Adaptation and evolution of drug-resistant Mycobacterium tuberculosis
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Citation for published version (APA):

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CHAPTER
one

Introduction
It’s a complicated story; lotta ins, lotta outs, lotta what-have-yous

- Jeff “The Dude” Lebowski
“On the basis of my extensive observations, I consider it as proven that in all tuberculous conditions of man and animals there exists a characteristic bacterium which I have designated as the tubercle bacillus [...]”

These were the words that Robert Koch spoke on March 24 1882 when he made public his discovery of the causative agent of tuberculosis (TB), *Mycobacterium tuberculosis*, for which he received the Nobel prize for Physiology or Medicine in 1905. Together with the recognition of TB as a single, transmittable disease (Jean-Antoine Villemin) and the finding that it is a curable disease, this was a major step forwards in the fight against TB.

Reports of people suffering from TB, or “phtisis” as it was dubbed by Hippocrates (ca. 460 BC), have been made as early as 4000-2000 BC [1], making it one of the oldest infectious diseases known to man. However, advanced palaeomicrobiological analysis of ancient (bacterial) DNA (aDNA) extracted from human remains [2] and, recently, comparison of whole bacterial genomes, indicate that *M. tuberculosis* has been infecting humans long before the Neolithic period (ca. 7000 BC) [3,4]. Evidence of typical lesions and spinal TB (Pott’s disease) in human fossils and mummies furthermore suggests that those ancient encounters with *M. tuberculosis* were not merely environmental but led to symptomatic disease and were often fatal.

Despite its infectious and lethal nature, it was not until the 18th century that TB took epidemic forms, most likely fuelled by the industrial revolution and the massive urbanization and poor hygiene/sanitation associated with it. This European epidemic coincided with the last period of the Renaissance and the Romantic Movement and many historic figures of that time were either victimized or inspired by TB. This resulted in a detailed description of “consumption” or the “white plague”, as TB was often referred to then, and the impact the disease had on society, literature and the arts [5].

Between the beginning of the 18th and the end of the 19th century, approximately one billion people died from TB globally, accounting for 25% of all deaths in this period. Around the turn of the 19th century, after Koch’s major discovery of the tubercle bacilli, the view on TB shifted from a romantic and even spiritual disease to a major killer that needs to be controlled. Efforts were made to cure TB patients and eliminate the disease, which ranged from quarantining infected individuals to collapsing or even surgical removal of the affected lung(s), with varying success rates.

In 1943 Schatz and Waksman isolated streptomycin, a secondary metabolite produced by the soil bacterium *Streptomyces griseus*, with antimicrobial activity against a broad range of microorganisms including *M. tuberculosis*. The successful cure of TB patients with streptomycin, during the first randomized controlled trial ever [6], was the
The beginning of chemotherapeutic treatment of TB. The cure rates of TB were increasing, although prior to these trials the incidence of TB had already been declining in most Western-European regions since the 1920s-1930s. It has been suggested that this decline was due to increased awareness of transmission risks as well as better care for TB patients [7–9].

The euphoria surrounding the first anti-TB drug was short-lived, however, as the first resistance-associated cases started emerging almost as soon as streptomycin came into clinical use. Introduction of new antibiotic compounds, such as isoniazid and para-aminosalicylic acid soon followed, but resistance was also quickly observed for these synthetic drugs [10]. Today resistance to all anti-TB drugs has been observed in all possible combinations. It was soon realised that treatment therapies with more than two drugs reduced the incidence of drug resistance and therefore made successful cure of the patient more feasible.

In the second half of the twentieth century TB was practically eliminated in the West until HIV/AIDS coinfection, poverty, failure of national TB Programmes and lack of appropriate treatment protocols caused TB to resurge in the late 1980's. The wake-up call came when New York was afflicted by an outbreak of multidrug-resistant TB in 1985, which through concerted action was controlled at the turn of the century, but at an estimated cost of 1 billion dollar. Despite these commendable local control measures, the worldwide TB epidemic continued to grow and spread, so that the World Health Organization (WHO) declared TB a global health emergency in 1993.

It is estimated that in 2010 alone over 8 million people developed tuberculosis (TB) and 1.4 million died from the consequences of a TB infection [11]. Due to improved global efforts to control TB, numbers have been declining almost everywhere in the world since 1996. However, the high prevalence, incidence and mortality of TB still make it the one of the most devastating infectious diseases, second only to HIV/AIDS (www.who.int). Uncomplicated pulmonary TB in adults or adolescents is, in principle, treatable with currently available drugs. Moreover, a vaccine is available that offers some protection against childhood TB, but that is highly efficacious against TB meningitis and miliary TB [12].

Unfortunately, circa 95% of TB cases and deaths occur in developing countries, where resources are typically limited, resulting in insufficient prevention, detection and treatment of TB. But also the long duration of treatment (6 months to over two years for multidrug or extensively drug resistant TB), development of drug resistant TB and the extent of the current globalization are obstructing the global eradication of TB.
Diagnosis

The typical appearance of a TB patient – pale, thin, weak, hollow-eyed – was often used to diagnose the disease until the end of the 19th century [1]. Other common symptoms include coughing (with or without blood), night sweats, loss of appetite, chest pains, short breath, fatigue and fever. Currently, anyone with an otherwise unexplainable persistent (productive) cough is suspected to suffer from TB and is therefore recommended to undergo TB diagnostic evaluation. Direct or indirect detection of TB bacilli in clinical samples collected from these persons confirms they are suffering from active TB.

After Koch discovered the causative bacterium and postulated his rules to prove the cause of an infectious disease, efforts were made to visualize tubercle bacilli in clinical specimens. The microscopic identification of TB bacteria is still the recommended method to diagnose TB suspects. Little has been changed on the staining procedures needed to identify mycobacteria, but efforts were made to improve the performance, of which fluorescent microscopy and the use of LEDs instead of mercury vapour lamps for illumination have led to an incremental improvement of the method [13–15].

In many high income countries TB screening is performed by radiography (chest x-rays), a method with low specificity. To identify infection, more and more use is made of immunological assays, such as the tuberculin skin test, or interferon gamma release assays [16,17], all of which have limited ability to discriminate active from latent TB. In contrast, light microscopic investigation of sputum samples remains the mainstay of TB diagnosis in many resource-limited settings, as is the often the only test for TB available.

Microscopy is specific when used on symptomatic suspects but lacks sensitivity. The low sensitivity of sputum smear microscopy [15], particularly in young children, people living with HIV/AIDS or extrapulmonary TB, has stimulated the development of other diagnostic tools, with the aim to improve the detection rates of TB.

It is recommended by the WHO that microscopy is always followed by culture. Traditionally this is done on solid Löwenstein-Jensen medium, with an average turn-around-time of 6-8 weeks. Rapid culture systems, based on the growth of TB bacteria in liquid medium, can reduce this time to 1,5 weeks (BACTEC 460) [18,19] and even combine with direct drug susceptibility testing for the four first-line drugs (BACTEC MGIT 960)[20,21]. The major drawback of culture methods is that a specialised laboratory, trained staff and a good infrastructure are required, making it difficult to implement in resource-limited countries. In addition, there is a considerable risk of contamination with other (myco)bacteria, most of which are naturally resistant to the majority of the TB drugs. This potential misclassification of the infective agent leads to
unwarranted elimination of effective drugs from the treatment regimen and implies post-culture identification of the cultured organism is essential when liquid culture is used and colony morphology cannot be seen.

The unravelling of the complete *M. tuberculosis* genome [22] has led to the development of many molecular tests based on the detection of species-specific genetic targets, shortening the time to definitive diagnosis to under a day. In the first instance these methods consisted of PCR amplification of selected discriminative *M. tuberculosis* genes (16S, IS6110) or, in contrast, of genes that were more conserved throughout the mycobacterial species (16S-23S ITS, *rpoB*), followed by sequencing. These assays proved highly specific, but obtained variable sensitivities compared to culture. The use of real-time PCR systems instead of traditional PCR, showed improvement of the sensitivity, but for both methods complex sample treatment and the expensive post-amplification analysis method prevented them from being implemented on a large scale. A major breakthrough in TB molecular diagnostics was the invention of line-probe assays, such as the commercially available InnoLiPa Mycobacteria [23] and the GenoType Mycobacterium [24]. These methods are able to discriminate *M. tuberculosis* complex and a range of other clinically relevant non-tuberculous mycobacteria (NTMs), by reverse hybridisation of PCR amplicons (16S-23S rRNA (InnoLiPa), gyrB or 23 rDNA (GenoType MTB)) to DNA probes that are immobilised on a strip. Both methods show very high specificity and sensitivity and can therefore be used directly on clinical samples or for the confirmation of (rapid) culture results [25,26]. As such, they have found their entry into many references laboratories in high income countries, but have been unable to replace microscopy or culture in low income countries. This is mainly due to the fact that line probe assays are technically demanding and relatively expensive and were, until very recently, only recommended for use on microscopy positive samples making initial microscopic examination a requirement.

Due to the high (and rising!) incidence of drug resistant TB, the WHO recommends that diagnosis of TB should preferentially be followed by testing the susceptibility of the isolated strains to the major anti-TB drugs. There is a plethora of phenotypic and molecular tests available, some of which I shall discuss below, that all have to be performed in addition to the diagnostic methods. Combined detection of the infecting mycobacteria and detection of drug resistance, preferably by using a single method, has the potential to reduce costs, time and errors that would otherwise hamper effective treatment.
Treatment

According to estimates made by the WHO, approximately one third of the people populating this earth is latently infected with \textit{M. tuberculosis}. When TB bacilli enter the naive host, they are assumed to establish a latent infection in the majority of the infected individuals. These latently infected individuals are at risk of developing active disease at some point in their lives. Some of the factors inducing reactivation of the bacteria have been identified, but the exact triggers that lead to active disease as well as if and when this will happen remain elusive. In resource-poor and/or endemic regions the proportion of infected people that develop active disease shortly after infection is considerably greater [27]. But even in this pool of symptomatic individuals, TB can still present as the typically chronic disease that it is known for, with intermittent periods of recovery during which the patient not only feels better, but the bacteria are also less prone to be transmitted [28].

Effective treatment of active TB therefore takes at least six months of chemotherapy. The first clinical use of streptomycin showed high rates of treatment failure due to the development of drug resistance. It is therefore recommended that TB patient be given at least two, but preferable three or even four drugs simultaneously. The effectiveness of combination therapy for TB was first proven in a clinical trial during which para-aminosalicylic acid (PAS) and streptomycin were administered simultaneously [29].

Another reason for combination therapy, apart from reducing the incidence of drug resistance, is to target all types of \textit{M. tuberculosis} populations inside the patient. TB is regarded as a heterogeneous disease; mycobacteria can be found in different structural foci that each have their specific environmental and physical conditions. The drugs included in the currently recommended first-line anti-TB therapy are each thought to be active against specific bacterial populations (See Table 1).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bactericidal/bacteriostatic</th>
<th>Spectrum of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>isoniazid</td>
<td>Bactericidal, early in therapy</td>
<td>Aerobically growing mycobacteria in pulmonary cavities</td>
</tr>
<tr>
<td>pyrazinamide</td>
<td>Bactericidal, until second month</td>
<td>Bacteria under low pH/in caseous necrotic foci, persisters</td>
</tr>
<tr>
<td>rifampicin</td>
<td>Bacteriostatic/bactericidal</td>
<td>Broad spectrum, is active against metabolically active bacteria throughout entire treatment</td>
</tr>
<tr>
<td>ethambutol</td>
<td>Bacteriostatic/bactericidal (depends on the dosage)</td>
<td>unknown</td>
</tr>
</tbody>
</table>

\textit{Table 1. Antibiotics included in first-line therapy and their suggested activity [30,31]}
Currently, the recommended treatment of uncomplicated cases entails administration of isoniazid, rifampicin, ethambutol and pyrazinamide daily for two months (intensive phase), followed by four months of isoniazid and rifampicin given three times a week (continuation phase). Treatment of drug-resistant TB is less standardized, because it depends on the resistance profiles in question, but also on the availability and effectiveness of second-line drugs. It is advised that at least one of the aminoglycosides (preferably not streptomycin) and one fluoroquinolone are included in the treatment regimen. Multidrug-resistant TB (MDR-TB), defined as TB resistant to at least rifampicin and isoniazid, can take up to 2-3 years to treat and is associated with lower success rates than TB susceptible to all major drugs. The treatment outcomes of extensively drug-resistant TB (XDR-TB) are even less favourable. This is partly due to the absence of effective drugs to treat XDR-TB and partly to the lack of appropriate tests to determine resistance against those drugs that are available. Since 1966, when rifampicin was marketed, no new drugs have been included in the standard first-line therapy; however, several clinical trials suggest that some newer second-line drugs (e.g. moxifloxacin) as well as new candidate anti-TB drugs can strengthen current first-line treatment regimens (REmxTB, http://www.ctu.mrc.ac.uk/remoxtb/default.asp). Some of these compounds (see Table 2) are new, others have previously been patented for the treatment of other ailments but have showed activity against TB and have been used on occasion compassionately or off-label for TB cases which have proven difficult to treat. Unfortunately, resistance has been observed in in vitro experiments for all of these compounds, indicating the need to use these drugs with the greatest of caution to ensure their efficacy for as long as possible.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>rifabutin</td>
<td>rifamycins</td>
</tr>
<tr>
<td>clarithromycin</td>
<td>macrolides</td>
</tr>
<tr>
<td>linezolid</td>
<td>oxazolidinones</td>
</tr>
<tr>
<td>cycloserine</td>
<td>d-alanine analogue</td>
</tr>
<tr>
<td>moxifloxacin</td>
<td>fluoroquinolones</td>
</tr>
<tr>
<td>thioridazine</td>
<td>phenothiazines</td>
</tr>
<tr>
<td>SQ109</td>
<td>ethambutol analogue</td>
</tr>
<tr>
<td>TMC207</td>
<td>diaryquinoline</td>
</tr>
<tr>
<td>PA-824</td>
<td>nitroimidazoles</td>
</tr>
</tbody>
</table>

Table 2. Several new potential TB drugs [30,32]. Rifabutin has a higher MIC than rifampicin and is preferred in case with HIV/AIDS coinfection, since it does not interfere with antiretroviral drugs. Linezolid and thioridazine are off-label drugs that have been used to treat MDR and XDR-TB. TMC207 and PA-824 are new compounds, currently in final phases of clinical trials. Moxifloxacin is a very powerful fourth generation quinolone; clinical trials suggest that duration of first-line therapy supplemented with moxifloxacin can be considerably reduced [33].
Drug resistance

From 1994 to 2010, 3.4% of new TB cases and 19.8% of previously treated cases globally involved multidrug-resistance. Treatment of MDR-TB consists of more expensive and more toxic compounds and has lower success (cure) rates than treatment for monoresistant or susceptible TB. The highest rates of MDR-TB among new (28.9%) and previously treated (65.1%) patients were found in former Soviet Union regions and the majority (>90%) of MDR-TB cases in the WHO European region are in Eastern European countries [11]. Mismanagement of MDR-TB can lead to extensively drug-resistant TB (XDR-TB), which is defined as MDR-TB with additional resistance to fluoroquinolones and at least one of the second-line injectable drugs. XDR-TB is associated with much higher mortality rates than MDR-TB, mostly because treatment options are very limited. According to estimates of the WHO, circa 9% of MDR-TB cases worldwide are in fact XDR-TB; most regions of the world had reported at least one XDR-TB case by the end of 2011. Misdiagnosis of MDR-TB and lack of effective treatment are thought to be the major causes for development of XDR-TB, whereas hospitalization and high HIV/AIDS prevalence are thought to facilitate the spread [11,34,35].

The initial trials with streptomycin in the 1940s showed that the combination of high mycobacterial load and monotherapy, treatment with a single drug, frequently results in the development of drug resistance. In the 1990’s the WHO adopted and endorsed a control strategy, DOTS, which stands for Directly Observed Therapy, Short-course. This strategy consists of five focal points, including governmental commitment, standardized treatment and observed intake of the drugs for the duration of the treatment. Under DOTS, the TB treatment was standardized and the duration was reduced from 1.5 years to six months. By ensuring a constant supply of quality drugs and stimulating adherence of the patient to the therapy, a considerable reduction in treatment failures and thereby increased control of TB was accomplished in the regions where DOTS was fully implemented. Incidence of drug resistance in new cases under DOTS is low, most likely because combination therapy is ensured for the duration of the treatment. In 1996, the DOTS strategy was extended to emphasize the control of MDR-TB, DOTS-plus, which requires that drug susceptibility testing is in place and quality assured second-line drugs are available [36,37]. DOTS is the most important component of the Stop TB Strategy 2006-2011, which was developed by the WHO in 2006.

Although the DOTS strategy has been implemented in over 80 countries in the world, there are still many patients that effectively receive monotherapy despite taking the full recommended set of drugs. This is partly due to the nature of the disease: active TB is heterogeneous or compartmentalized with different bacillary foci in the human body, in some cases even extrapulmonary. Consequently, not all administered drugs are able to reach the bacteria in sufficient doses to kill them. Malabsorption of anti-TB drugs, for
instance as a consequence of gastro-intestinal ailments or HIV/AIDS, has also been implicated in suboptimal therapy. In low-income countries, the main causes for drug resistance are intermittent therapy, scarcity or low quality of required drugs, inability to detect pre-existing (primary) drug resistance and poor compliance of the patient due to the cost, duration or adverse effects of the treatment.

In general, diversified treatment options and case management are thought to facilitate development of drug resistance, whereas areas with a strong, unified control programme, such as DOTS, have seen a decline in (MDR-)TB rates [11].

The compartmentalization of the bacilli in TB disease, as well as their lipid-rich outer cell wall does not only prevent most antibacterial chemicals from entering the mycobacterial cell, but also prevents the transfer of genetic material across the cell membrane. For many other bacterial pathogens this is a main source of acquisition of antibiotic resistance, whereas M. tuberculosis almost exclusively becomes resistant via spontaneous chromosomal mutations. Most of these mutations are single nucleotide polymorphisms (SNPs), but insertions, duplications and deletions have also been implicated in the acquisition of drug resistance. Drug resistance mutations mainly occur in the genes coding for the proteins that are directly targeted by the specific antibiotic compounds [38], responsible for activation of the drug or part of the pathway targeted by the drug [31,39–42]. See Table 3 for the currently used anti-TB drugs, the mechanisms of drug resistance and the most frequently affected regions in the mycobacterial genome.

The estimated rate of mutations conferring resistance to anti-TB drugs ranges from $10^{-6}$ to $10^{-9}$ mutations per replication, depending on the drug as well as the environment and growth stage of the bacilli. Since there is no proof of horizontal or vertical gene transfer in M. tuberculosis - M. tuberculosis is regarded as a clonal or monomorphic organism - once a mutation arises it is fixed in the genome of that particular cell and its entire offspring. Because the acquisition of drug resistance associated mutations are thought to be independent events, the chance of two mutations (and thus two antibiotics) occurring simultaneously would be $10^{-12}$-$10^{-18}$, in other words highly unlikely. This adds to the logic of the effectiveness of combination therapy, but also to the theory that polyresistant TB, MDR-TB and XDR-TB are the results of sequential mutagenesis and therefore emerge gradually rather than instantaneously.

Initially, when the first cases of MDR-TB presented, it was thought that the bacilli were too unfit to be transmitted, due to the impairment caused by at least two mutations. Emergence of MDR-TB was therefore regarded as a problem for (and of) the patient, rather than for the population [43,44]. However, as typing methods and molecular drug resistance detection methods advanced, it was shown that treatment failures were not only the result of de novo or acquired drug resistance (mutations occurred inside the patient) but also of primary drug resistance (infection with already resistant strains).
This knowledge has led to increased development and utilization of methods for
detection of drug resistance as well as typing of clinical TB specimens. Despite these
and other efforts to contain the transmission of drug-resistant TB, the incidence of
MDR-TB among new cases is rising faster than the incidence of TB in certain regions
[11]. Widespread transmission of XDR-TB has to date only been reported in association
with HIV/AIDS [35], but remains a cause for concern based on the experiences with the
expansion of MDR-TB.

An important factor that has contributed to the development and spread of drug
resistance is the delay in detecting drug resistance. In fact, most patients worldwide are
not tested for drug resistance at all, leading to inappropriate treatment in the presence
of (M)DR. Traditionally, susceptibility testing is performed by phenotypic methods in
which isolated TB strains are grown on selective medium. Susceptibility testing either
results in the minimal inhibitory concentration (MIC) of a certain compound for a strain
or the proportion of resistant bacteria present in a specimen [45]. Results of these tests
can take up to 3 weeks on LJ medium, but with automated culture systems [19,20] this
time can be shortened to 1-2 weeks. However, in this period from diagnosis to
determination of the proper treatment, patients are put on “empirical treatment”,
meaning that they are treated as if they were infected with a pansusceptible TB strain,
unless otherwise indicated. If, in fact, the case involves drug-resistant TB, this empirical
treatment can select for drug resistance and thereby cause treatment failure, instead of
preventing it. In addition to the long turn-around-time, drug susceptibility testing (DST)
requires the presence of specialized biosafety laboratories and personnel and it often
has a low predictive value, particularly for second-line drugs.

Many of the molecular mechanisms associated with drug resistance, mostly SNPs, have
been identified for all first-line and, to some extent, for the second-line drugs. For the
first-line drugs isoniazid, rifampicin and ethambutol, the most prevalent resistance-
conferring mutations found in resistant TB isolates are clustered in specific regions in
one or two genes. Many nucleic acid amplifications tests (NAATs), either based on in-
house protocols or commercially available [23,25], make use of this phenomenon by
identifying resistance mutations. By far the most widely used assays for molecular
detection of drug resistance to date, thanks to programmatic endorsement of the WHO,
are the GenoType assays which make use of multiplexed PCR amplification followed by
reverse hybridisation to a strip. Versions are available to detect resistance to isoniazid
and rifampicin [46,47] as well as for second-line drugs [48], although initial reports of
the predictive values of the latter are variable.

Molecular tests have the potential to significantly reduce the period from diagnosis to
drug resistance results and in addition make drug resistance testing possible in areas
where this was not done before. These tests can therefore reduce the amplification (by
shortening the empirical treatment period) and spread of resistance. However, there are
some major drawbacks that prevent them from being used routinely in all steps of the diagnostic chain. This is partly due to the fact that molecular testing is based on detection of drug resistance, not drug susceptibility. For some drugs the mutations conferring resistance appear in many regions of the genome and for some drugs not all mechanisms have been identified; molecular tests are therefore less than 100% sensitive. It also means that for full characterisation of a clinical isolate multiple tests (for diagnosis, differentiation, first-line and second-line drug resistance) have to be performed. Furthermore, the necessary equipment needs maintenance on a regular basis, is expensive and can only be operated by trained personnel. The performance is therefore mainly restricted to reference or regional labs and certainly not applicable at the point-of-care.

In the era of whole genome sequencing, genetic traits such as drug resistance mechanisms can be quickly identified and molecular tests are developing and evolving rapidly. In many labs these tests have only entered the stage very recently and as a consequence, clinicians are not always certain how to interpret the results to ensure the proper treatment. Studies to investigate the correlation of each molecular profile with clinical outcome are on-going.

It has been suggested that TB control would benefit on many levels from 1-day diagnosis combined with drug resistance testing [49]. A recently launched method that seems to fulfil this need is the GeneXpert MTB/RIF [50–52]. It consists of an automated closed system performing real time PCR for \( rpoB \), the gene involved with RIF resistance, and it can therefore detect both \( M. \) \( tuberculosis \) as well as drug resistance to RIF. Resistance to RIF is often strongly correlated with an MDR-TB phenotype and treatment failure with standard therapy. Results are produced within 2 hours and the assay has both a high specificity and sensitivity. It is furthermore a robust assay that has the ability to be performed close to the point of care and replace traditional methods and has therefore been recommended by the WHO.

Although the GeneXpert MTB/RIF is a huge step forward, the search continues for combined diagnosis, typing and drug resistance testing by methods that are rapid, easy to operate and more affordable.
<table>
<thead>
<tr>
<th>Drug</th>
<th>year of discovery</th>
<th>MIC (µg/ml)</th>
<th>Gene(s) Involved with resistance</th>
<th>Function of gene product</th>
<th>Mechanism of action</th>
<th>Most common resistance mutations at position (codon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>1952</td>
<td>0.02 – 0.2</td>
<td>katG, inhA</td>
<td>Catalase-peroxidase</td>
<td>Inhibition of mycolic acid synthesis and other effects</td>
<td>315</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1966</td>
<td>0.05 – 0.1</td>
<td>rpoB</td>
<td>β-subunit RNA polymerase</td>
<td>Inhibition of RNA synthesis</td>
<td>81-bp hotspot: 531, 526, 516</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>1952</td>
<td>16 – 50</td>
<td>pncA</td>
<td>Nicotinamidase/pyrazinamidase</td>
<td>Depletion of membrane energy</td>
<td>Scattered throughout regulatory and coding region</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>1961</td>
<td>1 – 5</td>
<td>embB</td>
<td>Arabinosyl transferase</td>
<td>Inhibition of arabinogalactan synthesis</td>
<td>306</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1944</td>
<td>2 – 8</td>
<td>rpsL, rrs, gidB</td>
<td>S12 ribosomal protein</td>
<td>Inhibition of protein synthesis</td>
<td></td>
</tr>
<tr>
<td>Amikacin/kanamycin</td>
<td>1957</td>
<td>2 – 4</td>
<td>rrs, eis</td>
<td>16S rRNA</td>
<td>Inhibition of protein synthesis</td>
<td>1401, 1484</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>1960</td>
<td>2 – 4</td>
<td>rrs, tlyA</td>
<td>16S rRNA</td>
<td>Inhibition of protein synthesis</td>
<td>1402, 1484</td>
</tr>
<tr>
<td>(Fluoro)quinolones</td>
<td>1963 (moxifloxacin since 1996)</td>
<td>0.5 – 2.5</td>
<td>gyrA, gyrb</td>
<td>DNA gyrase subunit A</td>
<td>Inhibition of DNA gyrase (topoisomerase?)</td>
<td>90, 91, 94</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>1956</td>
<td>2.5 – 10</td>
<td>ethA, inhA</td>
<td>Flavin monooxygenase</td>
<td>Inhibition of mycolic acid synthesis</td>
<td>Various deletions and SNPs</td>
</tr>
<tr>
<td>β-amino salicylic acid</td>
<td>1946</td>
<td>1 – 8</td>
<td>thyA</td>
<td>Thymidylate synthase A</td>
<td>Inhibition of folic acid and iron metabolism?</td>
<td>C(-)15 regulatory region, various SNPs in ORF</td>
</tr>
</tbody>
</table>

Table 3. MICs and mechanisms of resistance of current first- and second line anti-TB drugs. Adopted from refs [39,42].
Evolution and population structure

For a disease as old as TB it is nearly impossible to determine the origin of the disease, let alone “patient 0”. Until recently it was thought that humans contracted TB from cattle [5,53]. However, analysis of aDNA showed that humans suffered from TB long before the domestication of livestock [3]. In addition, extensive comparative genetic analysis has revealed that the causative agent of bovine TB, *Mycobacterium bovis*, and *M. tuberculosis* are related through a common ancestor, rather than that *M. bovis* evolved to *M. tuberculosis* directly [54–56].

*M. tuberculosis*, *M. bovis* and a group of related species, [22,55–57] which are all able to cause tuberculosis in humans, are collectively known as the *Mycobacterium tuberculosis* complex (MTBC). Already before the age of genomics it was determined that *M. tuberculosis* and *M. bovis* differed in the range of hosts they could cause disease in and it was therefore suggested they belonged to different species [58]. Currently, the MTBC includes different lineages that have been isolated from different animal sources. Over the course of time the various MTB lineages have each adapted to their primary host, to which they were named after [59], although the most recent common ancestor of the MTBC was most likely a human-adapted strain [55].

As genetic analysis advanced, more studies engaged in determining the population structure and phylogeny of *M. tuberculosis* [55,57,60–63] and it has been shown that all members of the MTBC share more than 99.95% genetic resemblance. Furthermore, it became evident that modern TB is not caused by a single strain, but rather that the population structure of *M. tuberculosis* shows genetic diversity. This diversity can be linked to human demography and migratory events and therefore suggests that the various *M. tuberculosis* strains have coevolved with their human hosts [56,57,63–67]. Various studies have investigated this divergence and based on the combined analysis of large sequence polymorphisms (LSPs), multilocus sequence typing (MLST) and single nucleotide polymorphisms (SNPs), six major phylogenetic lineages can be recognized within the human-adapted MTBC [55,60,63,64,68–72]. Each of these lineages is named after the geographical region where they were first identified and/or are most prevalent [57].

The comprehensive reconstruction of the evolution and the migration of the various strains highlight the clonal nature of the MTBC; the lack of horizontal gene transfer and the low recombination rate ensure that the majority of genomic changes, such as deletions or SNPs, are conserved throughout a genetic lineage. This trait has been the basis for the many molecular epidemiology and typing tools used to identify and differentiate clinical TB isolates [73]. The discriminatory ability and thereby the most appropriate use of these tools varies greatly and is directly linked to the stability of the
targeted biomarkers in the *M. tuberculosis* genome. Some comprehensive reviews have been published describing the range of molecular epidemiology and genotyping tools that are currently available, including the advantages and limitations of each of these methods [73–77].

The first molecular method that was standardized is IS6110 RFLP analysis [78], which involves determination of the copy number and position of IS6110, an MTBC-specific insertion sequence (mobile genetic element) with high diversity in the copy numbers and insertion sites among the different members of the MTBC. Although the transposition of IS6110 elements is to some extent random (in time and place in the genome), the method has proven to be quite suitable for the study of transmission dynamics and has been widely applied for cluster analysis. However, the ability to predict epidemiological clusters is rather limited in areas where TB is endemic or where circulating *M. tuberculosis* strains have low genetic diversity. In addition, for strains that carry less than six IS6110 copies the discriminatory power is low and a second method is usually required for more accurate genotyping. The method is furthermore technically demanding, requires subculturing of clinical isolates and interlaboratory comparison of obtained results is often problematic. Consequently, IS6110 RFLP analysis grew out of fashion as newer methods were developed that had the potential to replace it.

Currently, the most utilised typing method is spacer oligonucleotide typing, or spoligotyping [79]. This PCR-based method is based on the detection of 43 unique DNA spacer sequences in the direct repeat locus of the *M. tuberculosis* genome, which are interspersed by multiple 36-basepair direct repeats (DRs). Each of these spacers can be either present or deleted, resulting in characteristic 43-digit binary profiles (present/absent) by which specific genotypic strains can be recognised. For spoligotyping only a fraction of the bacterial DNA that is required for RFLP is needed to obtain a profile, allowing typing to be performed directly on patient material rather than waiting for cultures, which is the case for IS6110 RFLP. The turnaround-time for spoligotyping is also considerably faster than for IS6110 RFLP typing and highly reproducible results are produced that allow intra- as well as interlaboratory comparison. As a consequence, spoligotyping has been widely implemented and numerous strains derived from almost all regions of the world have been typed, resulting in a comprehensive database that allows newly typed strains to be allocated to genotypic families, based on their “spoligotype” [80].

With spoligotyping some of the ill-defined low IS6110 copy number strains can be further discriminated [81], but in general its resolution is lower than that of IS6110 RFLP, particularly for a distinct phylogenetic group referred to as “Beijing” strains [82,83] and in areas where specific strains are endemic or predominant. Together with an increased susceptibility for convergent evolution, the lower resolving ability makes spoligotyping less applicable for analysis of transmission chains and for surveillance.
Equivalent power as IS6110 RFLP can be obtained by MIRU-VNTR (mycobacterial interspersed repetitive unit – variable number tandem repeat) typing [84]. With this technique the number of tandem repeats or repetitive units at specific loci in the mycobacterial genome is determined. The number and size of the repeats are identified by PCR with primers specific to the flanking regions of each locus, followed by size determination via gel or capillary electrophoresis or even non-denaturing HPLC. Different sets of loci have been proposed and evaluated through the years, but the level of discrimination associated with these sets was often poor and additional typing methods were needed to accurately determine the genotype of clinical isolates [85–87].

In 2006, a standard set of 24 discriminative loci was suggested by a group of different laboratories [84]. The output of MIRU-VNTR typing results in a 24-digit numerical value, which in itself is not very informative except for transmission studies. As a result, it is not possible to generate a rooted tree with data provided by MIRU-VNTR. To resolve this issue a panel of 186 well-characterised strains, derived from various regions of the world and representing the main lineages within the MTBC, was typed with the standard set and results were entered into an online database for comparison purposes [88].

Because of its technical flexibility, relatively low price and rapid turn-around time, MIRU-VNTR typing is quickly becoming the standard tool for typing in many (reference) laboratories. However, despite its suitability for epidemiological purposes, it cannot be used for deep phylogenetic analyses or the determination of evolutionary descent. Additionally, as with spoligotyping the method is prone to homoplasies and determination of main lineages is not fully congruent with phylogenies inferred from sequence-based methods, like SNP or LSP analysis or MLST [89,90].

In the last decade, analysis of complete mycobacterial genomes has become increasingly feasible and affordable. The data that results from whole genome sequencing (WGS) can, however, be overwhelming and for an organism with such high clonality as \textit{M. tuberculosis} routine characterisation by WGS seems unnecessary. In contrast, WGS can be a very helpful research tool, not only to determine the rate of mutation in transmission lines, but also to select robust and informative genetic markers for typing purposes. A comprehensive phylogeny of the \textit{M. tuberculosis} complex has been created on the basis of discriminative genetic markers [55,60,63,64,68–72], giving a clear overview of the genetic diversity within this class of pathogens. WGS analysis of representatives of each lineage that is recognised could allow for further delineation within these lineages, although there is a risk of discovery bias [91]. MLST, LSP or SNP analysis are suitable methods for the multiplexed detection of various selected polymorphisms, each with their own “molecular clock”. Depending on the loci and number of markers included, a high resolution can be obtained, particularly with SNP typing. In addition, a high level of automation can be obtained and results are highly portable and allow for comparison between laboratories as well as between techniques.
The widespread implementation of each of the methods discussed here indicates that they have proven their usefulness. However, effective TB control does not rely on a unified approach or single genotyping system; rather, it is imperative to determine where each technique would be most suitable to ensure the information provided will be most beneficial [89,91].
Fitness

The most distantly related members of the MTBC still show more than 99% genetic resemblance and each lineage seems to be well-adapted to its host, suggesting an optimal interplay between host and pathogen factors [61,67,92]. However, certain MTBC lineages seem to challenge this apparent host tropism or geographical restriction; particularly members of the Beijing, Haarlem and LAM genotypic groups have been observed well outside of the geographical region they were named after and in patients of different ethnicities [63,65]. This is partly due to human migration and non-specific immune responses, but data from multiple epidemiological studies suggest that certain MTBC lineages are also more virulent or more “fit” than others. This increased virulence or fitness may lead to increased spread, acquisition of drug resistance, less vaccine-induced immunity, more severe disease or a combination of these [92–95].

In an attempt to elucidate the factors that underlie the seemingly evolutionary benefit, Beijing strains have been the subject of numerous studies conducted in the last decades. Many of these studies have shown that the Beijing family is highly associated with MDR and increased dissemination, amongst others [96–99]. Moreover, drug-resistant Beijing strains are often transmitted more efficiently than non-Beijing strains [100]. As drug resistance is often associated with an initial decrease in fitness (compared to drug susceptible counterparts in a non-selective environment), this either suggests that the fitness of Beijing strains is less affected by the acquisition of drug resistance mutations or that Beijing strains have a higher adaptive ability that allows them to restore the fitness deficit more rapidly. Many explanations have been suggested for this increased virulence and apparent lack of reduced fitness, including both host as well as pathogen factors [99].

One possible explanation is that certain bacterial populations have an increased adaptive ability due to an increased mutation rate, also referred to as “hypermutation”. In various common bacterial pathogens, this state of increased mutation is a direct consequence of less efficient post-replicative DNA repair; most hypermutators have mutations in one (or more) of the mutHLS genes, which are involved with mismatch repair [101–105]. In the genome of M. tuberculosis no homologues of the mutHLS system are present but several genes have been identified that play a role in DNA repair [106–108]. Many lineages of Beijing and some other lineages that have been associated with increased virulence, such as Haarlem, carry mutations in one or more of the so-called 3R genes (recombination, repair and replication) [109]. Possibly, impairment of DNA repair by mutations in 3R genes results in an increased mutation rate thereby increasing drug resistance rates.
Another mechanism that has been implicated in adaptive evolution is the SOS response, which is suggested to lead to a temporarily or transiently increased mutation rate [110]. This stress response is induced by DNA damage, caused by for instance mechanical stress or radiation, and is regulated by RecA and LexA [111]. It involves upregulation of dnaE2, an error-prone DNA polymerase with low fidelity that is not essential for survival of the bacteria, but that increases the genetic variety within the bacterial offspring. The SOS response is therefore thought to, transiently, promote (adaptive) evolution of a clone or a population. Some antibiotics, such as fluoroquinolones, have been shown to increase the SOS response and thereby the mutation rate in a wide range of bacterial species [112–115].

To all drugs used to treat TB resistance has emerged. The mutations that confer resistance to anti-TB drugs are mostly thought to confer a fitness deficit to the bacteria carrying them, a phenomenon that has been studied extensively in vitro [116,117] and in some cases by cluster analyses. Even though many resistant TB strains are regarded less fit than their susceptible counterparts, the high selection pressure exerted by the antibiotics in question causes the resistance-conferring mutations to be fixed in the gene pool, most often by acquisition of mutations that restore or even increase the fitness [118–121]. Bacteria that have a (temporary) increased ability to acquire mutations do not only have a higher chance of becoming resistant, but also of acquiring adaptive mutations that restore their fitness [122]. As a consequence, resistant bacteria would then be able to compete with susceptible strains in drug-free environments [44,123,124].

The extent of the fitness deficit conferred by de novo (drug resistance) mutations varies between the different mutations and the different strains; certain resistance mutations are found more often in specific genotypic families, suggesting that the fitness of any given mutation is also dependent on the genetic background of the organism [125–128]. Of all rifampicin resistance-conferring mutations observed in resistant mutants rpoB-S531L and rpoB-H526D/Y seem to have the least impact on fitness and, most likely as a consequence, are found most often in vivo as well as in vitro [116,129]. In the majority of isoniazid-resistant isolates a katG-S315T mutation is found. This mutation results in little to no loss of catalase activity and is associated with transmission [128,130–132]. Typically, all these mutations confer high levels of drug resistance.

As the number of drugs a strain has developed resistance to increases, the spectrum of viable mutations decreases, indicating that acquisition of resistance to a second or even a third drug forms a rather severe evolutionary bottleneck [133–135]. This convergent evolution provides the opportunity to identify polyresistant, MDR or XDR strains by targeting only a few resistance mutations.
Research questions and structure of thesis

TB is one of the oldest infectious diseases known to man and hence *Mycobacterium tuberculosis*, the infective agent, has had ample time to adapt to its preferred host and changing environment. This high level of adaptation led to the evolution of a successful pathogen, which is manifested in the chronic nature of the disease.

The population structure and detailed genetic analysis indicate that members of the *Mycobacterium tuberculosis* complex (MTBC) are well adapted to specific hosts; different genotypes can be recognised that are either geographically restricted or indicate human migration and animal types are restricted to their host.

Most *M. tuberculosis* clones are able to evade the host’s immune system and moreover to use it for the optimisation of the infection. Also chemotherapy is successfully evaded by genetically encoded resistance mechanisms that the mycobacteria have developed as a consequence of adaptive evolution. The widespread emergence of antibiotic resistant has challenged the view of TB as a treatable disease.

Many studies have been conducted on drug resistance and the evolution of *M. tuberculosis*. Notwithstanding, many molecular mechanisms facilitating the emergence, adaptation and spread of drug-resistant TB have yet to be discovered. This thesis reports studies of the adaptive mechanisms, using mostly in vitro based experiments and molecular tools to study the importance and influence of specific factors on the development of drug resistance and the evolution of drug-resistant strains of *M. tuberculosis*. The structure of this thesis is as follows:

**CHAPTER 2: Acquisition of rifabutin resistance by a rifampicin resistant mutant of *Mycobacterium tuberculosis* involves an unusual spectrum of mutations and elevated frequency.**

*Research question*: Does pre-existing rifamycin resistance influence the spectrum and frequency of subsequent rifamycin resistance?

*Synopsis*: In *Mycobacterium tuberculosis* mutations that confer resistance to rifampicin are mostly constrained to a 81-bp region of *rpoB*, with S531L and H526Y being the most prevalent resistance mutations found in clinical isolates. Mutants selected in vitro acquire the same mutations in *rpoB* as clinical isolates. We investigated the type and frequency of *rpoB* mutations that were acquired by a laboratory strain carrying a pre-existing *rpoB*-S522L mutation.
CHAPTER 3: Specific mutations in the *Mycobacterium tuberculosis* *rpoB* gene are associated with increased *dnaE2* expression.

**Research question:** Do clinically relevant rifampicin resistance-conferring mutations in *rpoB* elicit a stress response, as measured by *dnaE2* expression?

**Synopsis:** Survival of *de novo* mutants, for instance newly drug resistant *M. tuberculosis* clones, is possibly associated with (transient) stress responses. Increased expression of *dnaE2*, coding for an error-prone DNA polymerase in *M. tuberculosis*, is an indicator for stress and has been implicated with an increased mutation and survival rate. In a panel of isogenic *rpoB* mutants we determined the baseline *dnaE2* expression and compared these values to those obtained for the wildtype parent strain.

CHAPTER 4: Development of multiplex assay for rapid characterization of *Mycobacterium tuberculosis*.

**Research question:** Can detection of informative and discriminative genetic markers in the *M. tuberculosis* genome be combined with a multiplex molecular tool?

**Synopsis:** *M. tuberculosis* is a clonal organism with no evidence of transfer of genetic material across the cell wall and a low recombination rate. In addition, mutational events such as single nucleotide polymorphisms (SNPs) or deletions are mostly irreversible. As a consequence, distinct phylogenetic lineages as well as drug resistant strains can be recognised by specific mutational profiles. We developed a tool that enables multiplexed detection of multiple dispersed genetic markers in the *M. tuberculosis* genome, thereby combining identification of drug resistance and genotypic lineage. In this chapter the validation of this assay, based on Multiplex Ligation-dependent Probe Amplification (MLPA), is described.

CHAPTER 5: Resistant mutants of *Mycobacterium tuberculosis* selected *in vitro* do not reflect the *in vivo* mechanism of isoniazid resistance.

**Research question:** Can the high mutation rate associated with isoniazid resistance be explained by an *in vitro* model of the development of isoniazid resistance?

**Synopsis:** The high prevalence of isoniazid-resistant *M. tuberculosis* is often explained by a high mutation rate for this trait, although detailed information to support this theory is absent. We studied the development of isoniazid resistance *in vitro*, making use of a laboratory strain of *M. tuberculosis*. Spontaneous isoniazid-resistant mutants were characterized by molecular methods allowing identification of the most commonly encountered resistance-conferring mutations. Additionally, we determined the *in vitro* mutation rates for isoniazid and rifampicin resistance, and characterized the genome of a triple resistant strain.
CHAPTER 6: Pre-existing isoniazid resistance, but not the genotype of Mycobacterium tuberculosis drives rifampicin resistance codon preference in vitro.

Research question: Do genotypic lineage or pre-existing drug resistance influence the rate and/or spectrum of mutation for rifampicin resistance in vitro?

Synopsis: Both the probability of a mutation occurring and the ability of the mutant to persist will influence the distribution of mutants that arise in a population. We studied the interaction of these factors for the in vitro selection of rifampicin-resistant \textit{M. tuberculosis} mutants. We used well-characterised laboratory strains as well as clinical isolates, representing various \textit{M. tuberculosis} genotypes. Two distinct experimental approaches were used for the selection of in vitro resistant mutants, which were screened by MLPA or by sequencing of the \textit{rpoB} gene. For all strains the mutation rate for rifampicin resistance was determined with an adapted fluctuation assay.

CHAPTER 7: \textit{Mycobacterium tuberculosis} population structure determines the outcome of genetic based second-line drug resistance testing.

Research question: In the development of resistance within the patient, can heteroresistance be detected at a timely stage and will it be able to inform appropriate treatment regimens?

Synopsis: Drug resistance in \textit{M. tuberculosis} is mainly mediated by small genetic changes in target genes. Infection is often established in various infective foci within the patient, sometimes with distinct structural organization and populated by genetically different mycobacterial populations. It can thus be challenging to reach or maintain proper concentrations of all anti-TB drugs in all of these foci. Because of this compartmentalization there can be mixed infections with respect to genotype, but also with respect to populations that are susceptible and resistant to drugs. This heterogeneity can sometimes be observed by genotyping techniques or molecular resistance testing, but it is expected that in early stages of the development of drug resistance the detection limit of currently available tools is not sufficient to detect small (sub)populations of resistant clones. We have investigated the genetic basis of resistance to ofloxacin and amikacin in the first available \textit{M. tuberculosis} isolate as well as follow-up isolates from patients with pre-XDR and XDR-TB.

CHAPTER 8: Combined species identification, genotyping, and drug resistance detection of \textit{Mycobacterium tuberculosis} cultures by MLPA on a bead-based array.

Research question: Can the \textit{M. tuberculosis} MLPA assay be extended and analysis improved by transfer to a liquid bead-based array?

Synopsis: Previously, we developed an \textit{M. tuberculosis} specific MLPA assay allowing the multiplexed detection of 17 informative and discriminative genetic markers. This
method is based on final analysis by capillary electrophoresis, requiring expensive equipment and maintenance, which makes implementation of the MLPA assay in low-income countries less feasible. To address this issue, we transferred the analysis to a bead-based array, the Luminex MAGPIX system, which allows the simultaneous detection of 50 distinct analytes. The MLPA was expanded from a 17-plex to a 47-plex assay, including three controls. In this chapter the method and initial validation are described.

CHAPTER 9: General discussion.

In this chapter the main implications and overall conclusions resulting from the research presented in this thesis will be discussed.
Definitions and abbreviations

TB tuberculosis
DNA deoxyribonucleic acid
MDR-TB multidrug-resistant tuberculosis
WHO World Health Organization
HIV human immunodeficiency virus
AIDS acquired immunodeficiency syndrome
LED light emitting diode
PCR polymerase chain reaction
NTM non-tuberculous mycobacterium
RNA ribonucleic acid
XDR-TB extensively drug-resistant tuberculosis
DOTS directly observed therapy, short-course
polyresistant resistant to multiple drugs, but not MDR
SNP single nucleotide polymorphism
LJ Löwenstein-Jensen
pansusceptible susceptible to all (classes of) drugs
MTBC Mycobacterium tuberculosis complex
RFLP restriction fragment length polymorphism
aDNA ancient DNA
rDNA ribosomal DNA
rRNA ribosomal RNA
LSP large sequence polymorphism
MLST multi-locus sequence type/typing
DR direct repeat
HPLC high pressure/performance liquid chromatography
MIRU mycobacterial interspersed repeat unit
VNTR variable number tandem repeat
WGS whole genome sequence/sequencing
LAM Latin-American Mediterranean
in vitro in a test tube, culture dish, or elsewhere outside a living organism
in vivo within a living organism
de novo (mutation) newly acquired, not present in parental DNA
MLPA multiplex ligation-dependent probe amplification
BC before Christ
References


