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Molecular Detection and Characterization of Drug-Resistant *Mycobacterium tuberculosis*

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Cover photo: A close-up of nucleotide position 1349 of the gene *rpoB* in *Mycobacterium tuberculosis*. This is the second nucleotide position of the 450th codon, commonly referred to as the 531th codon. When substituted by a DNA replication error, resistance to the drug rifampicin arises.

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It is better to do nothing,
than to do what is wrong.
For whatever you do,
you do to yourself.

The Buddha

ABSTRACT

Tuberculosis (TB) is an ancient disease, but not a disease of the past. Despite the declaration of TB as a global emergency by the World Health Organization in 1993 the worldwide problem of TB has worsened. The increasing prevalence of drug-resistant strains of *Mycobacterium tuberculosis*, the causative agent of TB, demands new measures to combat the situation. Rapid and accurate diagnosis of the pathogen, and its drug susceptibility pattern, is essential for timely initiation of treatment, and ultimately, control of the disease. Furthermore, knowledge about which precise mutations confer drug resistance in *M. tuberculosis* does not only lead to a basic understanding of drug resistance mechanisms and drug actions, but is also important for the design and development of clinically sensitive and specific molecular methods aiming at detecting drug-resistant *M. tuberculosis*.

Paper I in this thesis investigated cross-resistance between the aminoglycosides amikacin (AMK) and kanamycin (KAN), and the cyclic peptide capreomycin (CAP). The results show that *tlyA* is neither a sensitive nor a specific genetic marker for detection of CAP resistance in *M. tuberculosis*, and that it is advisable to include *rrs* nucleotide position 1401 in a molecular-based assay for the detection of AMK-, KAN- and CAP-resistant *M. tuberculosis* clinical isolates. **Paper II** aimed at developing a pyrosequencing method for detection of first- and second-line resistance in *M. tuberculosis*. Pyrosequencing assays were developed for the genes *rpoB*, *katG*, *embB*, *rrs*, *gyrA* and the promoter regions of *inhA* and *eis*, which are associated with resistance to rifampicin (RIF), isoniazid, ethambutol, AMK, KAN, CAP and fluoroquinolones, respectively. Pyrosequencing is a highly throughput and robust method for detection of novel and a priori known mutations. The method can be used to screen a large sample volume, which is desired if aiming at investigating the prevalence of mutations in large sample collections. In **Paper III**, the utility of padlock probes for detecting drug resistance in *M. tuberculosis* was evaluated. The assay was developed for RIF resistance due to the importance of RIF in the standard TB treatment and its potential role as a surrogate marker for multidrug-resistant TB. The method proved to be robust for detection of specific mutations in the gene *rpoB*, and confirmation of loss of wild type as well as detection of *M. tuberculosis* complex DNA. The padlock probe assay was further extended in **Paper IV** to detection of extensively drug-resistant TB in a multiplexed fashion. Padlock probes were designed to target the most common mutations occurring in *rpoB*, *katG*, *rrs*, *gyrA* and in the promoter region of *inhA*. The analytical sensitivity achieved in **Paper IV** is comparable to that of PCR. The readout format employed in **Paper IV** eliminates the use of extensive equipment, but rather, signal can be detected by the naked eye.

This thesis has contributed to increased knowledge of drug resistance in TB, and has successfully developed new methods for rapid detection of drug-resistant *M. tuberculosis*. The results can guide future research and development of molecular methods aiming at detecting drug-resistant *M. tuberculosis*.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals.

I. **Engström, A.**, Perskvist, N., Werngren, J., Hoffner, S.E., Juréen, P. *Comparison of clinical isolates and in vitro selected mutants reveals that tlyA is not a sensitive genetic marker for capreomycin resistance in Mycobacterium tuberculosis.* J Antimicrob Chemother, 2011. **66**(6):1247-54.

II. **Engström, A.,** Morcillo, N., Imperiale, B., Hoffner, S.E., Juréen, P. *Detection of first- and second-line drug resistance in Mycobacterium tuberculosis clinical isolates by pyrosequencing.* J Clin Microbiol, 2012. **50**(6):2026-33.

III. **Engström, A.**, Zardán Gómez de la Torre, T., Strømme, M., Nilsson, M., Herthnek, D. *Detection of rifampicin resistance in Mycobacterium tuberculosis by padlock probes and magnetic nanobead-based readout.* PLoS ONE, 2013. (in press): e62015. doi:10.1371/journal.pone.0062015

IV. **Engström, A.**, Russell, C., Bandaru, M.K., Nilsson, M., Herthnek, D. *Detection of extensively drug-resistant Mycobacterium tuberculosis by padlock probes, rolling circle amplification and a low-density array.* Manuscript

Related work by the author not included in the thesis:

Martin, A., Paasch, F., Docx, S., Fissette, K., Imperiale, B., Ribón, W., González, L.A., Werngren, J., **Engström, A.**, Skenders, G., Juréen, P., Hoffner, S., Del Portillo, P., Morcillo, N., Palomino, J.C. *Multicentre laboratory validation of the colorimetric redox indicator (CRI) assay for the rapid detection of extensively drug-resistant (XDR) Mycobacterium tuberculosis.* J Antimicrob Chemother, 2011. **66**(4):827-33.

CONTENTS

LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AIDS	Acquired immunodeficiency syndrome
AMK	Amikacin
BCG	Bacillus Calmette–Guérin
bp	Base pair
C2CA	Circle-to-circle amplification
CAP	Capreomycin
CO	Capture oligonucleotide
DNA	Deoxyribonucleic acid
DO	Detection oligonucleotide
DST	Drug susceptibility testing
EMB	Ethambutol
FQ	Fluoroquinolone
HIV	Human immunodeficiency virus
INH	Isoniazid
ITS	Internal transcribed spacer
KAN	Kanamycin
LJ	Löwenstein–Jensen
LOD	Limit of detection
LPA	Line probe assay
MDR-TB	Multidrug-resistant tuberculosis
MIC	Minimum inhibitory concentration
MTC	*Mycobacterium tuberculosis* complex
NTM	Nontuberculous mycobacteria
PCR	Polymerase chain reaction
POC	Point-of-care
RCA	Rolling circle amplification
RIF	Rifampicin
RO	Restriction oligonucleotide
RRDR	Rifampicin resistance-determining region
rRNA	Ribosomal ribonucleic acid
TB	Tuberculosis
UTR	Untranslated region
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

1 INTRODUCTION

1.1 TUBERCULOSIS

1.1.1 Global burden

Tuberculosis (TB) is a common and in many cases a lethal infectious disease which has tormented mankind for millennia (1). The etiology and infectious character of TB was first described by the German physician and microbiologist Robert Koch in 1882 (2), for which he was awarded the Nobel Prize in Physiology or Medicine in 1905. More than a century later, despite advances in diagnostic, vaccination and treatment strategies, numerous challenges remain. Today, TB ranks number eight on the list of the most common causes of death worldwide, and it is the second most common cause of death from an infectious disease after the human immunodeficiency virus (HIV) (3).

When appropriately diagnosed and treated, TB is largely curable. Yet, in 2011, there were an estimated 8.8 million new TB cases and 1.4 million deaths, an equivalent of 3,800 deaths per day, caused by TB (3). The burden of TB is highest in Africa and Asia, and the five countries with the largest number of incident cases in 2011 were India, China, South Africa, Indonesia and Pakistan. China and India together account for almost 40% of the world's TB cases. The wide range of estimated per capita TB incidence rates can be exemplified by 6 per 100,000 population in Sweden (4), to alarmingly 993 per 100,000 population in South Africa (3), in 2011. Figure 1 shows estimated TB incidence rates by country in 2011. The majority of deaths caused by TB occur in developing countries (5), with case fatality rates of HIV-related TB being particularly high.

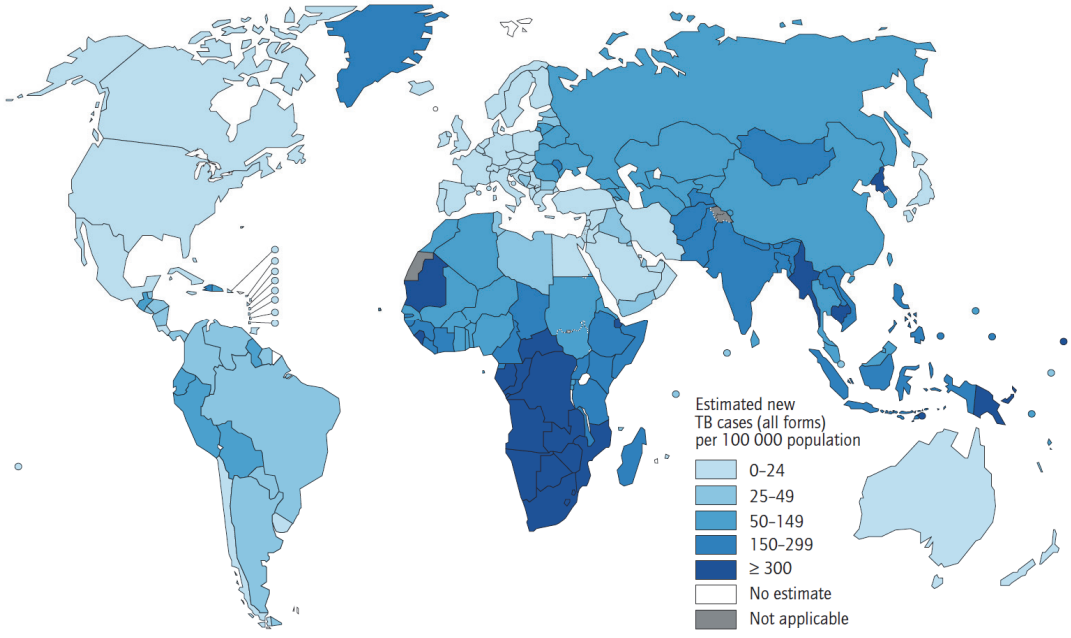

Figure 1. Estimated TB incidence rates by country in 2011. Reprinted with permission from the World Health Organization (3).

1.1.2 *Mycobacterium tuberculosis*

The causative agent of TB in humans was given its name *Mycobacterium tuberculosis* in 1886 (6). *M. tuberculosis* is sometimes referred to as the tubercle bacillus, as the bacterium found in round nodules known as tubercles in the lungs of TB patients. Mycobacteria can be divided into several major groups for purpose of diagnosis and treatment: the *M. tuberculosis* complex (MTC) that can cause TB, *M. leprae* that causes leprosy; and nontuberculous mycobacteria (NTM) includes all the other mycobacteria. NTMs are widely distributed in the environment, and are in most cases nonpathogenic for humans. Apart from the MTC and *M. lepeae*, the genus *Mycobacterium* comprises more than 140 species (7), of which approximately 50 are currently considered to be etiological agents of human disease (8). *M. avium* and *M. intracellulare* are two common NTM species primarily affecting immunocompromised individuals (9). The NTM *M. ulcerans* causes the necrotizing skin disease Buruli ulcer. This disease has the highest incidence in West Africa and is in many cases neglected despite being one of the most common human mycobacterial infection after TB and leprosy in some countries (10, 11).

The MTC is a group of closely related slow-growing mycobacteria. It traditionally comprises *M. tuberculosis*, the causative agent of the vast majority of human TB cases; *M. canettii* and *M. africanum*, agents of human TB in Africa; *M. microti*, an agent of TB in voles; and *M. bovis* which infects a wide variety of mammalian species including cattle and humans. Other additional spices are *M. caprae* and *M. pinnipedii* (12), which are causative agents of TB in animals. The near relatedness of the MTC members has been verified by high degree of similarity at the DNA level (12, 13). Despite this genetically close relationship, particular phenotypic characteristics, including different host preferences, have led researchers to maintain the traditional species names of these bacteria instead of classifying them as one species (6).

The sequence of the first complete genome of *M. tuberculosis*, that of the laboratory strain H37Rv, was published in 1998 by Cole and colleagues (14). The genome has a high guanine (G) and cytosine (C) content (65%), and comprises 4,411,532 base pairs (bp). The last re-annotation identified approximately 4,000 genes (15). Mobile genetic elements such as plasmids have been reported in some mycobacterial species, but not in *M. tuberculosis* (16). Nonetheless, insertion sequences (IS), and other repetitive elements, have been characterized in many mycobacterial species, including *M. tuberculosis*. Special attention has paid to the clustered regularly interspaced short palindromic repeat (CRISPR) sequences and IS6110 for molecular epidemiology, the former being an essential part of the bacterial adaptive immune system (17). The CRISPR system is used for spoligotyping (18), and IS6110 for restriction fragment length polymorphism analysis (19).

M. tuberculosis is a non-motile rod-shaped bacterium with a particularly slow generation time: 18-24 hours (20). Mycobacteria have a characteristic cell wall which is thicker than in most other bacteria. It consists of a peptidoglycan and polysaccharide

layer, and contains complex fatty acids, such as mycolic acids, which cause the waxy appearance and impermeability of the envelope. The three components alone make up 60% of the dry cell weight (21), whereof fatty acids comprise up to 30% (22). The cell wall of mycobacteria impedes classical Gram staining (23), but instead these organisms are classified as acid-fast bacilli (AFB).

In the human host, *M. tuberculosis* typically enters the alveolar passages of the lungs in the form of microscopic aerosolized droplets, known as aerosols, where it encounters cells of innate immunity, e.g. macrophages and dendritic cells, which are the main target cells for mycobacteria (24). *M. tuberculosis* is an obligate aerobe, i.e. requires oxygen to grow, but has the ability to survive in hypoxic conditions (25). The bacterium is phagocytosed by alveolar macrophages; however, has developed strategies to escape eradication and can thus survive within macrophages (26). From the initially infected phagocytes mycobacteria can spread to other parts of the lungs and the peripheral lymph nodes. Upon infection with *M. tuberculosis*, both antigen-specific humoral and cell-mediated immune responses are elicited by the host (27).

1.1.3 The Bacillus Calmette-Guérin vaccine

Bacillus Calmette-Guérin (BCG), the only vaccine available against TB, is one of the most widely used vaccines in the world (28). The live attenuated vaccine strain was derived from *M. bovis* by Albert Calmette and Camille Guérin at the Institute Pasteur of Lille, France, between 1906 and 1919 by 230 passages on potato slices soaked in ox bile and glycerol (29). Calmette and Guérin reported that during these passages, which each lasted for three weeks, the bacteria gradually lost their capacity to cause disease in various animal models (30), leading finally to the attempt to use it as vaccine for the prevention of TB in humans.

The vaccine, which has a low incidence of major side effects in immunocompetent individuals (28), protects children against disseminated forms of TB, such as the severe meningitis (31); however, the protection conferred against pulmonary TB in adults, representing the majority of the disease burden, has been highly variable in clinical trials, in fact ranging from 0 to 80% (32). The lowest levels of protection has been observed in countries with the highest incidence of TB (33).

Recently, a new BCG booster vaccine, the modified Vaccinia Ankara virus expressing antigen 85A (MVA85A) raised hope when it was evaluated in nearly 3,000 infants in South Africa. The vaccine was well tolerated; however, the results of the clinical trial also demonstrated that MVA85A did not elicit a protective effect against TB (34).

1.1.4 Clinical tuberculosis

TB is transmitted when an infected individual with active disease coughs, sneezes, or otherwise expels aerosols containing bacteria which are subsequently inhaled by another person into the lungs. The clinical presentation of *M. tuberculosis* infection is quite varied and depends on a number of both host- and bacterial-related factors. Host factors include age, immune status, malnutrition, immunization with BCG, and possibly genetic factors which are however currently unknown (35). In addition to host factors, there are probably factors related to the organism itself, such as virulence or tropism for specific tissues which influence the outcome and features of the infection. The host-microbe interaction also contributes to the clinical expression, for example, sites of involvement and severity of disease.

Among most immunocompetent individuals, infection with *M. tuberculosis* is highly likely to be asymptomatic. It has been estimated that the lifetime risk of developing active disease, i.e. clinically evident TB, after being infected is approximately 10% (36). In 90% of the cases the infection will remain latent or is possibly cleared, and will thus not cause disease (37). In certain subpopulations, individuals with immunodeficiency states for example, the proportion that develops active disease is much higher than in the general population. Among individuals who are co-infected with HIV and *M. tuberculosis*, the risk of developing TB is significantly increased to 5-15% per life year (38).

The site of involvement is an important factor influencing clinical expression of TB. Any organ can be the site of disease (39); however, approximately 85% of TB cases are limited to the lungs, with the remaining 15% involving extrapulmonary sites, or both pulmonary and extrapulmonary sites (40). This proportional distribution is substantially different among patients co-infected with *M. tuberculosis* and HIV, where extrapulmonary involvement is much more common. This is typical of what is seen in individuals with a compromised immune system, who have limited ability to contain the infection with *M. tuberculosis*, and as a consequence, hematogenous dissemination may occur with subsequent involvement of single or multiple extrapulmonary sites. Infants and the elderly are also more prone to develop disseminated disease, also referred to as miliary TB. Meningitis is the most frequent form of central nervous system TB with a peak incidence is in children younger than 4 years of age (35).

TB occurring at any site of the body causes certain symptoms that are not specifically related to the organ or tissue involved but are systemic in nature. Systemic manifestations, including fever, malaise, night sweats and weight loss, seem to be mediated by cytokines released by cells of the immune system (35). Cough is the most common symptom of pulmonary TB, which may be nonproductive during the early course of the disease. However, as inflammation and tissue necrosis proceed, sputum is usually produced with progressed disease. Abnormalities in the lungs, for example cavities, can be detected by radiographic examinations of the chest. Cavitation results from destruction of lung tissue and is common in pulmonary TB. Granuloma, the

hallmark of TB, creates an immune microenvironment in which the infection can be controlled. However, it also provides the bacteria with an environment in which they can survive over long periods of time. Macrophages are the dominant cell type in granulomas, but T cells are the organizers of granuloma structure and function. Healing of lesions in the lungs usually results in development of a scar with loss of lung volume. TB is a grim disease which must not be neglected. The prognosis of pulmonary TB is horrifying, if in fact left untreated it kills approximately 50-70% of those with active disease (41, 42).

1.2 HISTORY OF TUBERCULOSIS TREATMENT AND DRUG RESISTANCE

1.2.1 Sanatoria and discovery of antibiotics

During the pre-antibiotic era, i.e., before antibiotic treatment existed, a sanatorium offered the best chance for a TB patient to cure the disease. The first TB sanatorium was opened in 1859 in Poland (43), and subsequently became a common institution throughout Europe, in the United States and in other parts of the world. The treatment regimen consisted of bed rest, good nutrition and fresh air (42). Prior to the introduction of effective anti-TB drugs, there was a steady decline in TB. This has been attributed to many factors, including improvements in social conditions and nutrition (44).

Streptomycin (STR), discovered in 1943 (45), was the first drug used to treat TB. Antibiotic treatment proved to be more effective than the open-air treatment in a sanatorium and the introduction of TB chemotherapy led to the beginning of sanatoria demolishment. Para-aminosalicylic acid (PAS), discovered by the Swedish chemist Jörgen Lehmann, was the second antibiotic found to be effective against TB (46) and the drug was introduced in clinical use in 1948. STR was first administered as monotherapy; however, drug resistance became rapidly apparent (47). By the late 1940s, in order to circumvent drug resistance, a combinatory regimen with both STR and PAS was implemented. Isoniazid (INH), the third drug to be added to the combinatory TB regimen, was first synthesized over a century ago (48), but its activity against *M. tuberculosis* was not discovered until the early 1950s (49).

Despite the existence of drug-resistant M. *tuberculosis* since the introduction of anti-TB chemotherapy, the incidence of TB continued to decline in developed countries during the 1950s and 1960s (35). During this time, multidrug-resistant TB (MDR-TB) was known as resistance to STR, PAS and INH. In the early days of TB treatment, studies to monitor drug resistance were conducted in several countries (35, 50, 51). These surveys suggested occasionally high prevalence of resistance to STR, PAS and INH; however, generally stable levels were observed during the 1950s and 1960s (52). The high prevalence of STR resistance was attributed in great extent to wide use of STR monotherapy in the beginning of the TB treatment era. Only a few resource-limited countries monitored drug resistance trends by standard methods, nonetheless, reports from India and a few African countries during the 1950s and 1960s indicated high prevalence of INH resistance (51). Despite reports of drug resistance, the general decline in TB incidence suggested that the positive effect of combinatory anti-TB drug regimen was outweighing the negative impact of drug-resistant strains. Drug resistance was not considered to be a major problem (51).

During the early years of 1960, two additional anti-TB drugs, ethambutol (EMB) (53) and rifampicin (RIF) (54), were discovered and implemented in the treatment regimen a few years later. Pyrazinamide which was discovered already a decade earlier (55), was initially found to have little or no effect against TB *in vitro* (56). However, the drug had

a striking effect *in vivo* (56), and it was introduced in standard TB treatment during the 1980s. PZA along with RIF are believed to be important for shortening the duration of the TB treatment to 6 months (57).

During the 1970s and 1980s, parallel to the ongoing TB decline, the global interest in the disease was also diminishing. Funding was cut short, and TB control and surveillance activities were severely reduced and sometimes even completely stopped (35). At this time, RIF had been largely implemented in the standard TB regimen worldwide; yet the hazardous combination of resistance to RIF and INH was not envisaged a future problem. Only a few countries monitored trends on drug-resistant TB during 1970s and 1980s. Periodic surveys suggested an ambiguous situation, i.e. increasing as well as decreasing rates of resistance to anti-TB drugs (58, 59).

1.2.2 Multidrug-resistant tuberculosis

The 1980s marked the onset of the HIV and acquired immunodeficiency syndrome (AIDS) epidemic, and this, along with other factors such as immigration from high-incidence TB countries, homelessness and limited access to medical care led to a slowing decline or an increase in TB incidence in many high-income countries, after a steady decline. Several outbreaks of MDR-TB among hospitalized HIV/AIDS patients were documented in the United States and in parts of Europe in the late 1980s and early 1990s (60, 61). MDR-TB was at this time and still to date is defined as an *M. tuberculosis* strain that is resistant to at least the two main first-line anti-TB drugs RIF and INH. Treatment of MDR-TB is complicated; it requires the use of second-line anti-TB drugs which are less effective, more toxic and more costly. Moreover, treatment duration needs to be prolonged to 18-24 months (62).

Drug-resistant TB is in many cases the result of suboptimal antibiotic treatment. Poor treatment compliance, lack of high-quality drugs, misuse of drugs, poor follow-up of patients receiving treatment, lack of initiation of appropriate treatment regimens, and unsupervised usage of drugs obtained without prescription has been highlighted as the most important causes of resistance to anti-TB drugs.

The MDR-TB outbreaks in TB-HIV co-infected patients led to increased TB control actions in high-income countries. Diagnosis and treatment was standardized, drug susceptibility testing (DST) for culture-positive TB cases was performed, reporting and quality assurance was improved, directly observed treatment short-course (DOTS) was implemented and infection control procedures were enhanced. DOTS, lunched by the World Health Organization (WHO) in 1995, has five elements: political commitment, improved case detection, standardized treatment, reliable drug supply systems, and reliable monitoring, recording and reporting for supervision and evaluation (63). By the late 1980s and early 1990s, routine surveillance of drug-resistant TB was once again implemented, specifically to monitor the effects of renewed TB control efforts on drug resistance (35).

Most studies performed in resource-limited countries before the 1990s were not able to shed much light on the magnitude of problem with drug resistance and MDR-TB because of methodological limitations such as small sample size and lack of laboratory quality control. This led the Third World Congress on TB in 1992 to conclude that there was incomplete information about the global magnitude of MDR-TB and its impact on TB control (64). The following year, in 1993, the WHO declared TB to be a global emergency, specifically expressing that: "TB today is humanity's greatest killer, and it is out of control in many parts of the world. The disease, preventable and treatable, has been grossly neglected and no country is immune to it" (65, 66).

1.2.3 Global Project on Anti-TB Drug Resistance Surveillance

In 1994, the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) launched the Global Project on Anti-TB Drug Resistance Surveillance, aiming at assessing the global magnitude and monitoring trends of drug-resistant TB. Since then, country data are continuously collected, analyzed and disseminated to describe the global problem of drug-resistant TB. Evidence collected from 1994 and onwards within the framework of the Global Project on Anti-TB Drug Resistance Surveillance has confirmed previous assertions that in drug-resistant TB, including MDR-TB, is ubiquitous (67).

The first WHO/IUATLD global report on anti-TB drug resistance was published in 1997 (68). Until the early 2000s data consistently showed a low prevalence of MDR-TB among new TB cases in high-income countries, suggesting that MDR-TB was not a major problem in these settings (35). Drug resistance among previously treated patients occurred at a high prevalence in some countries, similar to the situation in the 1950s and 1960s; however, this was again not considered a troublesome situation since the proportion of patients with drug-resistant TB among all TB cases was estimated to be minimal (35). However, evidence did show that certain regions of Eastern Europe, Asia and other parts of the world endured a problem with drug-resistant TB that deserved full attention and close monitoring. During the 1990s, TB control was rapidly deteriorating in two global regions. Following the collapse of former Union of Soviet Socialist Republics (USSR), most of the newly independent states of Eastern Europe suffered rapid impoverishment and health system fragmentation. Between 1997 and 2000, the incidence of TB in the former USSR increased by 5 to 7% per year (69). The shift from a planned to a market economy, increasing poverty, collapse of the public health system, and continuation of outdated TB control measures have contributed to a reversal of the declining trends in TB in many of these countries. This increase, together with the lack of infection control activities, standardized treatment, and direct treatment supervision created the basis for the emergence of MDR-TB. At the same time in sub-Saharan Africa, the severity and consequences of the HIV epidemic on TB and drug-resistant TB were becoming increasingly apparent.

1.2.4 Extensively drug-resistant tuberculosis

In 2006, a new definition was announced, namely extensively drug-resistant TB (XDR-TB) (70). XDR-TB is defined as an MDR-TB strain that is also resistant to any fluoroquinolone (FQ) and at least one of the three injectable drugs amikacin (AMK), kanamycin (KAN), and capreomycin (CAP). FQs, AMK, KAN and CAP are important second-line drugs for the treatment of MDR-TB. Treatment is even more complicated for XDR-TB, and treatment success is low (71). In 2006 alarmingly high rates of MDR- and XDR-TB among TB-HIV co-infected patients were reported in South Africa (72). A mortality rate of 98% was reported for XDR-TB patients co-infected with HIV, and the majority of the patients died within two weeks of sputum collection (72). These observations called for urgent intervention since XDR-TB severely threatens the success of treatment programmes for TB and HIV.

1.2.5 Drug resistance today

Globally, 3.7% of new TB cases and 20% of previously treated cases were estimated to have MDR-TB in 2011. This translates to an estimated 310 000 new MDR-TB cases among notified TB patients with pulmonary TB. Almost 60% of these cases were detected in India, China and the Russian Federation. Figure 2 shows the percentage of MDR-TB among new TB cases in 2011. The highest rates of MDR-TB among new TB cases have been documented in Belarus (32%), Estonia (22%), Kazakhstan (30%), Kyrgyzstan (26%), and Uzbekistan (23%) (3). A survey recently conducted in Minsk, Belarus found MDR-TB among 35% of new TB cases and among 77% of those previously treated. This means that about half of the cases diagnosed in that setting harbor MDR-TB strains (73). XDR-TB has now been reported in 84 countries, and the average proportion of MDR-TB cases with XDR-TB is 9.0% (3). These observations have confirmed that MDR- and XDR-TB are severe problems and that specific control activities beyond the routine TB control are needed to address them.

As could be anticipated, the dreaded situation beyond XDR-TB has arisen. Recently, so called totally drug-resistant (TDR)-TB was reported in India, Italy and Iran (74-76). However, it should be noted that the term TDR has not been defined for TB and is not recommended by the WHO (77). While the concept is easily understood in general terms, in practice, *in vitro* DST can be technically challenging, and the term often reflects that a strain is resistant to a particular set of drugs available for DST at that time in a particular region or laboratory, which may not be all drugs available for treatment of TB.

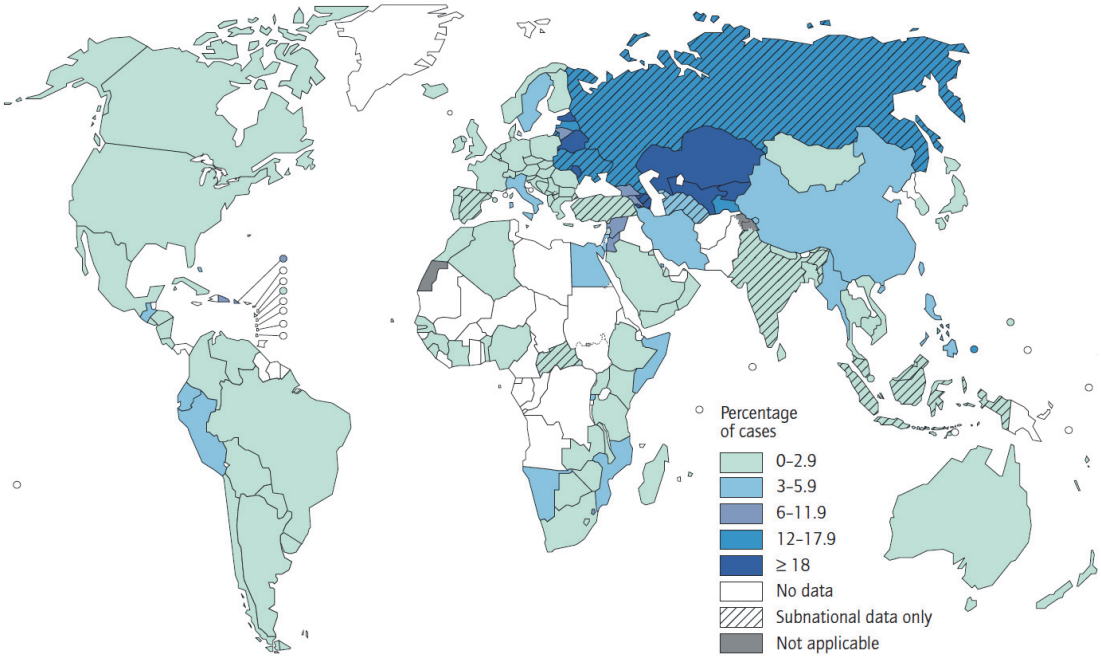

Figure 2. Percentage of new TB cases with MDR-TB in 2011. Figures are based on the most recent year for which data have been reported, which varies among countries. Reprinted with permission from the World Health Organization (3).

It must be realized that drug-resistant TB is serious global health problem. Inadequately treated patients become chronic carriers and can spread the strains further in the community. The findings of MDR- and XDR-TB at a low prevalence in a given region or setting does not imply that the issue can be ignored or forgotten there. Continuous transmission of drug-resistant strains by even a small number of individuals may develop into a serious problem if the issue is not addressed promptly. Ignored, it will mean more cases and higher costs to the health care system, which can be unmanageable in resource-limited countries. Drug-resistant TB is indeed very difficult to treat, and in fact, some cases may be practically impossible to treat. Public health measures to control or reverse the problem, where dissemination has already occurred, can be extremely expensive and are often beyond the means of countries with high prevalence of drug-resistant strains. It is clear that drug-resistant TB, when found at high prevalence, disrupts TB control efforts. Therefore, there is a critical need to find feasible and cost-effective solutions to tackle this global problem.

1.2.6 Current and future treatments

The current standard treatment for TB was implemented in the 1980s. The recommended treatment for drug-susceptible TB is a 6-month long combinatory regimen which consists of a 2-month intensive phase with the first-line drugs RIF, INH, EMB and PZA, followed by a 4-month continuous phase with the most effective drugs RIF and INH (62). A patient that is infected with a drug-resistant strain, especially in the case of MDR- and XDR-TB, must be prescribed drugs other than the standard regimen, as otherwise the number of effective anti-TB agents are low, and thus the

treatment is much less effective (78), and the risk of the bacteria developing resistance to other drugs is greatly increased (79). Preferably, DST results should guide treatment options; however, facilities enabling DST are largely not available in many settings around the world, and so, empirical MDR-TB treatment is unfortunately implemented in many cases. In conclusion, treatment of drug-resistant TB is much more complicated compared to its drug-susceptible counterpart, and patients are less likely to be cured (71). XDR-TB is very difficult to treat with currently available anti-TB drugs. In fact, treatment success of XDR-TB is below 30%, compared to over 90% for drug-susceptible TB (71). Thus, treatment of XDR-TB may be compared to a situation of no drug treatment at all.

Reports of alarming rates of drug resistance during the 1990s and 2000s accelerated the interest in developing new effective anti-TB drugs. Desired characteristics of a new anti-TB drug include a significantly decreased duration of treatment, lack of cross-resistance to other drugs, and compatibility with anti-retroviral therapy. In 2005, four decades after the discovery of the last anti-TB drug, Andries and colleagues reported the discovery of a new anti-TB drug, a diarylquinoline, named Bedaquiline (80). Bedaquiline is unique in its mode of action by inhibiting bacterial ATP production. The drug was approved by the Food and Drug Administration in the United States on December 28, 2012, and is recommended for the treatment of MDR-TB. Delamanid is another novel drug in late stage development, first reported in 2006 (81). The drug inhibits the synthesis of mycolic acids, a component of the mycobacterial cell wall (81). It is now evident that new, effective anti-TB drugs are urgently needed; however, they must be used wisely and carefully in order to avoid the emergence of drug resistance, and ultimately, loss of an effective agent. Ideally, not only one or a few new drugs are needed, but a whole new treatment regimen.

1.3 TUBERCULOSIS DIAGNOSTICS

Detection of the causative agent is a vital part of infection control. Prompt diagnosis is not only important for the individual patient, but also for the general public, since effective drug therapy will reduce the spread of the infectious agent in the community.

Clinical mycobacteriology laboratories play a key role in the control of the spread of TB through the timely detection, isolation, identification, and DST of *M. tuberculosis*. There is a great demand for reliable and rapid methods for diagnosing TB, and the emergence of MDR- and XDR-TB has during the last two decades generated a great interest in the development of molecular-based methods that enables rapid detection of *M. tuberculosis* and its drug susceptibility pattern. Still today, most TB patients globally are diagnosed by the method which was developed over a century years ago: microscopy.

1.3.1 Microscopy

In many African and Asia countries, where the TB prevalence is especially high, smear microscopy examination of nonconcentrated sputum specimens has been, and still remains the primary, or commonly, the only TB laboratory method available.

Smear microcopy is a low-cost method which is effective in detecting the most infectious cases and can be performed in basic laboratories. Smear-positive TB patients have high bacterial loads and have already developed cavitary lesions in the lungs. These patients are considered to be most infectious. Patients with smear-negative pulmonary TB have lower bacillary loads and generally lack cavitary lesions. The limit of detection (LOD) for microscopy is 5,000-10,000 AFB per ml sputum. Compared to culture-positive sputum specimens, the sensitivity of smear microscopy is approximately 50% (82); however, may be even lower in children and patients who are co-infected with HIV, who typically have a lower bacterial load. Thus, many cases remain undiagnosed by a laboratory method. The specificity of the technique is low, since a sputum smear-positive for AFB may represent either *M. tuberculosis*, an NTM, or another AFB.

AFB are difficult to detect using standard microbiological techniques (e.g. Gram stain), though they can be stained using concentrated dyes, particularly when the staining process is combined with heat (83). Contrary to other bacteria, AFB resist acid-based decolorization procedures, hence the name acid-fast. The high mycolic acid content of the mycobacterial cell walls is responsible for the high retention of the dye. The most common staining method used to identify AFB is the Ziehl-Neelsen staining technique. The bacteria are first stained with the pink dye carbolfuchsin and then decolorized with acid-alcohol. Mycobacteria retain the color after decolorization with the acid, but other bacteria are decolorized. The smear is subsequently counterstained with methylene-blue and analyzed in a light microscope. AFB are stained bright red and clearly stand out

against a blue background (84). AFB can also be visualized by fluorescence microscopy using specific fluorescent dyes (e.g. auramine-rhodamine stain), yielding better sensitivity (85).

Although smear microscopy may be considered a reliable tool for detecting the most infectious TB patients and for monitoring patient response to therapy (86), the problem of drug resistance, cannot be address with this technique.

1.3.2 Microbiological culture

Culture is considered the current diagnostic gold standard and is essential for detection of smear microscopy negative cases. The LOD of culture is considered to be 100 bacteria per ml sputum (87). Due to the particularly slow growth of *M. tuberculosis*, culture is a very time-consuming procedure. In fact, it may take weeks before colonies are obtained. Culture is compared to microscopy a relatively expensive method, and requires specialized laboratories and highly trained personnel. Due to the pathogenic nature of *M. tuberculosis*, culture isolation is not suitable for laboratories that do not have a proper biosafety level, including routines and appropriate equipment. An alternative for recourse-limited countries is to send specimens from the periphery to a regional or national reference laboratory for analysis. In middle-income countries that already have trained personnel and TB laboratories, abandoning culture isolation may not be advisable, but rather, emphasis should focus on upgrading existing TB laboratories. In other words, making them more efficient and safe. This is particular important in settings with high prevalence of drug resistance.

Globally, the egg-based culture medium Löwenstein-Jensen (LJ) is predominantly used for the isolation of *M. tuberculosis*. However, the agar-based culture medium Middlebrook is also used for culture. Both media require approximately three to six weeks from inoculation to show visible colonies (88). During the last three decades TB laboratories in resource-rich countries have implemented newer broth-based technologies for growth detection and DST. Mycobacteria have slightly faster growth rate in liquid media compared to solid media, consequently culturing methods involving liquid growth medium allows a more rapid identification of the bacteria (89).

1.3.3 Species identification

Differentiation of *M. tuberculosis* from other mycobacteria represents an important health issue in the diagnosis of TB. NTMs are increasingly recognized as causative agents of opportunistic infections in humans. *M. avium* is the most common and clinically relevant NTM species, especially among HIV-infected persons. The probability that the AFB detected by microscopy is actually *M. tuberculosis* depends on the frequency with which the laboratory isolates NTM. Intrinsic (natural) resistance to

antibiotics is not uncommon among mycobacteria, thus treatment should ideally be guided by species identification and DST results (90).

The classical laboratory approach to determination of bacterial species involves phenotypic characterization. Species identification is performed by observing colonial morphologic features, pigment production, growth rate and by employing biochemical tests for studying enzymatic characteristics. Biochemical tests involve for example investigation of reduction of nitrate and potassium tellurite, catalase production, and hydrolysis of Tween. Biochemical tests have successfully been used since the 1950s (91); however, using a battery of biochemical and other culture-based tests are both very labor- and time-consuming. Since the late 1980s high-performance liquid chromatography has been implemented for a more rapid identification of mycobacteria. The principle is based on the analysis of mycolic acids present in the cell wall (92).

More recently, rapid nucleic acid amplification tests have been developed to identify species. The AccuProbe Mycobacterium Tuberculosis Complex Culture Identification Test (Gen-Probe, Inc., San Diego, CA, USA) and COBAS TaqMan Mycobacterium Tuberculosis (MTB) Test (Roche Diagnostics, Basel, Switzerland) are two polymerase chain reaction (PCR)-based commercially available tests (93). COBAS TaqMan identifies *M. tuberculosis* specifically, whereas the AccuProbe test does not differentiate between different MTC species, but only detects the complex itself.

There are also commercially available line probe assays (LPAs) for detection of *M. tuberculosis*, the MTC or NTMs. The INNO-LiPA Mycobacteria v2 (Innogenetics, Ghent, Belgium) targets the 16S-23S ibosomal ribonucleic acid (rRNA) internal transcribed spacer (ITS) region and can be used to detect the genus *Mycobacterium* and 16 different mycobacterial species. The GenoType MTBC (Hain Lifescience GmbH, Nehren, Germany) is also a LPA for differentiation of the MTC. In addition, the tests GenoType Mycobacterium CM and the GenoType Mycobacterium AS (Hain Lifescience GmbH) can be used to identify the MTC and 40 NTMs.

1.4 DRUG RESISTANCE

1.4.1 Phenotypic characteristics

Resistance is a phenotype, or in other words, an observable characteristic. Resistance describes the ability of a bacterial cell to survive the presence of a drug at a concentration that normally kills or inhibits growth. Drug resistance is caused by genetic changes in the bacterium which are passed to subsequent generations. This can be differentiated from tolerance, which is a conditional phenotype typically mediated by the physiological state of the bacteria (94). Most antibiotics act against actively growing bacteria, and the lack of susceptibility of nongrowing bacteria to antibiotics is due to changes in bacterial metabolism or physiological state. Bacteria in the stationary phase become phenotypically resistant to many antibiotics. *M. tuberculosis'* phenotypic resistance to INH in the stationary phase is one example (95).

Another type of phenotypic resistance relates to the phenomenon of persisters and dormant bacteria. Persisters are a small number of bacteria, from actively growing cultures, that are not killed after exposure to antibiotics (96). The concept of persistence it not equivalent to dormancy, even though the terms in some cases can be used interchangeably. Dormant bacteria are in a state with no or low metabolic activity and do not form colonies directly on solid medium, however, can be resuscitated to form colonies on plate under appropriate conditions. On the other hand, persisters may or may not form colonies on plates, an outcome which depends on the environment (97). It is due to the survival of a small number of bacteria during antibiotic exposure despite lacking genetic resistance mechanisms that the minimum inhibitory concentration (MIC) is commonly defined as the lowest concentration of antibiotic that kills or inhibits growth of 99% of a bacterial population, but not 100% (98). The presence of persistent or dormant TB bacteria is believed to be one of the reasons for the lengthy duration of TB chemotherapy, since current drugs are not effective in eliminating persistent or dormant bacilli (99).

1.4.2 Genotypic characteristics

From the perspective of the pathogen, drug resistance can be either intrinsic or acquired. Intrinsic resistance refers to nonsusceptibility of a bacterium due to its unique characteristics. For example, *M. tuberculosis* is naturally resistant to penicillin due to its productions of beta-lactamases which deactivate the drug (100). Acquired resistance refers to susceptible bacteria becoming resistant to drugs as a result of genetic alterations. The emergence of drug-resistant bacteria demonstrates the survival of the fittest concept of Charles Darwin's theory of evolution (101). The host perspective of drug resistance in TB is summarized in Figure 3. Resistance is classified as acquired when drug-resistant mutants are selected as a result of suboptimal treatment, and as primary when an individual is infected with an already drug-resistant strain.

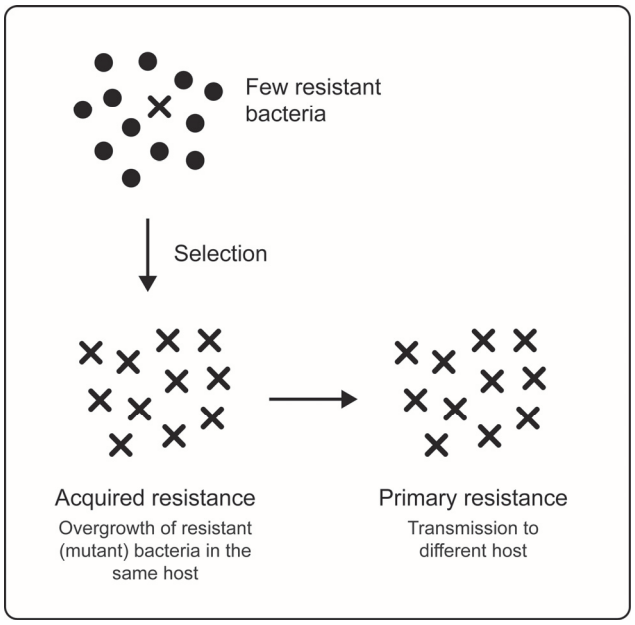

Figure 3. Selection of resistant bacteria during suboptimal antibiotic treatment. Continuous treatment could lead to acquired resistance. The remaining susceptible organisms in the population are eliminated. Primary resistance describes the scenario when an individual is infected with an already resistant strain.

To date, there are no reports of drug resistance in *M. tuberculosis* being mediated by mobile genetic elements, such as plasmids, transposons, or integrons, which are common in other bacterial species, but instead resistance seems to be conferred exclusively by spontaneous mutations in the chromosomal DNA (102). A point mutation is an alteration in the DNA sequence caused by a single nucleotide base change, insertion, or deletion. In the case when a gene codes for a protein, substitutions can be classified as nonsynonymous or synonymous, depending on whether the mutations lead to a change in amino acid sequence of the protein for which the sequence codes. Nonsynonymous or missense mutations are those that alter the amino acid sequence, and in the reversed scenario, synonymous or silent mutations are those that do not alter the amino acid sequence. Base substitutions most commonly confer drug resistance in *M. tuberculosis*; however, insertions and deletions of single bases, or longer regions, are also observed (103). *M. tuberculosis*, like other bacteria, becomes resistant by a number of strategies, including target modification, target overexpression, drug-inactivating enzymes, inactivation of drug-activating enzymes, and possibly efflux pump mechanisms (87, 104-106).

Pleiotropism refers to the phenomenon when a single gene affects several phenotypes (107). In *M. tuberculosis*, no pleiotropic mutation that mediates multidrug resistance has been reported; instead resistance to several drugs is caused by sequential accumulations of mutations in the chromosome. Cross-resistance arises when a strain acquires resistance to one drug through direct exposure, and at the same time also becomes resistant to one or more other drugs to which it has not been exposed to. Cross-resistance arises because the mechanism of resistance to several drugs is the same. The probability of a strain becoming resistant to two drugs with different

resistance mechanisms is given by the additive resistance rate for each drug. The mutation rate for RIF is approximately 1 in 10^8 organisms (108), and approximately 1 in 10^6 for INH (109), thus, the probability of a strain becoming resistant to both RIF and INH, i.e., and MDR-TB, in one strep is 1 in 10^{14}. It is unlikely that a bacterium becomes simultaneously resistant to both RIF and INH since areas of infiltration, granulomas, and even cavitary lesions are estimated to contain less bacteria (in average approximately 10^8-10^9 organisms) (87). However, it is important to note that the model breaks down when chemotherapy is suboptimal. Monotherapy, irregular drug intake, suboptimal dosage, poor drug quality, or insufficient number of active agents in the treatment regimen are examples of circumstances where resistant mutants can be selected, and remaining susceptible organisms in the population are eliminated, allowing the surviving drug-resistant organisms to eventually comprise the entire population (35, 110).

Mechanisms of drug action and resistance of the first- and second-line anti-TB drugs studied in this thesis are described below. The most frequent genes involved, their function and role, mechanisms of action, and reported frequencies of mutations are summarized in Table 1. Knowledge about which precise mutations confer drug resistance in *M. tuberculosis* does not only lead to a basic understanding of drug resistance mechanisms and drug actions, but is also important in order to design and develop clinically sensitive and specific molecular methods for the detection of drug-resistant *M. tuberculosis* isolates.

Table 1. Mechanisms of drug action and resistance in *Mycobacterium tuberculosis*

Drug	Mechanism of drug action	Genes involved in resistance[a]	Gene function	Role of gene product in drug resistance	Reported frequency in resistant strains	Reference
Rifampicin	Inhibition of transcription	*rpoB*	RNA polymerase β-subunit	Drug target	96%	116
Isoniazid	Inhibition of mycolic acid synthesis	*katG*	Catalase-peroxidase	Drug activation	50-95%	211
		inhA	Enoyl-acyl carrier protein reductase	Drug target	20-35%	211
Ethambutol	Inhibition of arabinan synthesis	*embB*	Arabinosyltransferase	Drug target	30-70%	151
Amikacin	Inhibition of protein synthesis	*rrs*	16S rRNA	Drug target	80%	155
Kanamycin	Inhibition of protein synthesis	*rrs*	16S rRNA	Drug target	60%	155
		eis	Aminoglycoside acetyltransferase	Drug degradation	20-40%	155
Capreomycin	Inhibition of protein synthesis	*rrs*	16S rRNA	Drug target	75%	155
		tlyA	Methyltransferase	Target methylation	1-3%	155
Fluoroquinolones	Inhibition of DNA supercoiling	*gyrA*	DNA gyrase subunit A	Drug target	70-90%	173

[a]The most predominant genes involved in drug resistance in *M. tuberculosis*

1.4.3 Rifampicin

RIF, a highly effective bactericidal rifamycin derivative, has been a key component of standard TB treatment regimen since it was introduced in 1966 (54). RIF is active against both continuously growing and semi-dormant bacteria in their spurts of metabolism (57).

RIF inhibits transcription by inhibiting the DNA-dependent RNA polymerase, an essential enzyme in the bacterium. The drug binds close to the catalytic center of the β-subunit of the RNA polymerase and blocks mRNA translocation (111, 112). RIF is a broad-spectrum antibiotic, and the resistance mechanisms in *M. tuberculosis* has been elucidated by analyzing analogous mutations in other bacterial species (113). The vast majority of RIF-resistant *M. tuberculosis* clinical isolates have mutations in the gene *rpoB*, which encodes the β-subunit of RNA polymerase. These mutations lead to structural changes in the drug target, which prevents RIF to bind to the target (108). The mutations are almost exclusively found within an 81-bp long region of the *rpoB*, termed the RIF resistance-determining region (RRDR) (114-116). The RRDR comprises codons 507 to 533; however, these codon numbers do in fact correspond to those found in the *Escherichia coli rpoB* sequence, designated on the alignment of the translated sequence, and do not represent the actual codon positions in *M. tuberculosis rpoB*. Nevertheless, this numbering system has become standard practice for *M. tuberculosis* (113, 117, 118), and will be used throughout this thesis.

The role of *rpoB* mutations in causing RIF resistance has been confirmed by genetic transformation experiments (118, 119). Commonly, resistance to RIF is achieved through point mutations; however, in frame deletions and insertions have also been reported (103). The most frequent mutations in *rpoB* among clinical isolates generally result in high-level resistance; however, more rare mutations, in codons 511 and 522 for example, are associated with low-level resistance to RIF (120, 121). Mutations associated with RIF resistance outside the RRDR have also been reported, for example in codon 146 (*E. coli* numbering system), although these are very rare (115, 122). Mutations in *rpoB* may modify the enzymatic activity and the fitness (123). The S531L mutation is the most prevalent missense mutation among RIF-resistant *M. tuberculosis* clinical isolates, and it has been shown that this particular mutation confers the smallest fitness cost *in vitro*, when compared to other mutations in *rpoB* (124).

1.4.4 Isoniazid

INH, which was introduced into TB therapy in 1952, is along with RIF, a very important first-line anti-TB drug (49). INH is bacteriostatic during the first 24 hours of treatment, after which the action is bacteriocidal (105). INH is only effective against actively growing *M. tuberculosis* (57). The mode of action of INH is highly complex, and although extensively studied, is yet not fully understood (105).

INH is a prodrug which is converted to its active form by the enzyme catalase-peroxidase KatG (125), which is encoded by the gene *katG*. The active chemical compund, an isonicotinic acyl radical, attacks the niotinamide group of nicotinamide adenine dinucleotide (NAD^+) to form an INH-NAD adduct (126). The primary target of INH in *M. tuberculosis* is believed to be the enoyl-acyl carrier protein reductase InhA (126-128). InhA is an NADH-specific enoyl-acyl carrier protein reductase (128), which is part of the fatty acid synthase type II (FASII) system (129). When the INH-NAD adduct inhibits InhA, the FASII system in a blocked, which leads to inhibition of mycolic acids synthesis, and the bacterial cell lyses (35).

INH-resistant *M. tuberculosis* mutants were selected *in vitro* shortly after the discovery of the drug (130). It was observed that many, but not all, mutants lost catalase activity (131). The role of catalase in INH resistance remained unclear for many years. It was hypothesized that the loss of catalase-peroxidase activity was an indication that the enzyme was an activator for INH (35). Gene transfer experiments later demonstrated that a loss of, or a mutation in, *katG* leads to INH resistance (132). A dysfunctional gene product (KatG) reduces its ability to activate INH, thus leading to INH resistance. Among various mutations, the S315T mutation in *katG* is most common (103), possibly because some of its catalase and peroxidase activity is still retained despite the mutation (133). The S315T mutation is associated with a relatively high level of INH resistance (134). Mutations conferring INH resistance have also been mapped to the promoter region of the *mabA-inhA* operon (hereafter denoted *inhA*) and, in rare cases, to the structural gene of *inhA* (135). *inhA* promoter mutations, most commonly at nucleotide position -15, lead to an overexpression of the target InhA and thus mediate resistance by reducing the effective drug concentration through drug titration (136). This mechanism leads to low-level INH resistance. Other genes such as *kasA*, *ndh* and *aphC* have been suggested to be involved in INH resistance; however, their exact role has not yet been determined (105, 137). Mutations in these genes have been found in both INH-resistant and INH-susceptible clinical isolates (138, 139). The genes are probably not directly involved in resistance, but may instead have secondary or compensatory effects (105, 140). Mutations in the promoter region of *aphC* may be important for restoring peroxidase homeostasis in the KatG-deficient organism (141).

1.4.5 Ethambutol

EMB is a bacteriostatic first line anti-TB drug that inhibits polymerization of mycobacterial arabinan, a polysaccharide constituent of the mycobacterial cell wall structural components arabinogalactan and lipoarabinomannan (142, 143). More specifically, EMB is thought to inhibit a set of arabinosyltransferase enzymes, EmbA, EmbB, and EmbC (144), which share 65% homology (145). These enzymes are critical for the synthesis of arabinan.

Mutations in the *embCAB* operon have been identified in EMB-resistant clinical isolates of *M. tuberculosis*, and in particular mutations in the *embB* gene seem to been

linked to EMB resistance despite sometimes discordant phenotypic and genotypic results. Mutations in *embB* typically yield a low to moderate resistance level (146). Several studies have demonstrated a strong association between mutations in codon 306 and resistance to EMB (147-149), and these observations have been confirmed by allelic exchange experiments (150-152). Mutations at *embB* codon 306 occur most frequently in clinical isolates (103); however, mutations in other codons, such as 406 and 497 have also been reported (153). Additional mutations in the *embC-embA* intergenic region have been found in strains that also had resistance-associated amino acid substitutions in EmbB (153), and it is possibly these mutations may play a secondary or compensatory role in resistance.

1.4.6 Amikacin, kanamycin and capreomycin

The aminoglycosides AMK and KAN are bactericidal drugs that are used to treat TB as well as other bacterial infections. The cyclic peptide CAP, also a bactericidal drug, is grouped with the aminoglycosides as second-line injectable drug, and it is recommended that one of these drugs is included in the treatment of MDR-TB (62). Despite belonging to different drug classes the three drugs share drug target. Cross-resistance has sometimes been observed between the drugs, although in varying degrees depending on the exact resistance mechanism present (154, 155). AMK, KAN and CAP inhibit protein synthesis by binding to the 30S ribosomal subunit, resulting in inhibition of translocation during protein synthesis (156-160).

Resistance to AMK, KAN and CAP is associated with mutations in *rrs*, the gene coding for the 16S rRNA, a component of the 30S ribosomal subunit (161). A mutation at nucleotide position 1401 is most commonly observed among clinical isolates and is associated with resistance to all three drugs, resulting in high-level resistance to AMK and KAN, and moderate-level resistance to CAP (162). Mutations at nucleotide positions 1402 and 1484 have also been reported among clinical isolates; however, not as frequent as the 1401 substitution (103). A mutation at nucleotide position 1402 is associated with high-level CAP resistance and low-level KAN resistance, whereas a substitution at nucleotide position 1484 leads to resistance to all three drugs (162).

It has also been shown that the gene *eis*, which encodes the aminoglycoside acetyltransferase Eis, is involved in KAN resistance (104). Overexpression of *eis*, mediated by mutations in the promoter region, confers low-level resistance. Eis acetylates and inactivates KAN (104). The bacteria thus become resistant via drug degradation. Recently, it was shown that mutations in the 5' untranslated region (UTR) of the transcriptional activator encoded by *whiB7* lead to an increased expression of *eis*, thus resulting in low-level KAN resistance (163).

Resistance to CAP is also associated with mutations in *tlyA* (164), which encodes the methyltransferase TlyA. It is believed that TlyA methylates nucleotide position 1402 in *rrs*, which enhances the antimicrobial activity of CAP. Disruption of *tlyA* by missense

or frameshift mutations leads to CAP resistance since the unmethylated ribosome seems to be insensitive to the drug (160). A nucleotide subsitution at position 1402 in the gene *rrs*, as mentioned above, also leads to CAP resistance since TlyA is unable to methylate this position if mutated.

1.4.7 Fluoroquinolones

FQs are bactericidal broad-spectrum antibiotics which, along with the second-line injectable drugs AMK, KAN and CAP, play an important role in the treatment of MDR-TB. FQs directly inhibit DNA topoisomerases which solve topological problems associated with DNA replication, transcription, recombination, and chromatin remodeling by introducing temporary single- or double-strand breaks in the DNA. The FQ forms a complex with the DNA and the topoisomerase, and generates double-strand DNA breaks, which is lethal for the bacteria (165). Studies with other bacterial species have revealed the presence of FQ resistance-associated mutations in genes encoding the two type II topoisomerases: DNA gyrase and topoisomerase IV, as well as in membrane proteins which regulate the intracellular concentration of the drug by mediating drug permeability and efflux (35). Contrary to other bacteria, mycobacteria do not harbor a topoisomerase IV homologue (ParC and ParE subunits) (166), but instead, FQs target DNA gyrase only.

DNA gyrase is a heterotetramer consisting of two A and two B subunits, encoded by the genes *gyrA* and *gyrB*, respectively (167, 168). FQ resistance-associated mutations in *M. tuberculosis* are predominantly found in codons 88-94 of *gyrA* (169-171), a region which is denoted the quinolone-resistance determining region. Most commonly missense mutations are observed in codons 90, 91 and 94, leading to moderate- to high-level FQ resistance (169, 170). Mutations in *gyrB* have also been detected in clinical isolates of *M. tuberculosis*; however, only very seldomly, and their exact role in FQ resistance remains unclear. Mutations in *gyrB* usually occur in association with *gyrA* mutations (172), and do not seem to be directly involved in FQ resistance (173). Low-level FQ resistance may be attributed to other drug resistance mechanisms, such as drug efflux mechanisms (174, 175).

1.5 PHENOTYPIC DRUG SUSCEPTIBILITY TESTING

The global epidemic of drug-resistant TB sets the importance of DST into a new perspective. For effective TB control, it is not only important to detect *M. tuberculosis*, but also its drug susceptibility pattern. This is particularly important in settings with high prevalence of drug-resistant TB, where the current standard TB treatment recommended by the WHO may be ineffective in a very large proportion of the cases (62, 176). Early detection of drug-resistant strains facilitates a timely shift to an effective drug regimen which not only improves treatment outcomes, but also reduces the spread of resistant TB in the community.

An alternative to guiding TB treatment by DST results is an empirical assumption based on the patient's failure to respond to the standard treatment regimen. However, such an option lacks accuracy, and it can be both dangerous and very costly. It can be dangerous for individual patients, who may receive inappropriate treatment, and for other individuals who also may be infected during the period of inappropriate treatment. Since treatment of drug-resistant TB is very costly compared to its drug-susceptible counterpart, empirical treatment may result in a situation which is much more expensive to handle than compared to the cost of performing initial DST.

A classical definition of a drug-resistant *M. tuberculosis* strain is that it is significantly different, in its degree of susceptibility to a particular drug, from a wild type strain that has never come into contact with the drug (177, 178). The purpose of DST is to determine if a particular strain is susceptible or resistant to a certain drug, and therefore, the requirement for DST is the ability to make a distinction between susceptible and resistant strains. This distinction is typically achieved by traditional culture-based methods.

To determine if a strain is susceptible and resistant to a certain drug, typically only one drug concentration is tested. This concentration, the so-called critical concentration, is used to distinguish between susceptible and resistant strains. It should be somewhere between the highest MIC of wild type strains and the lowest MIC of the strains considered resistant. This may be problematic for certain drugs, e.g. EMB, which have a narrow MIC range between susceptible and resistant organisms (146). In this case it may difficult to determine an appropriate critical concentration and commonly false resistant and false susceptible results may be obtained due to methodological difficulties.

Conventional phenotypic DST methods involve culturing bacteria on solid or in liquid media in the presence of drugs under standardized conditions to detect inhibition of growth. DST testing can be performed directly or indirectly. In the direct test, a set of drug-containing and drug-free media are inoculated directly with a specimen, for example a sputum sample. An indirect test involves inoculation of the media with a pure pre-grown culture and is typically performed with a bacterial suspension made from growth on solid media. Since an indirect test requires initial primary isolation as

well, the final DST results may be available after weeks to months. The advantages of the direct tests over the indirect tests are that the results are available more rapidly, and that the specimen better represent the original bacterial population in the patient. However, direct testing may involve insufficient or excess growth, or heavy contamination, and in such a case, the test must be repeated with a pure culture, i.e. as an indirect test.

Although a time-consuming procedure, due to the very slow growth of *M. tuberculosis*, conventional phenotypic DST methods are advantages in that they allow detection of drug resistance regardless of resistance mechanism. This is particularly important in cases when the molecular mechanism of drug resistance is unknown. Furthermore, phenotypic methods are not affected by mutations that do not cause drug resistance.

Globally, phenotypic DST is most commonly performed on solid culture medium. The proportion method, the resistance ratio method, and the absolute-concentration method are phenotypic DST methods on solid medium recommended by the WHO (178, 179). The proportion method is most commonly used however (179). In this method the percentage of resistant bacteria in the population is reported by comparing the number of colony forming units on the drug-containing and drug-free media. Most commonly an isolate is considered resistant if at least 1% of the bacteria are resistant.

The first commercial broth-based system for mycobacteria growth detection was introduced in the 1980s. This system, BACTEC 460 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), is a radiometric technique involving monitoring metabolism of bacteria during growth (180). Since it is broth-based, it can generate DST results more rapidly than methods employing solid media. The method was later further developed to the nonradiometric BACTEC MGIT 960 system (Becton, Dickinson and Company) (181). In the automated MGIT 960 system each test tube, containing liquid medium, has a fluorescence quenching-based oxygen sensor embedded in silicon at the bottom which fluoresces following the oxygen reduction induced by aerobically metabolizing bacteria. Thus consumption of oxygen during bacterial growth produces fluorescence when illuminated by a UV lamp, which is recorded by a camera. These commercial liquid culture DST methods have a relatively short turnaround time but are expensive and require specialist equipment.

In an effort to provide simple and inexpensive methods for detecting drug-resistant *M. tuberculosis*, several non-commercial culture-based methods have been developed. Examples of methods that have been endorsed by the WHO are the colorimetric redox indicator (CRI) assays (182), nitrate reductase assay (NRA) (183), and the microscopy-observation drug-susceptibility (MODS) assay (184). The CRI methods are based on the reduction of an indicator dye added to liquid culture medium in a microtitre plate after exposure of strains to anti-TB drugs. NRA is based on the ability of *M. tuberculosis* to reduce nitrate during growth, which is detected by a color change. In the MODS assay growth of *M. tuberculosis* is detected in a sealed microtitre plate containing liquid culture medium by microscopic observation of typical cord formation.

These techniques offer an attractive turnaround time, cost, and sensitivity, but there is limited evidence of the performance of the techniques for some first-line anti-TB drugs and for most second-line drugs (185).

1.6 MOLECULAR DETECTION OF DRUG-RESISTANT TUBERCULOSIS

Conventional culture-based diagnostic and DST methods are inherently time- and labor-intensive; therefore, there is an urgent need for new rapid TB diagnostic methods, specifically those that can detect drug-resistant *M. tuberculosis*. An alternative to culture-based diagnostic methods is the detection and analysis of bacterial nucleic acids, typically the genotype which causes a particular phenotype is analyzed.

Molecular-based methods that are designed to detect chromosomal mutations associated with drug resistance in *M. tuberculosis* offer several advantages over non-molecular methods. Although rapid and relatively cheap, sputum smear microscopy has poor sensitivity. Specificity may also be problematic if specimens are obtained from patients who are heavily colonized with or have chronic disease due to NTM. Culture remains the gold standard for detection of *M. tuberculosis*. The turnaround time for a molecular-based detection test is substantially shorter than culture, and in fact, the results may be available in a matter of hours or days instead of weeks or months. Even with automated liquid-culture diagnostic methods, isolation of *M. tuberculosis* may take a couple of weeks. Compared to culture, reproducibility may also be better for molecular-based methods, especially in the case of low to moderate level resistance (148). Molecular tests also offer the ability to work with poorly growing cultures. At the same time molecular-based methods offer a specific means of species identification.

Despite these advantages, molecular tests cannot completely replace microscopy or culture. Smear microscopy provides an index of the degree of infectiousness, thereby facilitating informed decisions regarding public health measures, such as the need of isolating a patient, in settings where resources allow. DNA is very stable molecule which can be readily detected in clinical specimens from patients who have received anti-TB chemotherapy. Therefore, the implementation of molecular tests to monitor treatment outcome is not recommended (35). Culture is essential for performing phenotypic DST and, if necessary, molecular epidemiology. Furthermore, molecular-based methods are compared to phenotypic DST methods often less sensitive to detect a resistant minority population in heteroresistant isolates (172, 186, 187).

Substantial progress has been made in the field of molecular biology over the last four decades. Sanger sequencing (188), a method for determination of the nucleotide order of a given DNA fragment, has revolutionized the analysis of genomes, and still remains widely used, especially for small-scale sequencing projects and for obtaining long contiguous DNA sequence reads in whole genome projects. The polymerase chain reaction (PCR) (189), which amplifies DNA, has been since its development in the late 1980s an indispensable technique in both medical and biological research fields. In order to be able to design and develop clinically sensitive and specific molecular tests it is essential to have a profound understanding of drug resistance mechanisms. Genetic manipulation of bacteria is a powerful approach for understanding the basic biology of the microbe. A genetic system that permits to transfer, mutate and express specific genes is a prerequisite for a detailed understanding of the basic molecular aspects of a

bacterium, including mechanisms of drug resistance. However, applications of these techniques in *M. tuberculosis* have been hampered due to several unique features of the organism. Its pathogenic nature and easy transmission by aerosols puts a demand for exquisite biosafety facilities, the lipid- and carbohydrate-rich cell wall of *M. tuberculosis* impedes uptake of macromolecules, illegitimate recombination, and low frequency of homologous recombination hampered the development of genetic systems for gene transfer (190). Advancements have however been made in this field allowing genetic manipulations and allelic exchange experiments (191, 192).

Ideally, a molecular test should have high clinical and analytical sensitivity and specificity, should be rapid, robust, compliant with decentralized use, inexpensive, have long shelf life, amenable to large-scale production, require minimal laboratory infrastructure and personnel training, and should be possible to apply on all types on specimens, not only sputum. This wish list has not yet been fulfilled, as no low-cost point-of-care (POC) test for TB exists. Nevertheless, these requirements should not stimulate resignation, but rather they should be regarded as a challenging inspiration to continue research and development.

Although a wide range of molecular diagnostic methods have been developed for the detection of drug-resistant *M. tuberculosis*, most are based on PCR amplification of a specific chromosomal region followed by analysis of the PCR product for detection of mutations associated with resistance to a particular drug. The presence or absence of a specific mutation is then regarded as an indication that the investigated isolate is susceptible or resistant to a particular drug. There is a number of scientific reports describing the development of methods employing various techniques for detection drug-resistant TB. These involve restriction enzyme digestion analysis (193), ligation-dependent probe amplification (194, 195), loop-mediated isothermal amplification (LAMP) (196), melting curve analysis (197), and PCR amplicon analysis by separation (198), or probe hybridization (199-202); however, the only method of these that do not rely on PCR is LAMP, a method that is difficult to perform in a multiplex manner.

Amplification of targets by PCR requires a set of general PCR amplification primers to hybridize upstream and downstream of the region of interest, followed by analysis of the product. Multiplex PCR employs multiple primer sets in a single reaction mixture to produce amplicons of varying sizes that are specific to different DNA sequences (203). Multiplex PCR, has the potential to decrease cost, time and effort in diagnostics. However, optimization of efficient multiplex PCR requires extensive laborious planning in primer design, nucleotide and primer concentrations, optimal salt and buffer conditions, and cycling temperatures (204), and is rarely capable of achieving high degrees of multiplexing (205).

Methods primarily used in **Paper I-IV** in this thesis, as well some commercially available PCR-based techniques, are described in further detail below.

1.6.1 DNA sequencing

DNA sequencing has become indispensable in various basic and applied research fields because it provides very valuable information: the nucleotide order of a given DNA fragment. Different sequencing methods can be used to determine the exact sequence of DNA; however, initially the genomic region of interest is amplified by PCR, and later subjected to a unique sequencing reaction. Sanger sequencing, developed in the late 1970s (188), is a chain-terminating sequencing method widely used in medical and research laboratories. Pyrosequencing, developed 20 years later (206), employs a different methodological principle: sequencing-by-synthesis.

Sequencing is generally very accurate and robust, and so, has widely been used for characterizing mutations in *M. tuberculosis*. In fact, it is often considered the gold standard for analysis of sequences and mutation detection. Sequencing offers screening of a priori known as well as novel mutations. It is specific and relatively rapid; however, it requires a high standard sequencing facility, something which may be a limiting factor in resource-limited settings. The principles of Sanger sequencing and pyrosequencing are outlined in Figure 4.

In Sanger sequencing, the nucleotide order is determined by preparing a sequencing reaction where regular deoxynucleotides (dNTPs) are combined with terminating dideoxynucleotides (ddNTPs), both which are incorporated during DNA polymerization. These chain-terminating nucleotides lack a 3′-hydroxyl group required for the formation of a phosphodiester bond between two nucleotides, causing the DNA polymerase to cease extension of DNA when a ddNTP is incorporated. The ddNTPs are commonly labeled with fluorophores, and the nucleotide sequence is analyzed by separating the fragments by capillary electrophoresis. The read length of Sanger sequencing is approximately 800-1000 bp.

Pyrosequencing differs from Sanger sequencing in that it relies on the detection of pyrophosphate (PPi) release upon nucleotide incorporation, rather than chain termination with ddNTPs. Pyrosequencing renders shorter sequences than Sanger sequencing, typically 50-60 bp, but has a shorter turnaround time and is more easily applicable in a large-scale fashion. In this method, one of the four dNTPs is added separately to the reaction. If the DNA polymerase incorporates the correct, complementary dNTPs onto the template, PPi is released (207). The enzyme adenosine 5'-triphosphate (ATP) sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5′ phosphosulfate. This ATP acts as fuel to the luciferase-mediated conversion of luciferin to oxyluciferin which generates visible light in amounts that are proportional to the amount of ATP, i.e. the number of nucleotides incorporated. The light produced in the luciferase-catalyzed reaction is detected by a camera. Unincorporated nucleotides and ATP are degraded by the apyrase, and the reaction can start again with another nucleotide.

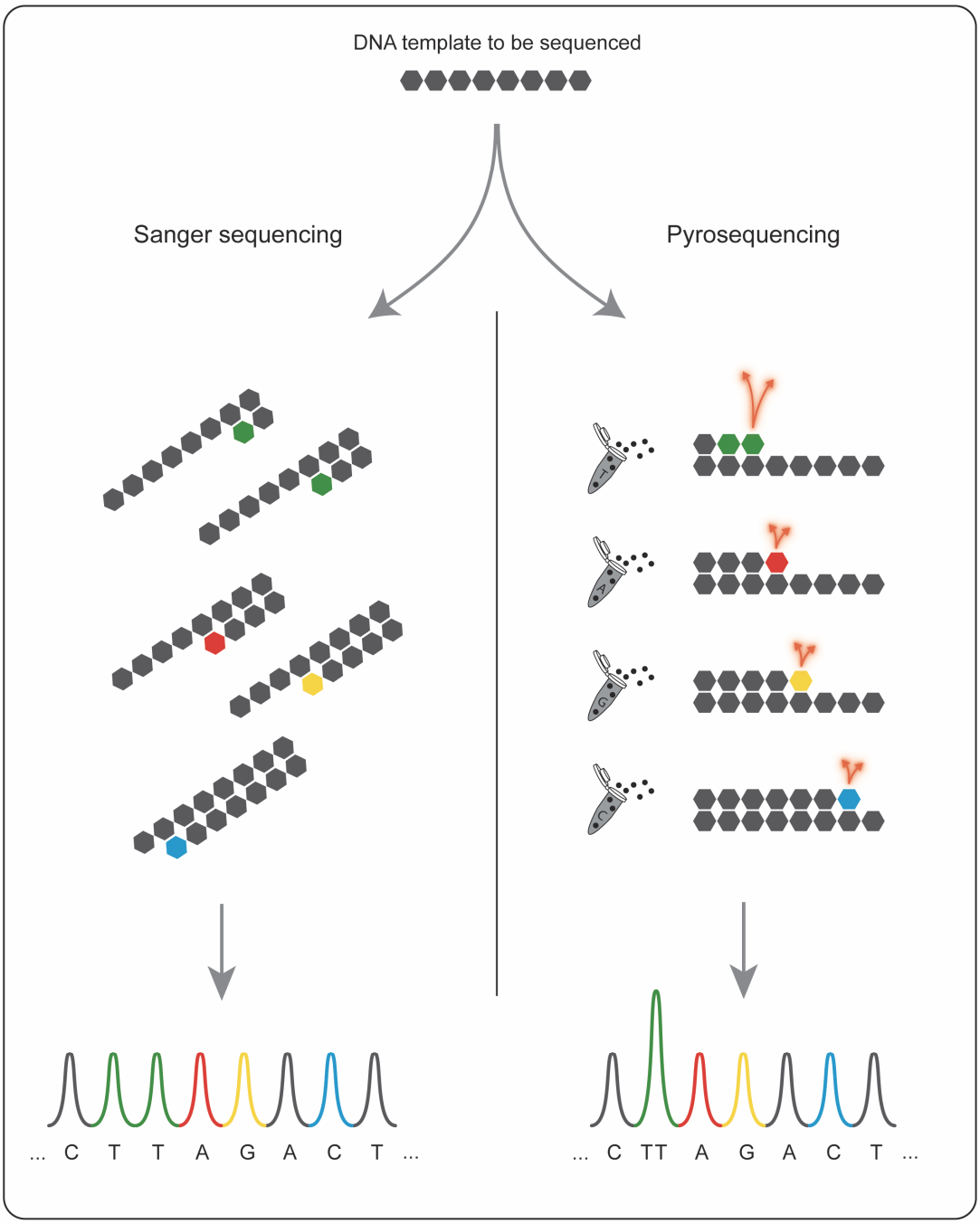

Figure 4. Schematic representation of Sanger sequencing and pyrosequencing. The nucleotide order is determined by Sanger sequencing by an elongation reaction containing both dNTPs and ddNTPs. The ddNTPs lack a 3′-hydroxyl group required for the formation of a phosphodiester bond between two nucleotides, causing the DNA polymerase to cease extension of DNA when a ddNTP is incorporated. The nucleotide sequence is analyzed by separating the fragments by electrophoresis and detecting the ddNTPs by fluorescence. Pyrosequencing relies on the detection of PPi release upon nucleotide incorporation. One of the four dNTPs is added separately to the reaction and when DNA polymerase incorporates the correct dNTP onto the template PPi is released. Another enzyme converts PPi to ATP which provides energy for luciferase to convert luciferin to oxyluciferin, a reaction which generates visible light proportional to the number of nucleotides incorporated. The light produced in the luciferase-catalyzed reaction is detected by a camera.

1.6.2 Line probe assays

Molecular line probe assays (LPAs) apply principles of nucleic acid amplification to detect both species and mutations associated with drug resistance. In these assays, similar to sequencing technologies, a genomic region is first subjected to PCR with biotinylated primers, followed by hybridization of the PCR product to oligonucleotide probes immobilized on a nitrocellulose strip. The presence of a mutation prevents hybridization of the PCR product to wild type probes, and conversely, a wild type sequence prevents hybridization to the mutant-specific probes. The biotinylated product enables visualization of colored bands by the naked eye. Mutations are detected by lack of hybridization to wild type probes, as well as by hybridization to specific probes designed for the most commonly occurring mutations. However, in some cases, a complete loss of signal is not observed when a mutation is present, but rather just a decrease of band intensity.

The WHO endorsed LPAs for the detection of mutations associated with drug resistance in *M. tuberculosis* in 2008 (208). The predominately used commercially available LPA assays are INNO-LiPA Rif.TB (Innogenetics NV, Gent, Belgium) (209), GenoType MTBDRplus and GenoType MTBDRsl (Hain Lifescience GmbH, Nehren, Germany) (210, 211). INNO-LiPA Rif.TB allows detection of mutations in RRDR associated with RIF resistance (209), and the GenoType MTBDRplus assay also detects, apart from RIF resistance, mutations in *katG* and the promoter region of *inhA*, allowing simultaneous detection of INH resistance. Thus, the GenoType MTBDRplus assay enables detection of MDR-TB. The GenoType MTBDRsl assay is developed for detection of FQ resistance by targeting the gene *gyrA*, and the injectable drugs AMK, KAN and CAP by analysis the gene *rrs*, enabling detection of XDR-TB. Analysis of the gene *embB*, linked to resistance to the first-line drug EMB, is also included in the GenoType MTBDRsl assay. All three assays include detection of the MTC. The GenoType MTBDRplus assay is also optimized for smear-negative pulmonary clinical specimens.

1.6.3 Real-time PCR based techniques

Real-time PCR has been employed for rapid detection of drug-resistant *M. tuberculosis* by applying different probes, including Taqman probes (212), locked nucleic acid probes (213), and molecular beacons (214). Real-time PCR allows simultaneous amplification of DNA sequences and detection of the product (215). The main advantages of real-time PCR techniques compared to conventional PCR are the speed of the test and lower risk of contamination if reaction tubes remain unopened after the PCR reaction.

The GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) is a commercially available automated cartridge-based DNA amplification test based on real-time PCR. The assay is designed to detect the MTC as well as RIF resistance (216). Species identification

and mutation detection is achieved through analysis of hybridization of five molecular beacons to the PCR product, specifically targeting the RRDR of *rpoB* (216). The assay does not detect specific mutations but instead indicates presence of mutations by loss of wild type. Since the endorsement of the test by the WHO in December 2010 (217), there has been a rapid roll-out of the product in many high burden TB countries in Africa and Asia (218). Despite its advantages, the roll-out of GeneXpert MTB/RIF is not without challenge. It is still a relatively expensive product, it is not a true POC test and it does not eliminate the need for phenotypic DST. In addition, its cost-effectiveness is only now being investigated, after a large scale roll-out (219, 220).

1.6.4 Padlock probes and rolling circle amplification

There is one report describing the detection of MTC DNA by a combination of padlock probes and pyrosequencing (221). However, there are no reports on the detection of drug-resistant *M. tuberculosis* employing padlock probes and utilizing isothermal amplification.

A padlock probe is a linear oligonucleotide that contains two target-complementary sequences at their 5' and 3' ends, joined by a target-non-complementary linker sequence (222). It is 60-100 bp long, of which the target-complementary sequences are 10-20 bp. The linker sequence, also referred to as the backbone, contains sequences for downstream identification, amplification and detection. Upon hybridization to the target, the two target-complementary ends will be brought into juxtaposition, forming a nick between the two padlock probe ends. The nick allows circularization of the probe by a DNA ligase, leaving the probe topologically wound around the target, resembling a padlock. The DNA ligase has a strong preference for a perfect hybridization between the probe and the target sequence, particularly near or at the very 3' end nucleotide, thus the ligation reaction is greatly hindered by mismatches (223). This allows a specific means of detecting single nucleotide variants (224). Due to its unimolecular character, padlock probe assays can be carried out in a highly multiplex fashion and alterations of the probe content does not require extensive protocol optimization (225). Padlock probes have successfully been used to detect both RNA and DNA (221, 226, 227), and applications of the technique are possible in aqueous solutions as well as *in situ* (228, 229).

For a sensitive assay, circularized padlock probes must be amplified prior to signal detection. Rolling circle amplification (RCA), also referred to as rolling circle replication, is an isothermal process of unidirectional nucleic acid replication based on circular DNA molecules. It is the process by which plasmids, bacteriophages as well as viral genomes can replicate (230, 231). Thus, circularized padlock probes can serve as a target molecule for RCA, which can be initiated by either an external primer or the target DNA itself. Enzymatic polymerization produces multiple copies of the circular template molecule, i.e., a long single stranded DNA molecule with tandem repeats complementary to the single stranded circle (232).

Phi29 DNA polymerase is an enzyme from the bacteriophage Phi29 of *Bacillus subtilis* (233). Phi29 DNA polymerase has several important properties: 3'-5' exonucleatic activity on single stranded DNA, strand displacement and processive DNA synthesis activity (234). The enzyme can be used to degrade non-base-paired nucleotides downstream of the padlock probe, and when it reaches the double-stranded DNA (i.e. the padlock probe hybridized to the DNA target molecule), it switches activity to polymerization. This process is referred to as target-initiated RCA. The resultant concatemer consisting of head-to-tail complementary copies of the circularized padlock probe spontaneously collapses into a coil-like structure, below referred to as an RCA product. Phi29 synthesizes DNA at a speed of approximately 1,700 nucleotides per minute (235). This means that if a padlock probe with a length of 100 bp serves as a target molecule for RCA, 1,000 head-to-tail copies are produced in one hour (235). This corresponds to an RCA product diameter of approximately 1 μm (236).

RCA alone does typically not yield an analytical sensitivity of clinical relevance. In order to apply RCA-based padlock probe assays on clinical specimens containing a pathogen of low abundance the so called circle-to-circle amplification (C2CA) method with better sensitivity can be employed. C2CA is a molecular method involving a series of enzymatic reactions for amplification of circular DNA molecules (237). The method is outlined in Figure 5. In the first step, padlock probes are added to the DNA target and a capture oligonucleotide (Figure 5a). The capture oligonucleotide is modified with biotin at its 5’ end which allows subsequent coupling to streptavidin-coated magnetic beads. Target-initiated RCA requires relatively short DNA target fragments; consequently, one capture oligonucleotide is necessary for each target fragment. The DNA ligase circularizes padlock probes which match the nucleotide sequence of the DNA target (Figure 5b). The DNA target is subsequently captured to streptavidin-coated magnetic beads via the biotinylated capture oligonucleotide, and unreacted padlock probes are removed from the reaction by a washing step with the aid of a magnet (Figure 5c). After the wash, Phi29 DNA polymerase is added to the reaction which initiates RCA after initial exonucleatic digestion of the protruding single stranded DNA target (Figure 5d). The RCA reaction is stopped by heat inactivation of the Phi29 polymerase, and RCA products are monomerized by addition of restriction oligonucleotides, which are complementary to the backbone of the padlock probes, and a restriction enzyme (Figure 5e), allowing digestion of the concatenated copies. The monomers are then re-ligated into new circle templates by hybridization of non-digested restriction oligonucleotides and ligation by a DNA ligase (Figure 5f). Phi29 is again added to the reaction and the second RCA is initiated by the restriction oligonucleotide now serving as a primer (Figure 5g). The procedure can be repeated multiple times, allowing an improved analytical sensitivity for each cycle (237).

After amplification by C2CA, RCA products can be detected by hybridizing labeled detection oligonucleotides to the backbone sequence of the padlock probe. Using a digital approach, individual labeled RCA products are detected in a confocal

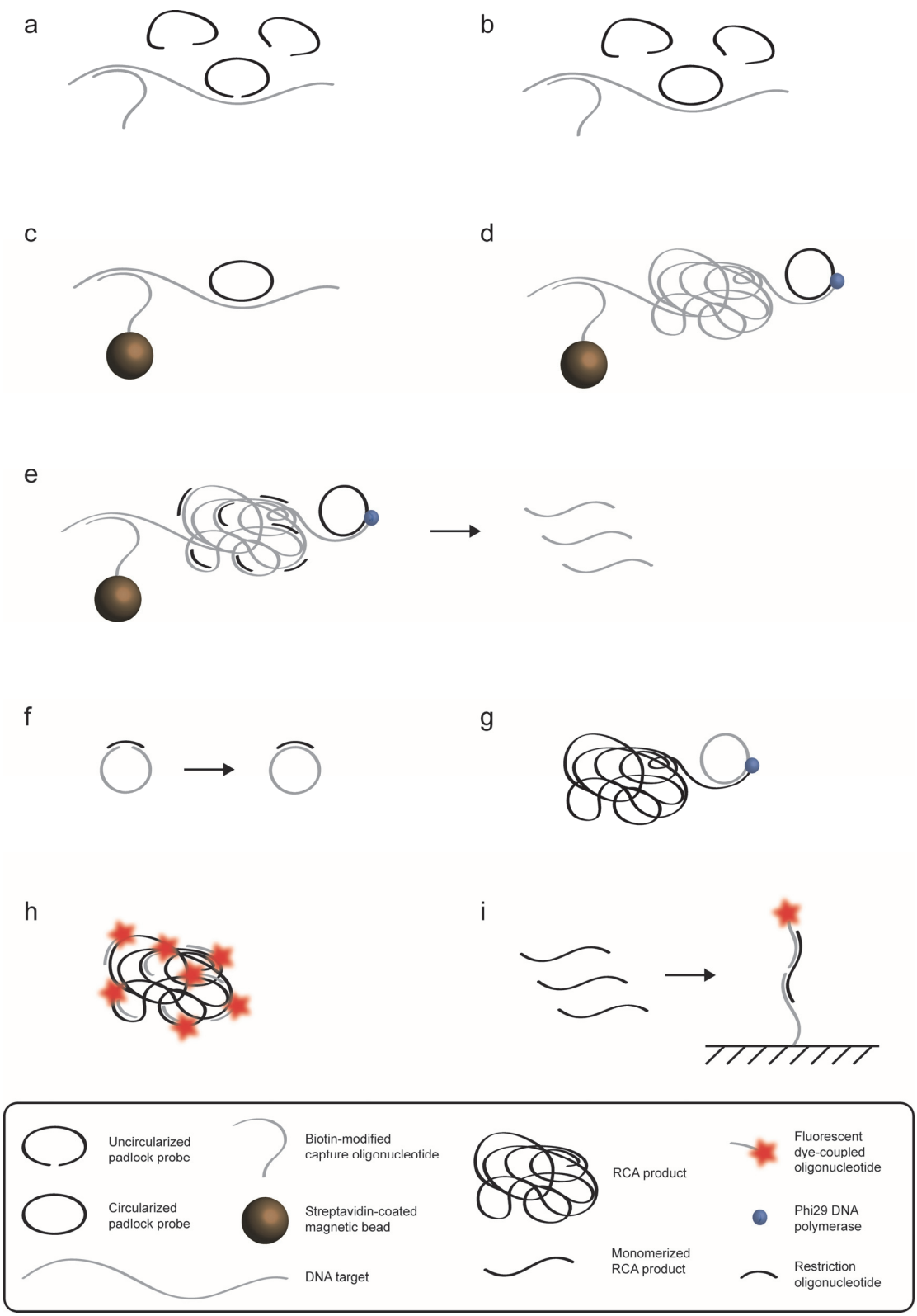

Figure 5. Schematic representation of C2CA. Hybridization of padlock probes and biotin-modified capture oligonucleotide to the DNA target (a), padlock probe ligation (b), target capture to magnetic beads (c), target-primed RCA (d), monomerization of RCA products (e), re-circularization of monomers (f), second RCA (g), detection of RCA products or monomers via fluorescent dye-coupled detection oligonucleotides; in solution (h) or after hybridization to an array (i).

microscope (Figure 5h), yielding a quantitative measurement (236). Alternatively, monomerized RCA products can be hybridized to unique immobilized tag oligonucleotides in an array format, allowing detection in a multiplexed fashion (Figure 5i).

2 STATISTICAL DEFINITIONS

The following definitions are used in this thesis:

Limit of detection (LOD) is expressed as the lower limit of detection which is defined as the concentration or amount of analyte that correspond to a signal that is above the background signal that is obtained when no analyte is present.

Analytical sensitivity describes the lowest concentration or amount of analyte that a given method can measure. It is numerically characterized by determination of LOD.

Analytical specificity describes the ability of a method to distinguish targeted molecules from any other molecules.

Clinical sensitivity (also referred to as diagnostic sensitivity) is defined as the number of true positives divided by the total number of true positives and false negatives. An ideal test should have a sensitivity of 100%, i.e., the test should always give a positive result in the case of a true positive. High clinical sensitivity corresponds to few false negatives.

Clinical specificity (also referred to as diagnostic specificity) is defined as the number of true negatives divided by the total number of true negatives and false positives. An ideal test should have a specificity of 100%, i.e., the test should always give a negative result in the case of a true negative. High clinical specificity corresponds to few false positives.

3 THESIS AIMS

The general aims of this thesis were to gain further understanding of drug resistance in *M. tuberculosis* and to develop molecular methods for the detection of drug-resistant *M. tuberculosis.*

Specific aims:

- To investigate cross-resistance between the aminoglycosides AMK and KAN and the cyclic peptide CAP (**Paper I**)

- To develop a pyrosequencing method for detection of XDR-TB (**Paper II**)

- To develop a molecular method for detection of RIF resistance in *M. tuberculosis* by padlock probes and a magnetic bead-based readout format (**Paper III**)

- To develop a multiplex molecular method for detection of XDR-TB by padlock probes and a low-density array (**Paper IV**)

4 RESULTS AND DISCUSSION

4.1 PAPER I

Treatment of TB should ideally be guided by DST results. Due to lengthy phenotypic DST procedures it is desirable to employ more rapid molecular-based methods tailored to detect mutations associated with drug resistance in *M. tuberculosis*. In order to develop a molecular method with high clinical sensitivity and specificity it is essential to have a profound understanding of drug resistance mechanisms. Resistance development to one drug can simultaneously lead to resistance to another drug, despite being structurally different and belonging to different drug classes. This is referred to as cross-resistance.

The aminoglycosides AMK and KAN, and the cyclic peptide CAP are efficient in eliminating bacteria, and thus, this group of second-line injectable drugs is important for the treatment of MDR-TB. Prescribing a drug that a strain resistant to, no matter via previous direct exposure to the drug or via cross-resistance, is detrimental. Likewise, it is unfortunate to abstain the use of drugs if possibly effective. Thus it is essential to have clear understanding of cross-resistance between drugs.

During the last decade there has been a debate on whether or not there is cross-resistance between AMK, KAN and CAP, and if so, if it is full or partial, and in particular which resistance mechanisms, i.e. mutations, lead to cross-resistance between the drugs. **Paper I** aimed at investigating this, specifically by analyzing genes and mutations previously reported to be involved in resistance to AMK, KAN and CAP, and by correlating these results with the MIC. The study aimed at investigating both susceptible and resistant clinical isolates and *in vitro* selected mutants. Nucleic acids sequences were investigated by Sanger sequencing and pyrosequencing, and the MIC was determined on an agar-based medium containing the drug diluted in 2-fold steps.

The first report describing the link between the gene *tlyA* and CAP resistance was primarily based on the results of *in vitro* selected mutants, indicating a strong association between mutations in this gene and resistance to CAP (164). This conclusion was only partially confirmed by the results of **Paper I**. In fact, the vast majority of CAP-resistant clinical isolates investigated in **Paper I** did not harbor a mutation in *tlyA*, which was expected. Instead, these isolates proved to have a mutation in the *rrs* gene.

In accordance with the observations made by Maus et al. (164), the results of **Paper I** to some extent confirm that the gene *tlyA* is involved in resistance to CAP in *M. tuberculosis*. The majority of mutants obtained by selection on CAP in **Paper I** did indeed harbor a mutation in this gene. However, mutations in *tlyA* were scantly observed among the clinical isolates, and it should also be noted that these mutations were found in both CAP-resistant and CAP-susceptible isolates. This implies that not

all mutations in *tlyA* actually do lead to CAP resistance, and this indication also emphasizes the importance of not only investigating resistant isolates in a study similar to **Paper I**, but that it is equally important to examine susceptible stains. Furthermore, in **Paper I**, *tlyA* mutations in mutants were scattered over a large section of the gene and it can be noted that *tlyA* involves a loss-of-function resistance mechanism, thus, contrary to what has been inferred in a review on the topic by Georghiou and colleagues (155), there is a strong indication that in fact no particular region of the gene is more important than another when it comes to its link to CAP resistance. This is very valuable and important information for the development of molecular-based methods which aim at detecting CAP-resistant *M. tuberculosis*.

A mutation at position 1401 in the gene *rrs* is associated with resistance to the drugs AMK and KAN. AMK, KAN and CAP share drug target, the ribosome, thus, it is anticipated that the same mutation can lead to resistance to all drugs, despite belonging to different drug classes. In fact, the vast majority of CAP-resistant clinical isolates in **Paper I** had a mutation at position 1401 in the *rrs* gene. In conjunction to these observations it should be noted that the results of **Paper I** may have originated due to the specific drugs used during treatment. It is possible that the CAP-resistant clinical isolates investigated in **Paper I** were selected with AMK or KAN, rather than CAP. Thus, in a scenario where CAP is primarily used in the treatment of MDR-TB, mutation in *tlyA* may be more common among CAP-resistant clinical isolates. Still, cross-resistance exists between all drugs in the group of second-line injectable drugs, and CAP treatment in a patient being infected with a strain harboring an *rrs* 1401 mutation may not be successful.

Not only mutations at nucleotide position 1401 in *rrs* were observed in **Paper I**, but also upstream and downstream of this position. A mutation at position 517 was observed particularly frequently among clinical isolates being low-level resistant to KAN. Interestingly, reports have implied discordances regarding this mutation being involved in resistance to KAN (162, 238, 239). After these studies had been published a report describing a previously unknown low-level KAN resistance mechanism was disclosed (104). This report showed that overexpression of Eis, via mutations in the promoter of *eis*, lead to degradation of aminoglycosides, and in particular KAN. This results in resistance via a drug titration mechanism. The gene *eis* was investigated in **Paper I**, and the results together with previously published data (162, 238, 239), suggest that the low-level KAN resistance observed in **Paper I** could be due to mutations in the promoter region of *eis*, rather than at position 517 in the gene *rrs*.

The notions that some CAP-resistant mutants did not have a mutation in *tlyA* but instead had a mutation in *rrs* at nucleotide position 1402 and that this particular mutation did not lead to AMK resistance, but only resistance to CAP and KAN, imply that the cross-resistance pattern may differ depending on the mutation, again underlining the important of thorough investigations of mutations and their potential role in drug resistance. **Paper I** also confirms previous assertions that mutations at nucleotide positions 1402 and 1484 are rare among clinical isolates of *M. tuberculosis*.

Yet another KAN resistance mechanism was recently described by Reeves and co-authors (163). The report presents evidence that *whiB7*, which is a transcriptional activator of *eis*, confers low-level resistance to KAN. Thus, building on the story of *eis*, mutations in the 5' UTR of *whiB7* lead to increased expression of *eis*, and consequently degradation of the KAN, despite no *eis* promoter mutations being present. This report was published after **Paper I**, and therefore, analysis of *whiB7* was not included, but could possibly explain variations in KAN MICs for isolates having identical *eis* mutations.

In conclusion, the results of **Paper I** show that *tlyA* is neither a sensitive nor a specific genetic marker for CAP resistance in *M. tuberculosis* clinical isolates. Instead, for the design of molecular-based test with good clinical sensitivity and specificity, it is advisable to include *rrs* nucleotide position 1401 for the detection of AMK-, KAN-, and CAP-resistant clinical isolates.

4.2 PAPER II

Thorough analysis of nucleic acid sequences is not only essential for determination of the precise nucleotide order of a given DNA fragment, but also allows robust detection of nucleotide variations. Screening for mutations which cause drug resistance can be performed by using molecular-based methods such as LPAs or other PCR-based methods (209-211, 216). However, current methods target only some, but not all, mutations linked to resistance. Thus, sequencing may yield a better clinical sensitivity and specificity. Furthermore, current commercially available methods are often unable to precisely determine which mutation is present in the target DNA. Not all mutations lead to drug resistance, and it is particular challenging when silent mutations are present. Silent mutations may cause loss of wild type signal, incorrectly indicating a resistant phenotype.

Sequencing does not discriminate against any types of mutations, no matter if novel or a priori known, or being a missense or silent mutation. Furthermore, sequencing can not only be used to detect mutations in *M. tuberculosis* clinical isolates, but can also be used to analyze large sample volumes for screening purposes. The latter information may be particularly important during development of molecular-based assays for detection of drug-resistant *M. tuberculosis*. A limitation of molecular assays is that it may for practical reasons be difficult to include all known mutations, but instead, the most common ones are chosen to be included in the method. Therefore, it is essential to have information about the prevalence of specific mutations associated with drug resistance in *M. tuberculosis*.

The aim of **Paper II** was to develop a pyrosequencing method for detection of first- and second-line drug resistance in *M. tuberculosis*. Genomic regions most commonly harboring mutations linked to RIF, INH, EMB, AMK, KAN, CAP and FQ resistance

were targeted in the study. A previously published *rpoB* assay was further optimized (240), which facilitated fewer sequencing reactions per sample. Novel pyrosequencing assays for *katG*, *embB*, *rrs*, *gyrA*, and the promoter regions of *inhA* and *eis*, were developed. Sequencing results were compared with phenotypic DST.

Despite rendering significantly shorter sequencing reads compared to Sanger sequencing, the clinical sensitivity and specificity of the pyrosequencing method in **Paper II** was highly similar to that of Sanger sequencing assays previously reported (137). Moreover, the Sanger sequencing methodology involves two PCR reactions, whereas pyrosequencing involves only one. Furthermore, Sanger sequencing also requires a clean-up procedure of the cycle sequencing reaction prior to analysis of the fragments. This means that less labor and time is required for preparation of the pyrosequencing reactions compared to Sanger sequencing. Furthermore, the preparation of the pyrosequencing reactions are performed in a 96-well format where essentially no more labor is required to prepare 96 samples compared to only one. Therefore, a large volume of samples can easily be analyzed by pyrosequencing.

A limitation with sequencing methods, which may not be evident, is the difference in ability to detect a minority population of a heteroresistant strain compared to phenotypic DST methods. Phenotypic methods commonly define a bacterial population as resistant if 1% or more of the bacteria are resistant. Sequencing methods though, cannot detect a minority population of 1%, but rather need up to 50% (187). This has implications for the interpretation of the results when comparing to phenotypic DST. Discordant results may not always be due to mutations being present in an unknown or not investigated region of the genome, but may in fact be present in the targeted region. Due to a low fraction of bacteria harboring a mutation, it may be presented as wild type despite a resistant phenotype. The low sensitivity does not only encompass sequencing, but also other molecular-based methods, involving both the GenoType MTBDRplus assay as well as methods employing molecular beacons (186, 187), which the GeneXpert MTB/RIF test is based on.

Molecular-based methods may be more robust in advising TB therapy, in particular for those drugs where the MICs of drug-susceptible and drug-resistant isolates to some extent overlap. Small variations in methodology of phenotypic-based methods may produce inconsistent results (148).

To summarize, **Paper II** aimed at developing a pyrosequencing method for detection of first- and second-line drug resistance in *M. tuberculosis*. The method is suitable for screening a large sample volume since pyrosequencing is easily performed in a high throughput format. The method can be used to detect mutations which cause drug resistance in *M. tuberculosis* as well as provide information about which mutations may be clinically useful to include in a more limited molecular-based test in terms of number of genomic regions or mutations that should be included.

4.3 PAPER III

The standard treatment regimen for TB is not considered to be effective if the bacteria are resistant to the first-line drug RIF (241), and therefore it is crucial to rapidly identify RIF-resistant isolates. In addition, resistance to INH often precedes resistance development to RIF, thus in settings with low prevalence of RIF monoresistance, resistance to RIF may be considered as a surrogate marker for MDR-TB (242).

Almost all of RIF-resistant strains have a mutation in the RRDR of *rpoB*, and several molecular-based assays have been developed to detect these mutations (197, 199, 202, 209, 211, 216). These methods are based on PCR though, a method which is inheritably difficult to perform in a multiplex manner. With increasing prevalence of XDR-TB and drug resistance beyond, analysis of several genomic regions is preferred.

There are no reports on the development of padlock probe assays for detecting drug-resistant *M. tuberculosis*. Therefore, the aim of **Paper III** was to evaluate to utility of padlock probes in detecting mutations associated with drug resistance in *M. tuberculosis*. Resistance to RIF was chosen in the study due to its importance in the treatment of TB, and since it may serve as a marker for MDR-TB.

In **Paper III**, padlock probes were designed to target the nine most common mutations in RRDR, i.e. at codons 516, 526 and 531. Together these account for approximately 85% of the mutations found in RIF-resistant strains (103). The objective was not only to detect specific mutations, but also to confirm loss of wild type signal. Since in most cases only one of the targeted codons is mutated, a novel type of padlock probe system for confirmation of loss of wild type was developed. The wild type probe system consists of a padlock probe and two gap-fill oligonucleotides, with their 3' ends targeting either the first or second nucleotide position of codons 516, 526 and 531. This system requires ligation at all three sites for circularization and subsequent amplification. The assay was also complemented with a probe targeting the 16S-23S ITS region for detection of MTC DNA.

The mutant-specific padlock probes were evaluated on genomic DNA in a nine-plex format. The results showed that there was no or very little reduction in signal for the multiplex reaction. Adding yet more probes to the system is not envisaged problematic, based on the results in **Paper III** and previous reports which successfully assessed 1,000- and 12,000-plex systems (225, 243).

A magnetic nanobead-based detection assay was evaluated as a readout format in **Paper III**. The complete assay involving the wild type probe system, the nine mutant-specific padlock probes and the species detection probe was tested on DNA obtained from RIF-susceptible and RIF-resistant *M. tuberculosis* strains. The method successfully detected mutations in RRDR and simultaneously confirmed loss of wild type as well as detected MTC DNA. The LOD of the method, 30 ng *M. tuberculosis* DNA, may not be considered clinically relevant for paucibacillary specimens, but

would require an initial pre-culture of the isolates. Nonetheless, the analytical sensitivity of the method may be improved by adding yet another RCA step to the C2CA protocol.

In conclusion, **Paper III** describes the development and evaluation of a padlock probe assay for detection of RIF-resistant *M. tuberculosis*. The study demonstrates that padlock probes can be successfully used to detect specific mutations in RRDR and simultaneously validate loss of wild type sequence and confirm MTC DNA.

4.4 PAPER IV

MDR-TB has become a reality, and now even the next generation of definitions, XDR-TB, is a real threat to current TB control measures. Reports of virtually untreatable strains of *M. tuberculosis* are alarming and their emergence must be taken seriously (74-76). The importance of rapidly identifying patients infected with drug-resistant *M. tuberculosis* cannot be underestimated as it is essential for implementation of appropriate actions.

Simultaneous investigation of resistance to several drugs is indeed desired. Thus, the aim of **Paper IV** was to develop a padlock probe assay for detection of XDR-TB. Padlock probes are not only advantageous over PCR in that they easily can be multiplexed, but also that an assay alteration does not require substantial re-design and strategic optimization. Furthermore, with continuous progress made in the research field of drug-resistant TB, a broader and more profound understanding of drug resistance mechanisms will arise. Therefore, the clinical sensitivity and specificity of an assay may be improved if the assay design is adjusted according to increased knowledge. Furthermore, the prevalence of drug-resistant strains varies in different settings. It may be advisable to implement different versions of an assay according to the different needs, thus flexibility is also valuable.

Recently, new hopes were raised with the successful development of a new anti-TB drug (80). However, the flip side of coin relates to drug resistance appearing. Despite careful use of new anti-TB drugs, it is likely that drug-resistant isolates will emerge, hence molecular-based methods designed to detect mutations associated with resistance in *M. tuberculosis* need to be flexible and adjustable according to new situations.

For its utility, the padlock probe assay in **Paper IV** requires a multiplex readout format. For ease of use it was envisaged that the readout format did not require advanced equipment, but rather that it could be read out directly by the naked eye. An array format was chosen, and in such a way, that each mutation would give an individual signal. This however could be adjusted, so that mutant signals are grouped according to drug or drug resistance mechanism, if desired. This would yield less detailed information, but could at the same also be more useful in some situations.

The analytical sensitivity achieved in **Paper IV** can be compared to that of PCR. In fact, signal was already obtained when applying as little target as 100 ymol, which is equivalent to 60 target molecules. A high analytical sensitivity is necessary when developing a molecular-based method for detection of TB, as it is beneficial to detect patients at an early stage of disease and not only the most infectious ones. Ideally, the method should be applicable on both smear-positive and smear-negative samples. A high sensitivity is also required for patients co-infected with HIV and pediatric TB cases.

Despite targeting the most prevalent mutations leading to resistance to RIF, INH, AMK, KAN, CAP and FQs, the method in **Paper IV** could be further developed by also targeting more rarely occurring mutations. This could improve the clinical sensitivity and specificity of the assay. Furthermore, other genes and genomic regions associated with drug resistance in *M. tuberculosis* may be considered as additions to the assay.

Paper IV aimed at developing a method for detection of XDR-TB by padlock probes and a low-density array enabling visualization of signal by the naked eye. The method proved to be robust for detection of specific mutations and wild type sequences, as well as simultaneous confirmation of MTC DNA.

5 CONCLUDING REMARKS

We have now come to realize that drug-resistant TB is not a possible scenario, in fact, it is the scenario. To decrease morbidity and mortality, appropriate actions must be undertaken. Ideally, antibiotic treatment should always be guided by DST results. Despite being generally robust, sensitive and specific, a major drawback with phenotypic DST methods is that they are inherently lengthy procedures. An alternative to examining the phenotype is to examine the genotype responsible for that particular phenotype, i.e. drug resistance. An advantage with molecular-based methods aiming at detecting mutations associated with drug resistance in *M. tuberculosis* is that their turnaround time is very short. Time may be crucial as patients with active disease may die or transmit drug-resistant bacteria to others before appropriate treatment has been implemented. Still, abandoning conventional phenotypic DST methods is not wise, since all methods have limitations. It is probably better to retain effectively operational mycobacterial laboratories, as the outcome probably benefits from the complement actions of different methods.

Empirical treatment is not an advisable substitute for DST, since using drugs which the bacteria are resistant to will not cure the patient, but rather only amplify the problem. Therefore, it is essential to work towards a solution which enables rapid detection of drug-resistant TB. This requires efforts of researchers, health care and laboratory personnel, as well as politicians, in individual countries and in the international community, since drug-resistant TB necessitates joint forces to accelerate the implementation of suitable TB control activities.

Rapid and proper diagnostics is an important part of TB infection control. Molecular-based methods offer a great chance to improve detection of drug-resistant TB, however, their development should be accompanied with a profound understanding of drug resistance mechanism, as otherwise, the usefulness of such a test may be limited.

Paper I investigated cross-resistance between the second-line anti-TB drugs AMK, KAN and CAP. The results show that *tlyA* is not a sensitive genetic marker for detection of CAP resistance in clinical isolates of *M. tuberculosis*, and that a mutation at nucleotide position 1401 in the gene *rrs* lead to resistance to all the three drugs. Thus, it is advisable to include *rrs* nucleotide position 1401 in a molecular-based method for detection of drug-resistant *M. tuberculosis*. **Paper II** aimed at developing a pyrosequencing method for detection of first- and second-line drug resistance in *M. tuberculosis*. Seven pyrosequencing assays were successfully developed for the genes *rpoB*, *katG*, *embB*, *rrs*, *gyrA* and the promoter regions of *inhA* and *eis*, which are associated with resistance to RIF, INH, EMB, AMK, KAN, CAP and FQs, respectively. Pyrosequencing is a throughput and robust method for detection of novel and a priori known mutations, specifically enabling detection of both missense and silent mutations. The method can be used to screen a large sample volume, which is desired when aiming at investigating the prevalence of mutations in a sample

collection. In **Paper III**, the utility of padlock probes for detection of drug-resistant *M. tuberculosis* was evaluated. The assay was developed for RIF resistance due to the importance of RIF in the standard TB treatment and its potential role as a surrogate marker for MDR-TB. The method proved to be robust for detection of specific mutations in the RRDR, and confirmation of loss of wild type as well as detection of MTC DNA. The padlock probe assay was further extended in **Paper IV** to detection of XDR-TB in a multiplexed fashion. Padlock probes were designed to target the most common mutations occurring in *rpoB*, *katG*, *rrs*, *gyrA* and in the promoter region of *inhA*. The analytical sensitivity achieved in **Paper IV** should enable application on both smear-negative and smear-positive samples. The readout format used in **Paper IV** eliminates the use of extensive equipment, but rather, signal can be detected by the naked eye.

It is evident that molecular-based methods have a great potential to enhance diagnostic capabilities; however, their results must be clearly interpreted within the clinical context and in the light of their performance characteristics demonstrated by the laboratory. In other words, a thorough understanding of the several parameters, including its procedural limitations, is critical for proper interpretation of results. Any molecular-based method must be validated in terms of both its analytical and its clinical sensitivity and specificity prior to implementation.

This thesis has contributed to increased knowledge of drug-resistant TB, and has successfully developed new methods for rapid detection of drug-resistant *M. tuberculosis*. The results of this thesis can guide future research and development of molecular methods for detection of drug-resistant *M. tuberculosis*.

Despite numerous obstacles, research and development in the field of drug-resistant TB should not lead to cessation of engagement, but rather should be regarded as an encouragement to continue to strive to understand TB fully and to continue to work towards a better situation for the TB patients and those at risk of contracting TB.

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