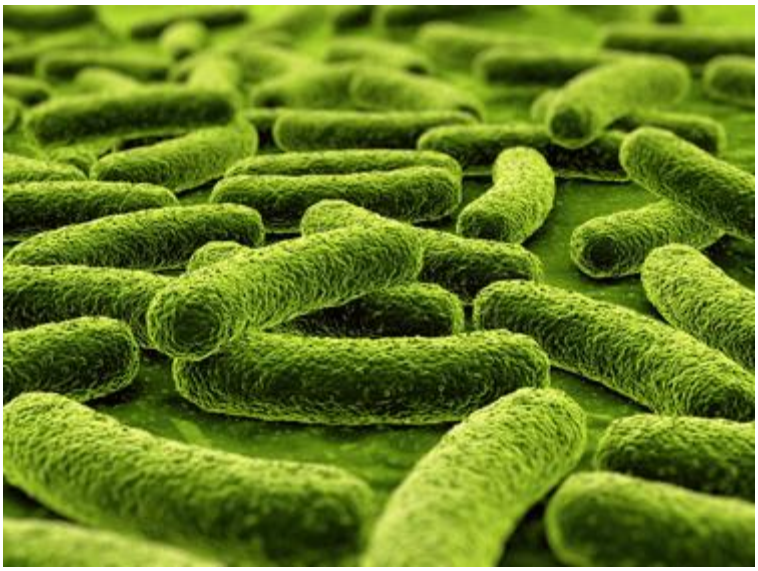
Prospects in the molecular screening for drug susceptibility of *Mycobacterium tuberculosis*

Where do we stand in the development of whole genome sequencing for drug resistant tuberculosis?



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Abstract

Tuberculosis (TB) is caused by an infection with *Mycobacterium tuberculosis*. Treatment of this disease is challenged by a growing problem of resistance. Whole genome sequencing techniques may play an important role in quick and cheap resistance screening in the future. However, before this technique can be used in the clinic, challenges lie ahead. This essay starts by giving an overview of the first- second- and new third-line drugs currently in use to battle TB. Currently, many researches are running to investigate possible new antitubercular agents. These consist of completely new agents, but also of antimicrobials that are or were already being used to treat other (lung)infections. The second chapter gives an overview of the currently known resistance genes and their mutations. Resistance genes must me mapped and all mutations causing resistance against all antitubercular drugs must be known before whole genome sequencing can be widely applied in the future. Mutations are better known for MDR-TB than they are for XDR-TB, while mutations for the new third-line drugs are poorly studied. The use of whole genome sequencing as a drug-susceptibility test would provide a much needed new tool in the treatment of TB, however, a lot of problems need to be solved before it can be widely applied. Possibly this technique may be applied to signal MDR-TB before it can also be applied to XDR-TB. Taking one step and one drug at the time, whole genome sequencing could become more and more valuable in the signalling of drug resistance and ultimately the treatment of TB.

Introduction

Tuberculosis (TB) is an infectious disease caused by mycobacteria and mostly affects the lungs. Most TB cases are caused by bacteria from the *Mycobacterium tuberculosis* complex (MTC). This complex entails four mycobacteria: *M. bovis*, *M. africanum* and *M. tuberculosis*. When a case of TB is caused by a bacterium from the MTC, this is called typical TB. Atypical TB is caused by a mycobacterium outside the MTC. In the majority of infections, the lungs are affected, causing lung TB. Another part of TB cases is an infection outside the lungs and is called extra pulmonary TB. Patients with TB get granulomas inside the lungs (in the case of lung TB) or elsewhere in the body (in extra pulmonary TB). Inside these granulomas, also called 'tubercles', necrosis of the tissue takes place. (Grosset, 2003) Today, TB mainly strikes in developing countries. Here, a large part of the people affected by the disease is HIV-positive. Kaplan et al., 2009)

A wide range of antibiotics is available for the treatment of TB. The first line of defence against TB is the group of first-line antibiotics. These include four antibiotics that are used when there are no signs of resistance yet. The second-line antibiotics are a group of antibiotic recommended by the World Health Organisation (WHO) as antibiotics to be reserved for the treatment of TB resistant to the first-line antibiotics. A part of these antibiotics is injectable, while the other part is administrated as a tablet or sachet. (WHO model list of essential medicines, 2011) Two types of resistance are distinguished: multidrug-resistant TB (MDR- TB) and extensively drug-resistant TB (XDR-TB). MDR-TB is resistance against at least two of the first-line drugs: isoniazid (INH) and rifampicin (RFP). XDR-TB isolates are also resistant to these two, with additional resistance to a fluoroquinolone and to at least one of the three injectable second-line antibiotics. (Hum Nath et al., 2013)

Currently -in 2013- many tests for identification of the TB-causing bacterium are available. These include microscopic examination after Ziehl coloration, analysis of fragment length polymorphisms with the IS6110 probe and 'matrix-assisted laser desorption and ionization-time-of-flight' mass spectrometry (MALDI-TOF MS).(Pignone et al., 2004) However, the antimicrobial resistance of these agents is still tested mainly by antibiograms that are time-consuming and not always effective, leading to increased resistance, mortality and nosocomial outbreaks. Technical advances have brought new methods for drug susceptibility testing, but none of them have yet been able to determine resistance within the timeframe of a single hospital visit. This stresses the need for more efficient, faster and cheaper methods for determination of drug susceptibility. In this context, genotyping methods are the most promising. (Helb et al., 2010)

One genotyping method that is already widely used to determine drug susceptibility is restriction fragment length polymorphism (RFLP) typing. In this typing method, the IS6110 restriction fragment is used to determine not only identity of the bacterium, but also resistance against rifampicin. Since resistance against rifampicin is commonly accompanied by resistance against INH, this method is a reliable indicator of MDR-TB. It can give results in a matter of hours with high specificities. (Bifani et al., 2009)

With the development of cheaper and quicker whole genome sequencing (WGS) methods over the past years, WGS seems to be the next step in quick determination of TB resistance. However, before WGS becomes useful, we need to know what mutations cause resistance against different antibiotics. For this purpose, this paper will first discuss the first-, second- and third-line antibiotics recommended by the WHO in the treatment of TB, MDR-TB and XDR-TB. Next, known mutations in

M. tuberculosis causing resistance against these antibiotics will be assessed. By doing so, this paper aims to provide insight in the possibilities of screening for drug resistant TB with the use of WGS methods. For this purpose, the struggles and problems that need to be overcome in order for WGS to be useful in the screening for drug resistance will be discussed in the final chapter.

Current antibiotics against tuberculosis

Since no effective vaccine is available yet, treatment of TB currently consists of the administration of antibiotics. However, due to the global emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) the treatment of TB is challenged. MDR-TB is defined as laboratory determined resistance to at least the first-line antibiotics isoniazid (INH) and rifampicin (RFP)) (Hum Nath et al., 2013). MDR-TB requires treatment with second-line antibiotics, which poses a problem because these medicines are costly and have more side effects. (Espinal et al., 2001)

Before moving on to the mutations causing this resistance, the following questions will be elaborated on in this chapter: what are the working mechanisms of the first-, second- and third-line drugs and what is their role in the treatment of TB?

First-line drugs

The first-line antibiotics are the first line of defence in the treatment of TB. These drugs are the first choice when treating patients with TB. Non-resistant TB is treated with four drugs for the first two months, after which a continuity phase starts in which only two drugs are administrated. Five first line drugs are recommended by the WHO as 'essential antituberculosis drugs'. These drugs are pyrazinamide, ethambutol, isoniazid, rifampicin or rifabutin and streptomycin. (WHO model list of essential medicines, 2011) These drugs will be the ones discussed in this section.

Pyrazinamide

The first of these drugs, Pyrazinamide (PZA), is a prodrug. It is hydrolysed inside the tuberculosis bacterium by pyrazinamidase (PZase), forming pyrazinoic acid (POA). In PZA resistant strains of *M. tuberculosis*, PZase activity can be lost. (Shi et al., 2011) Even though PZA is an important medical tool in the battle against TB, its working mechanisms are the least understood. It is thought to enter *M. tuberculosis* by passive diffusion. In the cell, due to its P_{ka} value of 2.9, it forms the carboxylate anion and could possibly be excreted or passively transported across the membrane (Zhang et al., 1999). It is proposed that in doing so, it makes the proton gradient collapse, reduce membrane potential and affect membrane transport. There is some evidence that supports this theory, but the real molecular targets of PZA still remain unknown (Zhang et al., 2003).

In the treatment of TB, the role of PZA is mainly the shortening of the treatment duration. Treatment duration has been brought down to 6 months instead of 9-12 months like before the discovery of PZA (Mitchison, 1985). Research is done on drugs to replace PZA, but treatment with PZA still is the best method. (Andries et al., 2005; Rosenthal et al., 2007; Tasneen et al., 2008; Nuermberget et al., 2008)

Ethambutol

Ethambutol (EMB) is a first-line antibiotic that is used to protect its companion drugs against resistance. However, in Drug Resistant TB (DR TB), 50-60% of bacteria are resistant to EMB. (Starks et al., 2009) The working mechanism of EMB is well described in literature: it inhibits the mycobacterial arabinosyl transferases. Arabinosyl transferase is an enzyme that is important in the polymerisation of arabinogalactan, a compound of the mycobacterial cell wall. (Dawei et al., 2011)

Isoniazid

The standard treatment for drug-susceptible TB consists of administration of rifampicin (RFP), PZA and a third drug, isoniazid (INH). This treatment takes 6–9 months and PZA is used only for the first 2 months, until susceptibility to INH and RFP is confirmed. (Blumberg et al., 2003)

Rifampicin and rifabutin

Rifamicines have allowed for the treatment of TB to be shortened from 18 to 24 months, to 9 months. By using them together with INH and PZA, treatment can be shortened to 6 months, without promoting drug resistance. (Mitchison, 2005).

The rifamicines kill TB by inhibiting bacterial DNA-dependent RNA polymerase inside the tuberculosis bacterium. RBT is a semisynthetic spiropiperidyl derivative of RFP. (Davies et al., 2007) Of the family of rifamicines, RFP is used the most, even though RBT has shown to be more effective in vitro. However, RFP is more cost effective. RBT is still used in the treatment of resistant TB. Approximately 12-20% of the RFP-resistant strains still remain susceptible to RBT, which makes it the favourable antibiotic to use in these cases. (Yew and Leung, 2008) In HIV patients, it is also the favoured treatment option, since it interferes less with HIV medication. (Nuermberger et al., 2010)

Streptomycin

Streptomycin (SM) was the first drugs to be discovered to treat TB. This aminoglycoside drug is derived from the actinobacterium *Streptomyces griseus.* It inhibits TB growth by binding to the 16S rRNA of the 30S rRNA subunit. By binding this rRNA, it causes misreading of the codon and eventual inhibition of protein synthesis. (Sharma et al., 2007) Not to be confused with the second-line injectables, SM must also be administrated through intramuscular injections, since it cannot be administrated orally. (Singh and Mitchison, 1954) SM belongs to the first-line drugs, but in practice is only used in settings where other first-line drugs are too expensive. (Sharma et al., 2007)

Second-line drugs

When patients cannot effectively be treated with the first-line antibiotics because of the occurrence of MDR-TB, the second-line antibiotics come in play. These antibiotics are recommended by the WHO to be reserved for the treatment of MDR-TB to minimalize the development of resistance to these antibiotics. The second-line antibiotics recommended by the WHO consist of three injectable medicines (amikacin, capreomycin and kanamycin), fluoroquinolones and three other drugs (ethionamide, p-aminosalicylic acid and cycloserine). (WHO model list of essential medicines, 2011)

For MDR-TB, treatment should consist of an intensive eight-month phase in which PZA (one of the first-line drugs) is supplemented with a minimum of four effective second-line drugs. These four should include a fluoroquinolone, an injectable, a thioamide and either cycloserine or teridizone. (Vora, 2010) When treatment is effective, after eight months, administration of the injectable should be stopped and the other for drugs should be administrated for a minimum of twenty more months. The drugs that are recommended in these groups by the WHO will be discussed: the three injectables, two fluoroquinolones, ethionamide, and cycloserine. In addition, para-aminosalicylic acid, which should be used when resistance is occurring in the first 8-month phase, will also be discussed. (Falzon et al. 2011)

Injectables

The injectables form a distinct group of medicines within the second-line drugs. The group consists of three antibiotics: amikacin (AMK), kanamycin (KAN) and capreomycin (CAP). AMK and KAN are aminoglycosides that bind to the 16S rRNA in the 30S ribosomal subunit of *M. tuberculosis* and thereby inhibit protein synthesis. The working mechanism of CAP is less well known, but it is thought to interfere with translation and thereby inhibit phenylalanine synthesis inside the *M. tuberculosis'* ribosome. It has been used since 1959 and is obtained from *Streptomyces capreolus.* (Handbook of Anti-Tuberculosis agents, 2008. page 89-91)

Fluoroquinolones

The fluoroquinolones are a big group of antibiotics. Their working mechanism relies on the inhibition of the *gyrA* and *gyrB* genes. These genes code for DNA gyrase, which makes the fluoroquinoles interfere with the mycobacterial chromosomal replication. Their MICs are lower than those of other (first-line) antibiotics. (Donald and Diacon, 2008) Fluoroquinolones can be used against active, replicating extracellular TB and latent intracellular TB. (Cole and Riccardi, 2011) Fluoroquinolones have the advantage that they usually cause fewer side effects than some other second-line antibiotics. (Yew and Leung, 2008)

The WHO recommends two drugs from the group of fluoroquinolones: ofloxacin (OFX) and levofloxacin. OFX is the favourable drug; however, levofloxacin can be its replacement. (WHO model list of essential medicines, 2011). OFX and levofloxacin (the L isomer of OFX) are favourable over the other fluoroquinolones because of their cost and effectiveness at higher dosing (750 mg or greater for levofloxacin). (Falzon et al., 2011) In addition, the administration of levofloxacin at high dose does not increase the amount of adverse effects. (Marra et al. 2005)

Fluroquinolones now belong to the second-line antibiotics that should be reserved for the treatment of MDR-TB. However, research is being done on the use of these antibiotics in the treatment of drug susceptible (DS) TB, in order to decrease the treatment duration of DS-TB from six to four or to three months. (Cole and Riccardi, 2011) In favourable settings, fluoroquinolones are associated with improved treatment outcomes when added to the treatment of MDR-TB. (Chan et al., 2004) However, in less favourable settings or situations, including HIV infected patients, outcomes have shown to be less positive. (Francis et al., 2008)

Other

Three other drugs are recommended by the WHO as second-line antibiotics in the battle against MDR-TB. These are ethionamide, para-aminosalicylic acid and cycloserine.

Ethionamide is part of a group of antimicrobial drugs called thionamides. Its working mechanism probably is the disruption of the mycolic acid of *M. tuberculosis*. This drug is recommended before the other second-line drugs like cycloserine, since it proved to be safe and active. (Prasad et al., 2006)

Para-aminosalicylic acid (PAS) is a drug that is recommended to be used when resistance or side effects are found in the intensive eight-month treatment phase. It has been in use since the 1940's, starting as a first-line drug in combination with INH and SM and after the first two decades as a second-line drugs. It should not be used in combination with ethionamide, since this combination can increase hepatotoxicity and the possibility of hypothyroidism. (World Health Organisation, 2008)

Cycloserine is an inhibitor of two enzymes in the mycobacterium: alanine racemase and D-alanine ligase. Alanine racemase produces D-alanine, which is the subunit of the terminal two amino acid residue of the peptidoglycan precursor lipid II. The two D-alanine residues are connected to each other by D-alanine ligase. By inhibition of these two enzymes, cell wall construction of the mycobacterium is disturbed. (Davis et al., 2005)

Third-line drugs

When resistance develops against the second-line antibiotics, this is called XDR-TB. The official definition of this XDR-TB is resistance against isoniazid and rifampicin, accompanied by resistance against any fluoroquinolone and to at least 1 of the 3 injectable second-line drugs.(Gandhi et al., 2010) The problem of XDR-TB calls for new drugs to be made and used. This is why, in addition to the first- and second-line drugs, a third category has been added recently: the third-line drugs. These consist of the most recently discovered agents against TB. The WHO has not recognised this category of medicines in its last version of the 'WHO model list of essential medicines' yet, but these new medications form the latest weapons in the battle against TB.

According to the Working Group on New TB Drugs, 21 new agents against TB are now (half-way in 2013) in (pre-)clinical trials. Some of these are entirely new antimicrobials, like bedaquiline, but they also include already known groups of antimicrobials like fluoroquinolones and rifamycines that first weren't used against TB.

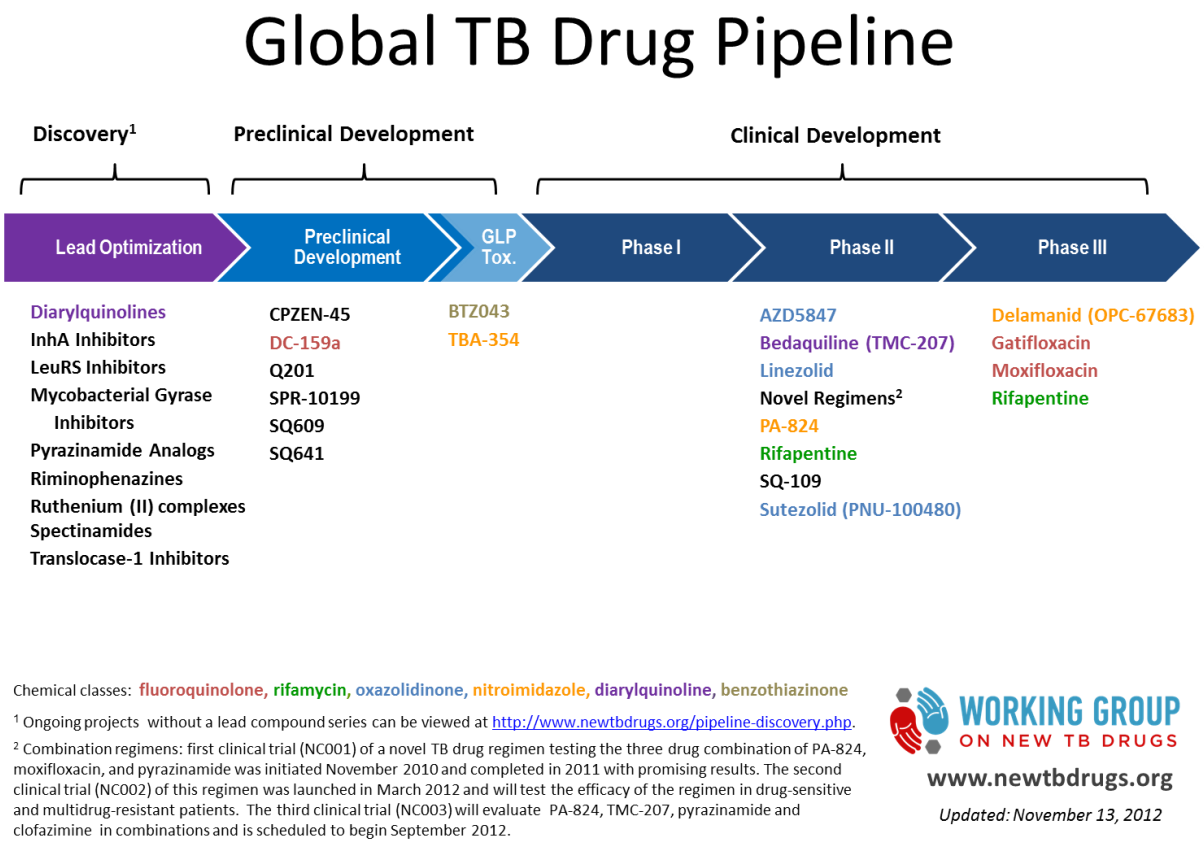

Figure 1 Antitubercular agents currently in (pre-) clinical trials. Obtained from http://www.newtbdrugs.org/pipeline.php on 30-04-2013

Most of these antibiotics are now in phase II or III of trials. Two of them, bedaquiline and delamanid, have been tested in clinical trials.

Bedaquiline

Of these drugs, bedaquiline (TMC207) was discovered in 2005 and has recently been the first antitubercular drug to be released onto the market in forty years, even though it is still in clinical trial. Currently, it is in phase II but it is expected to enter phase III in 2013. (Working group on new TB drugs, 2013) It is a member of the Diarylquinoline class of drugs and in clinical trial has shown to increase the proportion of patients with a sputum culture conversion from 9 to 48% when used in addition to standard therapy. Bedaquiline is a drug that targets mycobacterial ATP synthesis by sabotaging adenosine triphosphate synthase enzyme. It works against drug-sensitive and drug-resistant *M. tuberculosis* in culture and has also shown effect in patients with lung-TB. (Andreas et al., 2009) Current trials are running in Belgium and the United States and investigate the use of bedaquiline in the treatment of MDR-TB but also in combination with other standard medication to improve treatment of DS-TB. Bedaquiline has shown to better and speed up the treatment of TB, whether used with first- or second-line drugs. (Working group on new TB drugs, 2013)

Delamanid and PA-824

Delamanid is an antimicrobial currently in phase III of clinical trials. It is a nitroimidazole derivative that causes the cell wall to deteriorate by blocking the mycolic acid synthesis. It is currently in a clinical trial in which it is administrated orally to treat MDR-TB, to test the efficacy of this medicament in the treatment of HIV positive patients along with HIV negative patients. Former trials gave positive results and indicated that delamanid may be an effective tool in the bettering of MDR-TB treatment: delamanid was well tolerated and had low rates of discontinuation among the participants. (Zhang et al., 2013)

PA-824 is also a nitroimidazole derivative that is now being tested to treat DS-TB as well as MDR-TB and XDR-TB. (STOP TB Partnership, 2011) It is a prodrug that has shown to be active against latent and active TB. The conversion mechanism is not known yet. PA-824 works by the release of NO in the Mycobacterium, thereby killing it. The drug showed efficacy at low doses, without increased efficacy at increasing doses. (Tyagi et al., 2005)

Old drugs with new purposes

The problem of DR-TB calls for new medications but also for new use of known antimicrobials. Along with the completely new antimicrobial agents, there are also 'old' drugs currently under investigation for use in the battle against TB. Moxifloxacin and Gatifloxacin are fluoroquinolones that in the past have been and currently are used to treat other (respiratory tract) infections. However, they now are in the third phase of clinical trials in which they are used to treat TB. The advantage of fluoroquinolones is that they have less side-effect than other (second-line) drugs. The downside is the fact that resistance against one of the fluoroquinolones automatically means resistance against all others. (Working group on new TB drugs, 2013) In addition, moxifloxacin and gatifloxacin in the past have shown to not be highly potent antitubercular agents, which is why combination therapy will probably be required. (Drlica, 2004)

Also rifamicines that first weren't used against TB are now object of TB-research. Rifapentine is in the second and third phase of clinical trial for use in TB patients. The second-phase trial wants to test the

use of rifapentine to shorten the treatment of DS-TB. They use rifapentine in the intensive first eight weeks of treatment, instead of rifampicin. (Working group on new TB drugs, 2013)

Mutations causing resistance

Let us now look at the resistance genes that are now known or under investigation. This information is essential when we want to apply WGS to determine drug resistance.

Pyrazinamide

Although not much is known about the working mechanisms of PZA, a variety of mutations causing resistance in *M. tuberculosis* have been reported. First of these is the gene coding for the PZase that converts the PZA from prodrug to its active form: POA. This gene, *pncA*, when mutated, can form an inactive PZase, eventually causing a PZA-resistant *M. tuberculosis* bacterium. Another gene that is proposed to be involved in the working mechanism of PZA is *RpsA.* Mutated *RpsA* has been associated with resistance against PZA, however, no specific mutations have been reported. (Wanglian et al., 2011)

Ethambutol

EMB works by inhibiting arabinosyl transferase, which is encoded in the embCAB operon. This operon includes three different genes: *embC*, *embA* and *embB*. Up until now, many studies have been done on the association of mutations in these genes and resistance against EMB. Non-tuberculous mycobacteria with resistance against EMB have been investigated and showed substitutions of aminoacids in arabinosyl transferase encoded by *embB*. Many studies are done on mutations associated with EMB resistance. Most resistance-associated mutations were reported in *embB*. Some are connected to complete resistance and some to an increased minimal inhibition concentration (MIC). An overview of the exact mutations known to probably mark resistance is given in Table 2. (Starks AM et al., 2009; Safi H et al., 2008; Safi H et al.,2010) The most common mutation found in EMB resistant clinical strains is a mutation in *embB* at codon 306, making it the most reliable marker for resistance to EMB at the moment.

Table 1 Mutations in the mycobacterial embCAP operon associated with resistance against EMB

Gene	Mutation codon
embB	embB497, embB406, embB320 embB324, embB306 embB397 embB1024, embB44
embC	embC13

Isoniazid

INH is a prodrug activated by the KatG enzyme. Loss of function of this enzyme leads to resistance against INH. Also, mutations in inhA, the protein to which activated INH binds are reported to cause resistance against INH. Many studies have been performed to identify mutations causing the loss-of-function of KatG and the loss of ability of INH to bind to inhA. About 85% of INH resistant mycobacterial isolates have mutations in KatG and/or the inhA promoter. (Valvatne et al., 2009)

Rifampicin and rifabutin

Resistance against RFP and RFB are the most common types of resistance found in TB. Even in cases where RFP-resistant strains still are susceptible to RBT, the MICs are higher than in wild type strains, indicating a lower susceptibility to RBT also. However, these MICs can vary depending on the location of the mutations in the *rpoB* gene. The *rpoB* gene codes for a subunit of the RNA polymerase that RFP and RBT inhibit. Mutations in this gene may lead to a changed binding site, causing RFP and RBT to be unable or less able to inhibit the enzyme. (Yoshida et al., 2010)

Yoshida et al. examined the RBT MICs associated with different mutations in the 80 bp rifampicin resistance determining region (RRDR) in *rpoB*. They found 20 different mutations, of which the single-point mutations at codon 513, 525, 526, 531, 533, or 572 (detected in 72 of the total of 98 MDR-TB strains) influenced susceptibility to RBT. They also found that in these MDR-TB strains, novel mutations such as two strains with double-point mutations (Asp516Ala and Leu533Pro, or Ser512Ile and His526Pro) caused RBT resistance. In addition, they found one strain with an insertion (at codon 525), and one strain with a His526Ser mutation showing RBT resistance. Table 3 shows mutations now known to cause increased MICs or resistance to RBT. The researchers also found one mutations that was almost always associated with drug susceptibility to RBT: Asp516Va. Since resistance against RBT is always associated with resistance against RFP, mutations causing RBT resistance will also indicate RFP resistance. This is the same for INH, since resistance against RFP is almost always associated with resistance against INH. (Yoshida et al., 2010)

Previous studies also found a large part of these mutations. Especially mutations at codon 531, 526, 516 and 511 were found all over the world to be associated with RFP resistance. (Cengiz et al., 2002).Since the study described above also found these mutations associated with RBT, this again shows that RFP and RBT resistance is large caused by the same mutations.

Table 2 Mutations associated with resistance against RFP and RBT, or a higher MIC.

Gene	Location of mutations associated with increased MIC	Location of mutations associated with resistance
rpoB	rpoB513, rpoB525, rpoB526, rpoB531, rpoB533, rpoB572	rpoB516, rpoB526 rpoB516+rpoB533, rpoB512+rpoB526, rpoB525 insertion
rpoB, outside RRDR	Two mutations found by Yoshida et al., 2010 Exact location not reported	rpoB490 found by Cengiz et al., 2002

Streptomycin

The gene coding for 16S rRNA methyltransferase gidB, the *gidB* gene, is one of the genes reported to be involved in the development of resistance of TB against streptomycin. Mutations in this gene are found in resistant, as well as in susceptible strains though. (Wong et al., 2011)

The *rrs* gene is the second gene reported in the quest for mutations causing SM-resistance. It codes for the 16S rRNA in the 30S ribosomal subunit of *M. tuberculosis*. Mutations at lysine residue 42 or lysine residue 87 of the S12 ribosomal protein cause high-level resistance against SM. The S12 ribosomal protein interacts closely with the 16S rRNA near the binding site of SM. (Wong et al., 2011)

Second line drugs

Injectables

With the emerging problem of XDR-TB, it is becoming more and more important to rapidly determine drug susceptibility. However, screening for drug susceptibility in the injectables AMK, KAN and CAP with the help of cultures is complicated and can take weeks up to months, making the need for new and fast drug susceptibility tests even more important in the case of these medicines. (Pfyffer et al., 1999 and Shah et al., 2011) Where many studies have been performed on the mutations causing RFP resistance, less is known about the genetics of AMK, KAN and CAP resistant mycobacteria. (Boehme et al., 2010)

The genes *rrs, tlyA, gidB* and the *eis* promoter of *M. tuberculosis* are all found to be involved in resistance against the three injectable medicines used against TB: AMK, KAN and CAP. In these genes, studies have found mutations relative to the reference H37Rv genome. Among these are single nucleotide polymorphisms (SNPs), deletions and insertions; however, SNPs are most abundant. These mutations can cause the binding of the injectable drug to the target to fail or change the mechanism of action of the drug and thereby make a strain resistant. The well-studied mutations are found within the *rrs* and *tlyA* gene.

The first, *rrs*, is the gene coding for the 16S rRNA in the 30S ribosomal subunit of *M. tuberculosis*. The mostly reported mutations that are believed to cause resistance are A1401G, C1402T, and G1484T. A1401G is never observed in AMK- and KAN-susceptible mycobacteria, but was found in 7% of the CAP-susceptible bacteria. This makes A1401G a good potential indicator for AMK and KAN resistance, but a less reliable indicator for CAP-resistance.(Feuerriegel et al., 2009, Ioerger et al., 2009, Sirgel et al., 2011) SNP's G1484T and C1402T are commonly cited as being associated with resistance against all the injectables. However, others state that it is found in less than 1% of all isolates. Also, C1402T occurs in AMK and KAN susceptible strains as much as in AMK and KAN resistance strains, making it a less reliable marker for resistance. Another SNP which is found often in resistant strains, but much less in susceptible strains, is G1158T, making it a better marker for resistance than G1484T and C1402T.(Jugheli et al., 2009)

The second gene, *tlyA*, codes for a 29-O-methyltransferase that modifies nucleotide C1409 in helix 44 of the 16S rRNA and nucleotide C1920 in helix 69 of 23S rRNA. (Johansen et al., 2006) It is far less studied than the *rrs* gene. The *tlyA* gene has best been studied in research on CAP susceptibility. Mutations in this gene are extremely rare in CAP-resistant strains: a GT insertion at position 755 of the *tlyA* gene is the one mostly found. However, since all of the reported mutations are absent in CAP-susceptible strains, they are potential markers for CAP-resistance. (Maus et al., 2005, Feuerriegel et al., 2009 and Perdigao et al., 2010)

Relatively new is the association of a third gene with resistance against the injectable drugs: the *eis* promoter. The *eis* promoter enhances the intracellular survival of a *M. tuberculosis* related bacterium – *Mycobacterium smegmatis*. Now, it is also being investigated as a marker for resistance to KAN, when mutated in *M. tuberculosis*. The most frequently reported SNP is the G-10A SNP, which is found in about 20% of KAN resistant strains and in only 1% of KAN susceptible strains. The C-14T SNP is found in only 10% of KAN and AMK resistant strains, but also almost never in susceptible strains. Mutations were also reported in CAP resistance, but these appear to be less specific markers of resistance. (Engstrom et al., 2011 and Zaunbrecher et al., 2009)

A fourth gene has been identified that can cause resistance to the injectables when mutated. This gene, *gidB*, codes for a putative 16S rRNA methyltransferase and is also found to be a marker for streptomycin resistance. A G102 deletion is found with high frequency in AMK, KAN and CAP resistant isolates but almost never in susceptible strains. Some other mutations were found only in resistant strains, but these were far less frequent and thereby less reliable markers. (Wong et al., 2011)

Besides the mutations mentioned here, there are more mutations found that are associated with resistance against one or more of the injectables. However, whether they really cause resistance and therefor become useful as markers for resistance is not clear (enough) yet. (Via et al., 2010)

Compared to MDR-TB, the markers for XDR-TB are less well established and more research is needed to identify key-mutations causing resistance against KAN, AMK and CAP.

Table 3 Reliable known markers for resistance against injectables AMK, KAN and CAP

Gene	Mutation	Observed in resistance against
rrs	A1401G	AMK, CAP, KAN
	G1158T	AMK, CAP, KAN
tlyA	GT insertion at position 755	CAP
eis	G-10A	KAN
	C-14T	KAN, AMK
gidB	G120 deletion	AMK, CAP, KAN

Fluoroquinolones

TB resistance against fluoroquinolones in some regions can be caused by the extensive use of this group of antibiotics against respiratory tract infections. (Devasia et al. 2009; Ginsburg et al. 2003). Cross resistance can develop between the fluoroquinolones. [Ginsburg et al. 2003] The gene best studied in resistant strains is *gyrA*. Half of the fluoroquinolone-resistant strains have mutations in the quinolone resistance-determining region (QRDR) of *gyrA*. In g*yrB*, the mutations found are less reliable markers of resistance, since mutations are found in less than 20% of resistant strains. Mutations at *gyrA* codons 90, 91 or 94 are present in half of reported resistant isolates. g*yrA* and g*yrB* both need more extensive research on mutations, since mutations less frequently reported as gyrA90, gyrA91 and gyrA94 still could turn out to be good markers for resistance to fluoroquinolones. (Aubry et al., 2006)

Table 4 Mutations strongly associated with resistance against fluoroquinolones

Gene	Mutation codon
gyrA	gyrA90, gyrA91, gyrA94

Other

Ethionamide resistance has been associated with mutations in the *inhA* gene and the *ethA* gene. The mutations found in the *ethA* gene are not as closely associated with resistance as is the case with for example PZA and *pncA*. The role of the *ethA* gene is not understood yet, but the gene is highly conserved throughout the *Mycobacteria* species, suggesting that this gene is very important. (Glenn et al., 2003)

Para-aminosalicylic acid (PAS) resistance has been associated with mutations in the *thyA* gene. A fair amount of these mutations gave rise to amino acid substitutions in important sites of the enzyme it codes for: the catalytic site, binding site or co-factor binding site. (Mathys et al., 2009)

Third-line drugs

Since a part of the third-line drugs is fairly new and haven't been through all clinical trials yet, not a lot of research is done on the mutations causing their resistance so far. However, with knowledge of the working mechanism of these (new) medications and knowledge about resistance against them in other microorganisms, an educated guess can be made about their *M. tuberculosis* resistance genes. This would provide a starting point for future research on this subject.

Bedaquiline

For bedaquiline, research has already been performed to identify genes and mutations that are involved in resistance. For this first drug of the new class of diaryl quinolones, the mycobacterial ATP synthase is the target. A study identified six amino acid residues that can cause resistance when mutated. These amino acids are part of the subunit c that forms a C ring in the ATP synthase. The gene coding for this subunit c is the *atpE* gene. The researchers suggests that the six substitutions (Asp28→Gly, Asp28→Ala, Leu59→Val, Glu61→Asp, Ala63→Pro, and Ile66→Met), together with a tyrosine that is specifically conserved at position 64, form a cleft in the ATP synthase at which bedaquiline can bind. Here, it is anchored by ionic, hydrogen and halogen bonds at Glu61, Tyr64 and Asp28. Therefore, mutation of the codons coding for these amino acids can cause resistance. (Segala et al., 2012, Petrella et al., 2006)

Delamanid and PA-823

For delamanid, no studies were done to identify resistance genes yet. For PA-823, two resistance genes are known: *fgd1* and *ddn.* They code for a part of the five enzymes that are involved in the working mechanism of PA-823. No essential mutations are reported yet. Researchers do suggest that the genes causing resistance to PA-823, can also cause resistance against delamanid. (Feuerriegel et al., 2011)

Moxifloxacin, Gatifloxacin and rifamicines

For these drugs, no research has been performed to identify genes and mutations that cause resistance of *M. tuberculosis* against them. A starting point for these researches might be to look at prior research performed to determine these genes and mutations in other bacteria that are resistant against these drugs. Essential mechanisms and enzymes like ATP syntases that are found in most or all bacteria might have a good chance to also resistance in other types of bacteria when mutated.

Table 5 Overview of mycobacterial resistance genes

Antibiotic	**Resistance gene**	**Coding for**
PZA	*pncA*	Pyrazinamidase
RBT and RFP	*rpoB*	RNA polymerase B subunit
INH	*katG*	Catalase-peroxidase
	ahpC	Alkyl hydroperoxide reductase
	inhA	Mycolic acid biosynthetic pathway enzyme
EMB	*embB*	arabinosyltransferase
	embC	arabinosyltransferase
OFX	*gyrA*	DNAgyrase A-subunit
SM, KAN, AMK and CAP	*rrs*	16S rRNA
KAN	*eis*	Aminoglycoside acetyltransferase
	gidB	7-methylguanosine methyltransferase
SM	*rpsL*	Ribosomal protein S12
CAP	*tlyA*	A putative rRNA methyltransferase
PZA	*RpsA*	30S ribosomal protein S1
ETH	*ethA*	Unknown
PAS	*thyA*	Thymidylate synthase
Bedaquiline	*atpE*	ATP synthase
Delamanid, PA-823	*Fgd1, ddn*	

Whole genome sequencing: the future in drug susceptibility testing?

We have seen that much research is being done to develop new drugs and try new drug combinations to treat MDR- and XDR-TB. The mutations causing there resistance are subject of many researches also. These researches so far have given us many data, but how valuable are these and what remains to be done?

MIC: in need of definition?

For some drugs, whole genome sequencing for drug susceptibility testing is closer than other. For MDR-TB, for example, whole genome sequencing can be simplified. Since INH, RFP and RBT resistance are associated with each other, screening for mutations causing RBT resistance also immediately is screening for INH and RFP resistance. However, if no mutations are found that indicate RBT resistance, this does not automatically mean that the strain is also susceptible to INH and RFP. There for, mutations causing only INH and RFP resistance are also important. First step in whole genome sequencing would be to trace MDR-TB, though it still needs a lot of research, especially on pyrazinamide. Also, determining resistance to RBT remains problematic, since there are many mutations known to increase the MIC. However, for practical use, it remains unclear what it means for treatment if there is a higher MIC in contrary to complete resistance.

This problem of lack of consensus on MIC values is also a problem in the move towards whole genome sequencing. Different studies use different MIC values to determine impaired susceptibility and resistance. To be able to create international protocols for doctors and laboratories to determine resistance with WGS, consensus should be reached on what MICs indicate resistance. Only then, all researchers trying to identify mutations can work efficiently and make WGS a workable method.

Importance of mutation site

Some studies mention if the mutations in codons they find also cause changes in the amino acid they code for (for example Mathys et al., 2009), but some only mention they found a mutation. Even if a change in amino acid is found, there is no mentioning of the location of this amino acid. Mutations in the active site of an enzyme or the binding site for the antibiotic may cause resistance, just like deletions. However, no study reported in this essay mentioned the possible functional effects of the found mutations. Most articles just ‘associate’ their mutations with resistance, but do not attempt to explain the mechanism by which these mutations cause resistance. Attempts to elucidate the mechanism by which insertions, deletions or SNPs cause resistance could increase the reliability of certain mutations as markers for antimicrobial resistance. One of the other weaknesses now present in research on mutations causing resistance is the absence of research in which mutations are introduced in susceptible strains to test their resistance causing potential. In an ideal situation, frequently found mutations associated with resistance should be promoted in a drug susceptible strain. If this genetically modified mycobacterium then becomes resistant against one or more antimicrobials, it can be said for sure that this mutation causes resistance. However, these tests can be very costly, which makes it unlikely that this will ever be done for all mutations and drugs.

In vivo and in vitro correlation

As this chapter argues, connecting specific genotypes to resistance to certain antimicrobials brings along uncertainty. The first thing that must be carefully determined is the quality of in vitro testing to predict antimicrobial resistance. After all, these in vitro susceptibility tests are used to correlate mutations to resistance. If the correlation between in vitro and in vivo susceptibility of *M.*

tuberculosis to an antimicrobial is not 100%, the WGS-test will never be a completely accurate predictor of the efficacy of the antimicrobial in a patient, even if in vitro susceptibility can be accurately predicted by WGS.

Some studies have been performed to investigate the correlation between the outcomes of in vitro studies on the drug susceptibility and in vivo studies on the treatment success of non-tuberculous mycobacteria. Some of these have shown (for example Heginbothom, 2001) that predicting the treatment outcome by in vitro susceptibility tests remains problematic, although others (for example Fantin et al.(1991) on the correlation between in vitro and in vivo activity of antimicrobials against gram-negative bacilli) find the opposite and state that in vitro testing provides a good prediction on in vivo activity. No specific research was found on this correlation regarding the activity of antimicrobials used to treat tuberculosis. The lack of literature on this correlation for all antimicrobials used to treat TB is a problem that has to be tackled before in vitro susceptibility testing can become an entirely trustworthy method for in-patient activity prediction.

As we have seen, there are still many problems to be tackled before WGS can be implemented as a means to accurately predict drug susceptibility of TB. However, many attempts are already made and currently being made to create genome tests that can predict drug susceptibility.

For MDR-TB, some molecular detection methods are already available, but for XDR-TB these are less reliable. For the mutations found in XDR-TB there is less evidence for their resistance causing potential. The second and third-line drugs need a lot of research on the mutations causing resistance against them, even more so than MDR-TB. Fluoroquinolones are a special group in here, since cross resistance occurs. On one hand it means that even one detected mutation can cause resistance against an entire group, so even with minor information, resistance can be determined. On the other hand, it means that actually all mutations should be known in order for whole genome sequencing to be effective here: if no mutation is found but in reality there are resistance causing mutations present that have not been reported yet, this means that complete resistance against all of the fluoroquinolones might be missed.

Törör and Peacock (2012) look beyond all the limitations mentioned before and argue that the largest barrier to overcome before we can implement WGS for our purpose is ‘the lack of automated interpretation software that translates sequence data to provide information that can used by microbiologists (rather than bioinformaticians), that is clinically meaningful and that is generated with a short turnaround time’. Although this paper argues that there are far more details that should be worked on, Törör and Peacock make a point here: eventually, all the information we obtain about resistance genes should be gathered in one database, after which a computer program can scan these data for possible resistance gene matches when we have a patient with tuberculosis. Besides microbiologists and geneticists, bioinformaticians will be needed to develop databases and software to make WGS possible for our purpose.

Conclusion

Currently multiple corporate and academic groups worldwide are working to ultimately create genotyping tests that can be used to determine MDR- and XDR-TB quick and cheap. Taking together the research that has up until now been done on resistance genes in *M. tuberculosis* and the current status of whole genome sequencing techniques, it can be concluded that there is a long way to go until whole genome sequencing can replace the current drug susceptibility tests. Genome

sequencing costs drop quickly, but a lot of research remains to be done before all or most mutations causing resistance are identified. The first step would be to be able to identify MDR-TB. Still, even when all mutations are mapped, we should remember that what causes resistance in vitro does not have to do so in vivo or in all humans. This means it remains uncertain whether genome sequencing of *M. tuberculosis* will ever provide a fully 100% reliable method for screening for resistance. The use of whole genome sequencing as a drug-susceptibility test would provide a much needed new tool in the treatment of TB, however, a lot of problems need to be solved before it can be widely applied. Taking one step and one drug at the time and with the help of interdisciplinary research and approaches, whole genome sequencing can become more and more valuable in the signalling of drug resistance and ultimately the treatment of TB.

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Images

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