DRUG SUSCEPTIBILITY TESTING FOR OPTIMISING TUBERCULOSIS TREATMENT

SAMi SIMONS
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The research presented in this thesis was performed at National Tuberculosis Reference Laboratory of the National Institute for Public Health and the Environment (RIVM) in Bilthoven and at the Department of Respiratory Medicine of the Radboud University Medical Center in Nijmegen, the Netherlands.

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CHAPTER ONE

Introduction and outline of the thesis
The case of Andrew Speaker
In May 2007, Andrew Speaker hit the headlines when he took two transatlantic flights while presumably having extensively drug-resistant tuberculosis (XDR-TB). He had been diagnosed with multidrug-resistant tuberculosis (MDR-TB) two weeks before and had been advised by local health authorities not to travel. Speaker flew from Atlanta to Paris anyway and on to Greece and then to Italy for his honeymoon. Upon his return to the US, the CDC issued an international alarm asking airline passengers and cabin personnel from the two transatlantic flights to be tested for TB. This was quickly picked up by international media, creating an international scare for a deadly bug spreading across Europe. The history of drug-resistant tuberculosis
This scare of a patient with drug-resistant tuberculosis travelling through Europe proved to be a reality check on the way we looked at (multi)drug-resistant tuberculosis. Once the world’s most important cause of death, global tuberculosis incidence and case fatality rates fell thanks to the use of a standardised combination of different antituberculosis drugs under directly observed therapy (DOTS). These first-line antituberculosis drugs are rifampicin, isoniazid, pyrazinamide and ethambutol. Treatment success rates of drug-susceptible TB have steadily improved and cure rates reached their highest level of 87% in 2009. These advances in antituberculosis therapy led to the widespread believe that TB had been conquered and TB was a disease of the past. As TB cases dropped in wealthy countries, funding for research dried up and TB programs were cut by governments. In the resource-limited countries, national TB programs kept on relying on microscopy as the basis for case detection and the treatment approach was based on the empirical use of first-line drugs. Within these settings drug-resistant tuberculosis emerged. By the 1990s, the city of New York saw a foretaste of the TB threat the world was awaiting. A sharp rise of MDR-TB cases, i.e. Mycobacterium tuberculosis complex strains resistant to the two most effective first-line drugs rifampicin and isoniazid, was seen in New York City in the beginning of the 1990s. These notifications exceeded those of many low-income countries in some parts of the city and by 1991 New York City accounted for a remarkable 61% of all cases of MDR-TB in the United States. Public health care officials developed a plan to combat this MDR-TB epidemic which consisted of proper infection control methods, improved diagnostic laboratory methods, and directly observed therapy with at least four effective drugs. As a result, new cases decreased substantially within two years. The New York experience offered a blueprint for combating the MDR-TB threat globally, yet it was not taken up by international policy makers at that time. Treating MDR-TB was thought to be too expensive and complex. Moreover, a focus on treating MDR-TB would distract attention from the highly cost-effective DOTS strategy. Drug susceptibility testing was not widely advocated because it was thought that the long turn-around-time and subjective readings of tests on solid medium were insufficient to guide treatment decisions. As a consequence, new antituberculosis drugs, such as fluoroquinolones, were added to failing TB regimens without a concomitant upscale in laboratory diagnostic services. And by 2005, physicians treating multidrug-resistant tuberculosis were faced with an even bigger threat, extensively drug-resistant tuberculosis (XDR-TB). This form of tuberculosis is caused by strains of Mycobacterium tuberculosis complex that are not only resistant to rifampicin and isoniazid, but also to fluoroquinolones and second-line injectables (capreomycin, amikacin, kanamycin). Outbreaks of XDR-TB reporting high mortality rates in South Africa finally resulted in the highly needed changes in the way TB programs were organised. Since then, strategies such as the strengthening of health care services, the rollout of new technologies for detecting drug-resistant tuberculosis and the improved access to drugs have resulted in declining trends of MDR-TB in some regions of the world. Current challenges to global tuberculosis control
Today, MDR-TB and XDR-TB remain a threat to global tuberculosis control. In their 2012 global report, the WHO has estimated that approximately 4% of new TB cases and 20% of previously treated TB cases are diagnosed with MDR-TB. XDR-TB is seen in 9% of all MDR-TB notifications and the number of countries reporting XDR-TB cases is increasing. The MDR-TB notifications are often alarmingly high in countries of the former Soviet Union. In Minsk, Belarus, for example, MDR-TB is diagnosed in 35% of new cases and in 77% of previously treated cases. A major barrier for global TB control has been the lack of access to accurate tests for diagnosing MDR-TB and XDR-TB. First-line drug susceptibility testing (DST) is performed in less than 4% of new TB cases and 6% of previously treated cases. As a consequence, many MDR-TB cases remain undetected. New technologies based on identifying resistance-associated mutations in the DNA of M. tuberculosis complex bacteria may provide results within hours and may have the potential to serve as rapid screening tests for drug resistance. The recent introduction of the Xpert MTB/RIF assay, detecting both M. tuberculosis as well as mutations associated with rifampicin resistance, has been an important step up in improving rapid
diagnosis of MDR-TB. It is has been rolled out worldwide and is currently recommended as the first diagnostic assay for individuals suspected to have (MDR)-TB in high-burden settings.14

A second barrier has been the availability of quality assured antituberculosis drugs once the diagnosis of MDR-TB has been made.15 To increase the arsenal of anti-tuberculosis drugs, a lot of effort has been put into developing new compounds.16 New drugs effective against MDR-TB and XDR-TB, such as bedaquiline and delamanid, have passed the first regulatory hurdles and have reached the market. Another successful approach has been to repurpose existing drugs for tuberculosis treatment, such as linezolid, or to optimize the dose and thus its efficacy of existing antituberculosis drugs, such as rifampicin. Next-generation antituberculosis regimens are likely to consist of a combination of several of these new and already existing drugs. The ultimate goal of these next-generation treatment regimens will be to ensure that all patients with drug-resistant tuberculosis can be confident that their regimen will be safe and effective.17

**Aligning new treatment regimens with drug susceptibility testing**

To be safe and effective, the introduction of next-generation treatment regimens should be aligned with drug susceptibility testing (DST).18 Information on DST is essential because it can identify patients who will require early adaptation of the standard treatment. DST will also prevent patients from taking ineffective but toxic drugs for an extended period, which could lead to amplification of drug resistance. On a national level, DST can be used to monitor patterns of emerging drug resistance and may help to decide if introducing new treatment regimens will be successful. Ultimately, the alignment of DST with new treatment regimens is a prerequisite for reversing the global threat of MDR-TB and XDR-TB.

An optimal DST strategy should be sought for each new treatment regimen. Which DST strategy is best depends on the prevailing drug resistance level, the selection of patient population eligible for testing, and the health-care system implementing the algorithm.19 This should be preceded by a thorough understanding of the accuracy and performance of different diagnostic strategies, in real-life settings and under varying epidemiological situations. This is the main topic of this thesis.

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**Outline of the thesis**

This thesis is divided into two parts. The main part focuses on different strategies for optimising the diagnosis of drug-resistant tuberculosis. Chapter 2 introduces the different modalities for drug susceptibility testing (DST) in tuberculosis. It describes the history of DST from the pivotal article by Canetti in 1963 to the recently developed molecular assays such as the Xpert MTB/RIF and the MTBDRsl test. Chapters 3 to 5 address the difficulties in detecting pyrazinamide resistance, a first-line antituberculosis drug with increasing importance in the treatment of drug-resistant tuberculosis. Caveats in the phenotypic and molecular diagnosis of pyrazinamide resistance in both susceptible and multidrug-resistant tuberculosis are discussed. Chapters 6 and 7 focus on the accuracy of three DST assays for detecting drug-resistant tuberculosis. In chapter 6, we describe the diagnostic accuracy of the Genotype® MTBDRsl molecular assay and the MGIT960® liquid culture based method for detecting resistance to second-line antituberculosis drugs compared to the Middlebrook 7H10 agar dilution method. In chapter 7, we report on the performance of the Genotype® MTBDR®plus and MTBDRsl assay for detecting drug resistance in the Netherlands and discuss the role of these molecular tests in low-level drug resistance countries.

The second part of this thesis focuses on the potential of existing drugs for treating drug-resistant tuberculosis. The antimycobacterial activity of efflux pump inhibitors is described in chapter 8, where we present the in vitro activity of multiple phenothiazines and SILA421, an organosilicon compound, against a panel of M. tuberculosis strains with varying susceptibility profiles. The antimycobacterial activity of other drug classes is described in chapter 9, where we present the in vitro antimycobacterial activity of fusidic acid, nitrofurantoin, mefloquine, co-trimoxazole, amoxicillin with clavulanic acid and meropenem with clavulanic acid. The results provided within the framework of this thesis should contribute to a better understanding of the accuracy and performance of the different diagnostic modalities in real-life settings and under different epidemiological settings. This thesis concludes with a summary of the main results in chapters 10 and 11, together with a general discussion and suggestions for future research in chapter 12.
REFERENCES

CHAPTER TWO

Methods for drug susceptibility testing in tuberculosis

Partly based on:
Introduction

Tuberculosis (TB) has brought misery to the world since ancient times. It used to be one of the major causes of death worldwide with case fatality rates approximating 70%. And still today the global burden of TB remains enormous. Yearly, around 8.7 million new cases of TB are diagnosed and 1.4 million people die of TB. With this gloomy view in mind, the euphoria caused by the discovery of streptomycin in the early 1940s is not so surprising. It marked a new era in TB treatment and changed the prognosis from dismal to the expectation of cure. Soon after the discovery of streptomycin, studies noted the occurrence of streptomycin resistance during streptomycin monotherapy. Importantly, this resistance undermined treatment success for TB. Pyle and colleagues made a crucial observation; they showed that M. tuberculosis strains isolated from the sputum before treatment were not uniformly susceptible to streptomycin. Crofton and Mitchinson inferred from these observations that streptomycin monotherapy might favour the growth of resistant subpopulations. 8

The origins of drug resistance

This existence of resistant subpopulations still underlies our current idea on the emergence of primary drug resistance in M. tuberculosis. It is thought that all larger populations of M. tuberculosis naturally contain mutant subpopulations encoding resistance against all antituberculosis drugs and that in drug-resistant M. tuberculosis strains the proportion of resistance bacilli is considerably higher than in susceptible strains. Such resistant subpopulations arise through spontaneously occurring mutations and are generally considered not to be of clinical importance in wild type M. tuberculosis strains. It is during drug treatment that resistant subpopulations can multiply depending on the selective antibiotic pressure. Drug concentrations below the minimum inhibitory concentration (MIC) of a mutant subpopulation but above the MIC of a susceptible subpopulation favour the growth of the mutant subpopulation, thereby selecting for the development of a drug resistant M. tuberculosis strain.

Whether an M. tuberculosis isolate should be considered as resistant thus depends on the proportion of resistant subpopulations for a given concentration of an antituberculosis drug. It has been previously defined that a M. tuberculosis strain should be considered susceptible if this proportion does not exceed 1% of the total population. 8-10 For both pyrazinamide and ethambutol this proportion originally was 10%. 10 It could be determined by testing a M. tuberculosis culture against a series of drug concentrations and determining the minimal drug concentration inhibiting growth (the MIC). 8 Rather, mostly a single cut-off concentration is used to determine whether an isolate should be considered to be resistant or not, the so-called critical concentration. 11,12 Ideally this concentration should lie between the MIC of susceptible and resistant strains, but it is mostly derived from a MIC distribution of wild strains of M. tuberculosis that have never been exposed to drugs and determined as the concentration inhibiting the growth 95% (90% for pyrazinamide) of the wild type strains. 13,14,15

Drug susceptibility testing on solid media

The 1%-rule forms the basis of our current in vitro drug susceptibility testing (DST) and has already been laid out in 1963 by Canetti and colleagues. They proposed three methods for DST: the absolute concentration method, the resistance ratio method, and the proportion method. In the absolute concentration method M. tuberculosis bacteria are incubated on media that contain various drug concentrations including the critical concentration. 14,15 Growth at and above the critical concentration is interpreted as resistant. In the resistance ratio method, the MIC is divided by the MIC of the M. tuberculosis H37rv reference strain giving a ratio. A ratio of > 2 is considered as resistant. 16 In the proportion method, multiple standard dilutions of the inoculum are used. The number of colonies are then counted on drug containing medium from the most diluted inoculum and compared with the number of colonies in on drug free medium. 8-10 The isolate is considered to be resistant if the number of resistant bacillary units is more than 1% (or 10%) at the tested concentration. 8-10 Though initially described for DST in the egg-based Löwenstein-Jensen agar, the proportion method was later on adjusted to the Middlebrook 7H10 agar and is still propagated for second-line DST by the World Health Organization. 16

Drug susceptibility testing (DST) did not gain much popularity after its standardization. The first argument against the use of DST was that the extended turn-around-time of 6-8 weeks in the laboratory could not influence treatment decisions in a timely manner. 17 The second argument against the use of DST was related to the successful use of multiple drugs for treatment. 17 Because the antituberculosis regimen combining 4-5 drugs was very potent, it was thought that this would completely abolish failure and relapse resulting from initial drug resistance. 17 Together with the low prevalence of drug resistance among M. tuberculosis isolates, systematic surveys on drug resistance were not widely advocated.

Drug susceptibility testing in liquid medium

In the late 1970s liquid culture methods for DST were developed. In contrast to the earlier developed methods in which growth was detected by visual inspection, these liquid culture systems could detect growth of M. tuberculosis automatically. One of the first versions of this approach (the BacTec460, BD Biosciencem Sparks, Md, USA) utilized a radioactive label for the detection of growth. 18 MIC determination by liquid culture systems is performed by inoculating media with different drug concentrations together with a 1:100 inoculum dilution in a drug-free vial. 17 Its methodology resembles to proportion method and therefore is sometimes referred to as proportion method in liquid media. 17 To represent 99% inhibition,
the MIC is defined as the lowest drug concentration that yields growth index readings lower than those in the drug-free tube. 17

Its introduction was a major improvement in turn-around-time for DST in TB: an average of 18 days was required by the BacTec 460 radiometric method for complete recovery and drug susceptibility testing of \textit{M. tuberculosis}, as compared with 38 5 days for the conventional solid medium methods. 18 Furthermore, accuracy for detecting resistance to first-line and second-line antituberculosis drugs was excellent. 19-22 The BacTec 460 radiometric method, however, had its disadvantages and has largely been abandoned as a tool for drug susceptibility testing. Instead, nonradiometric liquid culture systems have been developed, such as the MB/BacT system and the Mycobacteria Growth Indicator Tube (MGIT) 960 system. The MGIT is most extensively studied and is likely to become the new gold standard. 23

Introduced in 1995, the MGIT 960 is a nonradiometric culture system to culture \textit{M. tuberculosis} from clinical specimens and to perform DST. 24 The MGIT medium consists of a modified Middlebrook 7H9 broth and uses a fluorescent technology to measure mycobacterial growth. 25 This technology is based on the indirect detection of the oxygen consumption of mycobacteria in the broth. The initial concentration of oxygen quenches fluorescence, but as mycobacterial growth occurs and oxygen is taken up, the lower oxygen concentration permits the indicator to fluoresce. This fluorescence is detected using a 365-nm UV transilluminator. Drug susceptibility testing in the MGIT was initially set up -and currently almost invariably used- as a qualitative system, 26 which means only one critical concentration of the drug is tested for growth of a clinical \textit{M. tuberculosis} isolate. Similar to the BacTec 460 system, drug susceptibility is measured by comparing fluorescence -and hence growth of \textit{M. tuberculosis}- in drug containing tube with that of a drug-free control tube incubated with a 1:100 diluted inoculum. If relative growth in de drug tube is equal or exceeds growth in the control tube, the isolate is considered resistant. If the relative growth is less than that in the growth control tube, the isolate is considered susceptible.

In 1999, a multi-centre study was published comparing the DST results of the MGIT system against DST results of the BacTec 460 for streptomycin, ethambutol, rifampicin and isoniazid. 27 The proportion method on solid medium was used as the gold standard in case of discordant results. A high agreement rate was observed (98.6%) among the 440 isolates tested. The authors concluded that drug susceptibility testing in the MGIT for first line drugs is as accurate as the BacTec 460. Recent meta-analyses have confirmed the accuracy and reliability of the MGIT 960 system to detect resistance to first-line antituberculosis drugs (Table 1). 28,29

Drug susceptibility testing to pyrazinamide for the MGIT system was introduced in 2002 by Pfyffer and colleagues. 30 Using a modified 7H9 broth with a pH value adjusted to 5.9, the MGIT pyrazinamide medium differs slightly of that for other first-line drugs. A review by Piersimoni and colleagues has shown that, at the critical concentration of 100 mg/l, sensitivity of the MGIT for detecting pyrazinamide resistance was between 96-100% and specificity was between 87-100%. 31 The lower specificity is caused by higher rates of false positives in the MGIT. This was confirmed in a recent study by Chedore and colleagues. 32 In their study on 743 isolates, 57 were found resistant using the MGIT 960. Repeated testing with the BacTec 460 and Wayne’s assay found that 24 isolates (4.2%) were in fact pyrazinamide susceptible.

In 2006, Rusch-Gerdes and colleagues demonstrated that the MGIT could also be used for DST to second-line antituberculosis drugs. 33 Validation of DST to second-line antituberculosis drugs in the MGIT system has been hampered however by differences in methodology between the various studies, the lack of standardisation in drug preparation and drug concentrations, and the lack of sufficient studies on clinical specimens. 34,35 Moreover, definite critical concentrations in the MGIT 960 system for most second-line drugs have not been well established. 36 This is because MICs of resistant and susceptible isolates of many second-line drugs are close to each other. 37 Also, data on the relationship of critical concentrations and clinical outcome are lacking for many second-line drugs. 38 As a consequence, DST to second-line antituberculosis drugs in the MGIT 960 system has not been sufficiently validated and standardized.

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>CCc mg/L</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>1.0</td>
<td>99.7 (74.3-100)</td>
<td>94.3 (76.7-98.8)</td>
<td>96.4 (94.0-98.9)</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.1</td>
<td>98.9 (94.4-99.8)</td>
<td>98.2 (95.4-99.3)</td>
<td>98.7 (97.7-99.7)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1.0</td>
<td>98.2 (92.9-99.6)</td>
<td>99.6 (98.5-99.9)</td>
<td>99.5 (98.6-100)</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>5.0</td>
<td>83.9 (72.7-91.1)</td>
<td>95.8 (80.9-99.2)</td>
<td>95.3 (92.5-98.0)</td>
</tr>
</tbody>
</table>

* Estimates are given in percentages with 95% confidence interval in brackets
  * CC, critical concentration given in mg/l

Diagnostic accuracy of the MGIT 960 system to detect resistance to first-line antituberculosis drugs a,b

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a Adapted from reference [29]

b Estimates are given in percentages with 95% confidence interval in brackets

c CC, critical concentration given in mg/l
Molecular detection of drug resistance

Primary drug resistance in *M. tuberculosis* arises via the selection of variants with spontaneously occurring mutations. This mutant selection depends on the available drug concentration. If these mutations occur in restricted genomic sites, they may be used as a surrogate marker for drug resistance. This association between point mutations and drug resistance forms the basis for our current molecular DST techniques and has markedly fastened the diagnosis of drug resistance.

Molecular targets for first-line antituberculosis drugs are listed in Table 2. Point mutations in the central region of the RNA polymerase beta subunit gene (*rpoB*), the main binding site for rifampicin, are seen in up to 96% of rifampicin resistant isolates. Isoniazid resistance is most often linked to mutations in the *katG* gene encoding the catalase-peroxidase enzyme which is necessary for isoniazid activation. Mutations in this gene are seen in 50–95% of isoniazid resistant isolates and are usually associated with high-level resistance. Low-level resistance is associated with mutations in the promoter region of *mabA/inhA* operon and occur in 8–43% of isoniazid resistance isolates. Resistance to the other first-line antituberculosis drugs has also been linked to certain target gene sequences, but are less frequently used for molecular drug susceptibility so far. Ethambutol resistance has been linked to mutations in the *embB* gene in 47–65% of isolates, and pyrazinamide resistance has been linked to mutations in the pyrazinamidase (*pncA*) gene in 72–95% of isolates.

The first commercially available molecular techniques have mainly focused on analysing the *rpoB* gene because of its high agreement with phenotypic resistance and because mutations in this gene are associated with resistance to first-line antituberculosis drugs. Solid-phase hybridization techniques, or line probe assays (LPA), were one of the first of such commercially available molecular assays which were widely deployed. LPAs are based on the reverse line blot hybridization of oligonucleotides on paper strips on which specific probes with wild type and mutant sequences have been immobilized. The target gene sequences in the *M. tuberculosis* gene are amplified, denatured to get them single stranded, and applied to the strips with the specific probes. Hybridization is revealed by the development of a coloured reaction on the strip.

The GenoType MTBDRplus (Hain Lifesciences) assay is an example of such a LPA for the diagnosis of rifampicin and isoniazid resistance. With this assay mutations are identified in the *rpoB* gene as well as mutations in the *katG* gene and in the *inhA* gene. A study in South-Africa showed that time to diagnosing MDR-TB could be shortened to 2 days by using the MTBDRplus assay. A recent meta-analysis confirmed that the Genotype MDRTBplus had an excellent diagnostic accuracy for direct drug susceptibility testing on clinical samples. For rifampicin, the pooled sensitivity was 99% (96–100%) and pooled specificity was 99% (95%– CI: 98%–100%). Direct susceptibility testing for isoniazid yielded similarly results. Pooled sensitivity was 96% (95%–CI: 93%–99%) and pooled specificity was 100% (95%–CI: 99%– 100%).

Given that the GenoType MTBDRplus is a LPA, it suffers from certain drawbacks. LPAs are only registered for use on sputum smear-positive specimens and on *M. tuberculosis* cultures. This is because its applicability in smear-negative sputum samples is relatively poor. Other drawbacks are its high costs and the requirement for separate clean room facilities to avoid contamination of the amplification reaction. Nonetheless, recent studies seem to indicate that the deployment of an LPA in the diagnosis of rifampicin resistance may be cost-effective and applicable in rural areas, and may even shorten time to diagnosis of MDR-TB by a median of 6 weeks.

Recently, a LPA for the detection of resistance to second-line antituberculosis drugs has been developed; the GenoType MTBDRsl assay. This test is based on the association between fluoroquinolone resistance and mutations in the *gyrA* gene and the relation between mutations in the *rrs* gene and resistance to second-line injectables. Mutations in the *gyrA* gene for instance have been detected in up to 92% of fluoroquinolone resistant isolates and mutations in the *rrs* gene have been detected in up to 85% of amikacin and capreomycin resistant isolates. The MTBDRsl LPA contains six probes targeting the most common *gyrA* mutations and two probes targeting *rrs* gene mutations. The MTBDRsl strip also contains two probes for detecting mutations in the *embB* gene that are associated with ethambutol resistance. Hillemann and colleagues have tested the Genotype MTBDRsl in 106 clinical samples, sixty-three isolates of which were multidrug resistant. Overall, sensitivity for

<table>
<thead>
<tr>
<th>Antituberculosis drug</th>
<th>Target gene</th>
<th>Product</th>
<th>Frequency among drug-resistant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td><em>rpoB</em></td>
<td>β subunit of RNA polymerase</td>
<td>&gt; 95%</td>
</tr>
<tr>
<td>Isoniazid</td>
<td><em>katG</em></td>
<td>Catalase-peroxidase</td>
<td>50-95%</td>
</tr>
<tr>
<td></td>
<td><em>inhA</em></td>
<td>Enoyl-ACP reductase</td>
<td>8-43%</td>
</tr>
<tr>
<td>Ethambutol</td>
<td><em>embB</em></td>
<td>Arabinosyltransferases</td>
<td>47-65%</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td><em>pncA</em></td>
<td>Pyrazinamidase</td>
<td>72-95%</td>
</tr>
</tbody>
</table>

* Adapted from reference [11]
diagnosing fluoroquinolone, amikacin, capreomycin, and ethambutol resistance was 90%, 83%, 87% and 59%, respectively. Specificity was 100% for diagnosing fluoroquinolone, amikacin and ethambutol resistance and 99% for diagnosing capreomycin resistance.

Though the LPAs have significantly reduced the time to detection of MDR-TB, it is the recent development of Xpert MTB/RIF test that has been an immense leap forward in the rapid diagnosis of MDR M. tuberculosis. The Xpert MTB/RIF test utilizes a hemi-nested real-time PCR assay to amplify a MTB-specific sequence in the rpoB gene after which the wild type or mutated sequences are detected by molecular beacons. The assay is fully automated; the only manual step is the addition of a bactericidal and liquefying buffer to the sputum before transferring it to a disposable plastic cartridge. A major advantage of this approach is that the whole procedure is performed in closed cartridges, thereby avoiding cross-contamination of amplicons.

The value of the Xpert MTB/RIF has been shown in two recent studies. The first study was a prospective, multi-country study of 1,730 patients suspected of having TB. Sensitivity of case detection for smear-positive patients was excellent (98.2%), but somewhat lower for smear-negative patients (72.5%). This increased to 90% when Xpert MTB/RIF was performed on three sputum samples. The Xpert MTB/RIF correctly detected rifampin resistance in 209 of 211 patients (99.1% sensitivity) and in all 506 patients with rifampin-susceptible TB (100% specificity).(18) A second, very recent study assessed the operational feasibility of the Xpert MTB/RIF assays in routine primary health-care centres in tuberculosis-endemic settings. In this second study, 6,648 participants with suspected (MDR) tuberculosis were enrolled. The Xpert MTB/RIF assay had a better sensitivity for detecting TB cases compared to direct microscopy (90.3% vs. 67.1%). Moreover sensitivity was not lower in HIV co-infected cases. Implementing the Xpert MTB/RIF assay reduced time to detection of rifampicin resistance to 1 day compared to line-probe assays (20 days) and phenotypic drug-susceptibility testing (106 days). This led to a reduced time-to-treatment of rifampicin resistant cases. This reduction was most clear in smear-negative, culture-positive cases, where time-to-treatment dropped from 56 to 5 day.

In summary, drug resistance in M. tuberculosis emerges through the selection of mutant subpopulations. These mutant subpopulations can be detected via drug susceptibility testing. Phenotypic testing using solid medium methods have currently been replaced by the more rapid liquid culture systems. It has been the introduction of molecular methods that has been a game changer though, reducing time-to-detection of drug-resistant TB to a single day. This has made point-of-care testing for drug-resistant tuberculosis well within our reach. Indeed, we are yet again on the verge of a new era of diagnosing and treating TB.
REFERENCES


Diagnosing pyrazinamide resistance in the Netherlands

Adapted from:
ABSTRACT

Background: Pyrazinamide is important in the treatment of tuberculosis. Unfortunately, the diagnosis of pyrazinamide resistance is hampered by technical difficulties. We hypothesized that mutation analysis combined with the Mycobacteria Growth Indicator Tube (MGIT) phenotypic method would be a good predictor for pyrazinamide resistance.

Design: We prospectively analyzed 1,650 M. tuberculosis isolates referred to our tuberculosis reference laboratory in 2008 and 2009. In our laboratory the MGIT 960 was used for pyrazinamide resistance screening. If a pyrazinamide resistant strain was detected, we performed a pncA gene mutation analysis. A second MGIT 960 susceptibility assay was performed afterwards to evaluate the accuracy of the pncA mutation analysis to detect true or false positive MGIT results.

Results: We observed pyrazinamide resistance in 69 samples using a first MGIT 960 analysis. In a second MGIT 960 analysis, 47 of the 69 samples proved susceptible (68% false positivity). Sensitivity of nonsynonymous pncA mutations in detecting resistant isolates was 73% (95% CI, 61%-73%) and specificity 100% (95% CI, 95%-100%).

Conclusions: A diagnostic algorithm combining phenotypic and molecular methods would have a 100% positive predictive value for detecting pyrazinamide-resistant isolates, indicating that such an algorithm, based on both methods, is a good predictor for pyrazinamide resistance in routine diagnostics.

INTRODUCTION

Pyrazinamide is one of the key components of primary drug therapy against tuberculosis, especially when multidrug-resistance has been diagnosed. The first clinical report of its antituberculosis activity dates to 1952. The addition of pyrazinamide and rifampicin to existing antituberculosis drug regimens has shortened therapy duration from 9 to 6 months and not using pyrazinamide is correlated with treatment relapse. It is a unique antituberculosis drug because of its activity against the slowly growing, semidormant bacilli in acidic environments.

The enzyme pyrazinamidase plays a crucial role in the mycobactericidal effect of pyrazinamide. This enzyme is expressed constitutively in the cytoplasm of M. tuberculosis. Only after conversion of pyrazinamide into pyrazinoic acid by this enzyme is its deleterious effect expressed on the tubercle bacilli by destabilizing the membrane potential and affecting membrane transport function. Consequently, loss of pyrazinamidase activity leads to pyrazinamide-resistant tuberculosis bacilli.

Nonsynonymous mutations in the gene encoding for pyrazinamidase, the pncA gene, lead to the loss of pyrazinamidase activity and are the major mechanism in the development of pyrazinamide resistance. Mutation analysis could thus be used to indirectly assess susceptibility to pyrazinamide. However, assessment of susceptibility based on pncA gene sequence analysis has its shortcomings. Mutations are highly diverse and widely dispersed throughout the pncA gene, limiting the chances of successful development of simple screening methods such as line probe assays. Furthermore, not all pyrazinamide-resistant M. tuberculosis isolates have mutations in this gene. For instance, mutations in the rpsA gene, encoding for ribosomal protein S1, have been described recently as a novel mechanism for pyrazinamide resistance. Phenotypic methods for testing susceptibility of M. tuberculosis to pyrazinamide remain the gold standard but also have their shortcomings. Both false-negative and false-positive resistant results are seen. In our experience false-positive resistant results (major errors) are seen most commonly. This observation has been noted by others also. In a study on susceptibility of 743 isolates tested in the Bactec Mycobacteria Growth Indicator Tube 960 (MGIT 960) method, Chedore et al. found that 42% of strains that tested pyrazinamide resistant at first appeared to be in fact susceptible when the test was repeated. It is assumed that a large inoculum size impairs pyrazinamidase activity and leads to false-positive cases of pyrazinamide resistance.
Because the gold standard—phenotypic pyrazinamide susceptibility testing—can be hampered by false-positive results and mutation analysis is not a validated alternative yet, diagnosis of pyrazinamide resistance remains difficult. We hypothesized that in routine diagnostics, mutation analysis added to culture-based methods might be a good predictor for pyrazinamide resistance. More specifically, based on the observation that susceptibility testing by the MGIT 960 method is mainly hampered by major errors, we hypothesized that nonsynonymous pncA mutations would be able to differentiate between true-resistant and false-resistant results.

METHODS

Setting
The National Institute for Public Health and the Environment (RIVM) is the national mycobacterial reference centre in the Netherlands. It receives all primary Mycobacterium tuberculosis complex isolates from the Netherlands. Annually, around 700 TB cases are culture positive which is around 70% of all TB notifications. Both multidrug resistance (MDR) and pyrazinamide resistance are estimated to be present in around 1% of these culture-positive cases.15

Drug susceptibility to pyrazinamide
Susceptibility to pyrazinamide was tested in the MGIT 960 method, according to the manufacturer’s instruction (Becton Dickinson, NJ, USA). Briefly, a pyrazinamide susceptibility test was prepared from a positive 7-ml MGIT tube using a direct inoculum obtained 1 to 2 days after a positivity signal. Two 7-ml Bactec MGIT 960 PZA medium tubes were used. One hundred µL of 8,000-mg/l pyrazinamide solution was added to one tube to achieve the recommended critical concentration of 100 mg/l (BD diagnostics). A 0.5-ml volume of the seed inoculum was aseptically pipetted in this drug-containing tube. A drug-free control tube was inoculated with 0.5-ml of a 1:10 dilution of the seed inoculum. Tubes were placed in the MGIT 960 and automatically read. Read-outs were analyzed using EpiCenter software package. Mycobacterium tuberculosis isolates were considered pyrazinamide resistant if the MGIT 960 system gave concordant resistant results on two separate occasions.

Figure 1
The PCR reading frame for the amplification of the pncA gene

Amplification and sequencing of the pncA gene
The entire pncA open reading frame, as well as 135 bp upstream and 79 bp downstream, were amplified by PCR. Two overlapping amplicons, covering a 773 bp contiguous sequence (Figure 1), were generated using the primers described in Table 1. PCR amplifications were carried out in a MBS 0.5S thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). Each reaction mixture (50 µl) contained 5 µl of 10-ng/µl template DNA, 25µl of HotStarTaq mastermix (Qiagen, Hilden, Germany), 10µl milliQ (Sigma-Aldrich, Irvine, Ayrshire, UK), 5µl of each primer (5 mM). The reaction mixtures were subjected to 15 min at 95°C, followed by 35 cycles of 60 s at 95°C for melting, 120 s at 60°C for annealing, 60 s at 72°C, and an elongation step at 72°C for 10 min. Unincorporated primers and dNTPs were removed from the reaction mixtures using EXOSAP_IT (USB Corporation, Cleveland, OH, USA) according to the manufacturer’s instructions. Automated DNA sequencing was performed using BigDye Terminator chemistry according to the protocol supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). All post-run analyses were performed using Bionumerics software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium).
Table 1
PncA primer sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’ to 3’</th>
<th>Position(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pncA_1F</td>
<td>GGC CGC GAT GAC ACC TCT</td>
<td>-133</td>
</tr>
<tr>
<td>pncA_1R</td>
<td>GCC GCA GCC AAT TCA GCA GT</td>
<td>305</td>
</tr>
<tr>
<td>pncA_2F</td>
<td>CGA AGC GGC GGA CTA CCA TCA CG</td>
<td>180</td>
</tr>
<tr>
<td>pncA_2R</td>
<td>CCC CAC CTG CGG CTG CGA ACC</td>
<td>639</td>
</tr>
</tbody>
</table>

(a) Numerical position on the primers as determined from the start codon of the pncA gene.

Genotyping
To assess potential associations between M. tuberculosis genotypes and pyrazinamide resistance, we performed IS6110 restriction fragment length polymorphism (RFLP) typing, spoligotyping and Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) typing of all isolates using previously published methods.19-21

Analysis
To answer our research question, we prospectively analyzed all M. tuberculosis isolates sent to our laboratory for resistance testing in 2008 and 2009. Mycobacterium bovis isolates were excluded since they are exclusively pyrazinamide resistant and carry only one specific pncA gene mutation. This study was set up as a pragmatic laboratory-based study using a diagnostic algorithm close to routine clinical practice. In our laboratory the MGIT 960 was used for pyrazinamide resistance screening. If a pyrazinamide-resistant strain was detected, we performed a pncA gene mutation analysis. A second MGIT 960 susceptibility assay was performed afterwards to evaluate the accuracy of the mutation analysis to detect true or false positive MGIT results.

The primary outcome was the diagnostic accuracy of pncA mutation analysis to detect true pyrazinamide resistance among M. tuberculosis isolates with a first resistant results from a MGIT 960 pyrazinamide susceptibility analysis. Diagnostic accuracy was expressed as sensitivity, specificity, positive and negative predictive value using 2 x 2 contingency tables. Estimates are given in percentages with a 95% confidence interval (95% CI).

RESULTS
Predicting pyrazinamide resistance
During 2008 and 2009, 1,650 M. tuberculosis isolates were sent for resistance testing to our laboratory (Figure 2). We observed pyrazinamide resistance in 69 out of 1,650 samples after the first MGIT 960 analysis. However, in the second MGIT 960 test, 47 of the 69 samples proved susceptible and 22 were confirmed to be resistant, indicating a false-positive rate of 68% in the first MGIT 960 test. Sensitivity of the nonsynonymous pncA mutation in detecting pyrazinamide true resistant isolates was 73% (95% CI, 61%-73%) and specificity was 100% (95% CI, 95%-100%). The positive predictive value of the nonsynonymous pncA mutation in detecting pyrazinamide resistance was 100% (95% CI, 85%-100%) and the negative predictive value was 89% (95% CI, 84%-89%). The overall accuracy of nonsynonymous pncA mutations for detecting pyrazinamide-resistant isolates was 91% (95% CI, 84%-92%).
Pyrazinamide-resistant cases in the Netherlands

Twenty-two isolates from 15 patients in 2008 and 2009 were pyrazinamide resistant; their baseline characteristics, MIRU-VNTR typing results, drug susceptibility profiles and pncA sequence analysis are described in Table 2. A wide variety of mutations was seen and one mutation was observed in the putative promoter region (NLA000801739). Some of the strains had the same type of mutations but different MIRU-VNTR patterns, indicating that they were truly different strains that had coincidentally acquired the same type of mutation. For example, patients NLA000800922 and NLA000800620 had the same mutation of his71 Gln, yet MIRU-VNTR analysis indicated that they were different M. tuberculosis strains.

Interestingly, 5 patients had pyrazinamide resistant isolates that did not carry any pncA mutation, neither in the gene itself, nor in the putative pncA promoter region. Three of these patients were in fact pyrazinamide monoresistant (NLA000800519, NLA000901231, NLA000801755). We performed both the MGIT 960 analysis as the pncA mutation three times to confirm these results. MIRU-VNTR analysis indicated that these were all different strains (Table 2).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex (M/V)</th>
<th>strain</th>
<th>VNTR</th>
<th>Resistances to other drugs</th>
<th>pncA mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H, RIF, E, CLR</td>
<td>A to C at 502</td>
</tr>
<tr>
<td>29</td>
<td>M</td>
<td>NLA000800326</td>
<td>9002568, 9002582</td>
<td>H, RIF, E, CLR</td>
<td>A to C at 502</td>
</tr>
<tr>
<td>84</td>
<td>M</td>
<td>NLA000800465</td>
<td>9002610</td>
<td>-</td>
<td>G deletion at 60</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>NLA000800620</td>
<td>9002653</td>
<td>-</td>
<td>T to A at 213</td>
</tr>
<tr>
<td>43</td>
<td>M</td>
<td>NLA000800922</td>
<td>9003512, 9003513</td>
<td>-</td>
<td>T to A at 213</td>
</tr>
<tr>
<td>26</td>
<td>V</td>
<td>NLA000801739</td>
<td>9002811</td>
<td>H</td>
<td>-12 promoter mutation T -&gt; C</td>
</tr>
<tr>
<td>41</td>
<td>M</td>
<td>NLA000801926</td>
<td>9002939</td>
<td>H</td>
<td>GAG deletion at 430</td>
</tr>
<tr>
<td>44</td>
<td>M</td>
<td>NLA000800594</td>
<td>9003531</td>
<td>H, RIF, E, AMK, CIP</td>
<td>G insertion at 218</td>
</tr>
<tr>
<td>29</td>
<td>V</td>
<td>NLA000900573</td>
<td>9003015</td>
<td>H, RIF, E, AMK, CIP, KAN, MOX</td>
<td>G insertion at 516</td>
</tr>
<tr>
<td>17</td>
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<td>NLA000901644</td>
<td>9000061</td>
<td>H, RIF</td>
<td>G to A at 3</td>
</tr>
<tr>
<td>25</td>
<td>M</td>
<td>NLA000902122</td>
<td>9000408</td>
<td>H, RIF, E, RFB</td>
<td>G to C at 289</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>NLA000800519</td>
<td>9003284</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>M</td>
<td>NLA000901231</td>
<td>9002622</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>V</td>
<td>NLA000801502</td>
<td>9002838</td>
<td>H</td>
<td>-</td>
</tr>
<tr>
<td>47</td>
<td>M</td>
<td>NLA000801595</td>
<td>9000055</td>
<td>H</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>M</td>
<td>NLA000801755</td>
<td>9002965</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Drug names: H, isoniazid; RIF, rifampicin; RFB, rifabutin; E, ethambutol; CLR, clarithromycin; AMK, amikacin; CIP, ciprofloxacin; KAN, kanamycin; MOX, moxifloxacin
Genotype family of pyrazinamide-resistant cases

Since we found some unusual pyrazinamide monoresistant cases, we wondered whether the respective M. tuberculosis isolates were clustered in certain genotype families. We therefore determined the genotype family of all resistant isolates (Figure 3). One spoligotype was not available (NLAAA000803926). Although a relatively high percentage of the Beijing genotype (5 out of 15, 33%) was noted in this sample, no specific genotype family clustering was seen among the pyrazinamide (mono)resistant cases.

DISCUSSION

This study shows that mutation analysis added to culture-based methods is a good predictor for pyrazinamide resistance in routine diagnostics. In our experiments, we have shown that in a series of 69 isolates found resistant at first instance by the MGIT 960, 68% were false-resistant and that nonsynonymous pncA mutations could identify accurately these false-positive results. Moreover, a diagnostic algorithm combining the MGIT 960 and mutation analysis could correctly identify pyrazinamide-resistant cases.

Our results are in concordance with a recent meta-analysis by Chang and co-workers, who also showed that nonsynonymous pncA mutations can detect pyrazinamide resistance accurately.8 The present study has some important strengths. First, we validated our results by retesting discordant pyrazinamide results, thereby minimizing major errors. Second, it was set up as a pragmatic trial enabling direct applicability of our diagnostic algorithm in everyday clinical diagnostics in a variety of settings.

Our results extend our knowledge on the role of molecular methods in the diagnosis of pyrazinamide resistance. The first studies on the association of pncA mutations and pyrazinamide resistance found mutations in up to 97% of pyrazinamide-resistant cases.22-24 However, these were mainly selected pyrazinamide-resistant cases with a high MIC. Subsequent studies have shown lower prevalence of pncA mutations in pyrazinamide-resistant cases.9,25 We also found a lower prevalence of nonsynonymous pncA mutations (67% of isolates). Given our experimental setup, our results may better reflect pyrazinamide resistance found in daily routine in a country with a low prevalence of pyrazinamide resistance. Zhang et al. have argued that such a finding may in part reflect incorrect pyrazinamide susceptibility testing.3 In our experiments both the MGIT 960 analysis as well as the mutation analysis yielded identical results three times at separate occasions. Hence, measurement errors cannot explain these findings. Pyrazinamide resistance in strains with wild type pncA sequences can alternatively be explained by the presence of a pncA regulatory gene outside our reading frame, or by pyrazinamide resistance mechanisms other than the effect on pyrazinamidase,3 such as the recent finding of mutations in the rpsA gene.10

Based on our results, we propose an algorithm, depicted in Figure 4, to assess pyrazinamide resistance in routine clinical diagnostics. After a first round of MGIT 960 testing, all isolates labeled resistant should undergo pncA gene sequence analysis. A nonsynonymous mutation has a positive predictive value of 100% for a true pyrazinamide resistant isolate. If a synonymous mutations or a wild type pncA gene is found, the MGIT 960 analysis should be
repeated. Given the shorter turnaround time of mutation analysis, incorporating molecular methods has the potential of shortening the diagnosis of pyrazinamide resistance. It is to be seen if such shortening will optimize treatment of tuberculosis patients, especially for multidrug-resistant TB patients in which pyrazinamide susceptibility testing is essential.1

Using the diagnostic algorithm proposed in this study (Figure 4), we found 15 cases of pyrazinamide resistance in the Netherlands in a two-year period (Table 4). The total number of cases diagnosed with culture-confirmed tuberculosis these 2 years was 1,504.15 The prevalence of pyrazinamide resistance among culture-positive M. tuberculosis cases in the Netherlands was therefore 1.0%. Five out of 15 (33%) cases had multidrug-resistant tuberculosis (MDR-TB) which is significantly higher than the general prevalence of MDR-TB in the Netherlands. Such higher prevalence of pyrazinamide resistance in multidrug-resistant tuberculosis is commonly seen.14 A relatively high number of Beijing genotype strains was seen because the Beijing genotype is associated with MDR-TB in Europe.15 Three out of 15 pyrazinamide-resistant cases found in this study were pyrazinamide monoresistant. Pyrazinamide monoresistance has been described previously but is rare.26-29 For instance, in a study from the United States, only 3 out of 1,916 isolates proved pyrazinamide monoresistant.30 which is a percentage similar to our findings (3 out of 1,650 isolates). However, though repeated analysis yielded the same result, we cannot rule out that we made a systematic measurement error giving repeated false-resistant MGIT results.

A limitation of this study is the use of the MGIT 960 as the gold standard. Though the proper gold standard for pyrazinamide resistance is not established yet, it is accepted that the Bactec radiometric method is probably most reliable.1 We choose the MGIT 960 as the gold standard since current meta-analyses suggest that the MGIT has comparable test performances and could therefore be used as reference DST assay.8,11 Moreover, there is a legitimate concern about the disposal of radioactive waste when the Bactec radiometric method is used. Third, Bactec 460 machinery is phased out, and supplies will no longer be available, limiting the applicability of a diagnostic algorithm incorporating this method. Last, other candidates for the gold standard are scarce. The 7H110 agar-based testing methods are considered less reliable.23 Susceptibility testing on solid Löwenstein-Jensen media is acceptable according to some experts,1 but is not used for drug susceptibility testing in most laboratories in the Western world anymore. The Wayne method might be another alternative.1 However, the Wayne method requires a sufficient number of bacilli for detecting pyrazinamidase activity making this test prone to false resistance testing which was exactly what we tried to minimize in our diagnostic setup. Moreover, at the time we undertook our study this assay was considered less sensitive for diagnosing pyrazinamide resistance.29

Though the MGIT can be regarded as a surrogate gold standard, this study confirms earlier observation by others that the MGIT reports false resistant isolates.12,13 Comparable high numbers of false positive resistant culture results have been reported by other researchers using the MGIT 960 technique. False phenotypic resistance is mainly caused by a well-known difficulty in pyrazinamide susceptibility testing: the use of large inoculums. A large inoculum size increases pH and thereby inactivates pyrazinamidase.24 An alternative explanation for the high false resistance rate could be the use a relatively low breakpoint in the MGIT (100 mg/l) thereby wrongly labelling susceptible or intermediate susceptible isolates as resistant.8 Others have suggested that 200 mg/l or 300 mg/l would be a more appropriate resistance breakpoint.1 Because we wanted to stay close to routine practice, we choose the 100 mg/l as prescribed by the manufacturer.17 Given the high rates of false positive results, a first notification of pyrazinamide resistance in liquid media, such as the MGIT 960, should be interpreted with caution. We would recommend to repeat the MGIT 960 giving

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**Figure 4**
Flow diagram to assess pyrazinamide resistance in a M. tuberculosis isolates in routine diagnostics, combining phenotypic and molecular methods

<table>
<thead>
<tr>
<th>Culture positive M. tuberculosis</th>
<th>1st MGIT</th>
<th>pncA analysis</th>
<th>2nd MGIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>prevalence of PZA resistance = 1%</td>
<td>positive predictive value (PPV) of resistance in first MGIT 960 = 32%</td>
<td>PPV of resistance if nonsynonymous pncA mutation found = 100%</td>
<td>PPV of resistant result in second MGIT 960 = 100%</td>
</tr>
<tr>
<td>1st MGIT 960 resistant</td>
<td>nonsynonymous pncA mutation</td>
<td>2nd MGIT 960 resistant</td>
<td>2nd MGIT 960 susceptible</td>
</tr>
<tr>
<td>culture positive M. tuberculosis</td>
<td>wild type or synonymous pncA mutation</td>
<td>susceptible</td>
<td>susceptible</td>
</tr>
</tbody>
</table>
special attention to the inoculum size and constitution. The present study highlights another possibility, namely, the use of a mutation analysis as an adjunct to the MGIT 960 as depicted in Figure 4.

The issue underlying these difficulties in interpreting pyrazinamide resistance testing, is the paucity of adequate in vivo data. Although pyrazinamide has been a drug available for fifty years, there are currently no studies published that linked in vitro pyrazinamide resistance to important in vivo clinical outcomes. There is some evidence that treatment outcome is worse in the presence of initial resistance in general, but this is not specified for pyrazinamide. The most important obstacle for sound clinical data are the technical difficulties and the limited standardization in drug susceptibility testing as described earlier. Our proposed flow diagram may help in standardizing pyrazinamide resistance testing and may be used in future studies on clinical outcome.

In summary, we have shown that a combination of MGIT 960 phenotypic pyrazinamide susceptibility testing and pncA mutation analysis provides a good predictor of pyrazinamide resistance in routine diagnostics. Nonsynonymous pncA mutations are able to differentiate between true resistant and falsely resistant MGIT 960 results. Given the high number of false positive results from phenotypic methods, our findings suggest that using mutation analysis improves and fastens pyrazinamide susceptibility testing. Based hereupon, we propose a diagnostic algorithm combining both phenotypic and molecular tests for the assessment of pyrazinamide resistance.
REFERENCES


The role of rpsA gene sequencing in the diagnosis of pyrazinamide resistance

Adapted from:
Simons S. O., Mulder A., van Ingen J., Boeree M. J., van Soolingen D.
Role of rpsA gene sequencing in the diagnosis of pyrazinamide resistance.
Journal of Clinical Microbiology 2013; 51: 382
Dear Sir,

We read with great interest the article by Alexander and colleagues on the frequency of rpsA mutations in pyrazinamide-susceptible and resistant isolates with a wild-type pncA gene.

In contrast to the report of Shi and colleagues, Alexander and colleagues could not find any rpsA mutations in pyrazinamide-resistant isolates and concluded that the analysis of the rpsA gene has no role in the diagnosis of pyrazinamide resistance.

Intrigued by their observations, we re-examined our sample of pyrazinamide-resistant isolates with wild-type pncA sequences, published previously in this journal (Table 1).

We read with great interest the article by Alexander and colleagues on the frequency of rpsA mutations in pyrazinamide-susceptible and resistant isolates with a wild-type pncA gene.1 In contrast to the report of Shi and colleagues, Alexander and colleagues could not find any rpsA mutations in pyrazinamide-resistant isolates and concluded that the analysis of the rpsA gene has no role in the diagnosis of pyrazinamide resistance.

Intrigued by their observations, we re-examined our sample of pyrazinamide-resistant isolates with wild-type pncA sequences, published previously in this journal (Table 1). This sample consisted of 5 isolates. rpsA gene sequencing was performed using the same methods as those of Alexander and colleagues.1 Sequencing the pncA gene revealed an G-to-A nucleotide change at position 778 in one isolate, leading to an amino acid change from valine to isoleucine. No changes in the rpsA gene were seen in the other four isolates. So, in contrast to the findings of Alexander and colleagues, we were able to find a non-synonymous rpsA mutation in pyrazinamide-resistant isolates with a wild-type pncA gene, albeit in a minority of cases.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Resistance profile</th>
<th>pncA gene</th>
<th>rpsA mutation</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
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<tr>
<td>NLA000800519</td>
<td>Z</td>
<td>wild type</td>
<td>G to A at 778</td>
<td>Val260Ile</td>
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</tr>
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<td>NLA000901231</td>
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<td>wild type</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NLA000801502</td>
<td>Z, H</td>
<td>wild type</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NLA000801595</td>
<td>Z, H</td>
<td>wild type</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>NLA000801755</td>
<td>Z</td>
<td>wild type</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Drug names: H = isoniazid, Z = Pyrazinamide

Table 1 RpsA mutations in pyrazinamide resistant isolates with a wild type pncA gene

Does this mean that rpsA sequencing could have a role in pyrazinamide resistance testing? To answer this question, we also reanalyzed our data on the accuracy of pncA gene sequencing in the diagnosis of pyrazinamide resistance. Incorporating rpsA gene sequencing in our proposed diagnostic algorithm would increase sensitivity from 72% to 77%, indicating that rpsA gene sequencing has only a modest effect on sensitivity. The finding of additional mutations correlated with pyrazinamide resistance, such as in the rpsA gene, is fascinating and offers important insights into the intracellular targets and behaviours of pyrazinamide. With the increasing possibilities for efficient determination of mutations, this could upgrade the reliability of testing resistance to this increasingly important antituberculosis drug. However, we agree with Alexander and colleagues that the role of rpsA gene sequencing itself in the diagnosis of pyrazinamide resistance is modest at best.

We appreciate the comments made by Simons and colleagues regarding the value of rpsA gene sequencing in the diagnosis of pyrazinamide resistance. The diversity of mutations associated with drug resistance in Mycobacterium tuberculosis has been a challenge to the sensitivity and predictive value of molecular diagnostic tools. For most clinical laboratories, full-gene sequencing of all resistance-associated loci is not a feasible approach.8 Until rapid, cost-effective methods for identifying every mutation in every strain are developed, operational demands dictate that routine diagnostic algorithms focus on the most common and informative targets. For example, most molecular assays target only the short “rifampin resistance-determining region” of rpoB, even though mutations outside this region can confer resistance to rifampin.5 Consistently, studies have identified pncA mutations in 80% of pyrazinamide-resistant Mycobacterium tuberculosis isolates.4 The paper by Shi and colleagues raised the possibility that rpsA mutations may explain resistance in wild-type pncA (pncAWT) strains.1 However, in our clinical collection of pyrazinamideresistant isolates, no non-synonymous mutations were observed. Although our colleagues report that 1 of the 5 pncAWT strains they examined contained a rpsA mutation, they do not indicate if they confirmed the phenotypic impact of the G778→A/Val260→Ile change (e.g., by cloning the mutant rpsA gene into a pyrazinamide-sensitive strain and measuring a decrease in susceptibility).

To be clear, we do not discount a role for rpsA, but the current data indicate that rpsA mutations account for resistance in only a small subset of strains. We also concede that a two-step approach, where only pncAWT isolates are subjected to rpsA sequencing, may be useful. However, our initial intent was routine analysis of both genes, just as common algorithms for investigating isoniazid resistance target both katG and inhA. Irrespective of the possible value of rpsA sequencing, it is evident that additional determinants of pyrazinamide resistance remain to be characterized. Notably, two of the isolates described by Simons and colleagues exhibited resistance to both pyrazinamide and isoniazid. We also observed this resistance pattern among pncAWT and rpsAWT isolates. Considering that pyrazinamide and isoniazid have somewhat similar chemical structures and that both are administered as prodrugs, we have wondered if some shared mechanism may mediate resistance to both agents. Thorough analysis of such strains may uncover novel determinants of resistance to pyrazinamide and other antimycobacterial agents and provide useful information for the effective treatment of tuberculosis.
REFERENCES


Detecting pyrazinamide resistance in multidrug-resistant tuberculosis

Adapted from:
Clinical Microbiology and Infection 2014; 20: 1015-1020
ABSTRACT

Background: There is an urgent need for rapid and accurate diagnosis of pyrazinamide-resistant multidrug-resistant tuberculosis (MDR-TB). No diagnostic algorithm has been validated in this population. We hypothesized that \textit{pncA} sequencing added to \textit{rpoB} mutation analysis can accurately identify patients with pyrazinamide-resistant MDR-TB.

Methods: We identified from the Dutch national database (2007-11) patients with a positive \textit{M. tuberculosis} culture containing a mutation in the \textit{rpoB} gene. In these cases, we prospectively sequenced the \textit{pncA} gene. Results from the \textit{rpoB} and \textit{pncA} mutation analysis (\textit{pncA} added to \textit{rpoB}) were compared with phenotypic susceptibility testing results to rifampicin, isoniazid and pyrazinamide (reference standard) using the Mycobacteria Growth Indicator Tube (MGIT) 960 system.

Results: We included 83 clinical \textit{M. tuberculosis} isolates containing \textit{rpoB} mutations in the primary analysis. Rifampicin resistance was seen in 72 isolates (87%), isoniazid resistance in 73 isolates (88%) and MDR-TB in 65 isolates (78%). Phenotypic reference testing identified pyrazinamide-resistant MDR-TB in 31 isolates (48%). Sensitivity of \textit{pncA} sequencing added to \textit{rpoB} mutation analysis for detecting pyrazinamide-resistant MDR-TB was 96.8%, the specificity was 94.2%, the positive predictive value was 90.9%, the negative predictive value was 98.0%, the positive likelihood was 16.8 and the negative likelihood was 0.03.

Conclusion: Pyrazinamide-resistant MDR-TB can be accurately detected using \textit{pncA} sequencing added to \textit{rpoB} mutation analysis. We propose to include \textit{pncA} sequencing in every isolate with a \textit{rpoB} mutation, allowing for stratification of MDR-TB treatment according to pyrazinamide susceptibility.

INTRODUCTION

Pyrazinamide-based treatment of multidrug-resistant tuberculosis (MDR-TB) has been the standard in the past decades and its importance has only become clearer in recent years. Studies have suggested that pyrazinamide (PZA) might work synergistically with other anti-tuberculosis (anti-TB) drugs such as fluoroquinolones and bedaquiline. Moreover, combining pyrazinamide in new anti-TB treatment regimens might have the potential to shorten MDR-TB treatment duration.

Despite this potential of PZA in MDR-TB treatment, the high prevalence of pyrazinamide resistance in MDR-TB and the troublesome drug susceptibility testing (DST) to PZA have dampened enthusiasm. Phenotypic DST using the MGIT 960 system is currently considered the gold standard, but it is prone to false-resistant errors. Screening for mutations in the \textit{pncA} gene might be an alternative proxy for PZA resistance. Indeed, a recent meta-analysis has suggested that within an MDR-TB population, \textit{pncA} sequencing has good diagnostic accuracy.

Sequencing the \textit{pncA} gene might therefore prove to be the DST assay to guide pyrazinamide-based MDR-TB treatment. However, no formal study has been published evaluating the accuracy of such a molecular-based diagnosis of PZA resistance in MDR-TB. We hypothesized that \textit{pncA} sequencing added to \textit{rpoB} mutation analysis can accurately identify these PZA-resistant MDR-TB patients. We chose \textit{rpoB} mutation analysis because this may be a good indicator for MDR-TB in high-burden settings. To test our hypothesis, we prospectively sequenced the \textit{pncA} gene in patient isolates with a known \textit{rpoB} mutation and compared results with phenotypic drug susceptibility results to rifampicin, isoniazid and pyrazinamide.
Materials and Methods

Study setting
This study was performed at the National Institute for Public Health and the Environment (RIVM), which receives all primary \textit{M. tuberculosis} complex isolates from the Netherlands.\textsuperscript{8} Yearly, around 1-2\% of all TB notifications in the Netherlands are considered to be MDR-TB.\textsuperscript{8} Pyrazinamide resistance in the Netherlands is detected in 1\% of all TB cultures\textsuperscript{6} and in 23\% of MDR-TB cases.\textsuperscript{9}

Study population
We retrospectively identified from our national database (2007-2011) all patients with a positive \textit{M. tuberculosis} culture containing a \textit{rpoB} mutation (\textit{rpoB}+ patients). We used the Genotype MTBDRplus kit (Hain Lifesciences, Nehren, Germany) to screen for mutations in the \textit{rpoB} gene. If an \textit{rpoB} mutation was found by the line probe assay, the hotspot region of the \textit{rpoB} gene was sequenced as previously described.\textsuperscript{10}

Phenotypic drug susceptibility testing
We tested susceptibility to rifampicin, isoniazid and pyrazinamide in all \textit{rpoB}+ patients using the Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson, Erembodegem, Belgium). Breakpoint concentrations were set at 1.0 mg/l (rifampicin), 0.1 mg/l (isoniazid) and 100 mg/l (pyrazinamide), according to the manufacturer’s instruction. The DST results for rifampicin and isoniazid resistance were extracted from our database for a first available isolate of an \textit{rpoB}+ patient. DST to these anti-TB drugs had been tested before this study within the context of routine diagnostic service. Multidrug-resistant \textit{M. tuberculosis} was defined as isolates being resistant at least to rifampicin and isoniazid.\textsuperscript{1}

For susceptibility testing to PZA, cultures from \textit{rpoB}+ patients were obtained from our \(-70\) °C freezer and sub-cultured in MGIT medium. Five experienced technicians prospectively tested for susceptibility to PZA using the MGIT PZA kit (pH 5.9) and an inoculum size of 0.5 ml.\textsuperscript{6} Test results were then compared with previous MGIT 960 results for PZA susceptibility testing from our database, which had been performed within the context of routine diagnostic service. Isolates were considered pyrazinamide resistant if the MGIT 960 testing yielded concordant resistant results and susceptible if testing yielded concordant susceptible results. Thirteen isolates with discordant results were subjected to a third PZA susceptibility testing and were sent to an external laboratory for confirmation by MGIT testing (ITM, Antwerp, Belgium). After this second round of testing, concordant DST results were seen in all isolates (11 resistant, 2 susceptible).

\textit{pncA} mutation analysis
The entire \textit{pncA} open reading frame, as well as 133 bp upstream and 79 bp downstream, were amplified by PCR, as described previously.\textsuperscript{6} Three experienced technicians carried out all procedures and readings. Blinding to the phenotypic reference test could not be guaranteed for every reading. Nonsynonymous \textit{pncA} mutations (\textit{pncA}+) were considered an indicator for pyrazinamide resistance.

Statistical analysis
The primary analysis was the diagnostic accuracy of finding a nonsynonymous \textit{pncA} mutation in \textit{rpoB}+ isolate (\textit{pncA} added to \textit{rpoB}) to identify PZA-resistant MDR-TB isolates. The study population included all patients with an \textit{M. tuberculosis} infection carrying an \textit{rpoB} mutation. The index test consisted of \textit{pncA} sequencing. The MGIT 960 test results for rifampicin, isoniazid and pyrazinamide were used as the phenotypic reference test. Only isolates with duplicate pyrazinamide test results were included in the analysis, limiting false-resistance results. Inconclusive test results from the \textit{pncA} sequencing were grouped with negative results, because in practice these patients would not be treated on the basis of mutation analysis but on the basis of results of the final phenotypic susceptibility tests.\textsuperscript{11}

Diagnostic accuracy (\textit{pncA} added to \textit{rpoB}) was expressed as sensitivity, specificity, positive/negative predictive value and positive/negative likelihood ratios using 2x2 contingency tables and OpenEpi software (version 2.3.1; Rollins School of Public Health, Emory University [http://www.OpenEpi.com]). The Wilson score method was used to calculate 95\% confidence intervals around the proportions. This report was written following STARD guidelines.\textsuperscript{12}
RESULTS

Study population

We identified 85 patients with a positive \textit{M. tuberculosis} culture containing an \textit{rpoB} mutation (\textit{rpoB}+ patients), of whom 83 were enrolled (Figure 1). The median age of the included patients was 27 years and 43\% were males (Table 1). The majority of patients were foreign-born and 23 patients came from high-burden MDR-TB countries. One patient was excluded because no source material was available for \textit{pncA} sequencing. This isolate was considered to be PZA and rifampicin susceptible, but isoniazid resistant during routine diagnostics. Another isolate was excluded because no source material was available anymore for duplicate pyrazinamide phenotypic susceptibility testing. This latter isolate had been designated as PZA-susceptible, MDR-TB during routine diagnostics.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic characteristic of the study patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (N=83)</td>
<td></td>
</tr>
<tr>
<td>Median age in years (IQR)</td>
<td>27 (23-33)</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>36 (43%)</td>
</tr>
<tr>
<td>Nationality</td>
<td></td>
</tr>
<tr>
<td>Foreign-born</td>
<td>78 (94%)</td>
</tr>
<tr>
<td>Born in a high-burden MDR-TB country$^a$</td>
<td>23 (28%)</td>
</tr>
<tr>
<td>TB localization</td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>56 (67%)</td>
</tr>
<tr>
<td>Extrapulmonary</td>
<td>27 (33%)</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>65 (78%)</td>
</tr>
<tr>
<td>Resistance to pyrazinamide$^b$</td>
<td>32 (39%)</td>
</tr>
</tbody>
</table>

$^a$ The following 27 countries are considered as high-burden MDR-TB countries by the WHO: Armenia, Azerbaijan, Bangladesh, Belarus, Bulgaria, China, Democratic Republic of Congo, Estonia, Ethiopia, Georgia, India, Indonesia, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Myanmar, Nigeria, Pakistan, Philippines, Republic of Moldova, Russian Federation, South Africa, Tajikistan, Ukraine, Uzbekistan, and Viet Nam.

$^b$ Thirty-one isolates were pyrazinamide-resistant MDR-TB. One isolate was pyrazinamide resistant, isoniazid resistant but rifampicin susceptible.

Abbreviations: FN, false-negative index test result; FP, false positive index test result; INH, isoniazid; MDR-TB, multidrug-resistant tuberculosis; MGIT, mycobacterial growth indicator tube; PZA, pyrazinamide; Rif, rifampicin; \textit{rpoB}+ patient, patients with a positive \textit{M. tuberculosis} culture carrying a mutation in the \textit{rpoB} gene; $\sim$, resistant; $\sim$, susceptible; TN, true-negative index test result; TP, true-positive index test result.
Phenotypic drug susceptibility testing (reference test)

We detected rifampicin resistance in 72 rpoB+ patients (87%), isoniazid resistance in 73 rpoB+ patients (88%) and pyrazinamide resistance in 32 (39%) rpoB+ patients. MDR-TB was identified in 65 rpoB+ patients (78%); isolates of eleven rpoB+ patients proved rifampicin susceptible in the MGIT system. Most of these isolates carried mutations at specific codon positions (Table 2). Phenotypic methods identified 31 (48%) PZA-resistant MDR-TB out of 65 MDR-TB isolates (Phenotypic reference test result).

Table 2
Association of non-synonymous mutations in the rpoB or pncA gene with phenotypic drug susceptibility (DST) to either rifampicin or pyrazinamide

<table>
<thead>
<tr>
<th>Drug</th>
<th>Locus</th>
<th>DST</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrazinamide</td>
<td>pncA</td>
<td>Resistant</td>
<td>Met11Le, Met1Thr, Ala28Thr, Ala46Val, Thr47Ala, Asp49Glu, His51Arg, His57Tyr, Pro52Leu, Pro59Ser, Thr76Pro, Leu85Arg, Phe94Cys, Gly97Arg, Tyr103stop, Ser104Arg, Thr135Pro, Thr168Pro, Met175Le, Val180Pro, Leu182Ser</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>rpoB</td>
<td>Susceptible</td>
<td>Asp129Asn, Val155Met</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
<td>Leu449Arg, Asp516Ala, Asp516Tyr, Pro520Ser, Ser522Leu, His526Ala, His526Asp, His526Leu, His526Tyr, Ser531Leu, Ser531Met, Ser531Phe, Leu533Pro</td>
</tr>
</tbody>
</table>

* Drug susceptibility was tested using the MGIT 960 system. Susceptibility to pyrazinamide was tested in duplicate.
* The pncA mutations not found in the DreamDB database (http://www.tbdreamdb.com) are highlighted in bold.
* The following mutations were only seen in combination with other mutations: Leu449Arg with His526Leu, Met515Leu with Leu511Pro, Pro520Ser with Ser522Leu and Leu533Pro with Asp516Ala.
* The following mutations were noted both in susceptible as well as in resistant cases: Asp516Tyr (1 susceptible, 1 resistant) and His526Leu (3 susceptible, 1 resistant).

Diagnostic accuracy of mutation analysis

We performed pncA mutation analysis in all isolates from the 83 rpoB+ patients (Figure 1). Forty-nine wild type pncA genes or genes with synonymous mutations were found and 33 nonsynonymous mutations (pncA+) were detected. One test result was not interpretable; this inconclusive isolate was classified as PZA-susceptible MDR-TB and grouped with the negative results (see Statistical analysis). The molecular algorithm based on finding a pncA mutation in a population of patients with an rpoB mutation (pncA added to rpoB mutation) detected 30 of the 31 PZA-resistant MDR-TB patients (Figure 1). Three patients would be incorrectly labelled as PZA-resistant MDR-TB. One of the isolates from these patients proved to be isoniazid and PZA-resistant but rifampicin susceptible. This isolate carried a T deletion at a position 12 bp downstream of the pncA gene and a Leu515Leu combined with a Met515Leu mutation in the rpoB gene.

The two other isolates, carrying a Val155Met and an Asp129Asn mutation in the pncA gene, were classified as MDR-TB, but were repeatedly PZA-susceptible in phenotypic testing. One patient with a PZA-resistant MDR-TB isolate carried a wild type pncA gene and would be incorrectly labelled as pyrazinamide-susceptible MDR-TB (Figure 1). Overall, the sensitivity of the index test (pncA added to rpoB mutation) for detecting pyrazinamide-resistant MDR-TB was 96.8%, the specificity was 94.2%, the positive predictive value was 90.9%, the negative predictive value was 98.0%, the positive likelihood was 16.8 and the negative likelihood was 0.03 (Table 3).

Table 3
Diagnostic accuracy of pncA sequencing added to rpoB mutation analysis for diagnosing pyrazinamide-resistant MDR-TB

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>95%-CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>96.8</td>
<td>83.8-99.4</td>
</tr>
<tr>
<td>Specificity</td>
<td>94.2</td>
<td>84.4-98.0</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>90.9</td>
<td>76.4-96.9</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>98.0</td>
<td>89.5-99.7</td>
</tr>
<tr>
<td>Positive likelihood ratio</td>
<td>16.8</td>
<td>8.7-32.3</td>
</tr>
<tr>
<td>Negative likelihood ratio</td>
<td>0.03</td>
<td>0.005-0.2</td>
</tr>
</tbody>
</table>
DISCUSSION

In this study we have found that pncA sequencing added to rpoB mutation analysis can accurately identify PZA-resistant MDR-TB. We showed that sequencing the pncA gene in M. tuberculosis isolates with an rpoB mutation had a sensitivity of 97% and specificity of 94% to detect pyrazinamide-resistant MDR-TB (Table 3). The rigorous DST testing to pyrazinamide and our set up as a diagnostic accuracy study according to STARD guidelines strengthens the validity of these results. Moreover, our results add to the growing evidence that screening for mutations in the pncA gene can be used as a solid proxy for pyrazinamide resistance in MDR-TB settings.6

In the present study, we wanted to set up a pragmatic diagnostic algorithm for high-burden MDR-TB settings, where screening for MDR-TB is preferably performed using rpoB gene mutation analysis.1 That is why this algorithm is different from our previously published algorithm, which is more suitable for diagnosing pyrazinamide resistance in low-burden MDR-TB and PZA-resistant settings.6 These two diagnostic algorithms together may help microbiologists in standardizing pyrazinamide resistance testing in low-burden as well as in high-burden MDR-TB settings.

In view of the central role of pyrazinamide in novel (MDR-)TB treatment regimens, such as in the NC-002 and NC-003 trials (www.tballiance.org), some authors have suggested stratifying MDR-TB according to PZA susceptibility.1 As such, we propose to perform pncA sequencing in every isolate with a mutation in the rpoB gene to rapidly and accurately detect pyrazinamide-resistant MDR-TB.1 Anecdotal evidence suggest that such strategy, when performed on direct sputum samples, may give accurate results within days.13

Other researchers have found lower proportions (between 72 and 88%) of nonsynonymous mutations in PZA-resistant strains highlighting the risk of false susceptibility, e.g., pyrazinamide resistance in wild-type pncA.6,14,15 However, contrary to our study, these studies were not designed as diagnostic accuracy studies, did not re-test isolates in the MGIT 960 system, or were not performed in an MDR-TB population.5 The occurrence of mutations in other genes, such as in the recently described rpsA gene and panD gene, could also explain why others found higher number of PZA resistant isolates without pncA mutations.16,17 In the present study, we did not systematically test for these mutations, since we rarely found PZA-resistant isolates with wild-type pncA and the clinical significance of these alternative mutations in routine pyrazinamide diagnostics is yet to be determined.6

Contrary to our expectations, we noted that mutations in the rpoB gene were not a perfect predictor for MDR-TB (Figure 1). On the one hand, we saw some rifampicin monoresistant isolates. This could be caused by the fact that our rpoB+ patients were drawn from a population with low levels of rifampicin resistance. It has been shown previously that in such situations rpoB mutations have a low positive predictive value for detecting MDR-TB.19 On the other hand, we noted that around 10% of rpoB mutations recorded in this study gave rise to a rifampicin-susceptible phenotype in the MGIT system. These rpoB mutations occurred mostly at specific positions of the rpoB gene (Table 2) that have been associated with discordant results for rifampicin in the MGIT 960 system.20 These specific mutations probably give rise to MICs for rifampicin that lay around its break point concentration, thereby leading to so-called low-level, or intermediate, rifampicin-resistant strains.21,22 This marks a flaw inherent to determining rifampicin susceptibility by rapid phenotypic DST assays, testing only at the breakpoint concentration. This is an important finding that poses a therapeutic challenge because the mutations that yield low-level resistance have been associated with treatment failure and relapse.21,22

In the present study, we presupposed that all pncA mutations were in fact resistance-determining mutations. And indeed, most of the pncA mutations seen in this study (Table 2) are in agreement with the resistance-determining mutations found in the DreamDB database (http://www.tbdreamdb.com, accessed February 17, 2014), seven of which are listed as high confidence for being resistance-determining.23 Seven mutations have not been described previously and two mutations –Thr168Pro and Met175Ile– were found in other publications (Table 2).24,25 The Met75Ile mutation has previously been associated with both susceptible and resistant pyrazinamide strains.26 Stoffels and co-workers have shown that these type of mutations encode for low-level pyrazinamide resistance,26 explaining why they might not be adequately detected using the MGIT 960 system. Low-level pyrazinamide resistance not detected by the MGIT system might also explain why we found two non-synonymous pncA mutations in pyrazinamide-susceptible strains (Table 2).24,25 The Met75Ile mutation has previously been associated with both susceptible and resistant pyrazinamide strains.26 Stoffels and co-workers have shown that these type of mutations encode for low-level pyrazinamide resistance,26 explaining why they might not be adequately detected using the MGIT 960 system. Low-level pyrazinamide resistance not detected by the MGIT system might also explain why we found two non-synonymous pncA mutations in pyrazinamide-susceptible strains (Table 2). On the other hand, these two might not be resistance-determining mutations; pncA mutant, but pyrazinamide susceptible isolates are occasionally seen.

Many factors interplay in a real-life diagnostic testing strategy and the present study should be cautiously interpreted as such owing to its design. First, some bias may have been introduced by the retrospective nature of our study using archived samples. Second, though the rigorous DST for pyrazinamide did strengthen the validity of our MGIT 960 results, such strategy is probably not feasible in routine diagnostics. Third, we performed indirect molecular DST using M. tuberculosis cultures, whereas in practice we would have preferred to perform
the molecular analysis directly on sputum samples. Future studies should examine the performance of this technique in programmatic settings.

In conclusion, we found that diagnosing pyrazinamide-resistant MDR-TB by rapid molecular assays is highly accurate. We therefore propose to include \textit{pncA} sequencing for every \textit{M. tuberculosis} isolate with a mutation in the \textit{rpoB} gene. This makes it possible to stratify MDR-TB according to pyrazinamide susceptibility, thereby paving the way for the urgently needed treatment efficacy studies within this population.
REFERENCES

Accuracy of genotypic and phenotypic methods for second-line drug susceptibility testing

Adapted from:
ABSTRACT

Background: The Mycobacteria Growth Indicator Tube (MGIT 960) automated liquid medium testing method is becoming the international gold standard for second-line drug susceptibility testing of multidrug- and extensively drug-resistant Mycobacterium tuberculosis complex isolates.

Design: We performed a comparative study of the current gold standard in the Netherlands, the Middlebrook 7H10 agar dilution method, the MGIT 960 system, and the GenoType MTBDRsl genotypic method for rapid screening of aminoglycoside and fluoroquinolone resistance. We selected 28 clinical multidrug- and extensively drug-resistant M. tuberculosis complex strains and M. tuberculosis H37Rv. We included amikacin, capreomycin, moxifloxacin, prothionamide, clofazimine, linezolid, and rifabutin in the phenotypic test panels. For prothionamide and moxifloxacin, the various proposed breakpoint concentrations were tested by using the MGIT 960 method.

Results: The MGIT 960 method yielded results 10 days faster than the agar dilution method. For amikacin, capreomycin, linezolid, and rifabutin, results obtained by all methods were fully concordant. Applying a breakpoint of 0.5 mg/l for moxifloxacin led to results concordant with those of both the agar dilution method and the genotypic method. For prothionamide, concordance was noted only at the lowest and highest MICs. The phenotypic methods yielded largely identical results, except for those for prothionamide.

Conclusions: Our study supports the following breakpoints for the MGIT 960 method: 1 mg/l for amikacin, linezolid, and clofazimine, 0.5 mg/l for moxifloxacin and rifabutin, and 2.5 mg/l for capreomycin. No breakpoint was previously proposed for clofazimine. For prothionamide, a division into susceptible, intermediate, and resistant seems warranted, although the boundaries require additional study. The genotypic assay proved a reliable and rapid method for predicting aminoglycoside and fluoroquinolone resistance.

INTRODUCTION

The emergence of multidrug-resistant tuberculosis (MDR-TB) in the 1990s, and more recently, extensively drug resistant tuberculosis (XDR-TB), has revealed the need for new drugs and alternative, second-line treatment regimens. Many of these second-line drugs are either old drugs that had not been frequently used because of side effects or unproven efficacy or newer drugs intended primarily for treatment of other infections.1 Their use necessitated an evaluation of drug susceptibility testing (DST) and a determination of the critical concentrations of these alternative drugs.

A variety of techniques is now available for second-line DST, of which the Mycobacteria Growth Indicator Tube automated liquid culture system (MGIT 960) is probably the most used and best validated at this moment.2 The latest addition to second-line DST are genotypic methods, which detect mutations in the gyrA gene of Mycobacterium tuberculosis that are associated with fluoroquinolone resistance and mutations in the rrs operon that are associated with resistance to capreomycin and the aminoglycosides.3,4,5

Despite the arrival of these novel tools, many uncertainties remain. Not all methods have been evaluated in comparative studies. Moreover, the critical concentrations for resistance to several second- and third-line drugs, including moxifloxacin and prothionamide, remain the subject of debate.4,5

In the Netherlands, the Middlebrook 7H10 agar dilution method has been used for second-line DST for 2 decades.4 In order to comply with international standardization requirements, a switch to the MGIT960 method has been initiated. In this study, we have compared the results of the MGIT960 method and the GenoType MTBDRsl assay, a commercially available genotypic second-line DST method, to our reference method, the Middlebrook 7H10 agar dilution method. For the MGIT960 method, we tested the various critical concentrations published for prothionamide and moxifloxacin but also included clofazimine in our drug panel. For the latter drug, which has become increasingly important in the treatment of MDR and XDR-TB, no in vitro DST data for the MGIT960 method were available.
From our laboratory database, we selected 28 multidrug-resistant clinical isolates (26 M. tuberculosis and 2 Mycobacterium bovis) and the M. tuberculosis H37Rv reference strain. Isolates were nonrandomly selected to include those previously designated susceptible and resistant to each of the drugs included in the test panel, except linezolid, for which no resistant strains are available. All strains were identified by a GenoType MTBC assay (Hain Lifescience, Nehren, Germany).

We performed DST using the 25-well Middlebrook 7H10 agar dilution method as previously described, the automated MGIT960 method with the TBeXist application and EpiCenter software package (BD Bioscience, Erebodegem, Belgium), and the genotypic GenoType MTBDRsl reverse line blot methodology (Hain Lifescience, Nehren, Germany). This molecular test detects mutations in the rrs (16S) gene that give rise to amikacin, kanamycin, and capreomycin resistance, as well as gyrA gene mutations that lead to fluoroquinolone resistance. The agar dilution method was considered the gold standard, and the selection of strains was based on previous results obtained by this method.

The applied drug concentrations and breakpoints, based on previous publications, are depicted in Table 1. For the agar dilution method, a MIC equal to the breakpoint concentration is reported as susceptible; plates were read after 4, 7, 10, and 14 days of incubation and on each working day thereafter. For the MGIT 960 method, growth at the breakpoint concentration is considered resistant; drug-containing tubes were read at the moment the growth control was signaled positive on the MGIT 960 system. Research was conducted in a blinded manner with respect to previous test results and to results obtained by the other methods in this study.

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentrations evaluated (in mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amikacin</strong></td>
<td>1, 2, 5, 10, 20</td>
</tr>
<tr>
<td><strong>Capreomycin</strong></td>
<td>2, 5, 10, 20, 40</td>
</tr>
<tr>
<td><strong>Prothionamide</strong></td>
<td>1, 2, 5, 10, 20</td>
</tr>
<tr>
<td><strong>Moxifloxacin</strong></td>
<td>0.125, 0.25, 0.5, 1, 2</td>
</tr>
<tr>
<td><strong>Linezolid</strong></td>
<td>0.125, 0.25, 0.5, 1, 2</td>
</tr>
<tr>
<td><strong>Rifabutin</strong></td>
<td>0.2, 0.5, 0.1, 2, 5</td>
</tr>
<tr>
<td><strong>Clofazimine</strong></td>
<td>0.5, 1, 2, 5</td>
</tr>
</tbody>
</table>

*Boldface* type indicates the breakpoint concentration (cut-off point for resistance).

### RESULTS

The turn-around-time of the MGIT 960 method is substantially shorter than that of the agar dilution method; after a mean of 8 days, the growth control tubes were positive (87.8 h; standard deviation [SD], 58.3 h) versus a mean duration of 18 days (SD, 3 days) for the agar dilution method (P < 0.01).

The DST results for amikacin, capreomycin, linezolid, and rifabutin are presented in Table 2. Phenotypic DST results for rifabutin, linezolid, and amikacin were fully concordant between the two methods. For capreomycin, 97% concordance was noted; one strain was borderline susceptible (MIC, 10 mg/l) when tested by the agar dilution method, but resistant (MIC, >2.5 mg/l) when tested by the MGIT 960 method (Table 2) (sensitivity, 95%; specificity, 100%); the genotypic assay detected an rrs gene mutation, which resolves this issue in favour of the MGIT 960 result. Complete cross-resistance between amikacin and capreomycin was noted when the MGIT method was used; there was 97% cross-resistance when the agar dilution method was used.
Table 2
Phenotypical drug susceptibility testing results for amikacin, capreomycin, rifabutin, and linezolid

<table>
<thead>
<tr>
<th>Drug</th>
<th>7H10 result (No. of isolates)</th>
<th>No. of isolates with indicated MGIT 960 result</th>
<th>Concordance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capreomycin</td>
<td>S (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifabutin</td>
<td>S (14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linezolid</td>
<td>S (29)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*s, susceptible; R, resistant; 7H10, Middlebrook 7H10 agar dilution method; MGIT 960, mycobacteria growth indicator tube.

*This strain harboured the A1401G rrs mutation, favouring the MGIT 960 result and a “resistant” designation.

The results for moxifloxacin are presented in Table 3. For moxifloxacin, the use of the 0.125-mg/l breakpoint concentration for the MGIT 960 method led to one false-resistance result, yielding a 97% concordance (sensitivity, 95%; specificity, 100%); the use of the 1.0-mg/l breakpoint led to two false-susceptibility results (93% concordance; sensitivity, 100%; specificity, 71%). Applying the 0.5-mg/l breakpoint, complete agreement was noted between results obtained by the MGIT 960 and agar dilution methods.

Table 3
Distribution of moxifloxacin MICs determined by two phenotypic methods

<table>
<thead>
<tr>
<th>7H10 MIC of moxifloxacin (mg/l)</th>
<th>No. of isolates with indicated MGIT 960 MIC (mg/l)</th>
<th>≤ 0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>&gt; 1</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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</tr>
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<tr>
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<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*Twenty-nine isolates were tested. Italics indicate the previously published breakpoint concentration and cut-off for resistance. Boldface indicates proposed breakpoints stemming from the current study: MICs of ≤ 2 (7H10) or ≤ 2.5 (MGIT 960) mg/l indicate prothionamide susceptibility, MICs of > 10 (7H10) or > 5 (MGIT 960) mg/l indicate resistance, and those in between indicate intermediate susceptibility.

b 7H10, Middlebrook 7H10 agar dilution method; MGIT 960, mycobacteria growth indicator tube.

The agreement between both phenotypical DST methods was low in prothionamide testing (Table 4). Applying the 2.5-mg/l breakpoint for the MGIT 960 method led to six false-resistance results and one false-susceptibility result (76% concordance; sensitivity, 73%; specificity, 86%; positive predictive value [PPV], 94%; negative predictive value [NPV], 50%). When applying the 5-mg/l breakpoint, three false resistances and one false susceptibility were noted (86% concordance; sensitivity, 86%; specificity, 86%; PPV, 95%; NPV, 67%). From these results, it is clear that at the lowest MICs (≤2 [7H10]/≤2.5 mg/l [MGIT 960]) and highest MICs (>10 [7H10]/>5 mg/l [MGIT 960]), concordance is the highest for the two phenotypic methods (Table 4).

Table 4
Distribution of prothionamide MICs determined by two phenotypic methods

<table>
<thead>
<tr>
<th>7H10 MIC of prothionamide (mg/l) (no. of strains)</th>
<th>No. of isolates with indicated MGIT 960 MIC (mg/l)</th>
<th>≤ 0.25</th>
<th>&gt; 0.25 and ≤ 5</th>
<th>&gt; 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 (11)</td>
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<td>0</td>
</tr>
<tr>
<td>2 (3)</td>
<td></td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5 (8)</td>
<td></td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10 (4)</td>
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<td>0</td>
<td>3</td>
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<tr>
<td>&gt;20 (3)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* Twenty-nine isolates were tested. Italics indicate the previously published breakpoint concentration and cut-off for resistance. Boldface indicates proposed breakpoints stemming from the current study: MICs of ≤ 2 (7H10) or ≤ 2.5 (MGIT 960) mg/l indicate prothionamide susceptibility, MICs of > 10 (7H10) or > 5 (MGIT 960) mg/l indicate resistance, and those in between indicate intermediate susceptibility.

b 7H10, Middlebrook 7H10 agar dilution method; MGIT 960, mycobacteria growth indicator tube.

All 29 strains proved susceptible to clofazimine by the 7H10 agar dilution method. By the MGIT 960 method, two strains were found to be resistant to 0.25 mg/l of clofazimine (i.e., a MIC of 0.5 mg/l); all others proved susceptible to this concentration or the lower concentrations (Table 5). From these data, a MIC90 of 0.5 mg/l for the agar dilution method can be calculated; for the MGIT960 method, the MIC90 is 0.25 mg/l.
The MTBDRsl assay revealed *rrs* mutations in eight strains (*M. tuberculosis*, 2 *M. bovis*) and *gyrA* mutations in seven strains (*M. tuberculosis*, 2 *M. bovis*). Five strains harboured the *A1401G rrs* (16S rRNA) gene mutation, and three harbored a *Cl402T* mutation (*M. tuberculosis*, 2 *M. bovis*). Hybridization with the probe for the *A1401G rrs* mutation was generally weak. All isolates with *rrs* mutations proved amikacin and capreomycin resistant, except for one strain with an *A1401G rrs* mutation that proved amikacin resistant by 7H10 agar dilution and MGIT960 testing but had a MIC of 0.1 mg/l for capreomycin, i.e., at the breakpoint, by the 7H10 agar dilution method. No false-positive or false-negative results were recorded.

Among the seven strains with *gyrA* mutations, one harboured an *A90V* mutation, three harboured an *S91P* mutation (including the 2 *M. bovis* strains), and one had the *D94G* mutation. Two strains were found to have multiple mutations; one had *A90V* and *D94A* mutations, and the other had *A90V*, *S91P*, and *D94N* mutations. For both, one mutant probe hybridized well and yielded a high-intensity band, whereas the others were weak. In 3 out of 8 strains (38%) with mutations in *gyrA*, wild-type sequences were also detected by the MTBDRsl assay. All seven isolates with mutations in *gyrA* proved moxifloxacin resistant by the 7H10 agar dilution method; no false-positive or false-negative results were recorded. All seven isolates with *gyrA* gene mutations had MICs above 0.5 mg/l for moxifloxacin when tested by the MGIT 960 method.

### Table 5

<table>
<thead>
<tr>
<th>7H10 MIC of clofazimine (mg/l)</th>
<th>No. of isolates with indicated MGIT 960 MIC (mg/l)</th>
<th>≤ 0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0.5</td>
<td></td>
<td>13</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td></td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Twenty-nine isolates were tested. Italics indicate the previously published breakpoint concentration and cut-off for resistance. **Boldface** indicates the proposed breakpoint stemming from the current study, 1 mg/l for clofazimine (MGIT 960).

### DISCUSSION

The results of second-line DST for *M. tuberculosis* complex bacteria obtained by the 7H10 agar dilution method and MGIT 960 method are largely similar. The much shorter turn-around-time for the MGIT 960 method (mean, 8 versus 18 days) is a major advantage over that of the previous standard, the 7H10 agar dilution method. Moreover, the reading in the MGIT system is done automatically, which is preferred over the reading by eye in the classical 7H10 agar method. Fast yet robust laboratory results are of paramount importance to guide the choice of drugs in MDR- and XDR-TB treatment. Before confidently switching to MGIT 960 as the gold standard for second-line DST, the uncertainties involving moxifloxacin and prothionamide breakpoints must be resolved.

Of all quinolones, moxifloxacin is considered the most promising antituberculosis drug.11,13 As a result, in vitro DST for this drug has become an important issue. However, the breakpoint concentration for the MGIT method is still a subject of debate, with published breakpoints ranging from 0.125 mg/l to 0.5 mg/l14 and 1.0 mg/l.15 Our comparison of the two phenotypic methods and the genotypic method supports the choice of 0.5 mg/l as the breakpoint. One strain without detectable *gyrA* mutations proved resistant to 0.125 mg/l of moxifloxacin, a previously proposed breakpoint;16 false-resistance results are major errors and could lead to unwarranted diagnoses of XDR-TB and unnecessary restrictions in the selection of active drugs for individual treatment regimens. Two strains with *gyrA* mutations grew at 0.125, 0.25, and 0.5 mg/l but not at 1 mg/l. Here, the 1.0-mg/l breakpoint16 would have led to false-susceptibility results; this can lead to the inclusion of presumably inactive drugs in treatment regimens, with serious consequences, and counts as a very major error.6

For moxifloxacin, the 0.5-mg/l MGIT 960 breakpoint concentration seems to correlate well with the drug’s bioavailability. In a recent study, the regular 400-mg once-daily dosage of moxifloxacin led to maximum serum concentrations of 4.7 mg/l, an area under the curve (AUC) of 48.2 mg·h/liter, and trough concentrations (24 h after intake) of 0.78 mg/l.11 The AUC/MIC ratio best describes the activity of moxifloxacin against *M. tuberculosis*, and should be >100 for optimal bactericidal activity, although this is rarely reached in TB treatment.19 Applying the 1.0-mg/l (7H10) and 0.5-mg/l (MGIT) to designate susceptible isolates, the MIC*₉₀₅₅₅₅ was 0.5 (7H10) or 0.25 (MGIT) mg/l, leading to an AUC/MIC₉₀₅₅₅₅ of 96.4 or 192.8, both compatible with substantial clinical activity.

Based on the suggested breakpoint concentrations for prothionamide, MGIT 960 test results compare poorly with those of the agar dilution method, at 76 to 86% concordance.
This discordance is not a novel observation, although its extent is larger than seen in previous studies. Two previous extensive studies noted more major and very major errors in prothionamide testing than for any other second-line drug.3,4 Owing to the inclusion of many susceptible strains, both studies still showed 96% and 97% percent agreement when applying the 2.5- and 5.0-mg/l breakpoints for the MGIT960 method, compared to results for the resistance ratio method6 or BACTEC 460 radiometric method.1 In our study, the concordance was highest at the lowest and highest MICs, which suggests that one breakpoint defining susceptibility and resistance may not be adequate for prothionamide. A division into susceptible, intermediate, and resistant may be preferable, at least if there are pharmacokinetic and clinical consequences. The existence of low-level resistance has also been suggested by previous investigators, on the basis of discrepancies in drug susceptibility testing.1,4 Larger studies are needed to define the suitability of this division and its MIC breakpoints as well as the genomic mutations underlying the different degrees of resistance.5 On the basis of our data, isolates with MICs of ≤ 2 (7H10) or ≤ 2.5 (MGIT) mg/l should be considered susceptible, those with MICs of >10 (7H10) or >5 (MGIT) mg/l should be considered resistant, and all those in between should be considered intermediate susceptible. For the intermediate group, pharmacokinetic and pharmacodynamic studies would be needed to determine the possible efficacy of prothionamide treatment.

Based on the MIC_{90} of 0.25 mg/l for clofazimine (Table 5), we propose a breakpoint concentration of 1 mg/l for the MGIT960 method, although the stability of clofazimine in 7H9 medium warrants separate investigation. However, the mechanism of action of clofazimine against M. tuberculosis remains uncertain,5 and the difficulties in isolating clofazimine-resistant strains6 raise a question about the existence of clofazimine resistance. In fact, the only isolate we previously found to be clofazimine resistant6 was intentionally included in this study and now proved susceptible by both phenotypic methods, rendering the previous observation most likely a laboratory mistake. The mutations needed to acquire clofazimine resistance may have an impact on the viability of M. tuberculosis. For nontuberculous mycobacteria, clofazimine resistance exists, mainly among rapid growers, though its underlying mechanism is not known.5

The molecular assay proved highly reliable in detecting amikacin, capreomycin, and moxifloxacin resistance. All rs mutations predicted amikacin and/or capreomycin resistance. Therefore, the positive predictive value of the genotypic tests may be considered very high. One strain with a “MUT1” (A1401G) rs mutation still tested capreomycin susceptible, albeit borderline, by the 7H10 agar dilution method. However, according to a previous study, this mutation induces capreomycin resistance.8 In contrast, for three strains, no hybridization with the wild-type probe was noted in the absence of MUT1 (i.e., the A1401G mutation) probe hybridization. This specific result indicates the presence of the C1402T mutation,4 which is supposed to result in amikacin susceptibility and capreomycin resistance.8 Our three strains all proved to be cross-resistant to both drugs; Hillemann and co-workers also noted this phenomenon for one strain in their study of the MTBDRsl assay’s performance.5 Since the hybridization of the MUT1 probe was generally weak, A1401G mutations may have potentially been misinterpreted as C1402T mutations.

For moxifloxacin resistance, the obtained MTBDRsl results were fully concordant with the MGIT960 results if the 0.5-mg/l breakpoint was applied. In fact, the molecular analysis provides further support for this breakpoint. Complete concordance with the 7H10 agar dilution was noted. Multiple mutations were found for two strains. Interestingly, in three out of eight strains with mutations in gyrA, wild-type sequences were also detected. This suggests that in these isolates, both wild-type (susceptible) and mutant (resistant) bacterial populations are present, in a ratio sufficient to allow hybridization of the wild-type and mutant probes. These results were also frequently found (21.9%) in the study by Hillemann and co-workers.5 This heteroresistance may result from the emergence of resistant subpopulations or infection by multiple strains, as has been noted for isoniazid and rifampin.19

While the MTBDRsl assay includes probes for embB gene mutation analysis, we decided not to compare these results with ethambutol results for the phenotypic methods, as low sensitivity of the molecular system has already been demonstrated6 and ethambutol is not a second-line drug.

The intricacies of second-line DST by phenotypic methods and their importance in clinical care underline the need for quality control efforts, both internal and external. The use of control strains may be preferable to minimize intertest variance; external quality control schemes are already in existence for second-line DST to ensure interlaboratory reproducibility. In summary, the 7H10 agar dilution and MGIT 960 phenotypical second-line DST methods for M. tuberculosis yielded largely identical results, except for those for prothionamide. For moxifloxacin and clofazimine, we propose 0.5 mg/l and 1 mg/l, respectively, as breakpoint concentrations for the MGIT 960 method. For amikacin, capreomycin, rifabutin, and linezolid, we support the 1.0-, 2.5-, 0.5-, and 1-mg/l breakpoints suggested previously.20 The MGIT 960 method has a much shorter turn-around-time than the conventional 7H10 agar method, which is essential for the timely optimization of patient treatment regimens. Here, the MTBDRsl molecular assay proved to be a reliable method for predicting aminoglycoside and fluoroquinolone resistance and thus for the rapid screening of (MDR)-TB strains for possible extensive drug resistance.
REFERENCES

Molecular drug susceptibility testing for first-line and second-line antituberculosis drugs in the Netherlands

Adapted from:
ABSTRACT

Background: The performance of molecular drug susceptibility tests in countries with low prevalence of drug resistance, such as the Netherlands, has not been adequately studied.

Objective: To evaluate the diagnostic accuracy of the GenoType® MTBDRplus and the MTBDRsl test to detect resistance to first-line and second-line antituberculosis drugs in the context of a nationwide screening program in the Netherlands.

Results: The MTBDRplus assay had a sensitivity, specificity, positive and negative predictive value of 100%, 99%, 80% and 100% for detecting rifampicin resistance. Sensitivity, specificity, positive and negative predictive value of either a katG or inhA mutation for detecting isoniazid resistance was 88%, 100%, 100% and 99%. The MTBDRsl assay had a sensitivity, specificity, positive and negative predictive value of 100%, 99%, 83%, and 100% for detecting moxifloxacin resistance. This was 62%, 71%, 58% and 74% for detecting ethambutol resistance. Sensitivity, specificity, positive and negative predictive value was 86%, 99%, 86%, and 99% for detecting amikacin resistance and 50%, 96%, 71%, and 91% for detecting capreomycin resistance.

Conclusion: The MTBDRplus and MTBDRsl assay may aid in decision making in tuberculosis treatment in low-level drug resistance settings and should preferably be used to exclude resistance.

INTRODUCTION

Molecular tests that detect specific resistance-determining mutations in the *M. tuberculosis* genome have aided in a rapid diagnosis of drug-resistant tuberculosis (TB). Examples of such rapid drug susceptibility tests (DST) are the GenoType® MTBDRplus and the Xpert® MTB/RIF for the detection of multidrug-resistant TB (MDR-TB) and the GenoType® MTBDRsl for the detection of resistance to second-line antituberculosis drugs and ethambutol. Implementation of these tests has provided substantial patient and programmatic benefits for diagnosing MDR-TB in high-burden settings. As a result, recent WHO guidelines have recommended that the Xpert® MTB/RIF should be used as the initial diagnostic test in adults suspected of having MDR-TB in high-burden settings.

There are only a few studies on the performance of these tests in countries with low levels of drug resistance however. Differences between high-burden and low-burden countries are important to appreciate the operational characteristics of these tests. These differences are the lower positive predictive value, the higher number of patients with paucibacillary disease and differences in logistics and resources. There is therefore a need to generate more evidence on diagnostic accuracy of these molecular DST in low-incidence, high-resource countries.

The Netherlands is such a low-level drug resistance country with 1% of new TB cases and 2% of previously treated cases being MDR-TB. The national tuberculosis reference laboratory receives all *M. tuberculosis* cultures from the Netherlands for phenotypic drug-susceptibility testing. The GenoType® MTBDRplus and the MTBDRsl test have been routinely used since 2007 and 2009 for the early detection of drug-resistant tuberculosis. The goal of the present study was to assess diagnostic accuracy of the GenoType® MTBDRplus and the MTBDRsl tests in the Netherlands. We therefore performed a retrospectively study to compare these two molecular tests with phenotypic drug resistance results to first-line and second-line antituberculosis drugs in the context of routine, non-trial diagnostics provided by a nationwide laboratory service.
MATERIALS AND METHODS

Sample collection
This study was performed at the National Tuberculosis Reference Laboratory, which serves as the national referral laboratory for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* complex isolates for the Netherlands. We retrospectively identified all *Mycobacterium tuberculosis* complex isolates that were sent to our laboratory for DST from 2007 to 2012. The GenoType® MTBC assay (Hain Lifescience GmbH, Nehren, Germany) was used for (sub) species identification.

Phenotypic drug susceptibility testing
Drug susceptibility testing to first-line antituberculosis drugs was carried out using the Mycobacteria Growth Indicator Tube (MGIT) 960 system (BD Bioscience, Erebodegem, Belgium) using break point concentrations of 1.0 mg/l (Streptomycin), 0.1 mg/l (isoniazid), 1.0 mg/l (rifampicin), 5.0 mg/l (ethambutol) and 100 mg/l (pyrazinamide). Drug susceptibility testing to second-line antituberculosis drugs was performed in multidrug-resistant isolates using either a Middlebrook 7H10 agar dilution method using break point concentrations of 1.0 mg/l (moxifloxacin), 10.0 mg/l (capreomycin), 5.0 mg/l (amikacin) or the MGIT 960 system using break point concentrations of 0.5 mg/l (moxifloxacin), 2.5 mg/l (capreomycin) and 1.0 mg/l (amikacin). Drug susceptibility testing to second-line antituberculosis drugs was performed in multidrug-resistant isolates using either a Middlebrook 7H10 agar dilution method or the MGIT 960 system using break point concentrations of 0.5 mg/l (moxifloxacin), 2.5 mg/l (capreomycin) and 1.0 mg/l (amikacin). Drug susceptibility testing to second-line antituberculosis drugs was performed in multidrug-resistant isolates using either a Middlebrook 7H10 agar dilution method or the MGIT 960 system using break point concentrations of 0.5 mg/l (moxifloxacin), 2.5 mg/l (capreomycin) and 1.0 mg/l (amikacin).

Molecular DST
We used the GenoType® MTBDRplus assay (Hain Lifesciences, Nehren, Germany) to screen for mutations in the *rpoB*, *inhA* and the *katG* genes.

Analysis
The primary analysis was the diagnostic accuracy of the MTBDRplus and MTBDRsl assay to detect phenotypic drug resistance to rifampicin (*rpoB*), isoniazid (*inhA/katG*), ethambutol (*embB*), moxifloxacin (*gyrA*), capreomycin (*rrs*) or amikacin (*rrs*). One isolate per patient was used. Diagnostic accuracy was expressed as sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) using 2x2 contingency tables and OpenEpi software version 2.3.1; [http://www.OpenEpi.com]).

The Netherlands Tuberculosis Registry provided data on patient characteristics after approval by the registry committee. These data were matched with microbiological results on the basis of a unique isolate number. The final dataset consisted of anonymous data. Ethical approval was therefore not required. A multivariate logistic regression analysis with MDR-TB as the independent variable and age < 45 year, sex, prior treatment and country-of-origin (foreign-born) as dependent variables was performed to identify risk groups for MDR-TB. Threshold for significance (P-value) was set at 0.05. Precision of the estimates was reported using 95% confidence intervals (95% CI). We used SPSS version 20.0 (IBM corporation, Armonk, NY) for all statistical analysis.

Two nationwide screening strategies have been applied during the study period for the MTBDRplus assay: routine testing of all new isolates (2009 and 2010) and testing upon request by submitting laboratories (2007, 2008, 2011 and 2012). We determined the potential clinical relevance of these two screening strategies by comparing the diagnostic accuracy. Moreover, we calculated whether screening high-risk groups would improve accuracy by defining a hypothetical cohort with high-risk patients (defined as age < 45 years, prior treatment or foreign-born). Accuracy of the MTBDRplus assay in this hypothetical cohort was then compared with the routine screening strategy.
RESULTS

Study population
Of the 5,305 isolates sent to the laboratory for DST, a comparison between the MTBDRplus assay and phenotypic DST was possible in 2,649 (Figure 1). A comparison between the MTBDRsl assay and phenotypic DST to second-line antituberculosis drugs was possible in 74 cases, except for DST to capreomycin, which was performed in 65 cases only.

Characteristics of the study population are shown in Table 1. Median age of the 2,649 cases was 37 years and the majority was male. Phenotypic MDR-TB was seen in 74 cases (3%). MDR-TB cases were younger, more often female and foreign-born (Table 1). Age under 45 years (OR 7.2, 95%-CI: 2.6-20.2), prior treatment (OR 5.0, 95%-CI: 2.4-10.4), foreign-born (OR 4.3, 95%-CI: 1.3-14.0) and female sex (OR 2.2, 95%-CI: 1.3-3.6) were all risk factors for MDR-TB.

Figure 1
Flow chart of M. tuberculosis complex samples sent to the National Institute for Public Health and the Environment (RIVM) for molecular analysis with either the MTBDRplus assay or the MTBDRsl assay (2007-12)

| 5,305 isolates sent to RIVM for analysis |
| 2,463 excluded: |
| - no DST (478) |
| - only DST (1,895) |
| - only molecular analysis (90) |
| - double isolates (193) |

| 2,842 isolates analyzed with MTBDRplus |
| 104 isolates with rpoB mutation |
| 2,738 isolates without rpoB mutation |

| 74 MDR-TB isolates analyzed with MDRTBsl assay |

| 30 no MDR |

| 74 MDR-TB isolates analyzed with MDRTBsl assay |

| Diagnostic accuracy of the MTBDRplus assay |
The MTBDRplus assay detected mutations in the rpoB gene of 104 isolates (Table 2), 83 of which were phenotypically rifampicin resistant and 74 of which were MDR-TB according to phenotypic methods.

Overall, sensitivity and specificity of the MTBDRplus assay to detect rifampicin resistance was 100% (95%-CI: 96-100%) and 99% (95%-CI: 99-100%), respectively (Table 3). Positive

<p>| Table 1 Characteristics of the study population |</p>
<table>
<thead>
<tr>
<th>All patients (N = 2,649)</th>
<th>MDR-TB patients (N=74)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
</tr>
<tr>
<td>Age in years (median, IQR)</td>
<td>37 [27, 55]</td>
</tr>
<tr>
<td>Sex (% males)</td>
<td>59%</td>
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<tr>
<td>Foreign born (%)</td>
<td>68%</td>
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<tr>
<td>From high-burden MDR-TB country (%)</td>
<td>18%</td>
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<tr>
<td>Prior TB treatment (%)</td>
<td>5%</td>
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<tr>
<td>Pulmonary TB (%)</td>
<td>55%</td>
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<tr>
<td><strong>MTB species (%)</strong></td>
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<tr>
<td>M tuberculosis</td>
<td>95%</td>
</tr>
<tr>
<td>M bovis (+ BCG)</td>
<td>4%</td>
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<tr>
<td>M africanum</td>
<td>1%</td>
</tr>
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<td><strong>Resistance profile (% of resistant isolates)</strong></td>
<td></td>
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<tr>
<td>Rifampicin</td>
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<tr>
<td>Isoniazid</td>
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<tr>
<td>Ethambutol</td>
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<tr>
<td>Pyrazinamide</td>
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<tr>
<td>Streptomycin</td>
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<td>MDR-TB</td>
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<td>Amikacin</td>
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<td>Capreomycin</td>
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</tr>
</tbody>
</table>

*a 23 isolates were not tested for susceptibility to pyrazinamide and 9 isolates were not tested for susceptibility to capreomycin. |

*b pre-XDR is defined as MDR-TB with either resistant to fluoroquinolones or second-line injectables but not to both.
predictive value was 80% (95%-CI: 71-86%) and negative predictive value 100% (95%-CI: 100-100%). Sensitivity of an rpoB mutation for detecting phenotypical MDR-TB was 100% (95%-CI: 96-100%), specificity was 99% (95%-CI: 98-99%), positive predictive value 71% (95%-CI: 62-79%) and negative predictive value 100% (95%-CI: 100-100%).

The MTBDR assay detected 178 mutations in the katG gene and 117 mutations in the inhA gene (Table 2). Sensitivity of a katG mutation for detecting isoniazid resistance was 55% (95%-CI: 50-61%), specificity was 100% (95%-CI: 100-100%), positive predictive value 100% (95%-CI: 100-100%) and negative predictive value 94% (95%-CI: 93-95%) (Table 3). The sensitivity of mutations in the inhA gene for detecting isoniazid resistance was poor (36%). Specificity was much better leading to a high positive predictive value (100%, 95%-CI: 98-100%). Diagnostic accuracy improved when results of the two molecular targets for isoniazid resistance were combined. The sensitivity of any mutation in either the katG or inhA gene for detecting isoniazid resistance was 88% (95%-CI: 84-91%), the specificity 100% (95%-CI: 100-100%), the positive predictive value 100% (95%-CI: 98-100%), and the negative predictive value 98% (95%-CI: 98-99%).

### Diagnostic accuracy of the MTBDRsl assay

The MTBDRsl assay was performed in every MDR-TB case diagnosed in the period 2007-2012. Mutations in the embB gene were encountered most often, followed by rrs mutations and gyrA mutations (42%, 9% and 8%, respectively) (Table 2). The diagnostic accuracy of gyrA mutations for detecting moxifloxacin resistance was good (Table 4); sensitivity was 100% (95%-CI: 51-100%), specificity was 99% (95%-CI: 92-100%), positive predictive value was 83% (95%-CI: 44-97%) and negative predictive value 100% (95%-CI: 95-100%).

Diagnostic accuracy of the MTBDRsl assay for detecting resistance to second-line injectables is shown in Table 4. Sensitivity of an rrs mutation for detecting phenotypic resistance to second-line injectables was mediocre at best (50% and 86% for capreomycin and amikacin resistance, respectively). Specificity was higher, reaching 96% for capreomycin (95%-CI: 88-99%) and 99% for amikacin (95%-CI: 92-100%). The positive predictive value of an rrs mutation for detecting resistance to capreomycin was 71% (95%-CI: 36-92%) and the negative predictive value was 91% (95%-CI: 91-96%). The positive predictive value or an rrs mutation for detecting resistance to amikacin was 86% (95%-CI: 49-97%) and the negative predictive value was 99% (95%-CI: 92-100%).

Four XDR-TB cases were seen. All these cases could be detected using gyrA mutations as a marker for XDR-TB (Table 4). Sensitivity and specificity of a gyrA mutation for detecting XDR-TB were 100% (95%-CI: 51-100%) and 97% (95%-CI: 90-99%). Two additional isolates
carrying a gyrA mutation were only pre-XDR, lowering positive predictive value of a gyrA mutation for XDR-TB to 67% (95%-CI: 30-90%). In contrast, the negative predictive value was excellent (100%, 95%-CI: 95-100%).

Table 4
Diagnostic accuracy of the MTBDR sl test for detecting phenotypic susceptibility to second-line antituberculosis drugs in the Netherlands

<table>
<thead>
<tr>
<th>Resistance profile</th>
<th>capreomycin</th>
<th>amikacin</th>
<th>moxifloxacin</th>
<th>ethambutol</th>
<th>XDR-TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>rs</td>
<td>rs</td>
<td>gyrA</td>
<td>embB</td>
<td>gyrA</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>50% (24-76%)</td>
<td>86% (49-97%)</td>
<td>100% (57-100%)</td>
<td>62% (44-77%)</td>
<td>100% (51-100%)</td>
</tr>
<tr>
<td>Specificity</td>
<td>96% (88-99%)</td>
<td>99% (92-100%)</td>
<td>99% (92-100%)</td>
<td>71% (57-82%)</td>
<td>97% (90-99%)</td>
</tr>
<tr>
<td>PPV</td>
<td>71% (36-92%)</td>
<td>86% (49-97%)</td>
<td>63% (44-97%)</td>
<td>58% (41-74%)</td>
<td>67% (30-90%)</td>
</tr>
<tr>
<td>NPV</td>
<td>91% (81-96%)</td>
<td>99% (92-100%)</td>
<td>100% (95-100%)</td>
<td>74% (60-85%)</td>
<td>100% (95-100%)</td>
</tr>
</tbody>
</table>

* Estimates are given as percentages with 95% confidence intervals in brackets

b MDR-TB, multidrug-resistant tuberculosis; PPV, positive predictive value; NPV, negative predictive value; XDR-TB, extensively-resistant tuberculosis

Clinical relevance of different screening strategies

Different real-life screening strategies were undertaken during the study period. These are shown in Table 5. Screening on request (strategy II) instead of a general screening (strategy I) did not change diagnostic accuracy, but would lower the number of isolates screened. We calculated whether screening in high-risk groups only would improve diagnostic accuracy (Table 5). Compared with strategy I, screening in high-risk groups (strategy III) did not change diagnostic accuracy of the MDRTB plus assay to detect MDR-TB. Implementing this algorithm would have missed one patient with an rifampicin resistant, isoniazid susceptible M. tuberculosis isolate but no MDR-TB case.

Table 5
Effect of different screening strategies on the diagnostic accuracy of the MTBDR plus assay to detect MDR-TB

<table>
<thead>
<tr>
<th></th>
<th>Strategy I</th>
<th>Strategy II</th>
<th>Strategy IIIa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening strategy</td>
<td>Screening every patient</td>
<td>On-demand of referring clinician</td>
<td>Screening risk groups</td>
</tr>
<tr>
<td>year</td>
<td>2009-2010</td>
<td>2011-2012</td>
<td>2009-2010</td>
</tr>
<tr>
<td>Total number</td>
<td>1569</td>
<td>918</td>
<td>1293</td>
</tr>
<tr>
<td>MDR-TB prevalence</td>
<td>32 (2%)</td>
<td>28 (3%)</td>
<td>32 (2%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100% [89-100%]</td>
<td>100% [88-100%]</td>
<td>100% [89-100%]</td>
</tr>
<tr>
<td>Specificity</td>
<td>99% [99-100%]</td>
<td>99% [98-99%]</td>
<td>99% [98-99%]</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>71% [57-82%]</td>
<td>72% [56-83%]</td>
<td>73% [58-84%]</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>100% [100-100%]</td>
<td>100% [100-100%]</td>
<td>100% [100-100%]</td>
</tr>
</tbody>
</table>

a This represents a hypothetical group from the 2009-2010 sample. Risk groups were: foreign-born, age < 45 years and prior TB-treatment

DISCUSSION

In the present study we determined the diagnostic accuracy of the GenoType® MTBDR plus and MTBDR sl assay to detect phenotypic drug resistance to antituberculosis drugs in the context of routine diagnostics in a high-resource, low-level drug resistance setting. We found that molecular DST had excellent accuracy for detecting phenotypic resistance to isoniazid, good accuracy for detecting resistance to rifampicin, amikacin and moxifloxacin, but modest to poor accuracy for detecting resistance to capreomycin and ethambutol.

The present study complements previous studies on the performance of the MTBDR plus assay in low-burden TB countries. Previous meta-analyses have raised questions on the applicability of the MTBDR plus assay to detect MDR-TB in such low-level drug resistant settings, suggesting that positive predictive value might be as low as 59%. In the present study the positive predictive value was 71%, probably because we used indirect DST. A recent laboratory study in the UK, another high-resource low-level drug resistance setting, showed similar accuracy for rifampicin resistance but found fewer false-positive results. This difference might be explained by their use of the LJ medium instead of the MGIT 960 system as the reference standard. We have previously reported that many of these presumably false-positive mutations occurred at positions of the rpoB gene that are associated with discordant, susceptible results in the MGIT 960 system and these mutations may in fact lead to low-level rifampicin resistance and treatment failure.
Our results are in line with a recent meta-analysis by Feng and co-workers on the performance of the MTBDRsl assay for detecting phenotypic drug resistance. In their meta-analysis, they found a similar performance of the MTBDRsl assay to detect fluoroquinolone and amikacin resistance but sensitivity to capreomycin was higher compared to the present study. The relative small number of isolates with \textit{rps} mutation in our sample might have caused this difference, as well as the higher proportion of \textit{A1401G} mutations we found in the \textit{rrs} gene. These mutations are associated with high-level amikacin resistance, but low-level resistance to capreomycin.\textsuperscript{39}

Many of the previous studies on the accuracy of the MTBDRsl assay do not well reflect low-level drug resistance settings.\textsuperscript{10,18-20} In our previously published article on the accuracy of the MTBDRsl assay for instance,\textsuperscript{10} prevalence of amikacin and moxifloxacin resistance was 28\% and 24\%, respectively, while corresponding proportions for MDR-TB isolates in the Netherlands are 10\% and 7\% (Table 1). Given the low prevalence and the concomitant high negative predictive value of both aminoglycosides and fluoroquinolones found in the present study, our results suggests that the MTBDRsl assay should best be used to rule out phenotypic resistance to these drugs in low-level drug resistance countries such as the Netherlands.

Our results also confirm the poor performance of \textit{embB} mutations for detecting ethambutol resistance. A recent meta-analysis by Cheng and co-workers showed a similar sensitivity and specificity for detecting ethambutol resistance of the MTBDRsl assay (55\% and 71\%).\textsuperscript{21} This poor performance of the MTBDRsl assay is probably caused by the inherent difficulties in phenotypic DST for ethambutol and by the fact that only mutations at position 306 are screened with this assay.\textsuperscript{21} Given the poor performance of the MTBDRsl assay, it seems that this assay can neither be used for detecting nor for ruling out ethambutol resistance accurately in settings such as the Netherlands and clinicians should await phenotypic DST before deciding to change treatment regimens.

The present study provides suggestions for applying molecular DST in the Netherlands. Given the low prevalence of MDR-TB (1\%) a case–detection strategy applying the MTBDR\textit{plus} assay (or any equivalent) for every suspected TB case might lead to unwanted overtreatment, might draw unnecessary financial burden on public health programs and might not be cost-effective.\textsuperscript{4,22} An alternative strategy would be to improve accuracy by screening in high-risk groups only. We showed that the common risk factors such as previous treatment, age and foreign-born were insufficient to increase predictive value in the Netherlands and might lead to missing drug-resistant TB cases with potential MDR-TB transmission, even when using indirect DST (reflex testing). This would tip the scale in favour for screening every proven \textit{M. tuberculosis} complex isolate in low-burden countries.

We would propose to include a MTBDRsl assay and a \textit{pncA} gene analysis in every isolate with an \textit{rpoB} mutation to further guide treatment initiation in these patients. The chances of additional resistance are negligible if an isolate would have a wild type \textit{gyrA}, \textit{rps} and \textit{pncA} gene. We would initiate first-line antituberculosis treatment together with moxifloxacin and amikacin in these patients. A concomitant \textit{katG} or \textit{inhA} mutation would strengthen the diagnosis of MDR-TB and favour initiating MDR-TB treatment without rifampicin and isoniazid. A \textit{pncA} mutation would suggest a pyrazinamide–resistant MDR \textit{M. tuberculosis},\textsuperscript{13} in which case we would propose to initiate MDR-TB treatment with second-line antituberculosis drugs only. A \textit{gyrA} mutation would suggest an XDR \textit{M. tuberculosis} in two-third of cases (Table 4), in which case we would wait further phenotypic DST before initiating treatment.

**Acknowledgements**

The authors would like to thank H. Schimmel for her assistance in providing the data from the Netherlands Tuberculosis Registry.

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Molecular drug susceptibility testing for first-line and second-line antituberculosis drugs in the Netherlands
Antimycobacterial activity of phenothiazines and SILA 421

Adapted from:
ABSTRACT

Background: Phenothiazines and SILA 421 are promising antituberculosis drugs targeting efflux pumps of both the mycobacteria and the macrophages that engulf them. They have proven activity against Mycobacterium tuberculosis in vitro as well as in vivo.

Design: In this study, we compared the in vitro activity of multiple phenothiazines and SILA 421 against 21 M. tuberculosis strains with different drug susceptibility patterns, using the Middlebrook 7H10 agar dilution method. Because the S-enantiomer of thioridazine might induce less neurotropism, we tested the activity of both enantiomers separately.

Results: Chlorpromazine, thioridazine and SILA 421 showed equal activity with an MIC\textsubscript{50} and MIC\textsubscript{90} of 4 and 16 mg/l, respectively. The S-enantiomer and R-enantiomer of thioridazine proved equally active in vitro. Desipramine and promazine showed little antimycobacterial activity.

Conclusions: This study confirms that phenothiazines and SILA 421 hold promise as antituberculosis drugs. The unabated activity of the S-enantiomer of thioridazine may enlarge the applicability of phenothiazines in tuberculosis treatment.

INTRODUCTION

The worldwide spread of multidrug-resistant (MDR) and extensively drug resistant (XDR) tuberculosis (TB) has been a major incentive in the search for new classes of antituberculosis drugs. Efflux pump inhibitors are one of those novel antituberculosis drug classes.\textsuperscript{7} The antimycobacterial effect of efflux pump inhibitors is postulated to be threefold. First, targeting mycobacterial efflux pumps decreases survival of M. tuberculosis.\textsuperscript{4} Second, efflux pump inhibitors increase susceptibility to other antituberculosis drugs by inhibiting efflux mechanisms partially responsible for antimycobacterial drug resistance.\textsuperscript{3} Third, efflux pumps can inhibit calcium efflux pumps of the phagolysosomes of macrophages thereby enhancing the killing activity of the pulmonary macrophages containing the entrapped M. tuberculosis bacteria.\textsuperscript{4}

Among the efflux pump inhibitors capable of killing M. tuberculosis are organosilicon compounds, of which SILA 421 has been shown to have potential as an antituberculosis drug.\textsuperscript{5} It shares the three pathways of antimycobacterial killing with other efflux pump inhibitors: it revealed direct in vitro activity against M. tuberculosis;\textsuperscript{5} it has been shown to modify resistance by inhibiting mdr-1 efflux pumps\textsuperscript{6} and has shown to enhance killing of M. tuberculosis by macrophages.\textsuperscript{5}

Another class of efflux pump inhibitors enhancing the killing of M. tuberculosis are the neuroleptic phenothiazines and the phenothiazine analogues thioxanthenes.\textsuperscript{7-9} The MICs of phenothiazines against M. tuberculosis vary from 4 to 16 mg/l depending on the medium used.\textsuperscript{10} Thioridazine, one of the phenothiazines, revealed the highest in vitro activity.\textsuperscript{7,10} Subsequent animal studies have shown that thioridazine, as a sole drug and in combination with other antituberculosis drugs, was able to reduce bacterial loads in mice infected with drug-susceptible or MDR-TB.\textsuperscript{11,12} Moreover, thioridazine has been used on a compassionate basis in MDR and XDR-TB patients.\textsuperscript{1,13}

The use of thioridazine has been limited by its neurological and cardiac side-effects.\textsuperscript{1,13} The neurological side-effects are caused by blocking of the dopamine (D2) receptor by thioridazine.\textsuperscript{14} It has hypothesized that the stereoselective S-enantiomer of thioridazine might induces less neurological side effects, putatively favouring the S-enantiomer to be used as antituberculosis drug instead of its racemate.\textsuperscript{14,15}

SILA 421 and the stereoselective S-enantiomer of thioridazine are promising additions to the spectrum of antituberculosis efflux pump inhibitors. However, these have only been
tested against a small number of *M. tuberculosis* isolates.5,15 We therefore assessed the antimycobacterial activity of SILA 421 against an extended set of *M. tuberculosis* strains with varying drug susceptibility profiles. Second, we compared the antimycobacterial activity of SILA 421 with that of thioridazine, its enantiomers and other phenothiazines.

**MATERIALS AND METHODS**

**Bacterial strains**

We selected 21 clinical *M. tuberculosis* isolates to serve as a panel for the evaluation of the *in vitro* antimycobacterial activity of SILA 421, the phenothiazines and both thioridazine enantiomers: Five pansusceptible, 11 monoresistant (6 isoniazid monoresistant, 4 rifampin monoresistant and 1 streptomycin monoresistant) and 5 multidrug-resistant clinical isolates were selected. These originated from the collection of the National Tuberculosis Reference Laboratory (Bilthoven, the Netherlands). *Mycobacterium tuberculosis* strains had been identified using the Genotype MTBC assay (Hain Lifescience, Nehren, Germany) and susceptibility testing to antituberculosis drugs had been performed using the Mycobacteria Growth Inhibitor Tube 960 system (MGIT 960) according to the manufacturer's instruction (Becton Dickinson, NJ, USA).

**Antimicrobial agents**

All drugs were received as chemically pure powder. Chlorpromazine, desipramine, promazine and thioridazine (TZsigma) were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). The two thioridazine enantiomers were prepared by Dr. J.B. Christensen (Dept. Chemistry, Copenhagen, Denmark) according to the procedure described by Bourquin previously.16 The racemic form was prepared by mixing equal 1:1 amounts of the two enantiomers. SILA 421 was provided by Dr. G. Hajos (Institute for Biomolecular Chemistry, Budapest, Hungary). All drugs were dissolved in distilled water to a stock solution of 10 g/l after which dilutions of anti-tuberculosis drugs were prepared, ranging from 1 mg/l to 16 mg/l.

**Drug susceptibility testing**

The Minimal Inhibitory Concentration (MIC) was determined using a Middlebrook 7H10 agar dilution method as described previously.17 In short, isolates were extracted from a -70°C freezer collection and sub-cultured on Ogawa medium. 25-Well plates containing 23 wells with different concentrations of the antimicrobial drugs (1, 2, 4, 8, 16 mg/l), as well as one control well with drug free medium were inoculated with 10 µL of *M. tuberculosis* suspension aiming at 10 x 10³ to 10 x 10⁴ CFU. Another control well was inoculated with a 1:100-diluted suspension. The MIC was defined as the lowest concentration of an antituberculosis drug that inhibits more than 99% of the growth of the mycobacterial culture and was determined by comparing the growth of the drug enriched well with the 1:100-diluted control well.

**RESULTS**

The MICs could be determined after a median of 14 days (range 14–18 days). Table 1 gives the MIC₅₀ (MIC at which 50% of the isolates are inhibited) and MIC₉₀ (MIC at which 90% of the isolates are inhibited) values of SILA 421 and the various phenothiazines. The MIC₅₀ of SILA 421 was similar to the MIC₅₀ of thioridazine and chlorpromazine, namely 4 mg/l. SILA 421 showed lowest MIC₉₀. In contrast, desipramine and promazine revealed limited antimycobacterial activity with MIC₅₀ and MIC₉₀ > 16 mg/l. Table 1 also depicts the effect of chirality of thioridazine on the activity against *M. tuberculosis*. The antimycobacterial activity of the S-enantiomer did not differ from the R-enantiomer. The MIC₅₀ of these enantiomers amounted to 8 mg/l and the MIC₉₀ to 16 mg/l.

<table>
<thead>
<tr>
<th>Efflux pump inhibitor</th>
<th>MIC₅₀ (mg/l)</th>
<th>MIC₉₀ (mg/l)</th>
<th>Range (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SILA 421</td>
<td>4</td>
<td>8</td>
<td>2 - 16</td>
</tr>
<tr>
<td>Thioridazine</td>
<td>4</td>
<td>16</td>
<td>2 - 16</td>
</tr>
<tr>
<td>Thioridazine, S-enantiomer</td>
<td>8</td>
<td>16</td>
<td>4 - 16</td>
</tr>
<tr>
<td>Thioridazine, R-enantiomer</td>
<td>8</td>
<td>16</td>
<td>4 - 16</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>4</td>
<td>16</td>
<td>&lt;1 - 16</td>
</tr>
<tr>
<td>Desipramine</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>16 - &gt;16</td>
</tr>
<tr>
<td>Promazine</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>16 - &gt;16</td>
</tr>
</tbody>
</table>

* MIC₅₀, MIC at which ≥ 50% of isolates are inhibited.  
* MIC₉₀, MIC at which ≥ 90% of isolates are inhibited.  
* MIC distribution was based on 20 instead of 21 isolates.
DISCUSSION

In the present study, we showed that Sila 421 has antimycobacterial activity similar to that of thioridazine in vitro, when tested against a wide variety of *M. tuberculosis* strains. Second, we confirmed the antimycobacterial activity of the phenothiazines and showed that the thioridazine S-enantiomer proved as effective as its racemate.

In a previous study, SILA 421 has been proposed to be more potent than thioridazine. However, only two *M. tuberculosis* isolates were tested. The present study confirms that SILA 421 may be a promising antituberculosis drug with activity against a wide variety of *M. tuberculosis* strains comparable to that of thioridazine. Further studies are warranted to investigate the potency of SILA 421 as an antituberculosis drug in animals and in humans. Besides its antimycobacterial activity, SILA 421 might also have another appealing feature. Like all efflux pump inhibitors, SILA 421 will inhibit the activity of efflux pumps of MDR mycobacteria, presumptively rendering mycobacteria more susceptible to the antituberculosis drugs to which it was initially resistant as a consequence of their extrusion from the cell. Thus, these drug resistance modifiers might be of interest not only for their immediate antimycobacterial activity but also as an adjunctive agent to be added to new or existing antituberculosis regimens.

Our study confirms that phenothiazines and their derivatives are active against *M. tuberculosis*. Other studies have found similar results for promazine, thioxanthenes, thioridazine, and the thioridazine enantiomers. By providing a MIC distribution of these drugs in a wider variety of *M. tuberculosis* strains and by showing that the MIC for all drugs was relatively constant with a MIC$_{50}$ of 4 mg/l for most drugs, the present study extends the knowledge on the antimycobacterial activity of this class of drugs.

Of additional interest is our finding that the S-enantiomer of thioridazine is as effective as thioridazine itself against *M. tuberculosis*. It is thought this S-enantiomer might induce less neurological side effects owing to a lower affinity to the D$_2$ dopamine receptor, putatively favoring the S-enantiomer over the racemate as an antituberculosis drug. However, it is unknown if the D$_2$ dopamine receptor is the only target for neurological side-effects of thioridazine and it is unclear whether the pharmacokinetic and pharmacodynamic properties of the thioridazine enantiomers are equal. Notwithstanding these limitations, our findings raise hope that structural optimization of thioridazine-derivatives is possible without losing antimycobacterial activity.

In conclusion, the present study provides confirmatory and incremental evidence of the antimycobacterial activity of SILA 421 and the phenothiazines, such as thioridazine. Therapeutic opportunities lay in the structural optimization of these drugs as well as in their drug resistance modifying effect. Future studies should bring SILA 421 and the phenothiazines further up the drug development pipeline.
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Reappraising approved antimicrobial agents as potential drugs against MDR-TB and XDR-TB

Manuscript in preparation
Repositioning approved antimicrobial drugs is gaining attention as means to speed up anti-tuberculosis drug development. We determined the MIC\textsubscript{50} of 6 licensed antimicrobial agents in 13 drug-resistant Mycobacterium tuberculosis strains using the MGIT 960 system. Fusidic acid (8 mg/l), mefloquine (16 mg/l), nitrofurantoin (32 mg/l), co-trimoxazole (2:38 mg/l), amoxicillin with clavulanic acid (8/8 mg/l), and meropenem with clavulanic acid (2/2.5 mg/l) all showed antimycobacterial activity suggesting that they, or another class representative, could be repositioned as antituberculosis drugs. This calls for a reappraisal of other approved antimicrobial agents as potential anti-TB drugs.

In view of this, the concept of repurposing existing drugs for the treatment of drug-resistant TB is gaining attention.\textsuperscript{3} It is the new use for existing approved drugs as anti-TB drugs.\textsuperscript{3} Examples of such repurposed drugs currently in use for treating MDR-TB and XDR-TB are linezolid and meropenem with clavulanic acid.\textsuperscript{4} Linezolid was originally introduced for treating nosocomial pneumonia and skin infections, but also proved to be active against \textit{M. tuberculosis} in 1991.\textsuperscript{4} Recent meta-analyses have shown that the use of linezolid-containing treatment regimens is associated with favorable outcomes.\textsuperscript{5,6,7} The first report of the antimycobacterial activity of meropenem with clavulanic acid dates to 2009.\textsuperscript{4} Since then case-control studies have reported that regimens including meropenem with clavulanic acid are associated with a better sputum-smear conversion at 3 months.\textsuperscript{5} As a result, the most recent TBNET consensus statement has suggested that both linezolid and meropenem/clavulanic acid may be used for the treatment of MDR- and XDR-TB.\textsuperscript{4}

Besides these two drugs there are other antimicrobial agents on the market with some effectiveness against TB. These are amoxicillin with clavulanic acid, thioridazine, metronidazole, doxycycline and co-trimoxazole.\textsuperscript{10} All these drugs have proven \textit{in vitro} activity against \textit{M. tuberculosis} and some have been studied in (MDR)-TB patients, albeit with varying success.\textsuperscript{10-12} There has also been some anecdotal evidence of the antimycobacterial activity of other antimicrobial agents, such as fusidic acid,\textsuperscript{13} nitrofurantoin\textsuperscript{14} and mefloquine.\textsuperscript{15} The antimycobacterial activity of these existing antimicrobial agents are hints suggesting that we are currently not using all potentially available drug classes. The relevance of the antimycobacterial activity of these agents may be underappreciated owing to limited \textit{in vitro} data on clinical isolates; proper MIC distributions have only been published for thioridazine and co-trimoxazole.\textsuperscript{16-18} The goal of the present study was to extend the knowledge of the antimycobacterial activity of these agents by providing a MIC distribution from a panel of various clinical \textit{M. tuberculosis} isolates.
MATERIALS AND METHODS

Sample collection
We selected 12 clinical M. tuberculosis isolates and the H37Rv reference strain to serve as a panel for the *in vitro* activity against M. tuberculosis. The clinical isolates originated from the collection of the National Tuberculosis Reference Laboratory (Bilthoven, the Netherlands) and some of these isolates have been used previously as a panel for testing susceptibility for other (novel) antituberculosis drugs.\(^{16,18}\)

*Mycobacterium tuberculosis* strains had been identified using the Genotype MTBC assay (Hain Lifescience, Nehren, Germany). The 13 strains had a known susceptibility pattern to first- and second-line drugs, as determined using the Mycobacteria Growth Indicator Tube 960 (MGIT 960; Becton Dickinson, Erebodegem, Belgium) and Middlebrook 7H10 agar dilution method.\(^{19,20}\) Multidrug-resistant (MDR) tuberculosis was defined as isolates that were resistant to at least rifampicin and isoniazid. Extensively resistant (XDR) tuberculosis was defined as isolates that were resistant to rifampicin, isoniazid, a second-line injectables and a fluoroquinolone.

Antimicrobial agents
The six antimicrobials agents were all acquired through Sigma Aldrich (Zwijndrecht, the Netherlands). The drug concentrations that were tested are depicted in Table 1. These drug concentrations were chosen on the basis of previous publications using liquid media.\(^{8,14,16-23}\) We used trimethoprim and sulfamethoxazole in a 1:19 ratio.\(^{22}\) Amoxicillin was tested using a fixed clavulanic acid concentration of 8 mg/l.\(^{8}\) We tested meropenem with and without a fixed clavulanic acid concentration of 2.5 mg/l.\(^{8}\) Based on previous observations (D. van Soolingen, *personal communication*) meropenem without clavulanic acid was tested in concentrations from 4 to 64 mg/l, while meropenem with clavulanic acid was tested in concentrations from 0.25 to 8 mg/l.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentrations evaluated (in mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin with clavulanic acid(^a)</td>
<td>2, 4, 8, 16, 32</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>0.5:9.5, 1:19, 2:38, 4:76, 8:152</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>4, 8, 16, 32, 64</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>2, 4, 8, 16, 32</td>
</tr>
<tr>
<td>Meropenem, with and without clavulanic acid(^b)</td>
<td>0.25, 0.5, 1, 2, 4, 8, 16, 32</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>4, 8, 16, 32, 64</td>
</tr>
</tbody>
</table>

\(^a\) The MGIT 960 system was used for drug susceptibility testing.

\(^b\) Amoxicillin was tested with a fixed clavulanic acid concentration of 8 mg/l.

\(^c\) Meropenem was tested with and without a fixed clavulanic acid concentration of 2.5 mg/l.

Drug susceptibility testing
The minimum inhibitory concentrations (MIC) were determined using the MGIT 960 system. To prepare inocula, isolates were extracted from a -70°C freezer collection an sub-cultured in MGIT medium. One or two days after the MGIT tube turned positive, the culture was mixed by vortexing and allowed to settle for 5-10 minutes as instructed by the manufacturer (Becton Dickinson, Erebodegem, Belgium). We then added 0.5 ml of *M. tuberculosis* broth supernatant, 0.8 ml of Bactec MGIT growth supplement (oleic acid, albumin, dextrose, catalase; OADC) and 0.1 ml of appropriate drug solution to a new MGIT tube. A growth control tube was simultaneously prepared with a 1:100-diluted 0.5 ml inoculum. All tubes were incubated at 37°C and read when the growth control tube had reached a growth value (GU) of 400.\(^{24}\) The minimum inhibitory concentration (MIC) was defined as the lowest concentration in which the GU of the drug-containing culture tube was <100.\(^{24}\) Results were summarized for each drug using the MIC\(^{50}\) and MIC\(^{90}\). Some of these results have been published previously as an abstract.
RESULTS

The susceptibility pattern and the individual MICs of the 12 clinical isolates and the H37Rv reference strain is shown in Table 2. Three isolates were fully susceptible, one isolate was ethambutol monoresistant, 2 isolates were isoniazid and ethambutol resistant, five were MDR *M. tuberculosis* isolates and three XDR *M. tuberculosis* isolates.

Table 2

The MICs (in mg/l) of six antimicrobial agents in 13 susceptible and drug-resistant *M. tuberculosis* strains

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Susceptibilitya</th>
<th>Antimicrobial agentb,c</th>
<th>AMC</th>
<th>MEM</th>
<th>MEC</th>
<th>SXT</th>
<th>FUS</th>
<th>MEF</th>
<th>NIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>Pansusceptible</td>
<td></td>
<td>16</td>
<td>&lt; 2</td>
<td>0.5</td>
<td>&lt; 0.5:9.5</td>
<td>&lt; 4</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>NLA009600108</td>
<td>Pansusceptible</td>
<td></td>
<td>&lt; 2</td>
<td>8</td>
<td>1</td>
<td>1:19</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>NLA009700234</td>
<td>Pansusceptible</td>
<td></td>
<td>16</td>
<td>16</td>
<td>1</td>
<td>4:76</td>
<td>&lt; 4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>NLA009800610</td>
<td>E resistant</td>
<td></td>
<td>&lt; 2</td>
<td>8</td>
<td>2</td>
<td>&lt; 0.5:9.5</td>
<td>&lt; 4</td>
<td>16</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>NLA000401745</td>
<td>H &amp; S resistant</td>
<td></td>
<td>16</td>
<td>&gt; 32</td>
<td>&gt; 4</td>
<td>&lt; 0.5:9.5</td>
<td>8</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>NLA000201682</td>
<td>MDR</td>
<td></td>
<td>4</td>
<td>32</td>
<td>2</td>
<td>1:19</td>
<td>&lt; 4</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>NLA000301128</td>
<td>MDR</td>
<td></td>
<td>8</td>
<td>16</td>
<td>1</td>
<td>&lt; 0.5:9.5</td>
<td>4</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>NLA000401230</td>
<td>MDR</td>
<td></td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>2:38</td>
<td>8</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>NLA000801695</td>
<td>MDR</td>
<td></td>
<td>&lt; 2</td>
<td>4</td>
<td>0.5</td>
<td>4:76</td>
<td>16</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>NLA000801810</td>
<td>MDR</td>
<td></td>
<td>&lt; 2</td>
<td>16</td>
<td>2</td>
<td>2:38</td>
<td>&lt; 4</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>NLA00090268</td>
<td>XDR</td>
<td></td>
<td>&gt; 32</td>
<td>&gt; 32</td>
<td>&gt; 4</td>
<td>2:38</td>
<td>32</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>NLA000901542</td>
<td>XDR</td>
<td></td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>4:76</td>
<td>32</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>NLA000901844</td>
<td>XDR</td>
<td></td>
<td>8</td>
<td>32</td>
<td>2</td>
<td>2:38</td>
<td>8</td>
<td>8</td>
<td>32</td>
</tr>
</tbody>
</table>

*a, E, ethambutol; H, isoniazid; MDR, multidrug-resistant; S, streptomycin; XDR, extensively drug-resistant.

*b, AMC, amoxicillin with clavulanic acid; MEM, meropenem; MEC, meropenem with clavulanic acid; SXT, trimethoprim–sulfamethoxazole; FUS, fusidic acid; MEF, mefloquine; NIT, nitrofurantoin.

*c, Amoxicillin (AMC) and meropenem (MEC) were tested with a fixed clavulanic acid concentration of 8 mg/l and 2.5 mg/l, respectively.

We observed *in vitro* antimycobacterial activity against both susceptible and drug-resistant *M. tuberculosis* for all six antimicrobial agents (Table 2). The MIC<sub>50</sub> (MIC at which 50% of the isolates are inhibited) and MIC<sub>90</sub> (MIC at which 90% of the isolates are inhibited) for amoxicillin with clavulanic acid were 8 mg/l and 16 mg/l respectively. We first tested meropenem without adding clavulanic acid because meropenem is a poor substrate for the BlaC β-lactamase of *M. tuberculosis*. We noted a MIC<sub>50</sub> of 16 mg/l and a MIC<sub>90</sub> of > 32 mg/l. Addition of 2.5 mg/l clavulanic acid lowered the MIC<sub>50</sub> to 2 mg/l. Clavulanic acid itself had no antimycobacterial activity (data not shown).

Besides β-lactam antibiotics, we also tested susceptibility to co-trimoxazole. We found that the MIC<sub>50</sub> was 2:38 mg/l and the MIC<sub>90</sub> was 4:76 mg/l. We additionally tested the antimycobacterial activity of fusidic acid, mefloquine and nitrofurantoin (Table 2). The MIC<sub>50</sub> for fusidic acid was 8 mg/l and the MIC<sub>90</sub> was 32 mg/l. For mefloquine, the MIC<sub>50</sub> was 16 mg/l and the MIC<sub>90</sub> was 16 mg/l. The MIC<sub>50</sub> of nitrofurantoin amounted to 32 mg/l and the MIC<sub>90</sub> to 64 mg/l.
DISCUSSION

The present study provides confirmatory and incremental evidence that amoxicillin with clavulanic acid, meropenem with clavulanic acid and co-trimoxazole could be repurposed as antituberculosis drugs against MDR/XDR-TB. Secondly, fusidic acid, mefloquine and nitrofurantoin also showed antimycobacterial activity suggesting that class representatives could be used as novel antituberculosis drugs.

We measured MICs to meropenem/clavulanic acid using the MGIT960 system and found that MICs were higher than those reported by Hugonnet and colleagues. Using a Middlebrook 7H9 broth medium, they reported an MIC$_{50}$ of 0.625 mg/l and an MIC$_{90}$ of 1.25 mg/l, whereas in the present study MIC$_{50}$ and MIC$_{90}$ were 2 mg/l and > 4 mg/l. This discrepancy may be partly explained by the fact that Hugonnet and colleagues used a microdilution assay and tested additional intermediate concentrations for more exact MIC titration. Also, meropenem may lose activity with time in the 7H9 medium, thereby complicating accurate interpretation of the MICs. Nonetheless, our results suggest that the activity of meropenem with clavulanic acid might be lower than previously anticipated.

The present study confirms that co-trimoxazole has potential as an antituberculosis drug. It is an attractive alternative because of its pharmacokinetic profile, its low costs and good tolerance in TB patients. Its mechanism of acting is through inhibition of the folic acid metabolism. The present MIC distribution in MDR/XDR M. tuberculosis isolates might help future pharmacokinetic and –dynamic studies in setting the optimal dose.

The other antimicrobial agents also revealed in vitro activity against M. tuberculosis. The MICs found for fusidic acid were lower than those in previous studies, while the MICs of mefloquine and nitrofurantoin were similar to those found previously. These drugs should be viewed as examplars of their respective drug classes, providing clues for drug engineering of novel antituberculosis drugs (Table 3). Fusidic acid, for instance, binds to elongation factor G thereby preventing its release from the ribosome. Nitrofurantoin, in turn, is converted into a reactive intermediate via type I oxygen insensitive nitroreductases, rendering nitrofurans attractive drugs against dormant mycobacteria. Mefloquine inhibits F$_{0}$F$_{1}$H+-ATPase echoing the mode of action of bedaquiline.

The present study provided a range of MICs for the six agents in 12 clinical drug-resistant M. tuberculosis isolates. Future studies in larger strain collections should therefore aim to correlate these MICs to PK/PD parameters. If these yield encouraging results, the next step would be to test these drugs in preclinical models, e.g. hollow fiber models and mouse models, before progressing to clinical trials. Only the latter will ascertain the true potential of these drugs as antituberculosis drugs.

In conclusion, our findings suggest that existing drugs indeed may have new uses in the treatment of drug-resistant TB. All six approved antimicrobial agents tested in the present study showed in vitro antimycobacterial activity suggesting that they, or another class representative, could be repositioned as antituberculosis drugs. This calls for a reappraisal of other approved antimicrobial agents as potential antituberculosis drugs.

Reappraising approved antimicrobial agents as potential drugs against MDR-TB and XDR-TB
REFERENCES


CHAPTER TEN

Summary
Introduction
The emergence of multidrug-resistant tuberculosis (MDR-TB) threatens global tuberculosis control. This has sparked a renewed interest in developing new antituberculosis drugs and regimens. To be safe and effective, the introduction of these new treatment regimens should be aligned with drug susceptibility testing (DST). Drug susceptibility testing is essential because it can identify patients who will benefit from treatment and because it can be used to monitor patterns of emerging drug resistance in the community. Which DST strategy is best depends on the prevailing drug resistance level, the selection of patient population eligible for testing, and the health-care system implementing the algorithm. This should be preceded by a thorough understanding of the accuracy and performance of the different diagnostic strategies, in real-life settings and under different epidemiological situations. This has been the main topic of this thesis.

Methods for drug susceptibility testing
We provided an introduction to the different methods for DST in TB in chapter 2. DST has classically been performed on solid media, such as the Löwenstein-Jensen medium. Semi-automated non-radiometric liquid culture systems, such as the Mycobacteria Growth Indicator Tube (MGIT) 960 system, have reduced the turn-around-time from months to weeks and will become the new gold standard in phenotypic DST. Unfortunately, DST in liquid media is still not fast enough for rapid DST. To this end, a molecular approach should be undertaken. This consists of detecting mutations in the M. tuberculosis genome that are associated with resistance to specific antituberculosis drugs. Examples of such resistance-determining mutations are mutations in the rpoB gene associated with rifampicin resistance and mutations in the pncA gene encoding for the enzyme pyrazinamidase, which is essential for the activity of pyrazinamide.

Detecting pyrazinamide resistance
Pyrazinamide is an important first-line drug in the treatment of tuberculosis. The diagnosis of pyrazinamide resistance is challenging owing to a high proportion of false-resistance results in phenotypic DST. In chapter 3, we describe an algorithm for diagnosing pyrazinamide resistance that combines mutation analysis with culture-based methods. We hypothesized that nonsynonymous pncA mutations would be able to differentiate between true-resistant and false-resistant phenotypic (MGIT 960) susceptibility test results. To answer this question, we analysed 1,650 M. tuberculosis isolates sent for resistance testing to the Dutch National Tuberculosis Reference Laboratory from 2008 to 2009. We observed pyrazinamide resistance in 69 out of 1,650 samples after a first MGIT 960 analysis. However, 47 of the 69 samples proved susceptible in a second MGIT test indicating a false-positive rate of 68% in the first MGIT 960 test. Sensitivity of nonsynonymous pncA mutations for detecting true pyrazinamide-resistant isolates was 73%, and specificity was 100%. The positive predictive value of a pncA mutation in detecting true pyrazinamide resistance was 100%, and the negative predictive value was 89%. We concluded that nonsynonymous pncA mutations were able to differentiate between true-resistant and false-resistant MGIT 960 results and proposed that all isolates labelled resistant after a first round of MGIT testing should undergo pncA gene sequence analysis.

Pyrazinamide resistance is seen in an minority of M. tuberculosis strains carrying a wild type pncA gene and hence a normal functioning pyrazinamidase enzyme. It has recently been demonstrated that pyrazinamide might also inhibit the trans-translation by the ribosomal protein S1, which is required for efficient protein synthesis. Mutations in rpsA gene, which encodes for this protein, might therefore mediate pyrazinamide resistance in these pncA wild type strains. In chapter 4, we re-examined 5 pyrazinamide-resistant isolates with a wild-type pncA gene. Sequencing the rpsA gene revealed an A-to-G nucleotide change in codon 260 in one isolate only, leading to an amino acid change from valine to isoleucine. Incorporating rpsA gene sequencing in our proposed diagnostic algorithm (chapter 3) would modestly increase sensitivity from 73% to 77%.

While screening for pyrazinamide resistance can be carried out using the MGIT 960 system, molecular screening assays are preferred in high-burden countries. We describe such a molecular approach for identifying pyrazinamide-resistant MDR M. tuberculosis, applicable for high-burden settings in chapter 5. We hypothesized that pncA sequencing added to rpoB mutation analysis would accurately identify pyrazinamide-resistant MDR M. tuberculosis. To test our hypothesis, we sequenced the pncA gene in patient isolates with a known rpoB mutation and compared results with phenotypic drug susceptibility results to rifampicin, isoniazid and pyrazinamide. We included clinical M. tuberculosis isolates from 83 patients that were sent to our laboratory from 2007 to 2011. Phenotypic reference testing identified pyrazinamide-resistant MDR-TB in 31 isolates (48%). Sensitivity of pncA sequencing added to rpoB mutation analysis for detecting pyrazinamide-resistant MDR-TB was 97%, the specificity was 94%, the positive predictive value was 91%, and the negative predictive value was 98%. We concluded that pyrazinamide-resistant MDR-TB could be accurately detected using pncA sequencing added to rpoB mutation analysis and proposed to include pncA sequencing in every isolate with an rpoB mutation.
Drug susceptibility testing to second-line drugs

A variety of techniques are now available for DST to second-line antituberculosis drugs. In Chapter 6, we compared the Middlebrook 7H10 agar dilution method with the MGIT 960 system and the GenoType MTBDRsl molecular assay. We selected 28 clinical multidrug- and extensively drug-resistant M. tuberculosis complex strains and M. tuberculosis H37Rv. We included amikacin, capreomycin, moxifloxacin, prothionamide, clofazimine, linezolid, and rifabutin in the phenotypic test panels. For amikacin, capreomycin, linezolid, and rifabutin, results obtained by all methods were fully concordant. All capreomycin- and amikacin-resistant isolates carried rrs mutations. For prothionamide, concordance was noted only at the lowest and highest MICs. Applying a MGIT 960 breakpoint of 0.5 mg/l for moxifloxacin led to results concordant with those of both the agar dilution method and the genotypic method. Our study supports the following breakpoints for the MGIT 960 method: 1 mg/l for amikacin, linezolid, and clofazimine, 0.5 mg/l for moxifloxacin and rifabutin, and 2.5 mg/l for capreomycin. For prothionamide, a division into susceptible, intermediate, and resistant seems warranted.

Rapid molecular DST in the Netherlands

Studies on the performance of molecular screening tests for drug resistance, such as the MTBDRplus and MTBDRsl assay, have mostly been performed in high-burden TB countries. There is a need to generate more evidence on diagnostic accuracy and health-program related outcomes of these tests in low-incidence, high-resource countries. The Netherlands is such a low-level drug resistance country. In Chapter 7, we evaluated the diagnostic accuracy of the MTBDRplus and the MTBDRsl test in 2,649 and 74 clinical isolates from a national-wide screening program (2007-2012) in the Netherlands to detect resistance to first-line and second-line antituberculosis drugs. The MTBDRplus assay had a sensitivity, specificity, positive and negative predictive value of 100%, 99%, 80% and 100% for detecting rifampicin resistance. Sensitivity, specificity, positive and negative predictive value of a katG mutation for detecting isoniazid resistance was 95%, 100%, 100% and 94%. Sensitivity, specificity, positive and negative predictive value of a inhA or katG mutation for detecting isoniazid resistance was 88%, 100%, 100%, and 98%. The MTBDRsl assay had a sensitivity, specificity, positive and negative predictive value of 100%, 99%, 83%, and 100% for detecting moxifloxacin resistance. The MTBDRsl assay had a sensitivity, specificity, positive and negative predictive value of 86%, 99%, 86%, and 99% for detecting amikacin resistance. Sensitivity, specificity, positive and negative predictive value was 50%, 96%, 71% and 91% for detecting capreomycin resistance and 62%, 71%, 58% and 74% for detecting ethambutol resistance. In conclusion, the MTBDRplus and MTBDRsl assay can be used as a tool for rapid susceptibility testing to first-line and second-line antituberculosis drugs in the Netherlands. The high negative predictive suggests that these tests should preferably be used to exclude resistance in low-level drug resistance settings, such as in the Netherlands.

Repurposing existing drugs as antituberculosis agents

To increase the arsenal of antituberculosis drugs, a successful approach has been to repurpose existing drugs for tuberculosis treatment. Phenothiazines are examples of such repositioned antituberculosis drugs, targeting efflux pumps of both the mycobacteria and the macrophages that engulf them. In Chapter 8, we compared the in vitro activity of multiple phenothiazines and SILA 421, an organosilicon compound, against 21 M. tuberculosis strains with different drug susceptibility patterns, using the Middlebrook 7H10 agar dilution method. Because the S-enantiomer might induce less neurotoxicity than the R-enantiomer of thioridazine, we also tested both enantiomers separately. Chlorpromazine, thioridazine and SILA 421 showed equal activity with an MIC<sub>S</sub> and MIC<sub>R</sub> of 4 and 16 mg/l, respectively. The S-enantiomer and R-enantiomer of thioridazine proved equally active in vitro. In conclusion, this study provided incremental evidence of the antimycobacterial activity of SILA 421 and the phenothiazines such as thioridazine. Therapeutic opportunities lie in the structural optimisation of these drugs as well as in their drug resistance-modifying effect. In Chapter 9, we determined the MIC<sub>S</sub> of 6 licensed antimicrobial agents in 12 drug-resistant M. tuberculosis strains and the H37Rv reference strain using the MGIT 960 system. Fusidic acid (8 mg/l), mefloquine (16 mg/l), nitrofurantoin (32 mg/l), trimethoprim-sulfamethoxazole (2:38 mg/l), amoxicillin/clavulanate (8/8mg/l), and meropenem/clavulanate (2/2.5 mg/l) all showed antimycobacterial activity suggesting that they, or another class representative, could be repositioned as antituberculosis drugs. This calls for a reappraisal of other approved antimicrobial agents as potential antituberculosis drugs.
CHAPTER ELEVEN

Samenvatting
**Introductie**

De opkomst van multiresistente tuberculose vormt een bedreiging voor de wereldwijde controle van tuberculose. Dit heeft geleid tot een hernieuwde interesse in het ontwikkelen van nieuwe antituberculose medicijnen en behandelingsschema’s. Een veilige en effectieve introductie van dergelijke nieuwe medicijnen dient hand in hand te gaan met bepalen van resistentie tegen deze medicijnen. Resistentiebepalingen zijn essentieel omdat daarmee patiënten geïdentificeerd kunnen worden die baat zullen hebben bij de therapie en omdat hiermee resistentie op populatienniveau gevolgd kan worden. Welke algoritme het beste gebruikt kan worden hangt af van het resistentieniveau in de populatie, de patiëntenselectie, en het gezondheidszorgsysteem dat het algoritme moet borgen. Dit dient voorafgegaan te worden door het bepalen van de betrouwbaarheid en validiteit van de verschillende resistentiemethoden, onder praktijkomstandigheden en in verschillende epidemiologische settings. Dit is het belangrijkste onderwerp van dit proefschrift.

**Resistentiebepalingen**


**Het bepalen van pyrazinamideresistentie**

Pyrazinamide is een belangrijk eerstelijns antituberculose medicijn. De diagnostiek naar pyrazinamide resistentie wordt bemoeilijkt door een hoog percentage fout-positieve uitslagen in de fenotypische resistentiemethoden. In hoofdstuk 3 beschrijven we een algoritme voor het bepalen van pyrazinamide resistentie op basis van een combinatie van moleculaire en fenotypische resistentiebepalingen. Onze hypothese was dat mutaties in het pncA gen konden voorspellen of een fenotypische kweekuitslag (MGIT 960) fout-positief of terecht-positief zou zijn. Dit hebben we onderzocht in 1.650 M. tuberculosis isolaten die tussen 2008 en 2009 naar het RIVM waren gestuurd voor resistentiebepalingen. In 69 van de 1.650 isolaten werd resistentie tegen pyrazinamide waargenomen na een eerste MGIT analyse. Echter 47 van de 69 bleken bij hernieuwde analyse toch pyrazinamide gevoelig te zijn (percentage fout-positieve 68%). De sensitiviteit van een pncA mutatie voor het bepalen van een terecht-positieve pyrazinamide resiste MGIT uitslag was 75% en de specificiteit was 100%. De positief voorspellende waarde was 100% en de negatief voorspellende waarde was 89%. We concluderen dat pncA mutaties resistentie tegen pyrazinamide nauwkeurig kunnen voorspellen en we adviseren om een pncA mutatieanalyse in te zetten in elk M. tuberculosis isolaat met een eerste pyrazinamideresistente MGIT uitslag.

Recent is een nieuw mechanisme voor pyrazinamideresistentie beschreven in M. tuberculosis isolaten met een wild type pncA gen en daardoor een functionerend pyrazinamidase enzym. Pyrazinamide zou de trans-translation van het ribosomaal eiwit S1 belemmeren, wat noodzakelijk is voor de eiwitsynthese in M. tuberculosis. Mutaties in het rpoB gen, dat codeert voor het ribosomaal eiwit S1, zouden dan ook geassocieerd kunnen zijn met pyrazinamideresistentie in isolaten met een wild type pncA gen. Om dit te onderzoeken hebben we het rpsA gen gesequenst in vijf isolaten met een wild type pncA gen. Dit wordt beschreven in hoofdstuk 4. In slechts één van de vijf isolaten vonden we een nucleotide verandering van adenine naar guanine in codon 260 van het rpsA gen. Dit leidde tot een aminozuur verandering van valine naar isoleucine. Het toevoegen van rpsA mutatie analyse aan het diagnostisch algoritme uit hoofdstuk 3 leidde tot een marginale verbetering van de sensitiviteit van 73% naar 77%.

Screening naar pyrazinamideresistentie gebeurt voornamelijk met het MGIT 960 systeem. Snellere, moleculaire screeningsmethoden worden bij voorkeur gebruikt in landen met een hoge prevalentie voor multiresistente tuberculose. In hoofdstuk 5 beschrijven we een diagnostisch algoritme op basis van moleculaire resistentiebepalingen waar mee pyrazinamideresistentie, multiresistente M. tuberculosis gediagnosticeerd kan worden. Onze hypothese was dat een mutatie in zowel het rpoB als het pncA gen voorspelling zou zijn voor een pyrazinamideresistente, multiresistente M. tuberculosis stam. Om dit te onderzoeken hebben we het pncA gen gesequenst van 83 M. tuberculosis isolaten met een rpoB mutatie. Deze waren tussen 2007 en 2011 ingestaat naar het RIVM. De moleculaire uitslag werd vergeleken met fenotypische resistentiebepalingen tegen rifampicin, isoniazide en pyrazinamide. Pyrazinamideresistente, multiresistente tuberculose werd gezien in 31 isolaten (48%). De sensitiviteit van de moleculaire analyse voor de detectie van pyrazinamideresistentie, multiresistente M. tuberculosis was 97%, de specificiteit was 94%, de positief voorspellende waarde was 91% en de negatief voorspellende waarde was 98%. Pyrazinamideresistente multiresistente M. tuberculosis kan dus nauwkeurig worden bepaald.
met moleculaire technieken. We adviseren dan ook een \textit{pmcA} mutatieanalyse uit te voeren in elke isoaat met een \textit{rpoB} mutatie.

**Resistentiebepalingen tegen tweedelijns antituberculose medicijnen**

Er zijn verschillende technieken beschikbaar voor het bepalen van resistentie tegen tweedelijns antituberculose medicatie. We hebben in \textbf{hoofdstuk 6} de Middlebrook 7H10 agar dilutiemethode vergeleken met het MGIT 960 systeem en de GenoType MTBDRsl moleculaire methode. Hiervoor hebben we 28 klinische multi- en extensieve resistentie M. tuberculosis complex stammen en de H37Rv referentie stam geselecteerd. Resistentie is getest tegen amikacine, capreomycine, moxifloxacine, prothionamide, clofazimine, linezold en rifabutin. De resultaten van de verschillende technieken kwamen volledig overeen voor amikacine, capreomycine, linezold en rifabutin. Alle capreomycine en amikacine resistentie stammen hadden een mutatie in het \textit{rrs} gen. Voor protonamideresistentie waren de resultaten voor de MGIT960 en de Middlebrook 7H10 alleen hetzelfde voor de hoogste en de laagste MIC waarden. Het toepassen van een 0.5 mg/l berekenings concentratie voor moxifloxacine leidde tot een volledige overeenstemming met de Middlebrook agar dilutie methode en de moleculaire analyse van het \textit{gyrA} gen. Op basis van deze studie werden de volgende berekenings concentraties voorgesteld voor de MGIT 960 methode: 1 mg/l voor amikacine, linezold en clofazimine, 0.5 mg/l voor moxifloxacine en rifabutin en 2.5 mg/l voor capreomycine. Voor prophthionamide zou een onderscheid gemaakt moeten worden tussen gevoelig, intermediair en resistentie stammen.

**Snelle moleculaire screening naar resistentie in Nederland**

De meeste studies naar de effectiviteit van moleculaire screeningsmethodes voor het bepalen van resistentie, zoals de MTBDR\textit{plus} en de MTBDR\textit{s} test, zijn verricht in landen met een hoog prevalentie voor resistentie. Er zijn meer onderzoeken nodig voor het bepalen van de validiteit en klinische relevantie van deze methode in landen met een lage prevalentie voor resistentie. Nederland is een dergelijk laag prevalentie land. We hebben in \textbf{hoofdstuk 7} de nauwkeurigheid van de MTBDR\textit{plus} en de MTBDR\textit{s} test bepaald in 2.649 en 74 klinische isolaten die waren verkregen via een landelijk screeningsprogramma in Nederland (2007-12) naar het bepalen van resistentie tegen eerste- en tweedelijns antituberculose medicatie. De MTBDR\textit{plus} test had een sensitiviteit, specificiteit, positief en negatief voorspellende waarde van 100\%, 99\%, 80\% en 100\% voor het detecteren van rifampicine resistentie. Sensitiviteit, specificiteit, positief en negatief voorspellende waarde van een \textit{inhA} of een \textit{katG} mutatie voor de detectie van isoniazide resistentie was 55\%, 100\%, 100\% en 94\%. De MTBDR\textit{s} test had een sensitiviteit, specificiteit, positief en negatief voorspellende waarde van 100\%, 99\%, 81\%, en 100\% voor het detecteren van moxifloxacine resistentie. Sensitiviteit, specificiteit, positief en negatief voorspellende waarde van de MTBDR\textit{s} test voor de detectie van amikacine resistentie was 86\%, 99\%, 86\% en 99\%. Dit was 50\%, 96\%, 71\% en 91\% voor de detectie van capreomycine resistentie. Sensitiviteit, specificiteit, positief en negatief voorspellende waarde voor het detecteren van ethambutol resistentie was 62\%, 71\%, 58\% en 74\%. Deze resultaten geven aan dat zowel de MTBDR\textit{plus} als de MTBDR\textit{s} testen gebruikt kunnen worden voor de snelle detectie van resistentie tegen eerste- en tweedelijns antituberculose medicatie. De hoge negatief voorspellende waarde suggereert dat deze testen bij voorkeur ingezet moeten worden om resistentie uit te sluiten in landen met een lage prevalentie van resistentie, zoals in Nederland.

**Herpositioneren van bestaande medicatie als antituberculose medicijnen**

Het herpositioneren van bestaande medicatie voor het gebruik als antituberculose medicijn is een succesvolle manier om het arsenal aan antituberculose medicatie te vergroten. Phenothiazines zijn een voorbeeld van dergelijke medicijnen, omdat zij efflux pomp kunnen blokkeren zowel in mycobacteriën als macrofagen. In \textbf{hoofdstuk 8} hebben we de \textit{in vitro} activiteit vergeleken van verschillende phenothiazines en van SILA421, een organicsilicon, tegen 21 M. tuberculosis stammen. Hiervoor gebruikten we een Middlebrook 7H10 agar dilutie methode. Daarnaast testten we activiteit van de verschillende enantiomeren van thioridazine, omdat het S-enantiomeer mogelijk minder neurotoxiciteit zou geven; een bekende bijwerking van thioridazine. Chloorpromazine, thioridazine en SILA 421 veroorden vergelijkbare antimycobacteriële activiteit met een MIC\textsubscript{50} van 4 mg/l en een MIC\textsubscript{90} van 16 mg/l. De \textit{in vitro} antimycobacteriële activiteit van het thioridazine S-enantiomeer was vergelijkbaar met die van het R-enantiomeer. Deze studie toont het potentieel aan van phenothiazines en SILA 421 als antituberculose medicijnen. Mogelijkheden tot optimalisatie van deze middelen liggen op het gebied van “drug engineering”. Deze efflux pomp remmers zouden ook ingezet kunnen worden om de gevoeligheid voor andere antituberculose medicatie te verhogen. Naast phenothiazines tonen andere medicatieklassen ook antimycobacteriële activiteit. In \textbf{hoofdstuk 9} hebben we de antimycobacteriële activiteit onderzocht van zes geregistreerde antimicrobiële medicijnen. Hiervoor werden 12 resistentie M. tuberculosis stammen en de H37Rv referentiestam gebruikt. Alle middelen toonden enige antimycobacteriële activiteit: de MIC\textsubscript{50} van fusidinezuur was 8 mg/l, van nitrofurantoin 32 mg/l, van co-trimoxazol 2:38 mg/l, van mefloquine 16 mg/l, van amoxicilline met clavulaanzuur 8/8 mg/l, en van meropenem 2/2.5 mg/l. De uitkomsten van deze studie suggereren dat er nog mogelijkheden zijn deze medicijnen, of een structuuranaaloge, te herpositioneren als antituberculose medicatie. Bovendien vraagt dit om een herwaardering van andere medicijnklassen als potentiële antituberculose medicijnen.
CHAPTER TWELVE

Conclusions and future directives
Introduction

Drug susceptibility testing (DST) is a prerequisite for reversing the global threat of multidrug-resistant and extensively drug-resistant tuberculosis. In this thesis we looked at the diagnostic accuracy and performance of different (molecular) DST algorithms. A subsequent task would be to align these DST algorithms with next-generation treatment regimens. But which drugs are components of these next-generation treatment regimens? Ideally, they should be oral drugs that are effective against susceptible and drug-resistant M. tuberculosis and that have minimal interaction with antiretrovirals. A regimen consisting of PA-824 (pretonamid), moxifloxacin and pyrazinamide (PaMZ) is the first of such a novel regimen undergoing clinical testing in phase III. In a 14-day EBA study, this regimen killed mycobacteria as fast as the current first-line treatment regimen. Results of a 8-week phase II trial are expected to be published soon (http://www.tballiance.org/portfolio/trial/nc002). A phase III trial will start in 2015 (the STAND trial). For an accurate assessment of the impact of these next-generation treatment regimens, in particular the PaMZ regimen, proper data on pyrazinamide and fluoroquinolone resistance is highly needed. Currently, surveillance of pyrazinamide resistance is lacking and data on fluoroquinolone resistance is collected in a minority of patients only.

Detecting pyrazinamide resistance

There is increasing evidence that pyrazinamide plays a predominant role in the sputum conversion and sterilizing activity of the current first-line antituberculosis treatment regimen. This is because pyrazinamide works better in the interaction with other drugs than it does as an isolated component. It is this synergistic potential that makes this drug appealing as a component in next-generation regimens. In particular, it has been shown that pyrazinamide potentiates the activity of important next-generation drugs such as fluoroquinolones and bedaquiline.

A recent meta-analysis has indeed shown that susceptibility to pyrazinamide is associated with treatment success in MDR-TB treatment. Drug susceptibility testing to pyrazinamide is therefore important to perform. Though the breakpoint concentration has traditionally been set at 100 mg/L, recent pharmacokinetic/-dynamic studies based on pyrazinamide AUC/MIC data have suggested that 50 mg/L might be a more clinically relevant susceptibility breakpoint predictive for treatment success.

Drug susceptibility testing to pyrazinamide poses a lot of challenges. False-resistant results are frequently seen due to large inoculum sizes causing inactivation of the pyrazinamidase enzyme. We have shown that false-resistance may be a problem in up to 68% of samples using the MGIT 960 system. This means that a first resistant result from the MGIT 960 system must be interpreted with caution. This has important consequences for the DST to pyrazinamide because the MGIT 960 may well be the future gold standard. Some authors have therefore proposed to use reduced inoculums size. We propose to increase reliability by performing a pncA mutation analysis in each resistant MGIT 960 result. We have shown that pyrazinamide resistance can reliably be detected in this way (chapter 3). The proposed flow diagram may therefore help standardize pyrazinamide resistance testing.

In high-burden MDR-TB settings a molecular approach would likely be more appropriate to accelerate DST. Recent WHO guidelines have recommended that the Xpert® MTB/RIF must be used as the initial diagnostic test in adults suspected of having MDR-TB in high-burden settings. We have shown that in the case of a rpoB mutation a pncA mutation analysis should follow and that a pncA mutation indicates a pyrazinamide-resistant M. tuberculosis. Such a strategy, when performed on direct sputum samples, may give accurate results within days. It could help to stratify MDR-TB treatment according to pyrazinamide susceptibility rapidly and accurately, for instance in clinical studies evaluating next-generation treatments such as the next PaMZ phase III trial (STAND trial).

The drawback of performing an pncA mutation analysis is the wide spread of resistance-determining mutations along the 561bp long pncA gene, making simple molecular DST technologies such as line probe assays difficult to design. Moreover, not every pncA mutation is strongly associated with phenotypic resistance. A database with all resistance-determining mutations, such as recently published, may aid clinical decision making. An alternative strategy could be to detect the wild type pncA gene as a correlate for pyrazinamide susceptibility. Likewise, this strategy will depend on the proportion of pyrazinamide resistant M. tuberculosis strains with a wild type pncA gene, which could be as high as 15%. Other mechanisms, such as inhibiting trans-translation and interfering with the pantothenate synthesis, might be responsible for inducing pyrazinamide resistance in these isolates. The value of molecular markers for these pathways, such as rpsA mutations and panD mutations, remains to be determined (Chapter 4).

Detecting fluoroquinolone resistance

Detecting fluoroquinolone resistance would be another important DST strategy to focus on. Fluoroquinolones are important second-line drugs in the MDR-TB treatment and will probably be a component of next-generation treatment regimens as well. Line-probe assays, such as the MTBDRsl assay, can be used to detect fluoroquinolone resistance. We have shown that the MTBDRsl assay may indeed aid in the diagnosis of fluoroquinolone and amikacin
resistance in MDR *M. tuberculosis* isolates (chapter 7). Results from this thesis suggest for instance that a *gyrA* mutation would have a sensitivity and specificity for detecting an XDR *M. tuberculosis* 100% and 97% (chapter 7), which might be cost-effective in high-burden, low-income settings. That’s why we would propose to include the MTBDRsl assay – together with *pncA* mutation analysis – in every isolate with a *rpoB* mutation to guide treatment initiation.

At present, the WHO does not recommend molecular assays for any second-line drug owing to a moderate sensitivity and a subsequently lower negative predictive value. Sensitivity of the MTBDRsl assay for detecting fluoroquinolone resistance is approximately 89% in recent meta-analyses. It will probably not exceed 90% since 10-40% of fluoroquinolone-resistant *M. tuberculosis* strains do not carry target mutations (in codons 90, 91 and 94 of the *gyrA* gene) detected by the MTBDRsl assay. Other proposed resistance mechanisms are mutations outside the quinolone resistance-determining region of the *gyrA* gene, mutations in the *gyrB* gene, mutations in other genes such as the *parC* and *parE* genes, or efflux pump mechanisms.

Notwithstanding this limitation of the MTBDRsl assay, clinicians should not solely focus on sensitivity and specificity to appreciate the performance of this test, but should also take into account the prevalence of fluoroquinolone resistance. Based on a prevalence of fluoroquinolone resistance of 20% in MDR-TB samples, found in surveillance of the WHO European Region, positive and negative predictive value of a *gyrA* mutation would be 92% and 97%. This means that this test could be safely used for detecting and excluding fluoroquinolone resistance in MDR-TB patients in the WHO European region. Only when the prevalence exceeds 30%, will the negative predictive value drop below 95%.

**DST to novel antituberculosis drugs**

There are currently several new drug classes that are beyond phase I assessment, such as diarylquinolines (bedaquiline), nitroimidazoles (delamanid, pretonamid), oxazolidinones (sutezolid, AZD5847), and ethylenediamines (SQ-109). Bedaquiline and delamanid are the only new drugs that have passed EMA and FDA approval. Rapid DST to these two drugs will be a challenge however. DST in the MGIT 960 system is not feasible at the moment for both drugs owing to technical restraints (bedaquiline attaches to the MGIT tube and various other plastics) and restrictions imposed by the manufacturer in the case of delamanid. As a result, DST can only be performed via the slower agar dilution method in few selected reference laboratories. There is thus a need to develop faster phenotypic DST platforms for both these drugs since they have already been introduced in clinical care. Though resistance-determining mutations for bedaquiline and delamanid have been identified, it is not expected that molecular DST will provide an alternative method for these drugs in the near future; the majority of bedaquiline resistant isolates do not carry mutation in the *atpE* gene31 and the majority mutations in the *ddr* gene are not associated with resistance to nitroimidazoles.

Besides the development of these new antituberculosis drugs, we have highlighted the potential of existing drug as repurposed antituberculosis drugs. We tested a variety of drugs, from phenothiazines (chapter 8) to established antimicrobial agents (chapter 9), and showed that they all had more or less antimycobacterial activity. It illustrates that we are currently not using all potential drug classes in the fight against tuberculosis and this calls for a appraisal of other approved (antimicrobial) drugs as potential antituberculosis drugs.

**Aligning DST with treatment regimens**

In general, DST can be deployed as case-detection assays such as the Xpert MTB/RIF assay or as reflex testing after the diagnosis of TB has been made, such as phenotypic DST to second-line drugs. At present, a case-detection strategy using molecular DST (Xpert MTB/RIF) has been prioritized to diagnose MDR-TB in high-burden settings. The recent WHO policy update from April 2013 has recommended that the Xpert MTB/RIF should replace conventional microscopy, culture and DST as the initial diagnostic test in adults suspected of having MDR-TB in high-burden settings.

There are two important considerations regarding the deployment of such rapid molecular DST platforms. The first factor is the health care level implementing the DST algorithm. Preferably, case-detection tests should be simple diagnostic tools so they can be used at peripheral levels of the health care system. The Xpert MTB/RIF is an example of such a test. Such a peripheral focus does not necessitate setting-up an extensive sample transport and allows for a rapid turn-around-time so doctors can actually act on DST results immediately before transmission of drug-resistant isolates occur. Reflex testing is likely more appropriate in centralised laboratories that can handle more complex platforms. It requires setting up rapid and reliable sample transport and necessitates more patient visits with potential lost to follow-ups and treatment delays.

The second important factor to consider when deciding on diagnostic algorithms is the prevailing drug resistance level. In high-burden countries there is good evidence that molecular DST as a case-detection strategy for MDR-TB is cost effective, reduces time-to-diagnosis and treatment initiation. Contrarily, the advantage of such a strategy is equivocal in low-burden countries such as the Netherlands. Though it is estimated that a case-detection strategy for MDR-TB would be cost-effective if prevalence of MDR-TB among
smear-positive TB cases is as low as 2%, a moderate to high sensitivity of the molecular DST is assumed in such cost-effectiveness studies. In low-burden, high resource countries this might not the case owing to a high percentage of smear-negative TB cases; sensitivity of detecting TB in smear-negative, culture positive TB may be as low as 46% in low-burden settings, thereby limiting the applicability of such a test as a screening assay. Another concern is the limited positive predictive value for MDR-TB in these low-burden countries. It has been estimated that positive predictive value diminishes to less than 70% in countries with MDR-TB prevalence lower than 5%. In our sample positive predictive value was higher (80%), probably because we used indirect DST in *M. tuberculosis* cultures from the Netherlands. The positive predictive value could be improved by a better risk assessment of the patient. However, we showed that common risk factors such as previous treatment, age and foreign-born were insufficient to increase predictive value (chapter 7). We would therefore argue against a case-detection strategy in the Netherlands. Rather, since there a rapid and reliable sample transport in our country, we would recommend reflex testing in centralised laboratories.

The development of DST should not be delayed. Implementing next-generation treatment regimens without any back-up surveillance strategy will eventually amplify drug resistance and will sacrifice every drug development made so far. The resurgence of MDR-TB cases in the New York City outbreak and the high fatality rates in XDR-TB cases in South Africa are a vivid reminder of the dangers of relaxing DST development. Nonetheless, balancing between this risk of emerging drug resistance and the unabated necessity to provide effective MDR-TB treatment as soon as possible is a challenge. It requires political courage and commitment. All in all, the costs of averting this global threat are huge, but the price of failure will be immense.

Conclusions and future directives
Conclusions and future directives

CHAPTER THIRTEEN

Biography and list of publications
Sami Olavi Simons was born on April 15th, 1980 in Alphen aan den Rijn, the Netherlands. He grew up in the city of ’s-Hertogenbosch and attended secondary school in Sint-Michielsgestel (Gymnasium Beekvliet). The numerus fixus caused him to study psychology and chemistry after graduating from secondary school. Thanks to changes in legislation, he could start medical school at the Radboud University Nijmegen in 1999. As a part of his medical training, he spent 6 months in Dakar, Senegal, for a research traineeship (dr. C. Boudin) studying transmission-blocking immunity against *P. falciparum*.

He obtained his medical degree in 2005, after which he started his internal medicine residency training at the Rijnstate Hospital in Arnhem (dr. L. Verschoor). In 2008, he began his fellowship training in respiratory medicine at the Radboud University Medical Centre in Nijmegen (prof. dr. P. N. R. Dekhuijzen). During his fellowship training he was an committee member of the Dutch Union for Respiratory Fellows (Professional Society for Respiratory Physicians), participating in the School of Pulmonary Medicine, the Bronkhorst committee and the Dutch Tuberculosis committee.

Besides these extracurricular activities, he started to work with dr. Jakko van Ingen, dr. Martin Boeree and prof. dr. Dick van Soolingen in the field mycobacterial diseases, which led to the start of this PhD candidacy in 2010. For his PhD thesis on drug-resistant *M. tuberculosis*, he travelled once a week to the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands) to work at the Tuberculosis Reference Laboratory. In 2013, he was selected for the Radboud Da Vinci Talent program for the personal development of PhD candidates.

Sami has been working as a pulmonologist since November 2012, combining his clinical work on respiratory physiology and immunology with his research activities. Besides his work on multidrug-resistant tuberculosis, his research activities currently focus on exacerbation susceptibility in COPD patients.

Sami lives in Nijmegen, together with Ieke Schillings and their three daughters Floortje, Isa and Roos.
LIST OF PUBLICATIONS


• Simons SO, Snijders SL. All that glitters is not gold in pursuing the diagnosis of pulmonary embolism. Chest. 2012;141(1):276-7.


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