“Whiskey is for drinking and water is for fighting over”
Mark Twain

Department of Microbiology, Tumour and Cellbiology (MTC)
Karolinska Institutet, Stockholm and Swedish Institute for Infectious Disease Control, Solna Sweden

Rapid assessment of drug susceptibility and mutation to resistance in *Mycobacterium tuberculosis* Beijing type

Jim Werngren

Karolinska Institutet

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ABSTRACT

The increasing rates of multidrug resistant tuberculosis (MDR-TB) worldwide, are seriously threatening the TB control efforts. The present thesis was aimed at increasing the knowledge about the role of different factors, such as the development of drug resistance and the efficacy of drug susceptibility assessments, in the problem with MDR-TB. We showed that the two novel commercial techniques, BacT/ALERT (bioMérieux) and INNO-LiPA Rif TB (Innogenetics), are sensitive and reliable methods for the determination of drug susceptibility in *Mycobacterium tuberculosis* (TB). Two different test protocols of the culture-based BacT/ALERT system were evaluated at separate times in our laboratory. After the first test episode (2002-2003), we concluded this system to be applicable for isolation of *M. tuberculosis* but that further optimization was needed for the assessment of drug susceptibility, since the system had too low sensitivity in detecting resistance to rifampicin and streptomycin. The results from the second test episode (2005-2006), using a novel protocol, showed improvement of detecting drug resistance to the three first line drugs; rifampicin, isoniazid and ethambutol. Apart from the high accuracy in determining drug resistance, advantages like the automated standardization of inoculum, recording and saving of data make this system a valid alternative for drug susceptibility testing of *M. tuberculosis*. The LiPA detects resistance to rifampicin by the identification of mutations in the rifampicin resistance-determining region of the *rpoB* gene. We evaluated this assay since a rapid detection of rifampicin resistance, and indirectly multi- drug resistance (MDR), is of great importance to ensure an effective treatment that will break the chain of transmission and prevent the development of drug resistance. When using the BACTEC 460 methodology as the reference, the LiPA correctly determined all 27 rifampicin resistant strains and 24 of the 26 susceptible strains. The two aberrant samples were both determined to harbour a $Leu_{511}Pro$ mutation, thus confirming LiPA's correctness in finding mutations. MIC determination did, however, reveal susceptibility to rifampicin of the two strains. Due to LiPA's rapidity, easiness and high accuracy, it is a valuable tool that offers an early warning system for MDR-TB. The detection and identification of *M. tuberculosis* strains with increased potential to develop and spread drug resistance is important to notice, since such clones challenge the control and treatment regimens of TB. During the last decade attention has been paid to the *M. tuberculosis* Beijing genotype in particular. Strains belonging to the Beijing genotype have been responsible for massive spread and outbreaks of MDR-TB, especially in the regions of the Former Soviet Union. Suggestions that these strains easier gain drug resistance and/ or are more virulent have been made. We determined the rate at which 13 *M. tuberculosis* strains (including Beijing) mutated to rifampicin resistance. All strains, irrespective the genotype, showed a mutation rate of $\sim 1 \times 10^{-8}$ mutations/cell/generation. Furthermore, we characterized the type and frequency of *rpoB* mutations in 189 rifampicin resistant mutants selected *in vitro*. Eighty-nine mutants were of the Beijing genotype. In summary, the $Ser_{531}Leu$ and $His_{526}Tyr$ mutations were predominant in both groups of genotypes, which also mirror the distribution in clinically rifampicin resistant strains. Interestingly, the only multiple mutations we selected, were exclusively present in the Beijing mutants. From this work new knowledge about rifampicin resistance in Beijing strains has been achieved on the genomic level.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Ängeby K, **Werngren J**, Toro JC, Hedström G, Petrini B and Hoffner SE. Evaluation of the BacT/ALERT 3D System for Recovery and Drug Susceptibility Testing of *Mycobacterium tuberculosis*. Clinical Microbiology and Infection, 2003; 9:1148-1152.

II. **Werngren J**, Klintz L and Hoffner SE. Evaluation of a Novel Kit for Use with the BacT/ALERT 3D System for Drug Susceptibility Testing of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology, 2006; 44:2130-2132.

III. Juréen P, **Werngren J** and Hoffner SE. Evaluation of the Line Probe Assay (LiPA) for Rapid Detection of Rifampicin Resistance in *Mycobacterium tuberculosis*. Tuberculosis, 2004; 84:311-316.

IV. **Werngren J** and Hoffner SE. Drug-Susceptible *Mycobacterium tuberculosis* Beijing Genotype Does Not Develop Mutation-Conferred Resistance to Rifampin at an Elevated Rate. Journal of Clinical Microbiology, 2003; 41:1520-1524.

V. Huitric E, **Werngren J**, Juréen P and Hoffner SE. Resistance Levels and *rpoB* Gene Mutations among *In Vitro*-Selected Rifampin-Resistant *Mycobacterium tuberculosis* Mutants. Antimicrobial Agents and Chemotherapy, 2006; 50:2860-2862.

CONTENTS

LIST OF ABBREVIATIONS

BCG	Bacille Calmette Guérin
CFU	Colony Forming Unit
DST	Drug Susceptibility Testing
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
rpoB	RNA Polymerase B
RRDR	Rifampicin Resistance-Determining Region
SLD	Second Line Drug
XDR	Extensively Drug Resistance
TTD	Time To Detection

1 INTRODUCTION

The tuberculosis (TB) incidence declined steadily during the 1900s in the developed countries in Europe and North America, mainly due to improved socioeconomic conditions, isolation of contagious individuals in sanatoria, pasteurization of milk, BCG vaccination programs and the introduction of effective anti-tuberculosis drugs (95). Although less historical data are available from the so-called developing countries, the incidence in these areas has been assumed to decline as the public welfare has increased and become increasingly accessible for poor people. Unfortunately the positive progression was drastically interrupted by the onset of the HIV (human immunodeficiency virus) pandemic, the breakdown of some national TB control programs and the emergence of multidrug-resistance (MDR), and in the 1993 WHO (World Health Organization) declared tuberculosis a global emergency. The WHO estimates that about one third of the world's population is infected with TB. Globally, there are eight million new cases and two million deaths from TB each year. The increase in world population is a main factor for the rising number of TB cases. The population growth is especially rapid in areas where TB is most prevalent, particularly middle Africa and South Asia. Secondly, the increase of HIV, which renders the host uniquely susceptible to TB, is occurring in the same areas of the world and is already causing an up to tenfold increase in tuberculosis case rates (22).

1.1 THE EMERGENCE OF DRUG RESISTANT TUBERCULOSIS

TB control efforts are today seriously threatened by the widespread occurrence of drug resistant strains. A particularly dangerous form of drug resistant TB is multidrug-resistant TB (MDR-TB), which is defined as resistance to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. *M. tuberculosis* strains resistant to anti-TB drugs were found in all 77 geographic sites studied by the WHO between 1999-2002. The median prevalence of MDR among new cases of TB was 1%, but the prevalence in Israel and Kazakhstan was as high as 14%. Other areas with a high prevalence of MDR among new cases were Estonia (12%), Lithuania and Latvia (9%) and Tomsk Oblasts (14%), in the Russian Federation, and Liaoning Province (10%) in China (107). A major problem was also identified in the Russian prison population, where 10% of one million prisoners had active TB (40, 110). Portaels *et al*. (84) reported a rate of MDR-TB of 24.6 % among prisoners in Baku, Azarbaijan, and Mariinsk, Siberia. Kimerling *et al*. found that 75% of new TB cases among prisoners in a Siberian prison hospital, were drug resistant, with ~40% MDR-TB (47).

Of special concern are the high rates of MDR-TB in the two most populous countries of the world, China and India, which account for 40% of all TB cases worldwide (28).

MDR-TB treatment requires the use of second-line drugs (SLDs) that are less effective, more toxic, and costlier than first-line isoniazid- and rifampicin-based regimens.

This year the Center for Disease Control and Prevention, Atlanta (CDC) and the WHO surveyed an international

network of 25 reference TB laboratories on six continents to assess the worldwide frequency and distribution of extensively drug-resistant (XDR) TB cases, i.e. MDR strains that also have resistance to one fluoroquinolone and one injectable drug such as amikacin or kanamacin. The survey revealed that, during 2000-2004, of 17,690 TB isolates, 20% were MDR and 2% were XDR. XDR-TB has emerged worldwide as a threat to public health and TB control, raising concerns of a future epidemic of virtually untreatable TB (15).

1.1.1 Anti-tuberculosis drugs and treatment regimen

In 1944, the first anti-TB drug, streptomycin (SM), was discovered in the USA and brought in to clinical use soon after. Despite the apparent high efficacy of SM, the monotherapeutic treatment regimen many times ended up with relapses due to the development of drug resistance (71). In Sweden scientists were also working on a cure for TB and developed para-aminosalycilic acid (PAS), which was brought into use in the late 1940s. By combining SM and PAS in the regimen the emergence of drug resistant bacteria was largely prevented and cure became the norm. The optimum length of treatment for pulmonary TB using SM and PAS was two years (66).

The powerful anti-TB activity of isoniazid (INH) was discovered in 1951 but still the development of resistant mutants was problematic (67). By adding this drug to PAS and SM therapy the cure rate amongst the patients became 90-95%. Two years of continual treatment was still required due to the presence of live bacilli in tissues even after sputum samples turned negative (43). Over the next decade further anti-TB drugs were discovered which could be added to the treatment regimen. Examples of these are pyrazinamide (PZA) and ethambutol (EMB), which are key drugs in the modern standard treatment. Yet others such as ethionamide and cycloserine, both with poor bacterial killing ability and troublesome side effects, are useful as reserve drugs for patients who have developed bacteria resistant to the better drugs.
In 1967, a new and perhaps the most important drug in the treatment of TB was discovered: Rifampicin (RIF). The introduction of this drug in the 1970s was an important landmark since a cure rate of >95% could be achieved after only nine months of combination therapy (17, 18). Today, the WHO recommends the following standard treatment regimen based on the first line drugs: two months of daily treatment with RIF, INH, PZA with or without SM or EMB followed by a continuation phase of 4 months during which RIF and INH are given three times a week. This short course treatment regimen is conveniently abbreviated to 2HRZ/4HR.
Unfortunately the development of the drug treatment of TB has also been the catalyst for the emergence of a new wave of drug resistance. Patients have been allowed to take their medication at home completely unsupervised. There is a risk that a patient, who has been sent home with three separate drugs, might take a single drug at a time or, for various reasons, simply does not follow or complete the strict treatment regimen. In this way a combination of poor compliance, poor medical supervision and also the use of drugs with low quality may result in multidrug resistance.

1.1.2 Genetics of drug resistance in *M. tuberculosis*

Drug resistance has been observed for all five of the first-line anti-TB drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), streptomycin (SM) and ethambutol (EMB) and for the second-line drugs. These observations have lead to a great deal of research interest to understand the molecular mechanisms of drug resistance in *M. tuberculosis*.

The first molecular study on drug resistance mechanisms in *M. tuberculosis* was on the mechanism of INH resistance (112). Subsequent studies have described the resistance mechanisms for other major anti-TB drugs such as RIF (97), SM (29, 41, 75), PZA (92) and EMB (6, 98). The genetics of the drug resistance is described below for the major important anti-TB drugs INH, RIF, PZA, SM, EMB and Fluoroquinolones.

Isoniazid

The molecular basis for INH resistance in TB is complex. The ***katG***, ***ahpC***, ***inhA***, and ***kasA*** genes have been associated with INH resistance.
INH is a pro-drug that requires activation by the *M. tuberculosis* catalase-peroxidase enzyme, encoded by the ***katG***, to become active (5, 112). Beside that ***katG*** activates INH, it is also considered to be important for protection against reactive oxygen and nitrogen intermediates produced by phagocytic cells (64). Mutations in the ***katG*** reduce the production of catalase-peroxidase and INH is activated only to a small degree.
M. tuberculosis may compensate for ***katG*** mutations by overexpressing the ***ahpC*** gene (which also is important for protection against oxidative stress) coding for alkylhydroperoxidase,

which can not activate INH. Mutations in ***ahpC*** are associated with INH resistance (5, 39).
Activated INH inhibits an enzyme, encoded by the ***inhA*** gene that is involved in the synthesis of mycolic acids. Mutations in ***inhA*** can modify the target (enzyme) and thus confer resistance to INH (89).
Mutations in the ***kasA***, involved in the biosynthetic pathway of mycolic acids, have also been described in INH resistant strains (55).

Rifampicin

RIF acts by binding to the beta subunit of the bacterial RNA polymerase, thereby inhibiting the synthesis of RNA, which thus cannot be transcribed from DNA (65). Resistance to RIF is almost exclusively due to mutations in a specific region of the ***rpoB*** gene, which codes for the RNA polymerase (97).

Pyrazinamide

The mechanism of action of PZA is yet not fully understood. PZA is a pro-drug that requires activation to its active form, pyrazinoic acid, by the enzyme pyrazinamidase (PZase) encoded by the ***pncA*** gene. Resistance to PZA occurs often together with mutations in a ***pncA***, which results in a loss or reduction of PZase (113).

Streptomycin

SM inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome, which will misread the mRNA message during translation and thereby perturbing protein synthesis. Resistance to the aminoglycoside SM is typically through mutations in ***rpsL*** and ***rrs***

genes coding for two SM targets sites on the ribosome (21, 34).

Ethambutol

EMB inhibits the synthesis of arabinogalactan, a major cell wall component of mycobacteria. The target of EMB is various arabinosyl transferases, coded by ***EmbA, B and C***, which are enzymes necessary for cell wall synthesis. Mutations in the embCAB operon are associated with EMB resistance (88, 96).

Fluoroquinolones

This group of antibiotics binds to DNA gyrase, which is responsible for DNA supercoiling. The DNA gyrase consists of two A and two B subunits encoded by ***gyrA*** and ***gyrB***. In *M. tuberculosis*, mutations in mainly ***gyrA*** are related to resistance to various fluoroquinolones such as ciprofoxacin, ofloxacin, mofloxacin and sparfloxacin (35).

1.1.3 Theoretical background to drug resistance in *M. tuberculosis*

Drug resistant TB in a setting derives from two plausible alternatives: suboptimal treatment and spread of drug resistant strains. Determination of clustering (number of genetically identical strains) and type of resistance mutations can be used to estimate the contribution of the two alternatives in the problem of drug resistance in an area. Four scenarios may be possible (let us only use RIF in the examples):

Same strain-same mutations

Circumstances and/or special properties, such as high virulence, of a RIF resistant strain can make it become predominant among the drug resistant TB cases in a geographical region, which will lead to that most of the infected will harbour this particular strain with its unique *rpoB* mutation.

Same strain-different mutations

Another scenario may appear when a highly transmissible drug susceptible strain infects many persons that during treatment failures with RIF randomly select different *rpoB* genotypes.

Different strains-different mutations

Inadequate treatment of patients infected with different RIF susceptible strains may lead to selection of various *rpoB* mutations.

Different strains-same mutations

Some mutations occur more frequently than others, probably because they confer no or only a small decrease of the biological fitness of the bacterium. The $Ser_{531}Leu$ mutation has in general very high frequencies among RIF resistant *M. tuberculosis* strains. Treatment failures can thus lead to the occurrence of the same *rpoB* mutation in many different strains.

Primary drug resistance is defined as when a person becomes infected with a drug resistant strain while *acquired drug resistance* occurs from the development of drug resistance due to inadequate treatment.

In contrast to other bacteria, drug resistance in *M. tuberculosis* seems to be exclusively confined to mutations in the chromosomal DNA and is not transferred between bacterial cells by mobile genetic elements, such as plasmids.
The prevalence of resistant clones in populations of wild type *M.*

tuberculosis is extremely low (20). It is estimated that in a wild type population one INH resistant mutant occurs per 10^6 bacilli, one RIF resistant mutant per 10^8 bacilli, one SM resistant mutant per 10^5 bacilli and one EMB resistant mutant per 10^6 organisms. The probability that random mutations in a single bacillus will result in resistance to more than one drug (e.g., INH and RIF) is obtained by multiplying the frequencies for each individual drug. One MDR mutant would spontaneously occur per 10^{14} bacilli ($10^6 \times 10^8$). Given that the number of bacilli in a lung cavity of 2.5 cm in diameter is estimated to be 10^8, an MDR organism would be quite unlikely to be present (74).

As mentioned earlier, resistance to anti-TB drugs in clinical praxis occurs when a patient receive only one drug at a time, leading to selection of resistant mutants. If two or more active drugs are used at the same time, each one contributes to avoid the evolution of resistance to the other. Hence, drug resistance in *M. tuberculosis* is a man-made problem that can be prevented by good treatment practices.

1.2 ASSESSMENT OF DRUG SUSCEPTIBILITY

It is essential to assess the drug susceptibility of the infecting *M. tuberculosis* strain to ensure an effective treatment that will break the chain of transmission and prevent the development of drug resistance. Traditional techniques for mycobacterial drug susceptibility testing (DST) are time consuming. It usually takes 3-6 weeks to obtain a positive culture from the primary specimen with an additional 3 weeks for susceptibility testing. The molecular methods, which screen for resistance-associated mutations, are by far the most rapid ones; results are available within one to two days. However, there is always a risk that the detection of a mutation in a resistance-associated gene not necessarily confers phenotypic resistance, but this is generally the exception. The molecular techniques have largely been focused on RIF resistance because the genetic target is well described and the presence of RIF resistance is almost always synonymous with MDR-TB. Detection of mutations in the *rpoB* gene can reliably identify RIF resistant strains.

1.2.1 Non-molecular techiques

There are three classical methods for the DST of TB; the absolute concentration method, the resistance ratio method and the proportion method which are performed on solid media (13, 14).

The absolute concentration method This method utilizes a standardized inoculum grown on drug-free media and on media where the drug to be tested has been incorporated in a series of concentrations. Resistance is expressed as the minimal inhibitory concentration (MIC), i.e. the lowest concentration at which no bacterial growth is observed.

The resistance ratio method This method compares the MIC of the test isolate to the MIC of a drug susceptible reference laboratory strain. A ratio is obtained by dividing the MIC of the test isolate with the MIC of the reference strain. Resistance is defined as a ratio of ≥ 8 and susceptibility as a ratio of <2.

The proportion method This method gives the exact proportion of resistant bacilli to a particular drug. A set of 100-fold dilutions of the bacterial

suspension is prepared and inoculated on media with and without the drug. For a valid test it is necessary that one dilution give 50-100 CFU (colony forming units) and the resistance is expressed as the proportion of drug resistant bacilli. If the proportion is > 1%, the isolate is considered resistant.

The development of the broth-based radiometric *BACTEC 460 system* (Becton Dickinson, Sparks, MD) in the 1970s, which is a modified proportion method, has made it possible to test drug susceptibility of the first-line drugs in about seven days (94). To facilitate rapid therapeutic decisions for patients with MDR, critical concentrations have been established for second-line drugs as well such as ofloxacin, amikacin, kanamycin, ethionamid, rifabutin and capreomycin (82).

Some new broth-based, either manual or fully automated, non-radiometric systems have been developed. These include the *Mycobacteria Growth Indicator Tube 960* (MGIT; Becton Dickinson, Sparks, MD) (90), *ESP Culture System II* (Difco, Detroit) (8) and the *MB/BacT* (Organon-Teknika, Durham, NC) (25) and its successors *MB/BACT* (bioMérieux) (7) and *BacT/ALERT 3D system* (bioMérieux)(I, II).

The Nitrate Reductase Assay, the *Resazurin Microtite Assay plate method* and the *colorimetric method of MTT* are examples of inexpensive, simple and rapid DST methods, especially suitable for resource-poor settings (1, 4, 80).

1.2.2 Molecular techniques

Identification of mutations in resistance-conferring genes offers a rapid detection of drug resistant isolates. Although these techniques are fast, they usually require technical equipments, reagents and expertise that raises the costs beyond what is considered reasonable in settings where resources are inadequate.

Sequencing The resistance gene is amplified by PCR (Polymerase Chain Reaction) and the genetic code is read by DNA sequencing in order to identify mutations (109).

Probe-based hybridization methods A PCR amplification of the resistance gene is followed by a step where the amplicon will hybridize to probes with corresponding wild type sequence or to probes with specific mutations. These methods include *Dot-blot* (108), *INNO-LiPA* (Innogenetics, Belgium) (19) and (III) and the *Genotype MTBDR* (Hain Lifescience, Germany) (70).

PCR-restriction fragment length polymorphism (PCR-RFLP) The resistance gene is PCR amplified and the amplicon is digested with a restriction enzyme that cuts at specific sequences in the gene. If mutation is present this will change the size of the digested gene fragments and mutation is detected by gel electrophoresis (109).

Single stranded conformation polymorphism analysis (SSCP) This method detects small changes in a nucleotide sequence, which causes differences in the secondary structure of the mutated gene. Due to an altered conformation the mutated DNA will display changed mobility in a polyacrylamide gel (46).

Heteroduplex analysis (HA) HA depends on the conformation of duplex DNA when analyzed in gels.

Heteroduplexes are formed when PCR amplification products from wild type and unknown mutant sequences are heated and re-annealed. If there is a sequence difference between strands of the wild type and tested DNA, the DNA strands will form a mismatched heteroduplex (77).

Molecular beacons Molecular beacons are hairpin-shaped single-stranded oligonucleotide detector probes, which undergo a fluorogenic conformational change when they hybridize to specific targets matching their sequence. Each target (mutation) will give a different colour (83).

1.2.3 Evaluation of DST techniques: aspects to consider

When evaluating a novel DST method, the most important to bear in mind is to consider its clinical usefulness, costs, accessibility of trained staff and required equipment and facilities. The new technique should be compared to other well-established DST reference techniques by using different statistical parameters (93):

- Sensitivity: Percent of positive (e.g. drug resistant) tests among true positive observations.
- Specificity: Percent of negative (e.g. drug susceptible) tests among true negative observations.
- Positive Predicted Value: Percent of true positive tests among all positive tests. This describes the probability that a positive test means that the patient has the (drug resistant) disease.
- Negative Predicted Value: Percent of true negative tests among all negative tests. This describes the probability that a negative test means that the patient does not have the (drug resistant) disease.
- Accuracy: Percent of true positive tests and true negative tests among all tests. This describes the test's degree of certainty to correctly detect true positive and true negative patients.

1.3 MUTATION TO DRUG RESISTANCE

All populations of organisms, also bacteria, include variants with unusual traits, e.g. the ability to withstand antibiotics. The antibiotic resistant trait will become positively selected every time the specific antibiotic is present and bacteria carrying this trait will survive, increase in number and eventually predominate the population. Why do these natural mutants exist? Mutations in "resistance genes" occurred long before antibiotics were introduced in human medicine and is probably used for protection from antibiotics produced by other microorganisms as bacteria and fungi (38). Furthermore a heterogeneous population is not so sensitive to eradication when external conditions are changing and is thus a kind of insurance for a long-term survival. As mentioned previously bacteria can acquire resistance by either of two mechanisms: spontaneous mutation, which is the case in *Mycobacterium tuberculosis* or horizontal transfer.

1.3.1 Selection of antibiotic resistant mutants

The rate by which drug resistance mutations are formed is dependent on the target size, i.e. how many genes and base substitutions that can confer resistance (63). In other words, the selection of antibiotic resistant phenotypes can occur at different (mutation) rates, which depend on the number of sites in the resistance gene that can give rise to a resistance phenotype but also the number of resistance genes. In *E.coli*, for instance, seven point mutations in the *gyrA* gene confer fluoroquinolone resistance, whereas only three mutations in *parC* gene lead to resistance (42). Consequently, the mutation rate for the *gyrA* gene will be higher, since it contains a higher number of resistance-conferring mutational sites. There are several other factors that can increase drug resistance mutation rates, i.e. unstable gene sequences surrounding nucleotides involved in the mutant phenotype, low or high copy number of the resistance gene, mutagenic agents and the efficacy of DNA repair mechanisms (63). *M. tuberculosis* have higher mutation rates for INH to which resistance can be conferred by several genes whereas the mutation rate for RIF resistance, almost exclusively conferred by mutations in the *rpoB* gene, is lower.

1.3.2 Fluctuation assay

A fluctuation test begins by inoculating a small number of cells and dividing it up into a large number of parallel cultures. The cultures are allowed to grow, usually to saturation, and then each culture is plated on a selective medium that allows the mutants to produce colonies (Fig. 1). The total number of cells is determined by plating appropriate dilutions of a few cultures on nonselective medium. The distribution of the numbers of mutants among the parallel cultures is used to estimate the mutation rate, which describes the probability of a cell to be mutated (per generation) (20, 31) and (IV). There are a variety of methods to calculate the probability of a cell to mutate but all estimates of the mutation rate are based on the distribution of the sizes of mutant clones, called the Luria-Delbrück distribution (Fig. 2).

The fluctuation test shows that mutations occur randomly in a growing culture, and are not induced by the selection agent (57).

If mutations were induced, e.g. after plating on streptomycin-containing media, all plates would have equal numbers of mutants. Since the test is based on a series of parallel cultures, the first mutation can occur very early in some of them and very late or not at all in the others, there is a "fluctuation". The mean number of mutants per culture is simply referred to as the mutation frequency.

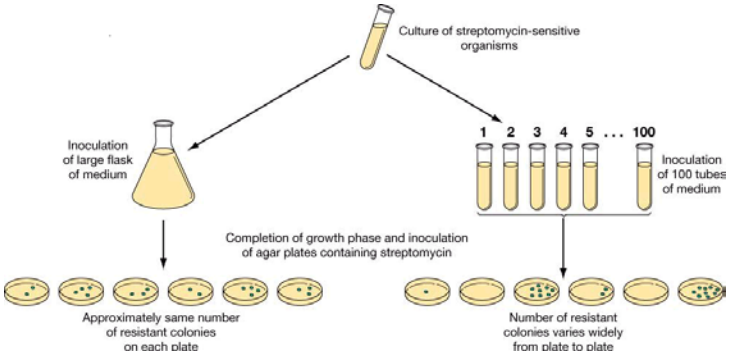

Figure 1. The fluctuation assay technique.

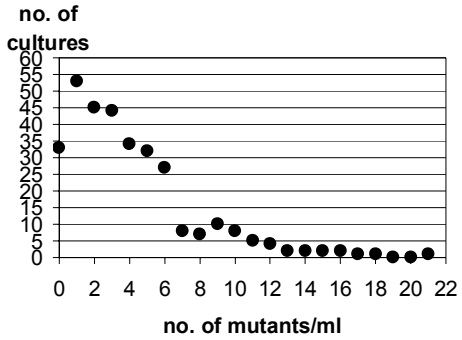

Figure 2. The Luria-Delbrück distribution (IV), showing the size (number of cultures) of the mutant clones (varying from 0 to 21 mutants per mL). The distribution indicates that probably one mutation occurred in the cultures during the test time.

1.3.3 DNA repair in *M. tuberculosis*

In the living cell, DNA undergoes frequent chemical changes. Most of these changes are quickly repaired. Those that are not result in a mutation. Thus, a mutation is a failure of DNA repair. A gene mutation is a permanent change in the DNA sequence that makes up a gene. Different molecular mechanisms cause mutations;
single base substitutions (point mutations) are single bases, which have been replaced by another base. A new nucleotide may alter the code of the gene, the codon, which will then code for a different amino acid in the protein product (missense mutation). Nucleotide substitutions that do not result in a change to the amino acid are called silent mutations. A mutation may also lead to the formation of a new stop codon (nonsense mutation) that terminates the translation (reading) of the mRNA leading to a truncated protein, which usually is not functional.
Extra base pairs may be added (insertions) or removed (deletions) from the DNA of a gene. Codons are read in triplets and both deletions and insertions therefore change the reading frame of the gene (frameshift mutation). To protect genome integrity, which is essential to ensure survival and further replication of the cell, all organisms have mechanisms to handle DNA damage. These are not perfect, but they do reduce greatly the frequency of mutation. Two important mechanisms are **proofreading** and **mismatch repair** (http://www.emunix.emich.edu/~rwinning/genetics/mutat.htm.).

Proofreading - works during DNA replication. As DNA polymerase III adds nucleotides to the growing chain, it checks each one for correct base pairing. If the correct nucleotide has not been inserted, the polymerase uses its 3' to 5' **exonuclease** activity to remove the incorrect nucleotide. The polymerase can then carry on and insert the correct nucleotide.

Mismatch Repair - this mechanism starts after replication, to correct errors that escaped proofreading. Because mismatched bases do not hydrogen bond properly, they create a distortion in the double helix, which can be

recognized and repaired by **excision repair.** The excision repair cut out the sequence with wrong matching, and the DNA polymerase fills in the gap with correct nucleotides. How does the repair system recognize which strand to repair?

There are two nucleotides (one on each DNA strand) that will not base pair - which one is the wrong nucleotide? The answer comes from **DNA methylation**. Under normal circumstances DNA is methylated; these methyl groups do not interfere with the function of the DNA in any way. Newly replicated DNA is not methylated however; the methyl groups are added enzymatically after replication. If mismatch repair is done immediately after replication (before methylation occurs), the original DNA strand will be methylated, and the newly synthesized strand (the one containing the error) will be unmethylated. The mismatch repair system therefore repairs the **unmethylated** strand.

DNA replication may begin before a lesion can be repaired. When the replication machinery hits the distortion (caused by the lesion) of the DNA, replication stops. The replication may reinitiate just beyond the lesion, leaving a gap in the DNA molecule. If the lesion is cut out by excision repair, there will be no template to use for resynthesis of the DNA. How, then, does the DNA get repaired? In this case, **recombination DNA repair** is initiated. First, the damaged region undergoes recombination with the complemenary strand from another DNA molecule. That is, the complementary sequence, covering the region with the lesion, is transferred from another DNA molecule to the damaged DNA molecule. This essentially transfers the gap to the DNA molecule that doesn't have the lesion but still have a complementary strand, which can be used to fill in the gap (by DNA polymerase), and the lesion on the other DNA molecule can be repaired by excision, since a template strand now exists.
If massive DNA damage overwhelms the mentioned repair mechanisms, DNA replication would stop and the cell would die. For these extreme cases, **SOS Repair** is the last repair maneuver. This mechanism allows replication to proceed across damaged templates, even though the template cannot be read accurately. Random nucleotides get inserted into the newly synthesized DNA strand. This mechanism is therefore error-prone, and leads to mutations, which could be deleterious. The alternative, however, would be death.

In order to identify the mechanisms of DNA repair in *M. tuberculosis*, homologues of genes known to be involved in the repair of DNA damage in *Escherichia coli* have been identified by bioinformatic analysis of its genome sequence. Genes coding nucleotide and base excision repair, recombination, and SOS repair were identified. Actually, homologues to *E. coli* genes known to be involved in the repair of oxidative and alkylative damage are present in *M. tuberculosis*. In contrast, no genes involved in mismatch repair (e.g., *mutS, mutL* or *mutH*) were identified (24). The ability to repair the DNA damage caused by exposure to reactive oxygen intermediates (ROIs) produced by the phagocytic host cell is likely to play a particularly important role in ensuring the intracellular survival *M. tuberculosis*. Indeed, open reading frames* (any sequence of DNA that can be translated into a protein),

similar to genes known to be responsible for the repair of DNA lesions resulting from the alkylation or oxidation of nucleotides, are present in the genome of *M. tuberculosis*.
*In a gene, ORFs are located between the start-code sequence (initiation codon) and the stop-code sequence (termination codon).

All DNA repair mechanisms are essential to keep the mutation rate as low as possible to reduce the risk of accumulation of mutations and deleterious mutations. Some bacteria display an unusually high mutation rate due, mainly, to the lack of a functional mismatch repair system. These bacteria are classified as mutator strains and have a mutation rate that is 10-1000-fold higher than for the wild type strain (68). Mutator strains are present in populations of *E. coli*, *S. typhimurium*, *P. aeruginosa* and *H. pylori* (11, 54, 79). Since mutations are of a central role in the development of drug resistance, mutators in a bacterial population could contribute to a more rapid and more extensive development of drug resistance (16, 36, 69). High bacterial mutation rate associated with faster resistance development have been observed in laboratory media and animal models (36, 91, 96). It is still unclear whether drug resistance development is more rapid in a patient infected with a mutator strain. Oliver *et al*. showed that cystic fibrosis patients harboring *P. aeruginosa* mutator strains developed more drug resistance and were more difficult to treat compared to patients infected with wild type strains (79).
No isolates of *M. tuberculosis* have yet been identified to have a true mutator phenotype (and to the best of my knowledge nobody has ever looked for them until recently). The DNA damaging effect of reactive oxygen and nitrogen intermediates, to which the intracellular bacteria is exposed to, and the long-term exposure to certain mutagenic drugs, could lead to mutations in genes involved in the function of DNA repair and thus the development of mutators. Missense mutations in putative mutator genes and their association with development of drug resistance have been identified in the Beijing genotype of *M. tuberculosis*. Mutator genotype did not appear to create drug resistance to a larger extent and yet no data on such mutator strains' mutation rate are available (52, 86). However, a *mutT1* knock out mutant of *M. tuberculosis* has been shown to acquire a 15-fold higher mutation frequency to RIF resistance compared to the wild type strain, showing this gene's important antimutator role (26).

1.3.4 Is there an emergence of clones with an increased potential to spread drug resistance?

It is known that different MDR-TB strains have the potential for transmission, even though resistance mutations often confer a fitness deficit to the bacterium (3, 62). By the implementation of molecular DNA fingerprinting techniques research have been able to unveil that in certain areas a small number of strains cause a disproportionate high number of cases of the disease. Several MDR-TB outbreaks have been reported worldwide over the past decade (9, 23, 27, 30, 58, 76, 81). The most extensive MDR-TB outbreak reported occurred in 267 patients from New York, who were infected by the W-Beijing genotype (32). This particular strain, which initially was fully drug susceptible, expanded clonally to result in a MDR phenotype, which disseminated to other

US cities (Atlanta, Las Vegas and Miami) and Paris (9). As will be discussed in the next section, Beijing strains often predominate among cases of MDR and this has contributed to the extensive investigation of this family of strains. The whole picture regarding the potential danger of this genotype is still not clear. Smaller outbreaks in the Czeck Republic and in Portugal have also involved other MDR genotypes of *M. tuberculosis* (50, 85). Little is known about circulating drug resistant strains in the developing countries that cannot routinely characterize strains on the genomic level (molecular DNA fingerprinting). It is therefore possible that other MDR genotypes with high potential for spreading have not been recognized.

1.3.5 The *M. tuberculosis* W-Beijing genotype

The most widely studied family of *M. tuberculosis* strains is the Beijing family, a group of strains that are closely related genetically, of which the first report came out in 1995 (105). In the early 1990s a multidrug resistant strain associated with large outbreaks and many deaths was identified in New York (2, 9). The strain was designated "W" and later recognized as a member of the Beijing genotype. Three methods identify Beijing genotype strains: spoligotyping (45), IS*6110* restriction fragment length polymorphism (RFLP) (104) and region A RFLP (51). As seen in Figure 3, the proportion of Beijing genotype strains is greatest in the Asian continent, where this genotype generally represents half of the TB cases but in the Beijing province >80% of the cases. In the states of the former Soviet Union the proportion is high: 45-56% in Russia and 29% in Estonia whereas its prevalence in Western Europe and sub-Saharan Africa is low (except in Cape Town). In North America and the Caribbean the proportion ranges from 8-14%.

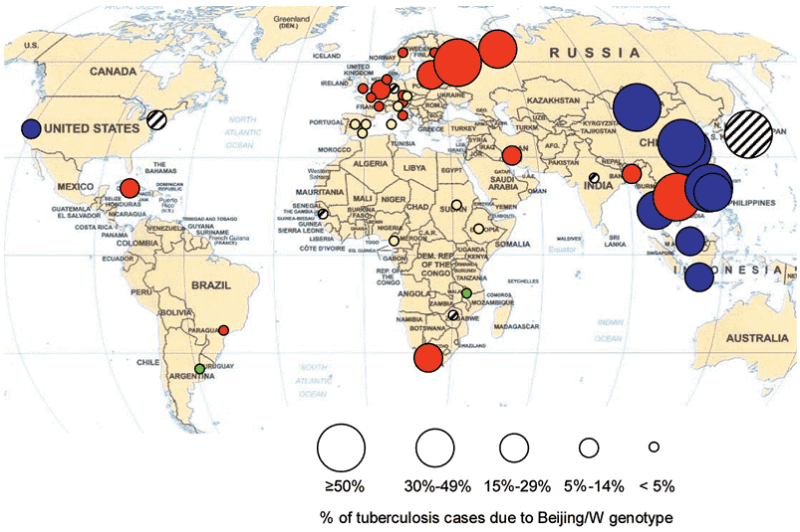

Figure 3. The proportion and association with drug resistance of Beijing genotype strains. Data represent 29, 259 TB patients in 35 countries. Size of circles indicates percentage of TB cases due to Beijing type; colour indicates drug sensitivity and distribution trend. Blue (stable, no drug res. assoc.), red (increasing, drug res. assoc.), green (increasing, drug sensitive), yellow (absent) and striped (trend or drug res.assoc. not known) (103).

Place	No of strains		% Drug resistance ANY		MDR	
	Beijing	non-Beijing	Beijing	non-Beijing	Beijing	non-Beijing
Austria	7	647	**14**	**7**	0	1
Denmark	16	1623	**12.5**	**10**	**6**	**0**
Finland	13	1102	**15**	**4**	**8**	**0**
Holland	199	3239	**9**	**6**	0.5	0.15
W Sweden	3	72	**33**	**3**	0	0
United K.						
London	5	145	0	3	0	0.7
Estonia	61	148	**70**	**14**	**34**	**2**
Russia						
S. Petersb.	133	103	**90**	**75**	**60**	**43**
Archangel	54	65	**80**	**37**	**46**	**8**
Azerbaijan	46	19	**89**	**68**	**61**	**32**
Africa						
Malawi	43	964	0	7	0	0
US						
San.Franc.	12	96	8	17	**8**	**0**
New York	273	1680			**93**	**0**
Cuba	22	136	**50**	**7**	0	1.5
Argentina	5	548	0	29	0	15
Brazil	4	416	**50**	**15**	**50**	**5**
Bangla-						
Desh	7	89	**71**	**20**	**29**	**3**
Indonesia	28	53	**36**	**17**	**7**	**4**
Malaysia	64	322	6	15	3	3
Bankok	98	106	30	31	**4**	**1**
Hanoi	33	20	**61**	**26**	**36**	**7**
Ho Chi						
Minh	81	87	**43**	**26**	**6**	**2**
Shanghai	25	14	44	57	**16**	**14**
Henan	36	16	**33**	**31**	**14**	**6**
Hong Kong	356	144	13.5	18	0.6	0.7
Mongolia	97	71	48.5	51	**2**	**0**
Taiwan	181	172	**50**	**35.5**	**19**	**16**

Table 1. Association between Beijing genotype and drug resistance in various areas. Bold figures showing Beijing type in association with drug resistance (103).

Associations of Beijing strains and drug resistance vary between countries (Table 1). In Estonia 1994 almost one third of all newly detected pulmonary TB patients were infected with Beijing strains, of which 70% exhibited some resistance and 34% were MDR. Of all MDR-TB patients in Estonia 87.5% were infected with Beijing strains (48). In Archangel Oblast, in the northwestern part of Russia, almost 45% of the pulmonary TB patients were infected with the Beijing type. Over forty percent of the Beijing strains were MDR (101). Researchers are concerned that the Beijing genotype may have a predilection for acquiring drug resistance (86) and may be spreading worldwide, perhaps as a result of increased virulence (56). The main hypotheses about the efficacy of the Beijing strain are as follows: BCG vaccination favouring the selection of this genotype (105), resistance to anti-TB drugs (48, 53, 78, 86, 101), increased potential to transmit (12, 49)

and increased virulence (56, 60, 111). Of course, these parameters do not only work separately but may combined lay behind the "successful" characteristic of the Beijing type. Also, to fully understand the epidemiology behind the Beijing family it is important to analyze the routes of human migration in the 20th century (73).

Drug resistance and clustering are often found together in association with the Beijing strain. As discussed in section 1.1.3, this could mean that Beijing strains are more likely to be MDR, leading to their spread, or that they transmit better so that the drug resistance spreads more effectively (59).

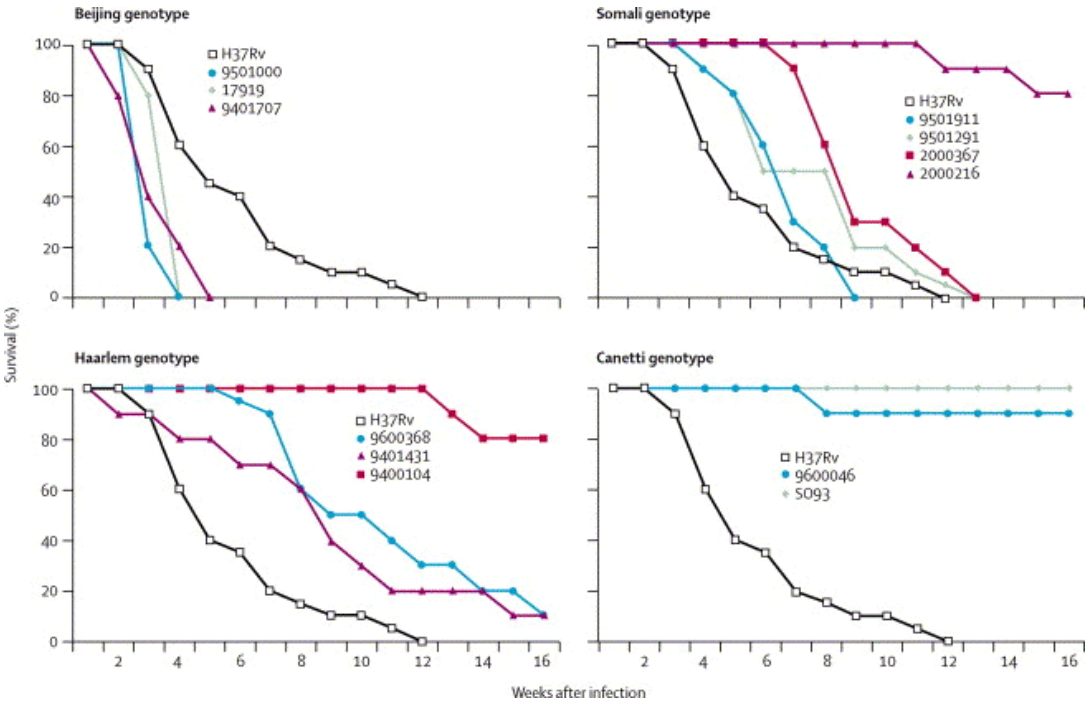

Figure 4. Survival of BALB/c mice (20 mice/strain) infected by intratracheal injection (2×10^5 bacilli) with *M tuberculosis* strains belonging to four different (worldwide) genotype families. Three different members of the Beijing type were tested. Reproduced with consent from the author (56)

2 THE PRESENT STUDY

2.1 GENERAL AIM

The purpose of this study was to increase the knowledge about the role of different components, such as mechanisms behind the development of resistant strains and the efficacy of drug susceptibility testing methods, contributing to the problem of drug resistant tuberculosis.

In order inhibit spread and give effective treatment it is of great importance to, as early as possible, detect drug resistant bacteria. Secondly, it is important to identify and characterize strains with increased potential to cause infection and to spread MDR-TB. We wanted to know whether Beijing strains mutate to drug resistance easier than other strains, making this genotype overrepresented among MDR-TB cases in many areas.

2.2 SPECIFIC AIMS

- To evaluate the commercial BacT/ALERT 3D system (bioMérieux, Durham, NC, USA) for isolation and drug susceptibility testing of *M. tuberculosis* [I, II].
- To evaluate the INNO-LiPA^{TM} Rif. TB (Innogenetics, Ghent, Belgium) for rapid detection of rifampicin resistance in *M. tuberculosis* [III].
- To study the *in vitro*-rate of mutation to drug resistance of various *M. tuberculosis* strains, including the Beijing genotype [IV].
- To determine the distribution of *rpoB* mutations and level of rifampicin resistance among *in vitro*-selected mutants of different *M. tuberculosis* strains, including the Beijing genotype [V].

3 GENERAL SUMMARY OF THE PAPERS

The clinical material and laboratory methods have been described in detail in the original papers (I-V).

3.1 RECOVERY OF THE TB COMPLEX USING THE BACT/ALERT (I)

Due to the slow growth of *Mycobacterium tuberculosis* (TB) isolation of the bacteria from primary specimens, e.g. broncheoalveolar lavage fluids, takes several weeks using solid media. It has been the common practice in most laboratories to use bacterial growth from solid media in order to ensure pure and abundant material for the preparation of standardised bacterial suspensions, used for the subsequent drug susceptibility testing (DST). To speed up the time needed for the recovery of TB, liquid media is an alternative, in which the mycobacterium grows faster.

Rapid and sensitive detection of *Mycobacterium tuberculosis* is of clinical importance for the treatment, control, and prevention of TB. Despite new nucleic acid amplification assays, unequivocal diagnosis of tuberculosis continues to rely on cultivation of *M. tuberculosis*. The BacT/ALERT 3D system is a fully automated liquid culture system, which allows the growth and detection of mycobacteria.

This study was performed at the Department of Clinical Microbiology, Karolinska Hospital, which receives all specimens for mycobacterial culture from central Sweden. During January to July 2002, 2659 consecutive clinical specimens including broncheoalveolar lavage fluids, fluids from joints, pleural, peritoneal and cerebrospinal fluids, secretions, blood, bone marrow and biopsies were cultured using the BacT/ALERT system and conventional LJ substrates. Specimens from non-sterile sites were decontaminated with the NAOH-sodium lauryl sulphate method (44). Then the samples were centrifuged and washed once with phosphate buffer. A portion of each specimen was inoculated on two LJ tubes and into one BacT/ALERT MP process bottle, following the manufacturer's instructions. The LJ tubes were incubated for seven weeks at 37°C and read every week. The process bottle was incubated at 37°C in the BacT/ALERT instrument that performs readings every 10 minutes.

M. tuberculosis was detected in 92 and 94 specimens in the BacT/ALERT and LJ medium, respectively and detection time was 25% shorter with the BacT/ALERT (Table 2).

	BacT/ALERT	Löwenstein-Jensen
Positive cultures (n)	92	94
Time (days), median (range)	18 (6-31)	24 (11-30)

Table 2. Recovery rate and time to detection of the two methods used for isolation of TB complex.

Of the 2659 mycobacterial cultures from the various specimens, the BacT/ALERT and LJ media detected 37 and 34 non-tuberculosis mycobacteria (NTM), repectively. The BacT/ALERT had a median detection time of 11 days and detected NTM twice as fast as the LJ substrates.

3.2 ASSESSMENT OF DRUG SUSCEPTIBILITY USING THE BACT/ALERT (I, II)

To limit the spread of TB and prevent the development of drug resistance, by ensuring appropriate therapy early in the course of the disease, experts at the Centers for Disease Control and Prevention (CDC) recommend that, for isolates of *Mycobacterium tuberculosis* complex (MTC), susceptibility test results should be available in 28 to 30 days from receipt of a specimen in the laboratory (99). The proportion method and the BACTEC 460 system (Becton Dickinson, Sparks, MD, USA, see 1.2.1) are two widely used DST techniques. The former procedure involves the inoculation of mycobacteria onto a solid medium with incubation at 35 to 37°C in a 10% CO_2 atmosphere. In such growth conditions, colonies of *M. tuberculosis* are not detected until 21 days after inoculation, which is too long for adherence to the CDC guidelines for efficiency. The BACTEC 460 MTB system was the first broth-based system that provided a more rapid result. The BACTEC bottles can be read radiometrically in as short time as 5 days, depending on the inoculum size.

As mentioned previously, the culture-based radiometric BACTEC 460 system has become a standard method to assess drug susceptibility of *M. tuberculosis*. However, the main drawbacks of this method, such as the production of radioactive waste, the need of daily manual loading and reading of results have been solved in newer systems. The BacT/ALERT 3D system (bioMérieux, Durham, NC, USA) is based on the detection of carbon dioxide (CO_2) released by actively proliferating mycobacteria. The elevated CO_2 concentration lowers the pH in the media, which in turn produces a color change, detected by a reflectometric unit in the instrument.

The BacT/ALERT automatically performs reading every 10 min and all data are transferred to and saved in the BacT/VIEW data manager program.

We have evaluated two different BacT/ALERT DST protocols at two separate occasions. The critical test concentrations of the antibiotics used in both kits were for RIF 0.9 mg/l, INH 0.09 and 0.4 mg/l and EMB 1.8 mg/l.

In the latter kit also PZA was included but preliminary DST results revealed shortcomings and the testing of this drug, using the present protocol, was interrupted. A novel BacT/ALERT kit for the test of PZA is under evaluation in our laboratory and will be reported separately in 2007.

Drug (conc.)	Res	Susc	Sensitivity	Specificity
RIF				
Correct:	**23/25**	**25/25**	**92%**	**100%**
INH				
Correct:	**26/27**	**23/23**	**96%**	**100%**
EMB				
Correct:	**6/6**	**44/44**	**100%**	**100%**
SM				
Correct:	**14/18**	**32/32**	**78%**	**100%**

Table 3a. BacT/ALERT DST results from the first episode (I). N=50

Drug (conc.)	Res	Susc	Sensitivity	Specificity
RIF				
Correct:	**36/37**	**43/43**	**97%**	**100%**
INH				
Correct:	**59/59**	**20/21**	**100%**	**95%**
EMB				
Correct:	**34/34**	**45/46**	**100%**	**98%**

Table 3b. BacT/ALERT DST results from the second episode (II). N=80

The major change in the latter protocol was that the 10-fold diluted (proportional) growth control, used in the earlier test, was excluded and drug resistance was exclusively determined as when the drug-containing test bottle gave a positive signal no later than 3.5 days after the undiluted control had flagged positive. Earlier a strain was considered drug resistant if growth was detected in the antibiotic-containing bottle before or at the same time as the proportional control. Contrary to previous BacT/ALERT susceptibility kits, streptomycin (SM) is no longer included. The latter BacT/ALERT protocol was shown to be more optimal for DST of *M. tuberculosis*, since the former kit tended to miss drug resistance, i.e. it had lower sensitivities (Table 3a and b). The BACTEC 460 system utilizes a 1/100 diluted growth control that is used for the interpretation of drug susceptibility. The 10-fold diluted growth control in the first BacT/ALERT protocol may become positive too fast, and thus some resistant strains will wrongly be interpreted as drug susceptible.

The turnaround times for DST ranged from 6.0 to 14.7 days (median: 7.8 days) when using the new modified protocol, compared to the earlier median time of 11 days (range: 7-26). Although almost all drug resistant strains gave a positive signal near the point of TTD (time to detection) of the direct (undiluted) control, the 3.5 days cut off used for interpretation of resistance may be too stringent for detection of some RIF resistant strains. This weakness was exemplified by one

RIF resistant strain that was falsely found to be susceptible due to a 1 day delay in the positive signal, which in a repeated run remained 0.5 days delayed.

Although DST results generally are not achieved faster when using the BacT/ALERT system instead of the BACTEC 460, there are other advantages like the standardization of inoculum (seed bottle), the automatic recording and saving of data that reduce the laboratory workload and no radioactive waste is produced. As is the case with the majority of novel techniques this system is not an alternative in settings where resources are limited (generally countries with high incidence of TB). Taken together our results suggest that the BacT/ALERT 3D system should be considered as a valid alternative for rapid drug susceptibility testing of *M. tuberculosis*.

3.3 RAPID DETECTION OF RIF RESISTANCE USING THE INNO-LIPA RIF TB (III)

As discussed in the introduction there are two major tools to combat the rise of MDR-TB: early case finding of drug resistant strains and an effective treatment regimen. Rapid detection of RIF-resistance (and indirectly MDR) would promptly lead to an adequate therapy, which improves the recovery of the patient, reduce the risk of development of even more highly resistant strains and reduce the time of infectiousness. In other words, early detection of RIF resistance counteracts both primary and acquired drug resistance (see 1.1.3). The extra cost for such laboratory examinations will eventually be refunded in terms of fewer numbers of drug resistant cases, (which need extended time of treatment with very expensive drugs) and shorter periods of hospitalisation. Keeping this in mind, there are good reasons to develop and evaluate techniques that offer rapid detection of drug resistance.
The line probe assay (LiPA) was evaluated for the early detection of RIF resistance in 53 *M. tuberculosis* clinical isolates. The LiPA is based on probes covering the RRDR (rifampicin resistance-determining region) sequence of *rpoB* (Fig. 5).

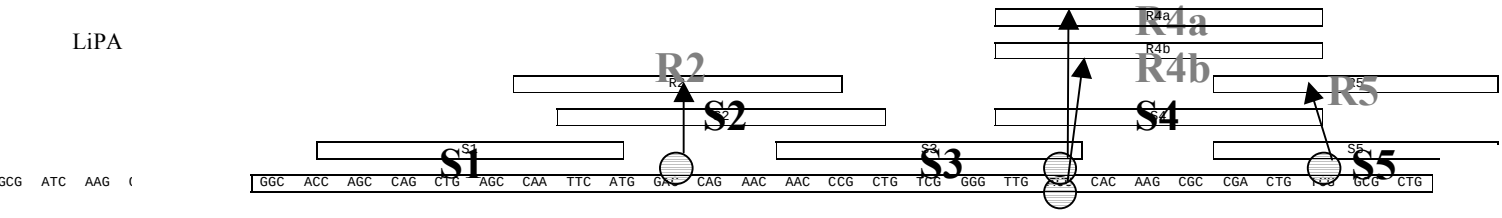

Figure 5. Position of the LiPA wild type probes S1-S5 and mutational probes R2, R4ab and R5 in the RRDR of *rpoB*. Grey circles represent the specific LiPA-determining mutation; R2-$Asp_{516}Val$, R4a-$His_{526}Tyr$, R4b-$His_{526}Asp$ and R5-$Ser_{531}Leu$.

The BACTEC 460 and Cycle sequencing were used as phenotypic and genotypic reference techniques, respectively. The two molecular methods detected wild type sequences in the RRDR of the *rpoB* gene in 24 strains whereas the remaining 29 strains were mutated. When the genotypic data was compared to the phenotypic drug susceptibility in BACTEC, 27 strains were true resistant and two were interpreted as sensitive to RIF (despite the presence of a mutation in RRDR). The two latter strains were determined to have a $Leu_{511}Pro$ mutation in the RRDR, which was indicated in the LiPA by the lack of hybridisation to the corresponding wild type probe (ΔS1). This mutation has earlier been reported to be present in some RIF susceptible strains (106). Furthermore these strains had MIC (minimum inhibitory concentration) to RIF of 0.5 mg/L and 0.25 mg/L, respectively. The $Leu_{511}Pro$ mutation, which cannot specifically be identified by the LiPA, demonstrates the disadvantage of this and similar methods.

Among the 29 mutated strains, 21 were determined by hybridisation to one of the R (mutational) probes (R2, R4a, R4b and R5). Of the remaining eight isolates, which were determined to be resistant due to the lack of hybridisation to one or more wild type probes, four were shown to have single nucleotide substitutions, three had double mutations and one strain harboured a deletion. Altogether, eleven different LiPA hybridisation patterns were seen among the 53 strains (Fig. 6). The $Ser_{531}Leu$ mutation (R5) occurred most frequently and were detected in 16 of the 27 (59%) RIF resistant strains followed by codon 526 mutations (R4a and b) having a frequency of 18.5%.

One RIF resistant bacterial sample (number 54 in Fig. 6) hybridised to the S1, S3 and S4 wild type probes and also to the mutational probes R4a and b. The LiPA result of this isolate is unlikely since hybridisation to both the wild type probe (S4) and its corresponding mutational probes (R4a and b) occurred simultaneously, which should be mutually impossible. Sequencing confirmed mutations in the S2 and S5 region of RRDR but no mutations were found to ratify the hybridisation to the R4a and b probes (see further discussion in the original Paper III).

The LiPA method only requires a thermal cycler for PCR and a shaking water-bath. The method is rapid, reliable and easy to use. Nevertheless the cost per test (~30 EURO) makes it too expensive to be run routinely in resource-poor settings, where DST methods like the Nitrate Reductase Assay (4) may be better alternatives for rapid assessment of drug susceptibility.

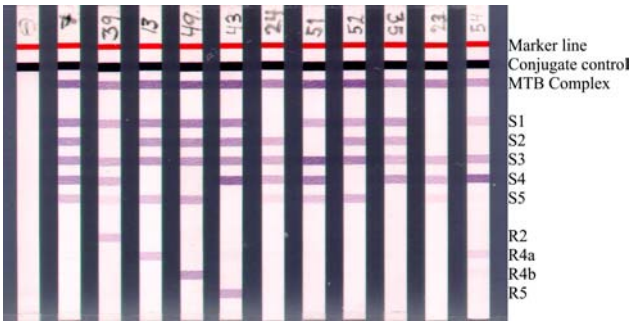

Figure 6. LiPA hybridisation patterns. From left to right; negative control, wild type, R2, R4a, R4b, R5, ΔS1, ΔS2, ΔS4, ΔS5, ΔS1+ΔS2 and ΔS2+ΔS5+R4a+R4b.

3.4 MUTATION TO RESISTANCE IN BEIJING GENOTYPE (IV, V)

Strains of *M. tuberculosis* with a reduced susceptibility to drugs may have a selective advantage if the physiological fitness of the bacteria is preserved after mutation. Patients infected with such strains may have a lower cure rate and could, if therapy is suboptimal, remain contagious for longer periods. This would enhance the risk of a continuous spread of the drug resistant disease. Beijing genotype strains have been spreading increasingly and are often associated with MDR-TB (37, 72, 103).
Furthermore, studies have shown that representatives of the Beijing family may be more virulent (56), have a better growth in human macrophages (111), do not necessarily become less fit in the drug resistant form (100) and carry mutations in putative mutator genes (86).

There were, however, no previous publications on the Beijing's capacity to develop drug resistance, i.e. no studies determining the rate of mutation to drug resistance. In fact, there were almost no studies characterizing *in vitro*-selected mutants of different *M. tuberculosis* strains and particularly not Beijing mutants. Using fluctuation assays, we determined the mutation rate of 13 different strains *in vitro*, using rifampicin as the marker. Six isolates were of the Beijing genotype and all strains were drug susceptible to first line drugs. By measuring the mutation rate we tested the hypothesis that Beijing strains have an increased capacity to generate resistant mutants, which more rapidly become selected during suboptimal treatment. All isolates showed similar mutation rates irrespective of genotype (Table 4a). The results suggest that Beijing strains do not develop resistance easier compared to other *M. tuberculosis* strains.

Strain	Cells/ml	Mut./ml	Mut.freq.	Mut.rate
Beijing	(x 10^8)		(x 10^{-8})	(x 10^{-8})
1	0.8	3.3	4.1	1.3
2	1.2	3.0	2.5	0.79
3	0.6	1.6	2.7	1.0
4	0.8	2.2	2.8	0.94
5	0.9	4.8	5.3	1.5
6	0.9	3.8	4.2	1.2
Average	0.9	3.1	3.6	1.1
Non-Beijing				
1	0.5	4.0	8.0	2.4
2	1.3	4.3	3.3	0.96
3	1.0	3.7	3.7	1.1
4	1.6	3.4	2.1	0.65
5	1.0	5.4	5.4	1.5
6	1.0	4.9	4.9	1.4
H37Rv	2.3	7.7	3.3	0.86
Average	1.2	4.7	4.4	1.3

Table 4a. The mutational frequency and rate of the 13 *M. tuberculosis* strains.

Strain	Cells/ml	Mut./ml	Mut.freq.
Beijing MDR+	**(X 10^8)**		**(X 10^{-9})**
1	**0.52**	**0.28**	**5.3**
2	**0.60**	**0.17**	**2.8**
Average	**0.56**	**0.23**	**4.1**
Beijing susc.			
1	**0.35**	**0.17**	**4.9**
2	**0.41**	**0.40**	**8,9**
Average	**0,38**	**0,29**	**6,9**

Table 4b. Mutation frequencies to ciprofloxacin resistance.

We also carried out a number of experiments on two MDR and two drug susceptible Beijing strains, using ciprofloxacin (2 mg/L) as the marker of their mutation frequencies (Table 4b, unpublished data). Ciprofloxacin resistant mutants appeared rarely, and because of this the mutation frequencies might have been inaccurate since too few cells (needed to establish the correct normal distribution of mutants) were plated.
No difference could be discerned between the drug resistant and susceptible strains. Anyhow, the test should be repeated with a higher inoculum and on more strains.

We used DNA sequencing to study specific *rpoB* mutations in 189 mutants that had been selected during the fluctuation assays. Eighty-nine of the mutants were of the Beijing type. Mutations were seen within the RRDR of the *rpoB*-gene in all but one mutant. Twelve different single nucleotide substitutions were distributed among 172 (91%) mutants. Also, 11 different deletions were detected among 14 (7.4%) mutants, and one double and one triple mutation occurred in two mutants. Similar to the findings in RIF-resistant clinical isolates (61, 87), the *in vitro* frequency of mutations was the highest for codons 526 (40%) and 531 (34%). Mutations in these two codons have been shown to confer high level of RIF-resistance but also a modest physiological cost, which could be reasons for their clinical predominance (10, 62).

However, contrary to clinical isolates, mutations in codon 516 were scarce (~1% of all mutants), and codon 522 mutations were the third most prevalent among our mutants (13%).
We determined the level of resistance to RIF for all mutants as the Minimum Inhibitory Concentration (MIC). In general, most mutations were coupled with a high level of RIF-resistance (MIC ≥ 32 mg/L). Several mutants with the $Ser_{522}Leu$ mutation did however show a lower level of RIF-resistance (MIC 8 ≤ 16 mg/L). The earlier reported fitness deficit (relative fitness being 0.54) (62), and the lower resistance level associated to the codon 522 mutation, could be the cause for its lower frequency in the clinical setting.
Theoretically Beijing strains could be associated with low cost mutations, making these strains more competitive in the spread of MDR-TB, but no noteworthy difference in the distribution of mutations, or in the level of resistance, was observed in our study

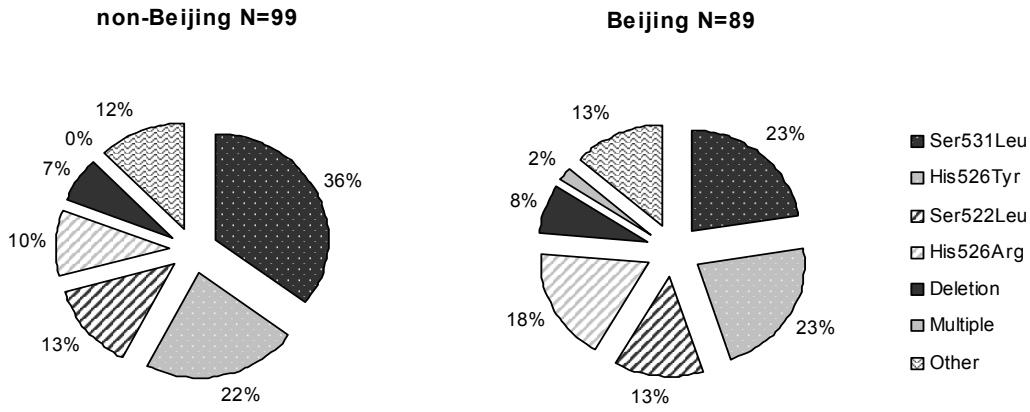

Figure 7. The distribution of *rpoB* mutations in the two groups of genotypes. The mutant with no mutation in the RRDR of *rpoB* is excluded. All rare single nucleotide substitutions (present in < 10 mutants) were put together in the sector "Other".

between strains of Beijing and non-Beijing genotypes (Fig. 7). The multiple mutations were, however, exclusively found in mutants of the *M. tuberculosis* Beijing genotype.

Although none of the parental strains showed increased mutation rates it would be of great interest to analyze the mutants with multiple *rpoB* mutations to determine the mutation rate and to screen the mutator genes for mutations. Although not significant, our correlation between multiple mutations and the Beijing genotype, is consistent with Tracevska *et al*'s observation that such mutations occurred at a higher rate in Beijing isolates (13%), compared to other genotype strains (2%) (102). This could be an indication of a defective DNA repair system (see 1.3.3) in strains of the Beijing type that would lead to a higher accumulation of mutations (including drug resistance mutations). Indeed, it has been suggested that Beijing strains may have DNA mutations conferring a mutator phenotype allowing an increased mutation rate, thus leading to a selective advantage during exposure to antibiotics (86).

Recently Gagneux *et al.* (33) studied *in vitro*-generated RIF resistant mutants of two different genetic lineages of *M. tuberculosis* (CDC 1551 strain and the T85 Beijing strain). Their data demonstrated that the *in vitro* relative fitness in RIF resistant *M. tuberculosis* depends on both the resistance-conferring mutation and the strain genetic background. The authors measured the relative fitness of RIF resistance in paired clinical isolates (the drug resistant isolate and its drug susceptible progenitor) from ten patients who acquired RIF resistance during treatment. All of the isolates belonged to one of the two strain lineages represented by CDC 1551 or Beijing T85. The $Ser_{531}Leu$ mutation was present in 5 of the 10 isolates and the other five harboured other *rpoB* mutations. The $Ser_{531}Leu$ mutants had a relative fitness equal or higher than the corresponding drug susceptible progenitor whereas the other mutants had a significantly lower physiological fitness. Additionally the $Ser_{531}Leu$

mutation was determined to cause only a small fitness deficit in laboratory-derived mutants of the two lineages of genotypes. These findings demonstrate that low-cost mutations are most prevalent among clinically resistant isolates and that these mutants overtime will have retained or even overcompensated fitness, probably through compensatory mutations.

Beside the correlation of Beijing strains and drug resistance in many countries, it has also been suggested that this genotype may be better at establishing an infection. Reinfections with drug resistant *M. tuberculosis* may be misinterpreted as a development of drug resistance during treatment, if molecular testing techniques are not used to discriminate between isolates. For instance, five Estonian patients with TB developed MDR-TB during therapy. DNA fingerprinting (RFLP) revealed that the drug resistance did not evolve from an endogenous reactivation (same strain causing a second round of disease) but that all were reinfected with a new MDR strain belonging the Beijing genotype (exogenous reinfection) (49). Such exogenous reinfections are probably more common in situations where contagious TB-patients are not isolated from other patients. Persons sharing room with TB-patients for long periods are continuously exposed to circulating strains. It still remains to be demonstrated whether exogenous reinfections with Beijing strains mainly are due to such circumstances, or if the genotype itself is more virulent.

To find out which components (virulence, immunogenicity, biological fitness and drug resistance), alone or in combination, are playing the most important role in making the Beijing type especially successful to spread drug resistant tuberculosis, future research should systematically characterise strains of Beijing genotype. The presence of mutator alleles, data on resistance mutations, DST data, virulence factors, epidemiological data (such as case linkages and patient history) and treatment outcome are important to register for patients infected with Beijing strains. By putting these pieces of information together we may clarify the role of the Beijing genotype in MDR-TB.

4 SUGGESTIONS FOR FUTURE RESEARCH

- The BacT/ALERT should now be considered a valid technique for drug susceptibility testing of *Mycobacterium tuberculosis* to the first line antituberculosis drugs rifampicin, isoniazid and ethambutol. There is, however, a need to further evaluate a novel protocol for the drug susceptibility determination of pyrazinamide and the important second line agents such as fluoroquinolones and aminoglycosides.

- More data on the prevalence of exogenous reinfections of *M. tuberculosis* Beijing strains are needed to support the hypothesis of high virulence and increased transmissibility of this group of strains.

- Competitive tests on clinically drug resistant Beijing strains are of great interest to determine the resistance-related cost of fitness in comparison to other *M. tuberculosis* genotypes.

- Identification of mutator genotypes in Beijing strains originating from several geographical regions to demonstrate the frequency of such mutations.

- The rate of mutation should be determined for Beijing strains with mutator alleles to correlate such genetic alterations with the development of drug resistance.

- Further studies on intracellular multiplications rates, immunogenicity and virulence should be carried out on larger numbers of genetically different Beijing strains to estimate the danger of these strains in general.

- The adaptation to intracellular conditions should be investigated for this family of strains to determine the degree of resistance to nitrogen and oxygen intermediates such as hydrogen peroxide, H_2O_2, whose mutagenic effect also could be determined *in vitro* (fluctuation assays). High resistance=enhanced ability to survive.

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7 APPENDIX (PAPER I-V)