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Molecular Characterisation of Antibiotic Resistance in *Mycobacterium tuberculosis*

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“Yes, I got the T.B.
And the T.B.'s all in my bones
All in my bones
Well, the doctor told me
That I ain't gon' be 'ere long”

Jack Dupree, T.B. Blues, in: Blues from the Gutter 1958

ABSTRACT

To counteract the emergence of multi-drug resistant tuberculosis (MDR-TB) a number of approaches have been proposed, amongst which are the sound usage of the present anti-TB drugs and the development of prompt and specific tools for the diagnosis of resistance.

To examine the role of drugs in the treatment of resistant TB, we investigated the general cross-resistance between kanamycin and amikacin, which has been reported in numerous studies. Forty clinical isolates were shown to be low-to intermediate resistant to kanamycin but fully sensitive to amikacin. Sequencing of the 16S rRNA gene, *rrs*, revealed no specific genotype correlating to this. We could however confirm the previously reported correlation between mutations at position 1400 and dually highly resistant strains. Since cross-resistance is not present in all strains, caution should thus be taken when extrapolating the results of susceptibility testing between the closely related drugs kanamycin and amikacin. A rapid detection of MDR-TB infections makes it possible to promptly change to a more effective treatment of the patient, which could shorten the patient's period of infectiousness and thus reduce the risk for additional new cases. We evaluated the usefulness of the commercially available hybridisation Line Probe Assay (INNO-LiPA^{TM} Rif.TB). We also developed an assay based on Pyrosequencing technology, for the identification of *rpoB* mutations, and thus the rapid detection of rifampicin resistance. Both methods detected mutations in all rifampicin resistant strains. Among the susceptible strains, the Pyrosequencing assay detected additional mutations whereas these could not be discriminated by LiPA. Since the *M. tuberculosis* Beijing family has been associated with major outbreaks and MDR-TB, we investigated whether rifampicin resistance-levels and *rpoB* mutations could be strain dependent. We studied 189 *in vitro* generated rifampicin resistant mutants, of which approximately half were of the Beijing family. There was no general difference in resistance-level or mutations between the two subsets of mutants. The two most common mutations found, irrespective of strain origin, were the Ser531Leu and His526Tyr, which reflect what is found among clinical isolates. Thus, the predominance of Beijing strains in terms of resistance and high prevalence in certain geographical areas are likely due to other factors than mutations in the *rpoB* gene. Lastly, we investigated the use of a sequencing assay directed to the *pncA* gene and sequences in its proximity, for the detection of pyrazinamide resistance. The phenotypic drug-susceptibility tests for the first-line agent pyrazinamide are cumbersome to use and known to be difficult to reproduce. Not only would a sequencing assay circumvent the obstacles associated with the phenotypic methods but also provide a shorter turnaround time. We identified mutations in all but one resistant strain. Among the susceptible strains, only a few mutations were found, and these were silent mutations. Thus, *pncA* sequencing seems to offer an attractive alternative to phenotypic tests.

In the present studies, we have shown that DNA-based methods directed to *rpoB* and *pncA* correlate well to the phenotypic methods for the detection of rifampicin and pyrazinamide resistance. A hybridisation-based method would be of second choice as there is a risk for false positive readings. Thus, we recommend the use of sequencing assays that will detect and directly define mutations. Unfortunately, for aminoglycosidic resistance there is yet no clear correlation between mutations and resistance, and only highly resistant strains could be identified using our sequencing assay. More importantly however, in contrast to what has earlier been described, we showed that cross-resistance between amikacin and kanamycin is not a general rule. Therefore we propose that, if these two drugs are being considered for treatment, drug-susceptibility testing on both drugs should be conducted simultaneously. These studies show that molecular techniques not only offer tools for a rapid detection of drug resistance, but also increase our understanding of how resistance is developed.

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Antimicrobial Agents and Chemotherapy, 2004, 47, 2971-2973

II. **Jureen P**, Werngren J, Hoffner SE.
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III. **Jureen P**, Engstrand L, Eriksson S, Alderborn A, Krabbe M, Hoffner SE.
Rapid Detection of Rifampin Resistance in Mycobaterium tuberculosis by Pyrosequencing Technology
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IV. Huitric E, Werngren J, **Jureen P**, Hoffner S.
Resistance Levels and rpoB Gene Mutations among In Vitro-Selected Rifampin-Resistant Mycobacterium tuberculosis Mutants
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V. **Jureen P**, Werngren J, Toro JC and Hoffner S
Pyrazinamide Resistance and *pncA* Gene Mutations in *Mycobacterium tuberculosis*
Manuscript in press (Antimicrobial Agents and Chemotherapy)

CONTENTS

LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AMK	Amikacin
bp	Base pairs
DNA	Deoxyribonucleic acid
DST	Drug susceptibility test
EMB	Ethambutol
HIV	Human immunodefiency virus
INH	Isoniazid
KAN	Kanamycin
katG	Catalase-peroxidase (gene)
LiPA	Line probe assay
M. tb	*Mycobacterium tuberculosis*
MDR-TB	Multi-drug resistant tuberculosis
MIC	Minimal inhibitory concentration
PCR	Polymerase chain reaction
pncA	Pyrazinamidase (gene)
PZA	Pyrazinamide
RFB	Rifabutin
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
RNA	Ribonucleic acid
RRDR	Rifampicin resistance determining region
rpoB	RNA-polymerase, β-subunit (gene)
rrs	16S rRNA (gene)
SNP	Single nucleotide polymorphisms
STR	Streptomycin
TB	Tuberculosis
WHO	World Health Organisation
XDR-TB	Extensively drug resistant tuberculosis

1 INTRODUCTION

1.1 TUBERCULOSIS YESTERDAY, TODAY AND TOMORROW

Tuberculosis (TB) is still one of the leading infectious causes of death. The bacteria is estimated to have infected one third of the global population with approximately 8.8 million new cases in year 2005 (143). Although it is a global epidemic, tuberculosis predominantly affects populations in resource poor countries, where 98% of all TB deaths occur (39). In the history of Western Europe and USA the disease was pronounced but gradually, as several social factors improved, public health measures were taken and the improvement of living standards led to the drastic decline in incidence rate during the nineteen hundreds (85). These measurements may have been even more important for the decline than the actual discovery of antibiotics. By the turn of the 19th Century, treating doctors had two important tools for diagnosis, namely the detection of the infectious agent of tuberculosis by microscopy (Robert Koch, Nobel prize in 1905) and chest x-rays (William Conrad, Röntgen Nobel prize in 1901). Still today, these two methods are cornerstones for the identification of tuberculosis.

As tuberculosis became recognized as a transmittable disease in the early 1900's, patients were isolated into sanatoriums. This was a very important step since it affected one of the corner stones of an epidemic's reproductive rate, namely the number of contacts between an infectious patient and their susceptible contacts. The improved living standards of a sanatorium stay for many patients may also have reduced the probability of the infected to become infectious, the second determinant of an epidemics reproductive rate. The third and last determinant, the duration of infectiousness of a patient, was not efficiently affected until the introduction of anti-tuberculosis drugs (17, 85). By then the importance of sanatorium stays had started to be questioned as it was showed that 10% of the patients abandoned these settings, probably due to the negative socio-economic consequences of such a stay. In contrast to this, less than 1% defaulted domiciliary treatment and there was no increased risk for infection of family members (98). In 1921, an attenuated strain of bovine tuberculosis, BCG (Bacillus Calmette-Guerin), was introduced as a vaccine against tuberculosis. Billions of people have since been vaccinated with BCG, but its efficacy is now questioned, as it seems to give little or no effect against pulmonary TB in the adult population (12).

The first antibiotics for TB treatment, streptomycin (STR) and PAS (*para*-aminosalicylic acid), were discovered in 1940's. These drugs were successfully separately used to treat patients, but soon after their introduction drug-resistance was frequently observed. By the late 1940's, combinatory treatment with both STR and PAS were used, thereby overcoming the resistance problems (51). In the beginning of the 1950's, isoniazid (INH) was discovered and added to the treatment regimens (85). Following this, it took some ten years for new TB drugs to be implemented in TB treatment, firstly ethambutol (EMB) and then, in the early 1970's, rifampicin (RIF). Pyrazinamide (PZA) was reintroduced during the 1980's and in many places it gradually came to replace the role of STR in the regimens (51). Thanks to these new drugs as well as the introduction of their analogues, several improvements in TB treatment could be done by the 1980's, such as reducing treatment duration by half to 6 months, however, since then there has been no new TB drug (98). After three decades of shallow interest from the pharmaceutical industry, several anti-TB drug-candidates have finally been identified and are under developed, of which some have already entered phase II/III clinical trials (35). This development has been greatly helped by the instigation of several non-profit, non-governmental organisations such as the Global TB-Alliance and FIND. These foundations, to

a big part supported by the Bill and Melinda Gates foundation, have initiated great efforts in not only trying to identify new or improve old drugs, but also improving present diagnostics as well as finding new methods (55). However, these efforts are working against the clock, as TB is now entering a new era in not only being resistant to the most important drugs, INH and RIF, but also becoming extensively resistant, being resistant to virtually all anti-tuberculosis drugs available.

1.2 TUBERCULOSIS AND HIV CO-INFECTION

Mycobacterium tuberculosis (*M. tb*), the causative agent of TB, is spread as an aerosol. The aerosol consists of small droplet nuclei containing bacteria and is created as an infected person coughs (50). These droplet nuclei, 1-5 μm in size, are small enough to get to the alveoli and can stay in the air for long periods of time. *M. tb* transmission is determined by; (a) the infectiousness of a coughing patient, (b) the probability of exposure to an uninfected individual, and (c) the immune status of the individual at risk (3). If the immune system is not impaired in any way, an infected person has a 10% risk *during their lifetime* of developing active TB, with the highest risk during the first 2 years after infection (50). An infection can thus remain asymptomatic (i.e. remain latent) for years, during which time the organism is presumably dormant, and the infected individual is not contagious (3).

One of the greatest challenges in the fight against the global TB-epidemic has been the global HIV-epidemic. The co-infection of HIV and TB poses numerous risk and difficulties in the control over TB (and HIV). HIV-positive individuals have a 10% risk *per year of life* of contracting TB (50). A dual infection is also associated with a higher mortality rate (38).

HIV-infected patients contract the classical lung TB to less extent, and thus is harder to detect by microscopy (3). This is further cumbersome since the latter is one the cornerstones for TB diagnosis in resource limited settings, i.e. in countries where the prevalence of co-infections are the highest (38). Furthermore, the treatment of the TB-HIV patients is more complicated due to the negative interactions between anti-retroviral and TB drugs. This is especially so for rifampicin, which increases the metabolism of several antiviral drugs. TB relapse rates are also higher among HIV-infected, especially among the severely immuno-compromised patients. Lastly, partly as a result of the elevated relapse rates, drug resistant TB is also more common among HIV-infected persons, which further affects the mortality rate (1).

1.3 TREATMENT AND DRUG RESISTANCE

1.3.1 Treatment and resistance

For the treatment of tuberculosis infections, combinatory regimens are used. This is to avoid the development of resistance to the few efficient drugs that are currently available. If resistance is not suspected or detected, the standard four-drug regimen is used. The drugs used are; rifampicin, isoniazid, pyrazinamide and ethambutol or streptomycin. The treatment is initiated with 4 drugs for two months and thereafter continued with RIF and INH for the following 4 months. In Western countries, PZA is used whereas STR is predominantly used in resource poor countries (44, 145). Although replaced by PZA in many countries, globally, STR still remains to be the second most common drug *M. tb* become resistant to (6.1%). The top position is taken by INH (6.6%) and RIF (2.2%) is the third most common form of M. tb

resistance (141). The importance of RIF and INH cannot be overestimated. In fact, the definition of multi-drug resistance (MDR-TB) is resistance to at least these two drugs.

If the strain is resistant to one drug or more, treatment is, if possible, altered. Such treatment regimens are complicated, prolonged and are much more prone to causing treatment failure. These treatments are both difficult and expensive and preferably follows an extended standardized treatment regime or being individualized in consultation with a clinician and expertise as well as being carefully monitored (142). The treatment success rate for strains resistant to both RIF and INH varies between 44-77%, which is to be compared to > 90% for fully susceptible strains (67). There are a number of different proposed second line agents for TB treatment, of which a few are; the fluoroquinolones, amikacin, kanamycin, capreomycin, ethionamide, PAS (*para*-aminosalicylic acid), cycliserine and thioacetazon. Since each compound has its own drawbacks e.g.; high cost, lesser efficacy or specificity, toxicity for the patient etc., there is a lack of good second-line agents (26, 83).

In October 2006 a new definition was announced, namely extensively drug resistant tuberculosis (XDR-TB). Such strains are defined as not only being MDR-TB but also resistant to any fluoroquinolone as well as one or more of the following injectable drugs: kanamycin, amikacin and capreomycin. There are in other words few efficient drugs left for treatment of such strains. With the recent detection of extensively drug-resistant TB strains in South Africa, a mortality rate as high as 98% was reported among co-infected TB-HIV patients. The majority of these patients died within 30 days after sputum collection (33).

1.3.2 Epidemiology of drug-resistance

Drug-resistance in tuberculosis originates from two possible sources; either initial or acquired drug resistance. Initial resistance refers to the spread of already resistant strains giving rise to primary resistance in a patient. Acquired resistance on the other hand originates from the selection for resistance due to inadequate treatment. This can be caused by several of reasons where interrupted drug supply and non-compliance to treatment are amongst a few. Another plausible scenario for acquired resistance is treatment with drugs that the strain already are resistant to; this enhances the risk of creating resistance to even more drugs (141).

A new TB-case is defined as a patient who has never taken TB-treatment or who has taken anti-TB drugs for less than one month. Contrarily, previously treated cases are defined as patients that previously have been treated for TB with combinatory chemotherapy for 4 weeks or more, excluding prophylactic therapy. The term combined cases, pools these two categories of TB-patients into a single group. Comparing new cases with previously treated, always show much higher prevalence of resistance in the latter group (141).

The World Health Organisation (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) monitor the global prevalence of tuberculosis drug-resistance. The WHO produces yearly reports, but also together with IUATLD, it estimates and analyses the prevalence of drug resistance trends in reports edited less frequently (138-141). Several conclusions can be drawn from these reports but more importantly they identify drug resistance hot spot regions, where specific efforts from the global society should be focused. Among these regions are the Baltic States and Russia where MDR-TB prevalence have remained exceedingly high from the first surveillances in 1994 up to the latest report in 2008 (138-141).

1.4 DIAGNOSIS

1.4.1 Identification of tuberculosis

1.4.1.1 AFB and clinical manifestations

Globally, the most common laboratory identification of TB is acid-fast bacilli (AFB) staining. The patient is asked to produce sputum, which is then smeared on a microscopy glass, stained by the AFB method and analysed in a light microscope. This method is cheap and easy to perform, but has a low detection rate. AFB-smear will furthermore not discriminate TB from several other mycobacterial infections. For a positive sputum smear about 5000 to10 000 bacilli per ml of sputum are needed. Patients with a pulmonary TB will have a positive smear in 50-80% of the cases (3). In order to increase the detection rate, concentration of soutum and fluorescence microscopy can be used, but this will still not be as efficient as culture. Clinical manifestations such as coughing for three weeks or more, night fever and weight loss are classical symptoms of TB, but neither universal nor specific for TB. Diagnosis is commonly based on several laboratory findings such as on the basis of symptoms, chest radiography, sputum microscopy, and culture. Bacteriological confirmation is not always possible, instead the patient response to treatment in combination with high clinical suspicion are in fact are the only sources of diagnosis for 15% of all verified TB cases in the United States.(124).

1.4.1.2 Culture

If economically possible, primary isolation by culture is the method of choice for detection of TB. It is furthermore also a requirement for drug susceptibility tests. The sputum is liquefied and decontaminated from other bacteria and subsequently inoculated. Culture can detect 100 organisms per ml of sputum and is either done on solid media or in liquid broth (124). There are different solid medias that can be used, but they all generally need four to six weeks from inoculation to show visible colonies. The method of choice for culture differs in different geographical settings. In Europe and many parts of Asia, the Löwenstein-Jensen (L-J) media is predominantly used, whereas in Japan, Ogawa egg medium is used and in the US agar based medium is predominately used for primary isolation. Primary isolation in broth is more expensive and more sensitive to contamination, but on the other hand its turnaround time for detection is usually much shorter and can be reduced to one to three weeks. There is furthermore also the bio-safety aspect that affects the choice of media; using broth-based media, aerosols are more easily created as compared to solid media (56).

1.4.1.3 Species identification

Globally, biochemical tests still are important for mycobacterial species identification but have in the western countries been replaced by probe based identification methods, since the latter are much faster, as well as being more accurate. These probe tests need a minimal amount (10^5 organisms) of bacteria and are thus used after primary isolation by culture. They are used to identify the most common, as well as the clinically important species of Mycobacteria. Commonly, these methods will not identify *M. tb,* but instead the members of the MTB-complex. The MTB-complex is the group of closely related bacteria and includes *M. tuberculosis, M. africanum, M. bovis* (also BCG) *M. microti* and *M. canetti* (3, 88). These bacteria are difficult to distinguish from each other being, amongst others, similar in their way of infection and treatment, but differing in their host preference. Most importantly however, *M. bovis* is intrinsically resistant to the first-line TB agent pyrazinamide making it important to distinguish it from the rest of the complex.

1.4.1.4 PCR

With the aim to both reduce time for species detection, as well as to enhance the detection-rate, several in-house polymerase chain reaction (PCR) methods were developed during the early 1990's. Joint evaluations on accuracy revealed extensive shortcomings with these methods, such as low specificity and sensitivity (86). These have now been replaced by two major commercial systems Amplicor (Roche) and E-MTD (Gen-Probe) where DNA or RNA are amplified and then identified by nucleic acid probes. Both methods have high accuracy in detecting the MTB-complex (as well as identifying *M. bovis*) in AFB-positive sputum samples with a sensitivity of 95%. In contrast the sensitivity can be as low as 48% if the AFB smear is negative. The specificity is approximately 95%, and thus, amplification methods are generally regarded as a complementary method to culture (3, 111).

1.4.2 Strain identification

1.4.2.1 Molecular typing

In order to monitor the spread of TB and to backtrack transmission routes, DNA based tools for TB strain identification were developed during the early 1990's. For tuberculosis there is now one predominant method called Restriction Fragment Length Polymorphism (RFLP) (133). The method is based on the copy-number and chromosomal location of the insertion sequence IS6110, which varies in number amongst different strains and slowly changes in number and location over time. This is an excellent method for epidemiology typing of TB, with a high resolution for most strains. Since RFLP has been in use for many years, a lot of data have been collected from the collaboration of several countries and are gathered in searchable databases. The major disadvantage with the RFLP method is the cumbersome laboratory work; not only are strain cultures needed, but the method itself takes a long time and needs trained personnel (91). In order to overcome this, spoligotyping was evolved. This PCR method is based on the variability in copy-number of direct-repeats at a single locus (134). The handling is much simpler than RFLP, but the discriminatory power is lower and is thus only used as a complement to RFLP.

Variable-number tandem repeats (VNTR), a method first used for human allelic studies, is yet another DNA marker that was used for identifying individuals. This technique has been adapted to tuberculosis strain detection and is denoted MIRU-VNTR (Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem repeat) (91). MIRU-VNTR analyses copy-numbers of repeats, however, in contrast to spoligotyping, these have several sites scattered around the genome. Since new potential MIRU-VNTR sites are continuously being found, this method is still regarded to be under development. Also, due to the extensive amount of historical data that currently exists for RFLP, this method is predominantly used. Nevertheless, considering that MIRU-VNTR enables a simpler handling of samples, as well as the possibility to be used on AFB-positive specimens, much speaks for this methods applicability in the future (61).

In addition, non-resistance dependent allelic variability has previously been used to discriminate members of the MTB complex as well as identifying specific lineages of *M. tb*. Rather recently Single Nucleotide Polymorphisms (SNP) have been proposed to be used as a molecular epidemiology tool (29).

1.4.2.2 The M. tb Beijing family

The Beijing strain was first recognised as a predominant genotype in the Beijing province of China and was denoted as *Beijing* according by European and Asian scientists (134). In New York City, USA, an outbreak was found to be caused by strains being predominantly MDR, and were denoted W-strains (13). The strains of these two separate regions were found to be genetically related by their molecular epidemiology patterns, but their original names were kept on each side of the Atlantic. These strains all show the same specific spoligo-pattern in the typing assay, they namely, lack the 34 first spacers of 43 in total (60). Another shared trait is that they seem to have an IS6110 insert near the origin of replication, whether this affects the bacteria still remains to be understood (64). These strains have been found to be present in almost all regions as they been searched for and seem to be most prevalent in Asia and the states of former soviet union (37). As these strains still show some degree of difference in their RFLP and spoligo patterns, but all still lacking spacer 1 to 34, they have been classified into the phylogenetic W-Beijing lineage (60). More importantly however, strains of the Beijing family have been associated with high prevalence of drug resistance and outbreaks (37, 62, 127). However, it has been shown that the strains of this family do not have an elevated mutations rate when selected for RIF-resistance (135).

1.4.3 Drug susceptibility testing

1.4.3.1 Culture

In order to confirm, or establish, an appropriate and efficient regimen the drug susceptibility testing (DST) should be carried out. The DST usually requires an additional two to four weeks after the primary isolation. If the patient converts to positive sputum or continues to be positive after three months, the DST should be repeated (3).

The WHO recommends four different methods for TB-DST. Three of these are performed on solid media and are the absolute concentration method, the resistance ratio method and the proportion method. The most widely used broth-based method is the radiometric BACTEC 460 and is a variant of the proportion method (141). All these four methods are used today and the method of choice is usually determined by tradition, as well as economic prerequisites. The BACTEC 460 system has the advantage of having a shorter turnaround time, requiring one week instead of three as compared to solid-media DST (50). BACTEC uses ^{14}C-labelled palmitic acid for the detection of bacterial growth, resulting in free $^{14}CO_2$, which is quantified daily (109).

Among the newer methods the, but two most credited are the BACTEC MGIT 960 and the BacT/ALERT (5, 50, 63). These have been evaluated and can be used for primary isolation of patient samples as well as for DST. These two methods do not reduce the DST turnaround time in comparison to BACTEC, but do not use radiolabeled substrate and have fully automated readings of the test bottles (5, 100). Other DST tests that are to date mostly used in research but have the potential to be used in the clinical laboratory includes the nitrate reductase assay and the resazurin assay (4, 71). Both being low cost methods and thus suitable to be implemented in high prevalence settings where resources are scarce.

1.4.4 DNA-based methods for drug susceptibility testing

Rapid drug susceptibility tests may prove very cost effective, as well as improving treatment more rapidly, and consequently reducing the development of resistance (28). The advances in molecular biology over the past decade have significantly enhanced our understanding of TB and drug resistance. This is especially so since the genome of the reference strain H37Rv (21) was sequenced in 1998 followed by that of the clinical isolate CDC1551 genome four years later (31). Today, several different Mycobacterial genomes are available, and a few other *M. tb* genomes have been annotated or are underway. Most method evaluations have been conducted on cultured strains with the ambition to later apply them directly on primary sputum specimens. These almost exclusively in house methods can be divided into two categories, those who directly scrutinise the DNA by sequencing and those who indirectly detect alterations in the DNA. The latter group of methods are based on indirect detection that is either based on hybridisation or different electrophoretic migration. All methods then compare the specimens' characteristics to that of *wild-type* DNA. The method of choice is likely a consideration of cost and availability to different machines in the local laboratory.

1.4.4.1 Hybridisation methods

Hybridisation methods are based on a set of DNA probes that will hybridise with the amplified DNA of a tested strain. A lack of hybridisation to wild-type probes, as well as plausible hybridisation to specific mutated probes, will indicate presence of resistance mutations. Rifampicin is often chosen for the development of resistance determination methods, as it is the key anti-TB drug and, together with INH-resistance, defines MDR-TB. The first commercial kit of impact for identifying RIF-resistance mutations in tuberculosis was the Line Probe Assay (INNO-LiPA^{TM} Rif. TB, Innogenetics), abbreviated as LiPA. This method contains 5 wild-type probes and four probes for the exclusive detection of the four most common RIF-resistance mutations (22). LiPA will be discussed in more detail in Chapter 2).

Microchip (79) is another evaluated solid-phase hybridisation method. Usually, only a limited number of mutations can be detected in studies using non solid-phase-hybridisation such as real-time PCR (125). These have varied numbers and lengths of the hybridisation probes. The more probes there are, the more detailed the results will be, but the more expensive and complicated the assay will be to handle.

A few years following the introduction of LiPA, a new hybridisation method was introduced by HAIN lifescience GmbH. At present it is more attractive than the LiPA as it is less costly and, besides detecting RIF-resistance mutations, it also detects INH resistance mutations. The HAIN, GT-MTBDR*plus* kit is directed towards codon 315 of the *katG* gene (amino acid S315T/T) and the regulatory region of the *inhA* gene (nucleotides; C15T, A16G and T8C/A). In collaboration with FIND, in the coming years the HAIN Company also plans to have a commercial kit for the detection XDR-TB. This kit will include the detection of RIF, INH and fluoroquinolone resistance mutations, but will most likely not be designed for the detection of resistance mutations to any the injectable drugs (personal communication with Dr. Paramasivan, Head of TB Laboratory Support, FIND).

1.4.4.2 Electrophoresis methods

Electrophoresis-based methods may be the cheapest available molecular methods, as they only require a PCR machine and electrophoresis equipment. A set of equipment that can be afforded by most national reference laboratories in the world. Multiplex PCR may be the simplest of these methods, but usually only detects one or two resistance mutations per reaction. This method uses a set of primers that are specific for the wild-type and one or a few resistance mutations, the resistance primers only giving a product whenever the corresponding mutation is present (82).

In order to detect more mutations, methods such as single-stranded conformation polymorphism (SSCP) (122), double gradient-denaturing gradient gel electrophoresis (DG-DGGE) and analogue methods have been evaluated (103). With these methods, a specific region of a gene is amplified by PCR and the product is either separated before loading or whilst being run on a gel. Such methods demand highly separating gels (commonly acryl-amide gels) and/or electrophoresis apparatus with specialised pH and/or charge gradients. The migration is compared to the migration of a wild-type fragment. These methods are beneficial from an economical point of view, but have been reported not to detect the most common mutations (129). Most importantly, they are hard to standardise and the results have been hard to reproduce in other laboratories; an indication of the limitation in using them as general methods (57, 129)

1.4.4.3 Sequencing methods

The gold standard for the molecular methods is sequencing by the Sanger sequencing method (101). The Sanger method is commonly a semi-automated 96-well analyser. Although it gives a high yield of very detailed data, this method has its disadvantages, mainly being costly, both in needing an expensive apparatus and reagents, but also the need for trained personnel for the laboratory work, as well as maintenance. In 1998, a new sequencing method was reported by a Swedish group at the Royal Technical Institute in Stockholm (95). The method was denoted Pyrosequencing due to one of the essential products in the reaction being pyrophosphate. Instead of separating the products on gels, this methods detects each nucleotide as it is inserted in the elongating DNA chain, giving a signal output much earlier than in Sanger sequencing. In Pyrosequencing each nucleotide is dispensed at time, and as a nucleotide is incorporated, pyrophosphate (PPi) will be released. The PPi is then converted into ATP by sulfurylase and the ATP is used by luciferase to produce light in proportion to the number of nucleotides inserted. The light is detected by a CCD camera and can be monitored in real time. Unincorporated nucleotides will be degraded by apyrase and the system is ready for another nucleotide to be dispensed. The pyrosequecing technology has been further developed and is today also available as a tool for large-scale sequencing (Genome Sequencer FLX, 454 Life Science/Roche). Other full genome sequencing technologies have recently become available such as the Illumina Genome Analyzer System (Solexa/Illumina) and SOLiD (Applied biosystem). Commonly, the laboratory part of all the different methods is a minor part, as data handling, follow up sequencing and bioinformatics are still issues to be solved for the application outside the research lab. Although prices have drastically been reduced over time, the costs of sequencing methods remain too high for them to be implemented in routine diagnostics.

1.5 MECHANISMS OF DRUG RESISTANCE

1.5.1 Origins of drug resistance in *M. tuberculosis*

A common mechanism for resistance acquisition of clinical relevance among bacteria is the uptake of foreign DNA. However, for *M. tb* is regarded not to be a clinical obstacle, instead, all resistance originates from random single-step spontaneous mutations at the gene loci of the chromosome at a low but predictable rate (153). Mutation rates for resistance differ between the drugs. For example, *M. tb* is 100 times more likely to become resistant to INH (resistance rate of 10^{-6}) than to RIF (resistance rate 10^{-8}). These figures are accumulative, i.e. the likelihood for resistance to both INH and RIF are 1 in 10^{14}, and this is to be compared to an average number of organisms in a tuberculosis cavity, which is estimated to be 10^{8}- 10^{9} (124). That resistance mechanisms to one drug are regarded not to be linked to resistance to another drug (unless they are chemically related e.g. the class of rifamycins), demonstrates why multiple drug regimens are so important. The likelihood of finding a strain resistant to multiple drugs that had not previously been exposed to any drug is extremely low and would require an immensely large bacterial population. Since resistance must originate from a stepwise acquisition of independent mutational events, events that can consequently be selected for during a sub-optimal treatment, resistant TB, MDR-TB and XDR-TB are regarded to be a man-made problem.

Mutation rates are closely related to the mechanisms of resistance. Resistance mutations to INH have so far been associated to at several different genes, and within each of these genes, several loci can be affected, thus giving several mechanisms of resistance and a relatively high INH-resistance rate (10^{-6}). RIF resistance mutations are on the other hand only associated to a small region of a single gene. These differences are also reflected in what is found in clinical isolates; INH-resistance is for example more commonly found than RIF-resistance. It is therefore proposed that detection of RIF resistance can be used as an MDR-TB surrogate marker (92, 141, 153).

1.5.2 Mechanisms for TB drugs and resistance

As the presented studies focused specifically on rifampicin (rifamycins), aminoglycosides and pyrazinamide, these will be discussed in greater detail later in this chapter. Below is a more general overview of drug-resistance mechanisms to the remaining main anti-TB drugs.

Although isoniazid is among the most studied anti-TB drugs several part of its mechanism of action remains unsolved. INH is a prodrug that needs to be activated by the *M. tb* catalase-peroxidase enzyme, which is encoded by the *katG* gene. Activation results in the formation of different radicals, which appear to target multiple sites in the bacterial cell (153). Mutations in the *katG* gene are present in 42-58% of INH resistant strains, with a clear majority having the amino acid Ser315 substituted (113). Furthermore, the active form of INH will form a ternary complex with the product of *inhA* and its cofactor, a constituent of the mycolic acid synthesis pathway. Resistance mutations have also been identified in this gene and the regulatory region of the *mabA-inhA* operon. These mutations have not only been associated with low-level INH-resistance, but they also seem to confer resistance to the second-line agent ethionamide (92). Mutations in the *kasA* gene have been related to INH resistance, but as they have also been found in INH-susceptible strains, the correlation to INH resistance needs to be further clarified (153). Mutations in the *ahpC* promotor region do not seem to confer INH resistance, instead it has provided an attractive model for *M. tb* compensatory evolution. The *ahpC* encodes an alkyl hydroperoxide reductase that, in a similar way as the catalase-peroxidase

enzyme, reduces reactive radicals in the bacteria. Thus an overexpression of *ahpC* is thought to compensate for the reduced activity in *katG* mutants (108). Ten percent of all INH resistant clinical isolates harbour *ahpC* promotor mutations and always seem to be associated with *katG* mutations (32, 93). The effect of the compensation has been questioned in other studies where an over-expression (45) or silencing (74) of *ahpC* did not alter the bacteria's fitness in mice. Finally, resistance-associated mutations are typically not identified in approximately 10-25% of INH resistant strains (93).

Ethambutol (EMB) is a bacteriostatic drug inhibiting the mycobacterial arabinosyl transferases, which are located in the bacterial cell membrane and are encoded by the *embCAB* operon (92, 153). As EMB inhibits the synthesis of the essential arabinogalactan, the cell wall stability is disturbed resulting in an uncontrolled movement of molecules into the cell, including drugs. Thus, EMB has a synergistic effect with other drugs used in TB-treatment (48, 50). Mutations in the *embB* gene are associated with a high-level EMB resistance, and mutations are predominantly (47-62%) found in codon 306 (93, 153)

Ciprofloxacin or ofloxacin are commonly used for the treatment of MDR-TB (36, 50, 145). These drugs belong to the fluoroquinolones, a group of broad-spectrum antibiotics active on several of the human bacterial pathogens. Fluoroquinolones target two bacterial topoisomerases: the DNA gyrase and topoisomerase IV (27, 50). The DNA gyrase is a heterotetramer and is constituted of two A subunits (*gyrA*) and two B sununits (*gyrB*) (92). In *M. tb*, only DNA gyrase is present (36) and resistance mutations are predominantly found in a short region, denoted quinolone resistance-determining region (codon 88 to 94; QRDR), of the *gyrA* gene (92, 118). The presence of QRDR mutations in resistant clinical isolates could vary as much as 42-90% and naturally other resistance markers and mechanisms have been suggested (20, 34, 36, 89). In a study by Kokagöz *et al.*, two-step ofloxacin resistant H37Ra mutants were selected for *in vitro*. In the first selection step, low-level resistant mutants with a single mutation in the *gyrA* were generated, whereas subsequent selection generated highly resistant strains with either an extra mutation in the *gyrA* or with a second mutation in *gyrB* (59). Mutations in the *gyrB* gene are rarely found among clinical isolates (10, 89). Other proposed mechanisms of fluoroquinolone resistance in TB are efflux pumps, which have previously been associated with low-level resistance in other bacteria (36, 153). Newer fluoroquinolones suchas gatifloxacin and moxifloxacin, are promising candidates to be used for the treatment of tuberculosis. These have the potential of reducing treatment time especially as they appear to have a greater sterilising activity than ofloxacin or ciprofloxacin (36).

Capreomycin is a basic polypeptide antibiotic that is frequently grouped together with the aminoglycosides, as they share the common feature of being injectable second line agents that targets the ribosome (142). Suspicion of crossresistace to amikacin and kanamycin has been speculated as isolates are frequently found to be resistant to all three drugs (130, 153). Maus *et al.* correlated *in vitro*-generated capreomycin resistance to the *tlyA* gene (76). Among clinical samples, isolates with a *tlyA* alteration were resistant to capreomycin but not necessarily to kanamycin and amikacin. However, if the isolates harboured either of the A1400G, C1401T or G1483T substitutions in the *rrs* gene (figure 1), resistance to all three drugs was seen (75, 76). In a later study, the same group showed that the *tlyA* gene product was a 2'-O-methyltransferase that modifies the C1401 of *rrs* (53). Thus, it seems that capreomycin resistance is caused by the lack of binding to the 1400-1483 loop of *rrs*, which can be induced either by silencing the *tlyA* or changing the target region in *rrs*.

1.5.3 Aminoglycosides

The aminoglycosides are bacteriostatic drugs that act by interfering with protein synthesis at the bacterial ribosome (92). For tuberculosis treatment, three drugs within this class are predominantly used; streptomycin (STR), kanamycin (KAN) and amikacin (AMK) (145) and only these will be referred to from here onwards. Streptomycin has traditionally been used as a first-line agent and still is in several settings. In Sweden, as well as several other countries, this drug has been replaced by PZA due to several advantages of the latter. One major drawback with the aminoglycosides is their poor absorption from the gastrointestinal tract, and is the reason for their being parenterally administered (107). Among the more pronounced adverse effects of aminoglycosides, is the intrinsic toxicity in the form of nephro-, vestibular- (STR) and auditory toxicity (AMK and KAN). These adverse reactions occur most frequently upon prolonged contact with the drugs (50). This does not agree well with the need for prolonged treatment of tuberculosis, especially the extended treatment period for drug resistant infections, where aminoglycosides are commonly used (142). In contrast to PZA, STR has its greatest effect in basic to neutral pH and becomes inactive in acidic environments (80). AMK is a semi-synthetic derivative of KAN, but is not toxic to the same extent as its parent molecule. Although both drugs are used as second line agents for the treatment of resistant tuberculosis, in many countries KAN is preferred as AMK is regarded as too expensive (107).

1.5.3.1 Mechanism of resistance

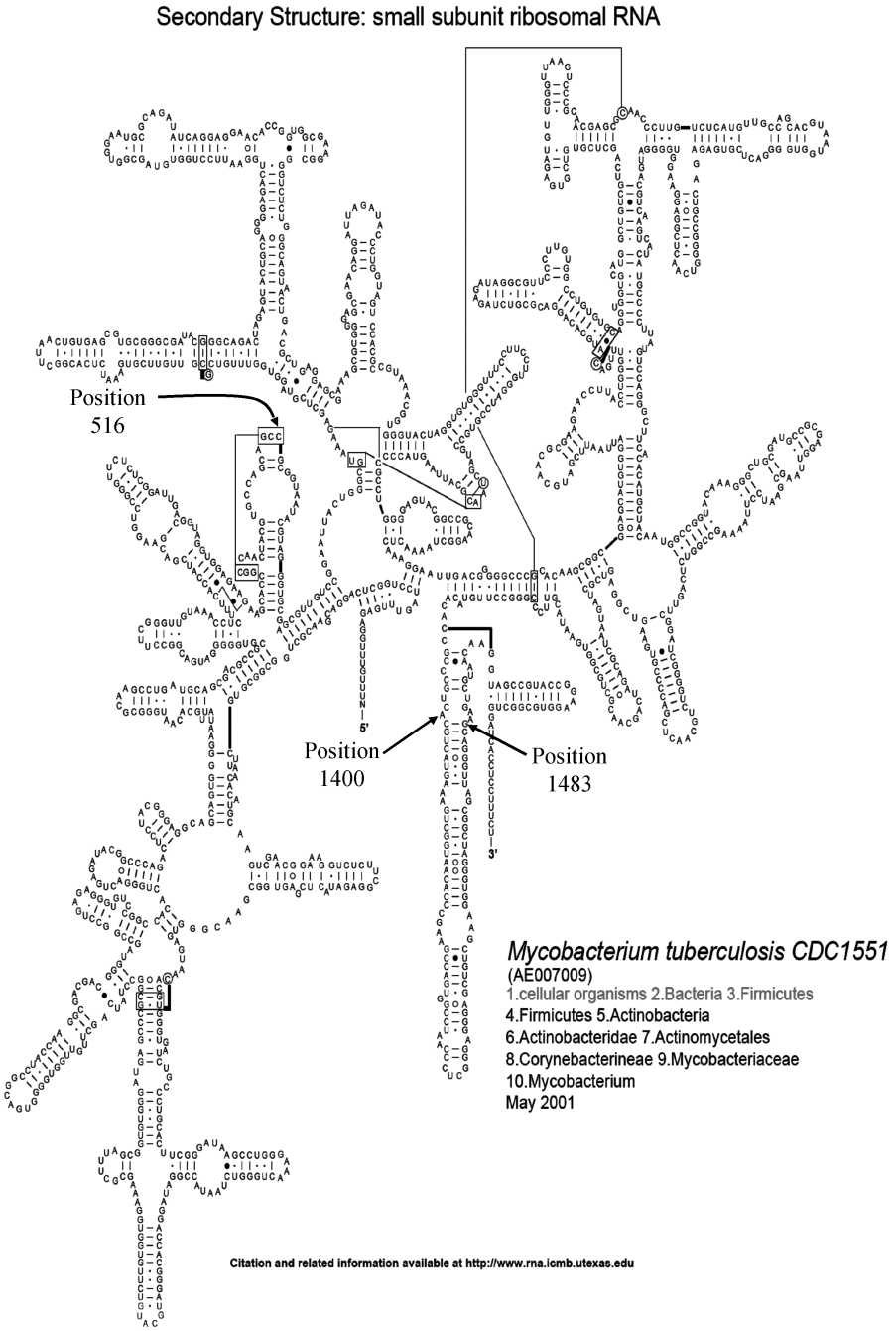
The ribosome (70S) is constituted of two major subunits 50S and 30S. The 30S in turn, consists of the 16S rRNA (encode by the *rrs* gene) and a number of proteins: from S1 to S21. Streptomycin binding of the 30S subunit will introduce misreading of the genetic code and thus increase the levels of misfolded proteins in the cell (99). The primary target for STR-resistance in TB is found in the S12 protein (*rpsL*). Within this gene, the most common amino acid substitution is at codon 43 whilst fewer are seen at position codon 88. The other major regions for STR resistance are mutations in the *rrs* gene at position 915 and in the 530-pseudoknot (*E. coli* numeration) (92). In earlier studies of the *M.tb* rRNA, the *rrs* of *Escherichia coli* was used as a model and several positions have remained according to this organism's nucleotide position. In *M. tb* the 530-pseudoknot is positioned around nucleotide 515 (Figure 1). In the functional ribosomal complex, nucleotides 514-516 fold back and pair with nucleotides 497-495 in another stem-loop structure (16, 30, 81).

Although STR, AMK and KAN are all directed towards the 30S subunit, STR seems to have a slightly different mechanism of action than the remaining two seeing that different sites for resistance mutations are observed. It is generally regarded that there is no cross-resistance between STR and either AMK or KAN in *M. tb*. However, cross-resistance between AMK and KAN is consensus, and usually only one of the two is tested in DST. High-level of resistance to AMK and KAN is typically correlated to mutations at position 1400 and its proximity (2, 92). Briefly, the 3' end of the 16S rRNA (position 1483) loops back and interacts with position 1401 (Figure 1). Position 1483 of the 16S rRNA is also a site for resistance mutations but at a lower frequency (92). Isolates with intermediate susceptibility to AMK or KAN seems to show no mutations in the *rrs* (2, 117, 120).

Besides chromosomal mutations, horizontal transfer of aminoglycoside-inactivating enzymes are a common resistance mechanism in many bacteria. This seems not to be the case in *M. tb*, which is consistent with the theory that tuberculosis does not have any gene transfer (93). Interestingly, intrinsic aminoglycoside-inactivating like enzymes have been identified in *M. tb*, but seem not to cause resistance to any of the aminoglycoside used for TB treatment.

Commonly, bacteria also have multiple copies of *rrs* genes, but this is not the case in *M. tb*, which contain only one copy of the essential gene (99).

Figure 1. The 16S rRNA of *M. tb*. (18)



1.5.4 Rifamycins

Rifamycins inhibit the RNA polymerisation by interacting with the DNA-dependent RNA polymerase. Rifamycins are a group of compounds that not only have an antimicrobial effect on mycobacteria, but also towards gram negative and gram-positive bacteria. They are also an important group of drugs for the treatment of leprosy. Since they were first discovered, hundreds of derivatives have since been isolated. The most important rifamycin for TB treatment is rifampicin (rifampin, RIF), which was discivered in the 1960's. Other rifamycins of importance are, rifapentine, rifabutin (RFB), rifalazil (KRM-1648) and rifamycin T9, some of which show higher bactericidal effect against TB than RIF. It is important to point out that most resistance mutations give cross-resistance to the different rifamycins. Nevertheless, some mutations seem to result in RIF resistance but not to one or several of the other rifamycins, wich will be deatlh with in detail when discussing paper IV. *In vitro*, RIF is active against *M. tb* at concentration from 1μg/ml and is is most efficient in inhibiting actively replicating bacteria. *In vivo*, it is regarded that RIF is not only effective in curing active TB, but also regarded to inhibit further spurts of metabolism among latent bacteria (80).

1.5.4.1 Mechanism of rifampicin resistance

The tuberculosis RNA-polymerase is comprised of five subunits of which the α, α', β, and β' make out the core enzyme. The last subunit (σ) is only shortly bound to the core when polymerisation is initiated and falls off as soon as elongation starts. When RIF binds to the β-subunit, the core can still be assembled to the DNA and the first phosphodiester bonds can be formed. However, RIF then bocks the further formation transcripts of three to four base pairs, and the elongation of RNA is inhibited (9).

The β-subunit of the RNA-polymerase is encoded by the 3.5 kbp *rpoB* gene. In *M. tb*, most resistance mutations involve single nucleotide substitutions in this gene, and (in frame) insertions or deletions are occasionally seen (92). Although being a highly essential gene, these mutations do not seem to affect the redundancy of the RNA-polymerase. Instead, these mutations are thought to mainly alter the amino acids to which the RIF molecule presumably binds (107). Recently, from structural studies of the *Thermus aquaticus* and *Thermus thermophilus*, a theory about rifamycin-binding was introduced (9). In this theory, rifamycins are classed into two groups, depending on their side groups from the shared ansa-core. Thus, mutations in the RNA polymerase can circumvent rifamycin binding in three ways; through (a) steric hinder, (b) reduced affinity and (c) allosteric modulation. The latter would probably not inhibit binding of the drug, but distort an inhibitory signal of the drug (9).

Over 96% of RIF resistant *M. tb* strains have mutations in an 81-bp region of the *rpoB* gene, referred to as Rifampicin Resistance Determining Region (RRDR) or cluster I region (40, 52, 92, 121) (Figure 3). The region comprises codon 507 to 533 according to the *E. coli* gene numbering (this numbering is used since the correlation was first identified in this organism). Mutations are found all over the RRDR, but some are more frequently occurring than others. For example, the Ser531Leu mutation has been reported to be present in 40% of clinical RIF-resistant strains (92). Rare mutations situated approximately 1000 bp up-streams the RRDR, have been associated with low-grade resistance to RIF (40).

1.5.5 Pyrazinamide

Although discovered already in the 1950's, several of obstacles surrounding pyrazinamide still remain to be solved. PZA was discovered to be effective against *M. tb* in the mouse models, but found to have little or no *in vitro* effect under normal culture conditions (150). However, through its introduction in physical therapy relapse-rates were strikingly reduced and PZA has since greatly contributed to the shortening of TB therapy from 9-12 months to 6 months (98). In contrast to other anti-tuberculosis drugs, PZA seems to be active against semi-dormant bacilli (42, 80). In line with this, it has also been shown that the drug has a greater activity in an anaerobic environment (131) and against stationary phase cultures (154). Pyrazinamide is exclusively active against the MTB-complex members, with the exception of *M. bovis*, who's intrinsic PZA-resistance is a characteristic feature for this member.

Pyrazinamide is a pro-drug, which thus needs to be converted into pyrazinoic acid (POA) in order to be effective against *M. tb*. POA itself is not used as a drug since it has been shown to be poorly effective *M. tb* infected mouse models (150). The breakpoint used today to define standard drug susceptibility to PZA is between 100-400 μg/ml (at pH 6.0), depending on method used (44, 87). The optimal pH for PZA-DST is a delicate matter. Not all tuberculosis bacteria will be able to grow at the originally set pH of 5.5, and an even more acidic environment will be more harmful for the bacterial resulting in lack of growth (44). On the opposite hand, in cultures at pH 5.5 the minimal inhibitory concentration (MIC) to PZA is 50 μg/ml, but the increase to pH 6.8 gives a 20-fold increase to an MIC of 1000 μg/ml (97, 151). This is to be compared with maximum serum concentration of 60-70 μg/ml upon oral administration of the drug (44, 87). The need for a precise pH further complicates the drug susceptibility testing, as several other factors in the available methods influence the pH. An increase in bacterial load will increase the pH, and will thus reduce the efficacy of the active drug (151). Also, seeing that the most commonly used *M. tb* culture methods are optimised for a certain (pH 6.6) as well as the fact that they contain the growth enrichment agent, bovine albumin (pH 6.9) (Middlebrook), further complicates the interpretation of PZA-DST. Not only will this alter the pH of the environment but the bovine albumin also seems to lower the drug availability by binding to the drug (151).

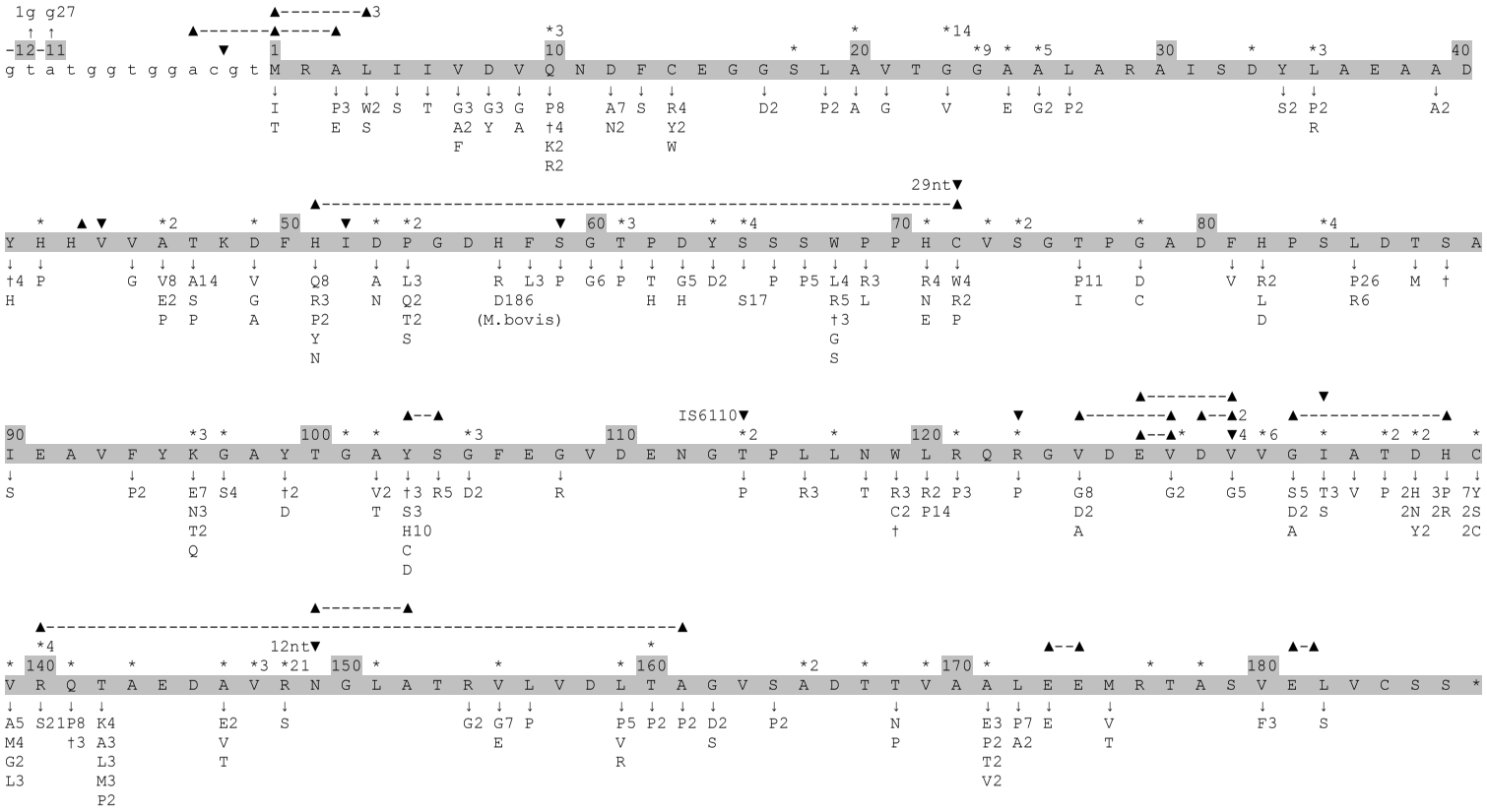
1.5.5.1 Mechanism of pyrazinamide resistance

In spite it's being widely used since the 1980's, PZA's actual mechanism of action remains to be found. In *M. tb* conversion of PZA into POA requires the action of the pyrazinamidase enzyme, encoded by the *pncA* gene (105). After conversion, there is no convincing evidence regarding the mechanism of action. The most predominant theory involves the acidification of the cytoplasm, a theory that has been developed by Zhang and co-authors (150). In this theory, it is thought that POA acts as an anion that is inefficiently pumped out of the cell and that will, in an acidic environment, be protonated to HPOA. The uncharged HPOA will thereafter efficiently permeate back into the bacterial cytoplasm and in the neutral cytoplasm will disintegrate into ionic form. The entry of POA with hydrogen ions is thus thought to acidify the cytoplasm as well as it de-energises the membrane and thus unspecifically affects the bacteria (150). This theory would explain the differences in MIC seen at different pH. By applying the Henderson–Hasselbach equation on the weak pyrazinoic acid (pKa 2.9), the slight increase from optimal pH will substantially decrease the HPOA form and therefore increase the drug tolerance of *M. tb* (97, 151). This is also supported by the observation that the efflux blocker reserpine will increase the levels of intracellular POA in *M. tb* (152).

Another theory is based on the pyrazinamide inhibition of the fatty acid synthetase I (FASI) shown by using the PZA analogue, 5-Cl-PZA (155). However, the actual role of PZA inhibition of FASI remains to be proven as it seem as 5-Cl-PZA and PZA/POA may have different targets (150).

Mutations in the *pncA* gene are the primary target for resistance. This is supported by the fact that the vast majority of PZA-resistant clinical isolates have alterations in this gene (figure 2) (8, 11, 20, 46, 49, 65, 66, 72, 73, 78, 90, 94, 96, 104-106, 112, 115, 128). In addition, the *M. bovis* as well as its vaccine derivate *M. bovis* BCG, are found to have a His57Asp substitution in the *pncA* gene (105). Strains resistant to PZA, including *M. bovis*, are nevertheless still sensitive to POA. One of the strongest evidence for this was the transformation of an *M. tb* PZA resistant isolate and a *M. bovis* BCG strain with the *M. tb* wild-type *pncA* gene. The formerly PZA-resistant strains became fully susceptible to PZA (105). Site-directed mutagenesis, changing nine specific amino acids of the *pncA* have further identified specific catalytic and metal ion binding sites within the protein (149). Although these seem essential for enzymatic function, several of these nine mutations are rare among the widely varied resistant clinical isolates. Instead, among the these isolates, mutations are found to be scattered over the 561 nucleotide-long gene, as well as in the upstream region (figure 2), with only loosely associated hot-spot regions (153).

Figure 2. Mutations located in the *pncA* gene in 808 PZA resistant tuberculosis strains reported in 20 different studies, including this. Silent mutations are also included.



2 THE PRESENT INVESTIGATION

2.1 PURPOSE OF THE STUDY

A correct and early detection of resistance in *Mycobacterium tuberculosis* would be a valuable tool for improved drug therapy and thus treatment outcome, which in turn could reduce the spread and emergence of resistance. To reach this objective, it is not only crucial to know the nature of resistance and its mechanisms, but also learn what methods can be used for rapid detection of such cases.

2.1.1 Specific objectives

- To study 16S rRNA mutations in kanamycin resistant but amikacin sensitive clinical isolates of *M. tuberculosis*.
- To evaluate the line probe assay (INNO-LiPA.Rif-TB), for rapid determination of rifampicin resistance.
- To design and evaluate a method for rapid detection of rifampicin resistance based on Pyrosequencing technology.
- To study resistance level and *rpoB* mutations from *in vitro* generated rifampicin resistant mutants.
- To design and evaluate a sequencing method of the *pncA* gene for the detection of pyrazinamide resistance.

2.2 MATERIALS AND METHODS

Below is a summary of the general methods used in study I – V. For a more detailed description of the methodology, please consult the specific manuscripts at the end of the thesis.

2.2.1 Strains and isolation of DNA

2.2.1.1 Tuberculosis isolates

All clinical *M. tb* isolates originated from the archive of the Estonian National Reference Laboratory as well as from the national strain collection at the Swedish Institute for Infectious Disease Control in Solna, Sweden. They were selected based on their previously determined drug susceptibility pattern. It was verified that none of the included isolates originated from the same patient. Subsets of isolates were also subjected to the molecular epidemiology tools RFLP and Spoligotyping. The fully susceptible *M. tb* reference strain, H37Rv (ATCC 25618) was used as a control in all studies except paper I. Prior to subsequent analysis, isolates were sub-cultured on Löwenstein-Jensen medium for 3-4 weeks at 37 ^{o}C.

2.2.1.2 In vitro selection of RIF mutants

The 189 RIF-resistant mutants used in paper IV were generated in a previous study (135) which was conducted with the aim to determine if Beijing strains, in contrast to other strains, have an increased capacity to generate resistance to rifampicin. The authors used 13 fully susceptible *M. tb* strains, including six different Beijing strains, H37Rv and the Harlinge strain. In brief, the experiments were performed as follows. For each strain, 25 low-density broth cultures (~10^3 cells/ml) were prepared and let to grow for 4 weeks. Subsequently, each culture was adjusted to an OD of 0.8 (600 nm), of which 1 ml was then pelleted and plated on 2 µg/ml of rifampicin (22 cultures) as well as on drug free media (3 cultures). After 28 days of incubation the colonies were counted and mutation rates, as well as mutation frequencies, were calculated. As all the strains showed similar mutation rates, the authors concluded that Beijing strains do not confer RIF resistance at an elevated rate.

2.2.1.3 Isolation of DNA

Strains were lysed for 30 minutes at 85 ºC in Tris-HCl. The suspension was let to stand in room temperature and then centrifuged. After removing the supernatant, the pellet was suspended in a 1:1 mixture of chloroform and water, vortexed and centrifuged again. The aqueous phase containing DNA was collected and quantified by optical density measure. Each sample was diluted to 5 ng/µl, and stored at -20ºC until further usage. For additional RFLP and Spoligotyping , DNA isolation was performed following other standard procedures (133, 134).

2.2.2 Drug susceptibility testing

2.2.2.1 Solid media

The MIC was determined in a two-fold dilution series of RIF (0.0625 µg/ml to 256 µg/ml), on Middlebrook 7H10 agarsupplemented with OADC and amphotericin (8 µg/ml). A replicator system, in which up to 46 samples could be simultaneously plated, was used. Bacterial suspensions (2 loops à 1 µl, in 500 µl PBS) were homogenized by ultrasound pulse-sonication, distributed to a 96-hole plate and replicated on the RIF-containing agar plates. Two drug-free control plates were also inoculated. Following a four-week incubation at 37°C, the MIC was determined as the first concentration at which no bacterial growth was visible. The cut-off for RIF-resistance was set at MIC ≥ 2 µg/ml. The MIC's of the susceptible parent strains were determined, and the H37Rv reference strain, as well as a highly RIF-resistant clinical isolate (MIC > 256 µg/ml), were used as controls in the experiments.

The MIC's to AMK and KAN were determined in the same manner but without using the replicator system. For this, two-fold serial dilutions of 2- 256 µg/ml were used and resistance to AMK and KAN was defined as an MIC of > 4 µg/ml.

2.2.2.2 BACTEC 460

Drug susceptibility was determined by standard BACTEC 460 procedures (Becton Dickinson, Sparks, MD), with the critical concentrations: RIF 2.0 µg /ml, INH 0.2 µg/ml, STR 4.0 µg/ml and EMB 5.0 µg/ml (109). These tests were performed as part of the routine diagnostics, and thus not performed by the author of this thesis.

For the present study, selected isolates were also tested for their MIC in the BACTEC 460 using two-fold dilutions (0.0625-256 µg/ml) of rifampicin. Susceptibility to PZA was determined by using the specific pyrazinamde kit for BACTEC 460. Briefly, bacteria were inoculated into BACTEC 12B medium and let to reach a growth index (GI) > 300. This was used as the inoculum source for the subsequent PZA test. For each strain, two BACTEC PZA test medium culture vials were inoculated, one drug free control vial supplemented with reconstitution fluid and one vial containing 100 µg/ml PZA (pH 6.0). The test was interpreted when the control vial reached a GI of 200. A strain was considered resistant if the GI of the PZA vial was > 11% of the GI of the control vial and was considered PZA susceptible if the GI was < 9% compared to the GI of the control (Becton Dickinson, Sparks, MD).

2.2.3 Genetic analysis

2.2.3.1 IS6110 RFLP and Spoligotyping

IS6110 RFLP: chromosomal DNA from each *M. tb* strain was genotyped by using a strictly standardized Southern blot hybridization method, based on the infrequent insertion or excision of the IS*6110* element in the *M. tb* chromosome (133). In brief, the DNA was extracted and digested with *Pvu*II. After electrophoresis of the digested DNA on an agarose gel, the 245-bp sequence of IS*6110* was chemiluminescence labelled. The gels were scanned and the results were analysed by computer with the Gelcompar software (Applied Maths, Kortrijk, Belgium).

Spoligotyping: This method generally has a lower discriminatory power than IS6110 RFLP, but spoligityping can separate certain families, such as the Beijing family of *M. tb*, where RFLP is insufficient. Spoligtyping uses primers directed to copy repeats interspersed by 35-41 nucleotide spacer regions of unique sequence. Infrequent variability in the number of spacers is used to discriminate strains (134). The biotinylated PCR products are hybridised to pre-spotted 43 spacer-region membranes (Isogen, Bioscience BV, Utrecht, Netherlands) (54). These tests were performed as part of the routine diagnostics, and thus were not performed by the author of this thesis.

2.2.3.2 Sanger sequencing

Apart from using different primers and annealing temperatures (Table 1), all Sanger sequencing assays in the present investigation were performed in a similar manner. The isolated DNA was amplified using standard PCRs. The PCR products were purified using spin-column kits and subsequently used as templates for the cycle-sequencing reaction. The samples were run in bi-directional reactions using the BigDye^{TM} DNA sequencing kit (Applied biosystems). All products were precipitated according to the Applied Biosystems protocol and analyzed in an ABI prism 3100 genetic analyzer (Applied biosystems). Retrieved sequences were then aligned to a published sequence of either the H37Rv or CDC1551, both being *M.tb* (Table 1). Alignments were done using either the NCBI blast 2 sequence tool (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) or the Vector NTI advance 9 software (InfoMax, Inc. USA).

In paper I, using primers (rrs.PCR.F123 and rrs.PCR.R535) directed to the putative hot spot region for amikacin and kanamycin resistance, all 49 strains were sequenced. A subset of 10 strains was thereafter selected for sequencing of the full *rrs*-gene. In paper II, III and IV the strains were sequenced with the OPRIF-F and OPRIF-R primers. Primer sets pncA_F3 and pncA_R4 were used for the PCR of all strains in paper V. In the subsequent sequencing assay of each strain, all four primers (Table 1) were used in two overlapping bi-directional sequencing reactions.

Table 1. Primers used for PCR, Sanger sequencing and Pyrosequencung

Gene (aligned to)	Primer name	Primer sequence	Ta in PCR	Product (bp)	Paper
rrs CDC 1551 MTr01	rrs.PCR.F123	aagggctgcgatgccgcgag	56°C	432	I
	rrs.PCR.R535	aagtccgagtgttgcctcagg			
	rrs.F133	tggccgtttgttttgtcaggata	55°C	615	I [a]
	rrs.R541	ttccccactgctgcctcccgtag			
	rrs.F490	cggcctgagagggtgtccggcca	58°C	458	I [a]
	rrs.R926	agcgtcagttactgcccagagac			
	rrs.F889	gcgcagatatcaggaggaacacc	56°C	476	I [a]
	rrs.R1343	gagttgaccccggcagtctctca			
	rrs.F1297	gcgcaacccttgtctcatgttgc	55°C	409	I [a]
	rrs.R1684	tacggctaccttgttacgacttc			
rpoB H37Rv Rv0667	OPRIF-F	cgg tcg gcg agc tga tcc	53°C	382	II, III and IV
	OPRIF-R	ttg acc cgc gcg tac acc			
rpoB H37Rv Rv0667	rpoB.PCR.SGS.Forw2	tttcgatcacaccgcagacgtt	63°C	175	III
	rpoB.biotin.Rev2.	[b]ggcacgctcacgtgacagac			
	F1	gcgatcaaggagt			III
	F7	cagccagctgagccaattca			III
	F9	accagaacaacccgctgtcg			III
	F13	tgacccacaagcgccgactg			III
pncA H37Rv Rv2043c	pncA_F3	aaggccgcgatgacacctct	54°C	931	V
	pncA_R4	gtgtcgtagaagcggccgat			
	P3-F	atcagcgactacctggccga			V
	P4-R	gattgccgacgtgtccagac			V

[a] 10 strains were analysed
[b] primer being biotinylated at its 5' end

2.2.3.3 Line Probe Assay

All strains were amplified with the specific enclosed primers to the INNO-LiPA^{TM} Rif. TB kit (Innogenetics, Ghent, Belgium) and handled according to the manual. The essence of LiPA kit is a stringent hybridisation of the biotinylated PCR products to probes on a nitrocellulose strip. After hybridisation, the biotinylated hybrids are detected by a colorimetric method and the strips are interpreted by the patterns received. Apart from a *M. tb*-complex specific probe, the Line probe assay comprises 5 wild-type overlapping hybridisation probes (~20 bp each) covering nearly the whole 81-bp RRDR in *rpoB,* as well as four probes exclusively detecting the four most common mutations conferring RIF- resistance (Figure 2). According to the kit manual, even if none of these four probes hybridize, a strain should still be regarded as resistant whenever at least one of the wild-type probes is not hybridising. In such cases one can only determine that a mutation is present within the region for the hybridization probe (approximately 20 bp) and no discrimination between silent, nonsense and missense mutations can be obtained.

2.2.3.4 Pyrosequencing

A combination of four sequencing primers, F1, F7, F9 and F13 (Table 1 and paper III, figure 1), were designed to detect all mutations in the entire 81-bp RRDR in four overlapping reactions (paper III, figure 1). For the pyrosequencing method, the RRDR was amplified in 180-bp fragments by using primers rpoB.PCR.SGS.Forw2 and rpoB.biotin.Rev2. The biotinylation of the 5' end of the latter primer was used to separate the PCR product into a single-stranded sequencing template. Each strain was analysed at least twice with each sequencing primer and collectively compared with the multiple clustalW alignments tool of the equipment. A strain was considered potentially resistant if a mutation that would alter the amino-acid sequence was present.

2.3 RESULTS AND DISCUSSION

2.3.1 Paper I

Full cross-resistance between the two aminoglycosides kanamycin and amikacin is believed to exist. Consequently only one of these two drugs is normally tested in the clinical laboratory. Despite this, 79 MDR-TB isolates were tested for both amikacin and kanamycin in parallel at the Tartu TB-laboratory in Estonia. Surprisingly, a difference in drug susceptibility patterns was seen among 43 of the isolates. These were characterised in further detail through MIC-determinations and sequencing of the *rrs* gene. Forty isolates were determined as sensitive to AMK (MIC ≤ 4 μg/ml) but low- to intermediate resistant to KAN (8-32 μg/ml). Two isolates failed to grow and one isolate was, in contrast to previous findings, resistant to both drugs. From the 79 originally tested isolates, 3 were highly resistant to both drugs (MICs > 256 μg/ml), and were included as controls, whereas full susceptibility to both drugs (MICs, ≤ 4.0 μg/ml) was seen in the five negative controls. The results from IS*6110* DNA fingerprinting showed a high degree of similarity among the kanamycin resistant but amikacin susceptible (KAN^R/ AMK^S) isolates, this in spite that they were all isolated from different patients.

None of the KAN^R/AMK^S isolates or the double susceptible isolates revealed any mutations in the 1400-1483 region of the *rrs* gene (figure 1). An A1400G substitution was on the other hand seen in all strains being highly resistant to both KAN and AMK. To investigate other possible resistance mutations in other parts of the *rrs* gene, 10 of the 49 isolates were selected in regards to MIC values and streptomycin DST pattern and their entire *rrs* gene was subsequently sequences. Only two of the ten isolates had mutations outside position 1400-1483. These isolates had mutations at position C516T (figure 1) and were resistant to KAN and sensitive to STR, but differed in their sensitivity to AMK.

Previous studies have reported that there is always full cross-resistance between KAN and AMK (2, 44, 93, 116, 144) but not to STR in *M. tb* (47, 77, 130). This observation is still what is stated in the WHO's literature regarding MDR-TB (142). Having studied several KAN^R/ AMK^S isolates, our findings that has also been confirmed in later studies (75), are in-compatible with these reports. It is a danger to make such a general statement, as it will nourish the routine use of cheaper KAN for both DST and treatment, while the better tolerated but more expensive AMK will be left unused if the strain is found resistant to KAN. If an isolate is KAN^R/ AMK^S such practice will lead to the withdrawal of a still active compound (AMK in this case) from a treatment that lacks few, if any, other optional drugs.

Although the majority of KAN-resistant isolates showed MIC's that were only one step higher (8 μg/ml) than the breakpoint (≤ 4.0 μg/ml), more than 1/3 of the isolates had MICs that were 2 or 3 (16-32 μg/ml) steps higher than the breakpoint. This shows that there are differences between isolates' MIC's. The clinical relevance of these findings should also be seen in the light of maximum serum concentrations, which for KAN is 14-29 μg/ml (50). In contrast, none of these isolates grew at any of the tested AMK concentrations on 7H10 agar (MIC at 2-256 μg/ml) or as originally tested, in BACTEC at 1 μg/ml respectively

The nucleotides around amino acids 1400 and 1483 (figure 1) are regarded as a hot spot region for aminoglycocide resistance. In spite of this, we could not find any mutations when sequencing the region for the KAN^R/ AMK^S isolates. The lack of mutations is in agreement with a previous study, analysing low-to intermediately cross-resistant isolates (MIC between 4-32 μg/ml) to AMK and KAN (2). Furthermore, in line with our findings, this study did find mutations in the region among the highly AMK/KAN resistant strains (> 256 μg/ml for both

drugs). Taken together, our data and the findings of three other studies (2, 117, 120), clearly implicate nucleotide substitutions at *rrs* position 1400 as important markers for high-level AMK-KAN resistance but not for low- to intermediate KAN resistance (8-64 μg/ml).

The second mutation we saw, the C516T (figure 1), is normally not associated with KAN resistance in *M.tb*, but instead has been observed in STR resistant isolates (24, 92). The latter would be consistent with the theory that the 530-pseudoknot interacts with S12, which is the most common site for STR resistance. This is however rather cumbersome since both the mutated isolates we identified with this mutation were sensitive to STR. In the reports where C516T has been associated with STR resistance, the sensitivity for KAN is uncertain, since this DST pattern has not been addressed in these report (24, 92). On the other hand, the C516T substitution has been found in two clinical isolates resistant to KAN (MIC 10 μg/ml and 40 μg/ml) and capreomycin (MIC 20 μg/ml and 80 μg/ml) but sensitive to AMK (MIC $\leq$ 4 μg/ml) and viomycin (MIC $\leq$ 10 μg/ml). Unfortunately, this recent study does not report the drug susceptibility for STR (75).

Another possible explanation for our differing results is that the C516T substitution might in fact be a polymorphism that is not associated with drug resistance. When a C491T transition was found in STR sensitive isolates it was proposed that these could be a neutral polymorphism (136), but again no DST pattern was reported for other aminoglycosides. This explanation is again not completely consistent with our findings, seeing that the C491T is not part of the 530-pseudoknot as the C516T substitution is. Further evidence, with full data on the relevant aminoglycosides for tuberculosis treatment, is needed to clarify the mechanism of this mutation. It is important that this question is met: although the use of STR as a first-line drug has been reduced, it is still widely used and most strains will become resistant to this drug before being exposed to the second line drugs AMK or KAN. Thus, only investigating isolates of clinical origin will only rarely show KAN and AMK resistance but STR susceptibility pattern.

In conclusion, an early detection of second line drug resistance could be used for the detection of treatment failure in an already complicated treatment regimen. It is therefore is unfortunate that the current novel genetic methods still fail to detect all clinically relevant levels of drug resistance to KAN and AMK, and only detect high level of resistance to KAN and AMK. If considering our phenotypic results, the DST results indicating ”low-to-intermediate level” resistances to KAN have the same consequences from the clinical point of view, as for isolates with “high-level” resistance, i.e. the drug may be excluded from treatment. In contrast to previous knowledge however, we have shown that full cross-resistance does not exist between KAN and AMK, and has been independently confirmed in a later study (75), thus both drugs should be tested for if both KAN and AMK are being considered for clinical usage.

2.3.2 Paper II and III

An early and correct detection of resistance to antibiotics in *M. tb* gives the opportunity in the initial phase of treatment to optimise the treatment regimen using only effective drugs. For the patient, this is evidently beneficial as the probability for treatment success increases. However, more importantly, a prompt initiation of optimal treatment will reduce the time of infectiousness as well as reduce the risk for the development of resistance to other drugs. This was recently communicated by the head of the UK tuberculosis control program: "The extra cost for such laboratory examinations would lead to substantial savings due to a lower number of patients developing resistant and MDR-TB. There are in other words good reasons to develop and evaluate techniques for laboratory tests that offer prompt detection of resistant *M. tb* isolates" (28).

Conventional TB drug susceptibility testing takes several of weeks to perform. Consequently, we wanted to investigate the possibilities of shortening the turnaround time of such testing by using DNA-based methods. In doing so we chose to concentrate on the first-line agent rifampicin. This drug is not only a key-drug in the treatment of TB, but resistance to this drug is also an indicator of treatment failure.

The hybridsation line probe assay (LiPA) and an in house Pyrosequencing assay were evaluated for the early detection of RIF resistance in 53 *M. tb* clinical isolates. The Bactec 460 and cycle-sequencing were used as phenotypic and genotypic gold standards. All three genetic methods detected wild-type DNA sequences in 24 strains and mutations in the remaining 29. When the genotypic results were compared to the phenotypic reference standard BACTEC, 27 of the 53 strains were found to be resistant to rifampin. Thus, two of the 53 samples were sensitive to RIF although they contained a mutation in the RRDR, as determined by the genotypic methods. Both sequencing methods revealed a $Leu_{511}Pro$ mutation, whereas in LiPA, these mutations were indicated by the lack of hybridisation to the corresponding wild-type probes. These two strains were further analysed and confirmed to be sensitive to RIF showing MICs of 0.5 μg /ml and 0.25 μg /ml, respectively.

One strain with two mutations in the RRDR ($Leu_{511}Pro$ + $Asp_{516}Gly$) was highly resistant with an MIC > 256 μg/ml. The most frequently occurring mutation detected was the $Ser_{531}Leu$, which was seen in 16 of the 27 (59%) RIF-resistant strains. One of the double mutated strains revealed a mutation that altered the amino acid sequence ($Ser_{531}Gln$), while the second mutation was a silent mutation ($GGG_{534}GGC$). Among the 29 strains lacking one or more wild-type hybridisations using LiPA, 21 were specifically determined by hybridising to either one of the resistance probes, whereas 8 isolates were determined to be resistant due to lack of hybridisation to one or more wild-type probes. One sample, although determined to be resistant in all methods, gave a ΔS2, ΔS5, R4a and R4b LiPA pattern. According to the recommendation of the LiPA manual, this should be interpreted as a mixture of strains. The absence of hybridisation to the S2 and the S5 probes was confirmed by both sequencing methods as an $Asp_{516}Tyr$ and an $Ala_{532}Val$ mutation respectively, but no mutation was found to ratify the hybridisation to the R4a and R4b probes. Lastly, all 53 strains were correctly identified to belong to the MTB-complex by the specific probes in LiPA.

The simplicity of LiPA speaks positively for such a system, as it only requires a PCR and a shaking water bath, which are affordable for most national TB reference laboratories in the world. However, each individual test is rather expensive. The Pyrosequencing apparatus, on the other hand, is expensive to purchase, but the cost per analysed well is fairly low. Thus,

neither of these two systems is suitable to be implemented at large scale in high incidence but low-income countries. In such settings, not even the broth-based DST methods used in Europe and the USA are feasible. Instead, these DNA based methods are intended to be used in mid- to high-income countries.

Despite one sample being wrongly shown to be a mixture of samples, LiPA was easy to run and read. Although correctly indicating resistance for this sample, such LiPA results are highly unlikely since the three patterns S4, R4a and R4b are mutually exclusive. If there had been a mixture of strains, all three strains should also have the other two additional mutations (ΔS2 and ΔS5 respectively). The hybridisation to the R4a and the R4b probes might be incorrect due to secondary structures in the PCR product, caused by the $Ala_{532}Val$ mutation (personal communication with Innogenetics).

The finding of Leu511Pro mutations in sensitive strains using LiPA exemplifies a disadvantage in using indirect mutation detection methods in comparison to direct methods such as sequencing. These types of mutations cannot be discriminated from other mutations affecting the same detection probe in LiPA, and would thereby be defined as resistant with this method. Furthermore, infrequent reports of silent mutations (Leu521Leu) and of mutations changing the amino-acid chain (Met515Val, Leu521Pro, Leu533Pro) but remaining RIF sensitivity do occur (119, 147), and are thus all at risk for being determined as resistant using LiPA.

Although posing a potential problem, a similar hybridisation system, PZA-LiPA, has been developed in house for PZA resistance detection (106). The wide variety of *pncA* mutations makes it impossible to include the specific detection of resistance mutations (figure 2). Instead this assay only detects mutations by the lack of hybridisation to wild type probes. It should be noted that the authors considered, at the time for their investigation, the two known silent mutations (G60G and S65S) that were detected by two additional specific probes (106). By the time their work was submitted, another study was published, which showed a novel silent mutation (A20A) in a PZA-sensitive strain (112). Among our PZA-sensitive strains included in Paper V, we found a G-insertion between –2▼–3. These two mentioned substitutions would in theory inhibit hybridisation to their corresponding wild-type probes using the PZA-LiPA, and would thereby be interpreted as PZA-resistant. As the majority of strains in *pncA* gene sequencing studies are resistant to PZA, new silent mutations are most likely to be found, as more PZA-sensitive strains will be scrutunised in future studies. Thus, an assay like this needs to be constantly updated, but more importantly, it leaves the interpreter of the strip in uncertainty as to whether a lack of hybridisation to the wild type probes is indeed due to a mutation giving PZA-resistance or not. Based on these observations, when using an indirect method as LiPA for RIF-resistance determination, the results should be confirmed through sequencing, especially in the case of novel mutations.

Sanger sequencing has frequently been used as a gold standard for mutation detection, but for large-scale routine diagnostics, this method is considered too costly and time consuming (123). Thus, our aim was to continue with Pyrosequencing to create a robust method with the same resolution as Sanger-sequencing but that is easier to handle and thus more suitable for screening high numbers of samples. Pyrosequencing does not require the same amount of maintenance as the other sequencing system. A major drawback however, is the shorter sequences retrieved, which have to be compensated for by overlapping reactions (paper III, figure 1). Considering the sequencing primers were designed to hybridise within the variable RRDR, we found the assay to be robust. In seven different sequencing reactions the primers

did anneal despite SNPs found being found at the annealing sites for these primers, none of which affected the sequence quality. On the other hand, a three-nucleotide deletion at the annealing site for the F9 primer did negatively affect the sequencing reaction for this primer. Although not satisfactorily sequenced by the F9 primer, this sample was regarded as resistant since the AAC_{518} deletion was detected by sequencing with the F7 primer (Table 1)

In conclusion, LiPA and Pyrosequencing are fast methods and can be performed within in a single day. Having found mutations that do not confer resistance, which lowers the specificity for LiPA, we suggest that between the two methods tested the pyrosequencing assay is the most suitable method for early detection of RIF resistance in TB. A further optimisation of the methods' throughput time, would be to apply these methods directly on clinical specimens and thus omit the lengthy bacterial isolation by culture.

2.3.3 Paper IV

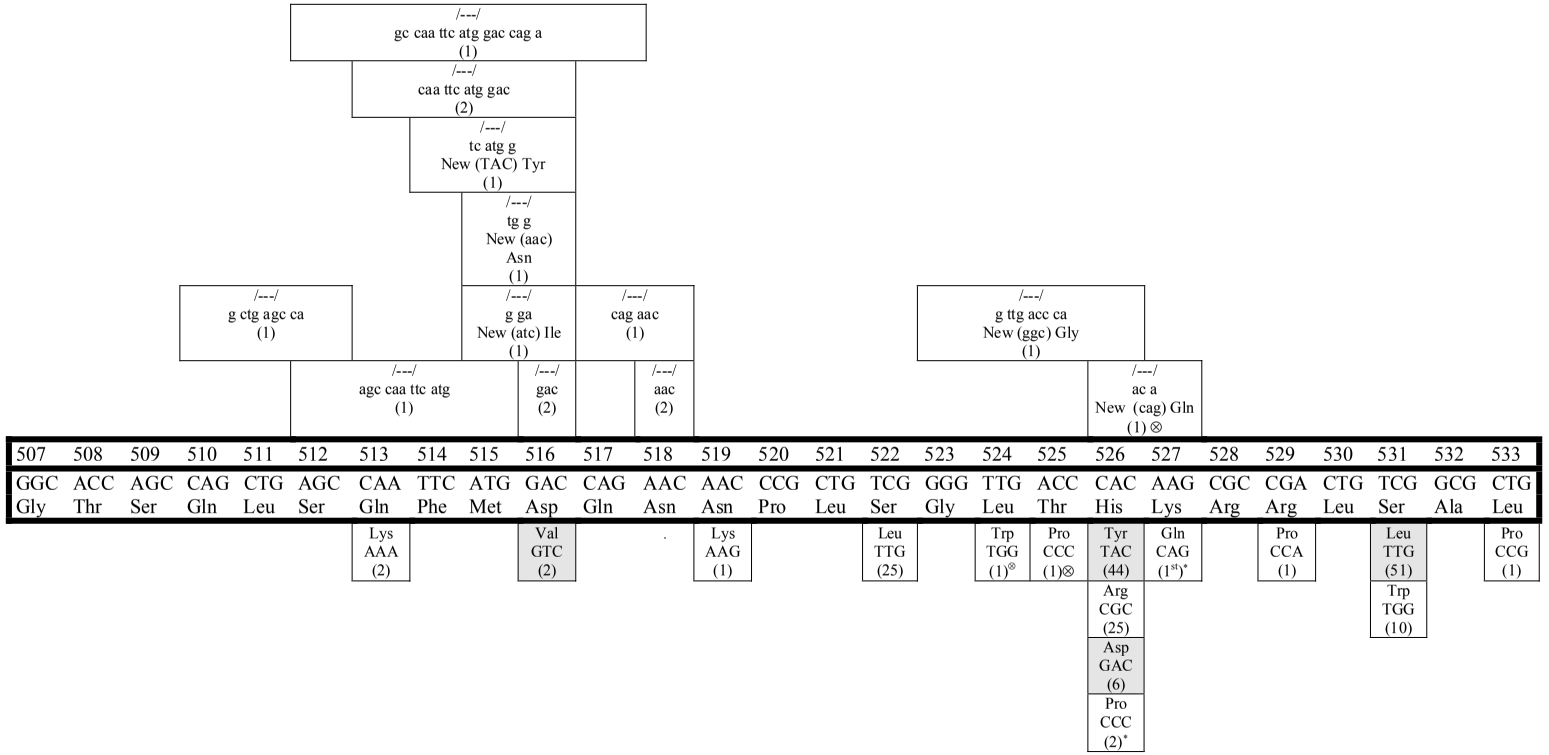
In this study we further investigated the mechanisms of rifampicin resistance and mutations in the *rpoB* gene. We wanted to determine whether RIF resistance-levels and *rpoB* mutations are correlated and further, if these parameters could be strain dependent, especially for strains of the Beijing family. Strains belonging to the *M. tb* Beijing family have been associated with major outbreaks and multi-drug resistance. We characterised 189 independently occurring, *in vitro*-selected, RIF resistant isolates that were previously isolated in a study showing that Beijing strains do not differ from non-Beijing strains with regard to their RIF-resistance mutation rate (135). In paper IV, these mutants were further characterised in regards of their level of RIF resistance and corresponding mutations in the *rpoB* gene.

In general, the mutants were highly resistant to RIF and only 16 of the 189 strains had an MIC ≤ 16 μg/ml. Sequencing of the RIF resistance-determining region showed alterations in all but one mutant. In total, 25 different RRDR alterations were seen (figure 3). The most common mutations were single nucleotide substitutions (172/189), whereas deletions occurred more seldom (14/189). The three most common single nucleotide substitution sites were codons 526, 531 and 522 (40%, 34% and 13% respectively). These three codons were the most commonly mutated in both Beijing and non-Beijing mutants. Also, no difference in resistance level was seen between these two groups of mutants. None of the 9 parent strains, from which the 189 mutants were selected, were resistant to RIF (MIC ≤ 1 μg/ml) nor did they have alteration in the RRDR.

As mentioned, the majority of the 189 mutants were found to have mutations at position 526 (41%) and 531 (32%). Mutations in these two codons are the most commonly affected in clinical isolates, representing more than 70% of RIF-resistant clinical isolates exhibiting an altered *rpoB* gene (figure 3). Overall, the range of mutations seen in this study resembles what is seen among clinical isolates (92). However, compared to previous studies in which RIF-resistant mutants have been *in vitro*-selected and characterized, the range of mutations we observed was slightly different (14, 70, 84). In one study, in which mutants were selected for from the H37Rv laboratory strain, only three different mutation sites were observed among a total of 153 mutants (14). These were His526Tyr, Ser531Leu and His526Arg respectively, and all substitutions were found to a similar extent. Morlock *et al*. reported 10 different mutations in 64 *in vitro*-selected RIF-resistant mutants, with an over representation of the Ser531Leu substitution (84). Again the H37Rv strain was used for the mutant selection. The fact that these two studies were based on mutant selection from the H37RV reference strain only, could account for the small range of mutation sites seen. In our study, apart from this strain, 8 drug-susceptible clinical isolates were used for the mutant selection, thus forming a better mimic of the clinical situation.

Lastly, using the Harlingen clinical isolate, *Mariam et al*. identified 3 different mutations among 27 *in vitro*-selected RIF-resistant *M. tb* mutants. In this study, none of the tested strains had the Ser531Leu mutation and a surprisingly high number of mutants (10/27) harboured no mutation in the RRDR (70). It should be noted that the one RIF-resistant mutant in our study that exhibited a wt RRDR and that was highly RIF-resistant, was also of Harlingen lineage.

Figure 3. Mutations in the *rpoB* gene for 188 *in-vitro* selected RIF resistant *M. tuberculosis* mutants. The four most common mutations found among clinical isolates are highlighted in grey.



⊗ Part of double mutation with a deletion also
* One of which is a part of a Double mutation
/---/ Deletion

Common for our study and two of these studies is the elevated prevalence of the Ser522Leu substitution (70, 84). In clinical isolates this mutation is rare, representing as little as 1-5% of the RRDR mutated RIF-resistant isolates (69, 92, 146). Among our mutants, this mutation site was correlated with varied MIC levels, ranging between 8-256 μg/ml, and with a median MIC of 32 μg/ml. The same variability in RIF-resistance has been noted among clinical isolates with the same mutation, but more importantly this mutation seems to be correlated with rifabutin susceptibility (137, 148). This has been confirmed in vitro by first selecting for RIF-resistance, to subsequently use these RIF mutants for a second selection for RFB-resistance (6). In the first selection, mutants were found to only have the Ser522Leu substitution, being RIF resistant but sensitive to RFB. After the second round of selection, these mutants were resistant to both drugs, and besides the Ser522Leu substitution had acquired different additional mutations. Several of these additional mutations were identified some 1000 nucleotides upstream position 522 and thus would not have been detected by the commonly used RRDR sequencing assays (6). Together with being sensitive to RFB, the combination of being associated to low RIF resistance and having a lower relative fitness (6, 70, 137, 148) may explain why this mutation is infrequently found in clinical isolates.

Mutations in codon 516 would be expected to be more frequent among our mutants (1.1%), as this mutation site is found in 7-16% of RIF -resistant clinical isolates (15, 19, 40, 69, 92, 114, 146). Mutations in this codon have also been associated with the phenotypic RIF-resistant but RFB-sensitive pattern (110, 137). From RNA polymerase structure studies, this site has been postulated to be part of an allosteric pathway. According to this theory, mutations at this codon would probably not inhibit the binding of RIF, but rather inhibit rifampicin's abortive signal on the RNA synthesis (9). As shown by Toungoussova *et al.*, this particular mutation seems to only pose a slightly reduced growth rate among clinical isolates and thus might only cause a small biological disadvantage to the bacteria. This would explain its higher frequency among clinical isolates (126).

All deletions were found to be in frame, although several of these were partially inside two codons, rendering a new amino acid besides the deletion. On the other hand, several deletions were identified (7.4%). among which several were surprisingly large. Considering the essentiality of the *rpoB* gene for the individual bacteria, and that it is a highly conserved sequence among all bacterial species, large deletions would pose a potential deleterious effect. Analogous with this, deletions are rarely found among clinical isolates, especially of the large category (15, 19, 40, 69, 92, 146). Thus, it is likely that such large deletions would be negatively selected had the bacteria been in its natural host environment.

We could not see any general difference in mutations among Beijing and non-Beijing mutants. As this lineage has been associated with outbreaks and a high frequency of multi-drug resistance (13, 62, 127), it has been postulated that these strains have an elevated mutation rate. However, in a previous study, it was shown that Beijing strains do not have an elevated RIF-resistance mutation rate *in vitro* (135). In Paper IV we could confirm that the generated Beijing mutants did not differ from the non-Beijing mutants with regards to RIF-resistance levels and *rpoB* mutations. In addition, we could not see any major difference in the mutations we found in the *in vitro*-selected material when compared to the mutations found among clinical isolates.

2.3.4 Paper V

The phenotypic drug-susceptible tests for the first-line agent pyrazinamide are cumbersome to use and frequently reported to be difficult to reproduce (7, 41, 43, 63, 150). Thus, a potential sequencing assay would not only circumvent the obstacles associated with the phenotypic methods but also to provide a tool with a shorter turnaround. Thus, we investigated the use of a sequencing assay directed to the *pncA* gene for the detection of pyrazinamide resistance and compared these results to a phenotypic PZA method. Using the standard protocol for the BACTEC 460 PZA kit, we found that 34 clinical isolates were resistant and 36 susceptible to PZA at 100 μg/ml. These isolates were further analysed for mutations in the *pncA* gene and its putative promotor. As controls, the PZA susceptible H37Rv strain was confirmed to be sensitive and without mutations. In contrast, the *M. bovis* had Ala39Ala and His57Asp substitutions whereas the *M. bovis* BCG only had the His57Asp substitution. Both strains were inluceded as intrinsically resistant controls and were also confirmed to be resistant by the BACTEC system.

Thirty-three of the 36 PZA sensitive strains lacked any mutation in the *pncA* gene, whereas one isolate was shown to contain a silent mutation (Ser65Ser) and two isolates were respectively found to have a unique insertion upstream of the gene. Furthermore, one strain was repeatedly determined to be intermediate resistant but showing a wild type *pncA* gene. On the other hand, only one of the 34 PZA-resistant strains was found to lack a mutation in the region investigated. Apart from one strain having an A to G substitution 11 nucleotides upstream the gene, all resistant isolates had mutations inside the *pncA*. Single nucleotide substitutions were found in 30 of the resistant strains whereas insertions and deletions were only found in a few strains.

Apart from RIF and INH, the WHO recommends the usage of PZA and ethambutol or streptomycin as first line agents for to the treatment of TB (145). Still, the most recent WHO publication on global drug resistance does not cover PZA resistance (138). Scrutinising the literature, surprisingly few studies on PZA-resistance prevalence are available. In one study, representing more than half of the German TB patients in the period 1996-2000, with an annual recording of 2720-3356 culture-confirmed cases, PZA resistance was present in approximately 1.5-2 % of the cases, whereas the rates for resistance to the other four first line agents were slightly lower but in line with the global report (67, 141). In the United Kingdom for the period 1993-1999, Djuretic *et al.* reported the DST patterns for 20947 *M. tb* isolates (i.e. excluding *M. bovis*) and found that among combined cases, 1.1 % were resistant to PZA, irrespectable of their resistance to other drugs (23). Apart from a somewhat elevated INH-resistance, the prevalence of drug resistance to the other four first line drugs in this study correlated well to what is found in the global population (141). Although not commented by the authors, a remarkably high number of PZA-DST (16%) were excluded due to failed testing (23).

Apart from a silent mutation, we did not find any mutations within the *pncA* of the sensitive strains. In comparison to the *rpoB* gene, silent mutations in *pncA* seem to be present more frequently but still few are reported (figure 2). At present it cannot be stated whether this is dependent on the relatively few sequenced PZA sensitive isolates or if the actual silent mutations in *pncA* are present at this frequencies. Furthermore, among the PZA sensitive strains we found two different strains with one-nucleotide insertions, three and 33 nucleotides upstream the gene. The nature of these mutations is unclear, as alterations in this region may not only

affect mRNA expression but also protein translation, a process that is not yet fully understood for *M. tb*. In addition, strains with the specific Thr47Ala mutation but that are at same time phenotypically sensitive to PZA, have been isolated. This phenomenon has been argued to correlate to certain strains such as those of the Beijing family (115). It has been further reasoned that these strains show a higher MIC but are still in the range of being susceptible strains (25, 112). However, although three Beijing isolates with the (Thr47Ala) were identified in our study, we could not confirm the above statements as all three strains were PZA resistant.

The substitution 11 nucleotides upstream of the *pncA* gene we found in a resistant strain probably hampers the protein expression and has been seen to be present in PZA resistant strains in several other studies (8, 11, 20, 73, 78, 90, 115).

The lack of mutations in the *pncA* gene and its upstream region, in PZA-resistant strains seems to be a general rule for a minor subset of the isolates, and has been reported in the majority of the previous studies (11, 20, 49, 65, 66, 73, 78, 90, 104, 112, 115, 128). Scorpio *et al.* found one such strain conferring low-level resistance (MIC 200-300 μg/ml in BACTEC). Interestingly, the strain was still responding to PZA treatment in mice at a dose comparable to what is used for humans, indicating that the borderline resistance may not have a clinical significance (58, 104). Subsequently, it has been argued that the drug concentration in BACTEC (100 μg/ml) is too low for the pH (6.0) and should be increased two to three times (44, 150).

In comparison to other drug resistance genes, it seems that the high diversity of *pncA* mutations (figure 3) is unique to PZA resistance (132). Although the basis for this high diversity is unclear, it is likely that since the *pncA* gene is not an essential gene for in vitro growth or spread of TB, there is no selective pressure for specific mutations and mutations anywhere in the *pncA* are well tolerated (20, 102).

Although far from explaining all unidentified cases, the lack of full concordance between sequencing and phenotypic DST can sometimes be explained by the fact that the sequencing design either, does not cover the complete *pncA* gene and its putative promotor (68, 96), or it does not contain overlapping sequencing reactions (94). However, the inconsistency between phenotypic and genotypic methods are commonly reduced by retesting, typically being beneficial for the original genotypic results, i.e. phenotypic assays are often the defaulting part (87, 104). Still, independent of the phenotypic methods used, sequencing of the *pncA* gene and its putative promotor has shown (72-99.9%) to be a reliable predictor for the detection of pyrazinamide resistance (112, 153).

In conclusion, with *pncA* sequencing we were able to identify mutations in all but one resistant strain. In addition, among the susceptible strains, only silent mutations if any were found. Thus, *pncA* sequencing provides an attractive complement to the phenotypic test for pyrazinamide resistance determinations.

3 CONCLUDING REMARKS

With the aim of improving existing *Mycobacterium tuberculosis* drug susceptibility tests (DST), we characterised the genetic resistance mechanisms for three important drugs classes. We further investigated the potential of our and others findings for the implementation of DNA-based methods in the detection of resistance in *M. tb*.

The three different drugs investigated in the present study represent different potentials and problems in the development of resistance diagnostics of *M. tb*.

(I) Rifampicin; DST to this drug generally causes no problems when tested for in the laboratory, and quality assurance evaluations generally show little if any disagreement between different laboratories. Resistance mutations in a short region of *rpoB,* as well as the low prevalence of mutations in sensitive strains, are well correlated to phenotypic drug susceptibility patterns.

(II) Amikacin and Kanamycin; Phenotypic drug susceptibility tests do not usually cause any problems when testing for these two drugs. Only for highly resistant strains are resistance mutations well correlated to mutations in the *rrs* gene. Low- to intermediate resistant strains do not have a sufficiently powerful genetic marker and could thus in our studies not be discriminated from sensitive strains. Also, we could not identify a common resistance marker for the amikacin susceptible but kanamycin resistant strains.

(III) Pyrazinamide; Ever since this drug began to be used for TB treatment the phenotypic methods of DST have been questioned and revised. Unfortunately this is still true today, with a great deal of difficulties in internal- and external-laboratory reproducibility. Mutations in the *pncA* gene usually show good correlation with the resistant phenotype, as well as seldomly being mutated among the susceptible strains. This is especially true when several different phenotypic assays are used in parallel and the original phenotypic data is corrected after retesting.

In conflict to what is previously reported, we have shown that the closely related drugs, KAN and AMK, do not share full cross-resistance. Instead, strains with a low-to intermediate KAN resistance may still be sensitive to AMK. Though these two drugs could be equivalently regarded for the treatment of MDR-TB, only one is typically tested in routine DST. According to our results however, the specific drug used in treatment should be specifically tested. Unfortunately, we could not identify a specific genetic marker for this KAN^R/ AMK^S phenotype, and thus until further genetic insights are generated, the DST should be performed by culture.

The three genes scrutinised in the present study (*rpoB, rrs* and *pncA*) are not equally essential for bacterial function. The two first, *rpoB* and *rrs*, represent genes that are crucial for the bacteria and would lead to cell death if fully deleted. The mutations found in these genes are strictly limited to certain hot spot regions, probably altering the drug interacting site and not considerably affecting the function of the gene product itself. Mutations may impair the proteins' function, rendering the bacteria less fit, but a functional RNA polymerase and ribosome respectively, will be maintained. Deviations from the tightly regulated areas of mutation were not seen, in spite using *in-vitro* generated mutants. Although the selective

pressure of host responses is avoided in *in vitro*-selection, these mutants were found to be highly RIF -resistant as well as containing mutations that are readily found in clinical isolates.

Mutations in the *pncA* gene on the other hand, do not obey this selective pressure for a specific region of the gene, seeing that PZA-resistant bacteria with a non-functional *pncA* gene are still infectious. As resistance is dependent on a non-functional *pncA*, the gene only needs to be silenced and an astonishingly high variety of mutations are thus found.

The development of a DNA based drug resistance method would give the possibility to drastically reduce the turnaround time of DST, especially if the method could be used directly on sputum. Two similar commercial kits are available today, these both being based on the hybridisation to probes representing both wild type DNA as well as a few, but relevant resistance mutations. With one of these kits, the Line probe assay, we have shown that the discriminatory power for RIF-resistance determination is not as good as would be expected. We subsequently hypothesised that by using a sequencing assay this issue could be circumvented. The Pyrosequencing method has beneficial features over the predominantly used Sanger sequencing in being more easily handled, as well as having a shorter turnaround time. We successfully designed an assay based on Pyrosequencing technology that was highly discriminatory for the detection of strains resistant, as well as sensitive, to RIF.

Pyrosequencing renders short sequences, and are thus a proper method for analysing mutations within a close range of a gene, as is the case for RIF-resistance. The method is however not suitable for sequencing whole genes, and thus not appropriate for the analysis of PZA-resistance. Instead, the *pncA* gene should be analysed by a method capable of analysing several hundred nucleotides in a single run. Although infrequent, new silent mutations are found in *pncA*, and thus a hybridisation method is not to be recommended. Instead, Sanger sequencing would be the method of choice.

The introduction of PCR analysis has raised the hopes for a shorter turnaround of *M. tb* drug susceptibility testing, especially if it could be implemented as a direct test. At present unfortunately, this is not possible for the combination of Amikacin and Kanamycin or for each of the drugs separately. On the other hand, we have shown through the use two sequencing assays, that a reasonably high discriminatory power for separating drug resistant from sensitive strains can be developed for both rifampicin and pyrazinamide, respectively.

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