Rv1808 | Rv2648 | Rv3325 | Rv3738c | rrs |
| Rv0797 | Rv1387 | Rv1809 | Rv2649 | Rv3326 | Rv3739c | Rv0340 |
| Rv0832 | Rv1396c | Rv1818c | Rv2650c | Rv3327 | Rv3746c | Rv1592c |
| Rv0833 | Rv1430 | Rv1840c | Rv2651c | Rv3343c | Rv3751 | Rv1772 |
| Rv0834c | Rv1441c | Rv1917c | Rv2652c | Rv3344c | Rv3798 | Rv2242 |
| Rv0850 | Rv1450c | Rv1918c | Rv2653c | Rv3345c | Rv3812 | Rv3124 |
| Rv0872c | Rv1452c | Rv1983 | Rv2654c | Rv3347c | Rv3827c | Rv3125c |
| Rv0878c | Rv1468c | Rv2013 | Rv2655c | Rv3348 | Rv3844 | Rv3126c |
| Rv0915c | Rv1548c | Rv2014 | Rv2656c | Rv3349c | Rv3872 | thyA |
| Rv0916c | Rv1573 | Rv2105 | Rv2657c | Rv3350c | Rv3873 | tlyA |
| Rv0920c | Rv1574 | Rv2106 | Rv2659c | Rv3367 | Rv3892c | accD6 |
| | | | Rv2666 | Rv3380c | Rv3893c | |

Table S2.2. Resistance profile for 51 strains with any drug resistance determined by WGS

| Study number | Pheno-type | WGS INH | | WGS RIF | | WGS EMB | | WGS STR | | WGS MDR | | Phen INH | | Phen EMB | | Phen STR | | Phen MDR | | Hist TB tr. | Lineage |
|--------------|------------|---------|---|---------|---|---------|---|---------|---|---------|---|----------|---|----------|---|----------|---|----------|---|-------------|--------------------------|
| | | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | | |
| 100884 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 100890 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 100913 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 100921 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 100926 | TBM | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 100962 | TBM | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 100964 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American |
| 100972 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 100989 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 1100217 | TBM | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 1100232 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Indo-Oceanic |
| 1100238 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American |
| 1100294 | TBM | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 1100607 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Indo-Oceanic |
| 1100707 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 1100773 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 1100797 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Indo-Oceanic |
| 1100903 | TBM | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 1100969 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (Cameroon) |
| 1101011 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 1101025 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American |
| 1101028 | PTB | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 1101054 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American |
| 1101303 | PTB | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |

| | | | | | | | | | | | | | | | | | | | | |
|---------|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|--------------------------|
| 1101323 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | Euro-American (X-type) |
| 1101355 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 1101572 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 1101628 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 900077 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | Euro-American (LAM) |
| 900130 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 900138 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | Euro-American (LAM) |
| 900161 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (X-type) |
| 900164 | TBM | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | Euro-American (X-type) |
| 900174 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 900178 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | Euro-American (mainly T) |
| 900181 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 900202 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 900221 | TBM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 900229 | TBM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 900319 | TBM | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (X-type) |
| 900365 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 900387 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 900603 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 900611 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 1100246 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 1100353 | PTB | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 1100907 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 1100943 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (LAM) |
| 1100995 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | East-Asian (Beijing) |
| 1101933 | PTB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| 1100909 | PTB | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Euro-American (mainly T) |
| | | | | | | | | | | | | | | | | | | | | East-Asian (Beijing) |

'1' indicates genotypic (WGS) or phenotypic (Phen) drug resistance detected, '0' no genotypic (WGS) or phenotypic (Phen) drug resistance detected, an empty cell indicates the data are missing; PTB: pulmonary TB; TBM: tuberculous meningitis; WGS: whole genome sequencing; Phen: phenotypic drug susceptibility.

Table S2.3. Heatmap of observed vs. expected genotypic drug resistance ratios per lineage and gene

| | East-Asian | Euro-American | Indo-Oceanic |
|-------|------------|---------------|--------------|
| katG | 1,23 | 0,90 | 0,00 |
| fabG1 | 0,62 | 1,26 | 0,00 |
| kasA | 0,00 | 0,00 | 40,25 |
| rpoB | 1,51 | 0,74 | 0,00 |
| rpoC | 2,78 | 0,00 | 0,00 |
| embB | 1,54 | 0,54 | 4,47 |
| pncA | 0,46 | 1,36 | 0,00 |
| rrs | 0,69 | 1,22 | 0,00 |
| rpsL | 2,22 | 0,33 | 0,00 |
| gyrA | 1,39 | 0,81 | 0,00 |
| eis | 2,78 | 0,00 | 0,00 |

Colours range from green (<1 , observed drug resistance lower than expected drug resistance) to red (>1 , observed drug resistance higher than expected drug resistance).

3

Linking minimum inhibitory concentrations to whole genome sequence-predicted drug resistance in *Mycobacterium tuberculosis* strains from Romania

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Abstract

Objectives

Mycobacterium tuberculosis drug resistance poses a major threat to tuberculosis control. Current phenotypic tests for drug susceptibility are time-consuming, technically complex, and expensive. Whole genome sequencing is a promising alternative, though the impact of different drug resistance mutations on the minimum inhibitory concentration (MIC) remains to be investigated.

Methods

We examined the genomes of 72 phenotypically drug-resistant *Mycobacterium tuberculosis* isolates from 72 Romanian patients for drug resistance mutations. MICs for first- and second-line drugs were determined using the MycoTB microdilution method. These MICs were compared to macrodilution critical concentration testing by the Mycobacterium Growth Indicator Tube (MGIT) platform and correlated to drug resistance mutations.

Results

Sixty-three (87.5%) isolates harboured drug resistance mutations; 48 (66.7%) were genotypically multidrug-resistant. Different drug resistance mutations were associated with different MIC ranges; *katG* S315T for isoniazid, and *rpoB* S450L for rifampicin were associated with high MICs. However, several mutations such as in *rpoB*, *rrs* and *rpsL*, or *embB* were associated with MIC ranges including the critical concentration for rifampicin, aminoglycosides or ethambutol, respectively.

Conclusions

Different resistance mutations lead to distinct MICs, some of which may still be overcome by increased dosing. Whole genome sequencing can aid in the timely diagnosis of *Mycobacterium tuberculosis* drug resistance and guide clinical decision-making.

Introduction

Drug-resistance poses a major threat to tuberculosis (TB) control. Global surveillance data suggest that in 2015, there were an estimated 480,000 new cases of multi-drug-resistant TB (MDR-TB), i.e. resistant to at least isoniazid (INH) and rifampicin (RIF), and that 9.5% of these cases were extensively drug-resistant (XDR), i.e. also resistant to amikacin (AMI), kanamycin (KAN), or capreomycin (CAP), and at least one fluoroquinolone (FLQ)¹. It is estimated that less than half of these cases are detected, and an even smaller proportion receive appropriate treatment¹. One of the main problems in the control of drug-resistant TB (DR-TB) is the lack of laboratory capacity to diagnose resistance². Conventional culture-based drug susceptibility testing (DST) is expensive, time-consuming and requires a specialized biosafety laboratory. Methods for DST for many of the second-line drugs have not yet been fully standardized³. In addition, these phenotypic methods often rely on growth of mycobacterial cultures in the presence of a single 'critical' drug concentration to distinguish resistant and susceptible strains based on epidemiological breakpoints, which are near the wild type minimum inhibitory concentration (MIC) distribution for some drugs⁴.

Molecular assays are now available and can overcome some of the disadvantages of phenotypic methods to diagnose DR-TB. Yet, these assays are expensive and cannot identify all of the genetic loci associated with drug resistance^{5,6}. Whole genome sequencing (WGS) of *M. tuberculosis*, tackles this problem and could enable the reliable prediction of the drug susceptibility phenotype within a clinically relevant timeframe. A fundamental issue in the application of WGS to predict drug resistance is how to interpret mutations in relation to phenotypic antibiotic resistance. The level of resistance, reflected by the MIC, a single nucleotide polymorphism (SNP) causes is most important for clinicians treating patients, in order to determine whether to increase the dosage or change the regimen.

Therefore, in the current study we provide this missing link by comparing MICs to drug resistance mutations determined by WGS. For this purpose we used a collection of drug-resistant strains from Romania, which had 20,000 new TB cases, and 800 new MDR-TB cases in 2012, making it one of the 18 high-priority countries for TB control in the World Health Organization (WHO) European Region⁷.

Methods

Selection of *M. tuberculosis* isolates

We used *M. tuberculosis* isolates from the bio-archive established at the TB laboratory of the Clinical Hospital of Infectious Diseases and Pneumophtisiology “Victor Babes”, Craiova, between 2014 and 2016. The laboratory runs approximately 7,000 sputum samples per year, covering much of the TB epidemic in Dolj county. Routine diagnostics as indicated by the National Control Programme include sputum microscopy and culture on Löwenstein-Jensen medium (liquid media introduced in May 2015). After primary culture on Löwenstein-Jensen medium, screening for INH and RIF-resistance is performed using INH 0.2 mg/l and RIF 40 mg/l-supplemented Löwenstein-Jensen slants.

All 73 isolates that were phenotypically resistant to INH and/or RIF were selected for the purpose of the present study. The 73 isolates were from different patients, and no serial isolates were included. Isolates stored in trypticase soy broth supplemented with glycerol were sub-cultured on Löwenstein-Jensen medium. Sub-cultured colonies were used for DNA extraction, and susceptibility testing.

Phenotypic drug susceptibility testing

All isolates were simultaneously subjected to MIC determinations of rifampicin, rifabutin, ethambutol, streptomycin, amikacin, kanamycin, ofloxacin, moxifloxacin, para-aminosalicylic acid (PAS), ethionamide and cycloserine by broth microdilution in Middlebrook 7H9 broth (SensiTitre MycoTB assay; Trek Diagnostics/ThermoFisher, Landsmeer, the Netherlands). Every isolate was tested once, unless contamination or no growth was observed; then the isolate was re-cultured and once more subjected to MIC testing. The MycoTB assay is a new method for susceptibility testing of *M. tuberculosis* complex^{8,9}. The plate uses a 96-well microtiter broth format and contains 12 lyophilized first- and second-line antimycobacterial drugs. In contrast to other *M. tuberculosis* complex susceptibility methods, which test one or two critical concentrations of a drug, the MycoTB assay examines a range of drug concentrations and produces an MIC result. In addition, critical concentration testing for streptomycin, isoniazid, rifampicin and ethambutol (SIRE assay; BD BioScience, Erembodegem, Belgium) was performed using the Mycobacterium Growth Indicator Tube (MGIT) macrodilution platform (BD BioScience, Erembodegem, Belgium). The critical concentrations used for MGIT testing were 0.10 µg/ml for INH, 1.00 µg/ml for RIF, 5.00 µg/ml for EMB, and 1.00 µg/ml for STR. Technicians performing susceptibility tests were blinded to the sequencing results.

Whole genome sequencing and *in silico* determination of drug resistance

DNA isolation using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) was followed by quantitative (Qubit v3.0) and qualitative assessment of the DNA (gel electrophoresis). We used the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) for library preparation. *M. tuberculosis* DNA was sequenced on an Illumina NextSeq500 instrument using 2 x 150 bp paired-end reads. One isolate was excluded due to failed library preparation, resulting in a total set of 72 isolates. No internal control was performed for WGS. After sequencing, the raw FASTQ sequence reads were filtered using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore), including removing of adapter sequences. Sequencing coverage was determined using the FASTQC quality control tool version 0.10.1, and the Genome Analysis Toolkit¹⁰. The average proportion of bases sequenced with a sequencing error rate of 0.1% or less per base was 77.8% per genome. The average coverage depth for the 72 sequenced strains was 165.1, and the average percentage of bases covered by at least one read was 99.5%. Quality control statistics are shown in Table S3.1.

Raw FASTQ sequencing files were uploaded to TB Profiler version 0.2.1, an online tool to determine drug resistance *in silico*¹¹. It uses raw sequence data as input, aligns these to the *M. tuberculosis* H37Rv reference genome, and compares identified single nucleotide polymorphisms (SNPs) and indels to a curated list of 1,325 drug resistance mutations. Although it allows examining heteroresistance, we focused on majority variants. In addition, it determines the *M. tuberculosis* lineage based on a 62-SNP barcode¹². The TB Profiler-predicted resistance mutations were validated using PhyResSE, version 1.0¹³, another online tool that maps raw sequencing reads to the *M. tuberculosis* H37Rv reference strain to report drug resistance and phylogenetic SNPs. We used the *M. tuberculosis* complex numbering system, based on the sequence of the reference strain: H37Rv¹⁴.

Phylogeny construction

A phylogeny was constructed to examine clustering of the isolates. The sequence reads were aligned to reference strain *M. tuberculosis* H37Rv, accession number NC_000962.3, and variants were called using Breseq software, version 0.27.1¹⁵. We extracted all 5,980 variable positions across the 72 *M. tuberculosis* sequences and concatenated them into a single alignment. Solely for the purpose of creating the phylogenetic tree, SNPs occurring in PE/PPE genes, genes related to mobile elements, as well as genes previously associated with drug resistance¹¹ were removed. The remaining 5,794 SNPs were used to construct the phylogenetic tree using PhyML, version 3.0¹⁶ using the HKY85 model with four categories for the gamma distribution, and using a hundred bootstraps.

Comparison of MGIT, minimum inhibitory concentrations and whole genome sequencing

For each isolate, susceptibility to STR, INH, RIF, and EMB as determined by MGIT was compared to the MICs determined by MycoTB. If MycoTB MICs were higher than the critical concentration (>0.25 $\mu\text{g/ml}$ for INH, >1.0 $\mu\text{g/ml}$ for RIF, >4.0 $\mu\text{g/ml}$ for EMB, and >2.0 $\mu\text{g/ml}$ for STR¹⁷, the isolate was considered resistant to the respective drug. Cohen's kappa statistic was calculated for each drug to determine the level of agreement between MycoTB- and MGIT-determined resistance. In addition, drug resistance mutations identified by TB Profiler and/or PhyResSE were correlated to MGIT results and MICs. We visualized the MIC for each drug in histograms, and subsequently determined the frequencies of the individual resistance-determining mutations and plotted their distributions against the MGIT results for INH, RIF, EMB, and STR, and against the MICs for first- and second-line drugs. For isolates with discrepant susceptibility results for INH or RIF between WGS and MGIT, we compared the MGIT to the DST performed on Löwenstein-Jensen and in case of genotypically resistant, phenotypically susceptible isolates we examined the quality of the variant call (Table S3.2). All analyses were performed in R, version 3.3.1 (<http://www.R-project.org>).

Data availability

The raw sequence files (FASTQ) were archived on the NCBI Sequence Read Archive and are available at: <http://www.ncbi.nlm.nih.gov/bioproject/475771>. The individual isolates can be accessed under the following Biosample accession numbers: SAMN09402650-SAMN09402721. The Bioproject accession number is: PRJNA475771.

Results

Phenotypic drug susceptibility testing

MGIT results for INH, STR, and EMB were available for 68 out of 72 isolates (four cultures being contaminated); RIF MGIT results were available for 69 isolates, with three cultures contaminated. Fifty-seven isolates were phenotypically resistant to INH, 34 to RIF, 24 to STR, and three to EMB. Using the MycoTB microdilution method, MICs for INH, RIF, STR, EMB, ETO, OFL, MXF, PAS, cycloserine, AMK, and KAN were successfully determined for 64 isolates; six samples were contaminated, and two repeatedly failed to grow in the MycoTB plate. By MycoTB, 51 isolates were resistant to INH, 31 to RIF, nine to STR, four to EMB, 40 to ETO, four to OFL, one to MXF, one to PAS, one to cycloserine, two to AMK, and three were resistant to KAN (Figure S3.1). Poly-drug resistance was observed in 47, and MDR-TB in 28 isolates, no isolates were XDR-TB according to MycoTB. The agreement between MGIT and MycoTB for INH, RIF, STR and EMB was greater than to be expected by chance (Table S3.3). Cohen's kappa indicated

excellent agreement for INH ($\kappa = 0.794$; $p < 0.001$) and RIF ($\kappa = 0.905$; $p < 0.001$), and little agreement for STR ($\kappa = 0.289$; $p = 0.006$) and EMB ($\kappa = 0.246$; $p = 0.045$).

Whole genome sequencing and *in silico* determination of drug resistance

Sixty-three (87.5%) isolates harboured drug resistance-conferring mutations, and 48 (66.7%) had INH and RIF resistance-conferring mutations and were genotypically multidrug-resistant. The identified drug resistance mutations are listed in Table 3.1. Resistance-conferring mutations to INH were identified in 61 (84.7%) isolates.

The most common INH resistance mutation was *katG* S315T, which was present in 38 (52.8%) isolates as a single mutation, and in 16 (22.2%) together with a mutation in the *fabG1* promoter region (synonym: *inhA* promoter region). Resistance-conferring mutations to RIF were found in 50 (69.4%) isolates. The most prevalent RIF resistance-conferring mutation was *rpoB* S450L, observed in 17 (23.6%) isolates as a single mutation, and in one isolate as a combined mutation. Mutations in genes related to EMB susceptibility were identified in 39 (54.2%) isolates; *embB* M306I was observed in 25 (34.7%) isolates, and in 14 of these as a single mutation. Resistance to STR due to mutations in the *rpsL* and *rrs* loci was found in 19 (26.4%) isolates; with *rrs* A1401G in 13 isolates being the most frequent. This mutation however is not considered causative of STR resistance according to expert knowledge^{18,19}. Mutations in the *pncA* gene, encoding the target of PZA were found in 23 (31.9%) isolates, 14 of which had a single *pncA* A146V mutation. Mutations to second-line drugs were less frequently observed, the *rrs* A1401G mutation associated with resistance to AMK, CAP, and KAN was most common, together with the C15T mutation in the promoter region of *fabG1* conferring resistance to ETO that was found in 20 (27.8%) isolates. The latter mutation is also associated with INH resistance. Only five isolates had one of four different mutations conferring resistance to FLQs.

Phylogenetic relatedness of the isolates

A phylogenetic tree was constructed based on 5,794 variable common nucleotide positions among the 72 *M. tuberculosis* isolates. Seventy-one isolates belonged to the Euro-American lineage and one belonged to the East-Asian lineage. The phylogeny showed that several isolates were closely related, but did not all harbour the same resistance-conferring mutations (Figure S3.2). Fourteen isolates within the same clade carried the *fabG1* (C-15T promoter), *katG* (S315T), *rpoB* (S450L), *embB* (M306I), and *pncA* (A146V) mutations, but they also harboured different mutations. Another 21 isolates clustered together in the tree and carried the S315T *katG* mutation, twenty of which had the H445N *rpoB* mutation, but apart from that their resistance profile differed. In addition, there were five pairs of neighbouring isolates that had similar resistance mutations.

Table 3.1. Frequency of *M. tuberculosis* resistance-conferring mutations

| Drug | Gene | Mutation | Frequency (%) | MGIT-resistant# | MIC > CC# | |
|--------------------------|----------------------------|------------------------------------|--|------------------|--------------|-------------|
| INH | <i>katG</i> | Ser315Thr | 38 (52.8) | 35/35 | 30/30 | |
| | <i>katG</i> + <i>ahpC</i> | Asp311Gly + G-48A promoter | 1 (1.4) | 1/1 | 1/1 | |
| | <i>katG</i> + <i>fabG1</i> | Ser315Thr + C-15T promoter | 16 (22.2) | 15/15 | 16/16 | |
| | <i>fabG1</i> | C-15T promoter | 4 (5.6) | 4/4 | 2/4 | |
| | <i>fabG1</i> + <i>inhA</i> | C-15T promoter + Ile21Val | 1 (1.4) | 1/1 | 0/1 | |
| | <i>ahpC</i> | C-52T promoter | 1 (1.4) | 1/1 | 1/1 | |
| RIF | <i>rpoB</i> | Asp435Val | 4 (5.6) | 3/4 | 3/4 | |
| | | Gln432Pro | 1 (1.4) | 1/1 | 1/1 | |
| | | His445Asn | 12 (16.7) | 2/11 | 1/7 | |
| | | His445Asp | 1 (1.4) | 1/1 | 1/1 | |
| | | His445Tyr | 4 (5.6) | 4/4 | 4/4 | |
| | | Leu430Pro | 2 (2.8) | 0/1 | 0/1 | |
| | | Ser450Leu | 17 (23.6) | 15/15 | 16/16 | |
| | | Ser450Trp | 1 (1.4) | 1/1 | 1/1 | |
| | | Asp435Gly + His445Asn | 1 (1.4) | 1/1 | 1/1 | |
| | | Asp435Val + His445Asn | 1 (1.4) | 1/1 | NA | |
| | | His445Asn + Pro454Leu | 2 (2.8) | 0/2 | 1/2 | |
| | | Leu430Pro + His445Asn | 1 (1.4) | 1/1 | NA | |
| | | Met434Ile + His445Asn | 1 (1.4) | 1/1 | 1/1 | |
| | | Phe424Leu + His445Asn | 1 (1.4) | 1/1 | 0/1 | |
| | | His445Asn + Ser450Leu + Pro-454Leu | 1 (1.4) | 1/1 | 0/1 | |
| EMB | <i>embA</i> | C-12T promoter | 1 (1.4) | 0/1 | 0/1 | |
| | | <i>embA</i> + <i>embB</i> | C-12T promoter + Asp354Ala | 1 (1.4) | 0/1 | 0/1 |
| | | | C-12T promoter + Glu504Asp | 1 (1.4) | 0/1 | 0/1 |
| | | | C-12T promoter + Met306Ile | 1 (1.4) | 0/1 | 0/1 |
| | | | C-12T promoter + Met306Leu + Asp354Ala | 1 (1.4) | 0/1 | 0/1 |
| | <i>embB</i> | Asp354Ala | 1 (1.4) | 0/1 | 0/1 | |
| | | Gly406Asp | 5 (6.9) | 0/5 | 0/1 | |
| | | Met306Ile | 14 (19.4) | 0/12 | 0/14 | |
| | | Met306Leu | 1 (1.4) | 0/1 | 0/1 | |
| | | Met306Val | 2 (2.8) | 0/2 | 0/1 | |
| | | Met306Ile + Gly406Asp | 9 (12.5) | 3/9 | 4/8 | |
| | | Met306Val + Asp328Tyr | 1 (1.4) | 0/1 | 0/1 | |
| | | Met306Ile + Tyr319Ser + Asp354Ala | 1 (1.4) | 0/1 | 0/1 | |
| | | STR | <i>rpsL</i> | Lys43Arg | 1 (1.4) | 1/1 |
| Lys88Arg | 1 (1.4) | | | 1/1 | 1/1 | |
| <i>rpsL</i> + <i>rrs</i> | <i>rrs</i> | | Lys43Arg + A1401G | 2 (2.8) | 2/2 | 2/2 |
| | | | A1401G* | 11 (15.3) | 6/10 | 1/10 |
| | | | A514C | 2 (2.8) | 2/2 | 2/2 |
| | | | C1402T* | 1 (1.4) | 1/1 | 0/1 |
| | | | C517T | 1 (1.4) | NA | 0/1 |

Table 3.1. Continued

| Drug | Gene | Mutation | Frequency (%) | MGIT-resistant [#] | MIC > CC [#] |
|----------------------|--|---------------------------|-------------------------|-----------------------------|--------------------------------|
| PZA | <i>pncA</i> | A1a146Val | 14 (19.4) | NA | NA |
| | | Gln10Arg | 1 (1.4) | | |
| | | Gln10Stop | 4 (5.6) | | |
| | | Gly17Asp | 2 (2.8) | | |
| | | Tyr34Stop | 1 (1.4) | | |
| Tyr34Stop + Gly17Asp | 1 (1.4) | | | | |
| ETO | <i>ethA</i> <i>fabG1</i> <i>fabG1 + inhA</i> | Thr61Met | 1 (1.4) | NA | 1/1 |
| | | C-15T promoter | 20 (27.8) | | 19/19 |
| | | C-15T promoter + Ile21Val | 1 (1.4) | | 1/1 |
| FQ | <i>gyrA</i> | Ala90Val | 1 (1.4) | NA | NA |
| | | Asp94Gly | 2 (2.8) | | 1/2 (OFL); 0/2 (MXF) |
| | Ser91Pro | 1 (1.4) | 0/1 (OFL); 0/1 (MXF) | | |
| | <i>gyrB</i> | Asp461Asn | 1 (1.4) | | 1/1 (OFL); 0/1 (MXF) |
| AMK | <i>rrs</i> | A1401G | 13 (18.1) | NA | 2/12 |
| | | A514C* | 2 (2.8) | | 0/2 |
| | | C1402T | 1 (1.4) | | 0/1 |
| | | C517T* | 1 (1.4) | | 0/1 |
| CAP | <i>rrs</i> | A1401G | 13 (18.1) | NA | NA |
| | | C1402T | 1 (1.4) | | |
| KAN | <i>eis</i> <i>rrs</i> | G-14A promoter | 2 (2.8) | NA | 1/2 |
| | | A1401G | 13 (18.1) | | 1/12 |
| | | C1402T | 1 (1.4) | | 0/1 |

*These mutations possibly have no causative role in conferring drug resistance to the respective drug (18, 19).

[#]Presented as N/N tested, because not all isolates with genotypic DST results had phenotypic DST results. The most frequently observed drug resistance mutations per drug are shown in bold. MGIT: Mycobacterium Growth Indicator Tube; MIC: minimum inhibitory concentration; CC: critical concentration; NA: not assessed.

Comparison of MGIT, minimum inhibitory concentrations and whole genome sequencing

First, whole genome sequencing was compared with phenotypic DST by MGIT. Figure S3.3 and Table 3.1 show the comparison of MGIT and WGS resistance prediction. For MGIT, all isolates with well-known INH resistance-conferring mutations were phenotypically resistant to INH, and all isolates without these mutations were susceptible to INH. For RIF, 13/69 (19%) discrepancies were observed between MGIT and WGS; all concerned WGS-resistant, MGIT-susceptible isolates. Eleven of these had a H445N *rpoB* mutation; eleven were susceptible according to DST performed on Löwenstein-Jensen medium, and variant call qualities were good (Table S3.2). For STR, WGS and MGIT did not match for 15/68 (22%) isolates; four had an A1401G *rrs* mutation but were susceptible

according to MGIT, and 11 were MGIT-resistant, but had no STR resistance-conferring mutations. For EMB, we observed 34/69 (49%) discrepancies; all isolates without an EMB resistance-conferring mutation were susceptible, but 34 isolates with an EMB resistance mutation were also susceptible. Only isolates with the combination of two *embB* mutations (M306I and G406D) were MGIT EMB-resistant, and this was only the case for three out of nine isolates with this combined mutation.

We next compared WGS with the MycoTB assay, which yields MICs (Figure 3.1 and Table 3.1). Different resistance mutations were associated with different MIC ranges. For INH, the *katG* S315T mutation was associated with clearly elevated MICs, as was the *ahpC* C52T promotor mutation although it only occurred in one isolate. Other mutations in *ahpC*, *fabG1*, and *inhA* were associated with MICs around or just above the critical concentration. For RIF, the *rpoB* S450L mutation was associated with elevated MICs, but other *rpoB* mutations were associated with a range of MICs including the critical concentration. Mutations in *rpsL* were all associated with MICs above the critical concentration for STR and the same applies to the *rrs* A514C mutation. However, other *rrs* mutations were associated with MICs around or below the critical concentration for STR. All found mutations in *embA* or *embB* were associated with elevated EMB MICs; their ranges included the critical concentration.

All isolates with resistance-conferring mutations to ETO had elevated MICs. The *fabG1* C15T promotor mutation was associated with a bimodal MIC distribution for ETO; some isolates had an MIC of twice the critical concentration; most others with the mutation had an eight times higher MIC than the critical concentration. For AMK and KAN, all observed mutations were associated with an MIC range below or including the critical concentration. OFL or MXF resistance mutations were uncommon, and these mutations were associated with an MIC above the critical concentration in only two isolates, and only for OFL. For STR and OFL, but especially for ETO, we observed isolates with no mutations conferring resistance to the respective drug, but with MICs above the critical concentration.

Discussion

In this study, we demonstrate that MIC determination using SensiTitre MycoTB is an excellent alternative to conventional DST methods; it is relatively rapid, straightforward, and it provides quantitative data on susceptibility to first- and second-line drugs, thus facilitating therapeutic decision-making and therapeutic drug monitoring to optimize regimen efficacy and minimize toxicity²⁰. Whole genome sequencing has been shown to be a sensitive, accurate, rapid, and financially feasible method for *M.*

tuberculosis drug susceptibility testing²¹. Here we show that WGS has additional added value in terms of predicting the level of resistance to different drugs. For key anti-tuberculosis drugs including rifampicin, isoniazid, ethambutol and fluoroquinolones, distinct genomic mutations are associated with particular MIC ranges, some including the critical concentration.

The MycoTB microdilution method is a commercially available method for MIC testing in *M. tuberculosis* and the first for second-line drugs. Previous studies have tested its performance compared to currently available methods for drug susceptibility testing, such as the BACTEC MGIT 960²², the indirect agar proportion method^{8,9,23}, the Löwenstein-Jensen proportion method²⁴, genotypic tests²⁵, or a combination of these¹⁷, and it has proven a rapid, convenient, quantitative, and accurate method for testing both first- and second-line antituberculosis drug susceptibility. We found discrepancies with WHO-endorsed phenotypic tests for first line drugs, especially STR and EMB, but phenotypic DST has been proven to be difficult for these drugs and the value of these tests as 'gold standard' is questionable. Compared to previous studies comparing MycoTB with MGIT, the MycoTB in the present study performed slightly better for INH and RIF, comparable for EMB, and slightly worse for STR^{17,22}. Furthermore, the microwell plate format without the need for equipment will allow its use in resource-poor settings, where it is most needed, provided that culture-based DST is possible, and proper biosafety measures are taken. Moreover, the visual reading of the plates requires some operator experience.

Whole genome sequencing is revolutionizing drug susceptibility testing in tuberculosis and has great potential in public health interventions. However, the various platforms used, as well as the shortcomings of critical concentrations for phenotypic DST hinder the comparison of genotypic and phenotypic DST, hence the validation of WGS-based susceptibility testing²⁶. The current critical concentrations have a limited evidence base and stem largely from observations on wild type MIC distributions, not from clinical or pharmacokinetics/pharmacodynamics studies²⁷. Data from several studies indicate that these critical concentrations need to be revised because they either bisect the wild-type distribution, or are substantially higher than the MICs of wild-type organisms resulting in potential false reporting of susceptibility²⁸. Indeed, in the current study, we observed that isolates with mutations associated with decreased susceptibility to RIF, STR, and EMB were reported susceptible according to MGIT, and MICs for these drugs were often around the critical concentration. These data support the notion of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) that critical concentrations should be defined by combining MIC distributions, preferably combined with clinical outcomes and pharmacokinetics/pharmacodynamics data^{28,29}. Even though WGS has already been successfully implemented in routine

diagnostic practice in some settings and has been shown to achieve generally high agreement with phenotypic first-line DST^{21,30,31}, MIC testing may help in more accurately assessing the performance of WGS for drug resistance detection and the role of this method in TB laboratory diagnosis.

The discrepancies between MGIT and MICs observed in our study support the current dogma that inconsistencies between phenotypic and genotypic DST found in important studies investigating the use of WGS for predicting *M. tuberculosis* drug susceptibility are partly attributed to shortcomings with currently endorsed methods of phenotypic DST, which essentially provide only qualitative results (sensitive or resistant), especially where mutations conferring low-level drug resistance are involved³². However, it is important to appreciate that unknown resistance mechanisms, inadequate limits of detection or artefacts of sequencing, random errors, and false associations between genotype and phenotype due to epistatic interactions could all play a role^{33–35}. The clinical impact of these discrepancies, and the effect of treatment regimen based on different DST methods are not clear yet, and clinical validation of the influence of MICs of single drugs on treatment outcome is warranted. The analysis of *embB* mutations has long been considered futile because they did not match well with phenotypic DST results³⁶, our data however show that these mutations do have an effect on MICs thus potentially influence treatment efficacy^{37,38} as has been shown previously in a mouse model of aerogenic tuberculosis³⁹.

Similar to what has been shown in a recent study by Heyckendorf *et al.*³², we observed that WGS did not miss phenotypically confirmed resistances to first-line drugs, except for STR. We discovered that the MIC-range for isolates without STR resistance-conferring mutations was wide and included the critical concentration. Unknown mutations in these isolates could have caused resistance. Critical concentration artefacts could also explain the discrepancies; 16 isolates were MGIT-resistant for STR, but had an MIC below the critical concentration. In addition, WGS predicted resistance in a number of phenotypically susceptible isolates. Especially *rpoB* H445N, *embB* G406D and M306I, as well as the *rrs* mutations did not correlate well with phenotypic resistance, in line with previous findings^{19,26,40–42}. We found that these mutations increased the MIC only slightly, or were associated with wide MIC-ranges. However, previous studies have shown that these mutations may still be clinically relevant^{37,39,41,43,44}.

This is the first study to investigate drug resistance in *M. tuberculosis* isolates from the greater Craiova area, Romania. In this selection of phenotypically drug-resistant isolates we observed low frequencies of resistance to second-line drugs, possibly as a result of limited availability of second-line antituberculosis drugs⁷. Surprising was the observation that all-but-one of the isolates belonged to the Euro-American lineage,

because there has been an extremely large cluster of MDR/XDR-TB *M. tuberculosis* strains in the EU, especially in the eastern part, which is significantly related to the spread of one strain or clone of the Beijing genotype⁴⁵.

The relatively small number of isolates does not permit drawing solid conclusions on the effect of most drug resistance mutations on drug susceptibility and the interrelatedness of different mutations, or their combined effects on the level of resistance. However, the findings we present are an important contribution to the field because of a lack of data correlating *M. tuberculosis* genotype and MICs⁴⁶. In addition, the phylogeny showed one clade of genetically closely related isolates. This could have affected the MIC distribution of the drugs these isolates are resistant to. However, the drug resistance mutations found in these isolates differed to an extent that makes it unlikely that these isolates represent the same strain. Also, we could not associate drug resistance mutations with the corresponding MICs for two strains that failed to grow on the MycoTB microtiter plate and six that were contaminated. Lastly, pyrazinamide is not included in the MycoTB plate, and we could not assess the MICs related to pyrazinamide resistance-associated mutations, which were discovered in 23 (32%) isolates.

In summary, we have shown that, especially for RIF, STR, and EMB, MICs near the critical concentration are common. Consequently, phenotypic DST based on critical concentration testing, e.g. the MGIT method, may provide inaccurate results, possibly leading to suboptimal treatment regimens. We compared WGS-predicted drug resistance mutations directly with MICs and found that different mutations lead to different levels of resistance; knowing the underlying mutations can guide clinical decision-making and facilitate therapeutic drug monitoring, ultimately leading to better treatment outcome.

Author Contributions

A.L.R., M.I., R.v.C., and J.v.I. designed the study, A.L.R., A.F., L.C., C.E., N.A., D.N., and V.G. performed the isolation, cultivation, and sequencing tasks, C.R., L.C., N.A., R.v.C., and J.v.I. analysed the data, C.R., A.L.R., L.C., R.v.C., and J.v.I. wrote the manuscript, and all authors read and approved the final version of the manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-27962-5>.

Competing Interests

The authors declare no competing interests.

Supplementary figures

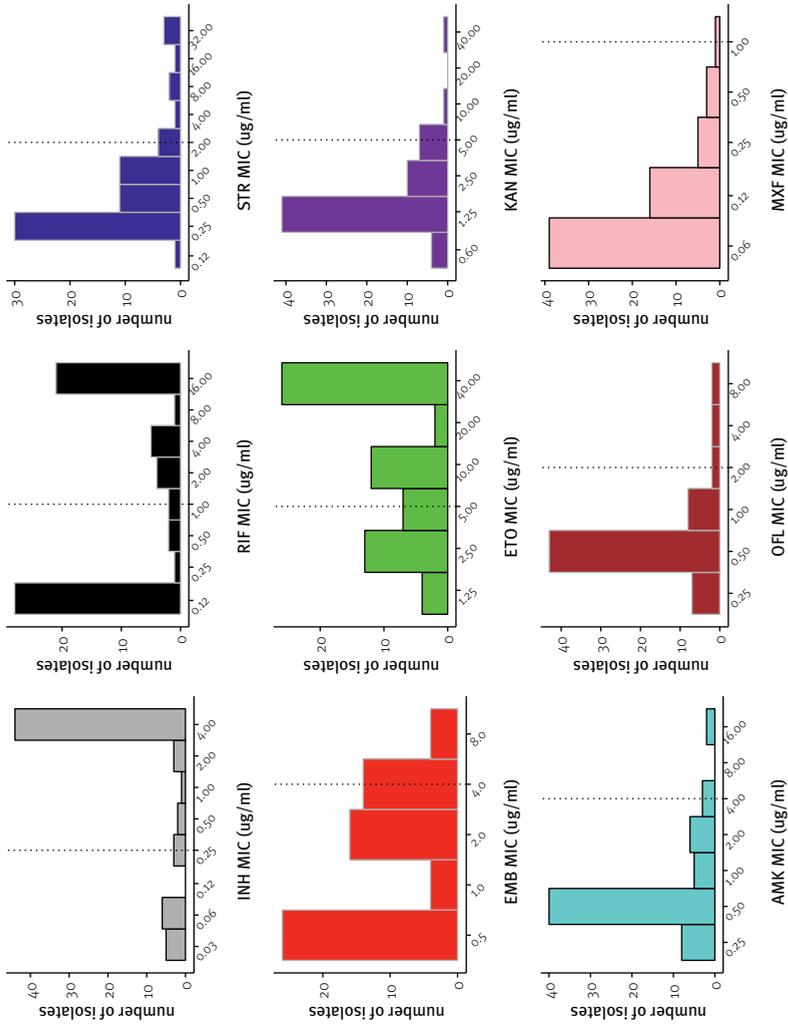


Figure S3.1. Overview of minimum inhibitory concentrations for nine antituberculous drugs. Minimum inhibitory concentrations were determined using the MycoTB microdilution method for 64 *M. tuberculosis* isolates.

Tree scale: 0.1

Lineage

- Euro-American
- East-Asian

Drug resistance mutations

- fabG1 (C-15T promoter)
- katG (Ser315Thr)
- katG (Ser315Arg)
- katG (Asp311Gly)
- inhA (Ile21Val)
- ahpC (C-52T promoter)
- ahpC (G-48A promoter)
- rpoB (Ser450Leu)
- rpoB (His445Asn)
- rpoB (Leu430Pro)
- rpoB (Met434Ile)
- rpoB (Pro454Leu)
- rpoB (Asp435Val)
- rpoB (Asp435Gly)
- rpoB (Phe424Leu)
- rpoB (His445Tyr)
- rpoB (Gln432Pro)
- rpoB (Ser450Trp)
- rpoB (His445Asp)
- rpoC (Gly332Arg)
- embA (C-12T promoter)
- embB (Met306Ile)
- embB (Gly406Asp)
- embB (Met306Val)
- embB (Iyr319Ser)
- embB (Asp354Ala)
- embB (Asp328Tyr)
- embB (Glu504Asp)
- pncA (Ala146Val)
- pncA (Gln10Stop)
- pncA (Gly17Asp)
- pncA (Tyr34Stop)
- pncA (Gln10Arg)
- rrs (C1402T)
- rrs (A1401G)
- rrs (A514C)
- rrs (C517T)
- rpsL (Lys43Arg)
- rpsL (Lys88Arg)
- ethA (Thr61Met)
- gyrB (Asp461Asn)
- gyrA (Ala90Val)
- gyrA (Asp94Gly)
- gyrA (Ser91Pro)

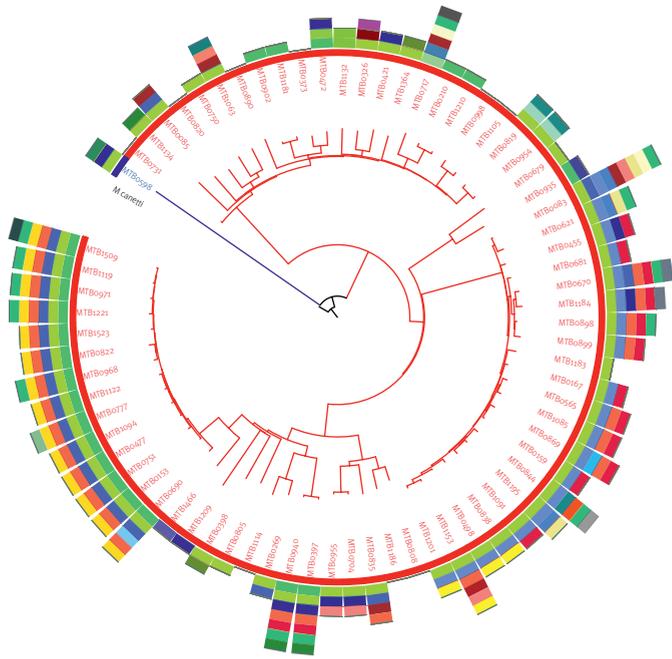


Figure S3.2. Phylogenetic tree showing the genetic relatedness of 72 *M. tuberculosis* strains isolated from 72 Romanian patients.

Supplementary tables

Table S3.1. Description of sequencing quality control parameters and statistics

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|------|-------------|---------------|-----------------|---------------------|
| MTBo083 | 5010168 | 98,8 | 729245204 | 155,8 | 160 | 99,6 |
| MTBo085 | 9244564 | 98,9 | 1331280683 | 292,3 | 299 | 99,4 |
| MTBo153 | 4980076 | 98,8 | 720076577 | 157,9 | 161 | 99,5 |
| MTBo159 | 4326888 | 98,8 | 628038812 | 138,1 | 142 | 99,6 |
| MTBo167 | 5439392 | 98,9 | 792319455 | 174,2 | 178 | 99,6 |
| MTBo210 | 5880320 | 98,9 | 852347773 | 187,4 | 192 | 99,5 |
| MTBo269 | 4613254 | 98,8 | 671851858 | 147,7 | 151 | 99,7 |
| MTBo326 | 5072238 | 98,8 | 730714611 | 160,2 | 164 | 99,5 |
| MTBo373 | 4397414 | 98,8 | 639876834 | 139,8 | 143 | 99,5 |
| MTBo397 | 2668762 | 98,8 | 388958930 | 82,6 | 85 | 99,8 |
| MTBo398 | 3282760 | 98,7 | 479249813 | 103,1 | 105 | 99,5 |
| MTBo421 | 5498680 | 98,8 | 797711420 | 174,6 | 179 | 99,5 |
| MTBo455 | 2581286 | 98,8 | 377936416 | 79,3 | 78 | 99,5 |
| MTBo472 | 6458404 | 98,8 | 937346421 | 204,5 | 210 | 99,5 |
| MTBo477 | 5466848 | 98,8 | 793482681 | 173,6 | 178 | 99,5 |
| MTBo498 | 3500310 | 98,7 | 509420826 | 108,6 | 112 | 99,5 |
| MTBo565 | 6226184 | 98,8 | 907091707 | 198,6 | 204 | 99,5 |
| MTBo598 | 5334456 | 98,7 | 776163802 | 169,7 | 173 | 99,3 |
| MTBo621 | 1315430 | 98,7 | 192150033 | 40,4 | 41 | 99,4 |
| MTBo670 | 1723318 | 98,7 | 252717866 | 51,7 | 53 | 99,5 |
| MTBo679 | 5047768 | 98,8 | 735251932 | 160,7 | 164 | 99,7 |
| MTBo681 | 2592134 | 98,3 | 374789416 | 81,0 | 82 | 99,5 |
| MTBo690 | 2788926 | 98,7 | 407670628 | 88,0 | 90 | 99,5 |
| MTBo704 | 4512874 | 98,8 | 655016172 | 143,0 | 146 | 99,4 |
| MTBo717 | 5180780 | 98,8 | 753070029 | 164,8 | 169 | 99,5 |
| MTBo731 | 6910880 | 98,8 | 995135438 | 218,9 | 225 | 99,7 |
| MTBo750 | 16678804 | 98,8 | 2289895133 | 501,3 | 500 | 99,6 |
| MTBo751 | 5024722 | 98,8 | 725893624 | 159,6 | 160 | 99,6 |
| MTBo777 | 6829294 | 98,8 | 984893721 | 216,8 | 222 | 99,6 |
| MTBo805 | 6349612 | 98,9 | 920541812 | 202,5 | 208 | 99,8 |
| MTBo808 | 13982680 | 98,9 | 1992277055 | 438,3 | 450 | 99,6 |
| MTBo819 | 7285102 | 98,8 | 1045110908 | 228,2 | 233 | 99,3 |
| MTBo820 | 6437616 | 98,8 | 928788036 | 203,9 | 209 | 99,6 |
| MTBo822 | 10553814 | 98,8 | 1498369699 | 329,4 | 335 | 99,6 |
| MTBo835 | 13273726 | 98,9 | 1867111990 | 410,9 | 422 | 99,5 |

Table S3.1. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|------|-------------|---------------|-----------------|---------------------|
| MTBo838 | 11449646 | 98,9 | 1631527546 | 358,9 | 368 | 99,6 |
| MTBo844 | 7042508 | 98,8 | 1014627854 | 223,5 | 229 | 99,6 |
| MTBo869 | 9514364 | 98,8 | 1348716932 | 296,8 | 304 | 99,6 |
| MTBo890 | 9986614 | 98,8 | 1426980119 | 312,9 | 321 | 99,5 |
| MTBo898 | 2673806 | 98,8 | 389093509 | 78,8 | 80 | 99,5 |
| MTBo899 | 3748466 | 98,8 | 543659909 | 110,8 | 111 | 99,6 |
| MTBo902 | 5105332 | 98,8 | 738012399 | 161,6 | 166 | 99,5 |
| MTBo935 | 4166006 | 98,8 | 600850392 | 131,6 | 134 | 99,9 |
| MTBo940 | 6345710 | 98,9 | 912105253 | 200,3 | 205 | 99,8 |
| MTBo954 | 5182548 | 98,9 | 749833435 | 164,1 | 169 | 99,3 |
| MTBo955 | 6342280 | 98,9 | 912264427 | 200,4 | 206 | 99,4 |
| MTBo968 | 5597674 | 98,8 | 808331240 | 177,3 | 182 | 99,6 |
| MTBo971 | 4810290 | 98,8 | 697511432 | 153,1 | 158 | 99,5 |
| MTBo998 | 6125292 | 98,8 | 882233413 | 193,8 | 200 | 99,5 |
| MTB1063 | 3131132 | 98,8 | 452357038 | 99,1 | 102 | 99,5 |
| MTB1085 | 4055568 | 98,8 | 586012462 | 127,2 | 131 | 99,5 |
| MTB1091 | 4496052 | 98,8 | 651171048 | 143,0 | 147 | 99,5 |
| MTB1094 | 5037192 | 98,8 | 726608727 | 159,5 | 164 | 99,5 |
| MTB1105 | 2766362 | 98,8 | 403334631 | 86,0 | 88 | 99,5 |
| MTB1114 | 7021708 | 98,8 | 1005915329 | 221,2 | 227 | 99,7 |
| MTB1119 | 4133620 | 98,7 | 598668119 | 131,1 | 134 | 99,5 |
| MTB1122 | 5510106 | 98,8 | 800687586 | 175,7 | 179 | 99,5 |
| MTB1132 | 2041418 | 98,7 | 297936222 | 64,9 | 66 | 99,2 |
| MTB1134 | 2636480 | 98,8 | 384682814 | 84,1 | 86 | 99,3 |
| MTB1153 | 2592910 | 98,8 | 377531940 | 82,8 | 85 | 99,5 |
| MTB1181 | 3687154 | 98,8 | 536107686 | 117,5 | 121 | 99,5 |
| MTB1183 | 4231256 | 98,8 | 614868223 | 135,1 | 138 | 99,5 |
| MTB1184 | 1520090 | 98,8 | 222159082 | 47,7 | 49 | 99,5 |
| MTB1186 | 2331846 | 98,7 | 339340987 | 74,3 | 76 | 99,5 |
| MTB1195 | 5434990 | 98,7 | 786889583 | 172,7 | 177 | 99,6 |
| MTB1201 | 3044342 | 98,7 | 439656581 | 96,5 | 99 | 99,5 |
| MTB1209 | 3420894 | 98,7 | 494659829 | 108,5 | 111 | 99,5 |
| MTB1221 | 2829856 | 98,8 | 411306820 | 89,8 | 91 | 99,5 |
| MTB1364 | 3977738 | 98,7 | 576100368 | 125,7 | 128 | 99,5 |
| MTB1466 | 2388624 | 98,7 | 347922516 | 75,6 | 77 | 99,6 |
| MTB1509 | 3914664 | 98,6 | 568644763 | 124,2 | 126 | 99,5 |
| MTB1523 | 2993102 | 98,8 | 435834604 | 95,7 | 98 | 99,5 |

Table S3.2. Discrepant drug susceptibility test results according to whole genome sequencing and Mycobacterium Growth Indicator Tube.

| Isolate | Discrepant WGS result | Discrepant MGIT result | LJ result | Sequencing statistics | | |
|---------|-----------------------|------------------------|-----------------|-----------------------|--|-----------------------------------|
| | | | | Underlying mutation | Sequencing coverage depth (no. of reads) | % of reads supporting the variant |
| MTBo159 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 147 | 100% |
| MTBo498 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 115 | 96.5% |
| MTBo598 | RIF-resistant | RIF-susceptible | Rif-resistant | rpoB (D435V) | 161 | 97.5% |
| MTBo621 | RIF-resistant | RIF-susceptible | Rif-resistant | rpoB (H445N) | 37 | 100% |
| | | | | rpoB (P454L) | 48 | 100% |
| MTBo681 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 123 | 97.6% |
| MTBo819 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (L430P) | 234 | 99.6% |
| MTBo838 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 383 | 98.4% |
| MTBo898 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 78 | 97.4% |
| MTBo899 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 122 | 99.2% |
| MTB1085 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 130 | 99.2% |
| MTB1091 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 136 | 99.3% |
| MTB1153 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 79 | 100% |
| MTB1195 | RIF-resistant | RIF-susceptible | RIF-susceptible | rpoB (H445N) | 183 | 99.5% |

Table S3.3. Concordance between Mycobacterium Growth Indicator Tube and MycoTB

| | | | MycoTB | | % Categorical agreement | Cohen's kappa (p-value) |
|------|-----|-------------|----------------------------------|------------------------------------|-------------------------|-------------------------|
| | | | MIC above breakpoint (resistant) | MIC below breakpoint (susceptible) | | |
| MGIT | INH | Resistant | 48 | 3 | 93.5 | 0.794 (p<0.001) |
| | | Susceptible | 1 | 10 | | |
| | RIF | Resistant | 29 | 2 | 95.2 | 0.905 (p<0.001) |
| | | Susceptible | 1 | 31 | | |
| | STR | Resistant | 7 | 16 | 71.0 | 0.289 (p=0.006) |
| | | Susceptible | 2 | 37 | | |
| | EMB | Resistant | 1 | 2 | 92.3 | 0.246 (p=0.045) |
| | | Susceptible | 3 | 59 | | |

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4

Diabetes is associated with genotypically drug-resistant tuberculosis

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Eur Resp J. To be adapted for resubmission.

Abstract

Background

Many tuberculosis (TB) patients with diabetes have poor treatment outcomes, but it is unclear whether this results from genotypic drug resistance of the infecting mycobacteria.

Methods

In this observational study we used whole genome sequencing to examine 1,365 known drug resistance mutations in 896 *Mycobacterium tuberculosis* isolates from TB patients from Indonesia and Peru with (n=159) and without (n=737) diabetes, 19% and 24% of whom respectively had been previously treated for TB. We used multilevel logistic regression to examine the relationship between diabetes and resistance mutations.

Results

In Indonesia, drug resistance mutations were found in *M. tuberculosis* isolates of 21 / 115 (18%) patients with and 43 / 292 (15%) patients without diabetes, respectively. In Peru, mutations were found in 18 / 44 (41%) patients with, compared to 149 / 445 (33%) without diabetes. Diabetes was independently associated with genotypic drug resistance (odds ratio (OR) = 1.69; 95% confidence interval (CI) = 1.04-2.77), in particular to rifampicin (OR = 2.52 (95% CI 1.19-5.34)). The association was stronger for those not previously treated for TB. Mutations in *Rv1482c-fabG1*, conferring resistance to isoniazid and ethionamide, and *gyrA*, conferring fluoroquinolone resistance, were overrepresented in isolates from Peruvian patients with diabetes ($p < 0.01$ and $p < 0.05$, respectively).

Conclusions

Diabetes is associated with genotypic drug resistance in *M. tuberculosis*, and this is not explained by prior TB treatment. Our findings support the importance of routine drug-susceptibility testing for TB patients with diabetes, and highlight the need for further study to understand the mechanism underlying the association.

Introduction

The convergence of the tuberculosis (TB) and type 2 diabetes epidemics¹ poses a great threat to global TB control. People with diabetes are three times more likely to develop active TB than people without diabetes, and diabetes is associated with increased death, TB treatment failure and recurrent TB²⁻⁵. A higher prevalence of drug-resistant TB among those with diabetes may be one contributing factor to their adverse clinical outcomes during and after TB treatment. Two recent meta-analyses examining the association between diabetes and multidrug-resistant TB (MDR-TB), including more than twenty-two thousand individuals with TB, showed that diabetes is an independent risk factor for MDR-TB^{3,6}. Drug-resistant TB is associated with increased morbidity and mortality and has emerged as a major threat to TB control worldwide⁷. However, the genetic characteristics of resistant *M. tuberculosis* strains causing disease in diabetes patients remain to be discovered.

In the current study we used whole genome sequencing to investigate genetic differences of *M. tuberculosis* isolates causing disease in two cohorts of TB patients with and without diabetes, focusing on the presence of drug resistance-associated mutations. As opposed to phenotypic drug susceptibility testing (DST), whole genome sequencing has the potential to unlock valuable information on the specific mutations underlying resistance, transmission clusters and the phylogenetic background of drug-resistant *M. tuberculosis* strains⁸, thereby increasing our understanding of the molecular basis of resistance of *M. tuberculosis* in these patients.

Methods

Subjects and isolates

We used *M. tuberculosis* isolates from two established TB cohorts of Indonesian and Peruvian patients who were screened for diabetes. Both cohorts were part of TANDEM, a multi-centre study focussing on the relationship between TB and diabetes⁹⁻¹¹. All participants underwent laboratory glycated hemoglobin (HbA_{1c}) testing (using the high-performance liquid chromatography method), as recommended by WHO for diabetes screening¹², regardless of their previous diabetes status. Further details on TB and diabetes case definitions are published elsewhere^{10,13} and are freely available in online appendices¹⁴. In Peru we selected all available *M. tuberculosis* isolates; in Indonesia we selected all available isolates from TB patients with diabetes plus a subset of isolates from patients without diabetes from the same clinics, during the same time period, frequency-matched by age. A single isolate from each patient, obtained at time of diagnosis, was selected for sequencing. Ethical approval was

received from the Observational/Interventions Research Ethics Committee, London School of Hygiene and Tropical Medicine on 18 December 2013 (LSHTM ethics ref: 6449, LSHTM amendment no: A473) and institutional review boards in Indonesia and Peru.

Cultured *M. tuberculosis* isolates underwent whole genome sequencing as described in the Supplementary methods. We used TB Profiler version 0.3.8¹⁵ to determine *M. tuberculosis* lineage and drug resistance, comparing identified single nucleotide polymorphisms (SNPs) and indels to a curated list of drug resistance mutations. All non-synonymous SNPs in *rpoA*, *rpoB* outside the rifampicin resistance-determining region (RRDR), *rpoC*, *ahpC* promoter region and *ubiA* were considered as mutations possibly compensating for the loss of fitness caused by drug resistance mutations¹⁶⁻¹⁸.

A phylogeny was constructed using PhyML version 3.0¹⁹. Based on the phylogeny, we calculated the distance to the genetically closest other isolate (minimum pairwise distance) for isolates from TB patients with and without diabetes separately. We compared the distribution of these distances using the Mann-Whitney U test, for Indonesia and Peru separately.

Statistical analysis

We compared patient- and isolate-related characteristics of TB patients with and without diabetes, stratified by country (Indonesia or Peru). We assessed the association between diabetes and genotypic drug resistance using multilevel logistic regression, correcting for age, gender, HIV-infection, previous TB treatment, and *M. tuberculosis* lineage. We expanded the analysis by examining differences in resistance to individual drugs and combined mutations accounting for MDR-TB, again using multilevel multi-variable logistic regression. To examine country-specific associations between diabetes and individual and multi-drug resistance, we used logistic regression stratified by country. We performed a sensitivity analysis to examine the effect of possible misclassification of patients with newly diagnosed diabetes on the association with any drug resistance. Furthermore, we investigated whether HbA_{1c} was associated with drug resistance and also analysed the data after excluding all patients with HIV.

We investigated whether resistance mutations in certain genes occurred more frequently in TB patients with diabetes. Per resistance gene, we summed the number of isolates with at least one known resistance mutation in the respective gene and compared these between patients with and without diabetes, for Indonesia and Peru separately.

Lastly, we examined if a possible association between diabetes and drug resistance could be explained by differences in potential compensatory mutations, hypothesizing that resistant isolates from patients with diabetes, who are thought to have a lower

host immune defence against *M. tuberculosis*²⁰, would need fewer mutations compensating for loss of fitness associated with drug resistance. We determined the association between isoniazid and rifampicin resistance mutations and the presence of previously proposed accompanying compensatory mutations^{16,18,21}, stratified for diabetes comorbidity, for Indonesian and Peruvian isolates separately.

Results

Patient and isolate characteristics

Diabetes was more common among the Indonesian (28%) compared to the Peruvian patients (9%), but genotypic drug resistance was more often observed in isolates from Peruvian (34%) compared to Indonesian patients (16%, Table 4.1). In both countries, TB patients with diabetes were older, more often female, and slightly heavier than TB patients without diabetes. Only 21% of the Indonesian and 14% of the Peruvian diabetes patients had been previously treated for TB, compared to 27% and 22% of the non-diabetes patients, respectively (Table 4.1).

A phylogenetic tree, based on 56,654 variable common nucleotide positions among 896 *M. tuberculosis* isolates, showed that isolates from TB patients with diabetes were spread evenly across the phylogeny, i.e. they were not clustered (Figure 4.1). The *M. tuberculosis* lineage distribution for isolates from patients with diabetes was significantly different from those without (Chi-square = 11.559; $p=0.009$), with East-Asian strains being more common (30% vs. 20%) and Euro-American strains less common among the TB patients with diabetes (67% vs. 77%). However, this difference was no longer apparent after stratifying by country (Chi-square = 4.692; $p=0.196$ for Indonesia and Chi-square = 0.241; $p=0.624$ for Peru); lineage was indeed highly associated with country (Chi-square = 90.383; $p<0.001$). With respect to clonality: for isolates from Indonesian TB patients with diabetes, the median minimum pairwise distance was 161 SNPs; for patients without diabetes this amounted to 164 SNPs ($p=0.867$). For isolates from Peruvian TB patients with diabetes, the median minimum pairwise distance was 98 SNPs, compared to 67 SNPs in patients without diabetes ($p=0.021$, Supplementary figure S4.1).

Genotypic drug resistance in TB patients with and without diabetes

In Indonesia and Peru, respectively 18% and 39% of the patients with diabetes were infected with *M. tuberculosis* with at least one drug resistance-conferring mutation, compared to 15% and 20%, respectively, of the patients without diabetes (Fisher's exact $p=0.454$ and $p=0.006$ respectively; Table 4.1). In multilevel logistic regression, genotypic drug resistance was significantly associated with diabetes, both in

Table 4.1. Baseline characteristics of tuberculosis patients with and without diabetes mellitus type 2

| | Indonesia | | Peru | |
|----------------------------------|---------------------|------------------------|--------------------|------------------------|
| | Diabetes (N=115) | No diabetes (N=292) | Diabetes (N=44) | No diabetes (N=445) |
| Male gender | 52 (45%) | 168 (58%) | 25 (57%) | 268 (60%) |
| Age (years) – median (IQR) | 50 (45-58) | 39 (33-48) | 52 (42-59) | 28 (22-39) |
| BMI | 21 (18-25) | 18 (16-20) | 24 (22-28) | 22 (20-24) |
| History of previous TB treatment | 24 (21%) | 79 (27%) | 6 (14%) | 96 (22%) |
| BCG scar | 51 (65%) | 108 (60%) | 38 (86%) | 342 (77%) |
| HIV-positive | 0 | 0 | 1 (3%) | 18 (5%) |
| <i>M. tuberculosis</i> lineage | | | | |
| East-Asian | 40 (35%) | 90 (31%) | 7 (16%) | 59 (13%) |
| Euro-American | 69 (60%) | 178 (61%) | 37 (84%) | 386 (87%) |
| Indo-Oceanic | 5 (4%) | 24 (8%) | 0 | 0 |
| <i>M. bovis</i> | 1 (1%) | 0 | 0 | 0 |
| Any resistance predicted by WGS | 21 (18%) | 44 (15%) | 17 (39%) | 88 (20%) |
| Treatment-naïve | 13 (11%) | 31 (11%) | 14 (32%) | 67 (15%) |
| Previously treated | 8 (7%) | 12 (4%) | 3 (7%) | 21 (5%) |
| MDR predicted by WGS | 6 (5%) | 8 (3%) | 6 (14%) | 38 (9%) |

Data are presented as N (%) unless indicated otherwise.

Data are missing for BMI (Indonesia: n = 149), History of previous TB treatment (Indonesia: n = 3), BCG scar (Indonesia: n = 149), HIV-status (Indonesia: n = 1; Peru: n = 75).

univariable (odds ratio (OR) 1.6; 95% confidence interval (CI) 1.1-2.5), and multivariable analysis (OR 1.8; 95% CI 1.1-2.9). No other factors were independently associated with genotypic resistance (Table 4.2).

Combining both countries and accounting for the country effect using multilevel multivariable regression, diabetes was significantly associated with rifampicin resistance (OR 2.5; 95% CI 1.2-5.3), also among those not previously treated for TB (OR 3.1; 95% CI 1.2-8.3). In addition, we found a trend towards more fluoroquinolone resistance in TB patients with diabetes compared to those without, which reached significance for Peru (OR 6.69; 95% CI 1.37-32.68). Moreover, although this association did not reach statistical significance, the odds of MDR-TB was twice as high in patients with diabetes, compared to patients without (OR 2.09; 95% CI 0.92-4.77).

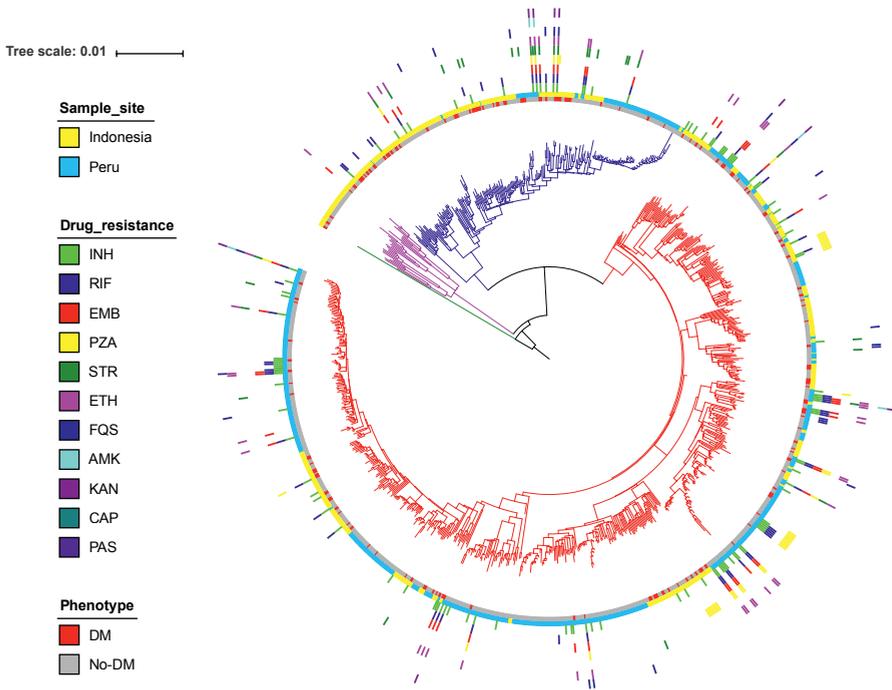


Figure 4.1. Phylogenetic tree of 896 *M. tuberculosis* isolates from TB patients with and without diabetes, showing that isolates from TB patients with diabetes were not clustered. Branch colours indicate whether isolates belong to the Indo-Oceanic (pink), East-Asian (blue), Euro-American (red) *M. tuberculosis* lineage, or to the *M. bovis* (green) lineage. The inner circle surrounding the phylogenetic tree indicates whether isolates originate from diabetes (red) or non-diabetes (grey) TB patients. The outer circle indicates isolates from Indonesian (yellow) and Peruvian (blue) patients. The bars outside the circles represent the drug resistance profile of the respective isolate.

In both countries, regardless of diabetes comorbidity, genotypic resistance was most common for isoniazid, followed by rifampicin (Table 4.3). In Peru, *Rv1482c-fabG1* (*inhA*-promoter) mutations that confer resistance to isoniazid and ethionamide, and *gyrA* mutations that confer resistance to fluoroquinolones, were significantly more common in diabetes compared to non-diabetes TB patients (Table 4.3). In Indonesia, drug resistance rates were lower and no significant differences in specific resistance mutations were observed between isolates from patients with and without diabetes, although a trend towards more frequent *rpoB* mutations was observed among TB patients with diabetes ($p=0.069$).

Table 4.2. Multilevel logistic regression model determining the association between diabetes and drug resistance, including a random intercept for site

| | Any drug resistance (N=169) | No drug resistance (N=722) | Univariate OR (95% CI) | Adjusted OR (95% CI) |
|---------------------------------------|--------------------------------|-------------------------------|---------------------------|-------------------------|
| Diabetes mellitus [^] | 38 (22%) | 120 (17%) | 1.6 (1.1-2.5)* | 1.8 (1.1-2.9)* |
| Diabetes mellitus [^] | | | | |
| No diabetes | 131 (18%) | 602 (82%) | REF. | REF. |
| Known diabetes | 27 (23%) | 88 (77%) | 1.6 (0.96-2.6) | 1.7 (0.98-2.9) |
| New diabetes | 11 (26%) | 32 (74%) | 1.8 (0.9-3.6) | 2.0 (0.9-4.4) |
| HbA1c (%) – median (IQR) [^] | 5.7 (5.4-6.4) | 5.7 (5.4-6.0) | 1.0 (0.97-1.1) | 1.05 (0.97-1.1) |
| No diabetes | 5.6 (5.3-5.8) | 5.6 (5.3-5.8) | 1.1 (0.7-1.7) | 1.4 (0.9-2.3) |
| Diabetes | 10.5 (9.0-11.8) | 11.3 (9.2-13.1) | 0.9 (0.8-1.1) | 0.9 (0.7-1.0) |
| Age ≤ 36 years | 89 (20%) | 353 (80%) | REF. | REF. |
| Age > 36 years | 81 (18%) | 372 (82%) | 0.9 (0.6-1.3) | 0.8 (0.5-1.1) |
| Male gender | 92 (54%) | 421 (58%) | 0.8 (0.6-1.2) | 0.9 (0.6-1.3) |
| HIV infection | 2 (1%) | 17 (3%) | 0.5 (0.1-2.1) | 0.5 (0.1-2.3) |
| Previous TB treatment | 44 (26%) | 161 (22%) | 1.2 (0.8-1.8) | 1.4 (0.9-2.1) |
| <i>M. tuberculosis</i> lineage | | | | |
| East-Asian | 34 (17%) | 162 (83%) | REF. | REF. |
| Euro-American | 135 (20%) | 535 (80%) | 1.2 (0.8-1.9) | 1.3 (0.8-2.1) |
| Indo-Oceanic | 1 (3%) | 28 (97%) | 0.2 (0.02-1.3) | 0.2 (0.03-1.6) |

The multivariable model was based on 816 patients without missing data (no drug resistance: n = 665; any drug resistance: n = 151). The patient with an *M. bovis* isolate was excluded from the analysis.

* P-value < 0.05

[^] Diabetes was included in the model as a binary (yes/no) variable, or as a categorical variable distinguishing between previously and newly diagnosed disease, or as continuous variable: HbA1c. The adjusted odds ratios represent the odds ratios from the model including the binary diabetes variable as the dependent variable and age, gender, HIV, previous TB treatment and *M. tuberculosis* lineage as independent variables.

Potential compensatory mutations

In Indonesia, three out of 37 (8%) isolates with isoniazid resistance mutations carried at least one potential isoniazid resistance-compensating mutation, compared to 39 out of 370 (11%) isolates without isoniazid resistance mutations (OR 0.7; 95% CI 0.2-2.6). In Peru, this was six out of 77 (8%) compared to nine out of 412 (2%) isolates, respectively (OR 3.8; 95% CI 1.3-11.0). Regarding rifampicin, ten out of 19 (53%) Indonesian isolates with rifampicin resistance mutations had at least one potential compensatory mutation in *rpoA*, *rpoC* and *rpoB* outside the RRDR, compared to 118 out of 388 (30%) isolates without rifampicin resistance mutations

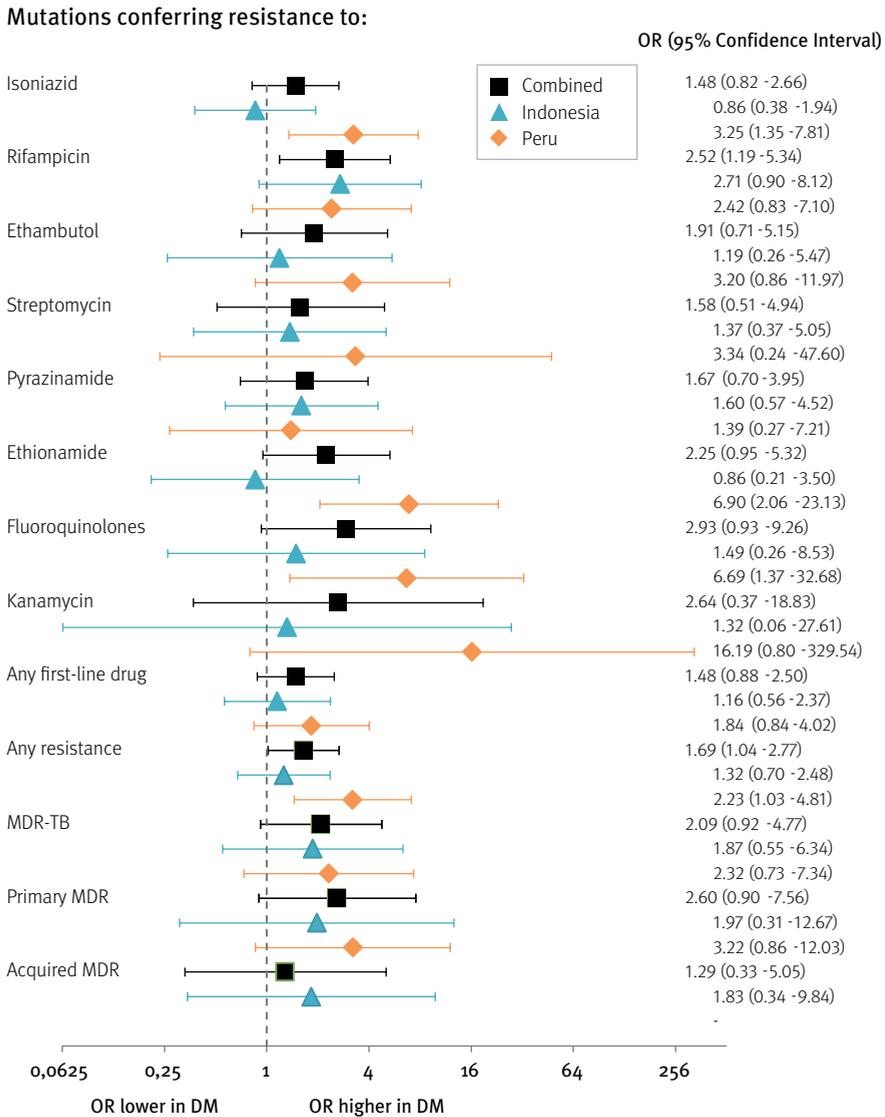


Figure 4.2. Forest plot showing the association between diabetes and genotypic drug resistance, adjusted for age, gender, site, HIV, previous treatment and *M. tuberculosis* lineage. * Odds ratios (ORs) could not be calculated for resistance to amikacin, aminoglycosides and acquired multidrug resistance (MDR) for Peru, due to too little resistant isolates. The patient with an *M. bovis* isolate was excluded from the analysis.

Table 4.3. Drug resistance genes with mutation(s) in *M. tuberculosis* isolates from patients with and without diabetes

| Drug | Gene | Indonesia | | Peru | |
|---------------------------|----------------------|------------------|---------------------|-----------------|---------------------|
| | | Diabetes (n=115) | No diabetes (n=292) | Diabetes (n=44) | No diabetes (n=445) |
| Isoniazid | <i>katG</i> | 8 (7.0%) | 19 (6.5%) | 6 (13.6%) | 48 (10.8%) |
| | <i>Rv1482c-fabG1</i> | 3 (2.6%) | 10 (3.4%) | 8 (18.2%)** | 23 (5.2%)** |
| | <i>ahpC</i> | 0 | 0 | 1 (2.3%) | 0 |
| Rifampicin | <i>rpoB</i> | 9 (7.8%) | 10 (3.4%) | 7 (15.9%) | 42 (9.4%) |
| Ethambutol | <i>embB</i> | 3 (2.6%) | 6 (2.1%) | 5 (11.4%) | 30 (6.7%) |
| | <i>embC-embA</i> | 0 | 0 | 1 (2.3%) | 6 (1.3%) |
| | <i>embR</i> | 0 | 1 (0.3%) | 0 | 0 |
| Streptomycin | <i>rpsL</i> | 4 (3.5%) | 5 (1.7%) | 1 (2.3%) | 16 (3.6%) |
| | <i>rrs</i> | 1 (0.9%) | 2 (0.7%) | 0 | 5 (1.1%) |
| Pyrazinamide | <i>pncA</i> | 7 (6.1%) | 14 (4.8%) | 3 (6.8%) | 22 (4.9%) |
| Ethionamide | <i>Rv1482c-fabG1</i> | 3 (2.6%) | 10 (3.4%) | 8 (18.2%)** | 22 (4.9%)** |
| | <i>ethA</i> | 0 | 0 | 0 | 1 (0.2%) |
| Fluoroquinolones | <i>gyrA</i> | 3 (2.6%) | 3 (1.0%) | 4 (9.1%)* | 7 (1.6%)* |
| | <i>gyrB</i> | 0 | 0 | 0 | 2 (0.4%) |
| Amikacin | <i>rrs</i> | 0 | 0 | 0 | 5 (1.1%) |
| Capreomycin | <i>tlyA</i> | 0 | 1 (0.3%) | 0 | 7 (1.6%) |
| | <i>rrs</i> | 0 | 0 | 0 | 5 (1.1%) |
| Kanamycin | <i>eis-Rv2417c</i> | 1 (0.9%) | 1 (0.3%) | 1 (2.3%) | 1 (0.2%) |
| | <i>rrs</i> | 0 | 0 | 0 | 5 (1.1%) |
| Para-amino-salicylic acid | <i>folC</i> | 0 | 2 (0.7%) | 1 (2.3%) | 2 (0.4%) |
| | <i>thyA</i> | 0 | 0 | 0 | 1 (0.2%) |

Data represent the number (%) of isolates with at least one mutation in the respective gene.

* P-value <0.05; ** P-value <0.01 (p-values are Chi-square p-values unless the expected number of resistant isolates equalled less than 5, in which case the Fisher's Exact Test p-value was calculated).

(OR 2.5; 95% CI 1.01-6.4). In Peru, this was 34 out of 49 (69%) compared to 211 out of 440 (48%) isolates, respectively (OR 2.5; 95% CI 1.3-4.6, Supplementary table S4.1). There were no differences in the association between isoniazid or rifampicin resistance mutations and potential compensatory mutations between patients with and without diabetes, or any interaction between diabetes and the presence of compensatory mutations (Supplementary table S4.1).

Sensitivity analysis

In the sensitivity analysis, distinguishing patients with newly and previously diagnosed diabetes, we confirmed the association between diabetes and drug resistance, although it was no longer significant with smaller numbers (adjusted OR 2.0; 95% CI 0.9-4.4 for those with newly, compared to OR 1.7; 95% CI 0.98-2.9 for those with previously diagnosed diabetes, Table 4.2). HbA1c was not associated with drug resistance. In addition, the association between diabetes and individual drug resistance differed per drug and per country (Figure 4.2). The association between diabetes and drug resistance remained similar after exclusion of the 19 Peruvian patients with HIV (adjusted OR 1.8; 95% CI 1.1-2.9).

Discussion

M. tuberculosis resistance mutations were more common in isolates from TB patients with diabetes, compared to those without. The association was evident for resistance to rifampicin, with a 2.5 times higher odds of rifampicin resistance in TB patients with diabetes, but was also strong for resistance to isoniazid and ethionamide, as well as fluoroquinolones, although these only reached statistical significance in the Peruvian cohort. The association of diabetes and drug resistance mutations was not explained by acquisition of drug resistance as a result of previous TB treatment since it was observed for both treatment-naïve, as well as previously treated TB patients.

Previous studies have investigated a possible link between diabetes and drug resistance, but most used phenotypic DST and focused on first-line drugs or MDR-TB (reviewed in Teegne *et al.*⁶). Whole genome sequencing allowed us not only to investigate the association between diabetes and drug resistance at the gene level, but also to take the diverse *M. tuberculosis* genetic background into account. The association between drug resistance and diabetes has been confirmed in some^{2,22}, but not all²³ previous studies. One possible explanation for discrepant results could be the different populations that were studied, the size of the studies, the extent to which account was taken of factors other than diabetes status, and lack of clarity with respect to the time of diabetes testing⁴. Here, we ascertained the association between diabetes and drug resistance, adjusted for possible confounders, and discovered that the association holds true regardless of the *M. tuberculosis* strain's phylogenetic background, and was not explained by clustering of drug-resistant strains among TB patients with diabetes, as pairwise distances were not shorter among the isolates from TB patients with diabetes (Supplementary figure S4.1).

Several factors might account for the observed association between diabetes and drug resistance mutations. First, people with diabetes might be at higher risk of nosocomial transmission of drug-resistant tuberculosis in low-resource settings²⁴. Second, lower rifampicin plasma concentrations among diabetes patients that were found in some²⁵ but not all²⁶ studies might lead to acquisition of drug resistance. However, all isolates in our study were collected before start of treatment, and only 26% of patients with a drug-resistant isolate reported an episode of previous TB treatment that might have resulted in acquired drug resistance mutations. Third, recent papers have found an interaction between drug resistance and cellular immunometabolism²⁷, and this interaction might be altered by diabetes. Fourth, similar to HIV²⁸, reduced host defence in people with diabetes might increase the risk of developing active TB caused by *Mycobacterium tuberculosis* strains with drug resistance mutations associated with loss of fitness⁷. Merker *et al.* have shown that the presence of mutations thought to compensate for bacterial fitness deficits was associated with transmission success and higher drug resistance rates¹⁸. As people with diabetes seem more likely to become infected and progress to active TB disease following infection, recently transmitted drug-resistant strains with compensatory mutations could be overrepresented among TB patients with diabetes. However, larger recent studies have challenged the paradigm of loss of fitness associated with drug resistance mutations^{29,30} and we did not find differences in potential resistance-compensating mutations among TB patients with diabetes compared to those without, neither did the pairwise distances indicate more recent transmission among TB patients with diabetes. The trend towards more fluoroquinolone resistance in TB patients with diabetes could be related to the prescription of broad-spectrum antibiotics, often fluoroquinolones, in patients with respiratory symptoms who are considered at increased risk of infections due to their diabetes³¹.

The current study has several limitations. First, the analysis was hindered by a 'noise' signal related to the different origin and phylogenetic backgrounds. Resistance was more common in Peru, and diabetes more common in Indonesia. However, we used multilevel regression analysis to account for this and to increase the likelihood of finding true associations. Second, we were underpowered for subgroup analyses. For instance, it is hard to draw conclusions regarding specific resistance mutations. We hope that the findings in this study may serve as a starting point for further, targeted research, powered for questions that may have arisen as a result of the presented data. Thirdly, we could not prove if diabetes is associated with more transmission of drug-resistance as the sampling fraction of *M. tuberculosis* isolates in the two high-endemic settings was likely to be too low, making it difficult to identify transmission clusters. Besides higher rates of transmission, diabetes may also lead to more TB reactivation caused by drug-resistant strains.

In summary, for the first time, we used *M. tuberculosis* whole genome sequencing data from two large cohorts of TB patients with and without diabetes to study the association between diabetes and genotypic drug resistance in TB patients. Diabetes was associated with an increased risk of disease caused by strains with resistance mutations, particularly those against rifampicin, but also against isoniazid, ethionamide and fluoroquinolones. Higher rates of resistance mutations among TB patients with diabetes were not explained by acquisition of drug resistance mutations during treatment, but might be related to increased susceptibility to opportunistic infection with drug-resistant TB. TB patients with diabetes should be prioritized for DST in settings where this is not performed for all patients.

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Supplementary methods

Whole genome sequencing

M. tuberculosis DNA was extracted after subculturing positive cultures on Ogawa (Indonesia) or Middlebrook 7H10-7H11 (Peru) solid medium using the cetyl trimethylammonium bromide (CTAB) method or the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA)³².

M. tuberculosis DNA from 148 Indonesian isolates was sequenced on an Illumina HiSeq 2000 instrument using 2 x 100 bp paired-end reads at the Beijing Genome Institute in Hong Kong, and on an Illumina NextSeq500 instrument using 2 x 150 bp paired-end reads at the department of Human Genetics at the Radboudumc, Nijmegen, the Netherlands for the remaining 259 Indonesian isolates. *M. tuberculosis* DNA from Peruvian isolates was sequenced on an Illumina HiSeq 4000 instrument using 2 x 150 bp paired-end reads at the Wellcome Centre for Human Genetics in Oxford, United Kingdom. Sequencing coverage was determined using the FastQC quality control tool version 0.10.1, and the Genome Analysis Toolkit³³. The average coverage depth for the 896 isolates was 110.7x, and the average percentage of bases covered by at least one read was 98.7%. Nine isolates failed the sequencing or mapping quality check and were excluded from further analysis. Sequencing quality control statistics are shown in Supplementary file 1.

We excluded the *rrs* C492T mutation from the resistance mutation database, because we considered this mutation to be a phylogenetic marker of the LAM3 sublineage rather than a resistance mutation³⁴. We used an allele frequency cut-off of $\geq 30\%$, as previously proposed³⁵. In addition, to identify potential compensatory mutations, variants were called with Breseq software, version 0.27.1³⁶ with a minimum threshold of 30x coverage. Mutations with low-quality evidence (i.e. possible mixed read alignment) were not included. We reported mutations according to the *M. tuberculosis* complex numbering system, based on the sequence of the reference strain, *M. tuberculosis* H37Rv³⁷.

Phylogeny construction

For phylogeny construction, sequence reads were aligned to reference strain *M. tuberculosis* H37Rv, accession number NC_000962.3, and variants were called using Breseq, version 0.27.1³⁶. We extracted all 58,041 variable positions across the 896 *M. tuberculosis* sequences and concatenated them into a multiple sequence alignment. Solely for the purpose of creating the phylogenetic tree, SNPs occurring in PE/PPE genes, genes related to mobile elements, as well as genes previously associated with drug resistance³⁸ were removed. The remaining 56,654 SNPs were

used to construct the phylogenetic tree using PhyML version 3.0¹⁹ using the HKY85 model with four gamma-distributed rate-categories, and using a hundred bootstraps.

Statistical analysis

We used the `glmer()` function from the `lme4` library³⁹ to assess the association between diabetes and genotypic drug resistance. We used a multilevel regression model with robust standard errors to account for clustering within countries⁴⁰. Diabetes, age, gender, HIV-infection, previous TB treatment, and *M. tuberculosis* lineage were included as categorical independent variables in the model. Age was included as a binary variable with the median age (36 years) as cut-off value for the best fit. The single patient with an *M. bovis* isolate was excluded from the multilevel logistic regression analysis, because it added complexity to the model and did not improve model fitness. Data were analysed using complete case analysis.

Significance of possible differences in resistance mutations between diabetes and non-diabetes TB patients were determined with the Fisher's exact test or Chi-square test if there were more than two categories per variable. To assess the association between isoniazid and rifampicin resistance mutations and the presence of previously proposed accompanying compensatory mutations, stratified for diabetes comorbidity, for Indonesian and Peruvian isolates separately, we compared crude odds ratios with stratum-specific and Mantel-Haenszel odds ratios. The Breslow-Day test was used to test the significance of interaction between diabetes and the presence of potential compensatory mutations. All analyses were performed in R, version 3.5.2.

Supplementary figure

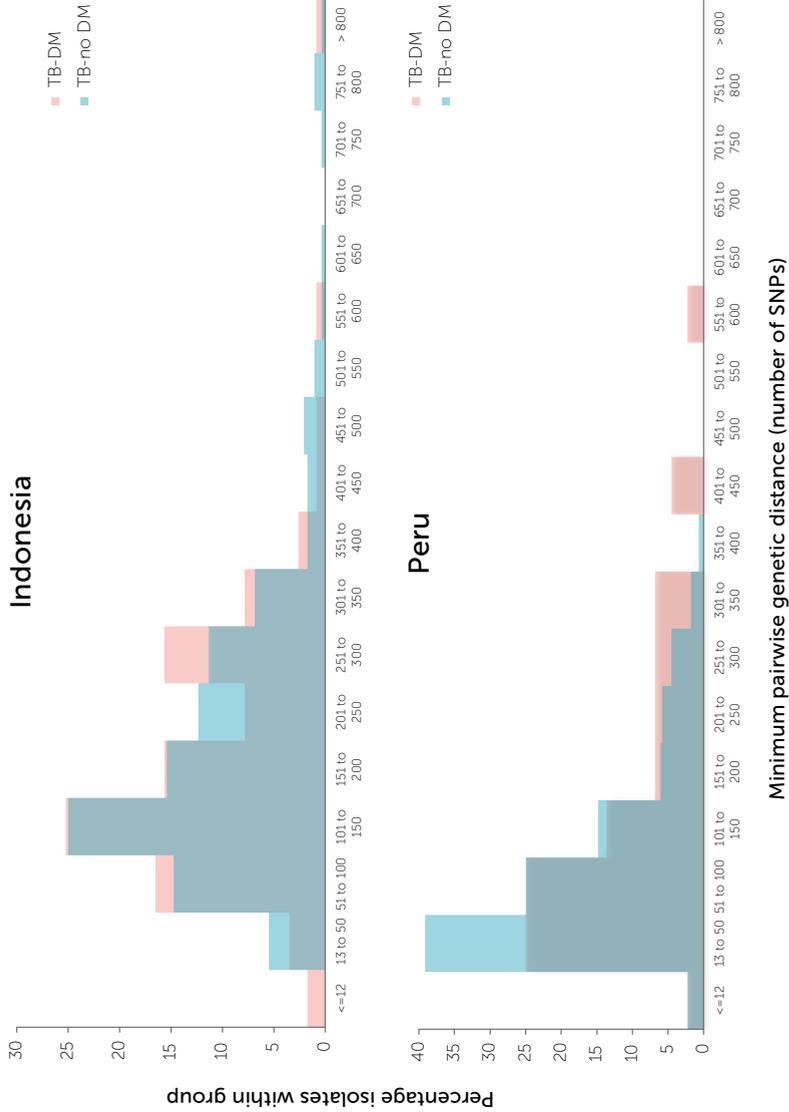


Figure S4.1. Histograms of minimum pairwise genetic distances for *M. tuberculosis* isolates from patients with and without diabetes.

Supplementary table

Table S4.1. Association between putative compensatory mutations and isoniazid and rifampicin resistance, stratified by diabetes comorbidity

| | | INH-resistant | INH-susceptible | Odds ratio (95% CI) | Crude & Mantel-Haenszel OR |
|-------------|--------------------------|---------------|-----------------|---------------------|----------------------------|
| No diabetes | Compensatory mutation(s) | 3 | 31 | 0.9 (0.3-3.3) | 0.7 (0.2-2.6) |
| | No compensatory mutation | 24 | 234 | | & |
| Diabetes | Compensatory mutation(s) | 0 | 8 | 0.6 (0.03-10.2) | 0.7 (0.2-2.5) |
| | No compensatory mutation | 10 | 97 | | |
| No diabetes | Compensatory mutation(s) | 5 | 8 | 4.0 (1.3-12.5)* | 3.8 (1.3-11.0)* |
| | No compensatory mutation | 59 | 373 | | & |
| Diabetes | Compensatory mutation(s) | 1 | 1 | 2.5 (0.1-43.4) | 3.7 (1.2-10.7)* |
| | No compensatory mutation | 12 | 30 | | |
| | | RIF-resistant | RIF-susceptible | Odds ratio (95% CI) | Crude & Mantel-Haenszel OR |
| No diabetes | Compensatory mutation(s) | 5 | 91 | 2.1 (0.6-7.4) | 2.5 (1.01-6.4)* |
| | No compensatory mutation | 5 | 191 | | & |
| Diabetes | Compensatory mutation(s) | 5 | 27 | 3.7 (0.9-14.6) | 2.7 (1.1-6.8)* |
| | No compensatory mutation | 4 | 79 | | |
| No diabetes | Compensatory mutation(s) | 29 | 195 | 2.4 (1.2-4.7)* | 2.5 (1.3-4.6)* |
| | No compensatory mutation | 13 | 208 | | & |
| Diabetes | Compensatory mutation(s) | 5 | 16 | 3.3 (0.6-19.2) | 2.5 (1.3-4.7)* |
| | No compensatory mutation | 2 | 21 | | |
| No diabetes | Compensatory mutation(s) | 34 | 286 | 2.6 (1.5-4.8)* | 2.8 (1.7-4.7)* |
| | No compensatory mutation | 18 | 399 | | & |
| Diabetes | Compensatory mutation(s) | 10 | 43 | 3.9 (1.3-11.3)* | 2.9 (1.7-4.8)* |
| | No compensatory mutation | 6 | 100 | | |

NOTE. Isoniazid and rifampicin resistance were defined as any mutations in genes known to confer resistance to isoniazid and rifampicin according to TB Profiler (19). For the top-half of the table, relating to isoniazid resistance, compensatory mutations concerned any mutations in *ubiA* and the *ahpC* promotor region; for the bottom-half of the table, relating to rifampicin resistance, compensatory mutations concerned any mutations in *rpoA*, *rpoB* outside the rifampicin resistance-determining region, and *rpoC*. * P-value < 0.05

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PART TWO

Tuberculosis disease phenotype
and transmission

5

Large-scale genomic analysis shows association between homoplastic genetic variation in *Mycobacterium tuberculosis* genes and meningeal or pulmonary tuberculosis

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Abstract

Background

Meningitis is the most severe manifestation of tuberculosis. It is largely unknown why some people develop pulmonary TB (PTB) and others TB meningitis (TBM); we examined if the genetic background of infecting *M. tuberculosis* strains may be relevant.

Methods

We whole-genome sequenced *M. tuberculosis* strains isolated from 322 HIV-negative tuberculosis patients from Indonesia and compared isolates from patients with TBM (n = 106) and PTB (n = 216). Using a phylogeny-adjusted genome-wide association method to count homoplasmy events we examined phenotype-related changes at specific loci or genes in parallel branches of the phylogenetic tree. Enrichment scores for the TB phenotype were calculated on single nucleotide polymorphism (SNP), gene, and pathway level. Genetic associations were validated in an independent set of isolates.

Results

Strains belonged to the East-Asian lineage (36.0%), Euro-American lineage (61.5%), and Indo-Oceanic lineage (2.5%). We found no association between lineage and phenotype (Chi-square = 4.556; p = 0.207). Large genomic differences were observed between isolates; the minimum pairwise genetic distance varied from 17 to 689 SNPs. Using the phylogenetic tree, based on 28,544 common variable positions, we selected 54 TBM and 54 PTB isolates in terminal branch sets with distinct phenotypes. Genetic variation in *Rvo218*, and absence of *Rv3343c*, and *nank* were significantly associated with disease phenotype in these terminal branch sets, and confirmed in the validation set of 214 unpaired isolates.

Conclusions

Using homoplasmy counting we identified genetic variation in three separate genes to be associated with the TB phenotype, including one (*Rvo218*) which encodes a secreted protein that could play a role in host-pathogen interaction by altering pathogen recognition or acting as virulence effector.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a major global health problem¹. Active TB mostly affects the lungs but may also spread to other organs. TB meningitis (TBM), which represents approximately 1–5% of all TB cases, is the most severe manifestation of TB, resulting in death or neurological disability in about half of those affected^{2,3}. It is largely unknown why certain people develop pulmonary TB (PTB) and others TBM. Host immune-related factors clearly play an important role, as shown by the increased risk of TBM for patients with advanced HIV infection, and the overrepresentation of young children among TBM patients. Host genetic factors may also play a role; single studies have linked susceptibility to TBM with variation in candidate genes^{4–8}.

Besides the host, genetic diversity of infecting *M. tuberculosis* strains may also affect disease phenotype. Even though *M. tuberculosis* is considered a clonal organism, there is considerable genetic variation in the genomes of infecting *M. tuberculosis* isolates^{9,10}. Epidemiological studies have reported significant differences among *M. tuberculosis* lineages in terms of virulence^{11,12}, transmission^{9,13,14}, progression to active disease after infection¹⁵, and response to treatment^{16,17}. *In vitro* studies have supported these findings by showing *M. tuberculosis* genotype-specific differences in the human immune response^{18–21}.

Animal studies have shown that *M. tuberculosis* strains differ in their ability to invade the central nervous system (CNS). Five *M. tuberculosis* genes (*Rvo311* (unknown function), *Rvo805* (intermediary metabolism and respiration), *pknD* (protein kinase D), *Rvo986* (cell wall and cell processes), and MT3280 (unknown function)) have been associated with invasion or survival in the CNS but not in lung tissues in mice²². Especially *M. tuberculosis pknD* was associated with invasion of brain, but not lung epithelia in guinea pigs²³, as was confirmed by another study showing that *pknD* vaccination offered significant protection against bacterial dissemination to the brain in guinea pigs²⁴. Similarly, in mice, clinical isolates from TBM patients disseminated extensively to cause meningitis, whereas *M. tuberculosis* H37Rv and clinical isolates from PTB patients did not²⁵. In rabbits, production of phenolic glycolipid has been linked with the increased propensity of East-Asian/Beijing strains to cause TBM²⁶. Finally, four *M. tuberculosis* genes were crucial for invading an artificial blood brain barrier in an *in vitro* model using primary human brain microvascular endothelial cells: *PE-PGRS18* (unknown function), *Rvo987* (cell wall and cell processes), *grcC2* (intermediary metabolism and respiration), and *PPE29* (unknown function)²⁷.

Much less is known about the role of *M. tuberculosis* genotype in TBM in humans. Most studies have examined associations of *M. tuberculosis* lineage with disease phenotype. Compared to other lineages, strains belonging to the East-Asian lineage were associated with extrapulmonary tuberculosis in one study²⁸, but not in another²⁹, while other studies found no association of *M. tuberculosis* lineage and disease localisation^{30,31}. Specifically looking at TBM, one study from Vietnam found the Euro-American lineage to be associated with PTB rather than TBM³². Only one study used whole genome sequencing to compare strains from TBM and PTB patients; large-scale and smaller genomic rearrangements, inversions, indels and single nucleotide polymorphisms (SNPs) in eight cerebrospinal fluid (CSF)-derived strains were not found in 69 comparison respiratory strains isolated from independent sputum samples³³. In the current study, we used a much larger set of isolates and a novel approach to examine the effect of the *M. tuberculosis* genotype on the susceptibility to TBM. We compared *M. tuberculosis* genomes isolated from 216 PTB patients and 106 TBM patients from Indonesia, all HIV-negative, to detect homoplastic genetic variants associated with either PTB or TBM.

Results

Lineage distribution and phylogeny construction

M. tuberculosis isolates from established patient cohorts in Bandung, Indonesia were selected for whole genome sequencing. All available *M. tuberculosis* strains isolated from HIV-negative TBM patients and randomly selected strains from twice as many PTB patients from the same setting were included, one strain was selected per patient. Compared to the 216 PTB patients, the 106 TBM patients were from a similar ethnic background, but slightly younger, more often male, and more often previously treated for TB (Table S5.1). Based on a 62-SNP barcode³⁴ 61.5% of the strains belonged to the Euro-American lineage (63.4% for PTB; 57.5% for TBM), 36% to the East-Asian lineage (33.3% for PTB; 41.5% for TBM), and 2.5% to the Indo-Oceanic lineage (3.2% for PTB; 0.9% for TBM). The lineage distribution did not differ significantly for strains isolated from TBM compared to PTB patients (Chi-square = 3.230; $p = 0.199$).

A phylogenetic tree was constructed based on 28,544 variable common nucleotide positions among the 322 *M. tuberculosis* isolates. The phylogeny showed that the TBM phenotype was not restricted to a certain *M. tuberculosis* lineage; instead it arose many times independently (Figure 5.1). In addition, the tree showed a high degree of strain heterogeneity. On average, two strains differed by about 1,000 SNPs, and this pairwise distance did not differ among PTB and TBM strains (data not shown), indicating that there was equal genetic diversity within PTB and within TBM strains. In

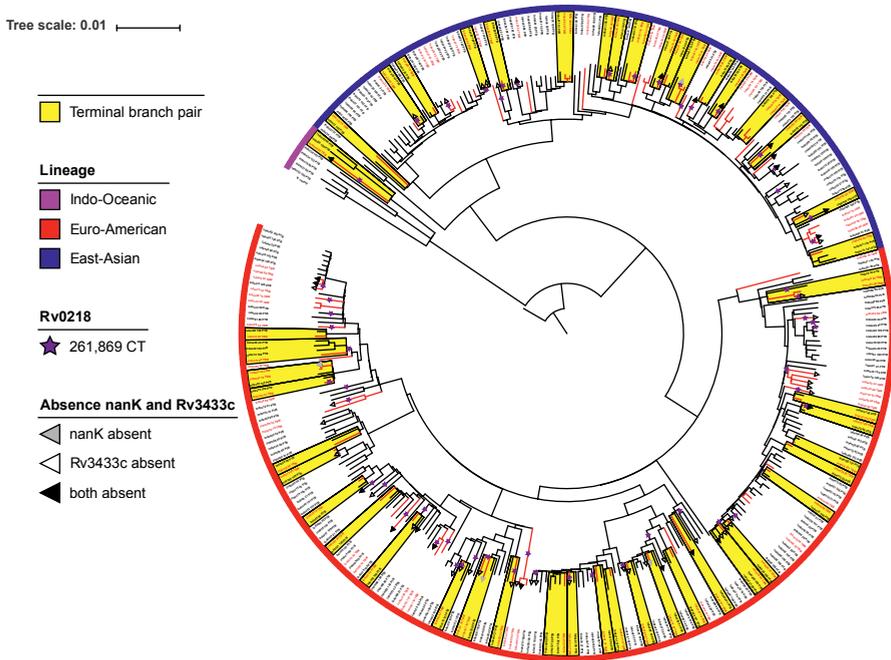


Figure 5.1. Phylogenetic tree of 322 *M. tuberculosis* strains isolated from TBM (red braches and leaves) and PTB (black branches and leaves) patients. The highlighted branches indicate the 108 strains in 47 terminal branch sets, together comprising the discovery set. The purple stars indicate the origin of the SNP in *Rv0218* according to the ancestral reconstruction. The grey and black triangles indicate the isolates in which *nanK* and/or *Rv3433c* are absent.

addition, the minimum pairwise genetic distance varied from 17 to 689 SNPs (data not shown), indicating that there was no clustering of strains (≤ 12 SNPs distance³⁵). For TBM strains the minimum genetic distance ranged from 17 to 1,785, and for PTB strains from 31 to 803 SNPs (data not shown).

TB phenotype-associated genetic variations

Genome-wide association approaches for bacteria can broadly be categorised into allele counting and homoplasmy counting methods³⁶. Allele counting methods are based on the overrepresentation of an allele at the same site in cases relative to controls, introducing a risk of false-positive findings due to population stratification. Homoplasmy counting on the other hand, counts repeated and independently emerging mutations that occur more often in branches of cases relative to controls. In the current study, we used a two-step approach: in the discovery phase, we used homoplasmy

counting by identifying terminal branch sets (TBSs) to maximize power to identify true associations with the TB disease phenotype, uncorrected for multiple tests. In the validation phase we examined associations identified in the discovery phase using allele counting with correction for multiple testing and for phylogenetic bias to distinguish true associations from false positives, and performed ancestral reconstruction to remove possible phylogenetic bias. To divide the genomes in a discovery and a validation set, we identified isolates in terminal branch pairs, trios and quartets (i.e. separated at a terminal or near terminal branch in the phylogenetic tree) with distinct phenotypes (Figure 5.2). Genetic differences between isolates within a TBS provide the strongest, homoplasy-corrected possible association with the phenotype.

The phylogenetic tree revealed a total of 47 TBSs containing 108 paired strains: 54 TBM and 54 PTB strains that make up the discovery set. The merged SNP lists consisted of 6,488 variable positions with the corresponding nucleotides for the 108 strains (Additional file 2). Using the homoplasy-based association analysis, we found individual nucleotide positions, genes, or pathways where TB disease phenotype-associated mutations repeatedly occur in different branches of the phylogenetic tree. These included 9 SNPs, 5 genes, and 1 pathway (Table 5.1).

We used the remaining 214 (52 TBM and 162 PTB) isolates not belonging to any of the TBSs (validation set) to verify these results. The discovery set showed a total of 6,488 different non-synonymous SNPs involving 6,483 dimorphic sites and 5 trimorphic sites across 2,778 genes; the validation set a total of 12,211 different nonsynonymous SNPs involving 12,185 dimorphic sites and 26 trimorphic sites across 3,359 genes (Additional file 3). There was an overlap of 2,694 non-synonymous SNPs and 1,564 affected genes. Out of 9 SNPs significantly associated with either TBM or PTB in the discovery set, one was confirmed in the validation set; the mutation in *Rvo218*. Similarly, out of 5 genes harbouring genetic variation associated with the TB phenotype, *Rvo218* was validated in the validation set (Table 5.1, Figure S5.1 and Figure S5.2). The pathway (ethylbenzene degradation) identified in the discovery set was not confirmed in the validation set.

To correct for potential phylogenetic bias in the validation set, we reconstructed the ancestral state for the SNP in *Rvo218* and compared the ratio of TBM vs. PTB isolates after the occurrence of this particular SNP with the ratio of TBM vs. PTB prior to the occurrence of this SNP in the validation set (Figure 5.1 and Figure S5.3). Three branches with back mutations (2 TBM, 1 PTB branch) were excluded from the analysis. Among the 33 nodes / leaves where the SNP occurred, the average unweighted proportion of TBM isolates among the child branches was 44.7%; among 166 isolates in the validation set not harbouring this SNP, 29 (17.5%) were from TBM patients (Table S5.2). The Z-score for the difference between proportions was -3.83 ($p < 0.001$).

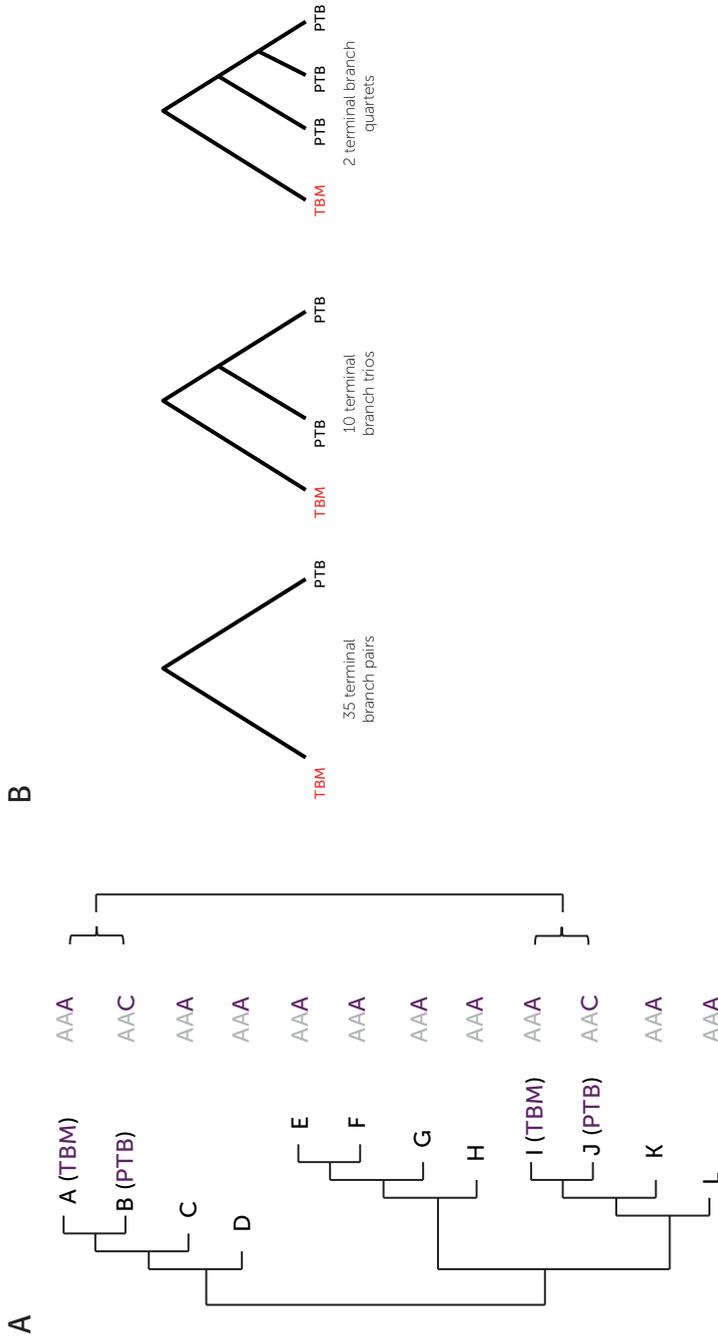


Figure 5.2. Homoplasly-based association analysis; detection of mutations occurring along disparate locations in the phylogenetic tree. Left part (A): schematic example of a tree containing two terminal branch sets (TBSs) with a homoplastic SNP. Right part (B): schematic overview of the composition of the 47 terminal branch sets.

Table 5.1. Significant SNPs, genes, and pathways identified by homoplasmy counting.

| | | Discovery dataset (N=108) | | | Validation dataset (N=214) | | |
|------------------------------------|-------------------|---------------------------|------------|---------|----------------------------|-------------|----------------------|
| SNP-level | | Strains with SNP (N) | | | Strains with SNP (N) | | |
| Gene (Rv-number) | Nucleotide change | TBM (N=54) | PTB (N=54) | P-value | TBM (N=52) | PTB (N=162) | P-value [^] |
| Unnamed (<i>Rv0218</i>) | 261,869CT | 25 | 10 | 0.002 | 21 | 24 | 0.001 |
| <i>PPE54</i> (<i>Rv3343c</i>) | 3,736,628TG | 22 | 35 | 0.008 | 27 | 87 | 0.472 |
| <i>PEPGRS19</i> (<i>Rv1067c</i>) | 1,190,093AC | 2 | 11 | 0.01 | 5 | 18 | 0.487 |
| <i>PEPGRS44</i> (<i>Rv2591</i>) | 2,922,848AT | 0 | 6 | 0.01 | 2 | 7 | 0.625 |
| <i>PPE3</i> (<i>Rv0280</i>) | 340,372TC | 2 | 10 | 0.025 | 4 | 13 | 0.589 |
| <i>PEPGRS9</i> (<i>Rv0746</i>) | 836,272AG | 7 | 1 | 0.029 | 3 | 9 | 0.603 |
| <i>PEPGRS26</i> (<i>Rv1441c</i>) | 16,18,978TC | 0 | 5 | 0.032 | 2 | 3 | 0.369 |
| Unnamed (<i>Rv0064</i>) | 713,36GC | 24 | 34 | 0.034 | 29 | 98 | 0.355 |
| <i>PEPGRS18</i> (<i>Rv0980c</i>) | 1,095,644CT | 0 | 4 | 0.044 | 2 | 2 | 0.238 |
| Gene-level | | SNPs in gene (N) | | | SNPs in gene (N) | | |
| Gene (Rv number) | | TBM | PTB | P-value | TBM | PTB | P-value |
| <i>PEPGRS19</i> (<i>Rv1067c</i>) | | 2 | 13 | 0.004 | 5 | 22 | 0.318 |
| Unnamed (<i>Rv0218</i>) | | 27 | 11 | 0.007 | 21 | 32 | 0.001 |
| <i>PEPGRS26</i> (<i>Rv1441c</i>) | | 0 | 5 | 0.031 | 2 | 4 | 0.491 |
| <i>glmS</i> (<i>Rv3436c</i>) | | 0 | 5 | 0.031 | 0 | 0 | n.a. |
| Unnamed (<i>Rv3740c</i>) | | 0 | 4 | 0.05 | 3 | 0 | 0.011 |
| Pathway-level | | Genes with mutation (N) | | | Genes with mutation (N) | | |
| Pathway name | | TBM | PTB | P-value | TBM | PTB | P-value |
| Ethylbenzene degradation | | 68 | 54 | 0.032 | 65 | 224 | 0.071 |

NOTE. P-values are based on permutation analysis; bold p-values indicate validated, Bonferroni-corrected significant enrichment [^] P-value thresholds in the validation set were Bonferroni-corrected for multiple testing by dividing them by the number of top hits in the discovery set: SNP-level: $p < 0.05/9$; gene-level: $p < 0.05/5$; pathway-level: $p < 0.05/1$

De novo genome assembly

Next, we used a reference-free *de novo* genome assembly approach to examine associations between presence/absence of genes and the TB phenotype, and to study sequences not present in the reference genome. The list of annotated coding sequences for the 108 assembled genomes in the discovery set contained 3,032 distinct genes that were present in at least one, but not all of the strains (Additional file 8). The permutation analysis revealed six genes that were significantly associated with the TB phenotype. Two of these genes, *Rv3433c* and *nank*, were validated in the

Table 5.2. Significant genes identified by the *de novo* genome assembly analysis

| Coding sequence | Discovery dataset (n=108) | | | Validation dataset (n=214) | | |
|---|------------------------------|---------------|---------|------------------------------|----------------|----------------------|
| | Strains with CDS present (N) | | | Strains with CDS present (N) | | |
| | TBM (n=54) | PTB (n=54) | P-value | TBM (n=52) | PTB (n=162) | P-value [^] |
| Bifunctional NAD(P)H-hydrate repair enzyme Nnr (<i>Rv3433c</i>) | 36 | 47 | 0.011 | 34 | 144 | 0.002 |
| TMAO/DMSO reductase | 46 | 53 | 0.016 | 49 | 152 | 0.592 |
| Antitoxin/MT2731 | 33 | 44 | 0.017 | 37 | 130 | 0.206 |
| NPCBM-associated, NEW ₃ domain of alpha-galactosidase | 15 | 6 | 0.022 | 14 | 19 | 0.063 |
| Oxidoreductase molybdopterin binding domain protein | 11 | 3 | 0.028 | 7 | 13 | 0.197 |
| N-acetylmannosamine kinase (<i>nanK</i>) | 44 | 51 | 0.036 | 44 | 157 | 0.001 |

NOTE. P-values are based on permutation analysis; bold p-values indicate validated significant enrichment
[^] P-value thresholds in the validation set were Bonferroni-corrected for multiple testing by dividing them by the number of top hits in the discovery set: $p < 0.05/6$

validation set: absence of these genes was associated with TBM (Figure 5.1, Table 5.2, Additional file 9). Together with *Rv0218* they bring the total number of genes associated with the TB phenotype to three.

Effect of detected SNPs on protein function and predicted function of phenotype-associated genes

We used published algorithms to predict the effects of identified mutations on protein structure and function. The SNP in *Rv0218*, a protein predicted to have transmembrane helices, likely leads to a decrease of stability of the protein (Table S5.3). For *Rv3433c*, and *nanK* no transmembrane helices or signaling peptides were predicted.

Discussion

To determine whether *M. tuberculosis* genetic variation is associated with the TB disease phenotype, we compared *M. tuberculosis* whole genome sequences from 216 PTB and 106 TBM patients and searched for homoplastic mutations. We identified three genes in *M. tuberculosis* (*Rvo218*, *Rv3433c*, and *nank*) to be associated with either TBM or PTB. Previous experimental studies have assessed the importance of *Rvo218*. This secretome gene encodes for a protein with multiple predicted transmembrane regions and a C-terminal molybdopterin binding domain that is often found in oxidoreductases and was shown to be essential for *M. tuberculosis in vivo* growth in C57BL/6 J mouse spleen³⁷. The SNP in *Rvo218* is predicted to decrease the stability of the respective protein. Secretome genes potentially influence pathogen recognition and host-pathogen interaction³⁸. If mutations in these genes alter the appearance of the *M. tuberculosis* surface, this could provide a mechanism by which *M. tuberculosis* could evade the immune response and enable dissemination to extrapulmonary sites. Secretome genes are more likely to contain false-positive associations as they are under selective pressure from the immune system and phages³⁹. How *Rv3433c* and *nank* could be related to the TB phenotype is not obvious, although functions have been predicted based on homology detection.

To our knowledge, this is only the second attempt to relate *M. tuberculosis* genetic variation to the TB disease phenotype in humans on a genome-wide scale. The other study, by Saw *et al.* showed large-scale rearrangements, short translocations, inversions, indels and SNPs in eight strains cultured from CSF³³. Non-synonymous SNPs in eight genes (*embR*, *lppD*, *PE-PGRS10*, *PE-PGRS19*, *PE-PGRS21*, *PE-PGRS49*, *PPE58*, and *Rvo278c*) were found in at least four of the eight CSF-derived strains, and in none of 69 strains isolated from sputum³³. We did not confirm this in our set of isolates, although *PE-PGRS19* was associated with the TB phenotype in the discovery set. Moreover, we used a two-step approach, based on homoplasmy counting as well as allele counting with a correction for phylogenetic bias to find mutations associated with the TB phenotype, and we performed ancestral reconstruction for the most discriminative SNP. Unlike a previous study from Vietnam³², we found no association between *M. tuberculosis* lineage and TBM. This is no surprise given the genetic diversity even within *M. tuberculosis* lineages⁹, and the observed pattern of TBM isolates scattered across the phylogenetic tree.

In concordance with previous findings⁹, we found considerable genetic diversity in *M. tuberculosis* in the current study. Two isolates differed on average by 1,000 SNPs, and this did not differ among PTB isolates and among TBM isolates. In addition, we did not observe any clustering, defined as two isolates differing by 12 SNPs or less³⁵. The lack

of clustering is probably a result of the low sampling fraction in this urban setting with thousands of incident TB cases each year.

Theoretically, two scenarios could explain the role of *M. tuberculosis* genetic variation in the development of TBM after infection with *M. tuberculosis*. First, upon infection the *M. tuberculosis* strain may carry certain mutations associated with dissemination and penetration of the blood-brain barrier. Second, a subpopulation of bacteria in the lungs of a PTB patient may develop such mutations, though it was recently shown for bacterial meningitis caused by *S. pneumonia* or *N. meningitides* that there is no evidence for differential selection between blood and CSF, and that any mutations between these two niches are likely due to mutation hotspots or forms of diversifying selection common to both niches⁴⁰. However, similar to the findings of Saw *et al.*³³, the genetic variants that we found to be associated with the TB disease phenotype were not exclusive for TBM or PTB, nor were they consistently present in all TBM or PTB strains. Therefore it seems that genetic variants may be part of a complex, multifactorial process leading to this devastating manifestation of TB, in which the human genotype or phenotype equally plays an important role^{32,41}.

This is the second, and by far largest study using whole genome sequencing to link *M. tuberculosis* genotype to TBM. In this large cohort of well-characterised patients we studied strains from HIV-negative, adult patients to control for the two most important known risk factors for TBM. In addition, both patient groups were similar with regard to gender, ethnicity, and previous episodes of TB. The *de novo* assembly adds to the strengths of this study because it enabled us to examine regions of the genome that do not map to the reference genome, allowing the investigation of associations between genetic variation in these genomic regions and the TB disease phenotype. The homoplasy-based association analysis has proven to be a successful method to detect *M. tuberculosis* loci associated with a certain phenotype (e.g. transmissible vs. non-transmissible, drug-resistant vs. sensitive)^{42,43}. The major advantage is that false-positive associations due to genetic relatedness of strains with the same phenotype (i.e. 'phylogenetic bias') are filtered out, thereby increasing statistical power to find true associations. In addition, the ancestral reconstruction in the validation step ruled out the possibility that the significant association for the SNP in *Rv0218* was due to population structure.

The current study has several limitations. Firstly, we only focused on mutations in coding regions of the genome, as they are more likely to have functional consequences, but mutations in non-coding regions could also affect function, for instance by transcriptional and translational regulation of protein-coding sequences⁴⁴. Secondly, the large number of genetic variants increases the risk of finding false-positive

associations, although homoplasmy counting enabled us to filter out many of these false-positives. We did not correct for multiple testing in the discovery set, but we used a validation set where we did correct for multiple testing for confirmation. Lastly, whether bacteria developed TBM-associated mutations before or after infecting a patient remains unclear. One way to investigate this is to compare the genomes of strains isolated from sputum and CSF from the same patient. Unfortunately we did not have the availability of paired isolates. Most TBM patients were too ill to expectorate sputum.

Conclusions

We present evidence from a homoplasmy-based association analysis that three *M. tuberculosis* genes, including *Rvo218*, a cell wall-associated and/or secretome gene, are associated with the TB disease phenotype. These findings serve as an important step forward in the quest for an improved understanding of the mycobacterial determinants of TB tissue tropism. Functional validation studies are warranted to further explore the effect of mutations in these genes on protein function.

Methods

Patients and isolates

We used *M. tuberculosis* isolates from two established cohorts of Indonesian patients with confirmed TB. The first group consisted of adult patients (≥ 15 years old) with TBM admitted at Hasan Sadikin Hospital between 2006 and 2013, with *M. tuberculosis* cultured from CSF. The second group was randomly selected from a cohort of culture-positive HIV-negative PTB patients (age ≥ 15 years) from the same setting recruited between 2012 and 2015. All patients were tested for HIV, and those who were HIV-positive were excluded.

Sequencing, alignment, and variant calling

Mycobacterial DNA was extracted from cultures using cetyl trimethylammonium bromide (CTAB) or using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories). A single isolate from each patient was selected for sequencing. *M. tuberculosis* DNA was sequenced on an Illumina HiSeq 2000 instrument using 2 x 100 bp paired-end reads at the Beijing Genome Institute in Hong Kong. After sequencing, the raw FASTQ sequence reads were filtered, including removing of adapter sequences, contamination, and low quality reads which have more than 10% N base calls, or where more than 40% of the bases have a quality score ≤ 4 . Quality control statistics are shown in Table S5.4. Five TBM strains and four PTB strains were contaminated, based on a low GC-content, and were excluded from further analyses. Sequencing coverage was

determined using the FASTQC quality control tool version 0.10.1. The proportion of bases sequenced with a sequencing error rate of 1% or less per base ranged from 93% to 97% per genome. The average coverage depth for the remaining 322 sequenced strains was 121.1, and the average percentage of bases covered by at least one read was 98.9%.

The sequence reads were aligned to reference strain *M. tuberculosis* H37Rv, accession number NC_000962.3, and variants were called using Breseq software, version 0.27.145 using a minimum threshold of 30x coverage. Mutations with low-quality evidence (i.e. possible mixed read alignment) were not included. The Breseq variant call output was converted to a tab-separated file for each sequence using customized Python and R scripts that are available upon request.

Phylogeny construction

A phylogeny was constructed to determine evolutionary relationships of the isolates. We extracted all 29,199 variable positions across the 322 *M. tuberculosis* sequences and concatenated them into a single alignment. Solely for the purpose of creating the phylogenetic tree, SNPs occurring in PE/PPE genes and genes related to mobile elements (genes listed in Table S5.5) were excluded to avoid any concern about inaccuracies in the read alignment in these parts of the genome. In addition, SNPs in an additional 40 genes previously associated with drug resistance⁴⁶ were removed to exclude the possibility that homoplasmy of drug resistance mutations would significantly affect the phylogeny⁴⁷. After applying these filters to the initial set of 29,199 SNPs, the 28,544 remaining SNPs were used to construct the phylogenetic tree using PhyML, version 3.048 using the HKY85 model with four categories for the gamma distribution, and using a hundred bootstraps.

To determine the lineage distribution of the strains and to evaluate whether an association exists between *M. tuberculosis* lineage and TB disease phenotype, we determined the lineage for each of the 322 strains using a 62-SNP barcode³⁴. The resulting classification in the main *M. tuberculosis* lineages also served as a quality check for the generated Maximum Likelihood (ML)-phylogenetic tree, as it enabled us to validate that isolates belonging to the same lineage clustered together in the tree. A Chi-square test was used to statistically test the association between *M. tuberculosis* lineage and TB disease phenotype.

Homoplasmy-based association test to identify associations between *M. tuberculosis* genotype and TB disease phenotype

We used a two-step approach: in the discovery step we aimed to maximize power by homoplasmy counting, without correction for multiple testing. In the subsequent validation step, aimed to distinguish true associations from false positives, we used allele counting with multiple testing correction, and performed ancestral reconstruction to remove possible phylogenetic bias.

To divide the genomes in a discovery and a validation set, we identified isolates in terminal branch pairs, trios and quartets (i.e. separated at a terminal or near terminal branch in the phylogenetic tree) with distinct phenotypes (Figure 5.2). These terminal branch sets (TBSs) together formed the discovery set. These provide the strongest, homoplasmy-corrected possible association with the phenotype. We used the remaining genomes to validate the association. For all isolates in the TBSs, we listed the non-synonymous SNPs to create a table with all variable positions in rows, the paired isolates in columns, and the corresponding nucleotide in the cells (Additional file 2). For every SNP, an enrichment score was calculated using the following formula:

$$\log \left(\frac{(\text{number of TBM isolates with SNP}/\text{total number of TBM isolates}) + 0.001}{(\text{number of PTB isolates with SNP}/\text{total number of PTB isolates}) + 0.001} \right)$$

A permutation p-value for each SNP was calculated by randomising the phenotypes over the isolates 1,000 times.

In parallel we grouped SNPs per gene, using the same empirical randomisation strategy to assess association, adjusted for gene length:

$$\log \left(\frac{((\text{number of SNPs in gene in TBM isolates}/\text{gene length})/\text{total number of TBM isolates}) + 0.001}{((\text{number of SNPs in gene in PTB isolates}/\text{gene length})/\text{total number of PTB isolates}) + 0.001} \right)$$

Similarly, we grouped genes with ≥ 1 SNP per *M. tuberculosis* pathway according to PATRIC⁴⁹, and calculated association using the aforementioned permutation analysis, adjusted for the number of genes in a particular pathway:

$$\log \left(\frac{((\text{no.of pathway genes with } \geq 1 \text{ SNP in TBM isolates}/\text{unique gene count})/\text{total no.of TBM isolates}) + 0.001}{((\text{no.of pathway genes with } \geq 1 \text{ SNP in PTB isolates}/\text{unique gene count})/\text{total no.of PTB isolates}) + 0.001} \right)$$

Significance of associations was determined by calculating a permutation p-value through randomization of the phenotypes over the isolates 1,000 times. All calculations were performed with customized Perl scripts that are available upon request.

We used the set of 214 strains that were not in TBSs to validate candidate SNPs, genes, and pathways identified in the discovery set, using the same permutation test as described above for the discovery set. We used a p-value threshold of 0.05 for the discovery set. The p-value thresholds in the validation set were Bonferroni-corrected for multiple testing by dividing them by the number of significant (candidate) hits in the discovery set. To correct for potential phylogenetic bias in the validation set, we performed ancestral reconstruction for validated TB phenotype-associated SNPs using FASTML⁵⁰ with default parameters, and compared the proportion of TBM vs. PTB isolates prior to (i.e. older than) and after (i.e. younger than) the occurrence of the SNP in the validation set. For each node / leave where the SNP occurred, we calculated the proportion of TBM isolates among the child branches and we calculated the (unweighted) average over all of these nodes and leaves to determine the proportion of TBM isolates after the SNP (Table S5.2). This way, every independent occurrence of the SNP contributes equally to the analysis, regardless of the number of child branches after the SNP, thus correcting for phylogenetic bias. The significance of the difference in proportion was determined by calculating the Z-score for 2 population proportions with accompanying p-value.

PE/PPE genes, a major challenge in the analysis of *M. tuberculosis* whole genome sequences due to the repetitive nature of these sequences, were included in the analysis. TB phenotype-associated SNPs in PE/PPE genes were manually examined to confirm that they did not fall within a repetitive region (for an example please see Figure S5.4).

De novo genome assembly

Sequence reads were *de novo* assembled using SPAdes, version 3.6.1⁵¹ with default parameters. All assemblies were evaluated, focussing on genome size, N50 length, number of contigs and scaffolds, and GC-content. The assembled genomes were annotated using Prokka, version 1.11⁵² with default parameters. Completeness and contamination of assemblies were determined with CheckM version 1.0.5⁵³. The assembly statistics are shown in Additional file 14. Presence or absence of annotated genes was determined for the 108 assembled genomes in the discovery set. An enrichment score per gene was calculated based on the frequency of occurrence in TBM vs. PTB strains using the following formula:

$$\log \left(\frac{(\text{number of TBM isolates with gene present} / \text{total number of TBM isolates}) + 0.001}{(\text{number of PTB isolates with gene present} / \text{total number of PTB isolates}) + 0.001} \right)$$

Statistical significance was again determined based on permutation by randomizing the phenotypes over the isolates 1,000 times. We repeated this permutation analysis

for the 214 genomes comprising the validation set, using Bonferroni-adjusted p-value thresholds. For the genes with a validated, significant enrichment for TBM or PTB, we confirmed their absence in the respective genomes by mapping the raw sequencing reads for these genomes back to the H37Rv reference sequence of the gene (Figure S5.5), and visualized this with integrative genomics viewer (IGV), version 2.3.32⁵⁴.

Prediction of mutation effects

We used two algorithms to predict the effect of the mutations on protein structure and function. I-Mutant version 2.0, which predicts the protein stability change upon single site mutation (<http://folding.biofold.org/imutant/i-mutant2.o.html>)⁵⁵ and PolyPhen-2, which predicts the possible impact of an amino acid substitution on the structure and function of a protein (<http://genetics.bwh.harvard.edu/pph2/>)⁵⁶ to predict the impact of the validated SNPs on protein structure and function. In addition, we used TargetP (<http://www.cbs.dtu.dk/services/TargetP/>)⁵⁷ to predict the subcellular location of the proteins encoded by the validated genes, and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>)⁵⁸ to predict transmembrane helices in these proteins.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Data generated or analysed during this study are included in this published article [and its supplementary information files],

or are available from the corresponding author on reasonable request. The raw sequence files (FASTQ) were archived on the NCBI Sequence Read Archive and are available at: <https://www.ncbi.nlm.nih.gov/sra/SRP130118>. The individual isolates can be accessed under the following Biosample accession numbers: SAMNo8376067-SAMNo8376388. The Bioproject accession number is: PRJNA430531. Phylogeny data have been uploaded to TreeBASE (<http://purl.org/phylo/treebase/phylovs/study/TB2:S22081>).

Authors' contributions

CR was responsible for conceptualization, data analysis, funding acquisition, and writing. LC was responsible for laboratory management, project administration, resources, and writing. AvL was responsible for conceptualization and writing. SD was responsible for inclusion of patients in the study and writing. ARG was responsible for patient management and writing. HNG was responsible for conceptualization and methodological guidance. MAH was responsible for conceptualization, methodology, resources, supervision, and writing. BA was responsible for funding acquisition, project administration, resources, and supervision. BED was responsible for conceptualization, data analysis, methodology, supervision, and writing. RvC was responsible for conceptualization, funding acquisition, project administration, supervision, and writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All adult patients provided written informed consent; from the age of 15, patients are no longer seen by a paediatrician⁵⁹ and parents provided informed consent for patients under 18. The consent procedure was approved by the local Institutional Review Board. The study protocols for the inclusions of patients and for bioanalysis were approved by the ethical committee of the Faculty of Medicine, Universitas Padjadjaran / Hasan Sadikin Hospital, Bandung, Indonesia under ethical registration number 0716040326.

Competing interests

The authors declare that they have no competing interests.

Supplementary figures

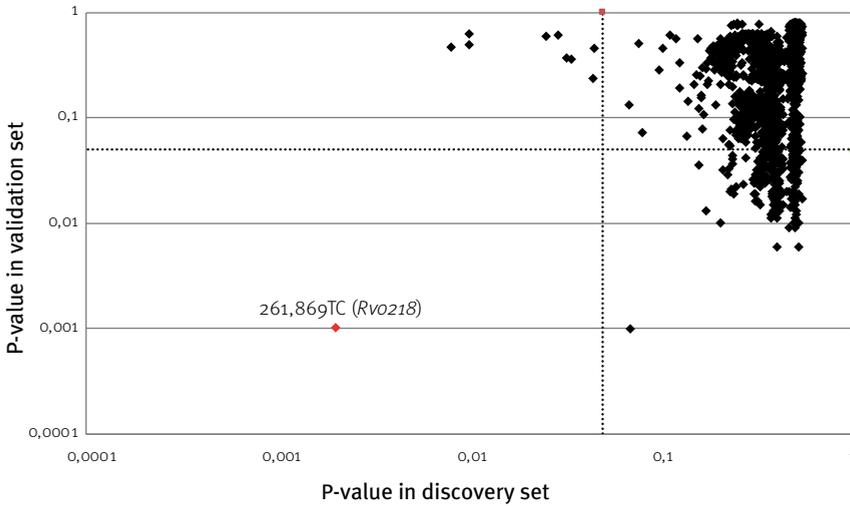


Figure S5.1. Scatterplot showing the p-values of the SNPs found in the discovery and the validation set. P-values in the discovery set are shown on the x-axis; p-values in the validation set are shown on the y-axis. Gene names are shown for SNPs significant in both the discovery and validation set.

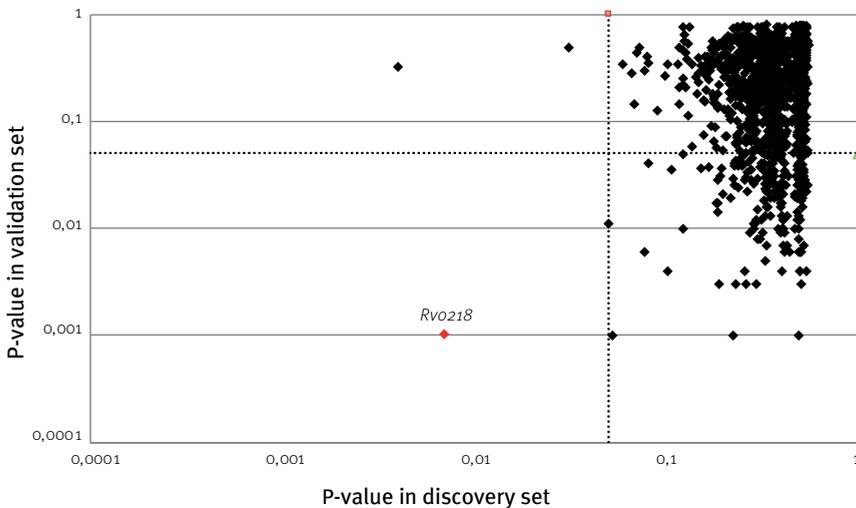


Figure S5.2. Scatterplot showing the p-values of the genes found in the discovery and the validation set. P-values in the discovery set are shown on the x-axis; p-values in the validation set are shown on the y-axis. Names are shown for genes significant in both the discovery and validation set.

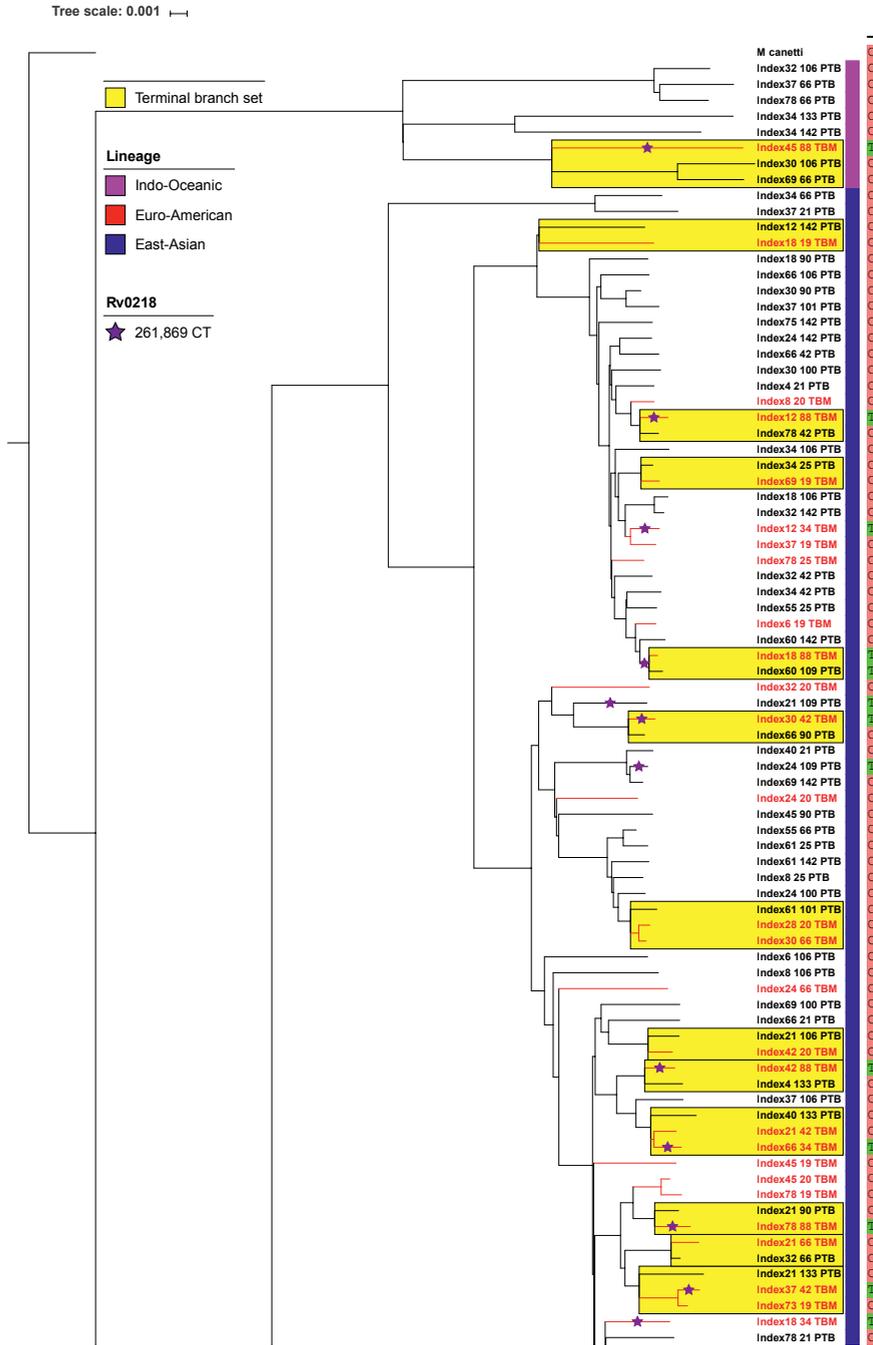


Figure S5.3.

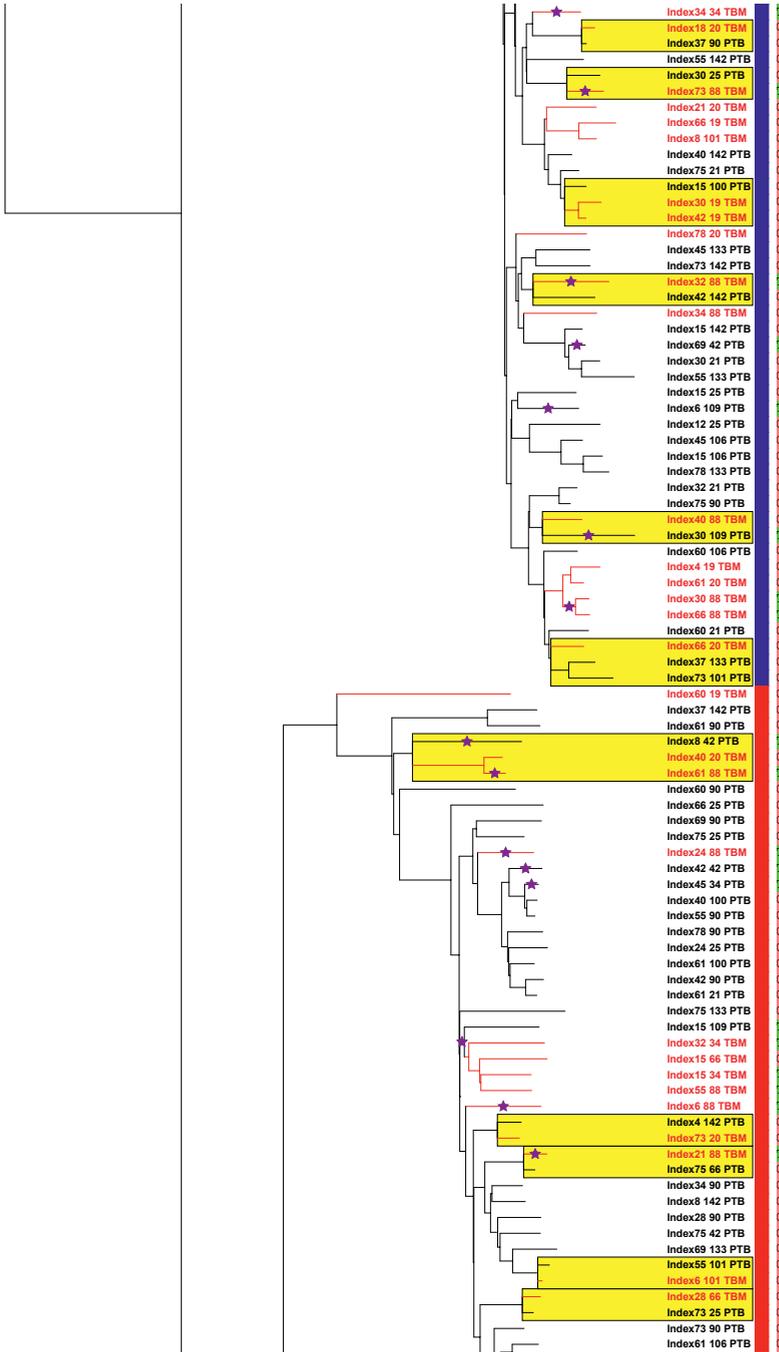


Figure S5.3. Continued.

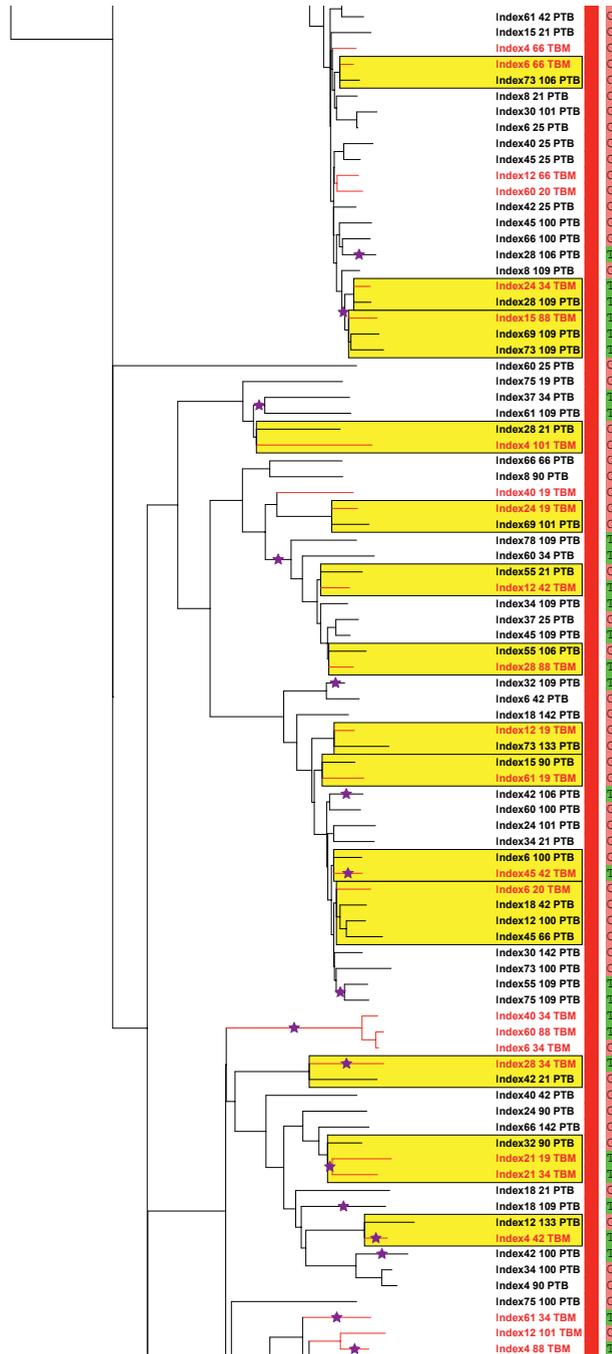


Figure S5.3. Continued.

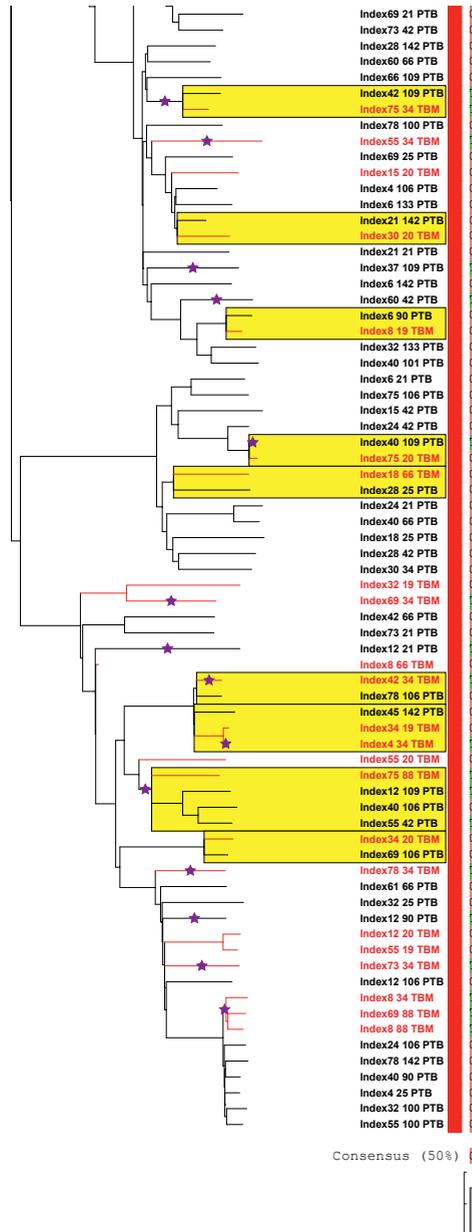


Figure S5.3. Phylogenetic tree of 322 *M. tuberculosis* strains isolated from TBM (red branches and leaves) and PTB (black branches and leaves) patients. The highlighted branches indicate the 108 strains in 47 terminal branch sets, together comprising the discovery set. The purple stars indicate the origin of the SNP in *Rvo218* according to the ancestral reconstruction. The nucleotide for SNP position 261,869 is indicated next to the leaf labels.



Figure S5.4. Diagram demonstrating breseq calling a SNP in the *PE_PGSR1* gene. Displayed are 60 Illumina sequencing reads mapping to the H37Rv reference genome (shown at the top and bottom). Visual inspection of the SNP confirms that it does not occur in a region containing uniformly lower base quality scores.



Figure S5.5. Read alignment demonstrating the absence of *nanK*. Displayed is the alignment of the raw sequencing reads against the H37Rv *nanK* gene. No reads are mapping to this gene, showing that it is absent in the sequenced genome.

Supplementary tables

Table S5.1. Description of baseline characteristics for PTB and TBM patients

| Characteristic | TBM patients (N=106) | PTB patients (N=216) |
|--------------------------------------|----------------------|----------------------|
| Male gender – N (%) | 58 (54.7) | 111 (51.4) |
| Age (yrs) – mean (SD) | 28.0 (8.9) | 39.1 (14.8) |
| Ethnicity – N (%) | | |
| Sundanese | 45 (93.7) | 187 (87.4) |
| Javanese | 3 (6.3) | 21 (9.8) |
| Sumatra | 0 (0) | 3 (1.4) |
| Other | 0 (0) | 3 (1.4) |
| Years after admission – median (IQR) | 6 (4-8) | 1 (1-2) |
| History of TB treatment - N (%) | 13 (13.1) | 37 (17.2) |

IQR, interquartile range; SD, standard deviation.

* Data were missing for history of TB treatment (TBM, n=7; PTB, n=1); ethnicity (TBM, n=58; PTB = 2).

Table S5.2. Ancestral reconstruction of SNP 261869TC in *Rvo218*

| Node/leave where SNP occurred | Number of child branches | Number of TBM branches among children | Percentage TBM branches among children |
|--|--------------------------|---------------------------------------|--|
| Index12_34_TBM | 1 | 1 | 100 |
| Index21_109_PTБ | 1 | 0 | 0 |
| Index24_109_PTБ | 1 | 0 | 0 |
| Index18_34_TBM | 1 | 1 | 100 |
| Index34_34_TBM | 1 | 1 | 100 |
| Index69_42_PTБ | 1 | 0 | 0 |
| Index6_109_PTБ | 1 | 0 | 0 |
| Index30_88_TBM Index66_88_TBM | 2 | 2 | 100 |
| Index24_88_TBM | 1 | 1 | 100 |
| Index42_42_PTБ | 1 | 0 | 0 |
| Index45_34_PTБ | 1 | 0 | 0 |
| Index15_109_PTБ Index32_34_TBM Index15_66_TBM | 4 | 3 | 75 |
| Index15_34_TBM Index55_88_TBM | | | |
| Index6_88_TBM | 1 | 1 | 100 |
| Index28_106_PTБ | 1 | 0 | 0 |
| Index37_34_PTБ Index61_109_PTБ | 2 | 0 | 0 |
| Index78_109_PTБ Index60_34_PTБ Index34_109_PTБ | 4 | 0 | 0 |
| Index37_25_PTБ Index45_109_PTБ | | | |
| Index32_109_PTБ | 1 | 0 | 0 |
| Index42_106_PTБ | 1 | 0 | 0 |
| Index55_109_PTБ Index75_109_PTБ | 2 | 0 | 0 |
| Index40_34_TBM Index60_88_TBM Index6_34_TBM | 2 | 2 | 100 |
| Index18_109_PTБ | 1 | 0 | 0 |
| Index42_100_PTБ | 1 | 0 | 0 |
| Index61_34_TBM | 1 | 1 | 100 |
| Index4_88_TBM | 1 | 1 | 100 |
| Index55_34_TBM | 1 | 1 | 100 |
| Index37_109_PTБ | 1 | 0 | 0 |
| Index60_42_PTБ | 1 | 0 | 0 |
| Index69_34_TBM | 1 | 1 | 100 |
| Index12_21_PTБ | 1 | 0 | 0 |
| Index78_34_TBM | 1 | 1 | 100 |
| Index12_90_PTБ | 1 | 0 | 0 |
| Index73_34_TBM | 1 | 1 | 100 |
| Index8_34_TBM Index69_88_TBM Index8_88_TBM | 3 | 3 | 100 |
| Sum | 45 | 21 | 1475 |
| Average | 1,36 | 0,64 | 44,70 |

Listed are the internal nodes and leaves where the SNP in *Rvo218* occurred according to the ancestral reconstruction of the SNP

Table S5.3. Protein prediction for genomic sites associated with the TB disease phenotype

| | Genomic position | Nucleotide change | Amino acid change | I-Mutant # | PolyPhen & | Predicted localization | Predicted number of transmembrane helices |
|------------|------------------|-------------------|-------------------|-----------------------|------------|------------------------|---|
| SNP-level | Rv0218 | | | | | | |
| | 261,869 | C=>T | R316C | Decrease of Stability | Benign | Other | 5 |
| Gene-level | Rv3433c | | | | | | |
| | nanK | | | | | Other | 0 |
| | | | | | | Other | 0 |

I-mutant predicts free energy changes of protein stability upon a point mutation under different conditions.
 & PolyPhen predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

^ TargetP predicts the subcellular location of proteins based on the predicted presence of any N-terminal signal peptides.

* TMHMM predicts transmembrane helices in proteins.

Table S5.4. Description of sequencing quality control parameters and statistics

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|-------|-------------|---------------|-----------------|---------------------|
| 100865 | 5393826 | 94,86 | 496846191 | 112,60 | 117 | 99,00 |
| 100866 | 2806938 | 95,62 | 258749297 | 58,70 | 61 | 99,10 |
| 100870 | 3469296 | 94,54 | 320142664 | 72,60 | 75 | 99,20 |
| 100874 | 8497168 | 95,62 | 783313115 | 177,60 | 184 | 99,70 |
| 100876 | 6903240 | 95,75 | 636549074 | 144,30 | 150 | 99,50 |
| 100878 | 8828962 | 95,59 | 814420068 | 184,60 | 191 | 99,50 |
| 100879 | 4471336 | 95,48 | 412040142 | 93,40 | 97 | 99,00 |
| 100883 | 11819814 | 95,73 | 1089505119 | 247,00 | 255 | 99,20 |
| 100884 | 3347584 | 95,08 | 309679647 | 70,20 | 73 | 99,50 |
| 100886 | 7686658 | 95,78 | 709079468 | 160,70 | 167 | 99,20 |
| 100887 | 4668126 | 95,28 | 430856934 | 97,70 | 101 | 99,40 |
| 100890 | 8814420 | 95,67 | 814649331 | 184,70 | 192 | 99,20 |
| 100891 | 5695014 | 95,52 | 524832508 | 119,00 | 123 | 99,10 |
| 100892 | 2735174 | 95,89 | 251826716 | 57,10 | 59 | 99,00 |
| 100894 | 6729510 | 95,83 | 620328102 | 140,60 | 145 | 99,50 |
| 100895 | 9433682 | 95,74 | 871716597 | 197,60 | 205 | 99,20 |
| 100896 | 8746936 | 95,72 | 806976809 | 182,90 | 190 | 99,80 |
| 100901 | 4186882 | 95,06 | 386788589 | 87,70 | 91 | 99,20 |
| 100904 | 8058216 | 95,56 | 744905643 | 168,90 | 175 | 99,40 |
| 100906 | 5458790 | 95,47 | 455994928 | 103,40 | 107 | 99,30 |
| 100907 | 9489138 | 95,51 | 876888230 | 198,80 | 207 | 98,70 |
| 100912 | 4186690 | 94,61 | 383674366 | 87,00 | 89 | 99,20 |
| 100913 | 6683380 | 94,67 | 616012210 | 139,60 | 146 | 98,80 |
| 100917 | 5527220 | 95,57 | 508711705 | 115,30 | 119 | 99,90 |
| 100918 | 6806576 | 94,53 | 628084709 | 142,40 | 148 | 98,80 |
| 100921 | 3123650 | 95,05 | 288755589 | 65,50 | 68 | 99,50 |
| 100923 | 10913652 | 94,92 | 1007150244 | 228,30 | 238 | 99,40 |
| 100925 | 8613960 | 94,75 | 795469596 | 180,30 | 188 | 98,80 |
| 100926 | 9149536 | 95,67 | 832817109 | 188,80 | 195 | 99,30 |
| 100927 | 7666522 | 95,70 | 707747568 | 160,40 | 165 | 99,90 |
| 100928 | 5247660 | 95,03 | 483371397 | 109,60 | 114 | 98,90 |
| 100929 | 6371238 | 95,54 | 587360512 | 133,10 | 138 | 99,00 |
| 100930 | 7564418 | 95,32 | 698275127 | 158,30 | 163 | 99,50 |
| 100931 | 9221108 | 94,85 | 852549574 | 193,30 | 202 | 99,20 |
| 100932 | 6497346 | 94,98 | 599473298 | 135,90 | 142 | 99,20 |
| 100933 | 6973496 | 94,98 | 642622186 | 145,70 | 152 | 99,40 |
| 100935 | 2267830 | 94,46 | 205539485 | 46,60 | 45 | 99,30 |
| 100941 | 2677256 | 95,41 | 244781931 | 55,50 | 54 | 99,10 |
| 100943 | 3170650 | 94,63 | 292561071 | 66,30 | 69 | 98,90 |
| 100946 | 5172654 | 94,71 | 477425025 | 108,20 | 113 | 98,90 |
| 100947 | 8855840 | 95,02 | 816620961 | 185,10 | 193 | 99,20 |
| 100951 | 2767780 | 95,39 | 255056580 | 57,80 | 57 | 99,10 |

Table S5.4. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|-------|-------------|---------------|-----------------|---------------------|
| 100960 | 4637948 | 95,43 | 425928568 | 96,60 | 99 | 99,10 |
| 100962 | 4310596 | 95,63 | 392051064 | 88,90 | 91 | 99,20 |
| 100963 | 1974408 | 94,21 | 182390070 | 41,30 | 43 | 98,80 |
| 100964 | 2262778 | 94,91 | 208683478 | 47,30 | 48 | 99,30 |
| 100967 | 2711596 | 95,22 | 248967022 | 56,40 | 58 | 99,40 |
| 100968 | 5123952 | 95,30 | 471987706 | 107,00 | 110 | 99,00 |
| 100972 | 7477410 | 95,21 | 689739641 | 156,40 | 161 | 99,20 |
| 100975 | 7998754 | 95,34 | 738615844 | 167,40 | 173 | 99,50 |
| 100976 | 3638594 | 94,75 | 335773295 | 76,10 | 80 | 98,80 |
| 100983 | 3669988 | 94,48 | 339045575 | 76,90 | 80 | 98,80 |
| 100984 | 6956916 | 95,35 | 641494402 | 145,40 | 145 | 99,40 |
| 100987 | 2629362 | 95,11 | 243150452 | 55,10 | 57 | 99,10 |
| 100989 | 11563038 | 95,77 | 1065686827 | 241,60 | 249 | 99,10 |
| 100991 | 6316160 | 94,96 | 582725768 | 132,10 | 138 | 98,90 |
| 100992 | 2859276 | 94,60 | 263619555 | 59,80 | 62 | 98,50 |
| 100993 | 8231110 | 95,79 | 758381553 | 171,90 | 178 | 99,20 |
| 100994 | 5536364 | 94,88 | 510337890 | 115,70 | 121 | 98,80 |
| 100996 | 7075788 | 95,07 | 651919072 | 147,80 | 153 | 99,20 |
| 100997 | 2132396 | 94,05 | 197331995 | 44,70 | 47 | 98,40 |
| 100999 | 6085482 | 95,58 | 556429986 | 126,10 | 129 | 99,60 |
| 900077 | 4657458 | 95,37 | 430691102 | 97,63 | 103 | 98,40 |
| 900083 | 6691044 | 95,56 | 617998403 | 140,09 | 147 | 98,20 |
| 900084 | 3248492 | 95,18 | 299922817 | 67,99 | 71 | 98,30 |
| 900089 | 3523302 | 95,20 | 324308817 | 73,51 | 76 | 99,30 |
| 900094 | 3387992 | 95,47 | 312171242 | 70,76 | 73 | 99,20 |
| 900095 | 4466868 | 94,91 | 411820079 | 93,40 | 94 | 99,10 |
| 900104 | 10781194 | 95,73 | 996184664 | 225,80 | 234 | 99,10 |
| 900110 | 6960366 | 96,63 | 642971426 | 145,75 | 150 | 99,80 |
| 900115 | 5065176 | 95,64 | 466750089 | 105,80 | 110 | 98,80 |
| 900126 | 4039714 | 95,29 | 373033196 | 84,56 | 89 | 98,00 |
| 900130 | 8172166 | 94,80 | 755611366 | 171,30 | 175 | 99,00 |
| 900135 | 6004426 | 95,27 | 555768696 | 126,00 | 130 | 99,30 |
| 900138 | 7583758 | 95,44 | 700928339 | 158,90 | 165 | 99,20 |
| 900144 | 4087780 | 95,28 | 376924265 | 85,44 | 88 | 99,10 |
| 900150 | 6564174 | 95,46 | 604493321 | 137,03 | 143 | 98,50 |
| 900157 | 13620988 | 95,57 | 1261781723 | 286,02 | 296 | 99,00 |
| 900159 | 5053700 | 95,72 | 465523074 | 105,50 | 109 | 99,00 |
| 900160 | 791534 | 94,83 | 72810185 | 16,50 | 17 | 98,90 |
| 900161 | 6767554 | 95,40 | 622483695 | 141,10 | 146 | 99,50 |
| 900164 | 5978184 | 94,93 | 551296262 | 125,00 | 130 | 99,00 |
| 900166 | 8463916 | 94,68 | 783004573 | 177,50 | 184 | 98,70 |
| 900169 | 3531234 | 95,09 | 324288155 | 73,50 | 73 | 99,20 |

Table S5.4. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|-------|-------------|---------------|-----------------|---------------------|
| 900173 | 4865874 | 94,96 | 448459089 | 101,70 | 105 | 99,10 |
| 900174 | 6456632 | 94,66 | 596399079 | 135,20 | 140 | 98,80 |
| 900176 | 5891534 | 94,74 | 545263881 | 123,60 | 129 | 98,60 |
| 900177 | 8367724 | 95,47 | 769016835 | 174,30 | 180 | 99,10 |
| 900178 | 5884278 | 94,75 | 544283139 | 123,40 | 128 | 98,80 |
| 900179 | 3757352 | 94,53 | 346790136 | 78,60 | 82 | 98,50 |
| 900181 | 3658838 | 94,80 | 338192325 | 76,66 | 80 | 98,30 |
| 900183 | 4112656 | 94,50 | 379449608 | 86,00 | 89 | 99,50 |
| 900190 | 5386042 | 95,48 | 496968260 | 112,65 | 117 | 99,60 |
| 900192 | 4411450 | 96,52 | 407585797 | 92,39 | 95 | 99,60 |
| 900199 | 9405206 | 95,68 | 869958388 | 197,20 | 204 | 99,30 |
| 900201 | 2187488 | 95,12 | 201682848 | 45,70 | 47 | 99,10 |
| 900202 | 8860806 | 95,83 | 818983627 | 185,70 | 192 | 99,20 |
| 900204 | 4196972 | 94,37 | 387936608 | 87,90 | 92 | 98,50 |
| 900206 | 6629018 | 94,70 | 613385999 | 139,00 | 145 | 98,60 |
| 900211 | 8882108 | 95,37 | 819024355 | 185,70 | 191 | 99,10 |
| 900212 | 8926712 | 95,66 | 822954141 | 186,60 | 193 | 99,00 |
| 900215 | 6848790 | 95,51 | 630580535 | 142,90 | 147 | 99,10 |
| 900221 | 2812730 | 95,25 | 260233372 | 58,99 | 62 | 98,30 |
| 900223 | 3083040 | 95,31 | 284289254 | 64,44 | 67 | 99,10 |
| 900225 | 4983966 | 95,07 | 460812198 | 104,50 | 108 | 99,50 |
| 900229 | 6520394 | 94,75 | 601645866 | 136,40 | 142 | 98,80 |
| 900236 | 12376840 | 96,27 | 1127089142 | 255,50 | 261 | 99,70 |
| 900238 | 9682506 | 95,50 | 895230730 | 202,93 | 213 | 98,30 |
| 900239 | 6366596 | 96,72 | 587033189 | 133,07 | 137 | 99,30 |
| 900241 | 5696554 | 96,58 | 527413573 | 119,55 | 123 | 99,40 |
| 900244 | 7550754 | 95,37 | 696142653 | 157,80 | 163 | 99,10 |
| 900251 | 5570528 | 95,79 | 513273278 | 116,40 | 120 | 99,30 |
| 900319 | 4327172 | 95,17 | 393895196 | 89,29 | 88 | 99,20 |
| 900343 | 4226624 | 95,62 | 390290590 | 88,47 | 93 | 97,90 |
| 900344 | 7305994 | 94,54 | 673506466 | 152,70 | 159 | 98,80 |
| 900365 | 3633014 | 95,68 | 336204924 | 76,21 | 80 | 98,40 |
| 900374 | 7682274 | 95,54 | 709433935 | 160,81 | 169 | 98,10 |
| 900387 | 4761330 | 95,25 | 438801669 | 99,50 | 99 | 99,30 |
| 900394 | 5895104 | 95,17 | 545589186 | 123,70 | 128 | 99,10 |
| 900397 | 6781232 | 95,65 | 626276164 | 141,96 | 149 | 98,50 |
| 900400 | 7612354 | 95,39 | 703612915 | 159,49 | 167 | 98,20 |
| 900406 | 7457526 | 95,41 | 685335643 | 155,40 | 159 | 99,20 |
| 900413 | 8074132 | 94,82 | 747137218 | 169,40 | 177 | 98,70 |
| 900416 | 5346728 | 94,62 | 494699921 | 112,10 | 117 | 98,70 |
| 900417 | 3282346 | 94,57 | 302572231 | 68,60 | 72 | 98,30 |
| 900421 | 6792232 | 94,41 | 628043107 | 142,40 | 149 | 98,60 |

Table S5.4. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|-------|-------------|---------------|-----------------|---------------------|
| 900428 | 6919752 | 94,64 | 640814487 | 145,30 | 153 | 98,00 |
| 900430 | 4576412 | 95,83 | 421902959 | 95,60 | 99 | 98,80 |
| 900437 | 10100234 | 94,68 | 932826377 | 211,50 | 220 | 98,40 |
| 900439 | 1719270 | 95,22 | 158735817 | 35,98 | 38 | 97,90 |
| 900443 | 6746410 | 95,67 | 622308095 | 141,10 | 146 | 99,30 |
| 900446 | 3568466 | 95,49 | 328026972 | 74,40 | 76 | 99,10 |
| 900459 | 3350726 | 94,33 | 309370206 | 70,10 | 73 | 98,70 |
| 900464 | 10104002 | 95,89 | 931474859 | 211,20 | 216 | 99,10 |
| 900469 | 5572320 | 95,63 | 514346967 | 116,60 | 120 | 99,60 |
| 900477 | 10793432 | 94,58 | 996500701 | 225,90 | 236 | 98,60 |
| 900489 | 5893196 | 95,85 | 543161841 | 123,10 | 128 | 98,80 |
| 900490 | 5546714 | 95,61 | 512275952 | 116,12 | 122 | 98,20 |
| 900517 | 5739918 | 94,60 | 531133943 | 120,40 | 126 | 98,60 |
| 900573 | 7510088 | 95,56 | 693375210 | 157,17 | 165 | 98,60 |
| 900574 | 7482078 | 95,25 | 688955525 | 156,17 | 162 | 99,10 |
| 900576 | 9422310 | 95,75 | 868555554 | 196,90 | 203 | 98,80 |
| 900580 | 2745100 | 95,70 | 232761189 | 52,80 | 54 | 99,00 |
| 900586 | 2581044 | 95,48 | 238334139 | 54,03 | 57 | 98,00 |
| 900601 | 9062300 | 95,53 | 836943089 | 189,72 | 199 | 98,20 |
| 900603 | 6675866 | 95,62 | 616819650 | 139,80 | 145 | 99,20 |
| 900609 | 4064352 | 94,30 | 375056975 | 85,00 | 89 | 98,30 |
| 900611 | 6260624 | 95,40 | 577124778 | 130,80 | 135 | 99,30 |
| 900612 | 5889964 | 95,58 | 542995499 | 123,10 | 127 | 99,00 |
| 900618 | 5136100 | 95,46 | 473770925 | 107,40 | 110 | 99,10 |
| 900637 | 2483164 | 95,21 | 229386411 | 52,00 | 54 | 99,20 |
| 900639 | 8184572 | 95,33 | 757111404 | 171,60 | 177 | 99,30 |
| 900654 | 4897320 | 95,37 | 450923632 | 102,20 | 105 | 99,30 |
| 900655 | 3036462 | 94,58 | 280487243 | 63,60 | 67 | 98,70 |
| 900666 | 5199786 | 95,85 | 478841323 | 108,50 | 112 | 99,00 |
| 900677 | 3880296 | 94,56 | 358103680 | 81,20 | 85 | 98,60 |
| 900680 | 5220468 | 94,67 | 481344407 | 109,10 | 114 | 98,70 |
| 900703 | 2435754 | 94,78 | 225517592 | 51,10 | 53 | 98,60 |
| 900706 | 4981156 | 95,53 | 461099564 | 104,52 | 109 | 98,20 |
| 900711 | 4677424 | 94,92 | 430474568 | 97,60 | 99 | 99,30 |
| 900716 | 10183442 | 94,59 | 938814696 | 212,80 | 222 | 98,80 |
| 900731 | 5091556 | 95,37 | 470191118 | 106,58 | 112 | 98,00 |
| 900746 | 6474714 | 95,36 | 598086075 | 135,57 | 142 | 98,10 |
| 900748 | 6584354 | 95,10 | 607985996 | 137,82 | 144 | 98,40 |
| 900751 | 3003642 | 95,37 | 276844453 | 62,75 | 65 | 99,00 |
| 900824 | 6534112 | 96,35 | 578749267 | 131,20 | 131 | 99,40 |
| 900845 | 3141548 | 94,36 | 290278154 | 65,80 | 69 | 98,60 |
| 900849 | 4992552 | 95,91 | 460361996 | 104,40 | 108 | 99,30 |

Table S5.4. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|-------|-------------|---------------|-----------------|---------------------|
| 1001002 | 8421510 | 94,85 | 779007911 | 176,60 | 184 | 99,20 |
| 1001003 | 3107372 | 95,52 | 286077988 | 64,90 | 67 | 99,10 |
| 1001009 | 4275054 | 95,13 | 394554574 | 89,40 | 93 | 99,30 |
| 1001011 | 5828312 | 94,85 | 537712611 | 121,90 | 127 | 99,30 |
| 1001012 | 5244854 | 95,36 | 483589099 | 109,60 | 113 | 99,60 |
| 1001013 | 8923730 | 95,41 | 822488632 | 186,40 | 193 | 99,70 |
| 1100213 | 4266778 | 94,92 | 394750818 | 89,48 | 93 | 98,90 |
| 1100217 | 5838902 | 95,61 | 539199148 | 122,22 | 125 | 98,80 |
| 1100231 | 4172814 | 95,42 | 384740674 | 87,21 | 90 | 98,90 |
| 1100232 | 6931214 | 95,41 | 637630282 | 144,54 | 149 | 99,40 |
| 1100233 | 6438244 | 96,59 | 591744287 | 134,14 | 136 | 99,60 |
| 1100234 | 2448826 | 95,04 | 226034251 | 51,24 | 54 | 97,90 |
| 1100238 | 5349728 | 95,45 | 493746431 | 111,92 | 115 | 99,20 |
| 1100246 | 4241148 | 94,92 | 392868330 | 89,05 | 92 | 99,00 |
| 1100250 | 5686092 | 95,38 | 525154268 | 119,04 | 125 | 98,10 |
| 1100254 | 691128 | 95,19 | 63775796 | 14,46 | 15 | 97,50 |
| 1100256 | 6861744 | 95,09 | 635722606 | 144,10 | 151 | 98,50 |
| 1100262 | 1867970 | 92,89 | 172516103 | 39,11 | 41 | 98,90 |
| 1100264 | 8528690 | 95,17 | 788677462 | 178,78 | 185 | 99,20 |
| 1100269 | 5137284 | 95,01 | 474920172 | 107,65 | 112 | 99,10 |
| 1100270 | 6399968 | 95,37 | 589507896 | 133,63 | 138 | 99,00 |
| 1100273 | 6762182 | 95,34 | 625911758 | 141,88 | 149 | 98,30 |
| 1100280 | 4627628 | 95,43 | 427362057 | 96,87 | 101 | 98,40 |
| 1100281 | 6110340 | 95,50 | 564048380 | 127,86 | 132 | 99,20 |
| 1100283 | 4638990 | 95,44 | 429132510 | 97,28 | 102 | 98,50 |
| 1100291 | 5293572 | 96,58 | 488900426 | 110,82 | 114 | 99,70 |
| 1100294 | 7450520 | 95,38 | 688696972 | 156,11 | 160 | 99,40 |
| 1100300 | 3092126 | 95,00 | 286048335 | 64,84 | 68 | 98,30 |
| 1100306 | 5766562 | 95,54 | 532393055 | 120,68 | 127 | 98,30 |
| 1100307 | 5090484 | 94,61 | 470152143 | 106,57 | 111 | 98,00 |
| 1100315 | 4590438 | 95,26 | 423829736 | 96,07 | 101 | 98,00 |
| 1100316 | 4639218 | 95,00 | 429207921 | 97,29 | 102 | 98,20 |
| 1100320 | 4273028 | 95,14 | 394124860 | 89,34 | 93 | 98,80 |
| 1100331 | 2893908 | 95,10 | 267254684 | 60,58 | 63 | 98,20 |
| 1100332 | 3547096 | 95,38 | 327284397 | 74,19 | 76 | 99,10 |
| 1100333 | 7640658 | 95,53 | 702530395 | 159,25 | 164 | 99,40 |
| 1100335 | 4719994 | 95,23 | 436845013 | 99,02 | 104 | 98,10 |
| 1100340 | 10582420 | 95,47 | 977856076 | 221,66 | 232 | 98,60 |
| 1100345 | 5664472 | 95,22 | 523635379 | 118,70 | 124 | 98,50 |
| 1100348 | 9478592 | 96,71 | 874252406 | 198,17 | 204 | 99,20 |
| 1100353 | 7157040 | 96,90 | 662329285 | 150,14 | 155 | 99,60 |
| 1100367 | 4378604 | 96,73 | 403022547 | 91,36 | 94 | 99,40 |

Table S5.4. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|-------|-------------|---------------|-----------------|---------------------|
| 1100369 | 3614860 | 95,09 | 334572793 | 75,84 | 79 | 98,40 |
| 1100373 | 7817932 | 96,89 | 721194175 | 163,48 | 169 | 99,30 |
| 1100374 | 5687648 | 95,12 | 524404441 | 118,87 | 123 | 99,30 |
| 1100396 | 4464604 | 96,87 | 412472505 | 93,50 | 96 | 99,60 |
| 1100397 | 7952458 | 95,27 | 734301221 | 166,45 | 172 | 99,10 |
| 1100400 | 4156544 | 94,85 | 384729079 | 87,21 | 91 | 98,50 |
| 1100421 | 8161636 | 96,93 | 744477220 | 168,76 | 174 | 99,30 |
| 1100607 | 6876462 | 95,38 | 632158432 | 143,30 | 148 | 99,40 |
| 1100707 | 4391668 | 95,52 | 405964237 | 92,02 | 96 | 98,90 |
| 1100708 | 5804762 | 95,37 | 535042353 | 121,28 | 126 | 98,90 |
| 1100716 | 8686226 | 95,58 | 800338659 | 181,42 | 188 | 98,80 |
| 1100730 | 8065486 | 96,76 | 746592857 | 169,24 | 174 | 99,70 |
| 1100738 | 5401532 | 95,40 | 500478912 | 113,45 | 118 | 98,30 |
| 1100746 | 3480194 | 95,27 | 321712017 | 72,93 | 75 | 98,80 |
| 1100757 | 6216226 | 95,37 | 572081247 | 129,68 | 130 | 98,90 |
| 1100760 | 3239756 | 95,38 | 298617464 | 67,69 | 71 | 98,50 |
| 1100763 | 5502764 | 95,21 | 506670287 | 114,85 | 117 | 98,70 |
| 1100764 | 5650758 | 96,92 | 521338278 | 118,18 | 122 | 99,30 |
| 1100773 | 5049028 | 95,31 | 466844260 | 105,82 | 109 | 99,10 |
| 1100774 | 1121332 | 95,44 | 103410546 | 23,44 | 25 | 98,50 |
| 1100786 | 5043614 | 95,42 | 463788799 | 105,13 | 109 | 99,10 |
| 1100797 | 6867872 | 95,63 | 631656757 | 143,18 | 147 | 99,30 |
| 1100903 | 3940012 | 95,42 | 363569155 | 82,41 | 85 | 98,60 |
| 1100907 | 6662502 | 96,74 | 616858915 | 139,83 | 144 | 99,40 |
| 1100908 | 4866202 | 95,41 | 447414934 | 101,42 | 101 | 99,00 |
| 1100909 | 4321576 | 95,18 | 398668836 | 90,37 | 94 | 98,70 |
| 1100917 | 1474708 | 92,91 | 136437753 | 30,93 | 32 | 98,40 |
| 1100918 | 8666842 | 95,55 | 800281865 | 181,41 | 190 | 98,40 |
| 1100921 | 5637528 | 96,81 | 521923240 | 118,31 | 122 | 99,60 |
| 1100929 | 4545226 | 95,44 | 419603339 | 95,12 | 98 | 99,20 |
| 1100930 | 3966012 | 95,49 | 367144208 | 83,22 | 87 | 98,30 |
| 1100931 | 4305168 | 95,24 | 398774103 | 90,39 | 94 | 99,40 |
| 1100933 | 1616234 | 92,92 | 149443084 | 33,88 | 35 | 98,40 |
| 1100936 | 4857908 | 95,07 | 448087877 | 101,57 | 105 | 98,60 |
| 1100937 | 4023822 | 95,29 | 371528087 | 84,22 | 86 | 98,90 |
| 1100942 | 3877632 | 95,55 | 357512894 | 81,04 | 84 | 98,70 |
| 1100943 | 4256638 | 95,07 | 391629990 | 88,77 | 92 | 99,00 |
| 1100945 | 3707196 | 95,44 | 342392133 | 77,61 | 81 | 98,50 |
| 1100969 | 9138662 | 95,44 | 843500045 | 191,20 | 197 | 99,20 |
| 1100977 | 5068004 | 95,36 | 467957429 | 106,08 | 111 | 98,20 |
| 1100981 | 5409442 | 95,53 | 499060319 | 113,13 | 117 | 98,70 |
| 1100984 | 5795768 | 95,60 | 535918638 | 121,48 | 126 | 99,00 |

Table S5.4. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--------------|-------------|-------|-------------|---------------|-----------------|---------------------|
| 1100987 | 2431690 | 96,80 | 392597634 | 88,99 | 92 | 99,00 |
| 1100995 | 4298726 | 95,24 | 397765175 | 90,16 | 93 | 99,10 |
| 1101003 | 8605192 | 95,75 | 793618170 | 179,90 | 186 | 99,20 |
| 1101004 | 5345552 | 95,18 | 493292026 | 111,82 | 116 | 99,20 |
| 1101008 | 6612834 | 96,66 | 610379624 | 138,36 | 142 | 99,30 |
| 1101011 | 5311910 | 95,44 | 490949034 | 111,29 | 115 | 98,90 |
| 1101012 | 4300000 | 95,19 | 397980899 | 90,21 | 93 | 98,90 |
| 1101015 | 8385930 | 95,79 | 774182342 | 175,49 | 179 | 98,90 |
| 1101018 | 2563844 | 95,70 | 237182128 | 53,76 | 56 | 98,80 |
| 1101025 | 9120244 | 95,95 | 843597287 | 191,23 | 198 | 98,90 |
| 1101028 | 1896992 | 95,80 | 175609777 | 39,81 | 41 | 98,80 |
| 1101037 | 5087396 | 95,77 | 471034289 | 106,77 | 111 | 99,20 |
| 1101047 | 7861958 | 95,42 | 727939191 | 165,01 | 173 | 98,30 |
| 1101054 | 7246156 | 95,44 | 670754167 | 152,05 | 159 | 98,40 |
| 1101061 | 8923668 | 96,99 | 823426144 | 186,65 | 193 | 99,30 |
| 1101064 | 4408312 | 95,68 | 406658628 | 92,18 | 95 | 98,50 |
| 1101067 | 8193818 | 96,70 | 756937816 | 171,58 | 177 | 99,60 |
| 1101074 | 3883520 | 95,62 | 358280741 | 81,21 | 85 | 98,50 |
| 1101077 | 3228516 | 95,68 | 298974274 | 67,77 | 70 | 98,70 |
| 1101080 | 4911704 | 95,80 | 453246251 | 102,74 | 107 | 98,70 |
| 1101084 | 5692096 | 95,77 | 525457900 | 119,11 | 124 | 98,60 |
| 1101085 | 4265798 | 94,95 | 394010661 | 89,31 | 93 | 98,80 |
| 1101088 | 3440936 | 95,98 | 318639444 | 72,23 | 75 | 98,70 |
| 1101089 | 6002548 | 96,96 | 556190844 | 126,08 | 130 | 99,60 |
| 1101253 | 3642584 | 95,74 | 336144308 | 76,20 | 79 | 98,80 |
| 1101292 | 6082440 | 95,86 | 560795868 | 127,12 | 132 | 98,80 |
| 1101303 | 9312806 | 95,99 | 861349125 | 195,25 | 202 | 99,20 |
| 1101304 | 2045310 | 93,60 | 188641178 | 42,76 | 44 | 98,50 |
| 1101316 | 10031646 | 95,64 | 923436067 | 209,32 | 214 | 99,00 |
| 1101317 | 4281276 | 94,97 | 393297145 | 89,15 | 92 | 99,00 |
| 1101318 | 8311646 | 95,94 | 767350601 | 173,94 | 180 | 99,10 |
| 1101319 | 5606644 | 95,88 | 518165835 | 117,46 | 122 | 99,00 |
| 1101321 | 13908338 | 95,81 | 1283563813 | 290,96 | 303 | 99,20 |
| 1101322 | 5388084 | 95,89 | 496954407 | 112,65 | 117 | 98,70 |
| 1101323 | 4780336 | 95,88 | 441188685 | 100,01 | 103 | 98,80 |
| 1101324 | 10556482 | 96,05 | 974353093 | 220,87 | 228 | 99,20 |
| 1101329 | 5265746 | 95,54 | 485933498 | 110,15 | 114 | 98,60 |
| 1101337 | 9036512 | 95,73 | 834483421 | 189,16 | 196 | 98,90 |
| 1101340 | 5004558 | 95,76 | 456531484 | 103,49 | 102 | 99,00 |
| 1101342 | 8516402 | 95,68 | 787938504 | 178,61 | 185 | 99,00 |
| 1101344 | 5945098 | 95,92 | 548757919 | 124,39 | 129 | 98,90 |
| 1101346 | 5892778 | 95,82 | 541723597 | 122,80 | 127 | 98,90 |

Table S5.4. Continued

| Study number | Clean reads | Q20% | Total bases | Mean coverage | Median coverage | Percentage bases >1 |
|--|-------------|-------|-------------|---------------|-----------------|---------------------|
| 1101348 | 7181066 | 95,79 | 662390301 | 150,15 | 156 | 98,80 |
| 1101350 | 5585560 | 95,70 | 517096265 | 117,21 | 122 | 98,80 |
| 1101355 | 5142926 | 95,59 | 475691952 | 107,83 | 112 | 98,70 |
| 1101359 | 8357208 | 95,68 | 769440613 | 174,42 | 180 | 99,00 |
| 1101360 | 6005832 | 95,75 | 554392328 | 125,67 | 130 | 99,10 |
| 1101367 | 2839034 | 95,73 | 262799618 | 59,57 | 62 | 98,80 |
| 1101368 | 5295318 | 95,94 | 488701549 | 110,78 | 115 | 98,40 |
| 1101382 | 2494612 | 95,72 | 229415793 | 52,00 | 54 | 98,50 |
| 1101386 | 9713036 | 95,80 | 896253841 | 203,16 | 211 | 99,10 |
| 1101554 | 8768224 | 95,99 | 809038817 | 183,39 | 190 | 99,00 |
| 1101556 | 2695552 | 93,65 | 248575069 | 56,35 | 58 | 98,30 |
| 1101557 | 5974612 | 95,54 | 552272734 | 125,19 | 130 | 98,80 |
| 1101572 | 9706246 | 95,94 | 895520927 | 203,00 | 211 | 98,80 |
| 1101576 | 4786726 | 95,86 | 441905771 | 100,17 | 104 | 98,70 |
| 1101580 | 7791782 | 95,82 | 718652884 | 162,90 | 169 | 98,90 |
| 1101600 | 3010808 | 95,87 | 278389255 | 63,10 | 65 | 98,70 |
| 1101602 | 3259454 | 95,92 | 299698944 | 67,94 | 71 | 98,80 |
| 1101603 | 7436708 | 96,04 | 686272907 | 155,56 | 161 | 98,90 |
| 1101604 | 6303476 | 95,18 | 581855643 | 131,89 | 136 | 98,90 |
| 1101610 | 5124546 | 96,59 | 472976032 | 107,21 | 111 | 99,30 |
| 1101623 | 5027232 | 95,51 | 463403211 | 105,04 | 108 | 98,70 |
| 1101628 | 5667660 | 95,74 | 524058595 | 118,79 | 123 | 99,10 |
| 1101652 | 7220436 | 96,62 | 666135610 | 151,00 | 156 | 99,30 |
| 1101933 | 4298652 | 95,27 | 397732878 | 90,16 | 93 | 99,10 |
| 1101942 | 4273636 | 96,65 | 395475578 | 89,65 | 92 | 99,60 |
| 1101947 | 5441952 | 96,70 | 499732433 | 113,28 | 115 | 99,30 |
| 1101984 | 4843912 | 96,79 | 446859913 | 101,29 | 105 | 99,20 |
| 1101997 | 5845746 | 96,62 | 538540400 | 122,08 | 126 | 99,80 |
| Samples excluded from further analysis | | | | | | |
| 100973 | 10916526 | 96,14 | 1042435 | 0,12 | 1 | 2,40 |
| 900089 | 6737346 | 96,70 | 289038979 | 32,76 | 34 | 99,25 |
| 900132 | 3437888 | 97,96 | 32333418 | 7,33 | 8 | 96,90 |
| 900248 | 1760232 | 96,56 | NA | NA | NA | NA |
| 900558 | 1303536 | 97,11 | NA | NA | NA | NA |
| 900625 | 5877240 | 95,53 | 1290461 | 0,29 | 1 | 10,70 |
| 1100343 | 8561266 | 95,67 | 6480416 | 1,47 | 2 | 60,60 |
| 1100772 | 1340688 | 94,87 | NA | NA | NA | NA |
| 1101001 | 8104650 | 96,05 | 275263806 | 62,30 | 65 | 98,30 |

Displayed are various measures of sequencing quality, used for the sequencing quality control check.

Table S5.5. PE / PPE genes and drug resistance genes excluded for the phylogeny construction.

| PE/PPE genes and genes in repetitive regions | | | Rv2107 | Rv2741 | Rv3381c | Known drug resistance genes |
|--|---------|---------|---------|---------|---------|-----------------------------|
| Rv0031 | Rv0922 | Rv1575 | Rv2108 | Rv2768c | Rv3386 | |
| Rv0096 | Rv0977 | Rv1576c | Rv2123 | Rv2769c | Rv3387 | |
| Rv0109 | Rv0978c | Rv1577c | Rv2126c | Rv2770c | Rv3388 | accD6 |
| Rv0124 | Rv0980c | Rv1578c | Rv2162c | Rv2791c | Rv3425 | ahpC |
| Rv0151c | Rv1034c | Rv1579c | Rv2167c | Rv2810c | Rv3426 | efpA |
| Rv0152c | Rv1035c | Rv1580c | Rv2168c | Rv2812 | Rv3427c | embA |
| Rv0159c | Rv1036c | Rv1581c | Rv2177c | Rv2814c | Rv3428c | embB |
| Rv0160c | Rv1039c | Rv1582c | Rv2278 | Rv2815c | Rv3429 | embC |
| Rv0256c | Rv1040c | Rv1583c | Rv2279 | Rv2853 | Rv3430c | embR |
| Rv0278c | Rv1041c | Rv1584c | Rv2328 | Rv2885c | Rv3474 | ethA |
| Rv0279c | Rv1042c | Rv1585c | Rv2340c | Rv2892c | Rv3475 | fabD |
| Rv0280 | Rv1047 | Rv1586c | Rv2352c | Rv2943 | Rv3477 | fadE24 |
| Rv0285 | Rv1054 | Rv1646 | Rv2353c | Rv2943A | Rv3478 | fbpC |
| Rv0286 | Rv1067c | Rv1651c | Rv2354 | Rv2944 | Rv3507 | furA |
| Rv0297 | Rv1068c | Rv1705c | Rv2355 | Rv2961 | Rv3508 | gid |
| Rv0304c | Rv1087 | Rv1706c | Rv2356c | Rv2978c | Rv3511 | gyrA |
| Rv0305c | Rv1088 | Rv1753c | Rv2371 | Rv3018A | Rv3512 | gyrB |
| Rv0335c | Rv1089 | Rv1756c | Rv2396 | Rv3018c | Rv3514 | inhA |
| Rv0354c | Rv1091 | Rv1757c | Rv2408 | Rv3021c | Rv3532 | iniA |
| Rv0355c | Rv1135c | Rv1763 | Rv2424c | Rv3022A | Rv3533c | iniB |
| Rv0387c | Rv1149 | Rv1764 | Rv2430c | Rv3022c | Rv3539 | iniC |
| Rv0388c | Rv1168c | Rv1765A | Rv2431c | Rv3023c | Rv3558 | kasA |
| Rv0442c | Rv1169c | Rv1768 | Rv2479c | Rv3115 | Rv3590c | katG |
| Rv0453 | Rv1172c | Rv1787 | Rv2480c | Rv3125c | Rv3595c | fabG1 |
| Rv0532 | Rv1195 | Rv1788 | Rv2487c | Rv3135 | Rv3621c | manB |
| Rv0578c | Rv1196 | Rv1789 | Rv2490c | Rv3136 | Rv3622c | ndh |
| Rv0741 | Rv1199c | Rv1790 | Rv2512c | Rv3144c | Rv3636 | nat |
| Rv0742 | Rv1214c | Rv1791 | Rv2519 | Rv3159c | Rv3637 | oxyR |
| Rv0746 | Rv1243c | Rv1800 | Rv2591 | Rv3184 | Rv3638 | pncA |
| Rv0747 | Rv1313c | Rv1801 | Rv2608 | Rv3185 | Rv3640c | rmlD |
| Rv0754 | Rv1325c | Rv1802 | Rv2615c | Rv3186 | Rv3650 | rpoB |
| Rv0755A | Rv1361c | Rv1803c | Rv2634c | Rv3187 | Rv3652 | rpsL |
| Rv0755c | Rv1369c | Rv1806 | Rv2646 | Rv3191c | Rv3653 | rrs |
| Rv0795 | Rv1370c | Rv1807 | Rv2648 | Rv3325 | Rv3738c | Rv0340 |
| Rv0796 | Rv1386 | Rv1808 | Rv2649 | Rv3326 | Rv3739c | Rv1592c |
| Rv0797 | Rv1387 | Rv1809 | Rv2650c | Rv3327 | Rv3746c | Rv1772 |
| Rv0832 | Rv1396c | Rv1818c | Rv2651c | Rv3343c | Rv3751 | Rv2242 |
| Rv0833 | Rv1430 | Rv1840c | Rv2652c | Rv3344c | Rv3798 | Rv3124 |
| Rv0834c | Rv1441c | Rv1917c | Rv2653c | Rv3345c | Rv3812 | Rv3125c |
| Rv0850 | Rv1450c | Rv1918c | Rv2654c | Rv3347c | Rv3827c | Rv3126c |
| Rv0872c | Rv1452c | Rv1983 | Rv2655c | Rv3348 | Rv3844 | thyA |
| Rv0878c | Rv1468c | Rv2013 | Rv2656c | Rv3349c | Rv3872 | tlyA |
| Rv0915c | Rv1548c | Rv2014 | Rv2657c | Rv3350c | Rv3873 | accD6 |
| Rv0916c | Rv1573 | Rv2105 | Rv2659c | Rv3367 | Rv3892c | |
| Rv0920c | Rv1574 | Rv2106 | Rv2666 | Rv3380c | Rv3893c | |

Listed are the genes that were excluded from the multiple alignment used to create the phylogenetic tree.

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6

Mycobacterium tuberculosis Beijing lineage evades BCG protection against infection

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In preparation

Abstract

Background

Many studies have examined the host and environmental factors that influence tuberculosis transmission and secondary cases of disease, but the *M. tuberculosis* genetic determinants of transmission remain poorly understood.

Methods

Using a household-based case-contact study in Bandung, Indonesia, we determined whether the genotype of the *M. tuberculosis* strain that a contact is exposed to influences their risk of infection, measured by interferon gamma release assay conversion. Generalised estimating equation for Poisson regression was used to investigate whether the *M. tuberculosis* genotype influences the disease phenotype in the contact. We calculated a transmission index reflecting the isolate's ability to transmit to contacts, adjusted for index case characteristics and exposure risk of the contact.

Results

M. tuberculosis genotype of 414 index case isolates was linked to IGRA conversion of 1,201 household contacts. The Beijing genotype was associated with a 39% increased risk of uninfected contacts to acquire *M. tuberculosis* infection compared to non-Beijing genotype strains (relative risk 1.39; 95% confidence interval 1.00 – 1.93; $p=0.048$). This was not explained by a higher bacillary load or higher frequency of pulmonary cavities in the index cases. In addition, BCG vaccination showed no protection against *M. tuberculosis* infection in contacts exposed to a Beijing genotype strain (RR=1.02; 95% CI 0.56 – 1.85; $p=0.9$), whereas a strong protection was observed against infection with non-Beijing genotype strains (RR 0.40; 95% CI 0.27 - 0.61; $p<0.001$).

Discussion

Beijing genotype strains are associated with increased transmission and BCG vaccination showed less protection in contacts exposed to Beijing genotype strains. Evading innate immune clearance in the contact, rather than inducing a disease phenotype in the index case that favours transmission, may explain the increased transmissibility of Beijing genotype strains.

Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), the number one cause of death from an infectious disease caused by a single pathogen¹. Mounting evidence from countries with high TB burdens points to ongoing transmission as the driving force maintaining TB incidence^{2,3}. Despite coordinated efforts to control TB transmission, an estimated 10 million people developed TB disease in 2017¹ and 50 million people worldwide are predicted to become newly infected with *M. tuberculosis* every year⁴. Transmission of *M. tuberculosis* occurs through aerosol droplets that are produced by coughing pulmonary TB patients and inhaled by exposed contacts. Tuberculosis transmission is dependent on the prevalence and infectiousness of pulmonary TB cases, the number and susceptibility of TB case contacts, the frequency and proximity of interactions between TB cases and contacts, as well as biological features of *M. tuberculosis*⁵. To date, considerable emphasis has been placed on the role of host and environmental factors associated with transmission, but far less effort has been put in understanding pathogen factors involved in manipulating and evading host immunity⁶. *M. tuberculosis* consists of seven human-adapted phylogenetic lineages and is subdivided into genetic families defined by genetic traits (single nucleotide polymorphisms (SNPs) or deletions)⁷. The East-Asian lineage (Lineage 2) is widespread globally and the Beijing genotype family is its major component⁸, which can be further subdivided into ancient and modern Beijing lineages⁹. Beijing strains have been associated with large outbreaks¹⁰⁻¹³, suggesting increased transmissibility for this genotype family.

M. tuberculosis genotype-specific traits could increase the risk of transmission in two ways: first; by inducing a disease phenotype in the index case that favours transmission, or second; by evading host immune responses that eradicate *M. tuberculosis* before an adaptive immune response develops¹⁴. In the current household case contact study, in which contacts are linked to a known index case, we sought to understand the influence of host and pathogen factors on *M. tuberculosis* transmission. Specifically we sought to test whether increased Beijing lineage transmission was related to a transmission-favourable disease phenotype or to evasion of host responses in the contact.

Methods

Setting and design

The Innate Factors and Early Clearance of *M. tuberculosis* (INFECT) study was conducted in Bandung, Indonesia (estimated TB incidence: 395/100,000)¹. The TANDEM project¹⁵, on the relationship between TB and diabetes mellitus, recruited sputum smear positive TB patients over 15 years of age with an abnormal chest x-ray. Patients were eligible for the INFECT study if they shared a household with others and had received less than two weeks of TB treatment. Their household contacts¹⁶ were eligible if they had lived with the index case for more than five hours a week and had no previous TB. Children under five years were ineligible and were referred to a primary care physician. The study was approved by the Health Research Ethics Committee Universitas Padjadjaran (14/UN6.C2.1.2/KEPK/PN/2014) and the Southern Health and Disability Ethics Committee New Zealand (13/STH/132). All participants gave written informed consent.

Study procedures

Patients with suspected TB disease were referred from primary care. Demographic characteristics, sputum smear grade¹⁷, and *M. tuberculosis* culture results were recorded¹⁸. Their chest x-rays were read by a physician who identified cavities and the extent of abnormalities, as previously described^{19,20}. They were treated for TB at no cost by the National TB Control Programme. Trained nurses recruited eligible contacts at home. They recorded demographic characteristics, smoking status, diabetes, history of human immunodeficiency virus (HIV) infection, TB symptoms, and sleep proximity to and hours spent with the index case the day before enrolment. Physical examination included assessment of the presence of a Bacillus Calmette-Guérin (BCG) vaccination scar, height and weight. Household contact *M. tuberculosis* infection status was assessed by QuantiFERON®-TB Gold In-Tube interferon gamma release assay (IGRA), interpreted according to the manufacturer's instructions²¹. Indeterminate tests were repeated, and the repeat value used. A random capillary blood glucose (RCBG) was performed, followed by venous glycosylated haemoglobin (HbA1c) for those with RCBG > 100 mg/dL (5.5 mmol/L). Contacts who were IGRA negative at baseline had a repeat IGRA at 14 weeks. Those with TB symptoms at baseline or follow up underwent chest x-ray and sputum examination.

M. tuberculosis isolates were suspended in liquid media and stored at -80°C until they were thawed and re-cultured for DNA extraction. *M. tuberculosis* DNA was extracted after subculturing of positive culture on Ogawa solid medium using the cetyl trimethylammonium bromide (CTAB) method or the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA).

M. tuberculosis DNA from 137 Indonesian isolates was sequenced on an Illumina HiSeq 2000 instrument using 2 x 100 bp paired-end reads at the Beijing Genome Institute in Hong Kong; the remaining 285 isolates were sequenced on an Illumina NextSeq 500 instrument using 2 x 150 bp paired-end reads at the department of Human Genetics at the Radboudumc, Nijmegen, the Netherlands. After sequencing, the raw FASTQ sequence reads were filtered, including removing of adapter sequences. All sequencing reads passed the Illumina base caller filter. Sequencing coverage was determined using the FastQC quality control tool version 0.10.1, and the Genome Analysis Toolkit²². Sequencing coverage statistics are shown in supplementary File 1. The sequence reads were aligned to reference strain *M. tuberculosis* H37Rv, accession number NC_000962.3, and variants were called using Breseq, version 0.33.2²³. We extracted all 57,045 variable positions across the 414 *M. tuberculosis* sequences and concatenated them into a multiple sequence alignment. Solely for the purpose of creating the phylogenetic tree, SNPs occurring in PE/PPE genes, genes related to mobile elements, as well as genes previously associated with drug resistance²⁴ were removed. The remaining 55,494 SNPs were used to construct the phylogenetic tree using PhyML version 3.3.20180621²⁵ using the HKY85 model with four gamma-distributed rate-categories, and using a hundred bootstraps. We used the R package ape²⁶ (version 5.3) to calculate the distance to the genetically closest other isolate (minimum pairwise distance) for all isolates, again excluding SNPs in PE/PPE genes, genes related to mobile elements and genes associated with drug resistance. We determined *M. tuberculosis* lineage based on a 62-single nucleotide polymorphisms (SNPs) barcode²⁷. To further classify isolates belonging to lineage 2 (East-Asian lineage) into non-Beijing, ancient Beijing and modern Beijing sublineages, we categorized isolates belonging to lineage 2.1 as non-Beijing (or proto-Beijing), those belonging to lineage 2.2.2 as ancient Beijing, and those belonging to lineage 2.2.1.1 or 2.2.1.2 as modern Beijing. The lineage 2.2.1 isolates, which cannot be unambiguously distinguished into ancient or modern Beijing according to the 62-SNP-barcode, were categorized according to the *M. tuberculosis* lineage 2 dendrogram published by Shitikov *et al*²⁸. We used TBProfiler version 2.1.1²⁴ to determine genotypic drug resistance. This tool uses raw sequence data as input, aligns these to the *M. tuberculosis* H37Rv reference genome, and compares identified SNPs and indels to a curated list of drug resistance mutations.

Data management and analysis

All data were double entered into a database (Microsoft Access) and checked for errors. IGRA conversion was defined as a change from a negative baseline test to positive at 14-week follow-up. Body mass index (BMI) was calculated according to the formula $BMI = \text{weight}/(\text{height}^2)$. Diabetes was defined as an HbA1c³6.5% and pre-diabetes as HbA1c 5.7-6.4%. To create one summary measure of exposure to

facilitate specific analyses described below, exposure risk scores were predicted from a logistic regression of *M. tuberculosis* exposure variables (index case: sputum smear grade, drug resistance mutations, cavities, extent of x-ray disease; contacts: hours spent with, and sleeping proximity to, the case) against IGRA results, as described previously²⁹.

The primary analysis was of risk of IGRA conversion for those with a negative IGRA at baseline, as incident conversions are almost certainly due to exposure to the index case. A sensitivity analysis of risk of a positive IGRA at baseline or follow up, compared to a persistently negative IGRA, was also performed.

Associations between host factors and IGRA were estimated using Poisson regression generalised estimating equations, clustering on case, and with robust standard errors to account for binomial data. Index case characteristics and exposure risk score were included as potential confounders. A modified backwards stepwise regression was used, with age and sex retained, and other variables retained if $p < 0.2$. Estimates for baseline associations are presented as prevalence ratios (PR) and those for conversion are presented as relative risks (RR).

To study pathogen genetic determinates of BCG evasion we calculated a transmission index that reflects the isolate's ability to transmit to contacts, accounting for the factors extrinsic to the pathogen such as contact susceptibility and proximity to the index case. The transmission index was calculated by subtracting expected instances of transmission to BCG-vaccinated contacts from the observed instances of transmission to BCG-vaccinated contacts in each household. To score observed transmission events we weighted a positive IGRA at baseline at 20% of a conversion, as there is less certainty that a positive IGRA at baseline resulted from exposure to the index case. The expected risk of transmission was predicted using the multivariable model for the primary analysis, but with the lineage term removed. All analyses were carried out using Stata version 15.

Results

TB household index cases and contacts

From home visits to 465 index case households, 2,090 contacts were screened and 1,620 were eligible (Figure 6.1). Of these, 1,347 (83%) contacts of 462 index cases gave informed consent for inclusion in the study. The whole genome sequencing was available on 414 index case isolates linked to 1,201 contacts. The remaining 48 index cases were not included because they did not have a culture submitted (n=13),

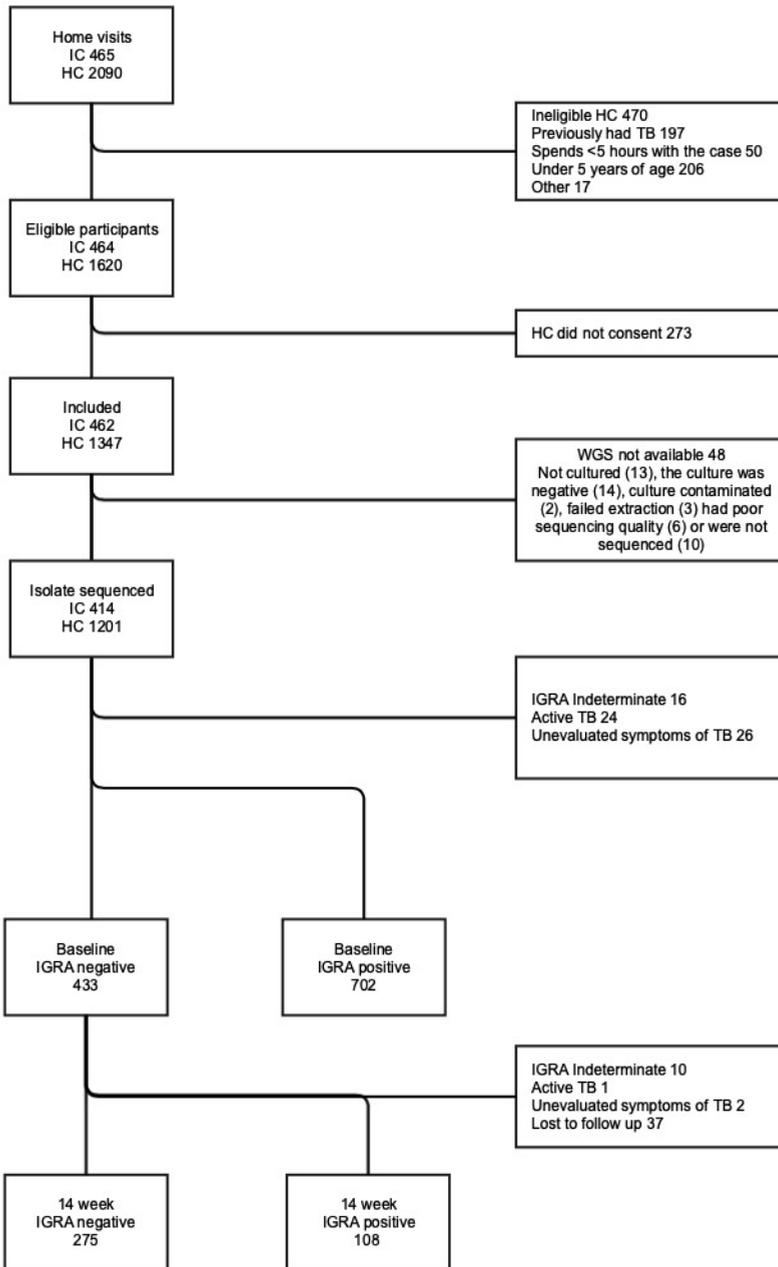


Figure 6.1. Recruitment and enrolment of participants into the study and outcome of participants included in the study.

the culture was negative (n=14), or contaminated (n=2), failed extraction (n=3) had poor sequencing quality (n=6) or were not sequenced (n=10). Contacts were classified into three groups as: baseline IGRA positive (n=709), persistently IGRA negative (n=277), and IGRA converters (n=108). For the 414 isolates that were whole genome sequenced, the median coverage depth was 96x, (range 15-363) and the median percentage of bases covered by at least one read was 99.2%.

The three classified groups of case contacts were similar with respect to median age, sex and duration of treatment of their respective index cases and with respect to their sex, smoking history and diabetes measurements (Table 6.1). However, persistently IGRA negative contacts had relatively lower measures of exposure to *M. tuberculosis* and, along with converters, were younger than those who were initially IGRA positive.

Of the sequenced index case isolates, 260 (62.8%) belonged to the Euro-American lineage, 133 (32.1%) were East-Asian (Beijing), seven (1.7%) were East-Asian lineage but not from the Beijing lineage and 14 (3.4%) were from the Indo-Oceanic lineage (Figure 6.2). Fifty-five (41%) of the East-Asian Beijing genotype isolates were ancient Beijing isolates, and 78 (59%) were modern Beijing isolates. Two (Euro-American) isolates differed by only ten SNPs, and could have been part of the same transmission cluster³⁰.

The clinical or radiological characteristics of index cases were similar for those infected with Beijing isolates compared to those infected with non-Beijing isolates (Table 6.2). Beijing isolates were more likely to be multi-drug resistant than non-Beijing isolates (5.3 vs. 1.8% respectively, $P=0.046$). Table S6.1 summarizes drug resistance mutations according to lineage. Patients infected with Beijing and non-Beijing isolates were similar with respect to the smear grade and presence of cavities. There was no difference in the dominant abnormality in each lung zone, according to lineage (Table S6.2).

Table 6.1. Characteristics of 1,085 contacts and their index case according to the contact's IGRA result

| Characteristic | Baseline IGRA Positive | Baseline IGRA Negative | |
|---|------------------------|------------------------|------------------------------------|
| | (N=702) | IGRA converter (N=108) | IGRA Persistently Negative (N=275) |
| Contact's index case^a | | | |
| Age (years) median (IQR) | 37.6 (28.6 - 49.8) | 38.4 (28.7 - 50.8) | 40.9 (30.5 - 51.9) |
| Female | 323 (46.0) | 56 (51.9) | 144 (52.4) |
| Days of treatment | | | |
| None | 574 (81.8) | 87 (80.6) | 224 (81.5) |
| <7 | 115 (16.4) | 20 (18.5) | 46 (16.7) |
| 7 to 13 | 13 (1.9) | 1 (0.9) | 5 (1.8) |
| Diabetes ^b | 169 (28.8) | 36 (37.9) | 84 (33.5) |
| Current smoker ^b | 66 (11.2) | 11 (11.6) | 31 (12.4) |
| HIV ^c | 1 (0.2) | 2 (2.2) | 0 (0.0) |
| Temperature ^b (C), median (IQR) | 36.8 (36.4 - 37.3) | 36.8 (36.4 - 37.3) | 36.8 (36.4 - 37.4) |
| X-ray ^d cavities (present) | 404 (59.5) | 55 (51.9) | 121 (46.7) |
| Extent of chest x-ray disease, median (IQR) | 50.0 (25.0 - 75.0) | 40.0 (25.0 - 55.0) | 40.0 (25.0 - 60.0) |
| Sputum smear grade | | | |
| 3+ | 345 (49.1) | 60 (55.6) | 90 (32.7) |
| 2+ | 199 (28.3) | 26 (24.1) | 76 (27.6) |
| Scanty/1+ | 158 (22.5) | 22 (20.4) | 109 (39.6) |
| Any drug resistance | 77 (11.0) | 17 (15.7) | 30 (10.9) |
| MDR | 17 (2.4) | 2 (1.9) | 10 (3.6) |
| Beijing lineage | 246 (35.0) | 39 (36.0) | 69 (25.0) |
| Contacts | | | |
| Hours with the case median (IQR) | 5.0 (2.0 - 10.0) | 5.0 (3.0 - 8.0) | 3.0 (1.0 - 7.0) |
| Sleep proximity to index case | | | |
| Same room | 482 (68.7) | 88 (81.5) | 216 (78.5) |
| Different room | 220 (31.3) | 20 (18.5) | 59 (21.5) |
| Age (years) median (IQR) | 30 (16 - 46) | 23 (15 - 35) | 23 (13 - 41) |
| Female | 408 (58.1) | 57 (52.8) | 149 (54.2) |
| BCG vaccination | 539 (76.8) | 80 (74.1) | 240 (87.3) |

Table 6.1. Continued.

| Characteristic | Baseline IGRA Positive | Baseline IGRA Negative | |
|-------------------------------------|------------------------|------------------------|------------------------------------|
| | (N=702) | IGRA converter (N=108) | IGRA Persistently Negative (N=275) |
| Smoking history | | | |
| Never smoked | 458 (65.2) | 70 (65.4) | 194 (70.5) |
| Quit >6 months ago | 30 (4.3) | 3 (2.8) | 13 (4.7) |
| Current smoker | 214 (30.5) | 34 (31.8) | 68 (24.7) |
| BMI (kg/m ²), mean (SD) | 21.9 (5.3) | 21.6 (5.3) | 21.0 (5.0) |
| Diabetes ^e | | | |
| No diabetes | 637 (90.7) | 96 (88.9) | 253 (92.0) |
| Pre-diabetes | 44 (6.3) | 7 (6.5) | 13 (4.7) |
| Diabetes | 21 (3.0) | 5 (4.6) | 9 (3.3) |

Figures are n (%) unless otherwise indicated.

^aIndex cases may be represented multiple times if they have more than one contact.

^bIndex case smoking, diabetes and temperature available for index cases of 1,036 contacts.

^cHIV result available for index cases of 1,025 contacts.

^dX-ray available for detailed reading in index cases of 1,158 contacts.

^eDiabetes defined as follows: No diabetes: RCBG<101 or HbA1c<5.7%; Pre-diabetes: HbA1c 5.7%-6.4%; Diabetes: HbA1c>6.5%

Abbreviations: PN: persistently negative; IGRA: interferon-gamma release assay; BMI: body mass index; HIV: human immunodeficiency virus; BCG: Bacillus Calmette–Guérin. SD: standard deviation; IQR: interquartile range; IGRA: interferon-gamma release assay

Index isolate and case contact IGRA results at follow-up

In a multivariable model, the relative risk (RR) of IGRA conversion was 1.39 (95% CI 1.00 – 1.93; $p=0.048$) for contacts exposed to Beijing versus non-Beijing isolates (Table 6.3). Risk of conversion was also increased in contacts exposed to high smear grade index cases and those who spent longer with the case, and was decreased in those who were BCG vaccinated. The effect of BCG vaccination was modified by the lineage of the isolate the contact was exposed to ($p_{\text{interaction}}=0.01$). BCG showed strong protection for contacts exposed to non-Beijing lineages (RR 0.40; 95% CI 0.27 - 0.61; $p<0.001$), whereas no protection was observed for contacts exposed to Beijing lineage (RR=1.02; 95% CI 0.56 – 1.85; $p=0.9$) (Table 6.4).

Table 6.2. Clinical and x-ray characteristics of 414 index cases according to lineage.

| Characteristic | Non-Beijing (N=282) | Beijing (N=132) | Total | P-value |
|------------------------------|------------------------|--------------------|----------------|---------|
| Age (years) | 40.7 (14.6) | 38.2 (12.9) | 39.9 (14.1) | 0.09 |
| Female sex | 139 (49.3) | 55 (41.7) | 194 (46.9) | 0.1 |
| Cough | 277 (98.2) | 131 (99.2) | 408 (98.6) | 0.4 |
| HIV | 0 (0.0) | 1 (0.9) | 1 (0.3) | 0.1 |
| Diabetes | 68 (27.8) | 34 (30.6) | 102 (28.7) | 0.5 |
| Current smoker | 28 (11.4) | 10 (9.0) | 38 (10.7) | 0.5 |
| Temperature | 36.9 (0.8) | 36.8 (0.7) | 36.9 (0.8) | 0.3 |
| Sputum smear grade | | | | 0.793 |
| 3+ | 85 (30.1) | 38 (28.8) | 123 (29.7) | |
| 2+ | 76 (27.0) | 36 (27.3) | 112 (27.1) | |
| Scanty/1+ | 121 (42.9) | 58 (43.9) | 179 (43.2) | |
| Any drug resistance | 40 (14.2) | 19 (14.4) | 59 (14.3) | 0.9 |
| MDR | 5 (1.8) | 7 (5.3) | 12 (2.9) | 0.046 |
| Cavities | 142 (52.4) | 70 (53.8) | 212 (52.9) | 0.8 |
| Temperature | 45.0 (25.0-70) | 40.0 (25.0-65) | 45.0 (25.0-70) | 0.3 |
| Hilar adenopathy | 50 (18.5) | 25 (19.2) | 75 (18.7) | 0.9 |
| Aorto pulmonary window | 42 (15.5) | 24 (18.5) | 66 (16.5) | 0.5 |
| Pleural effusion | 23 (8.5) | 7 (5.4) | 30 (7.5) | 0.3 |
| Pleural thickening | 14 (5.2) | 7 (5.4) | 21 (5.2) | 0.9 |
| Pleural calcification | 3 (1.1) | 0 (0.0) | 3 (0.7) | 0.2 |
| Cardiomegaly | 2 (0.7) | 0 (0.0) | 2 (0.5) | 0.3 |
| Tracheal deviation | 28 (10.3) | 18 (13.8) | 46 (11.5) | 0.3 |
| Right upper lobe collapse | 53 (19.6) | 30 (23.1) | 83 (20.7) | 0.4 |
| Left upper lobe collapse | 26 (9.6) | 12 (9.2) | 38 (9.5) | 0.9 |
| Miliary pattern | 3 (1.1) | 2 (1.5) | 5 (1.2) | 0.7 |
| Calcified granuloma | 233 (86.0) | 116 (89.2) | 349 (87.0) | 0.4 |
| Multiple calcified granuloma | 20 (7.4) | 8 (6.2) | 28 (7.0) | 0.7 |

X-ray available on 401 of 414 index cases. Smoking, diabetes and temperature available for 356 index cases. HIV result available for 351 index cases.

Abbreviations: MDR, multidrug-resistant; HIV, human immunodeficiency virus

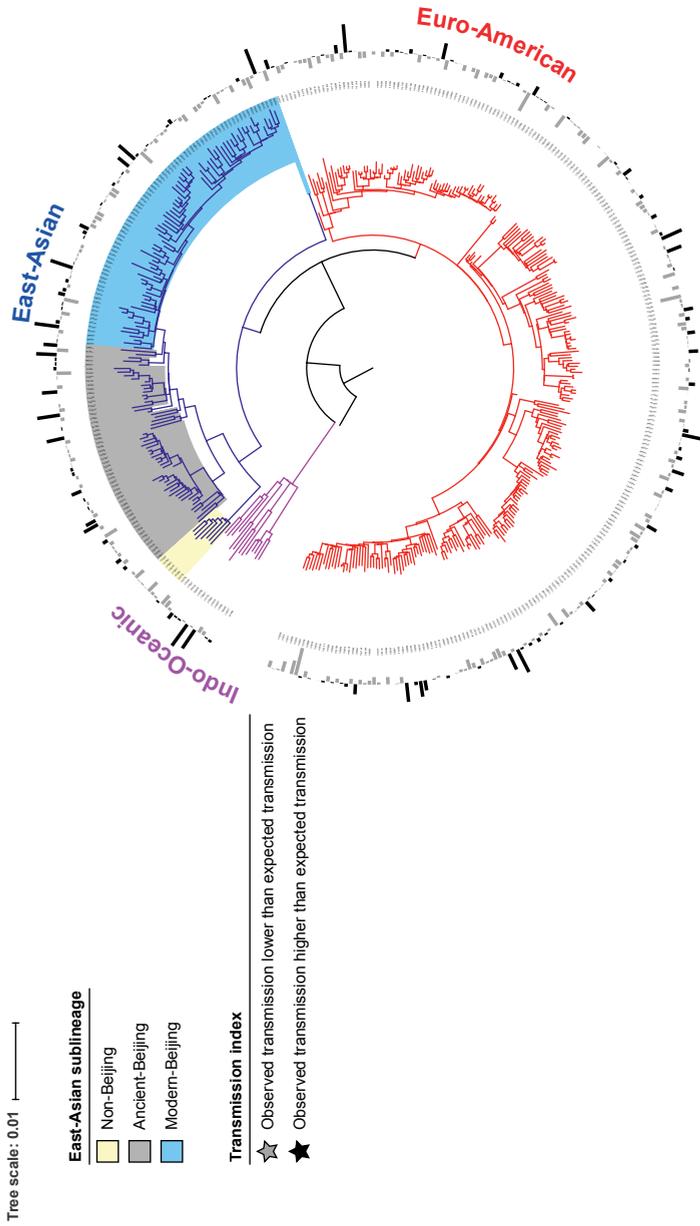


Figure 6.2. Phylogenetic tree of 414 successfully sequenced *M. tuberculosis* isolates with transmission indexes for the 373 isolates with BCG-vaccinated contacts. Bars in the outer ring indicate the transmission indexes.

Sensitivity analysis

The risk of a positive IGRA at either baseline or follow up was increased for contacts of index cases with a Beijing lineage isolate and decreased for BCG-vaccinated contacts (Table S6.3). In a multivariable model the effect of BCG vaccination was modified by the lineage of the index cases' isolate ($p_{\text{interaction}}=0.03$). Contacts of index cases with a non-Beijing lineage had a reduced risk of a positive IGRA if BCG-vaccinated (RR 0.82; 95% CI 0.76 - 0.89; $p<0.001$), whereas BCG vaccination was not associated with protection for contacts of index cases with a Beijing lineage isolate (RR=0.96; 95% CI 0.86 - 1.06; $p=0.4$) (Table S6.4).

Exploratory analysis

Of contacts with a negative IGRA at baseline, 25 (9.1%) were exposed to an ancient Beijing isolate, 44 (16.0%) to a modern Beijing isolate and 206 (74.9%) to non-Beijing isolates. Of contacts who converted, 19 (17.6%) were exposed to an ancient Beijing isolate, 20 (18.5%) to a modern Beijing isolate, and 69 (63.9%) to non-Beijing isolates. At follow up, the risk of IGRA conversion was 1.72 (95% CI 1.02 - 2.89; $P=0.041$) for contacts exposed to an ancient Beijing isolate versus non-Beijing and 1.25 (95% CI 0.77-2.01; $P=0.370$) for contacts exposed to a modern Beijing isolate versus non-Beijing (Table S6.5). Small numbers did not permit analysis of interaction with BCG.

Analysis of *M. tuberculosis* transmissibility

For the calculation of the *M. tuberculosis* transmission index, the total number of included households was 373; we excluded 41 without BCG-vaccinated contacts. We calculated an expected risk of conversion for 880 BCG-vaccinated contacts (BP: n=539; converter: n=80; PN: n=240; active TB: n=21). The mean transmission score for Beijing isolates was slightly higher but not significantly different from the mean transmission score for non-Beijing isolates (0.0037 vs. -0.0323, respectively; $p=0.251$, data not shown). Figure 6.2 shows the transmission indexes along the phylogenetic tree.

Table 6.3. Assessment of risk factors for IGRA conversion in household contacts who were IGRA negative at baseline (n=383)

| | IGRA PN (N=275) | IGRA positive (N=108) | Crude RR | 95% CI | P-value | ARR ^a | 95% CI | P-value |
|---|--------------------|--------------------------|----------|-------------|---------|------------------|-------------|---------|
| Age index ^e | 40.9 (30.3 - 51.9) | 38.4 (28.7 - 50.8) | 1.00 | 0.98 - 1.01 | 0.5 | 1.00 | 0.98 - 1.01 | 0.4 |
| Index case sex | | | | | | | | |
| Female | 144 (52.0) | 56 (51.9) | 1.00 | ref | | | | |
| Male | 133 (48.0) | 52 (48.1) | 1.01 | 0.71 - 1.45 | 0.9 | 0.97 | 0.69 - 1.34 | 0.8 |
| Diabetes index ^b | | | | | | | | |
| No | 167 (66.5) | 59 (62.1) | 1.00 | ref | | | | |
| Yes | 84 (33.5) | 36 (37.9) | 1.15 | 0.77 - 1.71 | 0.491 | | | |
| Smoking index ^b | | | | | | | | |
| Not currently | 220 (87.6) | 84 (88.4) | 1.00 | ref | | | | |
| Currently | 31 (12.4) | 11 (11.6) | 0.95 | 0.52 - 1.72 | 0.9 | | | |
| Cough duration (days) ^e | 30.0 (21.0 - 90.0) | 30.0 (30.0 - 90.0) | 1.00 | 1.00 - 1.00 | 0.9 | | | |
| Temperature ^b (°C) mean (SD) | 36.9 (0.8) | 36.8 (0.7) | 0.90 | 0.71 - 1.15 | 0.4 | | | |
| <i>M. tuberculosis</i> lineage | | | | | | | | |
| Non-Beijing | 208 (75.1) | 69 (63.9) | 1.00 | ref | | | | |
| Beijing | 69 (24.9) | 39 (36.1) | 1.44 | 0.98 - 2.10 | 0.06 | 1.39 | 1.00 - 1.93 | 0.048 |
| Index case highest smear grade | | | | | | | | |
| Scanty+ | 109 (39.4) | 22 (20.4) | 1.00 | ref | | | | |
| 2+ | 76 (27.4) | 26 (24.1) | 1.52 | 0.89 - 2.59 | 0.1 | 1.28 | 0.76 - 2.15 | 0.3 |
| 3+ | 92 (33.2) | 60 (55.6) | 2.38 | 1.48 - 3.83 | <0.05 | 2.26 | 1.43 - 3.59 | 0.001 |
| Any drug resistance | | | | | | | | |
| No | 245 (89.1) | 91 (84.3) | 1.00 | ref | | | | |
| Yes | 30 (10.9) | 17 (15.7) | 1.34 | 0.83 - 2.14 | 0.2 | | | |
| Multidrug resistance | | | | | | | | |
| No | 265 (96.4) | 106 (98.1) | 1.00 | ref | | | | |
| Yes | 10 (3.6) | 2 (1.9) | 0.58 | 0.19 - 1.75 | 0.3 | | | |
| Cavities ^c | | | | | | | | |
| No cavity | 138 (52.9) | 51 (48.1) | 1.00 | ref | | | | |
| Present | 123 (47.1) | 55 (51.9) | 1.16 | 0.82 - 1.64 | 0.4 | | | |

| | | | | | | |
|------------------------------------|--------------------|--------------------|------|-------------|-------|-------------------------|
| Extent x-ray disease ^{tc} | 40.0 (25.0 - 60.0) | 40.0 (25.0 - 55.0) | 1.00 | 0.99 - 1.01 | 0.8 | |
| Age contact ^e | 23.3 (12.6 - 41.5) | 22.8 (14.6 - 35.5) | 0.96 | 0.87 - 1.06 | 0.5 | 0.89 0.79 - 1.00 0.06 |
| Contact sex | | | | | | |
| Female | 150 (54.2) | 57 (52.8) | 1.00 | ref | | |
| Male | 127 (45.8) | 51 (47.2) | 1.04 | 0.75 - 1.45 | 0.8 | 0.99 0.68 - 1.43 0.9 |
| Contact ethnicity | | | | | | |
| Sunda | 254 (91.7) | 97 (89.8) | 1.00 | ref | | |
| Other | 23 (8.3) | 11 (10.2) | 1.03 | 0.95 - 1.11 | 0.5 | |
| Sleep proximity to index | | | | | | |
| Different Room | 218 (78.7) | 88 (81.5) | 1.00 | ref | | |
| Same Room | 59 (21.3) | 20 (18.5) | 0.87 | 0.57 - 1.34 | 0.5 | |
| Hours with Case ^e | 3.0 (1.0 - 7.0) | 5.0 (3.0 - 8.0) | 1.06 | 1.02 - 1.10 | 0.004 | 1.08 1.03 - 1.12 <0.001 |
| BCG vaccination | | | | | | |
| No | 35 (12.6) | 28 (25.9) | 1.00 | ref | | |
| Yes | 242 (87.4) | 80 (74.1) | 0.56 | 0.40 - 0.80 | 0.001 | 0.55 0.39 - 0.77 0.001 |
| Smoking | | | | | | |
| Non smoker | 208 (75.1) | 73 (68.2) | 1.00 | ref | | |
| Current smoker | 69 (24.9) | 34 (31.8) | 1.28 | 0.92, 1.78 | 0.1 | 1.43 0.97, 2.09 0.07 |
| Diabetes ^d | | | | | | |
| No diabetes | 254 (91.7) | 96 (88.9) | 1.00 | ref | | |
| Pre-diabetes | 14 (5.1) | 7 (6.5) | 1.27 | 0.68, 2.40 | 0.456 | |
| Diabetes | 9 (3.2) | 5 (4.6) | 1.30 | 0.63, 2.67 | 0.479 | |
| BMI ^e | 20.2 (17.0 - 24.3) | 21.0 (17.4 - 24.5) | 1.02 | 0.98, 1.05 | 0.337 | 1.03 1.00, 1.07 0.059 |

All figures are N (%) unless otherwise stated. Index cases maybe represented multiple times if they have more than one contact.

^aEstimates obtained by multiple regression, adjusted for index case age, sex and smear grade, and contact age, sex, hours spent with index case smoking and body mass index.

^bindex case smoking, diabetes and temperature available for index cases of 346 contacts.

^cx-ray available for detailed reading in index cases of 365 contacts.

^dDiabetes defined as follows: No diabetes: RCBG<101 or HbA1c<5.7%; Pre-diabetes: HbA1c 5.7-6.4%; Diabetes: HbA1c≥6.5%.

^eMedian (interquartile range).

Abbreviations: PN: persistently negative; IGRA: interferon-gamma release assay; RR: relative risk; ARR: adjusted relative risk; 95% CI: 95% confidence interval; BCG: Bacillus Calmette-Guérin.

Table 6.4. Association between BCG vaccination and IGRA at 14 weeks for those negative at baseline, according to index case lineage

| Lineage | BCG vaccination | IGRA PN N=275 | IGRA positive N=108 | RR ^a | 95% CI | P-value |
|-------------|-----------------|------------------|------------------------|-----------------|-------------|---------|
| Non-Beijing | No | 22 | 21 | 1.00 | ref | |
| | Yes | 184 | 48 | 0.40 | 0.27 - 0.61 | <0.001 |
| Beijing | No | 13 | 7 | 1.00 | ref | |
| | Yes | 56 | 32 | 1.02 | 0.56 - 1.85 | 0.946 |

*Data are presented as N (%) unless indicated otherwise.

^aEstimates obtained by multiple regression, adjusted for index case age, sex and smear grade, and contact age, sex, hours spent with index case smoking and body mass index.

Abbreviations: PN: persistently negative; IGRA: interferon-gamma release assay; BCG: Bacillus Calmette–Guérin; IGRA: interferon-gamma release assay; RR: relative risk calculated as risk ratios; 95% CI: 95% confidence interval.

Discussion

Here we have shown that both pathogen and host factors affect *M. tuberculosis* transmission. TB household contacts exposed to index cases infected with an *M. tuberculosis* Beijing isolate had a higher risk of becoming infected compared to those exposed to non-Beijing isolates. In addition, a history of BCG vaccination among TB household contacts was associated with a 60% lower risk of *M. tuberculosis* infection caused by non-Beijing isolates, but not with a lower risk of infection by Beijing family isolates. This relationship was independent of the presence of drug resistance mutations, or index case cavities or sputum smear grade. Therefore transmission of Beijing family isolates most likely arises from its ability to evade BCG-mediated protection in contacts, and not by inducing a more infectious disease phenotype in the index case³¹ or through its association with drug resistance.

People have speculated that BCG vaccination beginning in the mid 20th century drove the evolution of the virulent modern Beijing lineage^{32,33}. We observed that the isolates' transmissibility varied considerably along the phylogeny and within the Beijing lineage, and we did not find evidence of an evolutionary event after which the isolates became more transmissible (Figure 6.2). Rather, transmissibility seems to be a convergent trait, which has evolved independently multiple times. In addition, we did not find evidence to suggest that evasion of BCG protection was specific to modern Beijing family isolates. In fact, some studies have suggested that the evolution of

modern lineages predates the widespread use of BCG. Still, evasion of BCG-mediated immunity could contribute to the global emergence of the Beijing lineage. It may also contribute to the variability in estimates for protective effectiveness of BCG in different locations.

Our finding of an increased prevalence of *M. tuberculosis* infection in Indonesian case contacts of index cases with Beijing lineage isolates compared to others, mostly Euro-American lineage isolates, is consistent with the global emergence of the Beijing lineage over recent decades^{10-13,34}. However, the results of the few case contact studies that have assessed the impact of the pathogen on transmission are not consistent. In a cohort study of patients with TB and their household contacts in The Gambia, no difference in transmission between the MTBC lineages was observed³⁵; in addition, the ratio of infected child contacts among those exposed to a Beijing strain did not differ from those exposed to a non-Beijing strain in South-Africa³⁶; and the Beijing genotype was not associated with a higher number of tuberculin skin test (TST)-positive contacts in The Netherlands³⁷. In these countries however, the prevalence of the Beijing lineage is low (3 to 5% in Central America, Europe, and Africa³⁸) and the estimates for the Beijing lineage may lack precision. A recent study of households in Guangxi, China, where the prevalence of Beijing genotype strains is higher (as high as 45% in Far East Asia³⁸), showed that exposure to a Beijing strain was independently associated with a positive TST³⁹. The discrepancies between results from these studies might also result from differences in how and if host-related factors were controlled for.

Another method that has been used to study transmission is investigating transmission chains, usually by clustering strains based on genotype similarities. Several studies have found increased clustering of Beijing lineage isolates, assessed by whole genome sequencing^{10,13,40} or VNTR genotyping⁴¹. In contrast, two studies performed in low prevalence countries have not found increased clustering associated with the Beijing lineage^{42,43}. The Beijing genotype consists of a number of sub-lineages and so the discrepant findings could be due to the prevalence of different sub-lineages in the different study populations, as suggested previously⁴⁴. Within the Beijing lineage, modern Beijing strains were more often in a cluster than ancient Beijing strains in a study of 376 MDR-*M. tuberculosis* strains in China⁴⁵, and a study conducted in South Africa found significantly more clustering linked to recently evolved sub-lineages of the Beijing strain family compared to other sub-lineages⁴⁶. These studies have inferred transmission from the frequency of clustered isolates in surveillance culture collections where clinical and exposure data on cases are often limited. Furthermore, clustering in such studies may arise from more efficient *transmission* of a particular isolate but also from differences in *progression* of latent TB infection to active disease. A strength of

the present study is the direct measurement of *transmission*, defined as passing infection from index case to contact, and the ability to find detailed data on the index, contacts and their exposure to one another, at a level of detail that is not feasible for studies of clustered isolates. The prospectively defined groups enabled better measurement of confounding factors and accounted for misclassification of baseline positive patients, whose source of infection might have been different than the household index case⁴⁷, and whose infection might have happened in the distant past.

A study has found that BCG vaccination and BMI were risk factors for clustering among Beijing strains in China⁴⁸; both factors associated with a persistently negative IGRA in the present study. Adaptation of *M. tuberculosis* Beijing strains to their East-Asian host population as a result of host-pathogen co-evolution could have explained their higher transmissibility⁴⁹⁻⁵¹, but is unlikely to have caused the differential protective effect of BCG. It is difficult to speculate on the mechanism through which Beijing lineage strains evade BCG-mediated protection, when the mechanism of BCG protection itself is unknown. Beijing lineage strains could evade host immune response in the contact by evading recognition and reducing the pro-inflammatory cytokine production during infection, as seen in *ex vivo* studies⁵²⁻⁵⁴. In this cohort we previously showed that a persistently negative IGRA was associated with heterologous cytokine responses, similar to those activated during induction of trained innate immunity⁵⁵.

Without a safe human challenge model for tuberculosis, variation in BCG efficacy according to *M. tuberculosis* lineage cannot be assessed in experimental studies. Results from observational studies such as this need to be considered in light of their potential limitations. We determined BCG vaccination status by assessing the presence of a scar, as it is the most reliable method when dealing with a mixed age cohort. A minority of BCG vaccine recipients do not form a scar when administered at birth. Although this may introduce a bias whereby vaccination status inferred from scar formation leads to an underestimate of the effectiveness of BCG⁵⁶, it is not plausible that this misclassification differs according to the *M. tuberculosis* lineage of the index case, which was not determined until after the study concluded. Similarly, the IGRA is not a perfect test for *M. tuberculosis* infection, but there is no evidence that its performance differs according to infecting *M. tuberculosis* genotype. We have previously reported that more stringent definitions of conversions did not alter estimates of BCG mediated protection²⁹. A sensitivity analysis that included participants with a positive IGRA at baseline replicated the main finding, indicating that the exclusion of these participants from the primary analysis was not an important source of bias. Finally, the distribution of lineages and size of the study meant only an analysis of Beijing lineage versus all other lineages was possible.

In summary, *M. tuberculosis* Beijing genotype strains are associated with more transmission and may evade BCG-induced immunity. Whether this finding is generalizable to other ethnic host populations warrants further investigation. The impact of BCG vaccination on TB transmission may depend on the *M. tuberculosis* lineage distribution in the setting it is used. Ultimately, understanding the underlying mechanism of BCG evasion by Beijing family isolates could advance the knowledge on the development of new vaccines.

Supplementary tables

Table S6.1. Drug resistance mutations summarized per gene in Beijing and non-Beijing *M. tuberculosis* isolates

| Drug | Gene containing mutation(s) | Beijing (N=133) | Non-Beijing (N=281) |
|--------------------------|-----------------------------|-----------------|---------------------|
| Isoniazid | <i>katG</i> | 10 (8%) | 14 (5%) |
| | <i>Rv1482c-fabG1</i> | 2 (2%) | 13 (5%) |
| | <i>ahpC</i> | 1 (1%) | 0 |
| Rifampicin | <i>rpoB</i> | 9 (7%) | 6 (2%) |
| Ethambutol | <i>embB</i> | 5 (4%) | 3 (1%) |
| | <i>embC-embA</i> | 0 | 1 (0.4%) |
| Pyrazinamide | <i>pncA</i> | 4 (3%) | 13 (5%) |
| Streptomycin | <i>rpsL</i> | 5 (4%) | 1 (0.4%) |
| | <i>rrs</i> | 2 (2%) | 0 |
| | <i>gid</i> | 0 | 2 (1%) |
| Ethionamide | <i>Rv1482c-fabG1</i> | 2 (2%) | 13 (5%) |
| | <i>ethA</i> | 0 | 2 (1%) |
| Fluoroquinolones | <i>gyrA</i> | 2 (2%) | 1 (0.4%) |
| Amikacin | <i>rrs</i> | 1 (1%) | 0 |
| Capreomycin | <i>rrs</i> | 1 (1%) | 0 |
| | <i>tlyA</i> | 0 | 2 (1%) |
| Kanamycin | <i>eis-Rv2417c</i> | 1 (1%) | 0 |
| | <i>rrs</i> | 1 (1%) | 0 |
| Para-aminosalicylic acid | <i>folC</i> | 1 (1%) | 1 (0.4%) |

Data represent the number (%) of isolates with at least one known drug resistance-conferring mutation in the respective gene.

Table S6.2. Dominant x-ray abnormality according to lung zone and lineage for 401 index cases

| Dominant x-ray abnormality | Non-Beijing | | | | Beijing | | | |
|----------------------------|-------------|-------------|------------|-------------|-----------|-------------|-----------|-------------|
| | Any N (%) | Upper N (%) | Mid N (%) | Lower N (%) | Any N (%) | Upper N (%) | Mid N (%) | Lower N (%) |
| None | 2 (0.7) | 21 (7.7) | 32 (11.8) | 89 (32.8) | 2 (1.5) | 19 (14.6) | 26 (20.0) | 53 (40.8) |
| Cavity | 142 (52.4) | 112 (41.3) | 49 (18.1) | 6 (2.2) | 70 (53.8) | 60 (46.2) | 18 (13.8) | 2 (1.5) |
| Consolidation | 51 (18.8) | 42 (15.5) | 42 (15.5) | 35 (12.9) | 25 (19.2) | 18 (13.8) | 27 (20.8) | 19 (14.6) |
| Patchy Lesion | 74 (27.3) | 89 (32.8) | 137 (50.6) | 134 (49.4) | 32 (24.6) | 32 (24.6) | 56 (43.1) | 52 (40.0) |
| Nodules | 1 (0.4) | 1 (0.4) | 2 (0.7) | 1 (0.4) | 1 (0.8) | 1 (0.8) | 2 (1.5) | 2 (1.5) |
| Bullae | 1 (0.4) | 6 (2.2) | 9 (3.3) | 6 (2.2) | 0 (0.0) | 0 (0.0) | 1 (0.8) | 2 (1.5) |

Table S6.3. Assessment of risk factors for positive IGRA at baseline or follow-up (N=1,112)

| | IGRA PN (N=276) | Any IGRA positive (N=836) | RR | 95% CI | P-value | APR ^a | 95% CI | P-value |
|------------------------------------|--------------------|---------------------------|------|-------------|---------|------------------|-------------|---------|
| Age index ^b | 40.9 (30.5 - 51.9) | 37.6 (28.6 - 49.8) | 1.00 | 1.00 - 1.00 | 0.267 | 1.00 | 1.00 - 1.00 | 0.302 |
| Index case sex | | | | | | | | |
| Female | 145 (52.5) | 392 (46.9) | 1.00 | ref | | | | |
| Male | 131 (47.5) | 444 (53.1) | 1.06 | 0.96 - 1.16 | 0.232 | 1.03 | 0.95 - 1.12 | 0.43 |
| Diabetes index | | | | | | | | |
| No | 167 (66.3) | 495 (70.2) | 1.00 | ref | | | | |
| Yes | 85 (33.7) | 210 (29.8) | 0.95 | 0.85 - 1.07 | 0.412 | | | |
| Smoking index | | | | | | | | |
| Not currently | 220 (87.3) | 628 (89.1) | 1.00 | ref | | | | |
| Currently | 32 (12.7) | 77 (10.9) | 0.95 | 0.82 - 1.12 | 0.556 | | | |
| Cough duration (days) ^b | 30.0 (21.0 - 90.0) | 30.0 (30.0 - 90.0) | 1.00 | 1.00 - 1.00 | 0.658 | | | |
| <i>M. tuberculosis</i> lineage | | | | | | | | |
| Non-Beijing | 207 (75.0) | 540 (64.6) | 1.00 | ref | | | | |
| Beijing | 69 (25.0) | 296 (35.4) | 1.12 | 1.02 - 1.23 | 0.014 | 1.10 | 1.01 - 1.19 | 0.028 |
| Index case highest smear grade | | | | | | | | |
| Scanty [†] | 109 (39.5) | 184 (22.0) | 1.00 | ref | | | | |
| 2+ | 77 (27.9) | 236 (28.2) | 1.20 | 1.04 - 1.38 | 0.011 | 1.10 | 0.96 - 1.26 | 0.151 |
| 3+ | 90 (32.6) | 416 (49.8) | 1.31 | 1.15 - 1.49 | <0.05 | 1.23 | 1.09 - 1.39 | 0.001 |
| Any drug resistance | | | | | | | | |
| No | 246 (89.1) | 740 (88.5) | 1.00 | ref | | | | |
| Yes | 30 (10.9) | 96 (11.5) | 1.02 | 0.89 - 1.16 | 0.825 | | | |
| Multidrug resistance | | | | | | | | |
| No | 266 (96.4) | 816 (97.6) | 1.00 | ref | | | | |
| Yes | 10 (3.6) | 20 (2.4) | 0.88 | 0.70 - 1.11 | 0.296 | | | |
| Cavities | | | | | | | | |
| No cavity | 138 (53.1) | 334 (41.3) | 1.00 | ref | | | | |
| Present | 122 (46.9) | 475 (58.7) | 1.12 | 1.02 - 1.23 | 0.014 | 1.08 | 0.99 - 1.19 | 0.082 |

| | | | | | | | | |
|-----------------------------------|--------------------|--------------------|------|-------------|--------|------|-------------|-------|
| Extent x-ray disease ^b | 40.0 (25.0 - 60.0) | 45.0 (25.0 - 70.0) | 1.00 | 1.00 - 1.00 | 0.075 | 1.00 | 0.98 - 1.02 | 0.939 |
| Age contact ^b | 23.3 (12.5 - 41.4) | 28.9 (15.1 - 45.6) | 1.02 | 1.00 - 1.04 | 0.045 | 1.00 | 0.98 - 1.02 | 0.939 |
| Contact sex | | | | | | | | |
| Female | 149 (54.0) | 479 (57.3) | 1.00 | ref | | | | |
| Male | 127 (46.0) | 357 (42.7) | 0.97 | 0.90 - 1.04 | 0.35 | 0.97 | 0.88 - 1.05 | 0.433 |
| Contact ethnicity | | | | | | | | |
| Sunda | 254 (92.0) | 768 (91.9) | 1.00 | ref | | | | |
| Other | 22 (8.0) | 68 (8.1) | 1.00 | 0.98 - 1.02 | 0.933 | | | |
| Sleep proximity | | | | | | | | |
| Different room | 217 (78.6) | 586 (70.1) | 1.00 | ref | | | | |
| Same room | 59 (21.4) | 250 (29.9) | 1.11 | 1.03 - 1.19 | <0.001 | 1.07 | 1.00 - 1.15 | 0.055 |
| Hours with Case ^b | 3.0 (1.0 - 7.0) | 5.0 (2.0 - 10.0) | 1.02 | 1.01 - 1.03 | <0.001 | 1.02 | 1.01 - 1.03 | <0.05 |
| BCG vaccination | | | | | | | | |
| No | 35 (12.7) | 197 (23.6) | 1.00 | ref | | | | |
| Yes | 241 (87.3) | 639 (76.4) | 0.86 | 0.80 - 0.92 | <0.05 | 0.87 | 0.81 - 0.93 | <0.05 |
| Smoking | | | | | | | | |
| Non Smoker | 208 (75.4) | 578 (69.2) | 1.00 | ref | | | | |
| Current smoker | 68 (24.6) | 257 (30.8) | 1.08 | 1.00 - 1.15 | 0.042 | 1.10 | 1.01 - 1.20 | 0.026 |
| Diabetes ^c | | | | | | | | |
| No diabetes | 254 (92.0) | 757 (90.5) | 1.00 | ref | | | | |
| Pre-diabetes | 13 (4.7) | 53 (6.3) | 1.07 | 0.94 - 1.22 | 0.282 | | | |
| Diabetes | 9 (3.3) | 26 (3.1) | 0.99 | 0.81 - 1.21 | 0.938 | | | |
| BMI ^b | 20.2 (17.0 - 24.3) | 21.5 (17.8 - 25.2) | 1.01 | 1.00 - 1.01 | 0.017 | 1.01 | 1.00 - 1.01 | 0.068 |

*Data are presented as N (%) unless indicated otherwise.

^aEstimates obtained by multiple regression, adjusted for index case age, sex and smear grade, cavities and contact age, sex, sleep proximity to index case, hours spent with index case, smoking and body mass index.

^bMedian, interquartile range.

Abbreviations: PN: persistently negative; IGRA: interferon-gamma release assay; BCG: Bacillus Calmette–Guérin; IGRA: interferon-gamma release assay; APR: adjusted prevalence ratio calculated; 95% CI: 95% confidence interval.

Table S6.4. Association between BCG vaccination and IGRA at baseline and 14 weeks, according to index case lineage

| Lineage | BCG vaccination | IGRA-negative N=275 | IGRA-positive N=108 | APR ^a | 95% CI | P-value |
|-------------|-----------------|------------------------|------------------------|------------------|-------------|---------|
| Non-Beijing | No | 22 | 131 | 1.00 | ref | |
| | Yes | 185 | 409 | 0.82 | 0.76 - 0.89 | <0.001 |
| Beijing | No | 13 | 66 | 1.00 | ref | |
| | Yes | 56 | 230 | 0.96 | 0.86 - 1.06 | 0.4 |

^aEstimates obtained by multiple regression, adjusted for index case age, sex and smear grade, cavities and contact age, sex, sleep proximity to index case, hours spent with index case, smoking and body mass index. Abbreviations: BCG: Bacillus Calmette–Guérin; IGRA: interferon-gamma release assay; APR: adjusted prevalence ratio; 95% CI: 95% confidence interval.

Table S6.5. Association between the index cases' *M. tuberculosis* lineage and IGRA conversion in household contacts who were IGRA negative at baseline (N=383)

| | PN | Converter | RR | 95% CI | P-value |
|-----------------|------------|-----------|------|-------------|---------|
| Other isolates | 206 (74.9) | 69 (25.1) | 1,00 | ref | |
| Ancient Beijing | 25 (56.8) | 19 (43.2) | 1,72 | 1,02 - 2,89 | 0,041 |
| Modern Beijing | 44 (68.8) | 20 (31.3) | 1,25 | 0,77 - 2,01 | 0,37 |

*Abbreviations: PN: persistently negative; RR: relative risk; CI: confidence interval.

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7

General discussion

General discussion

The two parts of this thesis are discussed separately, followed by an outlook on the present and future challenges and opportunities of whole genome sequencing of *M. tuberculosis*.

Part one: drug resistance

The World Health Organization recommends drug-susceptibility testing of *M. tuberculosis* complex (MTBC) isolates for all patients with tuberculosis to guide treatment decisions and improve outcomes. Still, it is estimated that only 29% of the 558,000 people (range 483,000 – 639,000) who developed multidrug/rifampicin-resistant tuberculosis in 2017 were detected and notified to national tuberculosis programmes¹. These data come from 91 countries that have continuous surveillance systems, and 69 countries that rely on epidemiological surveys of bacterial isolates collected from representative samples of patients. In resource-limited settings where routine drug-susceptibility testing is not accessible to all tuberculosis patients owing to lack of laboratory capacity or resources, surveys conducted about every five years represent the most common approach to investigate the burden of drug resistance¹. In the first part of my thesis I have shown that whole genome sequencing is not only a promising alternative to phenotypic drug susceptibility testing in the diagnosis of drug-resistant tuberculosis, but that it can also guide individualized clinical decision-making, and add to our understanding of the epidemiology of drug-resistant tuberculosis.

Performance of genotypic compared to phenotypic drug susceptibility testing

Whole genome sequencing is an ideal technique for drug-susceptibility prediction in *M. tuberculosis*, in which drug resistance is largely determined by chromosomal mutations, especially now the costs of sequencing have come down substantially and tools to facilitate the analysis of whole genome sequencing data are becoming increasingly available^{2,3}. In **Chapter 2** of this thesis we showed that in a highly endemic setting in Indonesia the agreement of whole genome sequencing-based resistance prediction and phenotypic testing was high for isoniazid and rifampicin and lower for ethambutol and streptomycin. Recently, a large consortium showed, based on the analysis of more than 10,000 *M. tuberculosis* isolates collected from 16 countries across six continents and representing all major lineages, that whole genome sequencing can characterize susceptibility profiles to first-line antituberculosis drugs with high enough accuracy to be used in clinical practice⁴. The WHO targets of 90% sensitivity and 95% specificity for new molecular assays for *M. tuberculosis* were met

for all drugs, except the specificity for ethambutol. This study, like ours and most other studies, evaluated the performance of whole genome sequencing for drug susceptibility testing compared to phenotypic testing as the gold standard, but phenotyping is an imperfect standard^{5,6}, as we also showed in **Chapter 3**. Currently established procedures for *M. tuberculosis* phenotypic drug susceptibility testing classify clinical isolates as either drug-‘resistant’ or drug-‘susceptible’, on the basis of their ability to grow in the presence of a (mostly single) ‘critical drug concentration’⁶, defined as the lowest drug concentration that inhibits $\geq 95\%$ of wild-type strains of bacilli that have not been exposed to the drug previously. This definition of drug susceptibility is based on laboratory tests, and patients with strains that are flagged resistant in this manner do not necessarily fail to respond to treatment⁷. In addition, breakpoint artefacts, where critical concentrations are set too high, have been reported as a major cause for systematic phenotypic drug susceptibility testing errors⁵. Our findings in **Chapter 3** that mutations associated with ethambutol and streptomycin resistance only moderately increased the minimum inhibitory concentration and that breakpoints were set too high, could explain the discrepant results between genotypic and phenotypic drug susceptibility testing for these drugs observed in **Chapter 2**. Therefore, discrepancies between genotypic and phenotypic drug susceptibility testing should be studied cautiously and not automatically be judged in favour of the phenotypic method.

Precision medicine

Individualization of therapy based on whole genome sequencing for the treatment of drug-resistant tuberculosis will require an extensive knowledgebase in which the effect of genomic mutations (and their combinations) on all relevant drugs is characterized. In addition, a clear understanding of what the molecular-level data are predicting is essential. Studies to date have tried to link mutations to minimum inhibitory concentrations^{8,9}, or to a probability of one of two outcomes - phenotypic resistance or susceptibility¹⁰ for certain drugs. In **Chapter 3** we linked whole genome sequence-determined mutations to minimum inhibitory concentrations for all first- and most of the second-line drugs and found that different mutations lead to different levels of resistance; knowing the underlying mutations can guide clinical decision-making and facilitate therapeutic drug monitoring. The ultimate aim would be for molecular data to predict treatment outcome directly. The clinical implications of some mutations known to lead to high-level isoniazid (*katG* S315T), rifampicin (*rpoB* S450L) and fluoroquinolone (*gyrA* 94) resistance and treatment failure have been outlined in a consensus statement¹¹, and are in line with our findings. The data we presented in **Chapter 3** furthermore indicated that particular mutations in *rpoB* only cause a slight increase in the minimum inhibitory concentration for rifampicin and might justify the prescription of high-dose rifampicin in patients with these mutations. Similarly, a

recent paper by Farhat *et al.* discovered that promotor mutations have smaller average effects on resistance than gene body mutations⁹ and may be overcome by increased dosing. This way, whole genome sequencing can ultimately be used to personalize tuberculosis treatment, based on the detailed characterisation of the resistance profile of the bacterium¹² (**Figure 7.1**). HIV has already set an example, as nowadays genotypic resistance testing in developed countries is performed routinely as companion diagnostics in HIV therapy¹³.

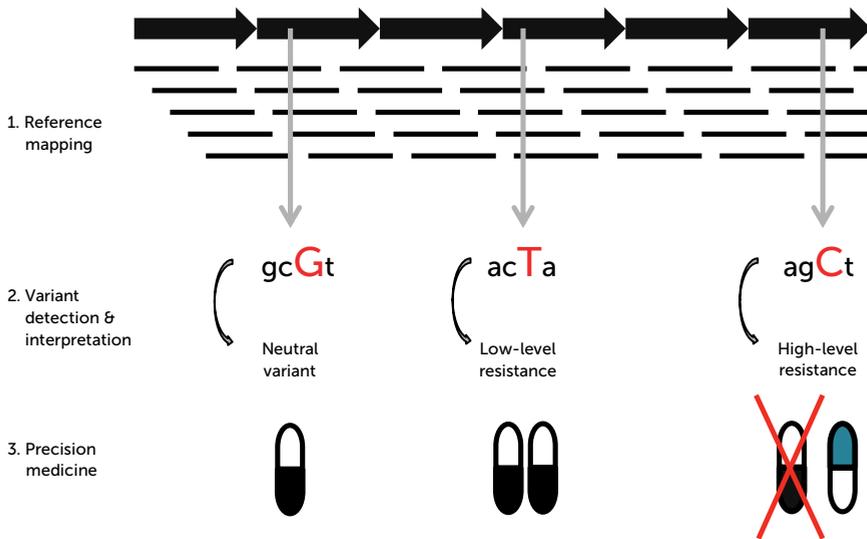


Figure 7.1. Drug resistance mutations can guide clinical decision-making. “Neutral variant” indicates that the mutation has no effect on the minimum inhibitory concentration for a specific drug.

Implementation in routine clinical diagnostics

Sequencing-based drug susceptibility testing offers other benefits for diagnosis and therapy besides the prospect of personalizing tuberculosis treatment. First, if all resistance-conferring mutations were known, this would facilitate individualized treatment by determining which drugs to give, not only which ones to avoid. Second, as drug resistance can be predicted from the genome sequence before phenotypic results become available, treatment with an appropriate regimen can be initiated earlier, reducing the risk of treatment failure, resistance amplification and transmitting drug-resistant tuberculosis. The potential of whole genome sequencing being performed directly on clinical samples would reduce the time to appropriate treatment initiation even further. Walker and colleagues¹⁴ have shown that data from whole

genome sequencing could be used clinically, to predict first-line drug resistance, drug susceptibility or to identify drug phenotypes that cannot yet be genetically predicted, and that this approach could be integrated into routine diagnostic workflows. Data on the routine use of genetic sequencing for antituberculosis drug resistance are scarce and only from industrialised countries. However, whole genome sequencing should be implemented particularly in low-income, high-burden settings where many still rely on empirical treatment regimens and the need is greatest, to actually revolutionize the diagnosis of drug-resistant tuberculosis. The studies that are part of this thesis are all performed in highly endemic, limited-resource countries, but whole genome sequencing was applied retrospectively, for research purposes. Research on the implementation of genome sequencing for routine drug-susceptibility testing in resource-limited settings has not been performed and is urgently needed.

Further study on possible loss-of-fitness-compensating mutations

Whole genome sequencing has facilitated significant progress in understanding the complex biology and epidemiology of drug-resistant tuberculosis, next to the clinical benefits of *M. tuberculosis* sequencing-based drug susceptibility testing. Mutations that lead to resistance can be deleterious or produce a fitness cost in the absence of the drug. One could therefore hypothesise that resistant strains are less virulent or less easily transmitted than drug-sensitive strains¹⁵⁻¹⁷. However, recent work has shown that additional mutations often follow or coincide with drug resistance mutations, and that these mutations can compensate for deleterious effects, restoring their initial growth capacity¹⁸⁻²⁰. Based on the data in **Chapter 4** one may hypothesize that less fit drug-resistant *M. tuberculosis* strains are more likely to cause active disease among people living with diabetes, because of their lower host defence²¹, and that these strains would have less mutations compensating for the resistance-associated loss of fitness. Our data did not support this hypothesis: we found no differences in the presence of potential rifampicin resistance-compensating mutations between strains infecting diabetes and non-diabetes patients.

A large number of putative compensatory mutations have been reported in the *rpoA*, *rpoB* and *rpoC* genes encoding the α -, β - and β' -subunits of the RNA polymerase^{22,23}. However, except for some notable exceptions²⁴, the evidence that they compensate for a loss of fitness caused by the primary resistance mutation has mainly been statistical^{23,25,26}. Therefore, in addition to the results presented in **Chapter 4**, where we focused on all non-synonymous mutations in *rpoA*, *rpoB* and *rpoC*, we sought to identify which of these mutations co-localized onto the 3D structure of the *Mycobacterium smegmatis* RNA polymerase. To this end, we counted the number of *rpoA/B/C* mutations in *M. tuberculosis* isolates with a known rifampicin-resistance mutation that converged in the 3D structure of the molecule as evidence for their role

in compensation for the loss of fitness caused by the resistance mutation. Conceptually this takes the approach of counting the number of “convergent” mutations that occur at the same location in the sequence a step further by examining it in the 3D structure and by putting it on a formal statistical basis (schematically represented in **Figure 7.2**). We projected the mutations that co-occur with the various rifampicin resistance mutations (separately for *rpoB* S450L, V170F and for *rpoB* mutations at position 435 and 445) onto the structure of the *M. smegmatis* RNA polymerase, which is 91% identical at the sequence level with *M. tuberculosis*²⁷. We used the tool mutation3D²⁸ that uses randomizations of the locations of mutations in the 3D structure to analyse whether certain regions are overrepresented, and explicitly includes convergent mutations in its analysis. For *rpoB* S450L we found one, highly significant ($E < 1E-5$) cluster that contains one mutation in *rpoA* (V183A) and five mutations in *rpoC* (V483G, V483A, W484S, I491V and V517L), distributed over two regions in the sequence (**Figure 7.3**). It should be noted that mutations in this region have been associated with resistance mutations before^{22,29} although not with all specific mutations that we found. For the other rifampicin resistance mutations we observed no significant clusters of compensating mutations in the 3D structure. The co-localizing mutations in the significant cluster occurred in rifampicin-resistant isolates from three tuberculosis patients with, and six without diabetes, and there was no difference in the presence of these mutations between strains infecting diabetes and non-diabetes patients.

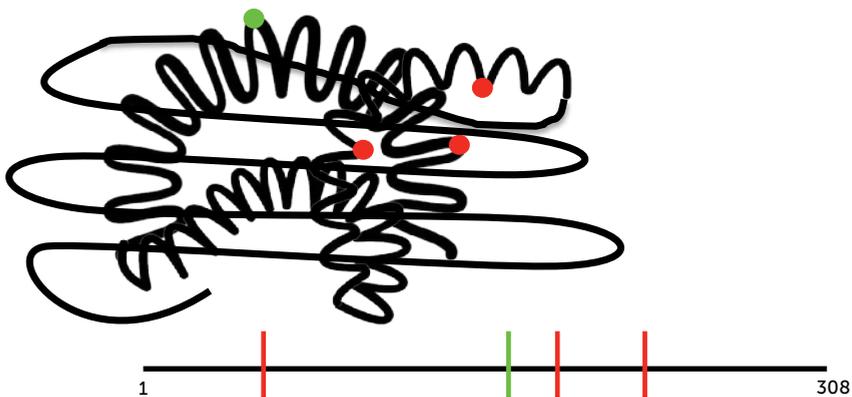


Figure 7.2. Missense mutations in a hypothetical protein. The linear protein model is given below the 3D structure to illustrate the importance of studying co-localisation of mutation in 3D structures. The mutations in red co-localize in the 3D model, whereas the mutation in green seems to cluster with the two red mutations in the linear protein model but falls outside the cluster in the 3D structure.

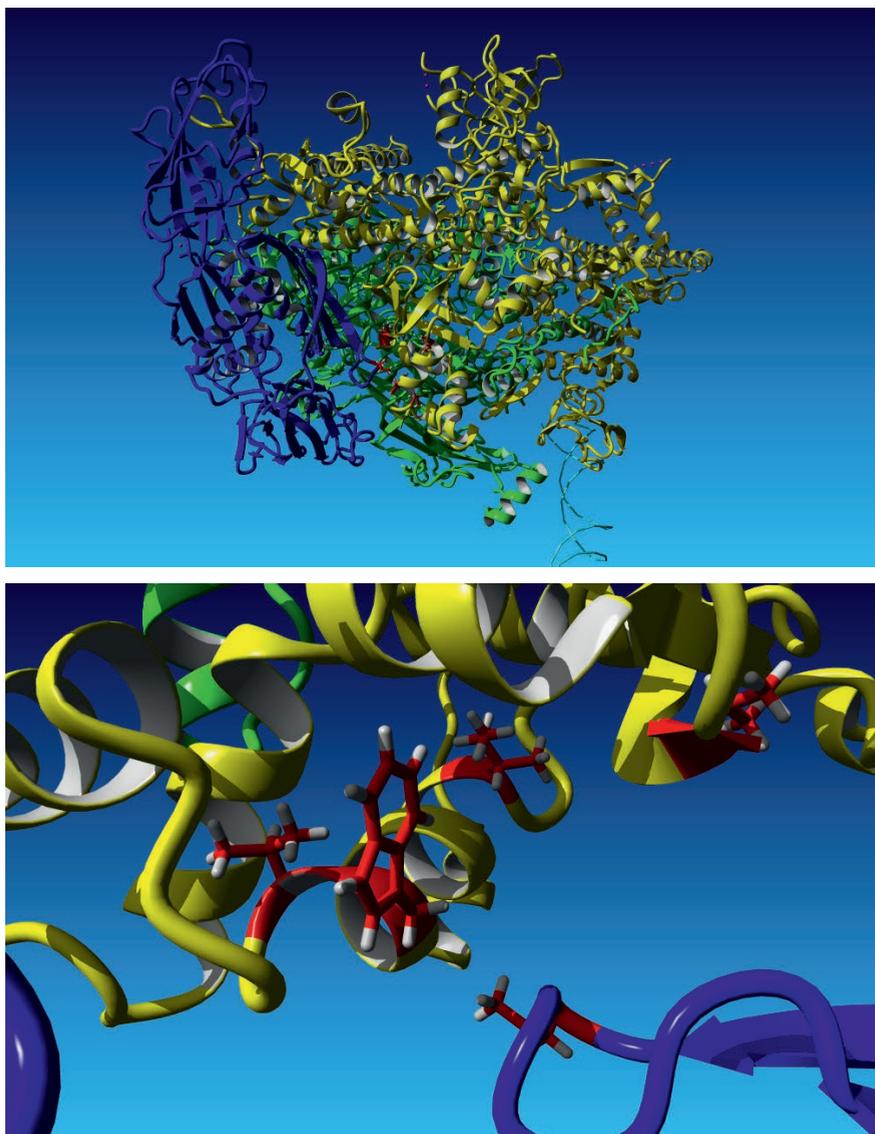


Figure 7.3. Potential rifampicin resistance-compensating mutations in *rpoA* and *rpoC* that co-localize on the 3D structure of *M. smegmatis* RNA polymerase. Mutations identified in *M. tuberculosis* isolates with rifampicin resistance mutations were mapped onto the RNA polymerase structure of *M. smegmatis*. The co-localised mutations (residues coloured in red) fall at the interface of *rpoA* (α - subunit, dark blue) and *rpoC* (β -subunit, yellow) of the *M. smegmatis* RNA polymerase. *RpoB* (β -subunit) is coloured in green.

Although the clustered mutations were not associated with the diabetes phenotype, our findings add evidence to the possible compensatory role of these mutations. Their interaction with the strain genetic background and the fitness cost of the resistance mutations may together influence the spread and maintenance of resistant variants in the population, a process called epistasis³⁰ (Figure 7.4).

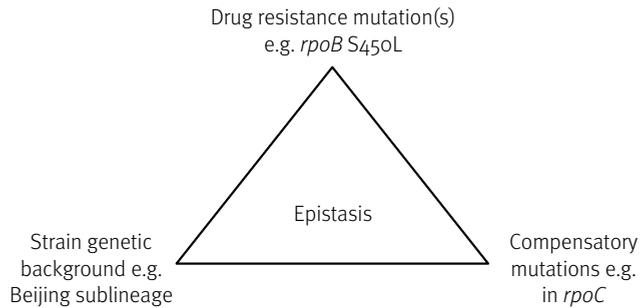


Figure 7.4. Epistatic interactions: the effect of drug resistance mutations alone or in combination as well as the presence of compensatory mutations affects the fitness of drug-resistant *M. tuberculosis* strains as a function of the strain genetic background.

Based on the findings in this thesis, and those of others, I think that whole genome sequencing-based drug susceptibility testing of *M. tuberculosis* can drastically improve the diagnosis of drug-resistant tuberculosis, but that there are important challenges to be addressed (**Box 7.1**).

Box 7.1. Conclusions and challenges of using whole genome sequencing for drug susceptibility testing

Conclusions

- Whole genome sequencing-based drug susceptibility testing of *M. tuberculosis* has the potential to drastically improve the diagnosis of drug-resistant tuberculosis:
 - Faster and more accurate diagnosis
 - Possibility to predict drug susceptibility in addition to resistance
- By generating whole genome sequencing data and linking these to relevant clinical parameters using state-of-the-art machine learning techniques, we can:
 - Increase our understanding of the molecular basis of antibiotic resistance
 - Reach better performance for several drugs while significantly reducing the number of candidate mutations to consider^{31,32}
 - Design PCR assays that target a small but optimal number of markers

Challenges

- Implementing whole genome sequencing in high-burden countries:
 - Need for expensive equipment and highly trained personnel
 - Requires complex bioinformatic procedures
- Lack of understanding of the genetic basis of antibiotic resistance complicates the interpretation of whole genome sequencing data

Part two: tuberculosis disease phenotype and transmission

The second part of this thesis focused on the link between *M. tuberculosis* genotype and tuberculosis disease phenotypes.

Exposure to *M. tuberculosis* can be followed by early clearance through innate immunity, direct development of active disease, or latent infection that may or may not reactivate up to several decades after initial exposure. Traditionally, these different phenotypes have been attributed to host and environmental variables³³. Because of the limited genetic diversity within MTBC (99.9% nucleotide identity³⁴) compared to other bacteria, the view has been that this “negligible” strain-to-strain genetic variation had no phenotypic relevance, i.e. lineage- or strain-specific differences in disease

manifestations. However, recent advances in whole genome sequencing of large MTBC clinical strain collections from global sources have revealed more genomic diversity than previously anticipated. In addition to the genomic diversity across MTBC clinical strains, findings from many experimental studies have led to a change in paradigm by demonstrating the phenotypic impact of this genetic diversity. For example, studies have reported differences between clinical strains with respect to their transcriptomic profiles^{35,36}, protein and metabolite levels^{36,37}, methylation profiles^{37,38}, drug susceptibility³⁹ and cell wall structure⁴⁰⁻⁴². In addition, MTBC genetic diversity has also been shown to influence disease severity and human to human transmission, with “modern” lineages showing a faster progression to disease and shorter latency periods compared to strains from the “ancestral” clades⁴³⁻⁴⁶. At the lineage level, the East-Asian lineage (Lineage 2) has been linked to increased transmission and extrapulmonary disease^{47,48} compared to the other lineages.

Associations beyond *M. tuberculosis* lineage level

However, associations reported between lineage and disease phenotype are not consistent⁴⁶ and considerable intra-lineage diversity exists. For example, Lineage 2 strains exhibit a range of inflammatory and virulence phenotypes^{49,50}. This could be due to the fact that Lineage 2 consists of a variety of sub-lineages, including various variants of “Beijing” strains, which might differ phenotypically⁴². Beijing strains have been proposed to possess selective advantages comprising an increased capacity to acquire drug resistance, increased transmissibility, hypervirulence and more rapid progression to disease after infection⁵¹. Still, also the association of Beijing strain infection with disease manifestations is not consistent⁵². This heterogeneity, together with evidence for higher virulence of modern Beijing strains in comparison with ancestral sublineage strains^{53,54}, suggests the existence of inter-strain variation within sublineages affecting the disease phenotype, or an important role for the interaction between the host and pathogen genotypes (co-evolution)⁵⁵.

In general, the genetic factors that are used to classify *M. tuberculosis* in phylogenetic groups, whether at supra-lineage, lineage, or sub-lineage level, do not necessarily provide clear genetic determinants of tuberculosis disease phenotype. For example, the genes that have been found to be associated with the invasion or survival of *M. tuberculosis* in the central nervous system⁵⁶, important for the development of tuberculous meningitis, can be found in all lineages of *M. tuberculosis*. Detecting the *M. tuberculosis* genetic determinants of phenotypic differences requires analytical methods that index genomic diversity in a more comprehensive manner. Many genotyping schemes have been developed for *M. tuberculosis* in the past⁵⁷, but only whole genome sequencing provides information about the whole genome, and can identify virtually all varieties of markers detected by other genotyping methods. It is

therefore highly accurate and precise in detecting variability among strains and provides a wealth of information at every level possible. In **Chapter 5**, we did not find an association between *M. tuberculosis* lineage and the tuberculous meningitis phenotype, but using whole genome sequencing we did find evidence for homoplastic genetic variation in three genes that were associated with the disease phenotype. The one study published on this topic after our study used a similar approach, and also identified variants – though different from the ones we found - associated with tuberculous meningitis in a lineage-independent fashion⁵⁸.

Challenges associated with microbial genome-wide association studies

The high resolution of whole genome sequencing also comes with analytical challenges in the quest to link genotype to phenotype. Instead of two categories, when linking ancient vs. modern lineages to differences in disease phenotype, the number of possible variations increases tremendously when examining all possible SNPs, insertions, and deletions across the entire genome, even in a clonal organism as *M. tuberculosis*. Whole genome sequencing generates thousands of potentially relevant mutations, the large majority of which will be noise that is not related to the phenotype of interest. In human genetics, genome-wide association studies (GWAS) have been developed and methods advanced significantly in the past fifteen years. The purpose of GWAS is to identify statistically significant associations that may indicate the presence of a causal relationship between genotype and phenotype while rejecting spurious associations arising from confounding factors. Compared to human GWAS, smaller genome sizes and the ability to manipulate the genomes in the laboratory may improve the power and computational ease of bacterial GWAS and facilitate the confirmation of candidate loci⁵⁹. Bacterial association mapping nonetheless remains a technical challenge in search of an optimal methodological approach. Microbial GWAS must overcome various confounding factors, such as the stronger population stratification⁶⁰, and the selection force on bacteria-related phenotypes. The problem of population stratification is especially critical in highly clonal (rarely recombining) bacteria, and in those with separate geographic- or host-associated subpopulations, such as *M. tuberculosis*⁵⁹.

Phylogenetic approach

Fortunately, population stratification and selection enable the adoption of a phylogenetic solution in microbial GWAS. Phylogenetic trees allow for the detailed identification of genetic relationships, not only at the level of population clusters, but also at the resolution of subpopulations and individual relationships. By combining a signal of selection with phenotypic associations, the phylogeny helps to decipher if an association is due to genetic relatedness rather than causality for the phenotype of interest (e.g. convergent mutations that occur only in cases and not in controls are

more likely to be causally related to the phenotype of interest). In **Chapter 5**, we used this ‘homoplasmy counting’ method to find *M. tuberculosis* genetic variations associated with the tuberculous meningitis phenotype by focusing on repeated and independently emerging mutations occurring more often on branches of isolates from tuberculous meningitis patients compared to those from pulmonary tuberculosis patients (**Figure 7.5**).

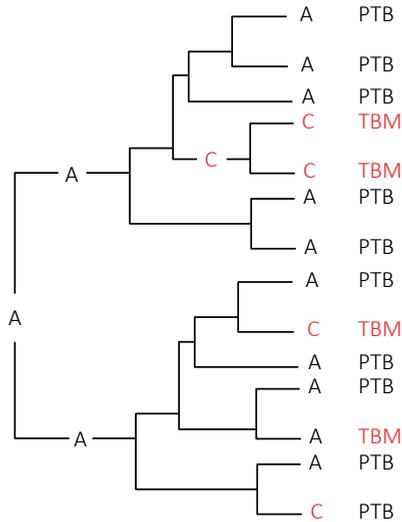


Figure 7.5. The homoplastic A→C mutation occurs independently in the tree and is associated with the tuberculous meningitis (TBM) phenotype.

The pitfall in using the phylogeny to adjust for associations due to population stratification (‘phylogenetic noise’) is the risk of masking causal variants, because differences between lineages account for large proportions of both phenotypic and genotypic variability. Correcting for lineage-effects will lead to overcorrection when these effects are biologically meaningful. In **Chapter 6**, we discovered that *M. tuberculosis* isolates belonging to the Beijing sublineage were associated with increased transmission. Additional analyses, including host-related variables, showed that this increased transmissibility was related to evasion of BCG-protection by these “Beijing” isolates. In this case, the observed lineage effect could, in a next step, guide the identification of loci that contributed to the significant lineage-level association. Disentangling the effects of a single variant from those related to lineage is potentially challenging, but has been shown to increase the power of microbial GWAS⁶¹. This strategy provides an alternative to prioritizing variants based solely on locus-specific significance, but it carries risks, because lineage-associated effects are more

susceptible to confounding with population-stratified differences in environment or sampling. Hence, dealing with population stratification represents a trade-off between the power to detect genuine associations of population-stratified variants and robustness to unmeasured, population stratified confounders.

Correction for multiple testing

Apart from the mentioned confounding factors, the other major source of false-positive associations is the multiple testing-problem that is intrinsic to GWAS. Various dimension-reduction approaches can be used to counter this problem. First, well-known methods to adjust for the large number of tests are the Bonferroni correction or the Benjamini-Hochberg procedure. Second, one can use independent datasets and test the most significant associations from the one set in the other. In **Chapter 5**, we used this approach and identified a list of top variants that were most likely associated with the tuberculous meningitis phenotype, and tested only these in a validation set of independent isolates, reducing the number of tests to correct for. A third method is the burden test, to aggregate information across several variant sites within a gene to enrich association signals and to reduce the penalty of multiple testing, which we also applied in **Chapter 5**. We also tested variants at pathway-level, an even higher level of aggregation. Fourth, a polygenic association test can be employed when one assumes that the trait in question is affected by a large number of variants on different genes. Fifth, one may choose to filter rare variants occurring in less than 1% of the isolates. Due to the highly clonal population structure of *M. tuberculosis*, many variants are rare and only occur in a very small fraction of the isolates (<0.01), increasing the number of variants to test for. Recent studies have found that 75% of the variants were found at a frequency of less than 0.0028 and 0.00054 in lineages 2 and 4, respectively in 4,408 sequenced genomes⁶², and that 83% of the variants had a frequency of ≤ 0.01 in 1,452 genomes⁹. Depending on the underlying assumption whether the cumulative effect of these rare variants together could explain a large amount of the variance in risk, they can be included in a burden test, a polygenic association test, or filtered-out to reduce the multiple testing issues.

Validation

We know from the experience of human GWAS that some loci found to be associated with a trait (after correction for multiple testing), can turn out to have little or no functional significance⁶³. Therefore, unless the associated locus has been previously shown to affect the phenotype, functional validation is desirable. The strongest evidence for the inference of functional associations remains experimental validation in the laboratory. The genetic variants we identified in **Chapter 5 and 6**, associated with tuberculous meningitis and transmissibility, respectively, could be validated experimentally. For example in a stimulation experiment where human macrophages

from tuberculosis patients are infected with *M. tuberculosis* strains with and without the identified mutations, a method that has been applied successfully in our laboratory before⁶⁴. Even stronger proof for a functional role would come from validation by genetic disruption and reconstruction of *M. tuberculosis* strains, coupled with loss and regain of the phenotype, using transposon insertion mutagenesis⁶⁵. With this ‘top down’ approach, findings from hypothesis-free GWAS can be used to generate hypotheses to be tested either in hypothesis-driven genetic association studies or experimentally in the laboratory.

Whole genome sequencing of *M. tuberculosis* has already, and will continue to improve our understanding of the evolutionary and molecular processes involved. Still, truly understanding the biological mechanisms underlying particular tuberculosis disease phenotypes studied in this thesis and by others remains extremely difficult. I believe that one of the major challenges is to define the phenotypic impact of specific *M. tuberculosis* genetic variants in the context of other bacterial determinants, the *M. tuberculosis* strain’s genetic background and other host and environmental factors also impacting the phenotype. This requires carefully considered study designs with an analytical approach integrating all these factors.

Future perspectives

Understanding the *M. tuberculosis* genome biology is a necessary step towards the control of tuberculosis. The *M. tuberculosis* genome was first sequenced in 1998 and thanks to progress in high-throughput sequencing and decreasing raw sequencing costs many more *M. tuberculosis* genomes have since been sequenced⁶⁶⁻⁶⁸, including the more than 1,000 in this thesis. The community consensus is that *M. tuberculosis* whole genome sequencing is now advanced enough to inform clinical decisions and public health policy⁶⁹. Several challenges associated with the standardization, validation and implementation of *M. tuberculosis* whole genome sequencing remain to be addressed (**Table 7.1**) that will require political commitment and the participation of supranational laboratories and regulatory authorities. In addition, the research community as a whole should continue to improve the technical and analytical aspects of whole genome sequencing. I would encourage health policy makers to commit resources to ensure access to standardized and validated whole genome sequencing methodologies, especially in high-burden countries, where they will have the largest impact on tuberculosis control.

Table 7.1. Overview of challenges and opportunities associated with applications of *M. tuberculosis* whole genome sequencing discussed in this thesis

| Challenges | Opportunities |
|---|--|
| General | |
| ! High costs | + Information on the entire genome |
| ! Data storage capacity | + High resolution |
| ! Bioinformatics support & IT infrastructure | + PacBio sequencing: repetitive regions and methylation sites ⁷¹ |
| ! Difficult to sequence regions with repetitive elements | + Can generate hypotheses to be tested experimentally |
| ! Maintenance of software tools ⁷⁰ | |
| Drug susceptibility testing | |
| ! Implementation in low-resource settings | + Faster than phenotypic DST |
| ! Relies on quality of mutation database | + Personalized medicine |
| ! Resistance mutation catalogues rely mostly on data from lineages 2 & 4 ^{72,73} | + Pipeline adaptation for NTM |
| ! Mixed infections or heteroresistance ⁷⁴ | + Resistance to many drugs at no extra costs |
| ! Incomplete understanding of the genetic basis of <i>M. tuberculosis</i> drug resistance | + Detection of novel resistance mutations ⁹ |
| ! Standardisation of methodology and genotype nomenclature ⁷⁵ | + Sequencing directly on patient samples will provide results faster ⁷⁶ |
| ! Implementation in routine clinical diagnostics | + Mapping transmission routes of drug-resistant tuberculosis |
| | + Input for designing PCR-based assays |
| | + Easy-to-use tools for raw sequencing files |
| Linking genotype to phenotype | |
| ! Confounding by host-related factors | + Phylogeny-based methods to correct for population stratification and selection ⁷⁸ |
| ! Strong population stratification ⁷⁷ | + Possibilities for dimension reduction (e.g. polygenic and burden tests) |
| ! Multiple testing problem | |
| ! Many rare variants | + Genome-to-genome approach ⁷⁹ |
| ! Validation | |

Abbreviations: DST: drug susceptibility testing; NTM: nontuberculous mycobacteria; PCR: polymerase chain reaction.

Implementation in routine clinical diagnostics

Whole genome sequencing has huge potential for applications in diagnostic microbiology including isolate characterization, drug susceptibility testing and establishing the source of recurrent infections and between-person transmissions. All of these have obvious clinical relevance and could provide additional information or even replace the knowledge obtained through standard clinical microbiology techniques. However, to date, *M. tuberculosis* whole genome sequencing has been mostly used for research purposes. One major limitation to rapidly obtaining useful information in a clinical setting is that analysis pipelines for microbial genomics have generally been

developed for fundamental research or public health epidemiology⁸⁰. This usually means that the pipeline consists of a very thorough and sophisticated workflow with a large number of options and parameters. While this is desirable from a researcher's perspective, it is clearly problematic for real-time analysis in a clinical setting. The user requires in-depth knowledge about the purpose each tool serves, the relative strengths and weaknesses of each approach, and a functional understanding of the important parameters. Furthermore, knowledge of the Linux operating system and command line is often essential for most of the advanced bioinformatic analyses; something clinical microbiologists are rarely trained for. Hence, there is a need for simpler software tools, free of charge, able to analyse data directly from the initial raw sequencing files, providing the basic functionalities all in one. Fortunately, online software tools are becoming increasingly available to support the clinical microbiologist in the analysis of *M. tuberculosis* whole genome sequencing data^{2,81} (Table 7.2).

Table 7.2. 'Easy-to-use' non-commercial genome analysis tools using raw sequencing data as input

| | Year of introduction | Web-based | Batch mode | Variants reported | Number of drugs tested |
|------------------------------------|----------------------|-----------|------------------------------|----------------------------------|------------------------|
| CASTB ⁸² | 2015 | Yes | No | None | 6 |
| KvarQ ⁸³ | 2014 | No | Yes | Only resistance mutations | 9 |
| Mykrobe Predictor TB ⁸⁴ | 2015 | No | In command line version only | Only resistance mutations | 12 |
| PhyResSE ⁸⁵ | 2015 | Yes | Yes | All mutations | 12 |
| TBProfiler ⁷² | 2015 | Yes | In command line version only | All mutations in candidate genes | 12 |
| TGS-TB ⁸⁶ | 2015 | Yes | Yes | All core genome mutations | 8 |

The implementation of whole genome sequencing in routine clinical diagnostics is particularly promising for drug susceptibility testing. The prediction of drug susceptibility based on drug resistance mutations has been shown reliable for first-line drugs^{4,87}, especially in settings with low drug resistance rates, and Public Health England has already decided to stop phenotypic drug susceptibility testing of isolates that are predicted to be genotypically susceptible to all first-line drugs. Similar decisions have been made in the Netherlands (planned for the end of 2019) and New York⁴. Ideally, genetic data should be linked to minimum inhibitory concentrations and, preferably, to patient outcomes. The aim would be to have a comprehensive,

standardized and curated resource for clinically relevant genetic mutations and associated metadata (geographic location, minimum inhibitory concentration, response to treatment)⁸⁸, similar to the in 2007 established Stanford HIV Drug Resistance Database⁸⁹. Whole genome sequencing on a large number of strains collected worldwide, coupled with these metadata can provide the appropriate statistical power to identify the subset of mutations predictive of treatment failure to any given drug. Furthermore, linking this with therapeutic drug monitoring results would enable clinicians to determine if treatment fails because of an increased minimum inhibitory concentration or because of a low blood concentration.

Understandably, efforts to develop whole genome sequencing-based microbial diagnostics have focussed on high-resource settings. Studies on the impact and cost effectiveness of routine whole genome sequencing in high burden settings are needed to determine the feasibility of whole genome sequencing in this setting. Currently, most hospitals in developing countries do not even benefit from a clinical microbiology laboratory. This presents an obvious challenge, but at the same time an outstanding opportunity for low- to middle-income countries to get up to speed with whole genome sequence-based developments in real-time clinical diagnostics, rather than adopting classical microbiological phenotypic drug susceptibility testing that may eventually be phased out in high-income countries. The installation of a molecular laboratory based around a benchtop sequencer might be an ideal investment, as it is neither far more expensive nor more complex than setting up a standard clinical microbiology laboratory. The introduction of easy-to-use software tools that predict drug resistance profiles based on raw sequence input files has already brought the implementation of sequence-based drug susceptibility testing in these settings a step closer. A possible alternative or intermediate solution to bridge the gap in drug susceptibility testing between high- and low-income countries is by translating the drug resistance markers discovered by whole genome sequencing studies into the development of new, improved molecular assays that test drug resistance based on these markers and are more affordable and easier to use and implement.

A thus far relatively unexplored area is the application of whole genome sequencing in routine clinical diagnostics of nontuberculous mycobacteria (NTM), several species of which are increasingly recognized as important opportunistic pathogens of humans⁹⁰. Despite the availability of whole genome sequencing technologies, limited effort has been put into the genetic characterization of NTM species. This year, we have started a project in the medical microbiology department to sequence all clinical NTM isolates and develop an automated bioinformatics pipeline for species identification and genotypic drug susceptibility testing. Both steps are crucial to guide treatment⁹¹; using whole genome sequencing can significantly speed up this process, leading to

more rapid diagnosis and streamlined treatment. Doing this in parallel with minimum inhibitory concentration determination will also enable the identification of novel resistance markers.

Linking genotype to phenotype

Future genetic association studies linking *M. tuberculosis* genotype to phenotype should include the human genotype as a variable of interest, because the global phylogeographic structure of *M. tuberculosis* and the association between *M. tuberculosis* strains and their human host populations provides solid evidence that these are closely related⁹²⁻⁹⁴. The interaction of the *M. tuberculosis* and human genotype might provide insight in the way the different *M. tuberculosis* lineages have adapted to specific human populations⁵⁵. Associations between particular human SNPs and specific *M. tuberculosis* genotypes^{48,95} support the concept of ‘co-evolution’ of *M. tuberculosis* and the human host. Such ‘genome-to-genome’ analyses have a great potential to uncover relevant interactions between the pathogen and host genome and their joint effects on phenotypes⁷⁹. For example, *M. tuberculosis* genetic variation *X* could have a different effect in a patient with genotype *Y*, than in a patient with genotype *Z*. The success of this approach has recently been shown in pneumococcal meningitis⁹⁶. We would like to use this method in follow-up studies, given that we have the availability of DNA samples for many of the patients who were included in the studies in this thesis.

The genes we found to be possibly related to the meningeal disease manifestation, and the phylogenetic association with a higher transmission rate through ‘BCG-escape’, need to be validated in follow-up studies. A genetic association does not prove causality, even though we adjusted for host-related factors and confounding by *M. tuberculosis* population stratification. To confirm that the found associations are functionally/biologically relevant, the next step would be to test the associations in another patient cohort, in a hypothesis-driven genetic association study, or experimentally in the laboratory. The advantage of functional validation in the laboratory is that conditions can be controlled in a way that other factors affecting the phenotype of interest are removed, kept constant, or varied on purpose. For instance to validate the genetic variants found to be associated with the tuberculous meningitis phenotype, macrophage infection models could be used to measure gene expression *in vitro* by infecting healthy macrophages with various *M. tuberculosis* strains with and without the identified mutations. In addition, macrophages from pulmonary tuberculosis and tuberculous meningitis patients could be infected with standard *M. tuberculosis* strains, or pulmonary tuberculosis and tuberculous meningitis patient macrophages could be infected with their ‘own’ *M. tuberculosis* strains to help disentangle the effect of *M. tuberculosis* or host genotype, or their interaction.

Functional genetics

Genetics does not “say it all”. In the end, the work described in this thesis can be used as a basis to work towards ‘functional genetics’ research, linking the *M. tuberculosis* genome to *M. tuberculosis* transcription, transcriptional regulation (e.g. epigenetics), lipidomics, proteomics, and/or metabolomics, in appropriate models (e.g. macrophages or blood or cerebrospinal fluid of healthy individuals or tuberculosis patients). Integrating multiple types of these quantitative molecular measurements creates a more holistic understanding of the functional relevance/consequences of *M. tuberculosis* genetic variation. It could also shed light on the biological role of *M. tuberculosis* genes, as the functions of 27% of the encoded proteins in the annotated version of the H37Rv strain have yet to be determined⁹⁷ while the functions of the remaining proteins have been based mostly on sequence comparison⁹⁸.

M. tuberculosis whole genome sequencing can be applied to the entire spectrum from molecule to man to population and will help to find answers to fundamental research questions, which can be translated in improved diagnostics and patient care, to ultimately be adopted for tuberculosis surveillance and control. I hope the findings in this thesis will help to develop better tools and strategies to control a disabling disease, for which the burden largely lies in resource-poor countries.

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8

Summary

Summary

The outcome of *M. tuberculosis* infection and the presentation and course of active tuberculosis disease are highly variable. Although many host and environmental factors have been identified that contribute to this variation, together they do not provide sufficient explanation for this variability. The growing knowledge of the global genomic diversity of *M. tuberculosis* complex (MTBC) affecting different cellular and immunological phenotypes supports that bacterial factors also play a role in the observed variation in clinical phenotypes of *M. tuberculosis* infection and active tuberculosis disease. Hence, in this thesis, containing whole genome sequencing data of more than 1,000 *M. tuberculosis* isolates and clinical metadata from tuberculosis patients from three different continents, I aimed to improve the understanding of bacterial factors determining drug resistance, disease presentation and transmission by studying genetic differences among *M. tuberculosis* strains on a genome-wide scale (summarized in **Table 8.1**).

Part one: drug resistance

The first part of this thesis focused on the use of whole genome sequencing to predict drug resistance in *M. tuberculosis* clinical isolates, and on the relation between diabetes and drug resistance mutations.

In **Chapter 2**, we sequenced a selection of 322 *M. tuberculosis* isolates from Indonesia to measure the distribution of drug resistance mutations and the concordance between phenotypic and genotypic drug susceptibility testing, which were until then unknown in Indonesia. We identified mutations associated with drug resistance to at least one tuberculosis drug in almost one sixth of the isolates. Most mutations were found in *katG*, *pncA*, *rpoB*, *fabG1* and *embB*. Agreement of whole genome sequence-based resistance prediction and phenotypic drug susceptibility testing to first-line drugs was high for isoniazid and rifampicin but was lower for ethambutol and streptomycin. Our findings support the potential benefit of using whole genome sequencing to generate an *in silico* drug susceptibility profile in Indonesia and show that mutations associated with drug resistance are highly predictive for phenotypic resistance to rifampicin and isoniazid in the region.

Whole genome sequencing may be a promising alternative to phenotypic drug susceptibility testing. However, the degree to which the various drug resistance mutations impact drug susceptibility remains to be investigated. Therefore, in **Chapter 3**, we examined drug resistance mutations in 72 phenotypically drug-resistant isolates from Romania and linked these to their measured minimum inhibitory concentrations. We observed that *katG* S315T for isoniazid, and *rpoB* S450L for rifampicin were associated with

Table 8.1. Summary of the main findings and implications of this thesis

| Findings | Implications for practice or research |
|--|---|
| Part one: drug resistance | |
| Agreement of drug resistance mutations and phenotypic DST was high for INH and RIF but low for EMB and STR (Chapter 2), because low DST quality and resolution for these drugs (Chapter 3) | Genotypic DST could offer a rapid and comprehensive diagnostic solution to phenotypic DST in Indonesia; larger studies are needed to confirm the clinical relevance of several uncommon mutations (Chapter 2) |
| Drug resistance mutations lead to varying levels of resistance; some may be overcome by increased dosing (Chapter 3) | Whole genome sequencing can aid in the timely diagnosis of <i>M. tuberculosis</i> drug resistance and guide clinical decision-making (Chapter 3) |
| Diabetes is associated with drug-resistant TB, especially among patients without prior TB treatment (Chapter 4) | TB patients with diabetes should be prioritized for DST in settings where this is not done for all patients (Chapter 4) |
| Part two: disease phenotype and transmission | |
| Three genes in <i>M. tuberculosis</i> (<i>Rvo218</i> , <i>Rv3433c</i> , and <i>nank</i>) are associated with the TB disease phenotype (Chapter 5) | Functional validation studies are warranted to further explore the effect of mutations in these genes on protein function (Chapter 5) |
| <i>M. tuberculosis</i> Beijing genotype strains may evade BCG vaccine-induced immunity (Chapter 6) | The impact of BCG vaccination on transmission may depend on the <i>M. tuberculosis</i> strain distribution in the setting where it is used (Chapter 6) |

Abbreviations: DST: drug susceptibility testing; EMB: ethambutol; STR: streptomycin; TB: tuberculosis; BCG: Bacillus Calmette-Guérin.

high-level resistance (8- to 32-fold and 4- to 16-fold increase in MIC, respectively), but several mutations such as in *rpoB*, *rrs* and *rpsL*, and *embB* were associated with minimum inhibitory concentration ranges including the critical concentration for rifampicin, aminoglycosides and ethambutol, respectively. This could explain the discrepant genotypic and phenotypic susceptibility results for streptomycin and ethambutol in **Chapter 2**, and taught us that resistance mutations induce varying levels of resistance, some of which may still be overcome by increased dosing. Hence, whole genome sequencing can guide clinical decision-making, by informing drug selection as well as drug dosing.

Diabetes increases the risk of developing active tuberculosis by threefold and the increasing prevalence of diabetes poses a great threat to tuberculosis control. Increased rates of drug resistance may contribute to poor treatment outcomes among tuberculosis patients with diabetes, complicating this even further. Still, little is known about the relation of diabetes and genotypic drug resistance. Therefore, in **Chapter 4**, we investigated the mutations underlying drug resistance in *M. tuberculosis* isolates from Indonesian and Peruvian tuberculosis patients with and without diabetes. Taking patients' country of origin into account and after adjusting for age, gender, previous tuberculosis treatment and *M. tuberculosis* lineage, we discovered that diabetes was associated with an approximately 1.7-fold increased risk of genotypic drug resistance, and to rifampicin in particular (2.5-fold increased risk). This association was especially evident for patients not previously treated for tuberculosis ('primary resistance'), and could not be explained by clustering of evolutionarily successful drug-resistant strains in patients with diabetes. Mutations in *Rv1482c-fabG1*, conferring resistance to isoniazid and ethionamide, and *gyrA*, conferring fluoroquinolone resistance, were overrepresented in isolates from Peruvian tuberculosis patients with diabetes. These results are important for the control of drug-resistant tuberculosis as they highlight the need for routine drug-susceptibility testing for tuberculosis patients with diabetes.

Part two: tuberculosis disease phenotype and transmission

The second part of this thesis focused on the role of *M. tuberculosis* genetic diversity in the presentation of disease as pulmonary or meningeal tuberculosis, and on disease transmission.

To determine whether *M. tuberculosis* genetic diversity is associated with the tuberculosis disease phenotype, we compared *M. tuberculosis* genomes from Indonesian pulmonary and meningeal tuberculosis patients and searched for homoplastic mutations (**Chapter 5**). We observed that the isolates from meningitis patients were scattered across the phylogenetic tree and that there was no association between lineage and disease localisation. At a higher level of resolution and using a homoplasmy-based association analysis, we found that genetic variation in *Rvo218* and absence of *Rv3343c* and *nanK* were significantly associated with disease phenotype, also in an independent validation set of isolates. It is unknown how *Rv3343c* and *nanK* might affect tuberculosis phenotype, but *Rvo218* is a predicted secretome gene with multiple transmembrane regions, possibly altering *M. tuberculosis* outer structure.

Similar to *M. tuberculosis* tissue tropism and supported by previous findings that *M. tuberculosis* virulence is associated with the isolate's genetic background, the risk of *M. tuberculosis* transmission could also be influenced by its genotype. Hence, we studied the effect of *M. tuberculosis* lineage on the risk of transmission from index

cases to household contacts in Bandung, Indonesia (**Chapter 6**). We discovered that *M. tuberculosis* Beijing genotype strains are associated with a higher risk of uninfected household contacts to acquire *M. tuberculosis* infection (measured with an interferon-gamma release assay). This was not explained by a higher bacillary load or higher frequency of pulmonary cavities in the index cases. In addition, a history of BCG vaccination among household contacts was associated with a 60% lower risk of *M. tuberculosis* infection caused by non-Beijing isolates, but no lower risk of infection by Beijing family isolates. Evading BCG-mediated protection in the contact, rather than inducing a disease phenotype in the index case that favours transmission, may increase the transmission rate in Beijing genotype strains.

9

Samenvatting

Samenvatting

Mycobacterium tuberculosis

Tuberculose is met 1,7 miljoen sterfgevallen in 2017 een van de top-10 doodsoorzaken ter wereld en de nummer één doodsoorzaak als gevolg van een infectieziekte. Naar schatting tien miljoen mensen wereldwijd kregen de ziekte in 2017. De ziekteverwekker, *Mycobacterium tuberculosis* behoort tot een grotere familie van aan elkaar verwante mycobacteriën: het *Mycobacterium tuberculosis* complex; het DNA van deze bacteriën lijkt sterk op elkaar. De bacteriën die tuberculose in de mens veroorzaken, kunnen weer onderverdeeld worden in 7 verschillende genotypen met elk specifieke genetische kenmerken. Lang dacht men dat er zo weinig genetische variatie binnen de tuberculosebacteriën was, dat dit onmogelijk effect kon hebben op de manier waarop de ziekte zich uit in de patiënt. Echter, mede door vooruitgang in de techniek waarmee het DNA bestudeerd kan worden, is aangetoond dat verschillen in het DNA van de bacterie wel degelijk van invloed zijn op het ziekteproces. In dit proefschrift heb ik door middel van 'whole genome sequencing' het complete DNA van *M. tuberculosis* onder de loep genomen om het effect van genetische variatie op antibioticaresistentie (deel 1), ziektefenotype (deel 2) en transmissie van tuberculose (deel 2) beter te begrijpen. In dit hoofdstuk vat ik de belangrijkste bevindingen van mijn proefschrift samen.

Deel één: antibioticaresistentie

Een van de grootste zorgen met betrekking tot tuberculosebestrijding is resistentie van de bacterie tegen een of meerdere geneesmiddelen. Hierdoor is de infectie minder goed of in zeldzame gevallen helemaal niet meer te behandelen. Slechts een kwart van de naar schatting 558,000 mensen die ziek werden van een multiresistente of rifampicine-resistente bacterie in 2017 werden met tweedelijnsmiddelen behandeld. Een belangrijke oorzaak hiervan is het niet, of niet op tijd diagnosticeren van infectie met een resistente bacterie. Wanneer ongediagnosticeerde resistente tuberculose niet volgens de juiste richtlijn wordt behandeld zal deze niet genezen en kan de resistente bacterie bovendien worden overgedragen op andere ongeïnfecteerde personen. Omdat antibioticaresistentie in *M. tuberculosis* veroorzaakt wordt door mutaties in bepaalde genen van de bacterie kan resistente tuberculose worden opgespoord door te zoeken naar deze mutaties in het DNA van de infecterende bacterie: genotypische gevoeligheidsbepaling. Veelal wordt dit echter nog gedaan door middel van de zogenaamde fenotypische gevoeligheidsbepaling, waarbij in het laboratorium wordt vastgesteld of de bacterie groeit bij een bepaalde concentratie antibioticum.

De fenotypische gevoeligheidsbepaling is complex en bemoeilijkt de diagnose van resistente tuberculose. Whole genome sequencing heeft de potentie om betrouwbaar antibioticaresistentie te kunnen voorspellen binnen een klinisch relevant tijdsbestek. In **hoofdstuk 2** hebben we met whole genome sequencing de mutaties in kaart gebracht die voor resistentie zorgen bij *M. tuberculosis* stammen van Indonesische patiënten. Daarnaast hebben we onderzocht in hoeverre de genotypische en fenotypische gevoeligheidsbepalingen overeen kwamen. We ontdekten bij bijna één op de zes *M. tuberculosis* stammen mutaties die geassocieerd zijn met antibioticaresistentie, vaak bij patiënten die niet eerder behandeld waren voor tuberculose. We vonden veel genetische varianten in verschillende genen. Mutaties in de genen *katG* (aminozuur 315), in de promotor van *fabG1* en in de bekende resistentieregio van *rpoB* lieten hoge overeenstemming zien met fenotypisch vastgestelde resistentie tegen respectievelijk isoniazide en rifampicine. Deze overeenkomst was veel lager voor mutaties geassocieerd met resistentie tegen ethambutol en streptomycine. We hebben hiermee voor het eerst de potentie van whole genome sequencing als voorspeller van resistente tuberculose laten zien in Indonesië, waar het fenotypisch bepalen van resistentie vaak niet mogelijk is. Met name mutaties geassocieerd met resistentie tegen de eerstelijns middelen isoniazide en rifampicine waren zeer voorspellend voor fenotypische resistentie in deze setting. Verder onderzoek is nodig om de impact van whole genome sequencing voor routine diagnostiek en management van resistente tuberculose in Indonesië te kunnen evalueren.

Whole genome sequencing kan in potentie de huidige, maar tijdrovende en complexe fenotypische gevoeligheidsbepaling van *M. tuberculosis* vervangen. Echter, er is nog weinig bekend over de mate van resistentie die de verschillende resistentiemutaties veroorzaken en over het effect van combinaties van resistentiemutaties. Daarom hebben we in **hoofdstuk 3** de met antibioticaresistentie geassocieerde mutaties in fenotypisch resistente *M. tuberculosis* stammen van Roemeense patiënten vergeleken met de mate van verminderde gevoeligheid tegen de verschillende antibiotica. We ontdekten dat de mutaties *katG* S315T en *rpoB* S450L geassocieerd waren met sterk verminderde gevoeligheid voor respectievelijk isoniazide en rifampicine en dat diverse mutaties in *rpoB*, *rrs* en *rpsL*, en *embB* geassocieerd waren met licht verminderde gevoeligheid tegen respectievelijk rifampicine, aminoglycosiden en ethambutol. Hierbij werden ook waarden gevonden rond het omslagpunt van gevoelig / resistent; dit zou mogelijk de lage overeenstemming tussen de fenotypische en genotypische gevoeligheidsbepaling voor ethambutol en streptomycine in **hoofdstuk 2** kunnen verklaren. Verschillende resistentiemutaties kunnen dus verschillende effecten hebben op de mate van gevoeligheid van *M. tuberculosis* voor antibiotica en in sommige gevallen kan de patiënt mogelijk nog effectief behandeld worden met een hogere dosis antibiotica. Whole genome sequencing kan hiermee richting geven aan de klinische besluitvorming met betrekking tot de keuze voor het juiste middel en de hoogte van de dosering.

Mensen met diabetes mellitus hebben een drie keer verhoogd risico op het ontwikkelen van actieve tuberculose. Het toenemende aantal diabetespatiënten wereldwijd heeft dus gevolgen voor de tuberculose-epidemie. Een groter aandeel resistente tuberculose onder diabetespatiënten vormt een verdere bedreiging voor de tuberculosebestrijding. Toch is er nog weinig bekend over de resistentiemutaties in deze specifieke patiëntenpopulatie. In **hoofdstuk 4** hebben we daarom de resistentiemutaties bestudeerd in *M. tuberculosis* stammen van Indonesische en Peruaanse tuberculosepatiënten met en zonder diabetes. We vonden dat diabetes geassocieerd was met een 1.7 keer verhoogd risico op genotypisch resistente tuberculose en met name resistentie tegen rifampicine. Dit kon niet verklaard worden door andere patiëntegereleerde factoren zoals leeftijd, geslacht en eerdere behandeling voor tuberculose, of het genotype waartoe de *M. tuberculosis* stam behoorde. Bovendien zagen we geen verband met transmissie van evolutionair gezien meer succesvolle resistente stammen in diabetespatiënten. Mutaties in met name de genen *Rv1482-fabG1*, geassocieerd met resistentie tegen isoniazide en ethionamide, en *gyrA*, geassocieerd met fluoroquinolonresistentie, kwamen vaker voor onder Peruaanse tuberculosepatiënten met diabetes. Deze resultaten zijn belangrijk omdat ze het belang van de invoering van een routine gevoeligheidsbepaling van *M. tuberculosis* in deze patiëntengroep aantonen.

Deel twee: ziektefenotypen en transmissie van tuberculose

M. tuberculosis wordt vooral verspreid via de lucht wanneer een tuberculosepatiënt hoest of niest. Wanneer iemand anders deze bacterie vervolgens inademt, zijn er verschillende scenario's mogelijk: een deel van de blootgestelde individuen ruikt *M. tuberculosis* op, nog voordat het afweersysteem hiertegen geheugen opbouwt. Een kleine minderheid wordt direct en vaak ernstig ziek, maar de meerderheid merkt niets van de besmetting. De bacterie verkeert dan in een 'slapende' staat en de patiënt is niet besmettelijk. De patiënt kan later in het leven alsnog ziek worden. De tuberculosebacterie kan namelijk tientallen jaren in slapende staat in het lichaam overleven en weer actief worden als de afweer van de geïnfecteerde persoon afneemt. Dat gebeurt bij ongeveer één op de tien besmette personen. Actieve tuberculose manifesteert zich doorgaans in de long, maar *M. tuberculosis* kan zich in alle organen nestelen en daar ziekte veroorzaken. Tuberculose in de hersenen, tuberculeuze meningitis, is de meest ernstige vorm van tuberculose. De helft van de patiënten houdt er zware neurologische handicaps aan over of overlijdt aan de aandoening. Uitzonderingen daargelaten, kunnen alleen patiënten met actieve longtuberculose de bacterie overdragen op anderen.

Verschillende factoren bepalen welk scenario plaatsvindt na blootstelling aan *M. tuberculosis* en hoe de ziekte zich eventueel uit in de patiënt. Veel van deze factoren zijn patiënt- of omgevingsgerelateerd. Echter, we komen er steeds meer achter dat ook de

bacterie een belangrijke rol speelt. De mate waarin bacteriën 'ziekmakend' (virulent) zijn verschilt, en genetische variatie binnen *M. tuberculosis* kan aan deze verschillen ten grondslag liggen. In **hoofdstuk 5** hebben we bekeken of genetische verschillen tussen *M. tuberculosis* bacteriën geassocieerd zijn met het meningitis of long-tuberculose fenotype door het DNA van bacteriën uit de hersenen van tuberculeuze meningitispatiënten te vergelijken met het DNA van bacteriën uit de longen van long-tuberculosepatiënten. We vonden dat het *M. tuberculosis* genotype niet bepalend was, maar dat subtielere genetische verschillen geassocieerd waren met het ziektefenotype. Mutaties in het gen *Rvo218* en de afwezigheid van gen *Rv3343c* of *nanK* hielden verband met uiting van de ziekte in de longen of in de hersenen. Gebaseerd op biologische eigenschappen van het gen *Rvo218* zou men kunnen veronderstellen dat dit gen invloed heeft op de buitenkant - en daarmee op de herkenning door het afweersysteem - van de tuberculosebacterie. Meer onderzoek is nodig naar de manier waarop variatie in deze genen invloed heeft op het ziektefenotype.

Naast het ziektefenotype kan genetische variatie binnen *M. tuberculosis* ook invloed hebben op de mate van overdraagbaarheid van tuberculose. Dit kan bijvoorbeeld doordat een bacterie meer schade in de longen aanricht waardoor de patiënt meer hoest, of doordat de bacterie kan ontsnappen aan het afweersysteem van een blootgesteld individu. In **hoofdstuk 6** onderzochten we de overdraagbaarheid van *M. tuberculosis* op mensen binnen hetzelfde huishouden in Bandung, Indonesië. We kwamen erachter dat ongeïnfecteerde huisgenoten die blootgesteld waren aan een patiënt besmet met een *M. tuberculosis* stam van het *Beijing* genotype, een hoger risico hadden om besmet te raken. Dit kon niet verklaard worden door een hogere concentratie tuberculosebacteriën in, of meer schade aan de longen van de patiënt. Bovendien ontdekten we dat huisgenoten die een BCG-vaccinatie hadden gehad, sterk beschermd waren tegen besmetting na blootstelling aan *M. tuberculosis*, maar dat BCG-vaccinatie helemaal niet beschermde tegen besmetting bij huisgenoten die blootgesteld waren aan *M. tuberculosis* genotype *Beijing*. Kortom, het ontsnappen aan BCG-gemedieerde bescherming lijkt de verhoogde overdraagbaarheid van *M. tuberculosis* genotype *Beijing* stammen te verklaren.

Al met al biedt het analyseren van het complete DNA van de tuberculosebacterie mogelijkheden, zowel voor de diagnostiek van resistente tuberculose als voor het begrijpen van deze complexe ziekte, om haar uiteindelijk beter te kunnen bestrijden. Dit vergt het blijvend bijhouden van de correlaties tussen het genotype van de bacterie en de verschillende fenotypische aspecten, zoals ik in mijn proefschrift heb gedaan. Ik hoop dat de bevindingen in mijn proefschrift bijdragen aan de ontwikkeling van betere hulpmiddelen en strategieën om tuberculose te kunnen beheersen. Een van de grootste uitdagingen zal liggen in het toegankelijk maken van deze technieken in

landen met weinig middelen, waar de ziektelast het hoogst is en de middelen het hardst nodig zijn.

Appendix

Dankwoord

Curriculum Vitae

RIHS PhD portfolio

Research data management

List of publications

Dankwoord

Mijn dank gaat uit naar iedereen die mij tijdens mijn promotie op welke manier dan ook heeft bijgestaan, gesteund, geïnspireerd, geholpen en gemotiveerd. Bedankt dat jullie me hebben vergezeld op deze wetenschappelijke ontdekkingsreis.

In het bijzonder wil ik mijn promotores en copromotores bedanken. Ik voel me gezegend met zo een divers gezelschap inspirerende mensen om me heen. Reinout van Crevel, je hebt me alle vrijheid en vertrouwen gegeven om de wereld van de tuberculose en de onderzoekswereld in bredere zin te verkennen. Met je aanstekelijke enthousiasme heb je me uitgedaagd en de mogelijkheden gegeven om letterlijk en figuurlijk mijn grenzen te verleggen. Dankjewel voor alles wat je me hebt gegeven, maar zeker ook voor alles wat je van me hebt gevraagd. Martijn Huynen, je hebt me geïntroduceerd en warm welkom geheten in de wonderde wereld van de bioinformatica. Je eindeloze nieuwsgierigheid en drang om alles te willen begrijpen werkt inspirerend. Voor jou is ieder antwoord slechts het begin van een nieuwe vraag. Jakko van Ingen, ik ben je bijzonder dankbaar voor de mogelijkheid die je me hebt geboden om mijn promotieonderzoek af te ronden. Met je bezielende positiviteit en enthousiasme creëer je een prettige sfeer en neem je anderen mee in je fascinatie voor de niet-tuberculeuze mycobacteriën. Lidya Chaidir, dear Lidya, I admire your dedication to tuberculosis research and your capability to combine so many different tasks; doing a PhD, teaching, managing the tuberculosis lab in Bandung, supervising and travelling. You have become a dear friend and I am proud to have you as my copromoter. Dank allen voor jullie vertrouwen in mij!

De leden van de manuscriptcommissie, Michel van den Heuvel, Wilbert Bitter en Jeannine Hautvast wil ik bedanken voor hun bereidheid om zitting te nemen in de manuscriptcommissie en voor het lezen en beoordelen van dit proefschrift.

Ik wil ook graag de mensen bedanken die mijn mentor en voorbeeld zijn geweest tijdens eerdere onderzoeksprojecten. Ann Ashburn, Vivian Weerdesteyn en Geert Verheyden namen me onder hun vleugels tijdens en voorafgaand aan mijn project in Southampton. Rob Aarnoutse bood me samen met Reinout van Crevel de kans om een dataset te analyseren en hierover te publiceren. Dick van Soolingen en Rianne van Gageldonk hebben me wegwijs gemaakt in de epidemiologie van tuberculose. Dick, ik vind het leuk dat we elkaar af en toe nog tegenkomen en ik hoop dat we elkaar tegen blijven komen. Marlies Hulscher was mijn mentor zowel tijdens mijn afstudeerproject als tijdens mijn promotieonderzoek. Marlies, ik bewonder je gedrevenheid in het verbeteren van de kwaliteit van zorg en ik mag je erg graag als mens.

The research I did during my PhD was in close collaboration with many inspiring colleagues from Bandung, where I worked and lived for seven months, and from many other countries. I am grateful for the lessons I learned from you, not only about science, but also about hospitality, modesty, and gratefulness. Dr. Bacht Alisjahbana, thank you for kindly welcoming me as a member of the TB/HIV research group and for looking after me, also outside the office. Professor Rovina Ruslami, thank you for being such a welcoming host. Sue McAllister, dear Sue, thank you for generously sharing your house with me. I learned a lot from you about doing research and living in a foreign country. I cherish the memories of our weekend breakfasts outside and the many motorbike rides. Professor Philip Hill, thank you for showing that doing high quality research and a good laugh can go hand in hand. Ayesha Verrall, dear Ayesha, you've inspired me both professionally as well as personally. I enjoyed our epidemiology discussions. Anca Riza, dear Anca, your expertise is human genetics but you have been a great help with mycobacterial genetics analyses, not to forget the shipments of mycobacterial DNA. My gratitude also goes out to 'the ladies' from the tuberculosis lab in Craiova for all their hard work on culturing and managing isolates. I would like to thank Julia Critchley, Cesar Ugarte, David Moore and other colleagues from the TANDEM consortium for contributing to Chapter 4 of my thesis. Thomas Kohl and Matthias Merker, thank you for introducing me to whole genome sequencing of *M. tuberculosis*. Dr. Ahmad Rizal Ganiem and Dr. Sofiat Dian, you are doing important scientific and clinical work on tuberculous meningitis. I learned a lot from you, also about the Indonesian cuisine. Dr. Darma Imran, thank you for giving me the opportunity to visit the emergency ward at the Cipto Mangunkusumo hospital in Jakarta. I would also like to thank my other Indonesian colleagues, Raspati Koesoemadinata, Intan Mauli, Tiara Pramaesya, Annisa 'Itcha' Rahmalia, Mawar Pohan, Ria Windyani, Bony Wiem Lestari, Jessi Annisa, Lika Apriani, my former roommates Ranni Trisnawati, Runi Rahmawati, Nopi Susilawati and Alif al Birru, the colleagues from the tuberculosis lab in Bandung who took great care of the *M. tuberculosis* isolates, and all others who contributed to the work presented in this thesis but are not mentioned here explicitly.

The time I spent in Bandung was not only an academic learning experience, it was a period of personal development. I am grateful for all the people I met and the friends I made, also outside the office. Misliani Intan Ardita, dear 'Dita', my football buddy. I really enjoyed our afternoon practices with the Rumah Cemara team. Thanks for welcoming me to your football teams and for introducing me to so many Indonesian traditions. Itcha, thank you for taking me by the hand on my quest for a football team to join in Bandung. Rizki Kurniawan, the way you use your hobby to do meaningful work is inspirational. Our visit to Persib Bandung was memorable! Ferlin Joswara, your work spirit is amazing and you create the most wonderful things. It was an honour to model for you. I am also grateful for the chance I got to play football with the girls

from Rumah Cemara, run with the Indorunners and discover the Bandung mountains with the Bandung biking group. You have helped keeping mind and body healthy. Arjan van Laarhoven en Suzanne van Dorp, dank jullie wel voor jullie grote dosis gastvrijheid tijdens mijn tweede verblijf in Bandung. Met de warmte van jullie persoonlijkheden en de verfrissing van koude biertjes en het zwembad voelde jullie huis als een tweede thuis.

Een van de leuke dingen aan promoveren is de vrijheid om je helemaal in een onderwerp te mogen verdiepen in een omgeving van talentvolle mensen van (en met) wie je veel kan leren. Ik heb het geluk gehad dat ik tijdens mijn promotieonderzoek heb mogen werken op drie verschillende afdelingen binnen het Radboudumc; de interne geneeskunde, het center for molecular and biomolecular informatics (CMBI) en de medische microbiologie. Van collega's met verschillende achtergronden heb ik geleerd om vraagstukken vanuit verschillende invalshoeken te benaderen. Met veel plezier heb ik samengewerkt met andere (ex-) promovendi aan tuberculose en niet-tuberculeuze mycobacteriën. Arjan van Laarhoven, Lidya Chaidir, Sofiati Dian, Lindsey te Brake, Valerie Koeken, Edwin Ardiansyah, Ekta Lachmandas, Hinta Meijerink, Anca Riza, Hanna Guimaraes, Jodie Schildkraut, Mike Ruth en Jordy Coolen, ik heb ervan genoten om met jullie na te denken over ingewikkelde concepten, vragen, scripts en analyses en bovenal ook van de gezelligheid. Daarnaast gaat mijn dank uit naar ervaren wetenschappers binnen en buiten het Radboudumc van wie ik veel heb geleerd, Mihai Netea, Jos van der Meer, Heiman Wertheim, Vinod Kumar, Alexander Hoischen, Jelle Goeman, Jan Buitelaar, Taco Kooij, Hester Korthals-Altes, Aldert Zomer, Sacha van Hijum, Han de Neeling en Bas Dutilh, zonder wie ik nooit had leren programmeren. Mijn nieuwe kamergenoten bij de medische microbiologie, John Verbeek, Marion Dinnissen-van Poppel, Nel Gerrits-Hofmans, Paul Rutten, Jordy Coolen, Tim Baltussen, Margriet Hokken en Jan Zoll ben ik dankbaar voor het warme welkom, net als Ingrid van Weerdenburg, Saskia Kuipers, Ellen Koenraad, Mariëlle Rockland, Lian Pennings, Mike Mientjes, Melanie Wattenberg, Nicole Aalders, Jasper Sangen, Twan Klaassen en Arjan de Jong. Veel bioinformatica-inzichten heb ik gekregen dankzij (oud-) collega's bij het CMBI; Peter-Bram 't Hoen, Gert Vriend, John van Dam, Wynand Alkema, Rob ter Horst, Jon Black, Wouter Touw, Xiaowen Lu, Selma van Esveld, Barbara van Kampen, Josh Gillard, William Leenders, Daniel Garza, Balaji Venkatasubramanian, Laurens van de Wiel, Joeri van Strien, Tom Ederveen, Hanka Venselaar, Charlotte Kaffa, Sander Bervoets, Coos Baakman, Jeron Venhuizen, Renee Salz en niet te vergeten Arthur Pistorius, die menig serverprobleem voor me heeft opgelost. In particular, I'd like to thank Dei Elurbe, Joanna Lange, Robin van der Lee and Lisette Meerstein-Kessel. You are not only skilled bioinformaticians, but also close friends. I hope we will stay in touch, wherever the wind may take us. Dear Lise, I am verry happy to have you as my paranimph.

Ik wil ook graag mijn dank betuigen aan Barbara van Kampen, Mieke Daalderop en Jeanine van der Wijst-Schrauwen, die ontzettend veel werk uit handen nemen van artsen en onderzoekers in de dynamische academische ziekenhuisomgeving.

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Soms moet je de halve wereld over om iemand die dichtbij woont te leren kennen. Lieve Rachel, in Bandung ontmoette ik je en intussen ben je een dierbare vriendin geworden. Je beseft zelf denk ik maar half hoeveel je me hebt gesteund de afgelopen jaren. Dankjewel voor al je positiviteit, lieve attenties en je vermogen om altijd een lach op mijn gezicht te kunnen toveren.

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Curriculum Vitae

Carolien Ruesen was born in Wehl, the Netherlands, on July 26th in 1988, and grew up in Duiven with her parents and sister. She graduated from the Stedelijk Gymnasium Arnhem, after which she studied Biomedical Sciences at the Radboud University in Nijmegen. Carolien went to Southampton, the United Kingdom, for her Bachelor's internship at the Southampton General Hospital where she studied spinal posture in chronic stroke patients under supervision of professor Ann Ashburn. She also participated in the Honours programme for Medical Sciences. During her Master Biomedical Sciences she did a major in Epidemiology, and gained her first experience with tuberculosis research during an extracurricular research project on tuberculosis together with dr. Rob Aarnoutse and professor Reinout van Crevel. Then, she performed an internship on drug-resistant tuberculosis at the Rijksinstituut voor Volksgezondheid en Milieu with professor Dick van Soolingen and dr. Rianne van Gageldonk. Carolien performed her graduate internship – studying determinants of HIV viral 'blips' - at the Radboud university medical center department of IQ Healthcare in Nijmegen, under supervision of professor Marlies Hulscher and professor Reinout van Crevel, for which she was awarded the Radboudumc Masterprijs Biomedische wetenschappen, and the Best Student Award Epidemiologie from the department for Health Evidence. After graduation, Carolien participated in the Radboud university medical center PhD Competition and received a scholarship to start a PhD project designed by herself, under supervision of professor Van Crevel. In preparation for her PhD, she worked for the TB-HIV research group led by dr. Bachtis Alisjahbana at the Padjadjaran University in Bandung, Indonesia, and lived there for seven months. Besides her research activities, Carolien has been a member of the Radboud Institute for Health Sciences PhD Council. She is currently working at the Radboudumc department of medical microbiology, in the mycobacteriology lab led by dr. Jakko van Ingen.

PhD portfolio

Institute for Health Sciences
Radboudumc

Name PhD candidate:

C.J. Ruesen, MSc.

Department:

Internal Medicine / Medical Microbiology

Graduate School:

Radboud Institute for Health Sciences

PhD period:

01-04-2014 – 01-07-2019

Promotor(s):

Prof. R. van Crevel, Prof. M.A. Huynen

Co-promotor(s): Dr L. Chaidir, Dr J. van Ingen

| | YEAR(S) | ECTS |
|---|---------|------|
| TRAINING ACTIVITIES | | |
| A) COURSES & WORKSHOPS | | |
| RIHS Introduction course for PhD students | 2014 | 1.0 |
| Introduction to R course, Maastricht University | 2014 | 1.0 |
| 5E005 Genetic epidemiology | 2014 | 5.5 |
| Scientific Integrity | 2015 | 1.0 |
| How to write a medical scientific paper | 2015 | 0.2 |
| Comparative genomics | 2016 | 5.5 |
| BEAST: divergence dating using genomic sequence data, Utrecht University | 2016 | 0.2 |
| The next step in my career | 2017 | 0.6 |
| Introduction to Python for biologists (IPYBo5), Glasgow, UK | 2018 | 1.5 |
| Advanced Python for biologists (APYBo2), Glasgow, UK | 2018 | 1.5 |
| Illustrator Workshop | 2019 | 0.1 |
| B) SEMINARS & LECTURES | | |
| RIHS seminar How to get the most out of your PhD part 1: industry | 2015 | 0.1 |
| RIHS seminar How to get the most out of your PhD part 2: a career in academia | 2015 | 0.1 |
| C) SYMPOSIA & CONGRESSES | | |
| Science day Internal Medicine | 2014 | 0.25 |
| RIHS PhD Retreat (poster) | 2014 | 0.5 |
| Radboud Science Day (e-poster) | 2015 | 0.25 |
| Center for Molecular and Biomolecular Informatics spring conference (poster) | 2015 | 0.2 |
| KNAW meeting PhD Students on Science 2.0 | 2015 | 0.2 |
| Center for Molecular and Biomolecular Informatics autumn conference (oral) | 2015 | 0.2 |
| RIHS PhD Retreat | 2015 | 0.5 |
| TANDEM progress meeting, Craiova, Romania (oral) | 2016 | 1.0 |
| European Molecular Biology Organization Conference Tuberculosis 2016, Paris, France (poster) | 2016 | 1.25 |
| Center for Molecular and Biomolecular Informatics spring conference | 2016 | 0.1 |
| Radboud Center for Infectious Diseases Science Day (e-poster) | 2016 | 0.25 |

| | YEAR(S) | ECTS |
|--|-----------|--------------|
| TRAINING ACTIVITIES | | |
| C) SYMPOSIA & CONGRESSES | | |
| RIHS PhD Retreat | 2016 | 0.2 |
| TBNET symposium on drug-resistant tuberculosis in Europe | | |
| Vereniging voor Epidemiologie symposium 'Go your own way' | 2016 | 0.5 |
| International Conference on the Pathogenesis of Mycobacterial Infections, Stockholm, Sweden (poster) | 2017 | 0.25 |
| RIHS PhD Retreat | 2017 | 0.1 |
| RIHS PhD Retreat | 2017 | 1.0 |
| European Congress of Clinical Microbiology and Infectious Diseases, Madrid, Spain (e-poster & poster) | 2017 | 0.5 |
| Center for Molecular and Biomolecular Informatics spring conference (oral) | 2018 | 1.0 |
| Union World Conference on Lung Health, The Hague, Netherlands (oral) | 2018 | 0.2 |
| Symposium 'Radboud New Frontiers 2018, Big data, better healthcare?' | 2018 | 0.2 |
| Science day Infectious Diseases & Global Health (oral) | 2018 | 0.25 |
| D) OTHER | | |
| Journal Club Center for Molecular and Biomolecular Informatics | 2014–2018 | 1.5 |
| Journal Club Health Evidence ('junior refereren') | 2014–2017 | 6.0 |
| Center for Molecular and Biomolecular Informatics comparative genomics lunch meetings | 2014-2018 | 6.5 |
| Cytokine meetings Internal Medicine | 2014-2017 | 2.0 |
| 'Broodje mycobacteriën' (tuberculosis / nontuberculous mycobacteria lunch meeting) | 2016-2018 | 0.5 |
| TEACHING ACTIVITIES | | |
| E) LECTURING | | |
| BSc course Global Health MED-MINo6 | 2014,2016 | 0.2 |
| BSc course Infection and Host Defense 5DT05 | 2015 | 0.1 |
| F) SUPERVISION OF INTERNSHIPS / OTHER | | |
| Project supervisor BMS Minor Clinical Research, Radboud University Hilde van Ras, Bo van Santvoort en Marloes Taken | 2015–2016 | 1.0 |
| Project supervisor BMS Minor Clinical Research, Radboud University Monse Wieland, Lieke Vissers, Mariken de Wit en Francine van Wifferen | 2016–2017 | 1.0 |
| OTHER | | |
| RIHS PhD Council member | 2015-2017 | 2.5 |
| Co-organizing a 2-day PhD Retreat | 2015-2017 | 3 |
| College Tour-like interview with Louise Gunning at RIHS PhD Retreat | 2016 | 0.3 |
| College Tour-like interview with Ronald Plasterk at RIHS PhD Retreat | 2017 | 0.3 |
| TOTAL | | 53.15 |

Research data management

The clinical data obtained during my PhD at the Radboud university medical center have been captured and stored in REDCap (Research Electronic Data Capture), a secure web application for building and managing online surveys and databases, which is managed by colleagues at the St George's University of London. The privacy of the participants in this study is warranted by use of encrypted and unique individual subject codes. Raw sequencing data described in Chapter 2, 3 and 5 are available through the Sequencing Read Archive (SRA) and are accessible via the sample numbers described in these chapters. The raw sequencing data used for Chapters 4 and 6 are stored on a server belonging to the Center for Molecular and Biomolecular Informatics (CMBI) at the Radboud university medical center, and will be made publically available through the SRA or the European Nucleotide Archive after publication of the respective manuscripts. We are also in the process of archiving these data in the new secured Digital Research Environment (DRE) of the Radboud university medical center. The scripts used to analyse the data are stored on Evernote, application software designed for note taking, organizing, task lists, and archiving. These will also be stored in the DRE. Published data generated or analyzed in this thesis are part of published articles and their additional files and scripts are available from the associated corresponding authors upon reasonable request.

List of publications

Ruth MM, van Rossum M, Koeken VACM, Pennings L, Svensson EM, **Ruesen C**, Bowles EC, Wertheim HFL, Hoefsloot W, van Ingen J. Auranofin activity exposes thioredoxin reductase as a viable drug target in *Mycobacterium abscessus*. *Antimicrob Agents Chemother*. 2019 Jul 1.

Zweijpfenning SMH, Schildkraut JA, Coolen JPM, **Ruesen C**, Koenraad E, Janssen A, Ruth MM, de Jong AS, Kuipers S, Aarnoutse RE, Magis-Escurra C, Hoefsloot W, van Ingen J. Failure with acquired resistance of an optimised bedaquiline-based treatment regimen for pulmonary *Mycobacterium avium* complex disease. *Eur Respir J*. 2019 Apr 18.

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