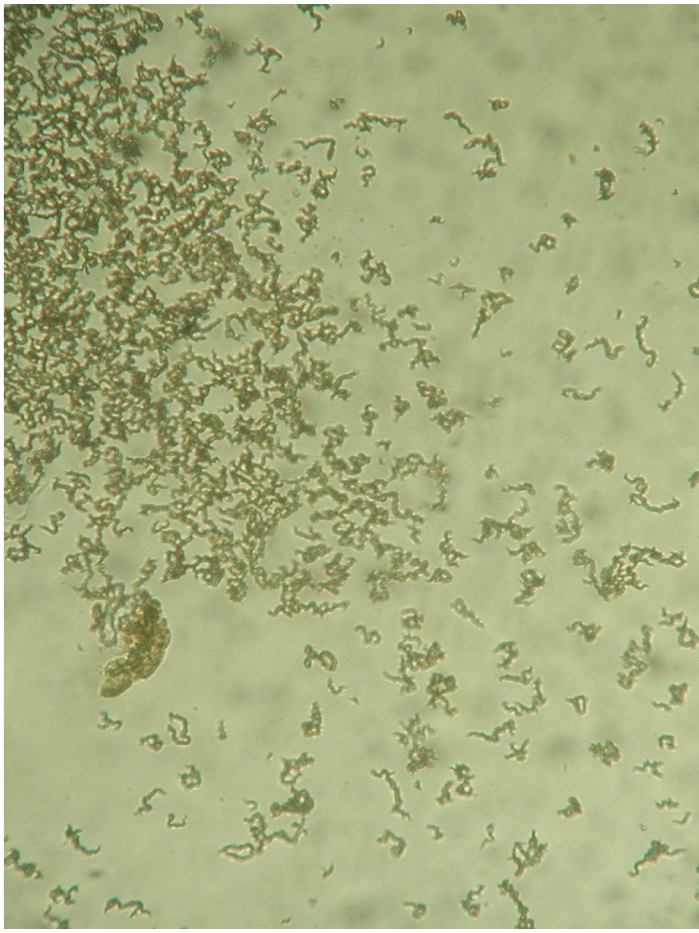
Improved diagnosis of drug-resistant tuberculosis:

from implementation of simple phenotypic testing to whole genome sequencing

Elisa Ardizzoni



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Verbeterde diagnose van geneesmiddelresistente tuberculose: van de implementatie van eenvoudige fenotypische testen tot het sequencen van het hele genoom

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Contents

List of abbreviations

BDQ	Bedaquiline
CC	Critical concentration
CPC	Cetyil-pyridinium Chlorite
CRI	Colorimetric redox indicator
DLM	Delamanid
DST	Drug-susceptibility testing
ECOFF	Epidemiological cut-off
FLDs	First-line drugs
FM	Fluorescent microscopy
FQ	Fluoroquinolones
gDST	Genotypic drug-susceptibility testing
GLI	Global Laboratory Initiative
GU	Growth unit
Indel	Insertion or deletion of nucleotides in genome
INH	Isoniazid
LAM	Lipoarabinomannan
LED	Light-emitting diodes
LJ	Löwenstein-Jensen
LZD	Linezolid
LPA	Line probe assay
LFX	Levofloxacin
MDR-TB	Multidrug-resistant tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimal inhibitory concentration
MODS	Microscopic observation drug susceptibility
MOX	Moxifloxacin
MTB	*Mycobacterium tuberculosis* complex
NAAT	Nucleic acid amplification test
NGS	Next generation sequencing
NRA	Nitrate reductase assay
OFX	Ofloxacin
pDST	Phenotypic drug-susceptibility testing
PZA	Pyrazinamide
RMP	Rifampicin
RR	Rifampicin-resistant
RS	Rifampicin-susceptible
RT	Room temperature
SDG	Sustainable Development Goals

SLDs	Second-line drugs
SLIs	Second-line injectables
SM	Smear microscopy
SNP	Single nucleotide polymorphism
STC	2, 3 diphenyl-5-(2-thienyl) tetrazolium chloride
TB	Tuberculosis
TLA	Thin layer agar
tNGS	Targeted next generation sequencing
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant
ZN	Ziehl-Neelsen

Summary

Until the coronavirus (COVID-19) pandemic, Tuberculosis (TB) was the leading cause of death from a single infectious agent, ranking above HIV/AIDS, with globally an estimated 10.6 million people developing TB in 2021 (1)**.** In the last years the indicators for TB incidence and new cases reported showed improvements, however COVID-19 pandemic has interrupted all efforts and even reversed this trend.

The gap between the estimated incidence of new TB cases and the number of people reported with TB is worrisome, despite major advancements in TB diagnostics made during the last decade.

The implementation of GeneXpert®MTB/RIF (Xpert MTB/RIF) and its updated versions has significantly improved the diagnosis of TB and drug resistance, especially in resource-limited areas. When this test was launched in 2011, Médecins sans Frontières (MSF) implemented Xpert MTB/RIF in 33 different locations with diverse epidemiological characteristics. From this large experience, documented in **Chapter II** of this thesis, appeared that Xpert significantly improved TB detection in all settings. Nevertheless, due to the high initial rate of errors occurring, the use of Xpert MTB/RIF as add-on test to microscopy seemed the best strategy. However, the high workload required by testing smear-negative samples with Xpert MTB/RIF, and the WHO recommendations that followed, have led to a progressive drop of microscopy as first test in most of the settings included in the study.

From the study also emerged that, despite being described as easy to implement, the introduction of Xpert MTB/RIF in decentralized settings required substantial efforts and resources, rather than being a plug-and-go process. Xpert MTB/RIF in fact has logistical constraints that can make its deployment in remote settings challenging. It requires stable power supply, controlled temperature for cartridge storage, and regular maintenance of equipment.

At the time of this study, the only drug investigated by Xpert MTB/RIF was rifampicin (RMP), and for this reason MSF focused on the development of a rapid and inexpensive diagnostic method applicable to remote settings, to detect resistance to other drugs. In **Chapter III, IV and V** we present results form studies that aimed to assess and improve thin-layer agar (TLA) testing, a phenotypic non-commercial method applied directly to sample sediments, for simultaneous detection of *Mycobacterium tuberculosis* (MTB) bacilli and its resistance to anti-TB drugs. As a first step we tried to identify the best decontamination method to be applied to samples undergoing long transport before culture to obtain MTB growth. We documented that adding cetylpyridinium chloride (CPC) to sputum followed by decontamination with NALC-NaOH and neutralization with Difco^{BD} neutralizing buffer was the preferred method, with the TLA positivity rate reaching 86.1%, close to the 88.2% obtained from non-CPC samples directly decontaminated with NALC-NaOH and inoculated in liquid medium (MGIT), considered the gold standard.

As a second step, we evaluated TLA as direct drug-susceptibility testing (DST) method at the Institute of Tropical Medicine (ITM) in controlled conditions, on smear-microscopy-positive samples arriving from the Georgia MSF project. TLA showed high sensitivity for the detection of

resistance to isoniazid (INH), RMP and fluoroquinolones (FQs) (94.7%, 98.2%, and 100% respectively) with 100% specificity. TLA-DST results were available after 7 days, versus 23 for indirect MGIT-DST. The ability to recover MTB was comparable to MGIT, albeit the recovery rate was affected by long sample transport and low mycobacterial load.

Subsequently, direct-on-sample TLA-DST was evaluated in field conditions in the Eswatini MSF project. Here, the TLA positivity rate was slightly lower compared to the study performed in ITM, while results still confirmed lower recovery rate for samples undergoing long transport. Another reason for the lower performance could be operator dependent. TLA requires multiple reading by microscopy, generating a high workload, difficult to sustain in laboratories performing routine tests for a large volume of samples. Considering only MTB-positive plates, TLA detected 90.3% of RMP-resistant MTB identified by their standard solid medium DST method, significantly higher than for Xpert MTB/RIF (51.6%). Interestingly, TLA showed an excellent ability to detect the *rpoB*_I491F mutation, responsible for RMP resistance but systematically missed by Xpert MTB/RIF, and to a lower extend also by MGIT. While this mutation is out of the RMP-resistance determining region of the *rpoB* gene targeted by all molecular rapid tests, it is very common in the South African region, representing 56% of all RMP-resistant strains.

Overall, the results emerging from these studies demonstrated that TLA correctly detects drug resistance. TLA presents several advantages, including the ability to provide rapid results and the possibility to be implemented in biosafety level 2 (BSL2) laboratories, making it suitable for district-level facilities. However, as limitations, TLA performance is highly operator dependent and may be affected by the need of multiple reading of the plates. In addition, MTB detection was lower when samples underwent long transport, compared to standard sample processing-growing conditions.

To improve MTB detection in TLA, we have investigated the use of OMNIgene®SPUTUM (OMNIgene), a preservative to be added to specimens with expected delayed transport prior to testing with Xpert MTB/RIF or MGIT. In our experience, the reagent had a detrimental effect on the MTB recovery rate in MGIT, while the MTB detection with Xpert was not affected by long storage and was comparable to samples without OMNIgene (**Chapter VI**). In addition, the reagent's high cost and the requirement for a centrifugation step was considered as another drawback. For these reasons the use of OMNIgene was not further investigated for its application to TLA-DST.

To address the need of rapid testing beyond RMP, a Xpert MTB/XDR now offers the possibility to detect resistance to FQs, INH and second-line injectables. However, rapid tests to detect resistance to new drugs, like bedaquiline (BDQ) and linezolid, are still lacking. To obtain DST results for these drugs, samples still need to be referred to high-skill laboratory levels for phenotypic DST or advanced molecular testing, limiting the decentralization of TB care. In addition, we still lack knowledge on the correct interpretation of the results for these new drugs. The association between phenotypic DST results and mutations found in candidate BDQ-resistance associated genes and their impact on treatment outcome, are difficult to interpret. Moreover, the role of prior exposure to clofazimine (CFZ) on amplification of BDQ resistance is unclear.

To contribute to the clarification of these questions, we conducted a study on amplification of BDQ resistance in patients from Armenia treated with BDQ in compassionate use prior to its registration by national regulatory authorities (**Chapter VII**). Prior exposure to CFZ did not correlate with presence of mutations in candidate BDQ-resistance associated genes, nor with BDQ phenotypic resistance at the start of BDQ-based treatment (baseline). Also, it did not impact acquired BDQ resistance during such treatment. Even for few patients, the presence of mutations in *Rv0678* at baseline was not correlated to unfavorable outcome.

Nevertheless, results showed that an increase in BDQ-MIC, amplification of phenotypic resistance or any amplification of mutations in candidate BDQ-associated resistance genes, regardless of their frequency occurring during treatment, should be seen as a predictor of resistance amplification and potential unfavorable treatment outcome. Another important finding was the low agreement between MGIT-DST and the determination of minimal inhibitory concentrations (MICs) on 7H11 agar; lowering the critical concentration in 7H11 from 0.25 µg/ml to 0.125µg/ml would improve the agreement without reducing specificity. Further testing on a larger sample is needed to corroborate this finding.

In conclusion, while Xpert MTB/RIF has revolutionized TB diagnosis, challenges persist in remote and resource-limited settings. Direct-on-sample TLA-DST shows encouraging results and further research is needed to optimize its implementation and inclusion of new drugs. A diagnostic algorithm which combines Xpert MTB/RIF for rapid diagnosis and baseline DST with TLA for comprehensive DST may be a way forward in improving TB care in such settings. Additionally, addressing the challenges of BDQ-resistance detection is crucial to guide treatment and preserve treatment options for future TB patients. Due to the variety of mutations found in these other studies, the development of a rapid test for detecting BDQ resistance does not seem close, so that in peripheral settings TLA could be a valid and rapid method to provide BDQ-DST/MIC results.

Samenvatting

Tot de pandemie van het coronavirus (COVID-19) was tuberculose (TB) de belangrijkste doodsoorzaak door één infectueus agens, boven HIV/AIDS, waarbij naar schatting 10,6 miljoen mensen TB zouden krijgen in 2021 (1)**.** In de afgelopen jaren lieten de indicatoren voor TB-incidentie en nieuwe gemelde gevallen verbeteringen zien, maar de COVID-19 pandemie heeft alle inspanningen onderbroken en deze trend zelfs omgekeerd.

De kloof tussen de geschatte incidentie van nieuwe tbc-gevallen en het aantal gerapporteerde mensen met tbc is zorgwekkend, ondanks de grote vooruitgang in tbc-diagnostiek in het afgelopen decennium.

De implementatie van GeneXpert®MTB/RIF (Xpert MTB/RIF) heeft de diagnose van tbc en geneesmiddelenresistentie aanzienlijk verbeterd, vooral in gebieden met beperkte middelen. Toen deze test in 2011 werd gelanceerd, implementeerde Artsen zonder Grenzen (AZG) Xpert MTB/RIF op 33 verschillende locaties met uiteenlopende epidemiologische kenmerken. Uit deze ervaring, gedocumenteerd in **Hoofdstuk II** van dit proefschrift, bleek dat Xpert MTB/RIF de detectie van tbc in alle settings aanzienlijk verbeterde. Desondanks leek het gebruik van Xpert MTB/RIF als aanvullende test op microscopie de beste strategie vanwege het hoge aantal fouten dat aanvankelijk optrad. De hoge werkdruk die het testen van uitstrijkjes-negatieve monsters met Xpert MTB/RIF met zich meebrengt en de aanbevelingen van de Wereldgezondheidsorganisatie (WGO) die later volgden, hebben er echter toe geleid dat microscopie als initële test in de meeste instellingen steeds minder wordt toegepast.

Uit het onderzoek kwam ook naar voren dat de introductie van Xpert MTB/RIF in gedecentraliseerde settings, ondanks het feit dat het als eenvoudig te implementeren werd omschreven, aanzienlijke inspanningen en middelen vergde, in plaats van dat het een plug-and-go proces was. Xpert MTB/RIF heeft een aantal logistieke beperkingen die het gebruik ervan in afgelegen omgevingen tot een uitdaging kunnen maken. Het vereist een stabiele stroomvoorziening, gecontroleerde temperatuur voor de opslag van de cartridges en regelmatig onderhoud van de apparatuur.

Ten tijde van dit onderzoek was het enige geneesmiddel dat door Xpert MTB/RIF werd onderzocht rifampicine (RMP), en daarom richtte AzG zich op de ontwikkeling van een snelle en goedkope diagnostische methode om resistentie tegen andere geneesmiddelen op te sporen die toepasbaar is op het tussenniveau. In **Hoofdstuk III, IV en V** presenteren we de resultaten van onderzoeken die gericht waren op het beoordelen en verbeteren van TLA-testen (thin-layer agar), een fenotypische niet-commerciële methode voor de gelijktijdige detectie van *Mycobacterium tuberculosis* (MTB) bacillen en hun resistentie tegen anti-TB-medicijnen, die rechtstreeks op monstersedimenten wordt toegepast. Als eerste stap probeerden we de beste decontaminatiemethode te identificeren die kon worden toegepast op monsters die lang werden getransporteerd voordat ze werden gekweekt om MTB-groei te verkrijgen. We hebben gedocumenteerd dat het toevoegen van cetylpyridiniumchloride (CPC) aan sputum gevolgd door ontsmetting met NALC-NaOH en neutralisatie met Difco^{BD} neutralisatiebuffer de

voorkeursmethode was, met een TLA-positiviteitspercentage van 86,1%, dicht bij de 88,2% die verkregen werd uit niet-CPC monsters die direct ontsmet waren met NALC-NaOH en geïnoculeerd in vloeibaar medium (MGIT), wat beschouwd wordt als de gouden standaard.
Als tweede stap evalueerden we TLA als directe antibitoticagevoeligheidstest (DST) in het Instituut voor Tropische Geneeskunde (ITG), dus in gecontroleerde omstandigheden. Dit deden we voor uitstrijk-microscopie-positieve monsters die binnenkwamen van het MSF-project in Georgië. TLA toonde een hoge gevoeligheid voor de detectie van resistentie tegen isoniazide (INH), RMP en fluoroquinolonen (FQ) (respectievelijk 94,7%, 98,2% en 100%) met 100% specificiteit. TLA-DST resultaten waren na 7 dagen beschikbaar, tegenover 23 dagen voor indirecte MGIT-DST. Het vermogen om MTB terug te vinden was vergelijkbaar met MGIT, hoewel dit werd beïnvloed door lang monstertransport en een lage mycobacteriële belasting.
Vervolgens werd TLA-DST direct op het monster geëvalueerd onder veldomstandigheden in het MSF-project in Eswatini. Hoewel het TLA-positiviteitspercentage in het algmeen iets lager lag vergeleken met het onderzoek dat op ITG werd uitgevoerd, vonden we ook hier een lagere positiviteitsgraad voor monsters met langdurig transport. Een andere reden voor de lagere prestaties zou uitvoerderafhankelijk kunnen zijn. TLA vereist meervoudig microscopisch aflezen, wat een hoge werkbelasting met zich meebrengt die moeilijk vol te houden is in laboratoria die routinetests uitvoeren voor een groot aantal monsters. Als we enkel de MTB-positieve platen beschowen, detecteerde TLA 90,3% van de RMP-resistente MTB's die met hun standaard DST-methode op vast medium werden geïdentificeerd, aanzienlijk meer dan de RMP-resistenten die met Xpert MTB/RIF (51,6%) werden gedetecteerd. Interessant genoeg bleek TLA uitstekend in staat om de *rpoB*_I491F-mutatie te detecteren, die verantwoordelijk is voor resistentie tegen RMP maar systematisch wordt gemist door Xpert MTB/RIF, en in mindere mate ook door MGIT. Deze mutatie valt buiten de RMP-resistentiebepalende regio van het *rpoB*-gen waarop alle moleculaire sneltesten zich richten, en komt zeer vaak voor in de Zuid-Afrikaanse regio waar ze 56% van alle RMP-resistente stammen vertegenwoordigt.
In het algemeen toonden de resultaten van deze onderzoeken aan dat TLA geneesmiddelenresistentie correct detecteert. TLA biedt verschillende voordelen, waaronder de mogelijkheid om snel resultaten te leveren en de mogelijkheid om te worden geïmplementeerd in laboratoria met bioveiligheidsniveau 2 (BSL2), waardoor het geschikt is voor faciliteiten op districtsniveau. De TLA-prestaties zijn echter sterk afhankelijk van de operator en kunnen worden beïnvloed door de noodzaak om de platen meerdere keren af te lezen. Bovendien was de MTB-detectie lager wanneer monsters lang werden getransporteerd, vergeleken met standaardomstandigheden voor monsterverwerking en kweek.
Om MTB-detectie in TLA te verbeteren, hebben we het gebruik van OMNIgene®SPUTUM (OMNIgene) onderzocht, een conserveermiddel dat moet worden toegevoegd aan monsters met verwacht vertraagd transport voorafgaand aan het testen met Xpert MTB/RIF of MGIT. Het reagens bleek een nadelig effect te hebben op de groei van MTB in MGIT, terwijl de MTB-detectie met Xpert MTB/RIF niet werd beïnvloed door lange opslag en vergelijkbaar was met monsters zonder OMNIgene **(Hoofdstuk VI)**. Verder werden de hoge kost van het reagens en de vereiste

centrifugatiestap als een ander nadeel beschouwd. Om deze redenen werd het gebruik van OMNIgene niet verder onderzocht voor toepassing op TLA-DST.

Om te voorzien in de behoefte aan snelle testen die verder gaan dan RMP, biedt Xpert MTB/XDR nu de mogelijkheid om resistentie tegen FQ's, INH en tweedelijns injecteerbare middelen te detecteren. Het ontbreekt echter nog steeds aan snelle tests om resistentie tegen nieuwe geneesmiddelen, zoals bedaquiline (BDQ) en linezolid, te detecteren. Om DST-resultaten voor deze geneesmiddelen te verkrijgen, moeten monsters nog steeds worden doorverwezen naar laboratoria met een hoog kwalificatieniveau voor fenotypische DST of geavanceerde moleculaire testen, waardoor de decentralisatie van tbc-zorg wordt beperkt. Daarnaast ontbreekt het ons nog steeds aan kennis over de juiste interpretatie van de resultaten voor deze nieuwe geneesmiddelen. De associatie tussen fenotypische DST-resultaten en gevonden mutaties in kandidaat-genen voor BDQ-resistentie en hun impact op het behandelingsresultaat zijn moeilijk te interpreteren. Bovendien is de rol van eerdere blootstelling aan clofazimine (CFZ) op toename van BDQ-resistentie onduidelijk.

Om bij te dragen aan de opheldering van deze vragen hebben we een onderzoek uitgevoerd naar toename van BDQ-resistentie bij patiënten uit Armenië die behandeld werden met BDQ als compassionate use voorafgaand aan de registratie ervan door de nationale regelgevende instanties (**Hoofdstuk VII**). Voorafgaande blootstelling aan CFZ correleerde niet met de aanwezigheid van mutaties in kandidaat-genen die geassocieerd zijn met BDQ-resistentie noch met fenotypische BDQ-resistentie aan het begin van de behandeling op basis van BDQ (baseline). Het had ook geen invloed op verworven BDQ-resistentie tijdens een dergelijke behandeling. Zelfs voor de enkele patiënten met aanwezigheid van mutaties in *Rv0678* op baseline isolaten, bleek dit niet gecorreleerd met een ongunstige uitkomst.

Niettemin toonde ons onderzoek aan dat een toename in de minimaal inhiberende concentratie (MIC) van BDQ tijdens de behandeling, amplificatie van fenotypische resistentie of eender welke toename van mutaties in kandidaat BDQ-geassocieerde resistentiegenen - ongeacht hun frequentie - gezien moet worden als een voorspeller van resistentieversterking en een mogelijk ongunstig behandelingsresultaat. Een andere belangrijke bevinding was de lage overeenkomst tussen MGIT-DST en de bepaling van MIC's op 7H11 agar; verlaging van de kritische concentratie in 7H11 van 0,25 µg/ml naar 0,125 µg/ml zou de overeenkomst verbeteren zonder de specificiteit te verminderen. Verdere testen op een groter aantal isolaten is nodig om deze bevinding te bevestigen.

Concluderend kan worden gesteld dat Xpert MTB/RIF een revolutie teweeg heeft gebracht in de diagnose van tbc, maar dat er nog steeds uitdagingen zijn in afgelegen en achtergestelde gebieden. Direct-on-sample TLA-DST laat bemoedigende resultaten zien en verder onderzoek is nodig om de implementatie en opname van nieuwe geneesmiddelen te optimaliseren. Een diagnostisch algoritme met Xpert MTB/RIF voor een snelle diagnose en basislijn DST in combinatie met TLA voor een uitgebreide DST kan een stap vooruit zijn in het verbeteren van de tbc-zorg in dergelijke omgevingen. Daarnaast is het van cruciaal belang om de uitdagingen van de detectie van BDQ-resistentie aan te pakken om de behandeling te sturen en behandelingsopties voor toekomstige tbc-patiënten te vrijwaren. Door de verscheidenheid aan

mutaties die in dit en andere onderzoeken zijn gevonden, lijkt de ontwikkeling van een snelle test voor het detecteren van BDQ-resistentie niet nabij, zodat TLA in perifere omgevingen een waardige en snelle methode zou kunnen zijn om BDQ-DST/MIC-resultaten te bekomen.

CHAPTER I

General Introduction

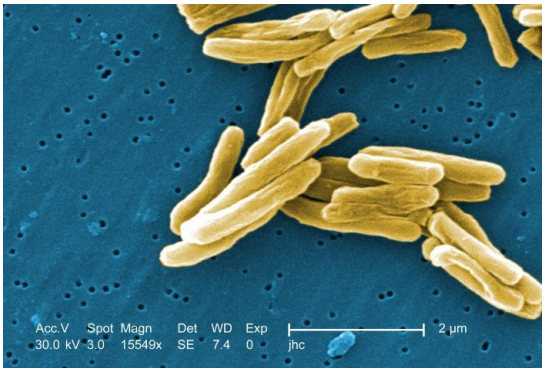
1.1 Mycobacterium tuberculosis complex

The first patient with tuberculosis (TB) was reported as early as 3.300 years ago in India (2). This ancient infectious disease remains the largest cause of mortality from infections today (with the exception of COVID-19 in 2021) (1), and is caused by members of the *M. tuberculosis* complex (MTB). The MTB comprises various species, mainly *M. tuberculosis* sensu stricto, *M. africanum*, *M. bovis* and *M. canettii,* which is part of the so called "smooth tuberculosis bacilli" group and lacks the typical cord forming colonies and rough aspect of the other members of the MTB (3)(4). Part of the MTB are also animal specific species such as *M. caprae, M. microti, M. pinnipedii, M. origys, M. mungi*, *M. suricattae.* The vaccine strain *M. bovis* Bacillus Calmette-Guerin (BCG), an attenuated strain of *M. bovis,* was developed a century ago and continues to be the most widely used vaccine in newborns worldwide.

To the *Mycobacterium* genus belong also other species called non-tuberculous mycobacteria (NTM). NTMs, distributed worldwide, are present in the environment, such as in water and soil. Some of these organisms, even if associated with less severe forms of disease, can cause significant morbidity (5).

Bacilli of the MTB have a typical rod-shape (Figure 1.1), and like other members of the genus *Mycobacterium*, are characterized by a very specific cell-wall composed of peptidoglycans, arabinogalactan, lipoarabinomannan (LAM) and mycolic acids.

Figure 1.1 Characteristic rod shape of *Mycobacterium tuberculosis* at electronic (left) and standard light microscopy after Ziehl-Neelsen stain (right)



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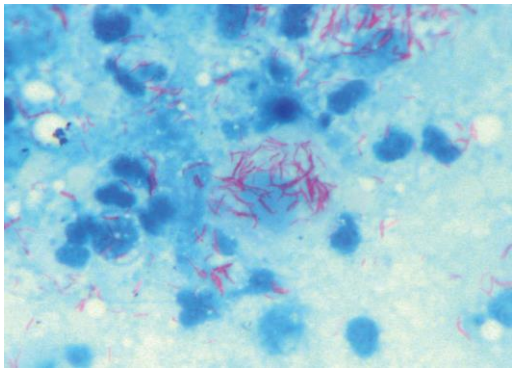

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This wax-like cell wall with high lipidic content makes mycobacteria resistant to antibiotics commonly used for other organisms, and to chemicals such as sodium hydroxide, acids and alcohol, unique characteristics on which diagnostic microscopy and culture techniques rely, as described later in section 1.4 (6).

One component of the cell wall is the glycolipid molecule, a cord factor responsible for the typical cord morphology visible at microscopic investigation after growth in liquid medium.

MTB is characterized by a slow replication rate, between 13 to 20 hours.

MTB is resistant to cold but killed by heat, hence the practice of milk pasteurization to prevent *M. bovis* infection. MTB is also sensitive to UV light (7). The MTB bacilli are generally aerobic with optimal growth in the presence of a high oxygen concentration such as in cavitary lesions, and facultative anaerobe, surviving in hypoxic granuloma (8).

1.2 Tuberculosis epidemiology advancement towards elimination

TB is the leading cause of death worldwide, worsened by the effect of COVID-19 during pandemic. Between 2020 and 2021 COVID-19 has interrupted or even reversed all efforts and achievements in fighting TB, highly affecting the access to TB diagnosis and treatment.
In 2021, globally an estimated 10.6 million people developed TB, reversing the slow decrease observed in the previous years (10.1 millions in 2020), and of them 1.6 million people died. In 2020 the number of reported people diagnosed with TB fell to 5.8 millions, decreasing compared to 7.1 millions in 2019, followed by slight improvement in 2021 with 6.4 million patients reported. The gap between estimated incidence of new TB cases and number of people reported with TB is worrisome, indicating that a large number of people with TB have remained undetected and untreated, increasing death the transmission of the disease.
Also resistance to rifampicin alone (RR-TB) or resistance to rifampicin in combination to isoniazid (multidrug-resistance, MDR-TB) has increased, with a total of 450 000 new cases, and only about one third could receive treatment for multidrug resistant TB (8).
One of the objectives of the UN Sustainable Development Goals (SDGs) is to end the TB epidemic by 2030. In response to this program, the World Health Organization (WHO) has launched the End TB Strategy, with three main objectives to be achieved by 2035: to reduce the TB incidence rate by 90% compared to 2015, to reduce by 95% the absolute number of TB deaths, and to eliminate TB-affected households experiencing catastrophic costs due to TB (9).
In 2021 the declines for TB incidence and death were are far from the target set by the End TB Strategy. TB incidence even increased of 3.6% between 2020 and 2021, reversing the decline of the previous years, as well as death rate, higher compare the 1.5 million estimates of 2020. As overall, between 2015 and 2021, the global TB incidence has decreased by 10%, half of the set milestone, while the reduction of deaths was only 5.9%, representing one sixth of the milestone for 2020 (Figure 1.2).

Figure 1.2 END TB strategy milestones and achievements in 2021

Targeted reductions relative to 2015

	Reduction in TB incidence		Reduction in deaths from TB	
	Set milestone	Achievement in 2021	Set milestone	Achievement in 2021
2020	20%	10%	35%	5.9%
2025	50%		75%	
2030	80%		90%	
2035	90%		95%	

Modified from WHO Global Tuberculosis Report 2021 (9)

In this strategy, prompt treatment and access to rapid molecular tests for TB detection and drug-susceptibility testing (DST) for anti-TB drugs are recognized as key steps. The past years have seen an unprecedented extensive implementation of the cartridge-based molecular GeneXpert®MTB/RIF assay (Cepheid, USA; hereafter "Xpert MTB/RIF") and line probe assays (LPAs), which have significantly changed the diagnosis of TB and its resistance (10) (see also section 1.4). The roll-out of molecular diagnostics and it's geographic coverage are however not yet complete.

A total of 5.3 million people were diagnosed with pulmonary TB in 2021, and of these 63% (3.4 million patients) were bacteriologically confirmed. Even if this represents and increase compared to the 59% in 2020, still the use of rapid molecular tests remains insufficient. Despite WHO recommendations, these tests were used as initial diagnostic for only 33% of newly diagnosed TB patients in 2020 versus 38% in 2021. In 2021, of the 3.4 million patients bacteriologically confirmed with TB, 141 953 were MDR/RR-TB and another 25 038 were diagnosed with pre-extensively drug-resistant TB (pre-XDR), defined as RR/MDR with additional resistance to a fluoroquinolone or XDR-TB, being pre-XDR with additional resistance to either bedaquiline (BDQ) or linezolid (LZD) (12).

While the roll-out of molecular diagnostics provides a more sensitive detection of TB than microscopy based diagnosis, it is logistically complex and leads to programmatic challenges, including high cost, need for training, maintenance of equipment and stock management. Together, accessibility to Xpert MTB/RIF remains inadequate in high burden settings, where these tests should be available as the initial diagnostic test for all people with signs and symptoms of pulmonary TB (11).

Like other organizations, Médecins sans Frontières (MSF) has been actively involved in the early roll-out of Xpert MTB/RIF in TB projects in settings that differed by TB and HIV prevalence and by the level of available facilities in the country laboratory network. The result of this large implementation effort, including improvement offered by Xpert MTB/RIF for patient diagnosis in MSF projects and associated challenges, is described in **Chapter II** of this thesis.

To improve TB detection, in addition to the introduction of molecular tests, in 2015 the WHO recommended the use of the lateral flow urine lipoarabinomannan assay (LF-LAM) for rapid TB detection in people living with HIV (12). However, this test, which is relatively simple and cheap (see section 1.4.3), has been introduced only in a limited number of countries.

Overall, TB diagnosis remains a challenge; hence a considerable proportion of TB cases are still clinically diagnosed rather than bacteriologically confirmed (13).

1.3 TB treatment and resistance development

Together with rapid diagnosis, swift initiation of effective treatment is crucial to cure TB and to contain the spread of the disease.

Before the introduction of anti-TB drugs, between the middle of the 19th and half the 20th century, the main approach to treat TB consisted of exposing patients to sunlight and increased caloric intake, with moderate physical exercise. During this period, sanatoria saw a big expansion

in Europe, with the first one opened in Germany in 1854, where patients could benefit from these treatments and were isolated to prevent TB transmission (14).

However, without antibiotic treatment, the death rate was about 65% (15) and TB remained a major challenge for public health. Alleviation of poverty and its associated malnutrition and crowded living conditions probably explains the decrease in TB incidence prior to effective treatment becoming available in the 1940's.

Streptomycin (STR), the first drug developed for TB, after initial success, resulted in a considerable proportion of amplification of drug resistance and relapses (16). A drastic improvement in the outcome of TB treatment arrived only with the use of streptomycin in combination with para-amino salicylic acid (PAS) and INH, which appeared soon after, followed by the introduction of pyrazinamide (PZA), ethambutol (EMB) and rifampicin (RMP) in the '70s (15). Fluoroquinolones (FQs) and second-line injectables (SLIs) were included in TB treatment only since the 80's, representing the key drugs against MDR-TB. Since then, the introduction of new drugs such as BDQ and delamanid (DLM) happened only in the last decade.

The treatment of TB is long and complex and relies on a combination of drugs with different activities because it needs to target bacilli at different metabolic stages: actively growing, semi-dormant or dormant. Drugs with high bactericidal activity like INH kill metabolically active bacilli and rapidly reduce the bacterial load. Hence their importance to interrupt transmissibility and to reduce the risk for selection of resistant mutants, which are associated with treatment failure (17). Sterilizing drugs such as RMP and PZA eliminate dormant bacilli to reduce the risk of relapse (17). Other "companion" drugs included in anti-TB treatment may only have bacteriostatic activity, and their main role is to prevent resistance to the core drugs.

The use of multidrug therapy is key to effective TB treatment, where single drugs should never be added to a failing treatment regimen, and treatment should be long enough to kill also dormant mycobacteria. Anti-TB treatment regimens in general are constituted by first-line drugs (FLD)s, used to treat rifampicin-susceptible (RS-)TB, and second-line drugs (SLDs), employed for treatment of RR/MDR-TB. SLDs are classified as group A, B or C drugs. The characteristics and activity of the drugs used for TB treatment are shown in Table 1.1.

Table 1.1 Drugs used for tuberculosis treatment

Class	Drug	Bactericidal activity	Sterilizing activity	Mechanism of action
First-line drug	Rifampicin	High in highly and slow metabolically active bacilli	Very high in high and slow metabolically active bacilli	Interact with the β subunit of RNA polymerase *rpoB* gene, preventing RNA elongation and protein synthesis
	Isoniazid	High against metabolically active mycobacteria	Moderate	Inhibits mycolic acid synthesis
	Ethambutol*£	Moderate/high against multiplying bacilli	Low	Inhibition of arabinogalactan synthesis
	Pyrazinamide*	Low	High against semi-dormant bacilli	Disruption of plasma membranes
Group A	Levofloxacin or moxifloxacin	High	High	Inhibition of DNA gyrase
	Bedaquiline	High	High	Inhibition of ATP synthase
	Linezolid	High	Low	Inhibition of protein synthesis
Group B	Clofazimine	Low	High	Inhibition of protein synthesis
	Cycloserine or Terizidone	Moderate	Low	Inhibition of protein synthesis
Group C	Ethionamide or Protionamide	Moderate/high	Low	Inhibition of cell wall synthesis
	Delamanid	High	unclear	Inhibition of mycolic acid synthesis
	Imipenem-Cilastatin or Meropenem	High	unclear	Inhibition of peptidoglycan synthesis
	Amikacin or Streptomycin	High	Low	Inhibition of protein synthesis
	p-amino salicylic-acid£	Low	Low	Inhibition of DNA precursor synthesis

Table modified after Van Deun (2018) and Dookie (2018) (18) (17); *Also part of the group C; £= mainly bacteriostatic activity

1.3.1 Treatment of drug-susceptible TB

Patients who did not receive TB drugs before or were exposed to TB treatment for less than one month (defined as newly diagnosed TB patients), receive first-line (category 1) treatment, preferably after laboratory confirmation of RS-TB. This regimen includes 2 months of intensive phase with 4 drugs and 4 months of continuation phase (2HRZE/4HR), with RMP and INH given throughout treatment. This regimen requires bacteriological monitoring with smear microscopy, plus DST in case of no clinical improvement, to assess for (amplification of) drug resistance. This 6-months combination therapy for RS-TB is highly effective. If adequately taken, patients achieve cure rates of >95% (19).

Other treatments have been used or proposed for RS-TB in the past but are not in use at present. Until 2017 for example, category 2 treatment (2HRZES/1HRZE/5HRE), was recommended for patients previously treated for more than one month with first-line drugs and confirmed to have RS-TB. This treatment however is no longer recommended, because of its major issue being the addition of STR only to a treatment potentially failing, which is against the principles for constructing/adapting anti-TB regimens. Also, cumulative ototoxicity of aminoglycosides (STR for RS-TB followed by SLIs if the patient develops RR/MDR-TB) would have jeopardized the "short treatment regimen", also known as the Bangladesh regimen, recommended by WHO in its 2016 guidelines (See 1.3.4), but now discontinued.

Several trials in the past have also investigated the possibility to include FQs in a 4-months short treatment for RS-TB. This option was initially discouraged by WHO, to avoid amplification of FQ resistance and losing FQs as core drugs for RR/MDR-TB (20). However, despite the initial concerns, since 2018, the treatment with RMP, EMB, PZA and levofloxacin (LFX) is now recommended for patients with confirmed RS but INH-resistant TB, (21). FQ-enhanced 4-monhs first line treatments are now also recommended as alternative to the standard DS-TB treatment (22) showing to be non-inferior to the standard 6 months treatment (23). However, when 'occult' RR- TB is missed, such as in eSwatini (see Chapter V), such regimens effectively result in FQ monotherapy, causing a serious risk for acquisition of FQ resistance.

To support good patient management and enhance the efficacy of first-line treatment, the directly observed treatment short course (DOTS) strategy was developed in the 90's. This strategy, which includes different elements such as political commitment, diagnosis of patients with smear microscopy and directly-observed treatment, has been recognized as one of the biggest public health interventions. However, its implementation required a considerable commitment, and despite the country investments done, insufficient patient management has led quickly to an increase in MDR-TB cases.

1.3.2 TB drug resistance

The characteristic of the lipidic cell wall of mycobacteria make the bacilli naturally resistant to antibiotics such as penicillin. Also, the dormant status of metabolically inactive mycobacteria can result in tolerance to anti-TB drugs. However, the most common mechanism for development of resistance for MTB consists in the random amplification of mutations. These mutations include non-synonymous single nucleotide polymorphisms (SNPs), or nucleotides insertions/deletions (referred as indels) in genes that either encode for drug targets, drug-activating enzymes or that are related to efflux pumps regulators.

The MTB population within a host is likely to contain rare naturally occurring drug-resistant mutants, proportional to the mutation rate and to the size of the bacterial population. In fact, the number of mutants in untreated patients is very low, but can increase through selective advantage during inappropriate treatment. The probability of these natural mutations to occur in MTB is about 1 in 10^8 for RMP and FQs and 1 in 10^6 for INH and EMB (18)(24). As spontaneous mutations occur independently for each drug, the risk of simultaneous occurrence of resistant mutants for two drugs corresponds to the product of the risks for each individual drug.

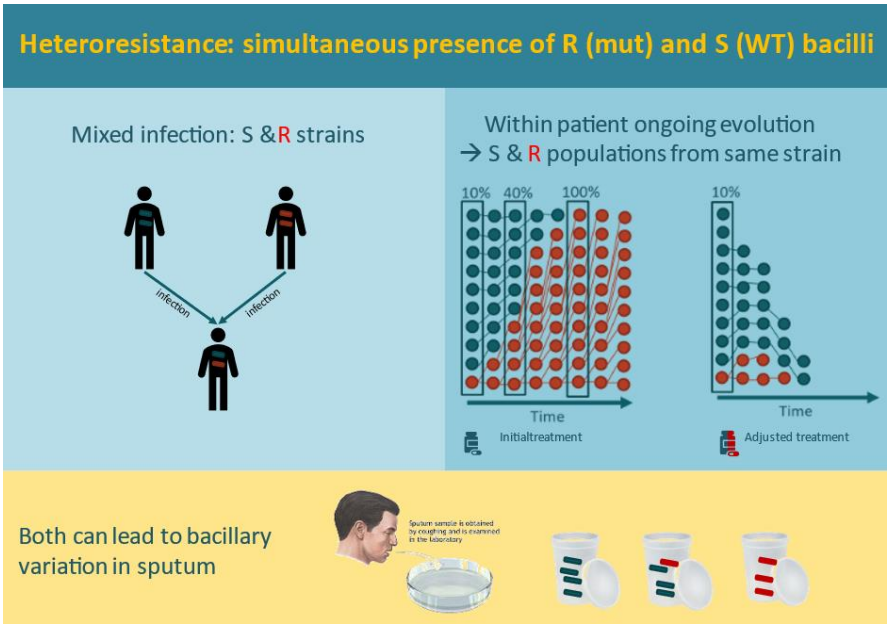
Drug-resistant TB can occur as acquired resistance or primary resistance. Acquired resistance is the situation described above, where during TB treatment susceptible bacilli are gradually replaced by resistant bacilli until they become the predominant mycobacterial population (24). Generally, patients receiving first-line treatment develop initially resistance to INH (with or without resistance to EMB and PZA), switching then subsequently from mono/poly-resistance to MDR, and (pre-)XDR (25). RR-TB without concomitant resistance to INH is considered relatively rare (26), and for this reason considered an indicator for MDR-TB even if INH resistance testing is not available.

Primary resistance is defined as infection with a strain already resistant, as a result of resistant TB transmission in the community. In good TB programs, transmission seems to be the major factor contributing to incident RR-TB, rather than acquisition of resistance during treatment (27) (28). Therefore, rapid detection of RR-TB is crucial, not only to provide a correct treatment to patients, but also to interrupt the spread of RR/MDR-TB. However, in specific settings, rapid detection of RR is challenging. In addition to difficult access to diagnostic tests for patients living in remote settings, various factors can contribute to the lack of RR detection, including mutations not targeted by the molecular tests used for routine diagnosis, or technical limitations of phenotypic methods (see section 1.4). The presence of heteroresistance poses an additional diagnostic challenge for molecular DST.

1.3.3 Heteroresistance

Heteroresistance is the situation in which both susceptible and resistant mycobacteria coexist in the same patient. Heteroresistance can be due to either co-infection with a resistant and a susceptible strain, or to the gradual clonal expansion of mutants in the infecting strain within a patient. If under continued drug pressure, the minority population will grow and may be detected as resistant, ultimately replacing the entire wild-type bacillary population. When all bacilli carry the mutation, it is considered “fixed”. (29), (Figure 1.3). While the clinically relevant level of heteroresistance is not yet known and may vary by drug, we hypothesize that the 1% cut-off used in phenotypic DST (pDST) is also a valid cut-off for genotypic DST (gDST), although few molecular assays will detect such a low proportion (29).

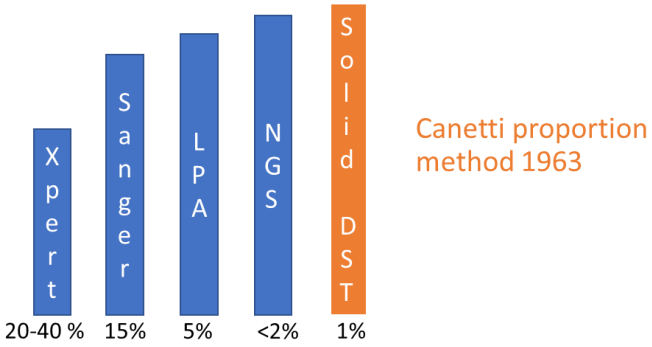
Figure 1.3 Mixed infection (A) and in-host evolution of heteroresistance (B)



From Rigouts presentations (2021)

Indeed diagnostic tests have different sensitivity to detect minority mutant/resistant populations, estimated as 1% for pDST and deep next generation sequencing (NGS) such as Deeplex®Myc-TB (Genoscreen), 5% for HainMTBDR*plus*, 20-40% for Xpert MTB/RIF, and 20% for Sanger sequencing (30)(31) (see also section 1.4). Thresholds for detection of heteroresistance by different tests is shown in Figure 1.4.

Figure 1.4 Proportion of mutants at which TB diagnostic tests detect heteroresistance



LPA= Line Probe Assay; deep NGS= Next Generation Sequencing; DST= drug susceptibility testing

Deep NGS and pDSTs have a very high sensitivity to detect heteroresistance. However, deep WGS (~1000x coverage) and indirect phenotypic DSTs are performed from mycobacterial cultures. This procedure represents a limitation to the sensitivity in detecting heteroresistance of these tests, as growing mycobacteria on culture medium, and even more so when multiple subcultures are

prepared for DNA extraction for WGS, may preselect one population that can overgrow another that is less “fit”, leading to incorrect DST results (32). In contrast, direct testing on clinical specimens such as sputum, including target deep sequencing (e.g. with Deeplex) or pDST like thin layer agar (TLA; described in section 1.5.1.3 and whose performance and implementation is described in **Chapters III, IV and V**) can better detect minority populations, limiting culture bias.

1.3.4 Treatment of multi-drug resistant TB

In resource-limited settings, an individualized approach for all TB patients may be difficult to implement, requiring a complex organization in drug management, staff training, patients reporting and individualized DST (33). To alleviate these challenging operational requirements, in 2000, the WHO has proposed the DOTS-Plus strategy as pilot project, focusing on the strengthening of laboratory capacity and introducing a standardized approach to MDR-TB treatment, based on countries’ drug resistance prevalence rather than on individual resistance patterns (34). Only starting from 2003 MDR-TB treatment has been standardized, resulting in a long treatment of 20 months or more, with an intensive phase of 6 months, which included SLIs, FQs (mainly ofloxacin and ciprofloxacin) and amongst other drugs EMB and PZA, followed by a 12-18 months continuation phase (35).

Since then, WHO guidelines on MDR-TB treatment have been revised numerous times. In 2011, the intensive phase with an injectable was extended to 8 months and EMB was no longer considered between the drugs to construct the treatment, but rather added if showing susceptibility (36). However, MDR-TB treatment was far from being optimal, showing cure rates as low as 50% (37), close to the natural history of untreated TB (38).

The main changes occurred in 2016, with the conditional recommendation of the “short-course” regimen (9-12 months), investigated in Bangladesh, for patients with confirmed susceptibility to FQs and SLIs. This regimen represents a milestone in MDR-TB treatment, with its shortened duration and a cure rate reported as high as 87.9% (39). The long MDR-TB treatment continued to be used for patients not eligible for the shorter regiment, and was revised in the same year (40) with ofloxacin (OFX) being phased out in favor of LFX or moxifloxacin (MFX), and with the introduction of new TB drugs such as BDQ (Sirturo^{TM}, Janssen Pharmaceutica) and DLM (DeltybaTM, Otsuka Pharmaceutical). At that time however the latter two were only considered as add-on drugs, due to poor accessibility, clinical trials still ongoing, and the limited experience regarding combination with other drugs.

In 2019, RR/MDR-TB treatment went through another major change, with the reclassification of anti-TB drugs (Table 1.1) and the introduction of an all-oral regimen of 9-12 months for patients whose isolate was confirmed susceptible to FQs. This treatment includes 6 months of BDQ intake, injectables are no longer considered key drugs due to their high ototoxicity, while FQs in combination with BDQ and LZD are considered the first treatment choices.

The long individualized RR/MDR-TB treatment with a duration of 18-20 months has also been maintained for patients with FQ resistance and previous exposure to other drugs.

Another two short regimens, the 6 month-regimen BPaL(M) composed by BDQ, pretomanid, LZD and moxifloxacin are recommended by WHO for MDR-TB and XDR-TB. Endorsed in August 2019

by the US Food and Drug Administration these treatment have a duration of 6 months, with a reported efficacy as high as 90% (41) (42).

The significant shortening of treatment duration constitutes a major evolution of the anti-TB treatments, having an impact on patient treatment compliance, and a potential influence on patients' financial situation, reducing loss of work, while reducing the financial burden on the health system(14).

One of the key components of these RR/MDR-TB regimens is the use of anti-TB drugs based on DST results. However, accessibility to reliable DST results is limited in most settings where these drugs are employed. In addition, for some drugs such as BDQ, critical drug concentrations to differentiate between drug-resistant and drug-susceptible bacilli are still not fully established, nor the correlation between the mutations detected and treatment outcomes. In most settings, treatment with BDQ is started based on the assumption that patients who did not receive this drug previously have susceptible TB. Nevertheless, occurrence of mutations potentially associated with BDQ resistance is reported to be as high as 6% (43)(44). However rapid detection of BDQ resistance is challenging. Rapid molecular tests as still not available due to the unclear correlation between BDQ minimal inhibitory concentration (MIC) and mutations found in BDQ resistance correlated genes (43), making access to rapid DST for BDQ an urgency.

1.3.5 Treatment outcomes

A patient is identified as having TB either bacteriologically, so when at least one sample is positive by smear microscopy, culture, or molecular tests, or clinically, in presence of TB symptoms and/or a suggestive X-ray, but without laboratory confirmation.

Upon treatment, patients are classified according to their response to treatment, which is evaluated clinically and bacteriologically, generally with smear microscopy for DS-TB and culture with DR-TB (45). In the past, treatment outcomes have always been defined in relation to a time threshold and in alignment with an intensive and continuation phase of treatment schemes. However, with the introduction of these short treatments leading to earlier conversion than in long regimens, and implementation of injectable-free short regimens, these definitions have been revised and simplified in 2020 (46) (Table 1.2). Amongst other changes, the time between two negative cultures required to define culture conversion was reduced from 1 month to 7 days.

Table 1.2 Treatment outcome definitions for pulmonary TB patients

Outcome	Definition
Cured	bacteriologically confirmed TB patient who completed treatment with evidence of bacteriological response and no evidence of failure.
Treatment completed	Patient who completed treatment, whose outcome does not meet the definition for cure or treatment failure.
Failed	A patient with treatment regimen terminated or permanently changed to a new regimen.
Died	A patient diagnosed with TB who died before starting treatment or during treatment.
Lost to follow up	Patient who did not start treatment or with treatment interrupted for 2 consecutive months or more.
Not evaluated	Patient transferred to another treatment unit and with unknown outcome.

Modified from WHO 2020 (46)

1.3.6 Detection of reinfection versus relapse

Recurrence of TB is defined as the return of signs of TB disease in a patient who was declared cured or who completed treatment and remained TB diseases-free for a minimum of six months after the end of the most recent anti-TB treatment.

Recurrence of TB can be due to true relapse, i.e. reactivation of TB after cure, or reinfection, i.e. infection by a new strain (47). On programmatic level, the differentiation is crucial to understand whether a regimen failed or transmission is ongoing. The estimate on how much relapse versus reinfection contributes to the overall burden of the disease is important to evaluate the efficiency of control measures against TB transmission.

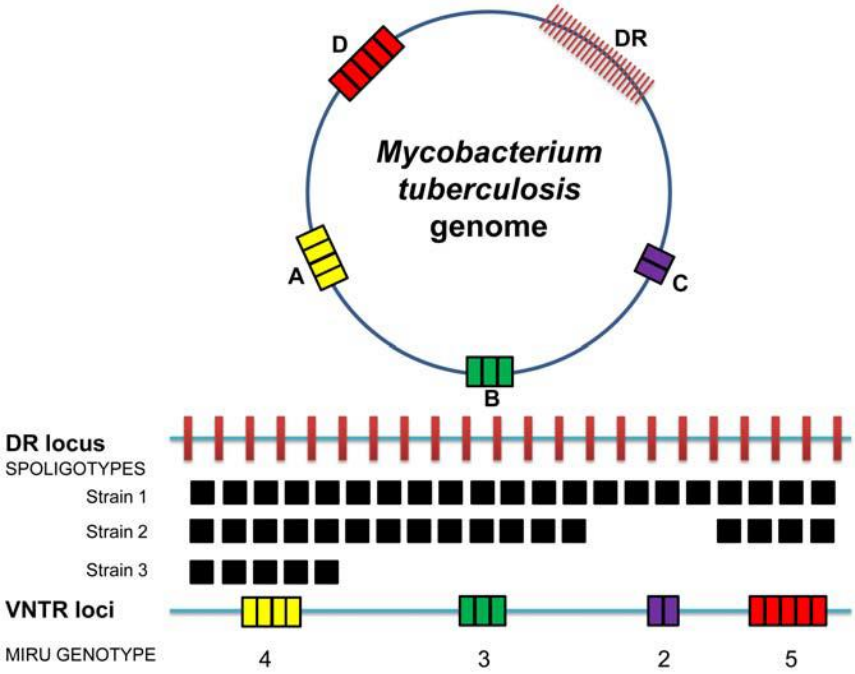
Before the application of molecular genotyping techniques, recurrence of TB was mainly interpreted as reactivation of the disease, rather than a new infection (48). Early methods used to distinguish different isolates, such as comparison of colony appearance, biochemical tests and phenotypic characteristics have shown insufficient resolution to distinguish strains (49). These techniques have been replaced by molecular techniques, described below.

Two “classical” molecular techniques are still widely used at present: spoligotyping and MIRU-VNTR typing, referring to the variable number of tandem repeats (VNTR) in multiple loci called mycobacterial interspersed repetitive units (MIRU).

Spoligotyping targets the variation in 43 spacers between the direct repeats in a well-conserved CRISPR based direct repeat (DR) locus of the MTB genome (Figure 1.5)(50). PCR products are hybridized to a membrane, showing signals when binding to the probes representing the

different spacers, and visualized as black squares indicating the presence of spacers (51). This technique is applicable directly to samples or to cultures, but is not highly discriminatory to differentiate strains across patients (49). MIRU-VNTR typing estimates the number of repeats in 12 or 24 MIRU loci spread over the genome (A,B,C,D in Figure 1.5). If two strains have a different number of repeats in maximum 1 locus, they are still considered identical (51). MIRU-VNTR typing is mostly done from cultures.

Figure 1.5 Targets for spoligotyping and MIRU-VNTR typing



From Comas 2009 (52). DR=direct repeat; VNTR= variable number of tandem repeats

These techniques, even if still in use, have progressively been replaced by NGS, especially WGS, with the assumption that strains with no or few SNPs are closely related. Relapse strains are usually identical or have one or few SNP differences. Typical cut offs to identify chains of transmission are 5 or 12 SNPs found in genes not associated with drug resistance, as such mutations may have occurred due to drug pressure rather than at the 'natural' molecular clock rate (53). However, clear cut-offs to distinguish reinfection versus relapse have not been fully defined . In addition, results should be interpreted also taking into consideration how common a strain is in a region, as predominant strains may result in similar NGS profiles, but with different phenotypic resistance patterns suggesting they are different strains. For a 'common genotype' such NGS-based approach thus overestimates the classification of relapse (54).

Therefore, when strains from sequential isolates from the same patient are compared, the distinction between reinfection and relapse remains challenging, also by WGS, and is further complicated by the difficulty to distinguish, among isolates with distinct genotypes, reinfection from administrative or laboratory errors.

1.3.6.1 Diagnosis of TB

The End TB strategy strengthens the importance of using molecular techniques to rapidly diagnose patients with symptoms of TB, and to ensure access to DST for RMP to all patients confirmed with TB. When RR/MDR-TB is confirmed, patients should be tested at least for FQ resistance. In addition, countries are encouraged to scale up capacities to also test new and repurposed drugs such as BDQ, LZD, CFZ and DLM, for which pDST is still considered the reference method.

For many years TB diagnosis has relied on microscopy and culture on Löwenstein-Jensen (LJ) or agar-based solid media, until 2007, when the use of liquid medium was endorsed by WHO (see 1.4.2.3). To date, culture in liquid medium remains the reference method for the diagnosis of TB. Culture is also still used for monitoring of patients on RR/MDR-TB treatment. The complexity of the biosafety and technical requirements for culture implementation, and the long time to obtain results, however limit its widespread application (55).

In recent years, an impressive effort has been made to improve TB diagnostic capacity, with the development of several molecular techniques to rapidly detect MTB and resistance to anti-TB drugs. Molecular tests, initially endorsed individually based on their characteristics, are now grouped by WHO in classes based on common parameters, such as the technology used, complexity (low, moderate, versus high), and test application, such as initial diagnosis of TB in combination with drug-resistance detection or not, or tests that are used during (post-) treatment follow up (56).

Among the phenotypic and molecular methods endorsed by WHO for TB diagnosis and to detect drug resistance, the paragraphs below focus on the tests that were used for the research described in this thesis, while other tests are only briefly mentioned.

In addition, we briefly describe microscopy techniques. While WHO recommends replacing microscopy with molecular tests for TB detection, this technique still has multiple applications. Microscopy remains crucial in remote settings where molecular techniques, even of low complexity, cannot be sustainably implemented due to logistic challenges and resource limitations. In addition, microscopy is still used for patient monitoring and to evaluate the mycobacterial load in samples to be tested with (advanced) molecular techniques.

1.3.7 Microscopy

Microscopy has been the most extensively used technique for many years, since the discovery of *M. tuberculosis* by Robert Koch in the 19th century (6). The first method developed, which uses standard light microscopy and Ziehl-Neelsen (ZN) staining, relies on the ability of carbol fuchsine to penetrate the mycobacterial wall, and at the same time on acid or alcohol fastness of the mycobacterial cell wall structure, so the ability to resist decolourisation with acid and alcohol (24). ZN can be implemented at all laboratory levels, is relatively simple to perform, is inexpensive and can rapidly detect acid-fact bacilli. However, microscopy presents several limitations. It cannot distinguish between viable and dead mycobacteria, nor between MTB and other mycobacteria, and does not provide information on drug resistance. In addition, the sensitivity

for the diagnosis of TB is low and variable compared to culture (from 20% to 80%) (57), with a limit of detection of 5000-10000 bacteria/ml of sputum, so that a third of the TB patients remains undetected (58). In general, microscopy-positive sputum specimens are referred to as smear-positive specimens.

Despite its simplicity, microscopy is rather labor-intensive, highly operator-dependent, and its quality is to be checked by a complex process of quality assurance, which includes slide rechecking, testing blinded panels of slides, and a continuous process of quality improvement (59).

In the last decades, microscopy has gone through several improvements. For many years, patients were requested to submit 3 samples collected over 2 days, including one sample collected in the morning. This has represented a considerable effort and cost for patients who travelled to health centers to submit multiple sputum specimens on multiple days (60), increasing the risk of loss to follow-up (61).

The ZN technique was substituted in 2005 by the use of fluorescent microscopy (FM), with slides stained with Auramine-O, a fluorochrome stain, and read by mercury vapour fluorescence microscopes (62). The advantage of this technique in a higher sensitivity and speed, as reading required a lower magnification.

Another major improvement was with the development and endorsement in 2010 of the light emitting diode (LED) microscopes, less expensive and with lower maintenance requirements compared to the mercury vapor microscopes (57). LED microscopy has 10% higher sensitivity for TB detection compared to standard microscopy, and detects TB in up to 67% of presumed TB patients (63). Even if LED-FM requires extensive training and supervision to distinguish artifacts from acid-fast bacilli, its introduction has significantly improved microscopic diagnosis. The method remains cheap, and as FM allows reading of slides at x400 magnification, it shortens the time for reading, allowing the reading of 60 slides as quickly as 25 ZN slides (64). WHO recommends to implement LED-FM in replacement of mercury vapour fluorescence microscopy and conventional ZN light microscopy in both high- and low-volume laboratories (57).

Despite the low sensitivity and the disadvantages described above, microscopy can be employed to detect the most infectious patients where Xpert assays are not yet implemented, and it is still used to monitor treatment response (15).

1.3.8 Culture methods for MTB detection

Despite the evolutions in TB diagnostics over the last decade, culture remains the reference standard for MTB detection (65). Due to its ability to distinguish viable from non-viable mycobacteria, culture remains indispensable for patient monitoring during treatment, especially for RR/MDR-TB. In addition, culture yields mycobacterial isolates to perform subsequent tests, such as pDST, including MIC determination, as well as molecular methods requiring sufficient quality and quantity of DNA, such as WGS.

Mycobacterial culture analysis is labour-intensive. The preparation of sputum for culture isolation starts with selective removal of other organisms from the sample, which would overtake growth of the slower growing MTB. This decontamination, to be performed as soon as possible

after sample collection, is followed by inoculation of the sample sediment on selective media and subsequent identification of mycobacterial growth.

1.3.8.1 Sample decontamination for culture

Generally referred to as decontamination, the preparation of samples prior to inoculation consists of two steps: digestion and decontamination. The purpose of digestion is to free bacilli from mucus and cells, while decontamination aims to reduce growth of competing organisms present in the sample. Decontamination is a critical step for cultures: if too strong, the process will not only kill the contaminant bacteria but also significantly decrease the number of mycobacteria, therefore decreasing the sensitivity of culture, while if too gentle, it will result in overgrowth by the 'contaminants'. Generally, for fresh samples a culture contamination rate of 2-3% for solid media and 5% in liquid media is considered acceptable, which can increase to 5-10% if samples take a few days to arrive at the laboratory, due to overgrowth of contaminants associated with transport delays (66). On the other hand, complete absence of contaminated cultures is a sign that samples may have been too harshly decontaminated, lowering the overall sensitivity of culture for the diagnosis of TB.

Several decontamination methods can be used. The Petroff method uses NaOH, which is added in equal volume to the sample to reach a final concentration of 1-2%, then neutralized before centrifugation with hydrochloric acid, with phenol red used as indicator. The modified Petroff approach is a simplified version of this technique, which uses NaOH followed by neutralization with distilled water (67). The most widely used method however is the NALC (N-acetyl-L-cysteine)-NaOH method, also aiming at a final NaOH concentration between 1-2%. Cysteine is added to NaOH because of its capacity to digest and liquefy mucus, and to reduce the toxic effect of NaOH. When NALC is used, loss of mycobacteria during decontamination decreases from 60% to 30% (68). The disadvantage of this method however is the very short shelf life of the cysteine solution: once reconstituted in NaOH, it must be used within 24 hours.

Cetyl-pyridinium chloride (CPC) is an ammonium compound added to samples for partial digestion and decontamination during storage transport. This reagent, when used alone as decontamination method, is not compatible with liquid nor agar based media, but only with egg based media, which contains phospholipids able to neutralize the ammonium (69). However, a second and shortened decontamination step may wash away CPC, allowing inoculation on any media (70). A comparison of these methods and improvement of compatibility of CPC versus agar media is presented in **Chapter III**.

1.3.8.2 Culture on solid media

The most common solid media used are LJ, in use in culture laboratories since the beginning of the 20th century, and Middlebrook 7H10 and 7H11 agar-based media developed in 1947 by Dubos and Middlebrook.

LJ is an egg-based selective medium and its formulation includes malachite green to inhibit growth of microorganisms other than mycobacteria. Fatty acids and proteins necessary for the

metabolism of mycobacteria are supplied by the egg, while glycerol provides the carbon source favored by *M. tuberculosis* but not *M. bovis* (71) .

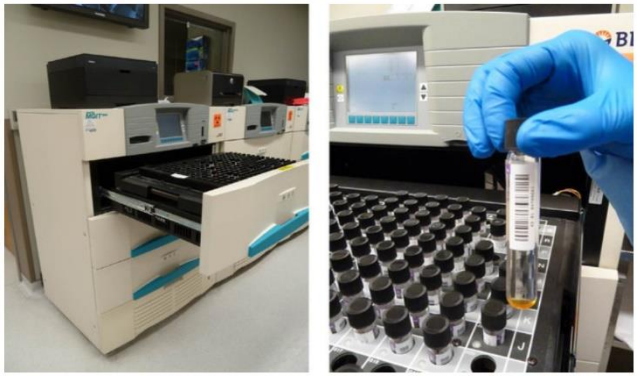
7H10 and 7H11 are modifications of the original formulation of oleic-acid albumin agar (72), where 7H11, compared to 7H10, includes casein hydrolysate that enhances growth of *M. tuberculosis*, especially drug-resistant strains that can be notoriously difficult to grow on 7H10 (73). These non-selective media are supplemented with OADC enrichment, which contains oleic acid, albumin, dextrose, catalase and sodium chloride to enhance mycobacterial growth.

Solid media are generally inexpensive and can be prepared locally in the laboratory, then stored ready to use for several months in a fridge (2-8°C). Inoculated slants are read by naked-eye weekly for up to eight weeks to give final negative results. Microscopy smear-positive samples tend to yield positive cultures on average after 10 days on 7H11 and 16 days on LJ (74).

1.3.8.3 Culture in liquid medium

Several systems use liquid medium for TB detection. The most common method used is Mycobacterium Growth Indicator Tube (MGIT, Becton Dickinson, USA) (Figure 1.6).

Figure 1.6 MGIT^{960} device and MGIT tube



The MGIT tube is filled with Middlebrook 7H9 and contains, amongst other reagents, Tween® 80 that facilitates nutrients to penetrate the mycobacterial wall. The principle consists of the presence of an oxygen-quenched fluorochrome embedded in silicone in the bottom of the tube. With microorganism growth in the tube, the decrease in free oxygen (replaced by carbon dioxide) causes the fluorochrome to be no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light. The extent of oxygen decrease is proportional to the fluorescence intensity (75).

Before inoculation, MGIT tubes are supplemented with OADC to enhance mycobacterial growth, and with PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin), a mixture of antibiotics added to control overgrowth by non-mycobacterial organisms at primary isolation.

MGIT tubes are incubated in an MGIT incubator, and automatically read hourly. On average smear-positive samples yield results within 10 days (76), while negative results are released after 42 days.

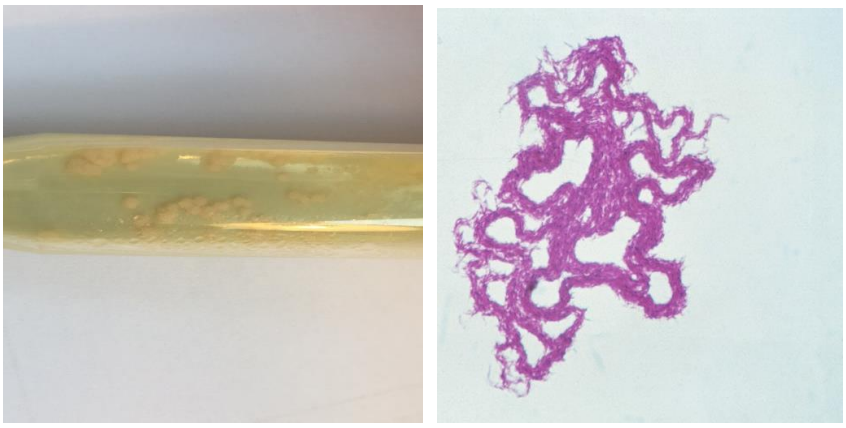
Its widespread use started in 2007, when the WHO recommended the introduction of MGIT-based culture in national reference laboratories first, then in regional laboratories. At the time of this recommendation there was insufficient experience to encourage the use of MGIT alone for culture isolation in laboratories without previous experience with solid culture, and parallel use of solid medium was still supported as back up, in case of MGIT consumables stock rupture or culture contamination. (77) Despite a wide experience with MGIT implementation achieved so far, this approach of parallel inoculation on solid medium in many countries has remained unchanged.

The MGIT technique has a higher sensitivity than LJ (78), with a limit of detection of ~10–100 bacteria/ml (79). However, this technique is expensive, and its implementation requires a laboratory with high level of biohazard containment due to high risk of material spillage when liquid cultures are manipulated (80). In addition, MGIT liquid medium is more prone to contamination than solid medium.

1.3.8.4 Mycobacterial identification

Once a culture is positive, growth is identified to distinguish between MTB and NTM. On solid medium, a preliminary identification is done by visual inspection, to check for the typical creamy 'cauliflower-like' appearance of the colonies (Figure 1.7, left picture).

Figure 1.7 Mycobacterial appearance on solid medium and in microscopic examination after growth in liquid medium.



On the left, Löwenstein-Jensen slants positive for *Mycobacterium tuberculosis*, on the right microscopic detection of typical cords for *M. tuberculosis* grown in liquid medium

Growth in liquid medium is checked for purity by inoculation on a blood agar plate and by investigation with smear microscopy for MTB characteristic cord-formations (Figure 1.7, right picture). Confirmation of MTB is usually performed with rapid immunochromatographic

identification tests (lateral flow assays). These rapid tests have the ability to detect the MPT64 protein released during MTB metabolism in cultures (81).
Another method used for mycobacterial identification consists of inoculating the bacilli in a medium containing p-nitrobenzoic acid (PNB), which is expected to inhibit MTB but to allow growth of NTMs (82).

1.3.9 Urine LF-LAM

The lateral flow urine lipoarabinomannan assay (LF-LAM, Abbott Laboratories, USA) is based on the detection of LAM antigen in urine, a lipopolysaccharide part of the cell wall that is released from mycobacteria. The test is very useful for TB detection in patients who cannot produce sputum. In patients with HIV and signs of TB, the test has a sensitivity of 42% and specificity of 91%, with sensitivity varying significantly according to the CD4 cell count (26% if ≤ 200 and 47% if ≤ 100). Due to its overall low sensitivity, particularly in non-immunocompromised persons, the test should not be used to screen for TB in the general population (83). LAM's invaluable advantage is that it is the only test can be considered as a point of care test and has been fully integrated in WHO algorithms that will be described later.

1.3.10 Nucleic acid amplification techniques (NAATs)

Compared to phenotypic methods described above, molecular tests can detect TB within a short time, ranging from a few hours to a few days, while ensuring high sensitivity and specificity. Detection of MTB by molecular techniques mainly relies on detection of specific sequence regions characteristic for MTB such as the most commonly used IS*6110* insertion sequence, 23SrRNA, 16SrRNA genes, or MPT64 gene, encoding for a protein typical of MTB. Alternatively, diagnostic tests use the *rpoB* gene, that encodes for the β subunit of bacterial RNA polymerase. This gene, also used as target for detection of RR, is present in all bacteria, showing specific characteristics for each bacterial species, including the sequence of the nucleotides and its length.
The nucleic acid amplification tests (NAATs) used only for MTB detection include low and moderate complexity tests such as Truenat MTB Plus (Molbio Diagnostics, Goa, India) and TB-LAMP (Eiken Chemical Company, Japan), a loop mediated isothermal amplification assay. These tests do not provide information on drug resistance. Other molecular tests provide information on drug resistance simultaneously with detection of TB. These techniques include low complexity tests such as Xpert MTB/RIF, Xpert Ultra) and Truenat MTB-RIF, or moderate complexity methods such as MTBDR*plus* and MTBDR*sl* (Bruker/Hain Lifescience, Germany) and others recently endorsed by WHO (Table 1.4).

1.3.10.1 Truenat MTB and Truenat MTB Plus

Truenat MTB Plus uses a real-time PCR on a chip for MTB detection, and can provide results in an hour. This technique uses two devices: one for extraction and a second for DNA amplification. Compared to culture, this method has a sensitivity and specificity of 83% and 99% respectively

to detect TB. Once the test is positive, the extracted DNA can be used on the MTB-RIF Dx assay, for detection of RMP resistance. These tests can be run at room temperature, up to 40°C with 80% humidity. Moreover it is battery operated, which make it suitable for peripheral settings. In 2020, Truenat MTB has been endorsed by the WHO as initial diagnostic test in patients with signs of TB, replacing microscopy and culture (84).

1.3.10.2 TB-LAMP assay

TB-LAMP is based on rapid nucleic acid amplification that occurs at a stable temperature of about 65°C. The test has about 80% sensitivity for detection of TB compared to culture and similar sensitivity to Xpert MTB/RIF (85) (86). TB-LAMP has the advantage to carry out DNA extraction and DNA amplification in a single device and to operate on battery power. Hence it is considered as a portable test requiring minimal infrastructure. Moreover, the instruments are more resistant to humidity compared to the GeneXpert device (see section 1.5.2.1). Thanks to low biosafety requirements similar to microscopy, ease of interpretation of results by naked eye with UV-light, and availability of results in an hour, this method is suitable for peripheral settings. For this reason, it has been endorsed by the WHO as replacement or as add-on test to microscopy for the initial diagnosis of TB in patients with signs of TB (85). However, this test should not replace other molecular techniques with higher sensitivity or those that are also able to detect drug resistant TB.

1.4 Diagnosis of drug resistance

1.4.1 Phenotypic methods for TB drug-resistant detection

The key role of the TB laboratory in the fight against RR/MDR-TB has been recognized by WHO since the early start of RR/MDR-TB treatment standardization in 2000 (35), highlighting the need to expand DST accessibility by the National TB Programs and encouraging the use of services offered by Supranational Reference Laboratories until these tests are in place at the national and/or regional level. Phenotypic DST remains the reference standard for the majority of the drugs (56). With the inclusion of new drugs such as BDQ and LZD in the scheme of treatment for RR/MDR- and XDR-TB, detection of resistance to these drugs is crucial. However, for BDQ, none of the mutations in genes known to be correlated with drug resistance have been significantly associated with BDQ resistance by WHO, mostly because of scarceness of data (87). For this reason, phenotypic DST to BDQ is considered a priority.

The “proportion method” is the most commonly used pDST method to define susceptibility to anti-TB drugs. It relies on the assumption that a mycobacterial population that shows less than 1% of growth on a drug-containing medium compared to a drug-free medium is considered as susceptible, and if equal to or above 1%, as resistant (88).

With this method a specific mycobacterial suspension is inoculated on the medium containing the drug (at the critical concentration) and compared to two controls (drug-free medium): one inoculated with the same suspension and one with its 10^{-2} dilution. The growth in presence of

the drug is compared to the 10^{-2} control, and if equal or more (so ≥1% of the control inoculated without dilution) the mycobacteria are defined as resistant.

With the proportion method mycobacteria are exposed to a specific drug at a critical concentration (CC), which can differ depending on the medium used. For MTB, the CC for each drug is based on the epidemiological cut-off value (ECOFF), which is the highest MIC that separates ≥99% of presumed susceptible (wild-type) bacilli from resistant (mutant) bacilli. The MIC for a specific mycobacterial population is the lowest drug concentration at which 99% of the mycobacteria do not show growth. The ECOFF distinguishes between susceptible and resistant isolates. However, resistant strains (above the CC) may show low and high level of resistance, reflecting two different mechanisms of resistance. Higher dosing of the drug correlates with therapeutic success in case of low-level resistant strains, but high-level resistance can not be overcome by further drug dosing increase. While the distinction between susceptible is defined as described above, increasing drug doses can only be done if PK/PD and safety data support such increase. The concentration that separates the low-level or intermediate from high-level resistance is referred to as “clinical breakpoint”(89).

Revised CCs from the WHO are shown in Table 1.3 (90).

Table 1.3 Recommended critical concentrations and clinical breakpoints (in µg/ml) for anti-TB drugs

Drug critical concentration (clinical breakpoint)	LJ	7H10	7H11	MGIT
rifampicin	40.0	0.5	1.0	0.5[(1)]
isoniazid	0.2	0.2	0.2	1.0
ethambutol	2.0	5.0	7.5	5.0
pyrazinamide	-	-	-	100.0
levofloxacin	2.0	1.0	-	1.0
moxifloxacin	1.0	0.5	0.5	0.25
(moxifloxacin)	-	2.0	-	1.0
amikacin	30.0	2.0	-	1.0
kanamycin	30.0	4.0	-	2.5
capreomycin	40.0	4.0	-	2.5
ethionamide	40.0	5.0	10.0	5.0
linezolid	-	1.0	1.0	1.0
clofazimine	-	-	-	1.0
Bedaquiline [(2)]	-	-	0.25	1.0
delamanid[(2)]	-	-	0.016	0.06

Modified from WHO 2018. Critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis); [(1)] 0.5 µg/ml revised in 2021 and replacing 1.0 µg/m; [(2)] CC proposed ad-interim.

pDST is applied to viable mycobacteria, either directly from samples (direct pDST) or from mycobacterial growth obtained from selective media (indirect pDST), and can be performed both on solid and in liquid medium. Genotypic methods rely on detection of mutations in genes associated with resistance to anti-TB drugs. These details will be described below for each technique.
Overall, pDST is considered highly reliable for RMP, INH, FQs or SLIs, but even here discordances between phenotypic and genotypic tests have been reported (see section 1.6.1). Also, due to technical limitations, for EMB for example, pDST produces inter- and intra-laboratory discordant results, even when testing the same isolate, due to drug instability in the medium and the MIC distributions of susceptible and resistant bacilli being close to the drug's ECOFF (91).
For new drugs like BDQ, DSTs have not been fully standardized yet, and the correlation between mutations and phenotypic resistance, as well as their significance in causing a worse clinical response are still unclear.

1.4.1.1 Drug-susceptibility testing on solid medium

The most common solid media used for DST are egg-based media and agar-based media, described in the culture section. The method used is the proportion method, described above. After inoculation, tubes are incubated at 37°C and interpreted after 4-6 weeks (92). The result is reported as S if growth on the drug containing medium is less than the 1/100 control, and R if equal or more. DST on solid media is overall accurate, when performed with precision, but with the disadvantage of a long turnaround time to results.

1.4.1.2 Drug susceptibility testing in MGIT liquid medium

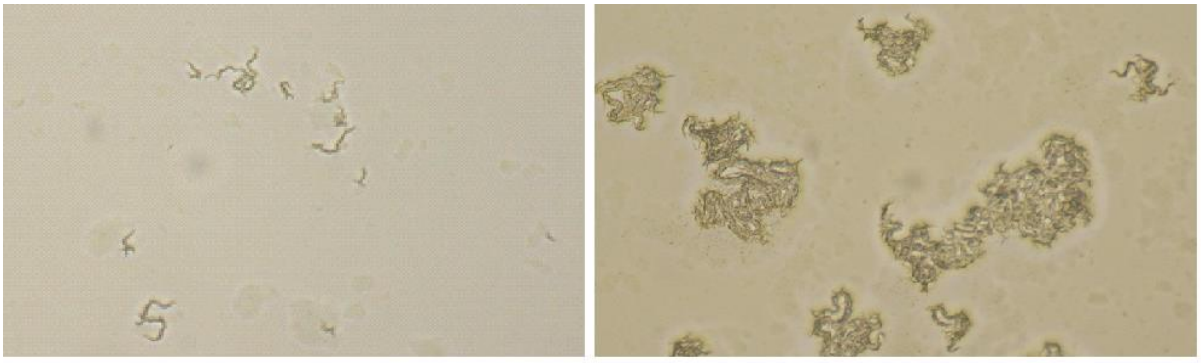
The MGIT^{960} system, which uses the same medium described above for culture, also relies on the proportion method, where the drug-free control tube is compared with a drug-containing tube inoculated with a bacterial suspension diluted 1:100. Results are interpreted by the instrument when the growth control reaches a growth unit (GU) value of >400 between 4-13 days (4-21 for PZA), and reported "resistant" if the GU in a drug-containing tube is >100, or "susceptible "if <100 (75).
The MGIT^{960} method is reported to be highly reliable, with high sensitivity for first line drugs (even if for RMP sensitivity is challenged by borderline mutations, see par. 1.6) and specificity varying from 89.8% to 100% for RMP, 100% sensitivity for ofloxacin and above 98.0% for kanamycin, and 100% specificity for both drugs (93).
Recognizing the higher sensitivity to detect mycobacterial growth compared to solid media, in 2007 WHO has encouraged the use of liquid medium over solid media for cultures and DST (77), conditional to appropriate training and the ability to bear the increased costs. As for solid media, implementation of DST on liquid media requires adequate biosafety measures in place, to contain bioaerosols produced during manipulation of a high concentration of mycobacteria (80).

1.4.1.3 Thin layer agar technique

The thin layer agar (TLA) assay is a non-commercial technique initially introduced as a culture method to detect TB (94)(95) that has been further investigated for rapid detection of resistance to anti-TB drugs directly from samples (96). Direct TLA is based on the inoculation of sputum sediment after decontamination on 7H11 in plates, and inspected for growth using a conventional light microscope (objective x10). Typically, TLA plates include a quadrant enriched with PNB, a PNB/drug-free control quadrant and quadrants with the medium containing the anti-TB drugs to be tested. This format allows simultaneous early detection of mycobacterial growth based on the observation of colony morphology, differentiation between NTM and MTB thanks to the PNB-quadrant, expected to show no or significantly less growth when compared to the growth control (95)(97), plus susceptibility of the drugs tested.
The typical mycobacterial colony morphology observed by microscopy varies according to the stage of growth of the TB bacilli. While initially colonies appear as single rods, fully mature colonies appear as a cohesion of curving strands, with a dark rough centre by microscopical investigation (98). Different colony stages are shown in Fig 1.8a and 1.8b.

Figure 1.8 Mycobacterial growth on a thin layer agar plate by microscopy reading

A Reading at day 5 **B Reading at day 10**



Reading by microscopy allows TLA to provide results with a shorter turnaround time to positivity (10 days versus 20) compared to conventional, indirect solid medium testing (95), with similar or higher sensitivity (96).
After sample decontamination and inoculation, TLA plates can remain closed until final interpretation, limiting biosafety requirements to L2 facilities for sample processing, compared to L3 facilities to manipulate isolates by standard, indirect pDST techniques. Due to the relatively lower biohazard risk and lower costs compared to commercialized liquid culture systems, this technique can be implemented in peripheral culture facilities, designed to contain moderate risk procedures (80).
In 2010, WHO evaluated the use of non-commercial methods such as colorimetric redox indicator (CRI), microscopically observed drug susceptibility (MODS), nitrate reductase assay (NRA) and TLA direct DST method. While WHO recognized CRI, MODS and NRA as rapid and inexpensive methods, due to drawbacks such as limited accessibility to specific consumables and equipment, high biosafety requirements, and lack of standardization that can more easily lead to errors, WHO

recommended to implement these techniques as ad-interim methods under specific operational conditions only at the reference laboratory level. TLA was excluded in this recommendation, due to insufficient evidence. To contribute to the evidence of TLA performance in providing accurate and timely results, we have conducted different studies, which focused on the improvement of the technique to detecting MTB and its resistance to different anti-TB drugs (**Chapters III, IV and V**).

TLA in fact shows several advantages. This technique, as explained above, can provide results in shorter time compared to standard pDST, and being a direct test, as mentioned in paragraph 1.3.3, can detect subpopulations of mycobacteria otherwise lost during subculture procedures.

1.4.2 Genotypic methods for TB drug-resistant detection

As mentioned above, this group of test includes a large number of techniques of different levels of complexity. Low complexity tests are Xpert MTB/RIF, Xpert Ultra, Xpert MTB/XDR, and Truenat MTB-RIF Dx assays. The moderate complexity NAATs include GenoType® MTBDR*plus* and GenoType®MTBDR*sl* assays (Hain LifeSciences GmbH, Nehren, Germany) , and a group of tests recently endorsed by WHO. These tests (Table 1.4) include Abbott RealTime MTB and Abbott RealTime MTB RIF/INH (Abbott), FluoroType MTBDR assay (Bruker/Hain Lifescience, Germany), BD MAX MDR-TB (Becton Dickinson) and Cobas MTB plus Cobas MTB-RIF/INH (Roche). High complexity NAAT include GenoScholar PZA-TB II.

1.4.2.1 Xpert MTB/RIF and Xpert MTB/RIF Ultra

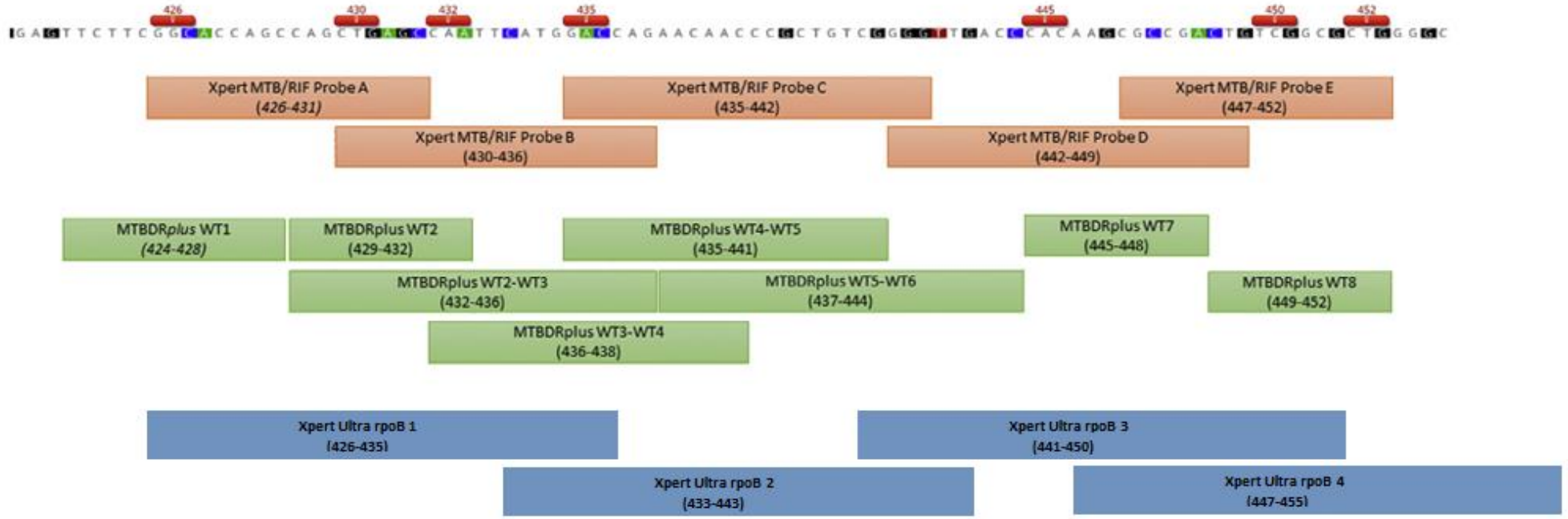
Xpert MTB/RIF, Xpert Ultra and other rapid molecular tests target the rifampicin-resistance determining region (RRDR), an 81 bp fragment of the *rpoB* gene in the MTB genome, for detection of RR. This area of the genome is not unique for MTB and also found in other bacteria, like *Escherichia coli*, whose sequence has for many years been used as surrogate for *rpoB* mutations nomenclature. This is gradually changing to address confusion derived by nucleotide sequence areas not completely overlapping (99). The positions of the most common mutations in *rpoB* are listed in both numerical systems in Figure 1.9.

Figure 1.9 Correlation between *Escherichia coli* and *Mycobacterium tuberculosis rpoB* numbering system (modified from Andre' 2018)

* The conversion of codon numbering between *E. coli* and *M.tuberculosis*

The probes developed to cover the whole 81bp *rpoB* fragment by Xpert MTB/RIF, Xpert Ultra and GenoType MTBDR*plus* are shown in Figure 1.10.

Figure 1.10 Xpert MTB/RIF and Hain MTBDR*plus* probe coverage of the *rpoB* gene



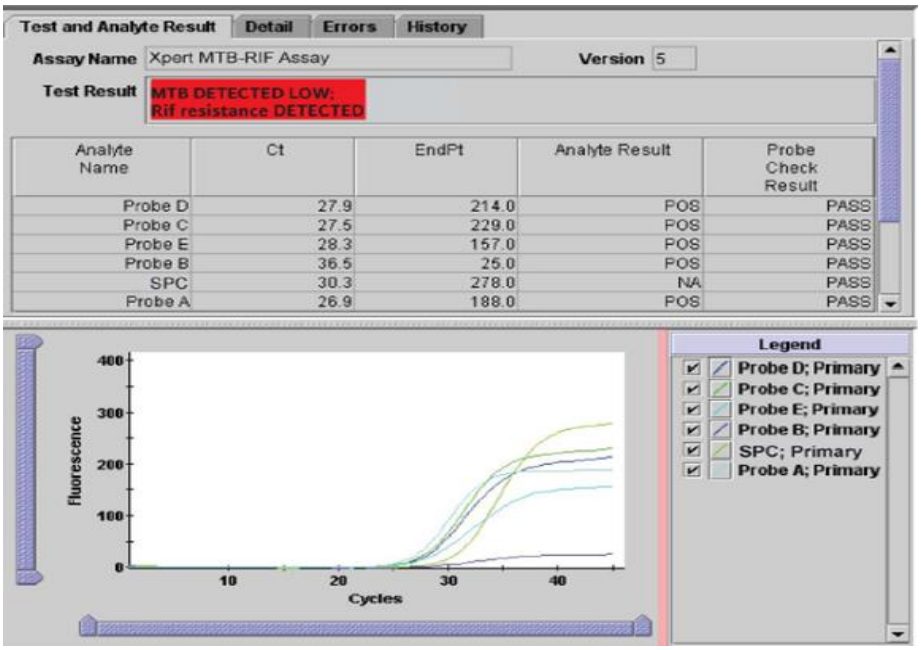
Modified from André, 2018 (99)

The Xpert MTB/RIF qPCR utilizes 5 molecular beacons that bind to different target segments to cover together the entire 81-bp *rpoB* core region (100) (Figure 1.10). The test provides an estimate of the amount of mycobacteria present in the sample by correlating the number of cycle threshold (Ct) values with a quantitative result: the sample is considered MTB positive with grade "High" if the first positive probe shows a Ct ≤ 16; "Medium" with Ct values between 16-22, "Low," with a range of 22-28; and "Very Low," with a Ct range of 28-38 (101). To ensure high specificity, the test is considered positive for MTB when at least 2 probes give a positive signal below Ct 38 that do not differ by more than two cycles.

The performance of the Xpert MTB/RIF test for MTB detection is slightly lower to that of culture, with a limit of detection of 131 CFU/ml versus 10-100 CFU/ml for liquid cultures (102). Results have shown that for pulmonary TB detection, the assay has a sensitivity of 98.2% for smear- and culture-positive samples, and a sensitivity of 72.5% for smear-negative patients, which can increase up to 90% if the test is repeated three times (103)(104).

The principle for RR-TB detection is based on the Ct values difference (ΔC_T) between the higher and lower values of the different probes, with a threshold set at a minimum of 4 cycles difference to declare a probe as absent or having delayed binding (Figure 1.11) (105). For samples with high Ct values, the RR status can not always be determined. If for instance the first probe has a Ct above 34.5 and the last above 38, the result is reported as indeterminate.

Figure 1.11 Example of XpertMTB/RIF report for a rifampicin-resistant tuberculosis result



From Cepheid training material

Since its launching, Xpert MTB/RIF has gone through different modifications for improvement. Initial evaluations reported 92.7% sensitivity and 99.0% specificity for RR detection (106). However, despite this high performance, the initial test version (G3) presented several limitations, including a reported high frequency of errors, mainly error "5011", indicating signal

loss in the amplification curve of the analyte, possibly due to loss of tube pressure (107). This error was virtually eliminated with the introduction of version G4.

In addition, false-susceptible cases were reported due to missed mutations in *rpoB* codon 452 correlated with probe E (108)(109), and false-resistant results correlated with imperfect binding of probe B (110) or silent mutations at codon 429 (111).

Despite improvements in the G4 cartridge, the problem related to missed L452P mutations by probe E (112) has not been fully resolved, as well as false-RR results due to delayed binding of probe D and E in paucibacillary samples (113).

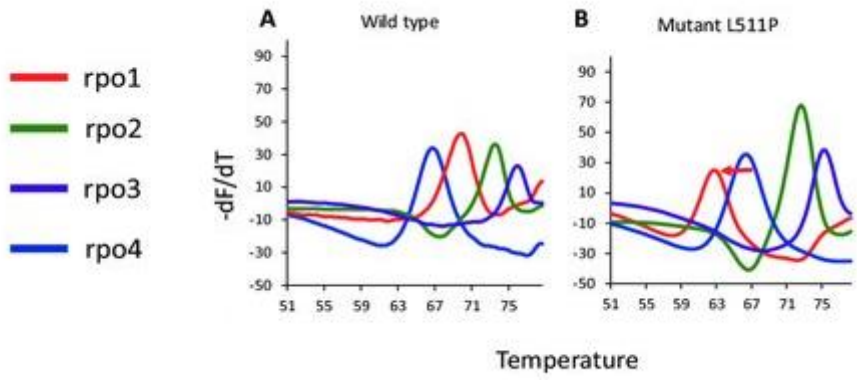
In 2017, a new version of the test, Xpert Ultra, has been launched. The test has a higher sensitivity to detect MTB than classical Xpert MTB/RIF, with a limit of detection as low as 16 colony forming units (CFU)/ml.

The increased MTB-detection sensitivity of Xpert Ultra has been achieved by the introduction of primers for two MTB specific multi-copy insertion sequences IS*6110* and IS*1081*, and the use of a larger PCR chamber (50 µl instead of 25 µl).

Reported results for detection of MTB use the same semiquantitative categories as Xpert MTB/RIF, with the addition of "trace" in case of paucibacillary samples, positive for IS*6110* and IS*1081* but negative for *rpoB* probes (15). These results contribute to a higher TB detection sensitivity but potentially at the cost of specificity, mainly due to detection of small amounts of residual DNA from no longer viable mycobacteria in patients treated in the previous five years. For this reason, "trace "results with Xpert MTB/RIF Ultra should be considered with caution for diagnosis in adults who were previously treated for TB (65).

The Xpert Ultra test uses 4 probes to cover the RRDR, and detection of RR is based on the assumption that melting temperatures (*Tm*) decrease in the presence of mutations in *rpoB* compared to the *Tm* for wild type sequences. One example is shown in Figure 1.12.

Figure 1.12 Example of melting temperature shift of an *rpoB* L511P (L430P) mutation



-dF/dT =change in fluorescence/increase in temperature

From Chakravorty 2017, (106)

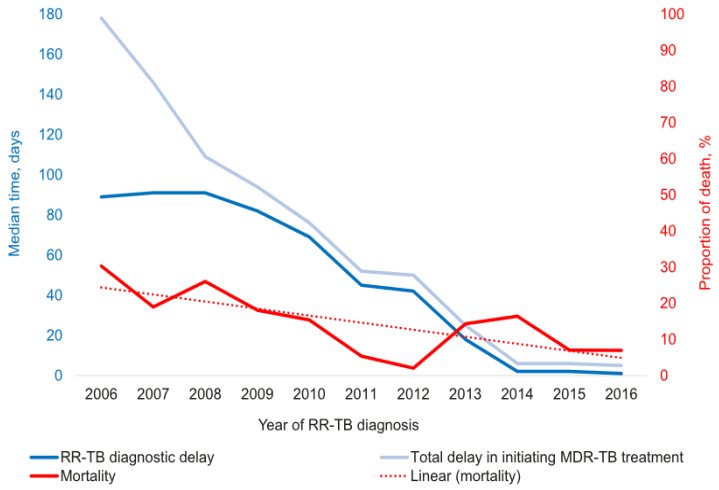
When MTB positivity is reported as “trace”, results for RMP are not interpretable, while for any other grade of positivity reported RMP “indeterminate” results may be due to the presence of multiple mutations and can be interpreted manually by analysing the melting curves (65). However, this interpretation is not standardized and requires high skills and expertise.

Sensitivity for RR detection is comparable to Xpert MTB/RIF, (106), with the advantage of an increased capacity to detect mutations in codon L452P, better differentiation of silent mutations, and increased specificity, avoiding the problem of false-resistant results in paucibacillary samples tested with Xpert MB/RIF (106).

Xpert systems, in contrast to culture and other molecular techniques, can be used in peripheral laboratories, given stabilized electricity is in place (114)(115). The procedure is simple and does not require highly qualified staff. Moreover, it does not create dangerous aerosols, as the reagent added to the sample kills 97% of the mycobacteria after 15 minutes of exposure, allowing Xpert assays to be implemented in a low level biohazard laboratory (116).

In 2011, WHO recommended to use Xpert MTB/RIF as the initial diagnostic test in patients presumed to have RR/MDR-TB or HIV- associated TB, and to consider it as a follow-on test to microscopy in settings where MDR-TB or HIV is of lesser concern. Since its endorsement, Xpert MTB/RIF has demonstrated to drastically improve TB detection (117) and shorten time to start of treatment (118)(119). In Figure 1.13 the impact of the Xpert MTB/RIF assay on delay to RR/MDR-TB treatment, and associated mortality in Rwanda is shown.

Figure 1.13 Trends of diagnostic/treatment delays vs mortality



From Ngabonziza (120); RR-TB= Rifampicin-resistant tuberculosis

For this reason, in 2013, the recommended use of Xpert MTB/RIF has been extended to all presumptive TB patients as initial diagnostic test, conditionally to resource availability (121). However, despite high performance of both Xpert MTB/RIF and Xpert MTB/RIF Ultra, culture remains the reference method for detection of active TB and drug resistance (105)(122)(123).

Xpert MTB/RIF has been implemented in many countries, but logistic constraints such as temperature control and power supply requirements still do not allow its use as a true point-of care test (114). MSF in collaboration with National TB Programs has supported the installation of Xpert MTB/RIF in different settings since the time of its endorsement. This experience has been capitalized through a large data collection (described in **Chapter II**) with the aim of describing not only the assay's performance but also to document practical challenges encountered during the test implementation in the field.

1.4.2.2 Xpert MTB/XDR Assay

This is a low complexity test for detection of resistance to INH, FQs, Eth and injectables. The test provides results in 90 minutes, with a sensitivity for INH and FQ resistance detection of 94% and 93% and a specificity of 98% (56). The Xpert MTB/XDR assay requires a 10-colour instrument, different than the initial versions typically used for Xpert MTB/RIF and Ultra, which represents an additional cost for laboratories that already use the 6-colour GeneXpert instrument for MTB and RR detection. The 10-colour device can also be used for Xpert Ultra (124).

1.4.3 MTBDR*plus* and GenoType®MTBDR*sl* assays

MTBDR*plus* and GenoType®MTBDR*sl* assays are currently the most widely used LPAs in TB laboratories. These tests detect MTB using a probe complementary to the 23SrRNA gene (125), as well as resistance to RMP, INH, FQs and SLIs.

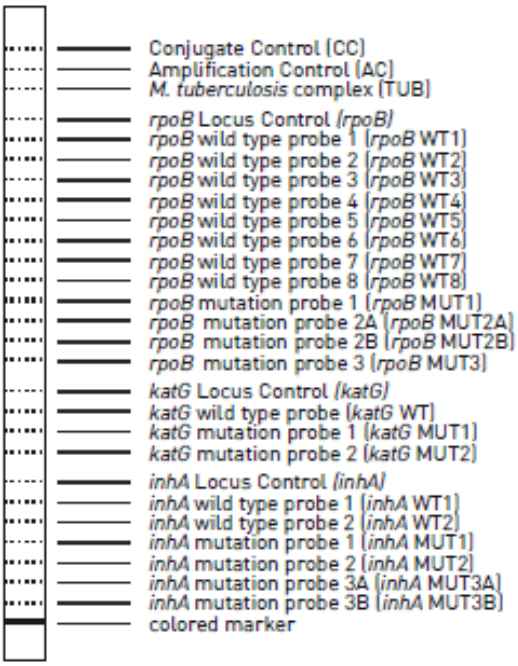
The GenoType MTBDR*plus* (V2.0) detects resistance to RMP using probes that target the RRDR region and to INH by detecting mutations in the *katG* gene *(*codon 315) plus *inhA* promoter (Figure 1a). The test has shown good performance as direct test among smear-positive patients, with sensitivity and specificity of 98.2% and 97.8% for RMP and 91.0% and 99.7%, for INH resistance compared to pDST (126).

GenoType®MTBDR*sl* (V2.0) detects resistance to FQs targeting the FQ-resistance determining region (QRDR) of *gyrA* (85-96 codons) and *gyrB* (536-541 codons). The *rrs* (nucleic acid position 1401, 1402 and 1484) plus the *eis* promoter region are included for detection of resistance to SLIs (Figure 1.14a and 1.14b, Figure 1.15) (127).

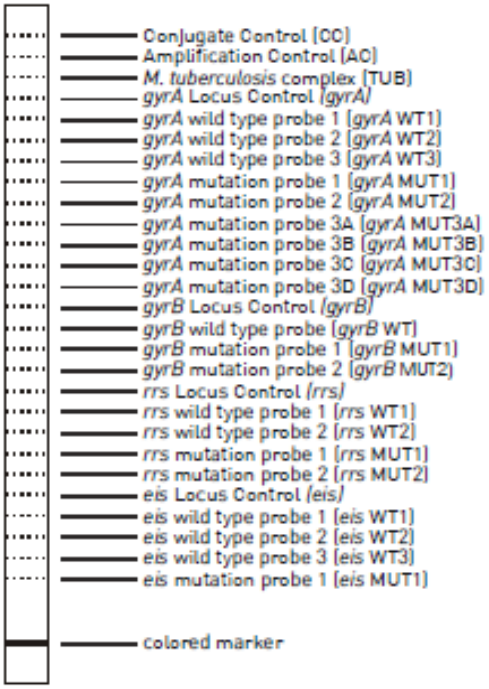
When used as direct test on smear-positive samples, sensitivity is 93% for FQs and 88.9% for injectables, while specificity is 98.3% and 91.7% respectively (128).

Figure 1.14 Layout of the GenoType MTBDR*plus* V2 and GenoType MTBDR*sl* V2 line probe assays strips for the detection of resistance to rifampicin and isoniazid, and fluoroquinolones and second-line injectables respectively

a MTBDR*plus*

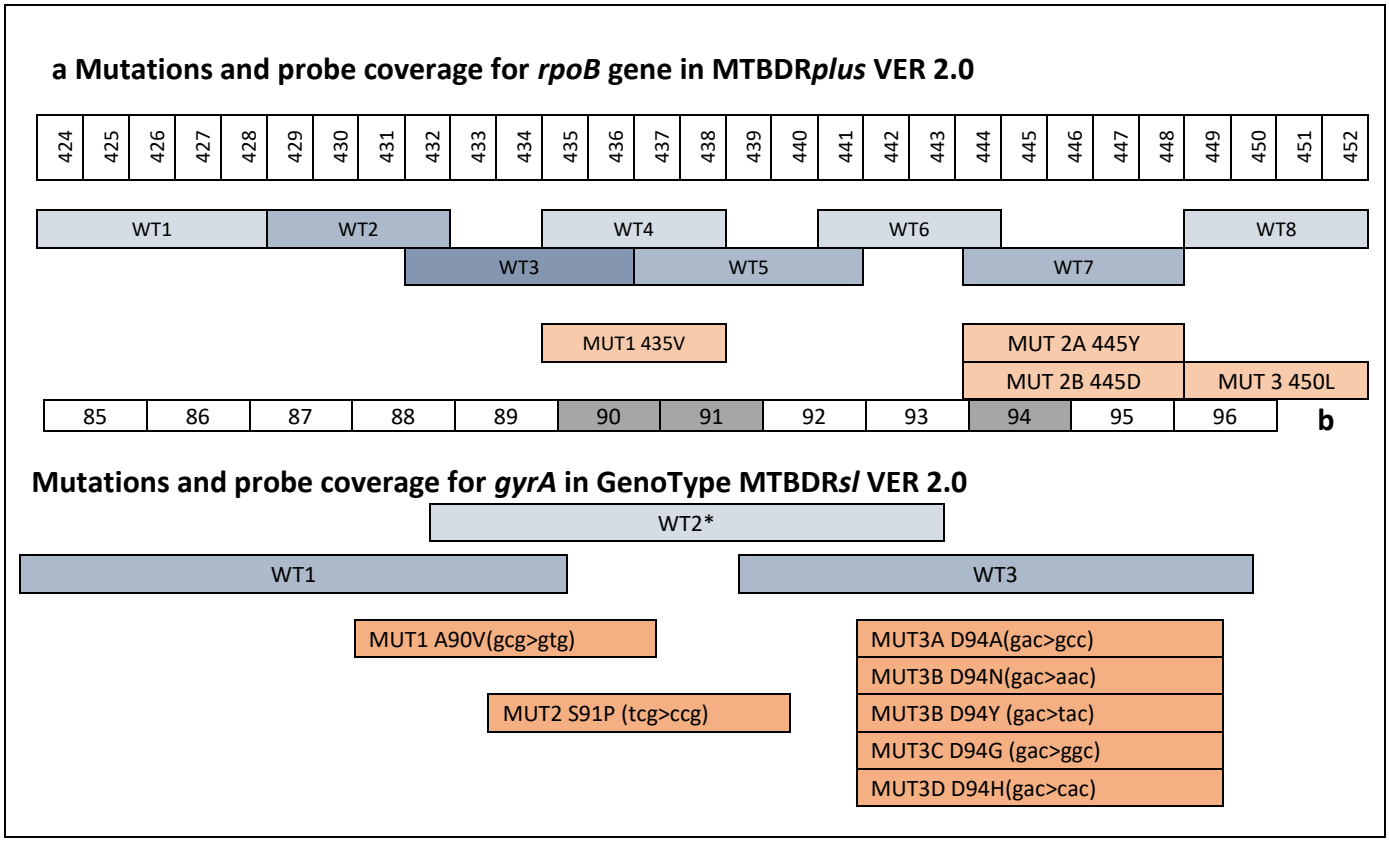


b MTBDR*sl*



The principle of both LPAs relies on the binding of the sample DNA either to the wild type (WT) probes or to probes with known mutations (MUT), visualized on the test strip as coloured bands after hybridization. Mutations conferring resistance are always characterized by one or more WT bands missing in the strip, while the correspondent MUT bands may be present only for common mutations. Figure 1.15 shows the gene coverage and mutations detected by both MTBDR*plus* Ver 2.0- and *gyrA* by MTBDRsl Ver 2.0 tests.

Figure 1.15 Mutations and probe coverage with MTBDR*plus* and MTBDR*sl*



Modified from "Line probe assays for drug-resistant tuberculosis detection "WHO 2018 (127)

The MDRTB*plus* and MDRTB*sl* have gone through modifications and improvements from their initial versions. Due to limited data and low sensitivity for INH detection, GenoType®MTBDR*plus* V 1.0 was initially endorsed by WHO in 2008 for smear-positive samples only. In addition, the test was found to miss the *rpoB* L452P mutation.

GenoType®MTBDR*sl,* in its first version included *gyrA* and *rrs*, and *embB* gene for EMB-resistance detection, and was endorsed only to rule in XDR patients, due to low detection of FQ and limited cross resistance between injectables (129).

Since then both tests have been revised. For GenoType®MTBDR*plus* the interpretation of the test was revised to include the mutation L452P as conferring resistance, while for the GenoType®MTBDR*sl* assay mutations in the *gyrB* and *eis* genes (correlated with FQ and low-level kanamycin resistance respectively), have been added, and *embB* for EMB has been removed. However, the relatively weak performance of the tests applied directly on smear-negative samples remains unchanged.

Acknowledging that these tests can provide rapid drug resistance detection, following a FIND evaluation in 2015, WHO now recommends them as initial tests to detect MDR, pre-XDR or XDR-TB for both smear-positive and -negative samples when resources are available, while recognizing that on smear-negative samples the rate of invalid results can be high (126)(130)(39)(131).

1.4.3.1 Moderate complexity nucleic acid amplification tests

This category includes tests recently endorsed by WHO that can only be performed by laboratories with molecular platforms and with strict quality assurance procedures in place. The tests characteristics, either as reported by the manufacturer or in literature, are summarized in the Table 1.4.

Table 1.4 Characteristics of moderate complexity nucleic acid amplification technologies (NAATs)

Test	MTB targets	Sensitivity; Specificity	N samples/ run	TAT (h)	LOD (CFU/mL)
Abbott RealTime MTB and MTB RIF/INH(132)					
	IS6110, *pab* gene 8 probes *rpoB*, 2 for *KatG*, 2 for *inhA*	RMP: 96.3%; 100% INH: 84.2%; 100%	MTB: 96 RMP/INH:24	10.5	MTB: 17 RMP/INH: 60
BD MAX MDR-TB					
	IS6110, IS1081 *rpoB* RRDR (codons 507-533), *inhA* promoter region, KatG 315 codon	RMP: 94.1%; 98.5% INH: 81.5%; 100%	24	4	MTB: 0.5 RMP/INH: 6
Roche Cobas MTB and MTB-RIF/INH					
	16S rRNA, 6 *esx* genes RRDR for *rpoB*, *inhA* promoter *KatG*	RMP: 97.3%; 98.6%; INH: 96.9%; 99.4 %	96	3.5	MTB: 8.8 RMP: 182 INH: 27.5
FluoroType MTBDR (133)					
	*IS6110* DNA *rpoB*, *inhA* and *KatG*	RMP: 100%; 97% INH: 100%; 98%	12	3	RMP: 9 INH: 14

TAT= turnaround time; LOD= limit of detection

In most cases, these methods require little manipulation, however, in contrast to the low complexity tests , the equipment requirements are considerable. In addition, to lower the cost, the throughput at each run should be high, making these tests more suitable at the central level, where samples from peripheral laboratories are combined.

1.4.3.2 GenoScholar PZA-TB II

GenoScholar PZA-TB II (Nipro, Japan) is classified between the high complexity tests, to be implemented in reference laboratories. The test is used for the detection of PZA resistance, and similar to the LPA uses a strip that binds with different probes to cover the *pncA* gene. However, in contrast to other LPAs, the strip does not include MUT probes and only relies on absence of

WT probes. Given the high number of probes (48) to cover the high variability of mutations seen over the entire *pncA* gene, the obtained profile is quite complex to read. Compared to sanger sequencing, the test has a sensitivity of 93.2% for the detection of PZA resistance and specificity of 91.2% (134).

1.4.3.3 Next generation sequencing

The molecular techniques described above are of great value to detect drug resistance. However, they are imperfect as they can miss mutations outside the targets covered, or do not include more recent drugs such as BDQ.

For many years Sanger sequencing has been used to identify mutations in selected genes. This technique can only provide results for one gene at the time and is cumbersome for a large number of tests, nevertheless it is very accurate and for this reason still used for resolution of specific technical issues (135).

However, none of these tests allows to distinguish relapse versus reinfection with another strain. NGS techniques include high-throughput sequencing methods that can identify any change in nucleotides, either SNPs, indels in one or more specific portions of the genome (target NGS), or the whole genome (WGS) in one single reaction.

In addition to identification of genomic sequence variants, NGS methods can also provide information on the relation between strains, allowing to distinguish between relapse with the same strains and reinfection with a new strain. In addition, WGS is very useful for the identification of clusters and outbreaks, whereby patient's isolates are defined as "close" applying a threshold of 5 or 12 SNPs difference (136)(137). Clustering is considered a proxy for recent transmission.

The most used platform for both NGS and WGS techniques is Illumina MiSeq™, also applicable to targeted sequencing with the commercial Deeplex®-MycTB assay (GenoScreen, Lille, France), which can run up to 96 samples in one run. A turnaround time of 3 days is reported, but this would require immediate DNA extraction and sufficient amount of samples to complete one run.

NGS techniques are mainly constituted by 4 steps. The first step consists of DNA extraction from clinical samples or isolates. When WGS is applied, DNA is checked for quality and quantity. Additional steps are library preparation, consisting of the preparation of DNA fragments to which oligonucleotide adaptors are linked to be able to identify distinct samples in the combined run, amplification of the fragments by PCR and library purification, followed by deep sequencing and bioinformatics analysis.

The high sensitivity of these methods to detect mutants is due to the multiple sequence reads at the same positions in the genome. The higher the number of reads per position (defined as depth of coverage), the more accurate is the identification of mutations encountered at this position. The quality of reads is assessed by the Phred program, which assigns the value of 20 for an accuracy of 99% (138).

WGS requires a large amount of good quality DNA and so far can only be successfully and consistently applied to MTB isolates. In contrast, Deeplex®-MycTB providing selective

amplification of the genes of interest instead of the entire genome, ensures reliable detection of variants as well as detection of heteroresistant populations, also in case of lower amounts of DNA, and thus also directly from smear-positive clinical samples (139).

Data analysis represents the main challenge for these techniques. Deeplex®-MycTB, compared to WGS, produces a relatively limited amount of data, and through an on-line platform the manufacturer provides a standard report that includes information on the quality of the procedure, such as depth of coverage and target coverage, species identification, strain lineage and mutations found, including an estimate of their correlation with drug resistance.

Analysis of results for WGS however is still quite complex and requires more skilled staff for the use of different on-line available "pipelines" such as TBprofiler (https://jodyphelan.gitbook.io/tb-profiler) and MTBSeq (140). These pipelines provide results for mycobacterial identification, lineage and drug resistance according to the frequency thresholds set to call for variants, generally 10% for TBprofiler and 75% for MTBSeq, below which limit heteroresistance will not be reported.

Deeplex®-MycTB can provide information on variants for 18 well-characterized genes known to be associated with resistance to streptomycin, RMP, INH, EMB, PZA, injectables, FQs, ethionamide, LZD, BDQ, and CFZ, on the contrary of WGS, that can potentially detect any mutation.

The mutations detected by genotypic testing are interpreted taking into consideration the level of confidence with which these mutations are correlated with the phenotypic drug-resistance profile, generally expressed as MICs ranges, for each specific drug. The level of confidence is considered high if the association is strong, well documented and supported by evidence (many data, high likelihood), moderate if evidence is incomplete, and minimal if evidence is weak (141). While for first-line drugs, FQs and SLIs these correlations are well established, for new drugs such as BDQ and DLM the correlation between mutations and MICs are still largely unknown, as well as their implications for patient's treatment response, i.e. the clinical breakpoint.

In general, given its applicability on smear-positive clinical samples, Deeplex®-MycTB is considered suitable for routine testing, even if the high cost limits its application, while WGS, in addition to the high cost, due to the complexity in interpreting the results and limited application directly on samples, is mainly used for research purposes. However several countries, such as UK and Netherland, have started using WGS as initial testing, followed by pDST to completely rule out resistance for specific drugs (142).

The high costs represents a challenge for the implementation of these technologies that can be considered cost effective only if applied in high throughput, so mainly suitable for reference laboratories (138), where in addition advanced molecular skills are available.

1.5 Association between phenotypic and genotypic tests

1.5.1 Discordance for rifampicin-resistance detection

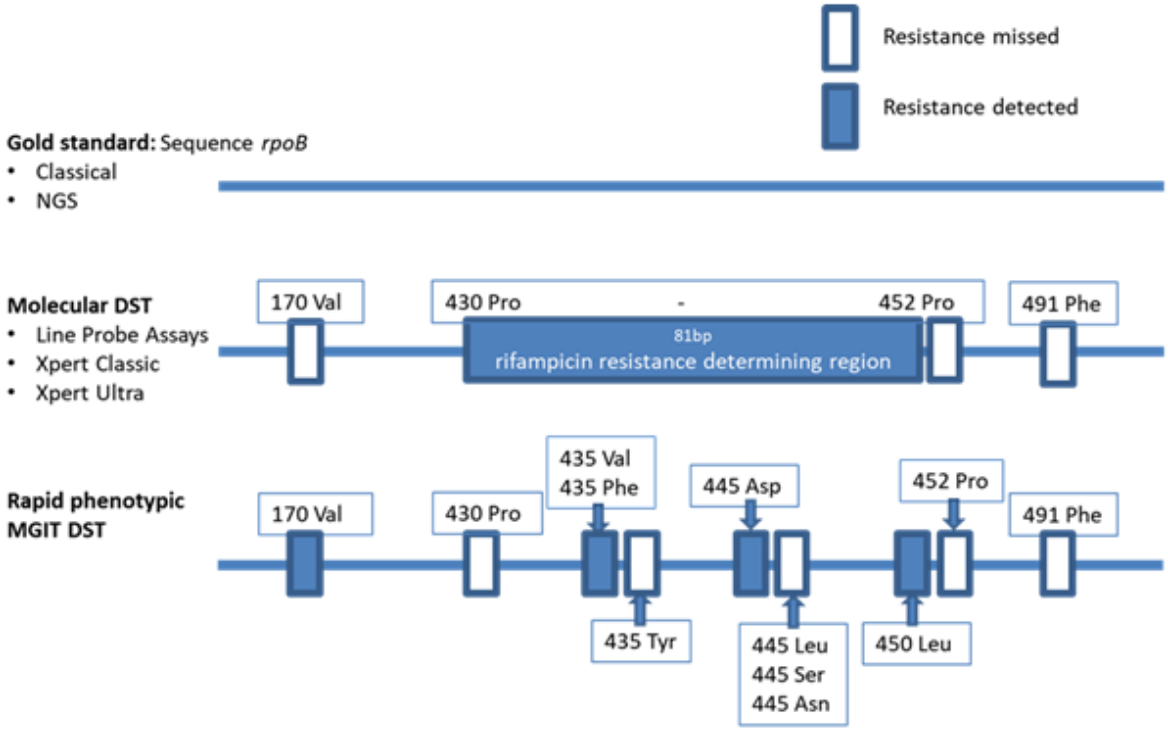
Discordance between phenotypic and genotypic DST is not infrequent, and challenges decisions on the design for best treatment. Discordance may happen for several reasons, including clerical errors (143) and variations between techniques. Discordance between molecular tests may be explained by probes covering different regions of the genome, while for phenotypic testing the different CCs used for different media may not give fully reproducible results, especially for strains with mutations that cause more fitness loss, which require longer incubation times to show resistance. Within the same method, strains may have an MICs very close to the critical concentration, such as for EMB, so that repeated results on pDST can switch between resistant and susceptible, and hence the association with gDST can become less clear (144).

The molecular mechanism of RR in MTB was initially reported in 1993 (145), after which subsequent DNA sequencing studies showed that more than 95% of RR-TB strains have mutations in an 81-base pair region (codons 426-452) of the *rpoB* gene, with more than 50 mutations within this region characterized by DNA sequencing. Of them, 435V/F, 445D/Y and 450L constitute the most frequent SNPs conferring high-level RR (144), all covered by the XpertMTB/RIF and LPA probes. However, the frequency estimates of these 'common' mutations were biased by pre-selection of isolates based on pDST. Unbiased estimates by sequencing directly on sputum revealed a higher frequency of less common disputed mutations, known as borderline *rpoB* mutations.

Xpert MTB/RIF and LPAs have been designed to reach high sensitivity and specificity against pDST (typically MGIT-based) as reference standard for RR detection. However recent studies have demonstrated that MGIT may be an imperfect gold standard for RR detection, due to RR-conferring mutations outside of the RRDR region, or mutations that confer low-level RR, or mutations that confer high-level resistance yet take a longer incubation time in rifampicin-containing medium to reveal themselves as resistant. Strains carrying such mutations do not grow at standard CC within the protocol of 13 days incubation in MGIT (146).

These challenging RRDR mutations are now referred to as "borderline" mutations, and are mainly represented, between others, by L430P and L452P, H445L, and D435Y (Figure 1.16) (144)(147).

Figure 1.16 Coverage of rifampicin-resistance detection for various *rpoB* mutants by different phenotypic and genotypic tests



Modified from de Jong presentation (2019)

Borderline mutations were initially considered as rare, but in fact are reported to be frequent, up to 10% or more, especially when tests are performed directly on sputum specimens (14). Their presence correlates with treatment failure rates (149)(150) like the 'common' mutations. These findings led WHO to reduce the CC for MGIT-DST from 1.0 to 0.5 µg/ml RMP (122).

At the time of XpertMTB/RIF development these discordances, regularly detected by the molecular test, were considered false RR against the MGIT gold standard. These cases were even more frequent in low MDR prevalence settings. To increase XpertMTB/RIF specificity, all RR results required confirmation by a second test (either repeat Xpert MTB/RIF or another phenotypic or genotypic test) (151).

Following the evidence that borderline mutations are in fact missed by MGIT, this recommendation has been reviewed, and the new approach is rather to consider as true RR any result, while follow up actions include sequencing, when possible, or pDST using solid media (56).

Discordance was also found between MGIT and Hain MTBDR*plus.* In the first version of the LPA, the borderline mutation L452P situated at the edge of the last probe WT8 (108) assay was not regularly detected and its detection was later removed from the test, to align results with the MGIT reference standard. It was then reintroduced in the MTBDR*plus* V.2 when the significance of the borderline mutations had become clear (148).

While these causes of presumed "false-RR" results have been resolved in favour of true RR, still false RR can occur. This is the case for RR results due to silent mutations and imperfect binding

of some probes such as probe B and E (152), or missed binding in paucibacillary samples defined by Xpert MTB/RIF as “very low” (113) (144). Such false RR may account for to up to 86% of paucibacillary samples (153), and may be frequent when using the assay for screening purposes in the general population.

In contrast, false-RS results by Xpert MTB/RIF are mainly due to mutations outside the RRDR region (Figure 11). One of the most reported is the I491F mutation, frequent in the Southern African region and missed completely also by MGIT (154)(155)(156), yet detected by agar- or LJ-based pDST with final reading at 6 weeks. These cases should be resolved by such ‘slow’ pDST or by sequencing. However, while pDST on LJ delays final results due to the long turnaround time, access to sequencing in remote settings remains a challenge. **We therefore investigated the performance of TLA for the detection of RR together with other anti-TB drugs, with the aim to propose TLA as simple method to detect drug resistance and specifically RR cases otherwise missed by routine techniques (Chapter IV, V)**.

Another reason for discordant results, as anticipated in section 1.3.3 is the different sensitivity of the diagnostic tests to detect heteroresistance. Several studies have shown that Xpert MTB/RIF has a threshold for the detection of minority populations with an *rpoB* mutation between 20-80% versus 20-70% for Xpert Ultra, depending on the specific mutation (31), where the LPA threshold is between 5-10% and pDST typically detects 1% by nature of its design (157).

While the intricacies of RR detection are thus quite complex, the Xpert system has revolutionized RR-TB diagnosis and retains high specificity, unless the bacterial load is very low in classic Xpert MTB/RIF. Clinicians facing patients with unexpected RMP DST results are recommended to repeat testing with the same test on a new sample from the same patient, to address potential administrative errors, and to err in favour of the resistant result, choosing RR/MDR-TB treatment whenever in doubt.

1.5.2 Detection of bedaquiline resistance

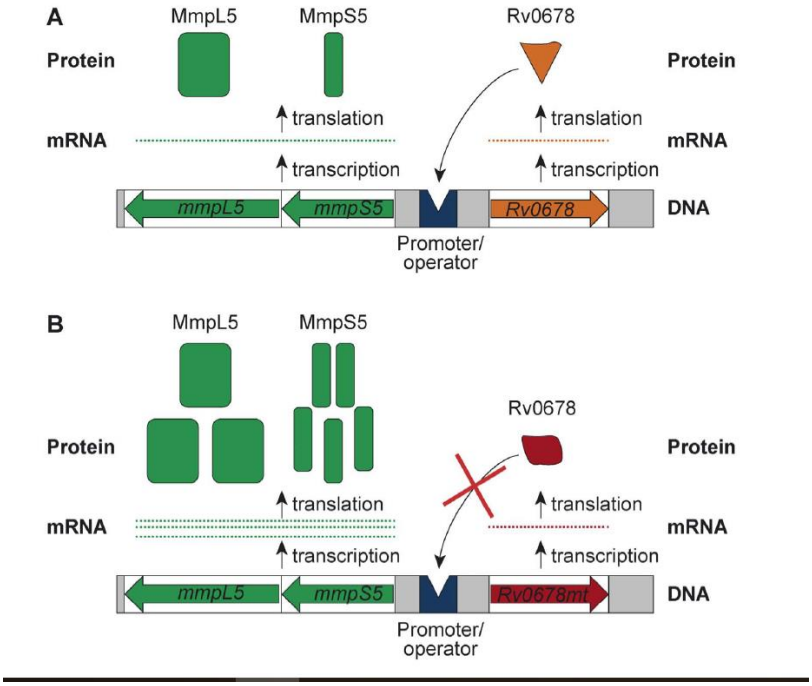
BDQ is part of a new class of drugs, the diarylquinolines, whose mechanism of action is the inhibition of ATP synthase (158). As BDQ has been only recently rolled out, it was assumed that isolates naïve to this drug did not require DST, and could be considered susceptible. However, several studies have shown more worrisome results. While baseline resistance to BDQ has been found also in isolates not exposed to this drug before (159), cross resistance between CFZ and BDQ exists, so that exposure to CFZ prior to BDQ containing regimens may contribute to baseline BDQ resistance (43).

Studies have identified different mechanisms of resistance, correlated with mutations in different genes. The *atpE* gene encodes for a transmembrane protein of the ATP synthase, which is the target of BDQ. These target-based mutations, so far rarely found in clinical isolates, have been mainly described among in vitro selected isolates and are generally correlated with high level resistance, up to 133-fold MIC increase (43).

Another gene implicated in BDQ resistance is *Rv0678,* a transcriptional repressor of genes encoding for the mmpS5-mmpL5 efflux pump, a mechanism of resistance shared with CFZ (Figure 1.17). These non-target based mutations generally confer lower increases in the BDQ-MIC level,

and some are associated with *lower* MICs. Even if these are the mutations most commonly found in clinical isolates, their correlation with poor clinical outcome remains unclear (160).

Figure 1.17 Mechanism of bedaquiline resistance in the presence of mutations in *Rv0678*



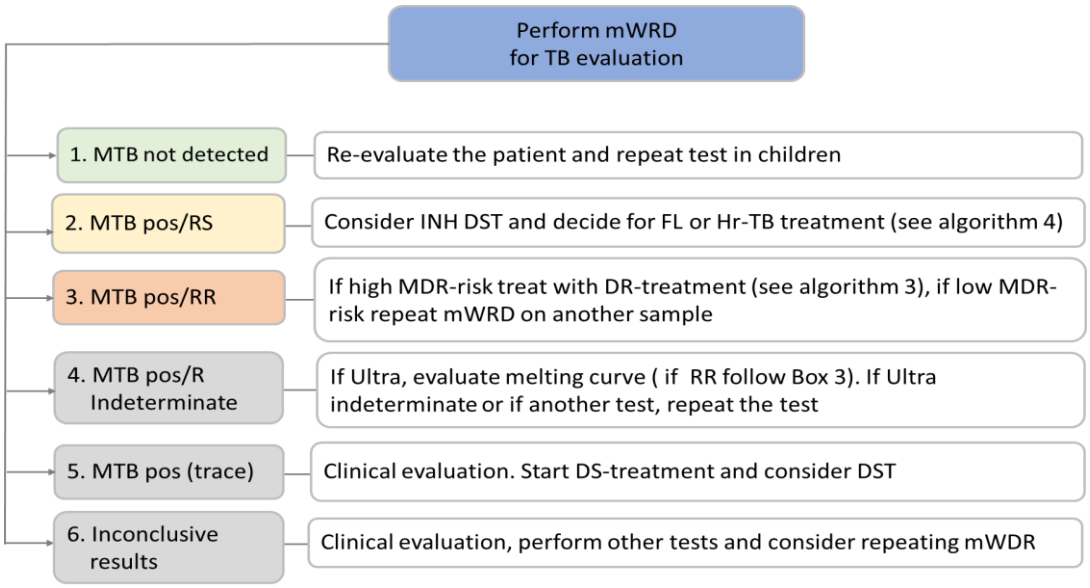
A. Rv0678 protein binds to the intergenic region between *Rv0678* and *mmpS5*, preventing transcription from the RNA polymerase and decreasing the expression of *MmpS5, MmpL5* and *Rv0678* proteins. B. Mutations in *Rv0678* will produce a modified repressor protein or with limited or no ability to bind to the DNA promotor, resulting in an increased expression of *MmpS5, MmpL5* and *Rv0678* proteins. From Andries, 2014 (159)

Mutations correlated with resistance to BDQ and CFZ have also been found in the *pepQ* gene, of which the function is unclear. The increase in MIC due to these mutations is reported to be low (160). How *Rv0678*mutations correlate with drug resistance in not fully clarified. The MIC increase conferred by these mutations is often very close to the CCs established for solid- and liquid media and isolates carrying these mutations may be classified as susceptible even if possibly clinically relevant (162). Moreover, gain and loss of mutations have been reported in sequential isolates during treatment. Diagnosis of BDQ resistance is challenged by all these factors, and by the fact that a wide range of mutations in *Rv0678* are found distributed across the whole gene (163), which complicates the development of a rapid molecular test. **With the aim to contribute to the clarification of the prevalence and impact of BDQ resistance on clinical outcome, we have conducted a study on isolates obtained from patients treated with BDQ (Chapter VII).** The study includes the description of mutations in *Rv0678* and their correlation with susceptibility testing, as at present detection of BDQ resistance mainly relies on lengthy pDST methods.

1.6 Diagnostic algorithms

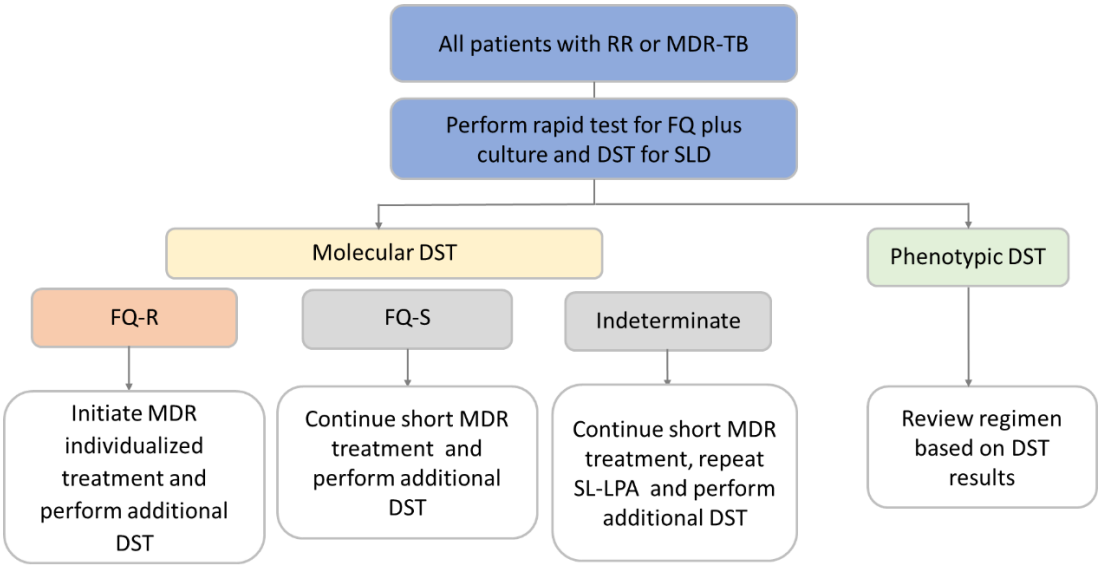
To date, Xpert MTB/RIF represents the simplest and fastest solution to diagnose (RR-)TB with a high sensitivity and specificity. Thanks to limited technical requirements and ease of use, the test can be implemented in peripheral and subdistrict level, and for this reason Xpert MTB/RIF is considered the first-line test for TB diagnosis. However, as described above, other molecular tests are endorsed by WHO, and referred to as WHO-recommended rapid diagnostics (mWDR).
Following TB detection, the testing algorithm recommended by WHO for patients with presumptive or confirmed RR-TB continues with at least DST to FQs, with either molecular or phenotypic methods. To support countries in optimizing the use of diagnostic tests available, in 2020, the WHO has provided 4 main algorithms for TB diagnosis (65)
Algorithm 1. All presumed pulmonary and extrapulmonary TB patients should be screened with mWDR and treated based on RMP results.

Figure 1.18 Algorithm 1: molecular rapid diagnostic methods as initial tests for *M. tuberculosis* detection (Modified from WHO 2020 (65)



Algorithm 2. This algorithm, complementary to algorithm 1, includes LF-LAM for patients with HIV, either with pulmonary or extrapulmonary TB symptoms, or without TB symptoms but low CD4 count. In this algorithm mWDRs are performed as add-on test after LF-LAM negative results or for patients with LF-LAM positive, to obtain results on drug resistance.
It needs to be considered that in patients presenting TB symptoms, neither LF-LAM or mWDR can exclude TB in case of a negative result, and other evaluations, including performance of cultures, should be conducted whenever possible.
Algorithms 3 and 4 focus on follow-up tests to be performed after algorithm 1 and 2, once patients are diagnosed with TB.
With algorithm 3, all patients identified with RR or MDR-TB from previous algorithms should be tested with molecular tests or pDST for second-line drug resistance

Figure 1.19 Algorithm 3: DST for second-line drugs included in all-oral treatment for RR-TB or MDR-TB patients



Modified from WHO 2021 (56)

Algorithm 4 is addressed to patients found to be RS-TB, for whom INH should be tested. If INH-S, patients are to be treated with first-line drugs, if INH-R or unknown, patients are treated according to the pattern of FQ resistance and the level of INH resistance.

While molecular tests in these algorithms represent the first-line test for TB diagnosis and rapid detection of drug resistance, and should be performed before initiating treatment, culture and pDST remain crucial. Their role is not only to monitor response to RR/MDR-TB treatment. Culture for example is also used to rule out TB for presumed TB patients with negative mWDR test results. pDST is used to resolve indeterminate drug-resistance results obtained by molecular testing for drugs other than RMP, where full *rpoB* sequence is the reference standard for RR. In settings where the prevalence of INH and FQ-resistance is high, pDST should be performed to identify resistance otherwise undetected, due to mutations missed by molecular methods (65). Indeed, pDST provides the reference standard for an extended range of anti-TB drugs for which the knowledge gap with gDST is too wide (164), including the new drugs like BDQ.

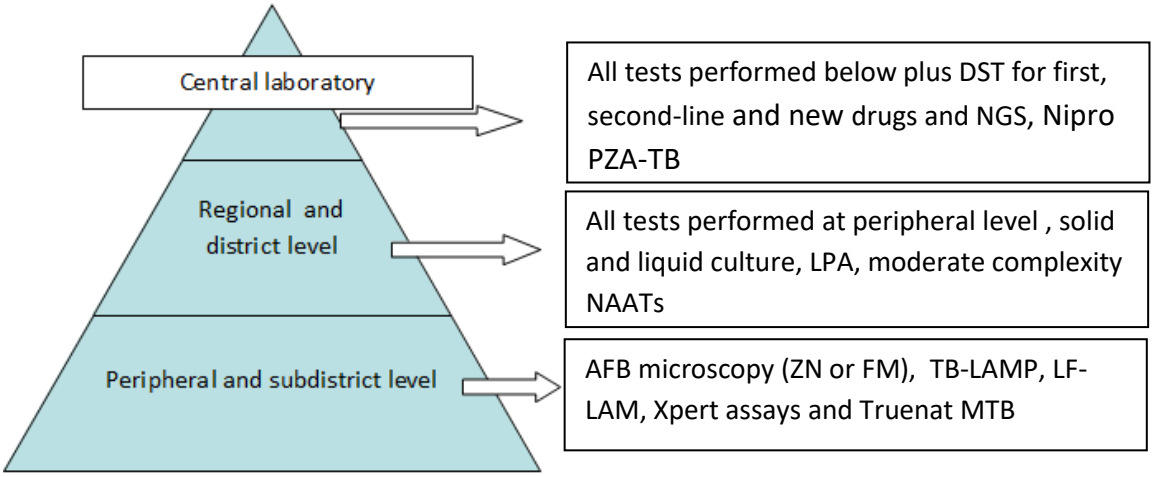
For these reasons, even if molecular techniques are preferred because they shorten the time to results and are more widely available, the use of phenotypic and genotypic methods should be seen as linked, to support the design of an adequate TB treatment.

The algorithms described above imply that mWDR for rapid detection of MTB and drug resistance are always available in peripheral facilities, close to patients, and that phenotypic methods are accessible. In remote settings, diagnosis may still rely on microscopy, and mWDR techniques are often implemented only at district levels, used as follow-on test rather than front-line tests (165). In addition, follow-up tests like culture and pDST, are mostly restricted to higher levels of the laboratory network (see section 1.8), challenging the access to this testing cascade.

1.7 The TB diagnostic laboratory network

In resource-limited settings, the laboratory network follows a pyramidal system, where tests are implemented based on test performance, time to result, ease to perform, logistic constraints, quality control requirements, cost and accessibility. WHO provides a road map for test implementation, recommending their introduction at specific levels of the TB laboratory network (Figure 1.20) (93).

Figure 1.20 Distribution of the diagnostic tests in the tuberculosis laboratory network



Modified from WHO 2020 (93); DST=drug-susceptibility testing; NGS=next generation sequencing; LPA=line probe assay, AFB=acid-fast bacilli, ZN=Ziehl-Neelsen, FM=fluorescent microscopy, NAATs=nucleic acid amplification technologies

1.7.1 Sample transport

As described in Figure 1.20, implementation of low complexity tests with limited logistic requirements is done at peripheral levels, while pDST mainly to new drugs remains confined to higher level. This distribution implies that the efficiency of this pyramidal structure depends on a good sample referral system, which can be challenging in resource-limited settings.

According to the type of samples and the tests to be performed, shipment requirements can vary largely.

According to manufacturer instructions, when transported from remote areas to centres for Xpert assays testing, samples should be kept for a maximum of 10 days between 2-8°C, and at ambient temperature (maximum of 35°C) for no longer than 3 days (107). However, these timelines are often violated, while there is scant literature on the effective impact of delays on molecular test performance.

Requirements for culture are stricter. Delay in sample processing is crucial for culture performance as high ambient temperatures increases contamination by faster growing bacteria, challenging the possibility to isolate mycobacteria. Samples shipped for cultures should be preferably kept at cool temperatures and processed as soon as possible, not later than 3 days

from collection. Respecting such timelines is very challenging, resulting in culture and pDST methods highly affected by transport delays and consequently high culture contamination rates. Transport in cold chain is expensive and logistically complex in resource-limited settings, and use of sample preservatives is an attractive alternative. The most used are ethanol and CPC, however the first cannot be applied on samples to be cultured, while the second is not compatible with liquid culture, if not removed by a washing step. Other transport reagents have been evaluated by WHO, including OMNIgene®SPUTUM (OMNIgene, DNA Genotek, Ottawa, Canada). The requirements for shipment and applicable tests according to the transport medium used and type of sample are shown in Table 1.5.

Table 1.5 Summary of tests performed by sample type and shipment characteristics

Shipping laboratory level	Sample type	Facility	Extra sample from patient	Minimal requirement	Sample treatment before shipping	MTBDRplus	MTBDRsl	Sanger sequencing	Deeplex/Spoligo	MIRU-VNTR	WGS	(sub-)culture	Phenotypic DST	IATA Category	Shipping restrictions	Time (days) lapse (collection - testing)
Peripheral + Central	Sputum	BSL1	√	0.5 ml	-	√	√	√	Sm≥1+	from culture	from culture	√	from culture	B		<3
	Sputum	BSL1	√	0.5 ml	CPC	√	√	√	Sm≥1+	from culture	from culture	√	from culture	B		<30
	Sputum	BSL1	√	0.5 ml	Heat	√	√	√	Sm≥1+	-	-	-	-	-		<30
	Sputum	BSL1	√	0.5 ml	EthOH	√	√	√	Sm≥1+	-	-	-	-	-	Limited quantity	-
	Xpert amplicon	BSL1	-	15 µl	Needle extraction	√	√	√	Sm≥1+	from culture	from culture	√	from culture	-		<5
	Sputum/SR mix	BSL1	-	0.5 ml	-	-	√	?	?	-	-	-	-	-		<12
	Sediment*	BSL2	√	0.5 ml	-	√	√	√	Sm≥1+	from culture	from culture	√	from culture	B		<3
	Sediment*	BSL2	√	0.5 ml	EthOH	√	√	√	Sm≥1+	-	-	-	-	-	Limited quantity	<30
Central	Isolate	BSL3	√	0.5 ml	-	√	√	√	√	from culture	from subculture	√	from subculture	A		<3
	Isolate	BSL3	√	0.5 ml	CPC	√	√	√	√	from culture	from subculture	√	from subculture	A		<3
	Isolate	BSL3	√	0.5 ml	EthOH	√	√	√	√	-	if high bacillary concentration	-	-	-	Limited quantity	-
	LPA DNA extract	BSL1	√	0.5 ml	-	√	√	√	Sm≥1+	-	-	-	-	-	-	-

1.7.1.1 Ethanol

Ethanol has the capacity to denature proteins, disrupting microorganisms cell walls. Because ethanol kills mycobacteria, this reagent is only used to preserve samples for testing with molecular methods. Its main advantage is the low cost, general availability and the ability to kill the mycobacteria present in the sample (166), which subsequently can be safely transported and manipulated. However, ethanol is considered a dangerous good, and shipment by air follows IATA transportation requirements.

1.7.1.2 Cetyl-pyridinium chloride

CPC is an ammonium compound generally adopted as antiseptic against numerous organisms. Due to positive charges in its structure, it is able to bind negative charges commonly found on bacterial membrane, with the effect of destabilizing the structure and interfere with osmoregulation (167). The main application for TB diagnosis is for sample decontamination during transport. CPC has limited toxicity towards MTB, and several studies have shown a higher yield for MTB recovery than the standard NALC-NaOH decontamination method when both methods are applied to samples transported several days at RT, and comparable to NALC-NaOH when samples are processed shortly after collection (168). CPC is cheap and compatible with molecular testing such as Xpert assays, and with culture using egg-based media, but it is not applicable to MGIT without additional washing steps, as described in section 1.4.2.1. In addition, samples containing CPC should not be stored at cool temperatures to avoid CPC crystallization, which makes it ineffective.

1.7.1.3 OMNIgene® SPUTUM

OMNIgene is a commercial reagent proposed by the manufacturer for application on samples during transport where refrigeration is not available. According to the company, samples can be stored between 4-40°C and up to 30 days before Xpert MTB/RIF testing and up to 8 days prior to MGIT culture inoculation.
According to the results in the literature, the reagent did not show to significantly improve performance of Xpert MTB/RIF versus untreated samples, moreover samples added with OMNIgene require centrifugation before processing, a major drawback compared to the limited biohazard and infrastructure requirements of XpertMTB/RIF.
While studies demonstrated that for samples treated with OMNIgene the LJ positivity rate and contamination rate improved, (169), this effect was not observed in MGIT- in some studies use of OMNIgene was correlated with false-negative primary isolation in MGIT (170)(171).
The recommendations for sample storage prior to testing with Xpert MTB/RIF have been very little investigated in literature. **To contribute to knowledge on OMNIgene performance and optimal sample storage, we conducted a study on the performance of Xpert MTB/RIF with and without OMNIgene on samples stored beyond these days, as well as on samples stored in**

ethanol (Chapter VI). Results did not encourage the use of OMNIgene, in line with what was reported in literature.
The initial version of OMNIgene was not endorsed by WHO (172). The reagent was revised by the company and evaluated in further studies, including the one mentioned above and included in this thesis, which confirmed the previous results and limited applicability.

1.8 Research objectives and study setting

Affordable and easily used techniques with limited logistic requirements are necessary in resource-limited settings, to allow access to TB diagnosis to all patients. In addition, the possibility to transport samples to reference laboratories is crucial to access more complex tests that cannot be implemented in peripheral facilities. Recent years have seen a major improvement and investment in the development of diagnostic tests, which represent a major improvement in sensitivity compared to microscopy, for many years the only test available in remote settings.
The diagnostic algorithms proposed by WHO recommend the use of rapid molecular techniques such as Xpert MTB/RIF or Ultra as initial tests for MTB detection and RR/MDR identification. To detect resistance to second-line drugs, WHO recommends LPAs and very recently Xpert MTB/XDR (173), while pDST is maintained to confirm results from molecular techniques or to test (new) drugs for which rapid molecular techniques are still not available. In addition, patients showing drug resistance should be monitored with culture during treatment.
While implementation of Xpert assays implies specific logistic requirements not always applicable in remote settings, access to pDST is even more complex and requires transport of samples collected from peripheral diagnostic centres to reference laboratories, which can lead to high contamination rates and considerable delay in obtaining results.
In addition, while Xpert assays have demonstrated a very high sensitivity in detecting MTB, in specific settings such as Southern Africa, specific *rpoB* mutations not covered by the standard molecular tests challenge the diagnosis of RR-TB.
In the last decade, new and repurposed drugs have been integrated in new TB regimens, improving patient outcomes and shortening the time to treatment. However, the alarming reports on resistance to BDQ for patients never exposed to the drug, and possible previous exposure to CFZ as a risk factor for amplification of BDQ resistance, highlight the need for rapid and accessible DST methods, which at present are not available.
The scope of this thesis is to contribute to the improvement of the diagnosis of drug-resistant TB in remote settings. Specifically, in the following chapters, I will:

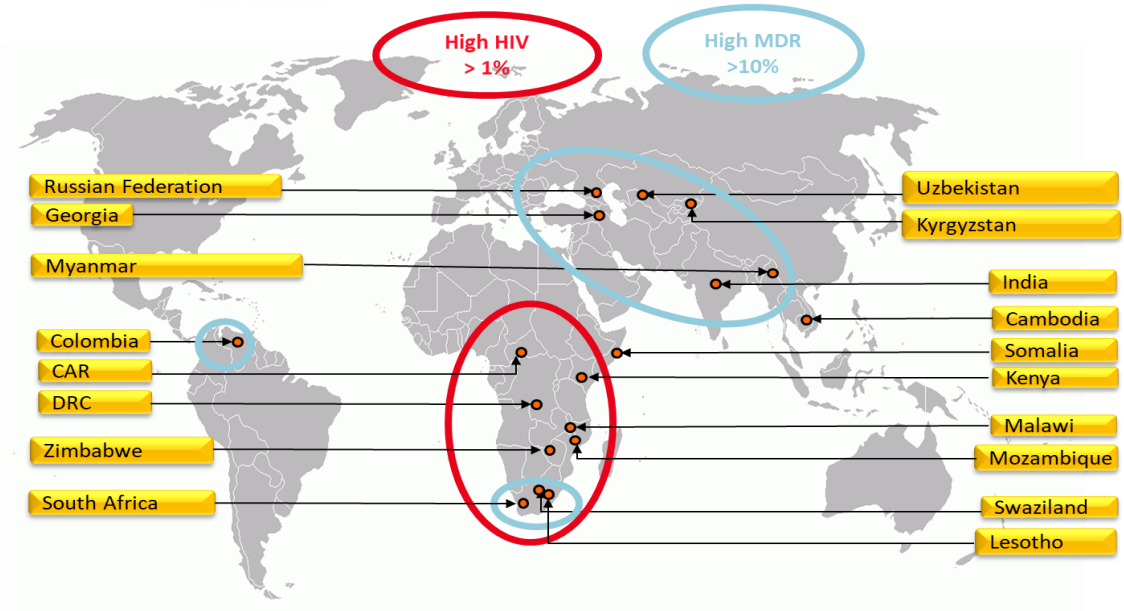
- Describe the practical experience of Xpert MTB/RIF implementation in the field, including the contribution of this technique to TB diagnosis, but also the challenges encountered, and the overall preparatory activity required to implement this technique.
- Describe the optimization of TLA technique, with the aim to propose this simple method for simultaneous detection of MTB and resistance to anti-TB drugs for peripheral settings, focusing on detection of RR cases otherwise missed by routine diagnostic methods in the South African region.

- Investigate, in sites where neither Xpert MTB/RIF or TLA can be implemented, the impact of sample storage on Xpert MTB/RIF results and the use of OMNIgene for sample transport from remote areas to laboratories where these tests are available.
- Describe the correlation of exposure to CFZ with mutations in genes implicated in BDQ resistance, and with BDQ-resistance amplification and treatment outcome.

The research took place at ITM and in multiple MSF project sites. Specifically:

Chapter II describes the results obtained from a collection of quantitative and qualitative data during Xpert MTB/RIF implementation in 33 sites from 18 countries (Figure 1.21). The included sites varied in MDR-TB – and in HIV prevalence, and in the facility level at which the test was implemented.

Figure 1.21 Distribution of Xpert MTB/RIF in 33 MSF projects



Since decades MSF collaborates with the National TB Programs in many countries on the setting up of TB diagnostic techniques, implementing innovative technologies, and supporting the development of non-commercial methods, like TLA. This technique has been implemented in two laboratories: Homabay, Kenya, where it has been used for years for TB diagnosis (Figure 1.22), and in Nhlangano, Eswatini, where TLA was performed for research purposes.

Figure 1.22 Reading of the TLA plates in the Homabay laboratory

The optimization of TLA was covered by 3 studies. The first, presented in **Chapter III**, describes the optimization of the decontamination method prior to TLA testing, applied to samples that underwent long transport. The study was carried out at ITM with the residual samples shipped to ITM for routine testing from five MSF projects in Georgia, Kenya, Uganda, Cambodia and the Democratic Republic of Congo.

The second study, presented in **Chapter IV**, investigates the performance of TLA as direct pDST. The study was carried out at ITM with samples shipped from an MSF project in Georgia. The country, listed among the high burden TB countries, was chosen because of the high MDR prevalence among TB patients of 11% (174).

The last of the three TLA studies, presented in **Chapter V**, was carried out in the laboratory of Nhlangano, Eswatini, to assess the performance of TLA in field conditions. Eswatini is characterized by a high TB and HIV prevalence, and by a high rate of *rpoB*_I491F mutations, which account for 56% of the total RR/MDR cases reported (175), representing the ideal setting to evaluate the ability of TLA to detect mutations otherwise missed by WHO endorsed techniques, such as Xpert MTB/RIF or Xpert Ultra or MGIT.

In **Chapter VI** we investigated the performance of OMNIgene to preserve samples for testing. The study was conducted in Kampala, Uganda, in a laboratory supported by Epicentre, using samples collected from patients with presumptive TB accessing the laboratory for TB diagnosis.

In the last study, unpublished, and whose results are presented in **Chapter VII**, we aim to contribute to the clarification on the correlation between decreased phenotypic sensitivity to BDQ, observed mutations in potentially associated genes, and treatment outcomes. In addition, we investigate exposure to CFZ as risk factor for amplification of BDQ resistance. This study was conducted at ITM on MTB isolates shipped from Armenia, a country with a high MDR-TB prevalence, where patients at the time of the sample collection (between 2013 and 2015) received BDQ in compassionate use.

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CHAPTER II

Implementing the Xpert® MTB/RIF diagnostic test for tuberculosis and rifampicin resistance: outcomes and lessons learned in 18 countries

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Abstract

Background: The Xpert®MTB/RIF (Xpert) is an automated molecular test for simultaneous detection of tuberculosis (TB) and rifampicin resistance, recommended by the World Health Organization as the preferred diagnostic method for individuals presumed to have multi-drug resistant TB (MDR-TB) or HIV-associated TB. We describe the performance of Xpert and key lessons learned during two years of implementation under routine conditions in 33 projects located in 18 countries supported by Médecins Sans Frontières across varied geographic, epidemiological and clinical settings.

Methods: Xpert was used following three strategies: the first being as the initial test, with microscopy in parallel, for all presumptive TB cases; the second being only for patients at risk of MDR-TB, or with HIV- associated TB, or presumptive paediatric TB; and the third being as the initial test for these high-risk patients plus as an add-on test to microscopy in others. Routine laboratory data were collected, using laboratory registers. Qualitative data such as logistic aspects, human resources, and tool acceptance were collected using a questionnaire.

Findings: In total, 52,863 samples underwent Xpert testing from April 2011 to December 2012. The average MTB detection rate was 18.5%, 22.3%, and 11.6% for the three different strategies respectively. Analysis of the results on samples tested in parallel showed that using Xpert as add-on test to microscopy would have increased laboratory TB confirmation by 49.7%, versus 42.3% for Xpert replacing microscopy.

The main limitation of the test was the high rate of inconclusive results, which correlated with factors such as defective modules, cartridge version (G3 vs. G4) and staff experience. Operational and logistical hurdles included infrastructure renovation, basic computer training, regular instrument troubleshooting and maintenance, all of which required substantial and continuous support.

Conclusion: The implementation of Xpert was feasible and significantly increased TB detection compared to microscopy, despite the high rate of inconclusive results. Xpert implementation was accompanied by considerable operational and logistical challenges. To further decentralize diagnosis, simpler, low-cost TB technologies well-suited to low-resource settings are still urgently needed.

Introduction

Tuberculosis (TB) remains a major public health problem, as evidenced by the estimated 9 million incident cases, 300 000 multi-drug resistant (MDR) cases and 1.5 million deaths worldwide in 2013.(1) However, only 58% of the new incident cases were bacteriologically confirmed by smear, culture, or Xpert® MTB/RIF (Xpert) (Cepheid, Sunnyvale, CA), while the remaining 42% were diagnosed clinically, including by X-ray.(2)

The Xpert test is an automated molecular system that allows for rapid, simultaneous detection of both *Mycobacterium tuberculosis* (MTB) and resistance to rifampicin, a key first-line anti-TB drug, directly from sputum (3) and extrapulmonary samples.(4) In 2010, the World Health

Organization (WHO) endorsed the Xpert test and recommended its use as the initial diagnostic test for people with HIV-associated TB or presumptive multidrug resistant TB (MDR-TB).(5) Three years later the recommendation was extended (conditional on availability of resources) to cover initial diagnostic testing for all adults presumed of having TB.(6) The 2011 guidelines released by WHO described implementation of Xpert as simple, requiring only minimal staff training, and feasible in diverse settings.(7) These guidelines helped trigger rapid worldwide adoption of Xpert: as of June 2014, a total of 3,269 Xpert instruments had been procured for the public sector in 108 of 145 countries eligible for concessional pricing.(2)

The diagnostic accuracy of Xpert in different settings and patient populations has been confirmed by several extensive validation studies, including a multicentre study carried out in six countries (8) and two systematic reviews and meta-analyses.(9),(10) However, nearly all these studies were conducted in controlled research environments that provide optimal conditions, and therefore may not reflect difficulties frequently encountered in routine programmatic contexts. Only one report, carried out in countries using different case detection strategies, reported on the performance of Xpert in a vast programmatic pilot project and provided comprehensive information on Xpert implementation under routine conditions.(11) However, the majority of the sites included used Xpert as an add-on test to microscopy, limiting the analysis of case detection provided by this technique as a first-line diagnostic test.(11)

In addition, a description of the difficulties experienced during the initial phases of technology implementation compared with a later stage of routine testing is still lacking. Yet knowledge of the common difficulties typically encountered during the initial implementation period in low-resource settings, and lessons learned in resolving them, are highly valuable to countries as they begin or continue scaling-up in new settings.

We present results from routine testing of pulmonary samples with Xpert following different diagnostic strategies adopted in TB programs supported by Médecins Sans Frontières (MSF). We also describe the key lessons learned during almost two years of implementation and routine use of Xpert under programmatic field conditions across varying ranges of geographic, epidemiological and clinical settings.

Methods

Study settings

From April 2011 to December 2012 a total of 38 Xpert four-module instruments were installed in 33 project sites in 18 countries supported by MSF, representing a diverse range of TB, MDR-TB and HIV prevalence. Sites were considered to have a high MDR-TB burden if the MDR prevalence previously reported in the patient cohort of the site exceeded 10% in newly diagnosed cases (12), while high HIV burden sites refers to settings with HIV prevalence ≥1%.(13)

Xpert devices were mainly placed in district and sub-district laboratories (21/33) apart from five regional, six peripheral and one penitentiary system facility. The site distribution of the Xpert instruments according to epidemiological setting and facility level per country is described in Table 1.

Table 1: Site distribution of GeneXpert instruments by epidemiological setting and facility level (n = 33)

	Site distribution by epidemiological setting				Distribution of sites by facility level			
	High MDR-TB /high HIV	High MDR-TB /low HIV	Low MDR-TB /high HIV	Low MDR-TB /low HIV	Regional	District or sub-district	Peripheral	Prison
Cambodia				1		1		
Central			1				1	
Colombia		1			1			
Democratic			1			1		
Georgia		1			1			
India		1				1		
Kenya			3			2	1	
Kyrgyzstan		2				1		1
Lesotho	1					1		
Malawi			2			2		
Mozambique			2			1	1	
Myanmar		1			1			
Russia		1			1			
Somalia				1	1			
South Africa	2					2		
Swaziland	5					5		
Uzbekistan		2				1	1	
Zimbabwe			5			3	2	
TOTAL	8	9	14	2	5	21	6	1

Laboratory procedures

Pulmonary samples collected from adults and children were tested with Xpert following the manufacturer's instructions.(14) In children (defined as below 15 years of age) unable to produce sputum, alternative respiratory samples such as gastric aspirates and induced sputum were obtained where facilities and expertise to carry out these procedures existed. Xpert results were interpreted according to the manufacturer's instructions. A test was reported as inconclusive if the Xpert instrument indicated a final automated result as invalid, error or no result which were not reported as desegregated data except for error 5011. In case of inconclusive results, Xpert was performed on the leftover sample or on a newly collected sample ; however these results were not provided for this data collection, which includes only results from the first testing. Initially, instruments at most sites used cartridge version G3. Version G4 was developed to reduce errors rate, mainly error 5011 rate, indicated by Cepheid as being due to signal loss detected in the amplification curve, and to improve test robustness by decreasing possible false rifampicin resistant results. This version was gradually introduced as it became available.(15)

Where culture techniques were available on site, samples were decontaminated and then tested with Xpert and inoculated in parallel either on Löwenstein–Jensen (LJ) culture medium, mycobacterial growth indicator tube (MGIT) (Becton Dickinson, Diagnostic Instrument Systems, Sparks, MD), or Thin Layer Agar (TLA) medium.

TB testing strategies

The study sites employed three testing strategies. Xpert as the initial diagnostic test for all presumptive TB cases was adopted in 23 sites, 22 of them performing Xpert in parallel with microscopy. Nine sites subsequently dropped the use of microscopy after a minimum of 300 tests, which was then employed only for Xpert positive cases to obtain a smear baseline result for follow-up and infection control purposes. The second strategy was used at 7 sites, where Xpert use was limited to patients at high risk of MDR-TB (previously treated, non-converting patients, treatment failures, symptomatic contacts of confirmed MDR cases)(5), HIV-associated TB, and presumed paediatric TB cases. The third strategy was used at the remaining 3 sites, where Xpert was employed as the first test for high-risk groups plus as an add-on test to microscopy in others.

Three sites performed Xpert in parallel to culture, each site following one of the three different strategies.

Data collection

Quantitative data and setting information

Data were collected between April 2011 and December 2012 by quarter and recorded in an electronic laboratory register developed for routine Xpert data collection. At each site, the period of use was divided into two phases: the "implementation phase", covering the first four months of Xpert use and the "routine activity phase" covering the subsequent months of routine testing that were covered by the monitoring period. Projects also provided information on the month in which the use of new G4 cartridge version began. G4 was considered introduced from the middle of the month onwards. Additionally, each project reported any module replacement throughout the monitoring period.

All projects provided information about the facility level of the laboratory where Xpert was implemented, including a description of infrastructure, environmental conditions, workload in the laboratory and sample collection strategy.

Qualitative data

Xpert was introduced in the sites following a period of preparation, which included training for laboratory technicians and implementation of logistical requirements, according to manufacturer and WHO recommendations (7). To identify the key lessons learned from implementing the GeneXpert system and Xpert testing from each study site, a questionnaire was distributed to all sites in December 2012, at which point the period of onsite Xpert testing ranged from four to 21 months. The questionnaire was completed by site laboratory coordinators. Questions focused on installation, daily use, maintenance of the equipment and calibration. Additional questions covered infrastructure requirements, requirement for training of laboratory staff, human resource issues related to the implementation of the GeneXpert system and overall impressions of GeneXpert instruments. The questionnaire requested answers in the form of yes/no, numbers, description of incidents, and included open responses.

Statistical analysis

To determine the statistical significance in group comparisons, p-values were calculated using the chi-square or Fisher's exact test for independent samples and McNemar's test for matched-pair samples. Statistical tests were two-sided at alpha = 0.05, and p-values <0.05 were considered statistically significant. All analyses were performed using Stata version 11 (StataCorp, College Station, TX, USA).

Comparison between Xpert and smear microscopy results is presented as relative gain, expressed for Xpert as an add-on test (calculated as the number of Xpert-positive and smear negative specimens divided by the number of smear positive specimens), and Xpert as a replacement test for microscopy (calculated as the number of Xpert positive specimens minus the number of smear positive specimens divided by number of smear positive specimens).

Ethics

The International Ethics Review Board of MSF reviewed the study protocol and determined that it did not require formal ethics board approval as it was based on retrospective analysis of routinely collected programmatic data.(16) None of the merged data can be linked to individuals and no patient identifiers were used, so confidentiality was preserved. The study did not require collection of additional patient samples or performance of any test additional to routine patient care procedures so the study did not constitute a risk for the patients. Due to the retrospective data analysis of merged results, the patients were not asked to provide informed consent.

Results

Detection of MTB complex in adult and paediatric samples

Between April 2011 and December 2012, results for a total of 52,863 Xpert tests were reported (Figure 1). Of the total 45,495 tests performed as the initial diagnostic test, the average MTB positivity rate was 18.5% (Table 2), with large variations between sites [range 9.7– 43.8%].

Of the 6,231 tests performed as the initial test only for high risk groups, the average MTB positivity rate was 22.3%, again with wide variation between sites [range 13.3–66.7%] (Table 3).

At the three sites where Xpert was used as the first test for high risk groups or as an add-on test to microscopy, of the 1,137 tests performed, the average positivity rate was 11.6% [range 4.1–14.1%] **(Table 4).**

Of 14 sites with data available for comparison, both for Xpert and microscopy the MTB positivity rate decreased significantly from the early implementation phase to the routine phase, for Xpert from 22.9% (818/3576) to 19.7% (2656/13479) (p=0.002), while the smear positivity rate decreased from 16.9% (606/3576) to 14% (1885/13479) (p=0.0002).

Twenty-four sites reported results from 1,278 samples collected from children, representing 2.4% of the total 52,863 samples tested. Of these, 61 were tested positive (4.7%) with Xpert.

Fig. 1: Xpert testing strategies and MTB detection

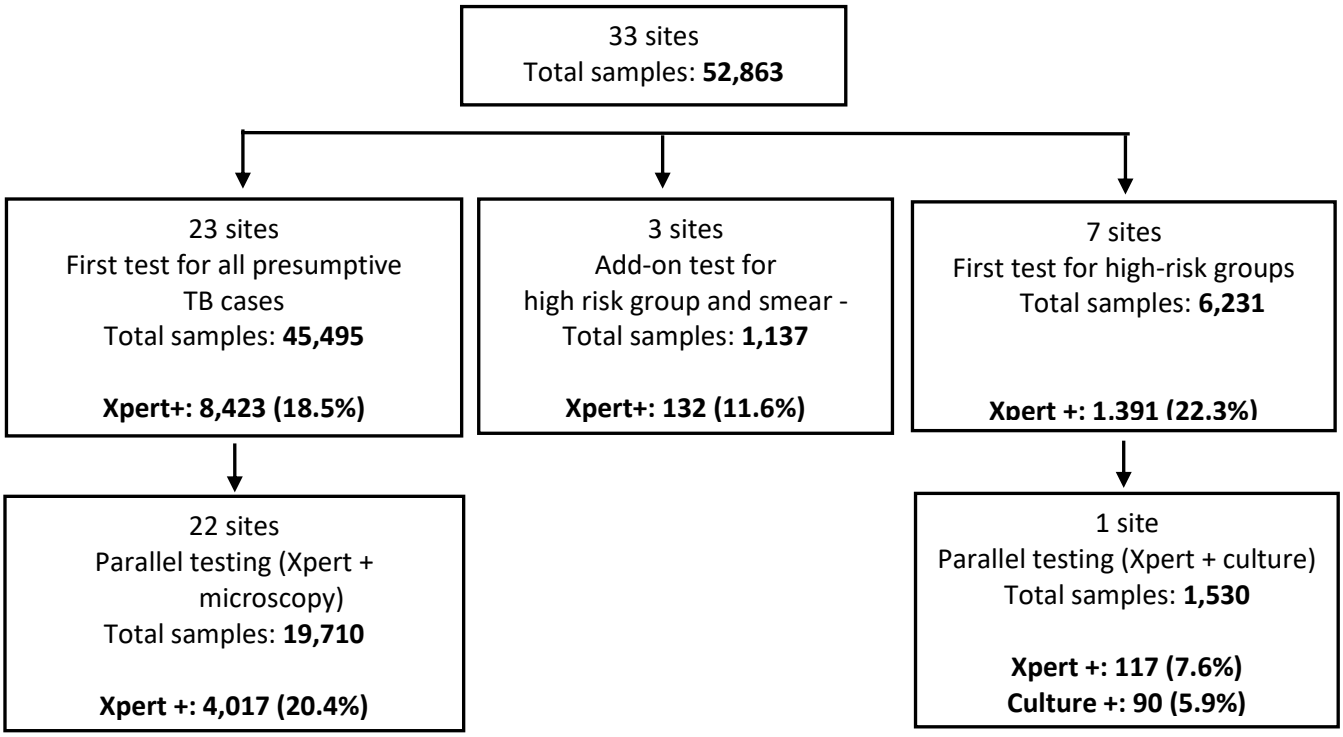

Table 2: Detection of MTB by Xpert in 23 sites using Xpert as first test

Project	Positive n (%)	Negative n (%)	Inconclusive n (%)	Total
CAR, Zemio	9 (18.4)	28 (57.1)	12 (24.5)	49
DRC, Kinshasa	144 (17.3)	656 (78.8)	33 (4.0)	833
Kenya, Kibera	437 (28.6)	1,023 (67.0)	67 (4.4)	1,527
Kenya, Mathare	589 (22.3)	1,668 (63.2)	381 (14.4)	2,638
Kyrgyzstan, Bishkek	309 (30.9)	641 (64.1)	50 (5.0)	1
Kyrgyzstan, Kara Suu	72 (23.8)	193 (63.7)	38 (12.5)	303
Lesotho, Roma	171 (19.2)	666 (74.8)	53 (6.0)	890
Mozambique, Mavalane	782 (31.1)	1,528 (60.8)	204 (8.1)	2,514
Mozambique, Moatize	487 (21.6)	1,531 (67.8)	241 (10.7)	2,259
Russia, Grozny	315 (43.8)	399 (55.5)	5 (0.7)	719
South Africa, Eshowe	1,309 (18.6)	5,542 (78.8)	181 (2.6)	7,032
South Africa, Mbongolwane	379 (9.7)	3,412 (87.7)	101 (2.6)	3,892
Swaziland, Hlatikulu	243 (13.9)	1,375 (78.5)	133 (7.6)	1,751
Swaziland, Mankayane	330 (12.8)	2,113 (81.9)	136 (5.3)	2,579
Swaziland, Matsanjeni	103 (13.6)	617 (81.7)	35 (4.6)	755
Swaziland, Matsapha	758 (13.9)	4392 (80.7)	294 (5.4)	5,444
Swaziland, Nhlangano	441 (13.9)	2,542 (80.1)	191 (6.0)	3,174
Uzbekistan, Chimbay	64 (19.5)	251 (76.5)	13 (4.0)	328
Zimbabwe, Epworth	627 (23.1)	1,882 (69.3)	205 (7.6)	2,714
Zimbabwe, Birchenough	195 (18.5)	802 (76.2)	55 (5.2)	1,052
Zimbabwe, Gokwe	5 (26.3)	9 (47.4)	5 (26.3)	19
Zimbabwe, Gutu	67 (18.3)	298 (81.4)	1 (0.3)	366
Zimbabwe, Murambinda	587 (16.1)	2,651 (72.5)	419 (11.5)	3,657
TOTAL	8,423 (18.5)	34,219 (75.2)	2,853 (6.3)	45,495

Table 3: Detection of MTB in 7 sites using Xpert as first test in high risk groups

Project	MTB Positive	MTB Negative	MTB Inconclusive	Total
	n (%)	n (%)	n (%)	
Colombia, Buenaventura	207 (42.2)	273 (55.7)	10 (2.0)	490
Cambodia, KC	383 (15.3)	1,884(75.3)	234 (9,4)	2,501
Georgia, Abkhazia	163 (25.7)	439 (69.1)	33 (5.2)	635
India, Manipur	14 (66.7)	3 (14.3)	4 (19.0)	21
Myanmar, Yangon	419 (38.6)	646 (59.5)	21 (1.9)	1,086
Somalia, Galcayo	8 (57.1)	6 (42.9)	0 (0.0)	14
Uzbekistan, Nukus	197 (13.3)	1,206 (81.3)	81 (5.5)	1,484
TOTAL	1,391 (22.3)	4,457 (71.5)	383 (6.1)	6,231

Table 4: Detection of MTB in 3 sites using Xpert as first test in high risk groups and as add-on test to microscopy for smear negative presumed TB

Project	MTB Positive n (%)	MTB Negative n (%)	MTB Inconclusive n (%)	Total
Kenya, Homa Bay	82 (14.1)	460 (79.3)	38 (6.6)	580
Malawi, Chiradzulu	40 (12.8)	224 (71.8)	48 (15.4)	312
Malawi, Thyolo	10 (4.1)	216 (88.2)	19 (7.8)	245
TOTAL	132 (11.6)	900 (79.1)	105(9.2)	1,137

Detection of rifampicin resistance

The rifampicin resistance detection rate among MTB-positive samples ranged from 0 to 41.4% (Table 5) across all 33 sites.

Overall, 123 of 9,929 (1.2%) Xpert-positive samples generated rifampicin-indeterminate results. Microscopy results were reported for 79 of the 123 Xpert rifampicin-indeterminate results, of which 63 (79.7%) were found to be smear-negative.

Table 5: Detection of rifampicin resistance by Xpert according to MDR-TB prevalence

MDR-TB prevalence	Country	MTB+/RIF resistant n (%)	MTB+/RIF susceptible n (%)	MTB+/RIF indeterminate n (%)	TOTAL MTB+ n
	Colombia	23 (11.1)	184 (88.9)	0 (0.0)	207
	Georgia	30 (18.4)	130 (79.8)	3 (1.8)	163
	India	5 (35.7)	9 (64.3)	0 (0.0)	14
	Kyrgyzstan	137 (36.0)	237 (62.2)	7 (1.8)	381
	Lesotho	13 (7.6)	158 (92.4)	0 (0.0)	171
High	Myanmar	71 (16.9)	339 (80.9)	9 (2.1)	419
	Russia	80 (25.4)	234 (74.3)	1 (0.3)	315
	South Africa	235 (13.9)	1,451 (86.0)	2 (0.1)	1,688
	Swaziland	197 (10.5)	1,636 (87.3)	42 (2.2)	1,875
	Uzbekistan	108 (41.4)	147 (56.3)	6 (2.3)	261
	SUBTOTAL	**899 (16.3)**	**4,525 (82.3)**	**70 (1.3)**	**5,494**
	DRC	17 (11.8)	122 (84.7)	5 (3.5)	144
	Cambodia	21 (5.5)	355 (92.7)	7 (1.8)	383
	Kenya	59 (5.3)	1,031 (93.1)	18 (1.6)	1,108
Low	Malawi	0 (0.0)	49 (98.0)	1 (2.0)	50
	Mozambique	99 (7.8)	1,163 (91.6)	7 (0.6)	1,269
	Zimbabwe	73 (4.9)	1,393 (94.1)	15 (1.0)	1,481
	SUBTOTAL	**269 (6.1)**	**4,113 (92.7)**	**53 (1.2)**	**4,435**
	TOTAL	**1,168 (11.7)**	**8,638 (86.9)**	**123 (1.2)**	**9,929**

Xpert results compared to sputum smear microscopy

Of the 22 sites that performed Xpert and microscopy in parallel for all presumptive TB cases, fluorescence microscopy (FM) and Ziehl-Neelsen (ZN) were performed in 12 and 10 sites, respectively. Of 19,710 samples tested in parallel, MTB was detected in 4,227 (21.4%) by either of the two techniques: 2,823 (14.3%) were positive by microscopy, and 4,017 (20.4%) were positive by Xpert, with a statistically significant difference ($p<0.0001$) and a kappa coefficient of 0.62 (Table 6).

Table 6: Results of Xpert compared to smear microscopy

		Xpert			
		Positive	Negative	Inconclusive	Total
Smear microscopy	Positive	2,613	73	137	2,823
	Negative	1,404	14,143	1,340	16,887
	Total	4,017	14,216	1,477	19,710

Using Xpert as an add-on test following microscopy would have resulted in an average 49.7% relative

gain in bacteriologically-confirmed TB, while replacing microscopy with Xpert as the first test in the diagnostic algorithm would have increased the laboratory detection of MTB compared to microscopy by 42.3%.

The relative gain of Xpert as add-on test versus microscopy varied widely between sites, from 9.7% to 110.4%, and was significantly higher in sites performing ZN (77.5%) compared to FM microscopy (39.6%, p<0.001) (Table 7).

Table 7: Relative gain by project for Xpert used as add-on test

		Xpert+/ Smear-	Xpert +/ Smear+	Sm+	Relative gain
FM	Kenya, Mathare	58	535	596	9,7%
	Kenya, Kibera	54	239	240	22,5%
	Malawi, Chiradzulu	13	27	27	48,1%
	Zimbabwe, Epworth	210	410	428	49,1%
	DRC, Kinshasa	46	82	88	52,3%
	Kenya, Homa Bay	29	48	53	54,7%
	Zimbabwe,	57	88	101	56,4%
	Swaziland, Hlatikulu	93	148	154	60,4%
	Swaziland, Nhlangano	177	245	269	65,8%
	Zimbabwe,	28	38	39	71,8%
	Swaziland, Matsanjeni	47	52	56	83,9%
	Zimbabwe, Gokwe	2	2	2	100,0%
	Total	814	1914	2053	39,6%
ZN	CAR, Zemio	2	7	11	18,2%
	Russia, Grozny	103	190	213	48,4%
	Zimbabwe, Gutu	25	42	43	58,1%
	Mozambique,	46	64	67	68,7%
	Kyrgystan, Bishkek	46	46	63	73,0%
	Swaziland, Mankayane	14	16	19	73,7%
	Uzbekystan, Chimbay	31	29	35	88,6%
	Lesotho, Roma	20	20	20	100,0%
	Mozambique, Moatize	250	230	242	103,3%
	Swaziland, Matsapha	53	46	48	110,4%
	Total	590	690	761	77,5%

Results of Xpert compared to culture

Of the three sites performing Xpert in parallel with culture, only Cambodia provided laboratory results for a total of 1,530 smear-negative samples tested in parallel with Xpert and MGIT **(Table 8**). Of a total of 157 TB patients detected by either test, culture detected 90 (57.3%) versus 117 (74.5%) detected by Xpert.

Table 8: Detection of MTB by Xpert compared to MGIT culture in smear-negative samples

	Culture				
Xpert	Positive	NTM*	Negative	Contaminated	Total
MTB positive	50	8	38	21	117
MTB negative	33	99	1009	138	1,279
MTB inconclusive	7	16	87	24	134
Total	90	123	1,134	183	1,530

*NTM: non-tuberculous mycobacteria

Xpert inconclusive results, module replacement and errors

Of the total 52,863 samples tested, 3,341 (6.3%) generated inconclusive results. The proportion varied between projects, with a median of 5.7% [range 0–26.3%]. Only six sites reported a level of inconclusive results below 3%.

To analyse factors that might have influenced the proportion of inconclusive results, we analysed their frequency by level of facility where Xpert was implemented, the module replacement, the cartridge version used (G4 versus G3), and staff experience in performing the test (routine versus implementation phase).

When analysed by level of facility, the proportion of inconclusive results was 2.5% (73/2965) at regional level, 6.4% (2680/42021) at district and sub-district level, 7.8% (538/6877) at peripheral level and 5.0% (50/1000) in the penitentiary system ($p<0.001$).

In total, 12 sites underwent module replacement due to high error rates, with an average of 8.2% (2332/27382) inconclusive results versus 4.4% (1109/25481) for projects that did not change module ($p<0.001$). However, none of the 12 projects reached a rate of inconclusive results below 3% after module replacement.

Of the 33 sites, 27 had a period of activity longer than 4 months, covering both implementation and routine phases, allowing a comparison of outcomes during these two time periods. Overall, the proportion of inconclusive results was significantly higher during the implementation compared with the routine activity phase (8.0% vs 5.8%, $p<0.001$).

The proportion of inconclusive results was 7.4% using the G3 cartridge and 5.8% using the G4 cartridge ($p<0.001$).

Stratification by phase and cartridge version showed that the phase had an impact only when the G3 cartridge was used, while the cartridge change from G3 to G4 reduced the proportion of inconclusive results irrespective of the phase of implementation (Table 9).

Thirty projects reported information on the frequency of error 5011. For G3, error 5011 accounted for 425 of 888 errors (47.9%), and 30.8% of the 1381 inconclusive results with this cartridge, while for G4 it represented 379 of 978 errors (38.8%) and 30.1% of the 1260 inconclusive results with G4. Although the decrease was significant ($p=0.03$), the frequency of error 5011 remained high.

Table 9: Inconclusive Xpert results by implementation phase and cartridge version

Cartridge version	Xpert inconclusive results			
	Phase 1	Phase 2	Total	p-value
G3	8.8% (557/6,360)	6.9% (1,199/17,278)	7.4% (1756/23,638)	P<0.001
G4	4.7% (69/1,466)	5.1% (1,365/26,845)	5.1% (1,434/28,311)	P=0.54
Total	8.5% 626/7,826	5.8% (2,564/44,123)	6.1% (3,190/51,949)	
p-value	p<0.001	p<0.001		

Identifying key lessons learned

In all sites logistical requirements were in place before implementation of the test; they were either already in place, or implemented expressly for Xpert introduction. Twenty eight questionnaires on implementation issues were received, providing information for all implementing sites (Table 10).

Three countries with multiple sites, Swaziland, Zimbabwe and South Africa, completed a single questionnaire with combined country information. Projects reported installation of air conditioning as one of the main logistical interventions (54%), followed by installation of a generator (39%), while the majority of the laboratories were already equipped with a biosafety cabinet prior to Xpert implementation (89%).

High rates of inconclusive results were reported as one of the main limitations by almost half of the respondents. Fourteen respondents mentioned having contacted the manufacturer (Cepheid) specifically regarding the high rate of inconclusive results. Eleven respondents reported having modules replaced, upon the manufacturer's advice, for a total of 21 replaced modules. Two modules also failed the installation check. In total, 15% [23/152] of all modules initially distributed were replaced.

Seven of 28 respondents reported changing their sputum collection strategy as a result of implementing Xpert, with two sites moving from the collection of three to two samples, and three sites from two to one sample. Two sites adopted spot-spot collection cf. spot-morning.

Lack of the user manual and software in Russian at the time of implementation (later addressed by the manufacturer) hindered Xpert usage in some settings.

Limited internet access was a barrier for annual calibration. Other challenges included the need for basic computer training in one third of the sites, where microscopists were not sufficiently familiar with their use.

All respondents reported being generally satisfied with the system. However, some commented that discordant results between Xpert and culture made interpretation of results difficult, that

bloody sputum resulted in inconclusive results, and that viruses occasionally infected the computer used with the system.

Table 10: Quantitative results from the lessons learned questionnaire (n = 28)

Infrastructure	**Yes**	**No**
	n (%)	
Laboratory renovation required	5 (18)	23 (82)
Air conditioning installed for test implementation	15 (54)	13 (46)
Generator installed for test implementation	11 (39)	17 (61)
Installation biosafety cabinet for test implementation	3 (11)	25 (89)
Equipment performance		
Failed installation check (one module per machine)	2 (7)	26 (93)
Experienced performance problems	9 (32)[1]	21 68)
Assay performance		
Staff computer training required	10 (36)	18 (64)
High error rates reported to Cepheid	14 (50)	14 (50)
Modules replaced on advice of Cepheid	11 (39)	17 (61)
Module calibration		
Module exchange-based calibration procedure followed	11 (39)[2]	17 (61)
Impact on programmes		
Sputum collection strategy changed	7 (25)	21 (75)
Overall impressions		
Satisfaction with the system due to: simplicity of procedure	17 (61)	11 (39)
Speed of assay	6 (21)	22 (79)
Increased sensitivity cf. smear microscopy	5 (18)	23 (82)
Frustrations due to: high error rates	17 (61)	11 (39)
Lack of Russian-language software	3 (11)	25 (89)
Lack of isoniazid resistance detection	2 (7)	26 (93)
Most positive aspects		
On-site rifampicin resistance detection	11 (39)	17 (61)
Increased sensitivity for tuberculosis detection	12 (43)	16 (57)
Speed to results	2 (7)	26 (93)
Simplicity of use	3 (11)	25 (89)

1. 5/9 experienced barcode scanning problems; 2/9 sites had GeneXpert machine failure when the ambient temperature exceeded 30 °C; 1/9 had a cartridge stuck in a module.
2. This process went smoothly for 8/11; 2/11 experienced customs problems, and 1/11 experienced a long delay in shipment of replacement modules.

Discussion

As countries embark on the implementation and scale up of the new Xpert technology, there is an increasing need to document and share the programmatic and operational lessons emerging from this logistically-intensive activity, especially across different settings in resource-constrained contexts.

Our experience showed that Xpert significantly contributed to TB detection when used as the first diagnostic test for all presumptive TB in parallel with microscopy, and that it would have led to a high relative gain if used as a replacement test. At one site Xpert and culture on MGIT equally contributed to detection of TB among smear-negative presumptive TB patients, and overall the test contributed to the detection of TB in paediatric samples, although analysis on this point is limited by the relatively small paediatric sample size. High rates of inconclusive results represented one of the major challenges, related to various factors including technical issues and staff experience. Xpert implementation required consistent laboratory support with costly logistical interventions.

In our study, when compared to microscopy, the Xpert relative gain varied according to the technique used, (i.e. higher compared with ZN cf. FM). In general Xpert relative gain varied between projects. Apart from the program in Central African Republic, for which the number of tests performed was very low, two sites in Kenya, Kibera and Mathare, showed lower relative gain. These results may be explained by the different testing strategies implemented and the heterogeneous epidemiological contexts in which the test was deployed. For example, if TB patients tend to wait before seeking medical care, they may have high bacillary loads which can be detected by both smear microscopy and Xpert. This might explain the comparatively low relative gain in the Mathare and Kibera projects, which are located in a slum, where the smear positivity rate was higher than for all other sites included in the study, which suggests that patients generally sought care at an advanced stage of the disease.

The positivity rate decreased significantly from the implementation to the routine phase. The positivity rate in the routine phase was similar to that reported by South Africa (16%), India (20%) and the TB REACH multicentric study (15%).(17),(18),(11) The decrease over time could be explained by an increased number of presumptive TB patients tested with Xpert over time, as also observed by other authors.(19)

Our results show that replacing microscopy with Xpert would have resulted in fewer cases detected rather than performing Xpert as an add-on test following microscopy, which may be explained by several factors. Firstly, among cases detected by microscopy but not Xpert, the majority were due to Xpert inconclusive results. In these cases repeating the test on a new sample may have produced a positive Xpert result; however, these results were not available, thus the proportion of cases detected by Xpert may be underestimated.(20) Secondly, Xpert negative, smear positive results could be due to the presence of non-tuberculosis mycobacteria (NTM) that can be detected by microscopy but are reported as negative by Xpert, as the assay is highly specific for the detection of *M. tuberculosis* complex, and cross-

reaction with NTM has not been reported. Thirdly, the quality of microscopy in these sites was high, performed under regularly supervised and controlled conditions. On the other hand, microscopy performance could also be underestimated, since the result of only one specimen was considered, whereas microscopy investigation is normally based on testing at least two samples. (21)It is known that microscopic investigation of a second sample can increase case detection by 10-14%.(22) Considering the increased detection from testing a second sample, the incremental yield of Xpert compared to conventional microscopy would have still accounted for an estimated 18.6% - 24.6%. While case detection would increase by the use of Xpert as an add-on test to smear negative samples, the low positivity rate of microscopy shows that 86% of the cases not detected by microscopy would still require testing with Xpert. This approach would represent a very high workload and investment compared to directly performing Xpert as the first diagnostic test, and should be carefully considered.

A multicentric field demonstration study carried out in six countries reported that a single Xpert test detected 90.3% of TB cases which were bacteriologically confirmed by liquid culture.(8) In our study, the comparison between Xpert and culture was only possible at one site, where the sample was tested in parallel with Xpert and the MGIT automated system. At this site, Xpert detected only 55.6% of cases confirmed by culture, which is lower than reported elsewhere (8), but comparable to values reported by Theron (23), who also reported a per sample analysis; while Boehme compared Xpert positivity to final culture results obtained by multiple testing, which may decrease Xpert positive/culture negative results. However, Xpert also detected a substantial number of cases that were missed by culture, either because of contamination, or harsh decontamination leading to negative results, or possible mixed infection with NTM, which would have been misclassified by culture as NTM positive.

For rifampicin resistance detection, rates of indeterminate results were lower than the 2.4% reported in a field demonstration study (8) and comparable with results reported by the TB REACH study.(11) As expected, indeterminate results were found mostly in smear negative samples, with low bacillary load; however, almost 20% occurred in smear-positive samples.

Overall the number of children screened with Xpert for TB was low at all sites, possibly due to the difficulty of obtaining an adequate sample for laboratory confirmation. Among the children tested, the detection of TB using Xpert was lower than in other studies.(24) However, in our study only one sample was tested per patient, while other authors report that to increase detection in children, algorithms including collection of samples from different body sites (e.g. gastric aspirates) should be included.(25)

The rate of inconclusive results in the first multi-country feasibility study conducted in seven countries was below 3%(8); South Africa achieved a similar rate.(17) However, in MSF-supported sites, half of the 33 sites had rates of more than double this benchmark. The TB REACH multicentric study reported rates in agreement with ours (10.6%)(11), possibly due to the related routine implementation conditions, compared with the Boehme evaluation study. This value is also comparable or higher than rates for interpretable results with other validated tests, such as Genotype MTBDRplus assay (92%) and conventional methods (78%).(26)

The high rate of inconclusive results in our study was a source of frustration, due to having to collect replacement samples from some patients, results being delayed, and the expense of having to use multiple cartridges for one patient. This unforeseen rate of usage in some sites led to stock rupture, therefore having a detrimental impact on patient diagnosis.

In MSF projects the algorithm included retesting Xpert for patients with inconclusive results, when possible using the leftover sample, or a newly collected sample, but results from retesting were not available and are not included in this analysis. Repeating Xpert testing for inconclusive results is reported to resolve inconclusive results(5), so this procedure should be included in the routine diagnostic algorithm, and results of retesting should be collected when possible.

Inconclusive results can occur for different reasons, including incorrect manipulation of the samples.(27) Our results suggested that several aspects may have been correlated with inconclusive results, showing that aside from technical limitations, which were partially resolved by module replacement and cartridge version, staff experience in performing the test contributed to decreasing the rate of inconclusive results. In a report published by FIND it is stated that due to the launch of the G4 cartridge, error 5011 was virtually eliminated.(15) In our experience, implementation of the new cartridge across our sites decreased the occurrence of error 5011; however it was still reported by the laboratories on occasion. Due to the lack of information regarding the distinction between the type of inconclusive result and error codes other than 5011, this retrospective analysis of routinely collected data could not account for other factors influencing the rate of inconclusive results, such as turnover of the staff, temperature fluctuations in the laboratory or power supply interruptions.

The Xpert system was initially described as easy to perform, requiring minimal training and set up, including in peripheral settings. However, in our experience the device was not uniformly easy to install and operate. Its implementation required costly interventions, including provision of air conditioning, provision of uninterrupted electricity and internet connection for calibration. Until a more robust system is available, these extra costs need to be taken into account prior to the decision to introduce the test. The feedback from users was overall positive, mainly due to the simplicity of the procedure. However, aside from logistical interventions, implementation required regular technical support, including training in results interpretation, which had to be adapted to the level of the facility, such as in the case of reference laboratories due to discordant results with culture techniques. Language issues, which initially hindered implementation in some sites, were eventually addressed by the manufacturer.

This study presents some limitations. In this per sample analysis the comparison of Xpert versus microscopy was based on investigation of one sample per patient, while routinely microscopy is performed on multiple samples and Xpert is repeated for inconclusive cases. The data analysis did not include results from repeated Xpert testing for inconclusive results, which may have increased the positivity rate for this test. The use of aggregated data collected routinely prevented us from undertaking a more precise and accurate investigation of the factors associated with proportion of inconclusive results.

Conclusions

The implementation of Xpert in diverse clinical settings was feasible and led to a significant increase in bacteriologically-confirmed pulmonary TB both for Xpert as first test, and as an add-on test. The choice of the best strategy should take into account the epidemiological setting, including prevalence of NTM, and the test cost which may represent a limitation in resource-constrained settings. However, the estimation of the cost should take into account that the major investment is often represented by the logistical support required for installation of the system, while the higher test cost compared to microscopy is offset by the higher sensitivity and specificity compared to microscopy.

In our experience the system was far from a "plug and play" device. High numbers of inconclusive results represented an extra expense. Significant infrastructure requirements, training, technical support and experience were indispensable to decrease errors and achieve good routine results, and time was needed for programmes to become more effective in applying sample collection strategies. To further decentralize diagnosis of drug-sensitive and drug-resistant TB strains, more robust, simpler technologies which are well-adapted to low-resource settings are still needed. In addition, as the GeneXpert system is now relatively widespread in many high-burden countries, development of a cartridge incorporating resistance detection for other drugs could boost the fight against drug-resistant tuberculosis.

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Conflicts of interest

None to declare

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CHAPTER III

Decontamination methods for samples preserved in cetylpyridinium chloride and cultured on thin-layer agar

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Abstract

Settings: Long transport times of samples to culture laboratories can lead to an increased contamination rate and significant loss of viability, resulting in a decreased culture positivity rate.
Objectives: Thin Layer Agar (TLA) is a sensitive culture method for isolation of *Mycobacterium tuberculosis* that has been optimized with *N*-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) decontaminated samples. Its combination with other decontamination procedures has not been extensively validated.
Design: In this study, conducted on a total of 390 smear-positive samples, we compared culture positivity for samples decontaminated with the Petroff method versus NALC-NaOH neutralized with phosphate buffer (PBS), applied to samples either preserved with cetylpyridinium chloride (CPC) or CPC-free, then for CPC-preserved samples decontaminated with NALC-NaOH neutralized with Difco neutralizing buffer. Sediments were inoculated on TLA, then MGIT^{960} or LJ gold standards.
Results: NALC-NaOH decontamination yielded higher culture positivity in TLA than Petroff, which was further enhanced by neutralization of the CPC with the Difco buffer. Surprisingly, culture positivity on LJ also increased after Difco buffer, suggesting that CPC may not be completely neutralized in egg-based medium.
Conclusions: After transport in CPC, NALC-NaOH decontamination followed by neutralization with Difco buffer resulted in the best recovery rates for samples inoculated on TLA and on LJ.

Introduction

Use of automated nucleic acid amplification techniques can speed up the diagnosis of tuberculosis (TB) and the detection of multi drug-resistant TB, but does not eliminate the need for conventional culture, both to perform phenotypic susceptibility testing for other drugs, and to monitor patients during treatment.[1] However, in most countries, samples need to be transported to central laboratories for comprehensive diagnostic services, which can take several days, resulting in increasing samples contamination and loss of viability of mycobacteria, with subsequent decreased of recovery rate.[2]
N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) method is commonly used to reduce contamination in specimens prior to inoculation on selective media. Alternative decontamination techniques include Petroff,[3] and cetylpyridinium chloride (CPC), an ammonium compound added to samples during storage or transport for partial digestion and decontamination.[4]
Thin Layer Agar (TLA) is an inexpensive and sensitive culture method for *M. tuberculosis* (MTB) based on micro colony detection.[5] When used for smear-positive samples, the median time required for mycobacterial detection is 14 days, versus 9.6 days with the liquid culture MGIT^{960} system, and 23 days with the solid medium Löwenstein-Jensen (LJ).[6,7,8]

However, TLA culture can yield high contamination rates when inoculated with samples previously treated with Petroff or NALC-NaOH alone.[6,7] The combined application of NALC-NaOH and CPC has rarely been described, and the combination of CPC with the Petroff method is discouraged.[9] On the other hand, the use of CPC is incompatible with agar based media, unless CPC is neutralized, limiting its application to TLA. Sediment containing CPC can be inoculated on LJ, as phospholipids present in egg based medium can neutralize the residual of this ammonium compound.[10,11] Difco neutralizing buffer (BD Diagnostics –US), contains monopotassium phosphate with buffering capability, then sodium thiosulfate and aryl sulfonate which respectively inactivate the effect of chlorine and quaternary ammonium compounds. Its effect on CPC has been described earlier, [10,11] although its applicability to agar medium needs further investigation.[12]The objective of this prospective study was to identify an improved method to recovery mycobacteria in TLA from samples that need to undergo prolonged transportation. To our knowledge, no studies have described decontamination with NALC-NaOH of preserved-CPC samples combined with the Difco buffer prior to inoculation on TLA plates.

We report the application of TLA to samples decontaminated with four different methods, NaOH neutralized with hydrochloric acid (HCl)(Petroff), NALC-NaOH alone neutralized with phosphate buffer pH 6.8 (PBS) (NALC-NaOH/PBS); NALC-NaOH in combination with CPC neutralized with PBS (NALC-NaOH-CPC/PBS) and NALC-NaOH with CPC neutralized by Difco buffer (NALC-NaOH-CPC/Difco). Decontamination with CPC alone was not used for routine testing, as previous experience suggested that this results in a high contamination rate (data not presented). The performance of TLA is analysed in terms of positivity rate, contamination rate, and time to positive results expressed as turnaround time (TAT), compared to culture results in MGIT^{960} or on LJ medium gold standards.

Matherials and methods

Study design

The Mycobacteriology Laboratory at the Institute of Tropical Medicine (ITM) in Antwerp receives diagnostic samples from various Médecins sans Frontières-TB projects for culture and drug susceptibility testing.

In the field, each patient submitted three samples for microscopy investigation, then two were chosen based on higher microscopy grade and stored at 2-8°C for a maximum of four days. Before shipment at room temperature (RT) to ITM, one of the two samples was added with an equal volume of 1% CPC and 2% NaCl.

At reception, samples CPC-free were randomly decontaminated with NALC-NaOH or Petroff, while samples containing CPC were treated only with NALC-NaOH. Sediments obtained from samples containing CPC, incompatible with liquid medium,[3] were inoculated on LJ and sediments from samples without CPC were inoculated in BACTEC MGIT^{960}(BD Diagnostic Instrument Systems, Sparks, MD). All portion of sediments from smear positive mucopurulent samples, to be discarded, were inoculated in parallel on TLA plates.

Firstly we compared three decontamination methods: Petroff, NALC-NaOH/PBS, and NALC-NaOH-CPC/PBS. Secondly, we substituted PBS with the Difco buffer to compare NALC-NaOH-CPC/PBS to NALC-NaOH-CPC/Difco. Results recorded on the study database were de-identified from patients. The study protocol was approved by the Ethical Review Board of ITM.

Decontamination methods

Petroff method

Reagents were prepared as described by Kent.[11] Samples were added with 3%-NaOH in equal volume, incubated at RT for 20 minutes, then neutralized with HCl. After centrifugation at 3000 x g for 20 minutes, the sediment was added with 1 ml sodium chlorite 0.85% (NaCl) solution, then inoculated in MGIT^{960} according to manufacturer procedures.[13] The remaining resuspended sediment was diluted with 2.5 ml of NaCl solution , then 0.1 ml of the 1:6 dilution was inoculated into the TLA medium, on each half of the plate.

NALC-NaOH/PBS method

NALC-3% sodium hydroxide citrate was prepared as described by Kent,[11] then added to the sample in an equal volume. After 20 minutes of incubation, the sample was neutralized with PBS then centrifuged. The sediment was resuspended with 1 ml NaCl solution, and then inoculated in MGIT^{960}. The remaining resuspended sediment was treated and inoculated on TLA as described above.

NALC-NaOH-CPC/ PBS method

The samples were processed similarly to NALC-NaOH/PBS method except for the use of distilled water instead of NaCl solution to resuspend the sediment, and a 10 minutes incubation time, to minimize killing of mycobacteria by the combined decontamination methods.

NALC-NaOH-CPC/Difco method

The decontamination procedure was as described above for samples with CPC, but PBS was substituted by Difco buffer in the neutralization step.

Inoculation on TLA plates

Plates were prepared using a plastic Petri dish (BD, Sparks MD US) divided in two: one half for growth control (GC) and the other half for mycobacterial identification. The GC contained 5 ml of Middlebrook 7H11 enriched with 10% oleic acid, albumin, dextrose and catalase (OADC) (BD), then piperacillin, trimethoprim and amphotericin (Sigma), all at 4μg/ml concentration.[14] The other half contained the same medium enriched with PNB (500μg/ml) (Sigma). After inoculation, the TLA plates were left to dry for 30 minutes in the biosafety cabinet, sealed with parafilm leaving 1 cm uncovered to allow ventilation, and then incubated at 37°C in a 5% CO_2 incubator. The plates were examined by microscopy at x100 magnification at day 5, 7, 9, 13, 15, 20, 25, 30, 35, 40. Detection of MTB was based on presence of growth only on the GC, confirmed by observation of typical cording. Plates were reported as contaminated when the contaminant growth hampered interpretation of the plate and negative if no growth was detected within 40 days.

Inoculation on BACTEC MGIT ^{960} and LJ

The cultures in MGIT^{960} were performed following the manufacturer's instructions. Positive tubes were checked by microscopy and blood agar plate, incubated for 48 hours at 37°C.

LJ medium was prepared according to international standards, then inoculated and incubated at 37°C and examined weekly for up to eight weeks.

The identification of mycobacterial growth detected either in MGIT^{960} or on LJ was performed using an LJ medium containing PNB (500 µg/ml) and PNB-free LJ tube, used as a control, both inoculated with 0.1 ml of the suspension from positive cultures.

Analysis of the data

Positivity rate and contamination rate were calculated, using the total number of samples inoculated as denominator. We used McNemar's tests to measure the agreement between TLA and the gold standard methods and Fisher's exact test to compare the positivity rate on different decontamination methods. TATs were compared using the Wilcoxon signed rank sum test or the Wilcoxon-Mann-Whitney test for paired and independent samples respectively. Pairwise comparisons of transport time were made by Mann-Whitney test, adjusting the level of significance to <0.01 by the Bonferroni correction for multiple pairwise comparisons.

TATs were calculated from day of inoculation to day of positive cultures. For MGIT^{960}, it included the time required for preliminary identification by smear microscopy and blood agar medium.

Results

Between February 2010 and March 2011, we received 390 smear-positive samples, 173 containing CPC and 217 without CPC. The 173 samples containing CPC were further decontaminated with NALC-NaOH; of these 94 were neutralized with PBS and 79 with Difco. Of the 217 specimens without CPC, 56 were decontaminated with Petroff and 161 with NALC-NaOH/PBS. The positivity rate was equally distributed among the decontamination groups. The median (interquartile range) transport time of samples decontaminated with Petroff was 13 days, significantly longer than 10 days for samples decontaminated with NALC-NaOH/PBS ($p<0.01$) and NALC-NaOH–CPC/ Difco ($p=0.03$), and 9 days with NALC-NaOH- CPC/PBS ($p <0.01$). All culture-positive samples were identified as MTB. Table 1 shows the tests performance when samples were decontaminated either with Petroff, NALC-NaOH/PBS or NALC-NaOH-CPC/PBS. Overall positive rate for TLA combined to the gold standard was 69.6% using Petroff, 90% using NALC-NaOH/PBS, and 81.9% using NALC-NaOH-CPC/PBS.

Culture on TLA yielded higher positivity rates for samples decontaminated with NALC-NaOH/PBS ($p=0.005$) and NALC-NaOH-CPC/PBS ($p=0.608$) compared to Petroff. However positivity rate was significantly reduced for NALC-NaOH-CPC/PBS compared to NALC-NaOH/PBS, from 77.6% to 61.7% ($p=0.009$), while no contamination was detected either on TLA or on LJ. Four TLA plates inoculated with sediment from decontamination with NALC-NaOH/PBS and one with NALC-NaOH-CPC/PBS showed partial contamination which did not prevent their interpretation. LJ or MGIT^{960} showed better positivity rate than TLA when using NALC-NaOH. TAT for TLA slightly increased for samples added with CPC.

All plates inoculated after decontamination with NALC-NaOH-CPC/PBS presented debris that interfered with microscopy reading. In order to assess if Difco would better neutralize the effect of CPC, we substituted PBS for Difco buffer (Table 2). Overall positive rate for TLA and LJ combined using NALC-NaOH-CPC/Difco was 93.7%. The positivity rate significantly increased for both media compared to samples treated with NALC-NaOH-CPC/PBS, from 61.7% to 86.1% ($p<0.001$) for TLA and from 78.7% to 93.7% ($p=0.008$) for LJ, and no plates were contaminated. The median TAT was reduced on both TLA and LJ.

All plates could be clearly readable, as Difco buffer completely eliminated the debris (Figure 1).

Table 1. Performance of TLA, MGIT^{960} and LJ inoculated after decontamination with Petroff or NALC-NaOH neutralized with PBS

	Petroff (n=56)		
	TLA	**MGIT^{960}**	**McNemar's test p-value**
Positivity rate (%)	32 (57.1%)	33 (58.9%)	0.7815
Contamination rate (%)	9 (16.1%)	10 (17.9%)	0.7630
TAT (median (IQR) days)	7 [5-28]	16 [9-49]	0.0059

	NALC-NaOH/PBS (n=161)		
	TLA	**MGIT^{960}**	**McNemar's test p-value**
Positivity rate (%)	125 (77.6%)	142 (88.2%)	0.0011
Contamination rate (%)	6 (3.7%)	8 (5.0%)	0.5271
TAT (median (IQR) days)	8 [5-32]	15 [8-69]	0.001

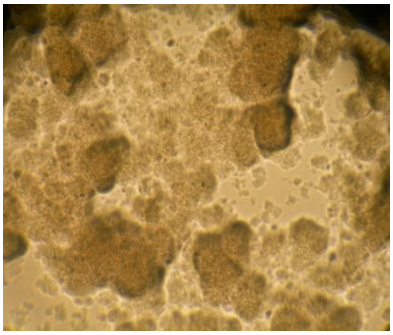
	NALC-NaOH -CPC/PBS (n=94)		
	TLA	**LJ**	**McNemar's test p-value**
Positivity rate (%)	58 (61.7%)	74 (78.7%)	0.0006
Contamination rate (%)	0 (0.0%)	0 (0.0%)	1
TAT (median (IQR) days)	12 [5-40]	28 [16-79]	<0.001

TLA: Thin Layer Agar; LJ: Löwenstein-Jansen; CPC: cetylpyridinium chloride; PBS: phosphate buffer; NALC-NaOH: *N*-Acetyl-L-Cysteine-Sodium Hydroxide; IQR: interquartile range; TAT: Turnaround time

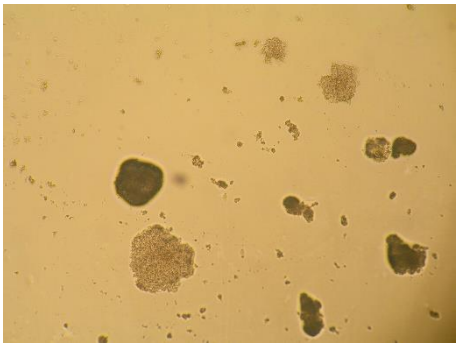
Table 2. Performance of TLA and LJ inoculated after decontamination with NALC–NaOH-CPC/Difco (n=79)

	TLA	**LJ**	**McNemar's test p-value**
Positivity rate (%)	68 (86.1%)	74 (93.7%)	0.0143
Contamination rate (%)	0 (0.0%)	0 (0.0%)	1
TAT (median (IQR) days)	8 [5-27]	26 [13-48]	<0.001

Fig. 1 TLA appearance by microscopy reading at x10 magnification with and without DIFCO neutralization



a. Sediment with CPC inoculated on TLA and neutralized with PBS



b. Sediment with CPC inoculated on TLA and neutralized with Difco buffer

Discussion

Thin Layer Agar (TLA) has been optimized for use with NALC-NaOH decontaminated samples. Use of other decontamination procedures had not yet been extensively validated.

In the first phase of our study samples processed with NALC-NaOH/PBS showed higher positivity rate than NALC-NaOH-CPC and Petroff, with contamination rate below the recommended threshold of 5-10 % for samples processed after long transport.[15] Petroff gave lower positivity rate for TLA, and higher contamination rate, in accordance with contamination rate of 17% on TLA and 21.1% in MGIT^{960} found by Martin et al.[7] However, in contrast to our results, positivity rate was very high, 97.3% on TLA and 97% on MGIT^{960} for smear positive samples. In our study Petroff also adversely affected performance of MGIT^{960}, which was not significantly different from TLA. These results for Petroff could be partially explained by the delay in samples processing, which was longer than for the samples decontaminated with other methods.

In our study, contamination rate for NALC-NaOH/PBS was lower than 16.5% and 26% reported by Mejia [8] and Martin.[6] Robledo, in a multicenter study, found rates between 2.7 to 9.5%.[16] These higher rates can be related to the lower concentration of antibiotics added to the medium, 0.02 µl/ml instead of the 4.0 µl/ml used in our study.

Smithwick reports attempts to find an alternative procedure to NALC-NaOH, failing to find a concentration of CPC that would allow growth of mycobacteria on agar while controlling contamination.[17] The author then concluded that CPC could be considered a valuable method if combined with LJ to recover mycobacteria. Selvakumar reported similar results, finding comparable positivity rates for samples treated with NaOH on the day of sampling and samples added with CPC and processed after 7 days, while performance of NaOH for samples CPC-free processed after one week was significantly lower.[4]

Our study demonstrated that CPC reduced contamination rate but increased TAT and significantly affected positivity, possibly due to the higher NaOH concentration we used

compared to the Smithwick study,[17] (2% versus 1% final concentration) and to the use of CPC in combination with NALC-NaOH, more harsh than CPC alone.

The use of Difco buffer to neutralize the effect of CPC greatly improved the performance of TLA in terms of positivity rate. The combination of TLA and Difco buffer resulted in recovery rates comparable to MGIT^{960} combined to NALC-NaOH/PBS, considered the gold standard method for decontaminating and culturing samples for mycobacterial investigation.

These results are similar to the findings from Pardini,[4] who reported higher positivity rate for samples containing CPC and neutralized with Difco buffer compared to NaOH alone, 63.8 % and 47.1% respectively, prior to inoculation on agar and LJ medium. Positivity rate increased to 78.3% after retreatment of sediments obtained from CPC-containing samples with NaOH alone. However in this study decontaminants where not applied simultaneously, results for this second retreatment are combined to results of CPC alone, and the author did not report the performance separately for agar and LJ.

In our experience Difco buffer also improved the reading of the plates by eliminating debris from CPC-containing samples, colonies were clearly visible by microscope at an earlier stage of growth, shortening the time to detection.

Recovery of mycobacteria on LJ from samples treated NALC-NaOH-CPC/PBS was lower than the rate reported in another study [18] Interestingly, the Difco buffer also improved the recovery rate on LJ from 78.8% to 93.7% (p=0.005), suggesting that CPC may not be completely neutralized by the egg-based medium during incubation.

However, except for Petroff, TLA positivity rate was significantly lower compared to the two gold standards. This could be due to the higher dilution of the inoculum on TLA compared to MGIT^{960} and LJ, required to permit microscopic interpretation, which could be decreased to improve positivity.

Even if CPC increased TAT for TLA plates, time to positivity for TLA was significantly shorter compared to MGIT^{960} and LJ, regardless the decontaminant used. In addition, NALC-NaOH-CPC/Difco decreased the time to detection to eight days, equal to the samples without CPC.

In conclusion, in our experience, NALC-NaOH-CPC/Difco represented the preferable method of decontamination of samples inoculated in TLA, improving also recovery of mycobacteria from LJ when applied to samples processed after transport in CPC. TLA showed a significant improvement in performance with this decontamination method, comparable to MGIT^{960}, and with a significantly shorter isolation time compared to LJ. The disadvantage of TLA is the lower positivity rate compared to LJ and the additional effort to read the plates using a microscope. However TLA, provides simultaneously detection and identification of culture growth without opening the plate, and limits the need for safety requirements in the laboratory compared to MGIT^{960} and LJ, which require manipulation of colonies to allow identification. As recovery rate on LJ for samples treated with Difco buffer showed to be the best method, the combination of TLA and LJ for samples treated with this reagent would represent the optimal strategy for both fast and sensitive mycobacterial recovery.

On CPC-containing samples, contamination was absent with both TLA and LJ methods, which suggests that it may be possible to reduce time of decontamination or NaOH concentration in order improve positivity.

Future studies can test whether Difco buffer on samples containing CPC can make these compatible with inoculation in liquid media to decrease the contamination rate while maintaining good recovery rates.

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CHAPTER IV

The thin-layer agar method for direct phenotypic detection of multi- and extensively drug-resistant tuberculosis

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Abstract

Background: Molecular techniques rapidly detect resistance to rifampicin (RMP) and isoniazid (INH) but do not eliminate the need for culture-based drug susceptibility testing (DST) to other drugs.

Objectives: The thin layer agar (TLA) test, a non-commercial direct-DST method, has demonstrated good performance for INH and RMP yet evidence is still limited, while its applicability for Ofloxacin (OFX) and Kanamycin (KAN) DST is unknown.

Design: We compared 279 TLA-DST to MGIT for INH and RMP, and 280 TLA-DST for OFX and KAN to 7H11 agar proportion method, obtained from 320 smear-positive samples from 165 Georgian TB patients. Discrepancies were solved by comparison to a composite reference standard. The MDR prevalence was 30 of 164 patients (18.3%) and 2 (6.7%) of those had XDR-TB.

Results: TLA showed 94.7%, 98.2%, 100% and 78.9% sensitivity for INH, RMP, OFX and KAN respectively with 100% specificity. Time to results was on average 7 days in TLA, 23 in MGIT and 49 for 7H11 agar.

Conclusion: In low resource settings TLA can be applied for the rapid detection of INH, RMP and fluoroquinolones resistance. Further studies are necessary to improve sensitivity for KAN, to further assess its performance for OFX and other drugs, and applicability in field conditions.

Introduction

To improve detection of multi-drug resistant tuberculosis (MDR-TB), WHO recommends the use of Xpert®MTB/RIF (Cepheid) and Line Probe Assays (LPA) like MTBDR*plus* (Hain LifeSciences).(1),(2) Whereas Xpert®MTB/RIF detects rifampicin (RMP) resistance directly from specimens, MTBDR*plus* detects resistance to both isoniazid (INH) and RMP, yet can be applied directly only to decontaminated smear-positive (Sm+) samples. Nucleic acid amplification tests (NAAT) are highly sensitive, yet they do not eliminate the need for culture and drug susceptibility testing (DST), still required to test other drugs and to monitor patients during treatment.(3) MTBDR*sl* (Hain LifeSciences) is the only commercial assay available for rapid detection of second–line drug resistance. However, its use to optimize patients' individualized regimens has not been endorsed yet(4), hence, DST for these drugs still relies on phenotypic testing.(2)

In 2009, WHO has assessed the performance of non-commercial methods such as the colorimetric redox indicator method (CRI), microscopic observation drug susceptibility assay (MODS), nitrate reductase assay (NRA) and thin layer agar (TLA). CRI, MODS and NRA have been recognized as valid methods, yet were recommended only as ad-interim solutions at reference laboratory.(5) For TLA, however, results were still considered insufficient to draw conclusions on performance and feasibility.(5)

TLA uses 7H11 agar plates directly inoculated with decontaminated Sm+ specimens. Reading by conventional light microscopy permits rapid detection of growth without opening the plates, reducing the biohazard risk.

Two studies report high performance of TLA for *Mycobacterium tuberculosis*-complex (MTBc) isolation and direct resistance detection, however, either the sample size was limited(6), or plates were read by naked eye, increasing time to detection.(7) Another study used TLA-DST as *indirect* test for RMP, ofloxacin (OFX) and kanamycin (KAN). (8) Hence, TLA performance as *direct* DST for OFX and KAN remains unknown.

In this study, we assessed the performance of direct TLA-DST in detecting INH and RMP resistance compared to indirect MGIT and OFX and KAN resistance compared to indirect 7H11 agar. Discrepant results were resolved using a composite reference standard (CRS) consisting of minimal inhibitory concentration (MIC) determinations and target gene sequencing. In addition, we compared the processing time and the cost for TLA to gold standard methods.

Matherials and methods

Study design

Every patient aged 15 years and older, with at least one Sm+ sputum by Ziehl-Neelsen (ZN) in the National Reference Laboratory (NRL) of Tbilisi and not having received TB treatment in the previous month, was eligible for the study. After signing the informed consent, patients submitted two additional expectorates that, if Sm+, were stored in the fridge until shipment at ambient temperature to the Mycobacteriology laboratory of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium. Only samples confirmed as Sm+ at ITM were included in the study.

This study protocol was approved by the Georgian- and Belgian ethical committees.

Laboratory procedures

In ITM, the samples were decontaminated with 1.5% Nalc-NaOH final concentration. After centrifugation for 20 min at 3000g the sediment was re-suspended in 1 ml distilled water. One drop was used to prepare a ZN smear and 500 µl was inoculated in MGIT culture according to the manufacturer's protocol. The remaining suspension was diluted 1:5 in sterile distilled water, and 100 µl inoculated in each of the six wells of a TLA plate (Corning Costar 3516), which consisted of one well for growth control (GC), one well for each drug and one well for p-nitrobenzoic acid (PNB) for differentiation between MTBc and non-tuberculous mycobacteria (NTM). Plates, prepared at ITM, contained 7H11 agar and OADC (BD 211886), plus amphotericin, piperacillin and trimethoprim, all at 4µg/ml concentration, to decrease contamination.(9) Drug-containing wells contained 0.2 µg/ml INH, 1µg/ml RMP, 2.0 µg/ml OFX (Sigma-Aldrich) and 6.0 µg/ml KAN (ICN) final concentrations. MTBc growth in MGIT, confirmed with SD-Bioline TB Ag MPT64 (Standard Diagnostic), was inoculated for INH and RMP DST in MGIT following manufacturer's procedure, and in parallel on Löwenstein-Jensen (LJ) for strain storage and for OFX and KAN DST by 7H11 agar indirect proportion method, according to international standards. The drug concentrations for DST on 7H11 agar were the same as for TLA, with resistance defined at a cut-off of 2.0 µg/ml for OFX and 6.0 µg/mg for KAN.

After inoculation, TLA plates were read on days 5, 7, 10, 13, 15, 20, 25, 30, 35, up to 40 days, adjusted to working days, using a conventional light microscope (objective 10 x). Growth in the

GC well paired with inhibition in the PNB well was considered positive for MTBc.(9) DST was interpreted if at least 10 colonies were present in the GC-well, otherwise considered invalid. Drug resistance was defined as any growth in the drug-wells.(7) TLA plates were read blinded to the MGIT and DST-7H11 agar results.

Discordant results were compared to a CRS consisting of MTBDR*plus* for INH and RMP, MTBDR*sl* for OFX, target gene sequencing (*rpoB, katG, inhA, gyrAB* and *rrs* 1400 region) and MIC, performed on LJ for INH and RMP, 7H11 agar for OFX and REMA for KAN, with cut-off at 0.2 µg/ml, 40µg/ml, 2µg/ml, 2.5 µg/ml respectively.(10) Finally, the CRS also included comparison of the results from the paired sample of the same patient, confirmed to have the same genotype by spoligotyping, to exclude laboratory errors. Test costs were calculated using manufacturers price list and FIND website. Time for tests performance was calculated based on the ITM laboratory technician's estimates for each step.

Results

Between November 2010 and February 2012, 362 Sm+ samples were received at ITM from 183 consecutive patients (Figure 1). Of them, 42 (11.6%) were found Sm- and excluded. Samples were decontaminated after a median of 12 days [IQR 9-14] from collection. Of the 305 (95.3%) MTBc-positive TLA plates, 7 showed partial contamination, which did not prevent interpretation of DST results. In MGIT, 306 (95.6%) cultures were positive, increasing to 313 (97.8%) after re-decontamination of 7 partially contaminated MGIT cultures. Of 320 Sm+ samples, direct TLA yielded valid DST results in 89%, indirect MGIT in 97.5% and indirect 7H11 agar in 97.2% of the samples (Table 1).

Table 1 TLA versus MGIT and 7H11 for provision of valid DST results

		MGIT DST		7H11 DST		
		DST valid	**others***	**DST valid**	**Others***	**Total**
TLA	**valid direct DST**	279	6	280	5	285 (89%)
	others*	33	2	31	4	35 (11%)
	Total	312 (97.5%)	8 (2.5%)	311 (97.2%)	9 (2.8%)	320

* Either culture negative, contaminated or invalid DSTs

In total, 279 DSTs were available for comparison for INH and RMP, with 35.1% resistance to either or both drugs, and 280 DST for OFX and KAN, with 8.2% resistance to either or both drugs. Sensitivity of TLA compared to indirect DST- prior to resolution of discordances- was 92.3%, 96.4%, 83.3% and 57.9% for INH, RMP, OFX and KAN respectively, while specificity was 100% for all drugs except for 99.6% for OFX (Table 2). Discrepant results between TLA and MGIT or 7H11 medium were compared to the CRS (Table 3). One discrepant DST result for INH and RMP was removed from the analysis as spoligotyping was discordant between the sediment used for TLA and the isolate tested in MGIT.

Table 2 Comparison of 279 DST results between by TLA , MGIT and 7H11 agar medium prior to resolution of discordances

		Thin Layer Agar				
		R	S	Tot	**Se**	**Sp**
MGIT	INH R	90	7	97	92.7% (95% CI 85.7-97.0)	100%
	INH S	0	182	182		
	Total	90	189	279		
	RMP R	54	2	56	96.4.% (95% CI 87.7-99.6)	100%
	RMP S	0	223	223		
	Total	54	225	279		
7H1	OFL R	5	1	6	83.3% (95% CI 35.9-99.6)	99.6% (95% CI 98.0-100)
	OFL S	1	273	274		
	Total	6	274	280		
	KAN R	11	8	19	57.9% (95% CI 33.5-79.7)	100%
	KAN S	0	261	261		
	Total	11	269	280		

Table 3 Confirmatory tests for discordant results

ID	ITM Sm result	Drug tested	Indirect DST	TLA	MTBDR*plus*	MIC (µg/ml)	Sequencing	Result from paired sample		Final result
								TLA	MGIT	
77B	3+	INH	R	S	*inhA* WT*; Kat*G delWT1,MUT1	>16	-	not available		R
51A	8AFB	INH	R	S	*inhA* WT; *Kat*G delWT1,MUT1	>16	-	not available		R
52B	3+	INH	R	S	*inhA* WT; *KatG* delWT1,MUT1	8	-	R	R	R
2B	1+	INH	R	S	*inhA* WT; *Kat*G delWT1,MUT1	>16	-	R	R	R
104 B	2+	INH	R	S	*inhA* WT; *Kat*G delWT1,MUT1	>16	-	R	R	R
19B	2+	INH	R	S	*inhA* WT*; Kat*G WT	0,1	*inhA* WT, *Kat*G WT	S	S	S
51A	8AFB	RMP	R	S	*rpo*B delWT8; MUT3	>160	*rpo*B Leu 531	not available		R
28A	4+	Ofx	R	S	*Gyr*A WT	4	*Gyr*AB WT	S	S	S
31A	3+	Ofx	S	R	Sediment and isolate not available			R	R	R
83A	2+	Kan	R	S	-	2,5	WT	DST invalid		S
96A	3+	Kan	R	S	-	2,5	WT		Sample A	S
96B	4+	Kan	R	S	-	2,5	WT		Sample B	S
4B	3+	Kan	R	S	-	2,5	WT	S	S	S
20A	4+	Kan	R	S	-	5	WT		Sample A	R
20B	4+	Kan	R	S	-	5	WT		Sample B	R
15A	3+	Kan	R	S	-	>5	WT	R	R	R
38A	2+	Kan	R	S	-	>5	A 1401 G	N/A	N/A	R

INH DST results were initially discordant for six samples, all resistant in MGIT and susceptible on TLA. Five isolates carried mutations in the *KatG* gene and showed an MIC above the cut-off, so were considered as false-susceptible on TLA. The sixth discrepancy was resolved in favour of TLA as the sediment was wild type (WT) by MDRTB*plus* and sequencing, the MIC was 0.1µg/ml and the paired sample confirmed INH susceptibility in both TLA and MGIT. All INH-resistant results on TLA were concordant with MGIT. While smear positivity was similar (p=0.2), time to TLA-GC positivity was significantly longer for discrepant DST results versus concordant INH results, 14 days (SD=3.14) versus 7 (SD=3.51) (p=0.04).

For RMP, one sample was TLA susceptible but MGIT resistant. MTBDR*plus* detected an *rpo*B gene mutation, confirmed by sequencing as Leu531, while the MIC was >160 µg/ml. This sample was considered as false-susceptible on TLA.

Of eight OFX-resistant results in either technique, two were discrepant. One isolate TLA susceptible but MGIT resistant had WT *gyrA* and *gyrB* profile by LPA and sequencing, with a MIC of 4µg/ml, one dilution above the critical concentration. The paired isolate was susceptible by both techniques, confirming OFX susceptibility in favour of TLA. From the remaining isolate resistant on TLA and susceptible on indirect DST, the sediment was not viable and the isolate was contaminated so not available for sequencing, however the paired isolate was resistant by both techniques, confirming OFX resistance in TLA.

Of the eight discordant KAN results, all TLA susceptible and 7H11 agar resistant, four were WT by sequencing, with MIC at 2.5µg/ml. Two of these WT isolates belonged to the same patient. Another patient yielded KAN susceptible results by both techniques for the paired isolate, whereas for the remaining patient paired DST results were not available. These four discrepant results were solved in favour of TLA. The remaining four samples showed an MIC ≥5 µg/ml, three were WT and one showed the *rrs* A 1401 G mutation. All four samples were considered false susceptible by TLA. To further investigate the reasons for KAN-discrepancy we established the MIC for all eleven concordant resistant results. All strains had an MIC of ≥5 µg/ml in REMA. Distribution of smear positivity grade and time to TLA-GC positivity did not significantly differ between concordant and discordant results.

After discrepancy resolving, the corrected TLA-DST sensitivity was 94.7%, 98.2% 100% and 78.9% for INH, RMP, OFX and KAN respectively with a specificity of 100% for all drugs (Table 4).

Table 4 Comparison of DST results between TLA and composite reference standard for INH, RMP, OFX and KAN after resolving discrepancies

Composite Reference standard	Thin Layer Agar					
		R	S	Tot	**Sensitivity**	**Specificity**
	INH R	90	5	95	94.7% (95% CI 88.2-97.7)	100%
	INH S	0	183	183		
	Tot	91	187	278		
	RMP R	55	1	56	98.2% (95% CI 90.4-100)	100%
	RMP S	0	222	222		
	Tot	55	223	278		
	OFL R	6	0	6	100%	100%
	OFL S	0	273	273		
	Tot	6	273	279		
	KAN R	15	4	19	78.9% (95% CI 54.5-93.9)	100%
	KAN S	0	260	260		
	Tot	15	264	279		

Of the 115 patients submitting two samples, 112 (97.4%) showed concordant TLA results on the paired samples while 3 (2.6%) were discordant for INH.

The delay between sample collection and processing was significantly longer (median 14 days [IQR 11-18]) for samples that yielded invalid TLA-DST results relative to those with valid results (median 12 days [IQR 9-14], p=0.009), while microscopy positivity was significantly higher (p<0.001) in samples yielding valid TLA-DST results. Valid TLA plates turned positive after a median of 7 days [IQR 6-10], versus 20 days [IQR 13-30] for the ones with invalid DST results. For conventional DST methods, MGIT results were available after a median of 23 days [IQR 20-26] including primary isolation and an additional 49 days for 7H11 including 21 days on average for LJ subcultures for a total delay of 72 days [IQR 69-75].

Excluding equipment and infrastructure costs, the consumables for TLA-DST are estimated at 2€ and 1.6€ for a smear (11), versus 7.5€ for MRDTB*plus*, 8.89€ for Xpert®MTB/RIF at FIND negotiated price, and 15€ for culture, identification and DSTs on MGIT and 7H11 agar, with MGIT consumables purchased at FIND negotiated prices. The total time for one TLA plate, including medium preparation, test processing and plate readings, is on average 30 minutes versus 50 minutes for the conventional DSTs, 2 hours for Xpert®MTB/RIF or 5 hours for MRDTB*plus* according to companies' instructions.

Discussion

This study shows that direct TLA-DST reliably detects INH and RMP resistance compared to the CRS and represents the first attempt to apply TLA as a direct method to detect OFX and KAN resistance, reducing the time to DST results from 72 to 7 days. TLA correctly detected OFX resistance when compared to the CRS, but performance for KAN was lower than for the other drugs. Several factors can contribute to KAN discrepancies, such as the MIC close to the cut-off or the possible drug adsorption by polypropylene tubes in the 7H11 agar test, while difficult inoculum standardization can only partially explain the fluctuation of results between techniques

as for the four KAN resistant strains missed by TLA, the results for other drugs tested were all concordant. While performance is lower compared to other phenotypic methods, the TLA sensitivity is higher than rapid methods such as indirect MDRTB*sl* (78.9% versus 66.9%). (12) Due to the high specificity, TLA results could be used to rapidly identify XDR-cases. Future studies on optimization of the TLA performance for injectable agents can test the effect of a less diluted inoculum, a lower drug concentration, additional plate readings after prolonged incubation, and comparing results between different injectables.

The culture positivity rate for TLA and MGIT was 95.3% and 97.8%, slightly higher compared to respectively 91.3 % and 96.7% found by Robledo. (6) Twenty TLA positive cultures showed insufficient growth on the GC to allow DST reading, with significantly lower microscopy positivity and longer sample processing delay than those with valid GC growth. Despite the significant impact of delay in sample processing on the mycobacterial viability and DST interpretation, also reported by others(13), contamination never prevented interpretation of the plates, while Robledo found a 4.1% contamination rate, which difference might be explained by our addition of antibiotic mixture. One pair of samples yielded different spoligotype patterns, suggesting either an administrative error, cross contamination during MGIT DST inoculation, or mixed infection in the patient.

Results for INH and RMP were slightly lower compared to the 100% sensitivity for both drugs on TLA reported by others(6),(7), but these studies included a limited number of drug resistant samples. Results are also in line with other non-commercial methods: 97% and 98% for INH and RMP using CRI and MODS, and 91% and 97% respectively with NRA, (5)while the specificity was lower than 100% for all techniques. (5)Comparing to other rapid methods, TLA sensitivity for INH was higher than MDRTB*plus* V.2 when applied to Sm+ (89.3%) (14) and comparable to MDRTB*plus* and Xpert®MTB/RIF (98.1% and 98.9% respectively) for RMP detection (15).

All five false-INH-susceptible isolates on TLA showed a high MIC and the missed RMP resistant isolate showed a 531Leu mutation, usually conferring high resistance levels.(16) False susceptibilities may be due to slower growth of some strains, to the short incubation time, or the difficulty in standardizing the inoculum for direct tests, however only one sample showed false susceptibility to more than one drug.

To decrease false susceptible results, Schaberg (7) suggests to limit dilution of the inoculum to ensure sufficient growth by diluting the sediment according to the sample microscopy grade. In our study sediments had different positivity grades (Table 3) but were all diluted 1:5 as re-suspension.

TLA provides results considerably faster than conventional DST and also compared to non-commercial direct DST methods, 7 days for TLA versus 10 days for NRA and 12 days for MODS. (17)(18)(19) Moreover, in contrast to conventional DST, TLA reduces biohazard risks as there is no need to open the plates, so can be implemented in moderate-risk TB laboratories.(20) In addition, TLA is less costly and presents a lower workload than gold standard techniques.

Conclusion

In low resource settings where Xpert®MTB/RIF is not available, TLA could present an affordable alternative to LPA to rapidly detect MDR and XDR-TB in high-MDR risk groups, while in combination to Xpert®MTB/RIF, TLA could be employed in Sm+ samples to rapidly detect resistance to INH and identify XDR-TB. In appropriate biosafety settings, the TLA GC would moreover provide isolates for extensive DST. TLA would also be of added value in patient monitoring, where NAAT are of little use, to detect drug resistance amplification in Sm+ and to provide information on culture conversion in Sm- cases. Further studies are needed to improve KAN performance, to extend OFX testing including different concentrations to detect high level FQ resistance, and to evaluate its application at field level.

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CHAPTER V

Thin layer agar-based direct phenotypic drug-susceptibility testing on sputum in Eswatini rapidly detects Mycobacterium tuberculosis growth and rifampicin resistance, otherwise missed by WHO endorsed diagnostic tests

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Abstract

Xpert MTB/RIF rapidly detects resistance to rifampicin (RR); however, this test misses I491F-R conferring rpoB mutation, common in southern Africa. In addition, Xpert MTB/RIF does not distinguish between viable and dead Mycobacterium tuberculosis (MTB). We aimed to investigate the ability of thin-layer agar (TLA) direct drug-susceptibility testing (DST) to detect MTB and its drug-resistance profiles in field conditions in Eswatini. Consecutive samples were tested in parallel with Xpert MTB/RIF and TLA for rifampicin (1.0mg/ml) and ofloxacin (2.0mg/ml). TLA results were compared at the Reference Laboratory in Antwerp with indirect-DST on Löwenstein-Jensen or 7H11 solid media and additional phenotypic and genotypic testing to resolve discordance. TLA showed a positivity rate for MTB detection of 7.1% versus 10.0% for Xpert MTB/RIF. Of a total of 4,547 samples included in the study, 200 isolates were available for comparison to the composite reference. Within a median of 18.4 days, TLA detected RR with 93.0% sensitivity (95% confidence interval [CI], 77.4 to 98.0) and 99.4% specificity (95% CI, 96.7 to 99.9) versus 62.5% (95% CI, 42.7 to 78.8) and 99.3% (95% CI, 96.2 to 99.9) for Xpert MTB/RIF. Eight isolates, 28.6% of all RR-confirmed isolates, carried the I491F mutation, all detected by TLA. TLA also correctly identified 183 of the 184 ofloxacin susceptible isolates (99.5% specificity; 95% CI, 97.0 to 99.9). In field conditions, TLA rapidly detects RR, and in this specific setting, it contributed to detection of additional RR patients over Xpert MTB/RIF, mainly but not exclusively due to I491F. TLA also accurately excluded fluoroquinolone resistance.
KEYWORDS resistance detection, TLA, Xpert MTB/RIF, tuberculosis, MDR, XDR, rpoB I491F mutant

Introduction

In 2019, about 10 million people globally developed tuberculosis (TB), and half a million people developed TB resistant to rifampicin (RR-TB) (1). Even if rifampicin (RMP) drug susceptibility testing (DST) coverage at TB diagnosis increased to 61%, a considerable number of patients with undetected RR-TB are still treated with an ineffective rifampicin-based treatment regimen. These patients are at high risk of treatment failure and continue spreading RR-TB (1). To enhance RR detection, the End TB strategy recommends improving case detection and DST coverage also with the use of molecular techniques. The tests used most commonly to detect drug resistance are Xpert MTB/RIF (Cepheid, USA), which simultaneously detects Mycobacterium tuberculosis complex (MTB) and RR, and line probe assays (LPA) such as GenoType MTBDRplus, (Hain Lifescience, Germany) for isoniazid (INH) and RMP and MTBDRsl for fluoroquinolone (FQ) and second-line injectables (2). The rollout of Xpert MTB/RIF, also in peripheral laboratories, substantially decreased diagnostic delay (3, 4). However, these rapid molecular techniques miss specific rpoB mutants at positions outside the RR determining region of the *rpoB* gene (RRDR), which are associated with equally poor treatment outcomes, as “common” *rpoB* mutants (5). One example is the I491F RR-conferring mutation among the so-called disputed mutations (6), which, in Eswatini, accounts for 56% of all RR-TB cases (7) and is reason for grave concern (8,9).

Furthermore I491F is, in most of the cases, tested as false rifampicin sensitive (RS) in mycobacterial growth indicator tube (MGIT) phenotypic DST (pDST) and is only detected by sequencing of the entire *rpoB* gene or slow pDST on solid media, such as Löwenstein-Jensen (LJ) (9–11). Isolates with these mutations are also partially missed by other non-commercial methods, such as microscopic observation direct susceptibility testing (MODS), with a reported sensitivity of 75% (12).

TLA has previously been described as an affordable method to detect MTB (13, 14). This technique has limited costs (15) and can provide results considerably faster than indirect pDST methods. Direct thin-layer agar DST (referred to as TLA below) is not among the non-commercial methods recommended by WHO (16). However, when TLA is used for simultaneous MTB and drug resistance detection, clinical samples are inoculated immediately after decontamination on the medium with and without antibiotics (17) avoiding the intermediate step of MTB isolation, which eliminates the need for high-level biohazard containment such as is needed for indirect pDST (18). These characteristics make this test suitable for low-resource settings (19), although studies on its applicability in routine practice are still limited. In settings where the prevalence of the I491F mutation is low, TLA has shown similar overall performance to indirect MGIT-DST in terms of sensitivity, specificity, and turnaround time (15, 17). However, the ability of TLA to detect RR due to mutations outside the RRDR is unknown.

The primary objective of this study was to investigate the use of TLA as a direct pDST test for RR-TB detection in a peripheral laboratory when applied to smear-positive (Sm+) and smear-negative (Sm-) sputum samples in a setting with a high prevalence of the rpoB I149F mutation. In addition, we describe the test performance for detection of MTB and resistance to ofloxacin (OFX) as an indicator for FQ resistance, a key class of drug in the treatment of RR-TB. DST results were evaluated in comparison to a composite reference standard that included genotypic plus phenotypic testing.

Results

MTB detection

Between January 2014 and December 2016, 3,097 patients provided a total of 4,547 samples. The overall MTB positivity rate was 7.1% (322/4,547) for TLA and 10.0% (456/4,547) for Xpert MTB/RIF (Table 1). Among Sm+ samples, TLA positivity was 68.6% (138/201) versus 90.7% (176/194) for Xpert MTB/RIF, compared to 3.7% (153/4,107) versus 5.7% (234/4,130), respectively, for Sm- samples (P=0.0001 in both groups). Among Xpert MTB/RIF-negative or inconclusive samples (n = 3,972), the relative gain of TLA for detection of MTB, when performed as a follow-on test after Xpert MTB/ RIF, was 7.5% (34/456), while the reverse, the relative gain of Xpert MTB/RIF over TLA for MTB detection, was 55.9% (180/322).

The median turnaround time (TAT) between sample collection and TLA inoculation was 4 days (interquartile range [IQR], 2 to 7), while the median time from inoculation to TLA positivity was 11 days (IQR, 7 to 19).

When inoculation was performed within 4 days, or from 5 days or later from sample collection, the TLA positivity rate for Sm+ samples decreased from 72.6% to 59.0% (P=0.053), while the

negative rate increased from 20.1% to 28.3.8% (P=0.16), though it did not reach statistical significance. Contamination varied from 7.4% to 12.1% (P=0.27).

Table 1 Detection of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria, for TLA versus Xpert®MTB/RIF

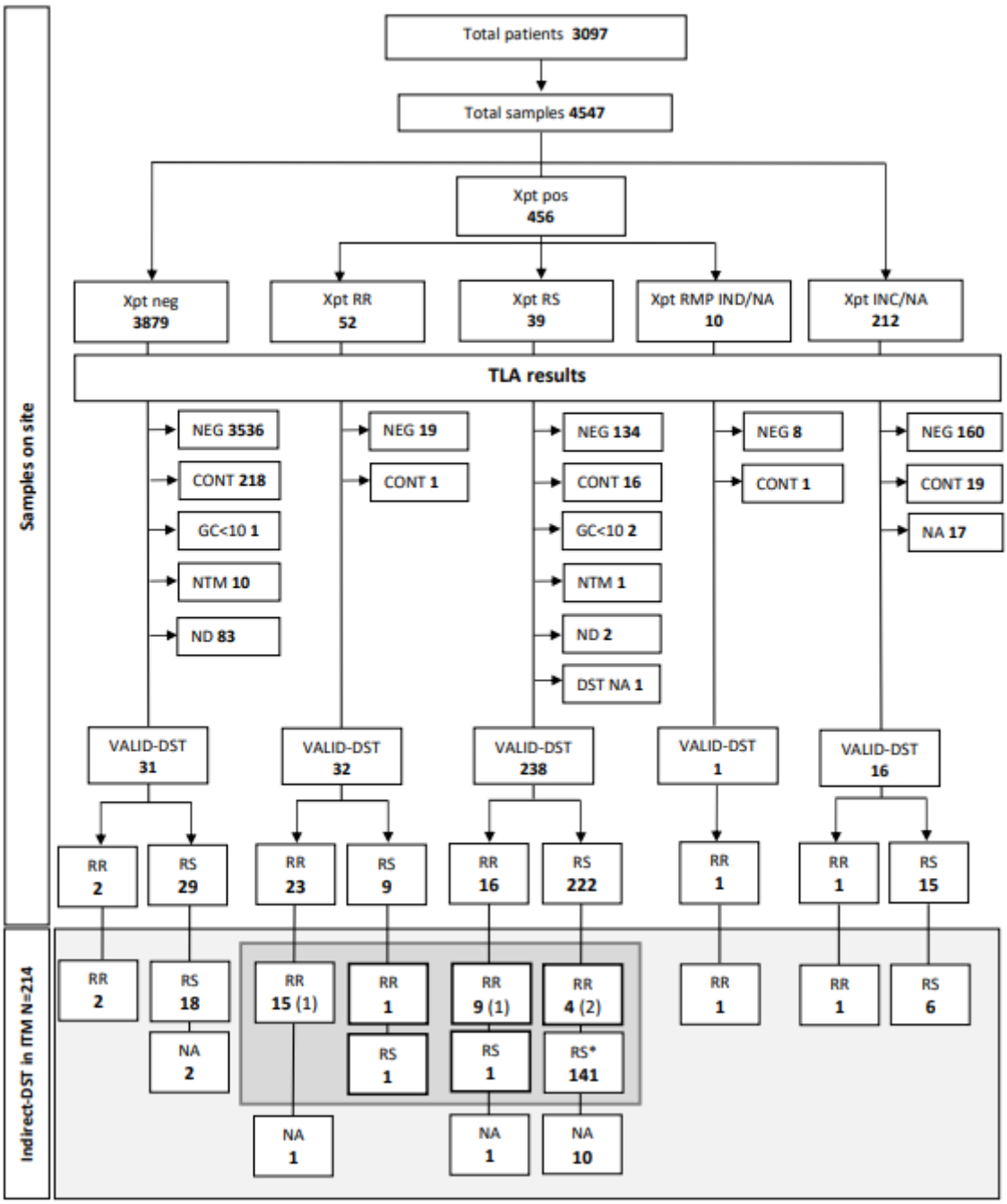
	TLA										
	Neg		**Pos**		**NTM**		**Cont**		**Unknown**		**Total**
	N	%	N	%	N	%	N	%	N	%	N
Total	**3857**	**84.8**	**322**	**7.1**	**11**	**0.2**	**255**	**5.6**	**102**	**2.2**	**4547**
Xpert											
Neg	3536	91.2	32	0.8	10	0.3	218	5.6	83	2.1	3879
Pos	161	35.3	274	60.1	1	0.2	18	3.9	2	0.4	456
High	24	15	124	77.5	1	0.6	11	6.9	0	0	160
Medium	39	35.1	70	63.1	0	0	2	1.8	0	0	111
Low	39	39.4	56	56.6	0	0	3	3	1	1	99
Very Low	59	68.6	24	27.9	0	0	2	2.3	1	1.2	86
Inconclusive	76	81.7	2	2.2	0	0	12	12.9	3	3.2	93
Unknown	84	70.6	14	11.8	0	0	7	5.8	14	11.8	119

TLA=thin layer agar; Xpert=Xpert®MTB/RIF; MTBc=Mycobacterium tuberculosis complex; NTM = non-tuberculous mycobacteria; Inconclusive= error, invalid, no result

Results available for evaluation of TLA for detection of drug resistance

Out of 322 MTB-positive TLA plates, 214 (66.5%) had the corresponding LJ slants sent to the Institute of Tropical Medicine (ITM) (Fig. 1). At ITM, 200 (93.5%) of the isolates grew after subculturing. Four (2.0%) isolates were classified as administrative errors (Table S1 in the supplemental material), leaving 196 (98%) isolates available for analysis.

Figure 1 Phenotypic rifampicin resistance testing results from the site (direct, TLA) and the reference laboratory (indirect, LJ)



Xpt, Xpert MTB/RIF; INC, result inconclusive (error, invalid, no result); RMP, rifampicin; GC, growth control; NTM, nontuberculous mycobacteria; IND, indeterminate; NA, not available; (n), administrative errors excluded; for details refer to Table S1. In the gray area, results for 172 isolates with indirect-DST, Xpert, and TLA results available, including administrative errors; *, 100 isolates selected for WGS, in addition to all RR detected by any method.

RMP resistance detection

Of 196 isolates included and with valid indirect-DST results at ITM, 168 (85.7%) had a valid initial Xpert MTB/RIF result. For another 28 (14.3%) samples, Xpert MTB/RIF results were either indeterminate or not available, while TLA was concordant with indirect-DST (Table 2).

After resolution versus the composite reference standard, a total of 168 (85.7%) isolates were finally classified as RS and 28 (14.3%) RR. Of the 168 samples with valid Xpert MTB/RIF results, 157 (93.5%), were concordant between Xpert MTB/RIF and TLA (142 RS and 15 RR), while 11 samples (6.5%) had discordant results between the two tests (Table 2). Of the 142 RS TLA and Xpert MTB/RIF concordant samples, one was RR by indirect-DST, making a total of 12 (6.1%) discordances for the 196 isolates tested between any two of the three tests.

With Xpert MTB/RIF, 9 (5.4%) isolates were falsely reported as RS, and one falsely showed RR, giving a sensitivity of 62.5% (15/24; 95% confidence interval [CI], 42.7 to 78.8) and specificity of 99.3% (143/144; 95% CI, 96.2 to 99.9). On TLA, RR was missed in two isolates, and in another isolate, TLA falsely showed RR. Hence, the sensitivity of TLA to detect RR was 93.0% (26/28; 95% CI, 77.4 to 98.0), and specificity was 99.4% (167/168; 95% CI, 96.7 to 99.9).

Most discrepancies between TLA and Xpert MTB/RIF RMP results were due to the presence of non-RRDR mutations outside the Xpert MTB/RIF target (five I491F and one V170F mutation) or wild-type (WT) rpoB genes with variable phenotypic results. All non-RRDR mutations were detected by TLA and had an RMP minimal inhibitory concentration (MIC) of 160mg/ml. While V170F in one isolate was detected by MGIT, only 2 of 5 I491F mutations were detected by the liquid medium (Table 2). In addition to the five I491F mutants found among TLA-Xpert MTB/RIF-discrepant results, another three I491F mutants were detected; one I491F in combination with M434I was RR by all tests, and two (one showing I491F mutation alone and one in combination with M434I and S450L) had no Xpert MTB/RIF RMP DST results (MTB not detected or RMP indeterminate). So, in total, 8/28 (28.6%) of the confirmed RR isolates had the I491F mutation, all of them showing also an S315T mutation in katG by whole genome sequencing (WGS).

Two paired isolates from the same patient were RR by all phenotypic tests and Xpert MTB/RIF yet had a WT rpoB sequence (Table 2).

Table 2. Rifampicin test results of TLA and Xpert®MTB/RIF, against composite reference standard

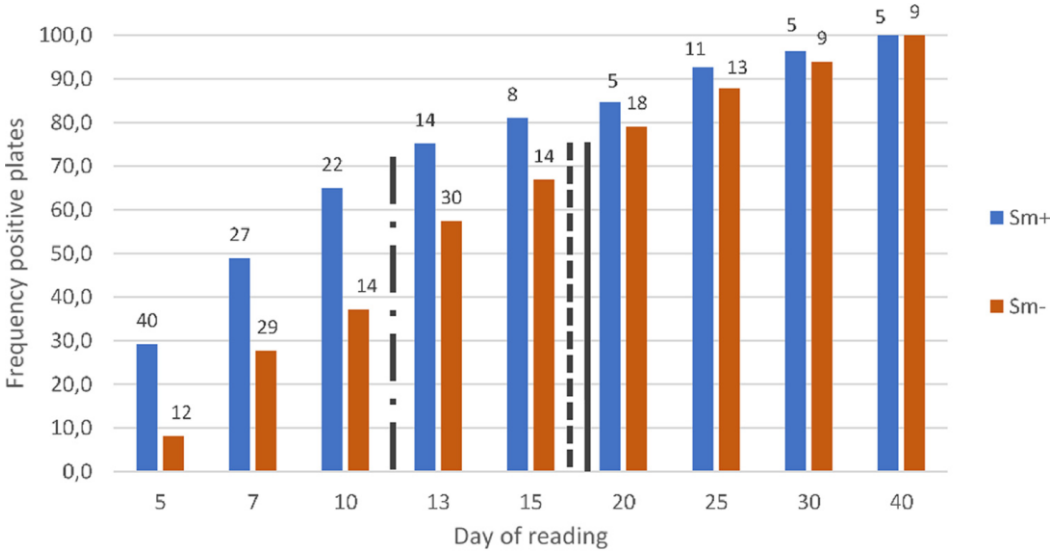
CRS	N	*rpoB* target Sanger/ WGS	LJ	Xpert	TLA RMP DST	MDRTBplus	LJ-MIC (µg/ml)	MGIT DST	TLA final interpretation	TLA – Xpert concordance
RMP R (n=28)	1	M434I; I491F/ M434I; I491F¥	R	R	R	na	na	na	TRUE RR	Concordant
	3	na/ H445L	R	R	R					
	8	na/ S450L	R	R	R					
	2	WT/WT€	R	R	R					
	1	H445D/H445D	R	NEG	R	na	na	na	TRUE RR	na
	1	I491F;S450L;M434I/	R	NEG	R					
	1	I491F/I491F¥	R	IND	R					
	1	S450L/S450L	R	na	R					
	5	I491F/I491F	R	S	R	WT	160-	3S, 2R	TRUE RR	Discordant
	1	H445L+WT/WT*	R	S	R	WT	160	S		
	1	V170F/V170F	R	S	R	WT	160	R		
	1	WT/WT°	R	S	R	WT	160	R		
	1	S450L/S450L	R	R	S	delWT,MUT3	160	R	FALSE RS	Discordant
	1	WT/WT°	R	S	S	WT	160	R	FALSE RS	Concordant
RMP S (n=168)	9	na/WT	S	S	S	na	na	na	TRUE RS	Concordant
	6	WT/nd	S	S	S					
	3	na/na	S	S	S					
	2	na/na	S	na	S	na	na	na	TRUE RS	na
	1	WT/WT	S	R	S	WT	20	S	TRUE RS	Discordant
	1	WT/WT	R	S	S	WT	20	S	TRUE RS	Concordant
	1	WT/WT	S	S	R	WT	20	S	FALSE RR	Discordant

CRS= composite reference standard; WGS=whole genome sequencing; Xpert=Xpert®MTB/RIF; LJ= Löwenstein-Jensen; TLA= thin layer agar; RMP=rifampicin; MIC=minimal inhibitory concentration; MGIT=mycobacterial growth indicator tube; na= not available; §=one result invalid; ¥= isolates with I491F mutations either detected also by Xpert, or without Xpert result; IND= RMP indeterminate; €= paired isolates with *rpoB* sequencing WT results; °=P631S mutation in the *ponA1* region;*=rpoB H445L+ L452P detected at low frequency mode.

Two additional isolates were consistently phenotypic RR (indirect-DST LJ, MIC LJ, MGIT, and TLA in one isolate), while all molecular assays (Xpert MTB/RIF, LPA, Sanger sequencing, and WGS) suggested a WT rpoB gene. In both isolates, WGS detected a P631S mutation in the ponA1 region (Table 2). One isolate carrying the H445L-elusive mutation was RS by Xpert MTB/RIF and RR by indirect-DST LJ.

Overall, the median time to detect RR was 18.4 days, similar to 17.0 days for resistance conferred by I491F alone (P = 0.8) and 18.3 days for isolates carrying other RR-conferring mutations, but significantly longer than the 12.2 days for RS isolates (P = 0.03) (Fig. 2).

Figure 2 Number of positive plates and cumulative percentage per day of reading



Sm+, sputum smear microscopy positive; Sm-, sputum smear microscopy negative. Indication of median positivity and drug susceptibility results for rifampicin-susceptible (S) isolates (black line with dashes and dots), rifampicin-resistant isolates with mutations different from I491F (solid black line), and rifampicin-resistant isolates with I491F mutation only (dashed black line).

OFX drug resistance detection. Overall, of the 196 isolates included in the analysis, 185 had valid indirect-DST results in ITM. One isolate carrying mutation D94N was OFX-R (2.0mg/ml) on indirect-DST and correctly detected by TLA. A total of 184 isolates were classified as OFX-S, all except one correctly identified by TLA (183/184; specificity 99.5%; 95% CI, 97.0 to 99.9) (Table 3), including three isolates that were OFX-R by indirect- DST but susceptible by molecular tests. One isolate was false OFX-R but WT for all molecular test.

Table 3 Thin layer agar results versus indirect-DST and composite reference standard for resistance to Ofloxacin

OFX result by CRS	N of isolates	*gyrA/B* target Sanger sequencing/WGS	LPA	LJ (µg/ml) OFX 2.0	TLA (µg/ml) OFX 2.0	TLA final interpretation
OFX-R (n=1)	1	na/D94N	na	R	R	TRUE OFX R
OFX-S (n=184)	117	na/WT	na	S	S	TRUE OFX S
	1	na/WT	na	S	R	FALSE OFX R
	62	na/na	na	S	S	TRUE OFX S
	1	WT/na	WT	S	S	TRUE OFX S
	3	WT/WT*	WT	R	S	TRUE OFX S

CRS= composite reference standard; TLA=Thin layer agar; OFX=ofloxacin; WGS=whole genome sequencing; na=not available;*= for 1 isolate WGS not done;

Discussion

This study evaluated the TLA performance for MTB detection and direct DST for RMP, alongside OFX testing. In Nhlangano, a peripheral and low-resource setting, TLA showed a relatively high positivity rate for MTB detection, albeit below the 83% between Sm+ samples reported by other studies (13, 14). In our study, culture positivity was slightly affected by a delay in sample processing, while the contamination rate did not significantly increase. These results suggest that TLA may be suitable in laboratories at peripheral levels, where samples collected from remote areas are transported for testing. TLA performed excellently to detect RR after a median of 18.2 days.

In our study, more than 80% of the discordances between initial Xpert MTB/RIF and TLA were resolved in favour of TLA, which also correctly detected all I491F mutations, which accounted for almost half of all discordances.

In Eswatini, the prevalence of I491F is reason for concern. This mutation is regularly missed by Xpert MTB/RIF, a limitation that persists in the new version, Xpert MTB/RIF Ultra (20), with patients misdiagnosed as having RS-TB and receiving repeated rounds of ineffective first-line treatment. The national drug resistance survey carried out in Eswatini from 2017 to 2018 (7) has shown that the prevalence of I491F in MDR isolates has reached 56% compared to 30% detected by the survey from 2009 (9, 11). In our study, I491F caused 28.6% of all RR, although our findings may not be representative of the entire country. Indeed, WGS analysis showed that isolates with I491F mutations belong to a particular outbreak clone that evolved over time and acquired further resistance to first- and second-line drugs (21, 22). Thus, isolates with this particular mutation, not detected by standard diagnostic tests, are an enormous public health problem.

To improve rapid detection of these missed RR cases, the new algorithm proposed by the National TB Program in 2019 includes starting empirical multidrug-resistant TB (MDR-TB) treatment for all detected INH-R cases while pursuing pDST on solid medium and sequencing of the rpoB gene to determine RR (23). This approach is supported by our results, where all the isolates with I491F mutations also carried mutations in the katG gene, correlated with INH resistance. In this algorithm, TLA could play a role to rapidly detect these RR cases at a peripheral

laboratory equipped for moderate hazardous containment (biosafety level 2 [BSL2]) (18) while waiting for sequencing results.

Partial fitness loss for isolates carrying the I491F mutation has been proposed as a reason for false RS results in MGIT due to the short incubation time (maximum, 14 days) (5, 10). Despite the relatively short turnaround time on TLA (median, 17.0 days), none of the I491F strains were false RS on TLA compared to three of the five MGIT tested. The time to detection for the I491F mutants did not differ from the ones carrying other RR-conferring mutations, albeit collectively, the rpoB mutants grew significantly slower than rpoB wild-type isolates on primary isolation.

Besides I491F, another non-RRDR mutation (V170F) detected in one isolate was missed by Xpert MTB/RIF and LPA, yet was detected by TLA. This mutation is globally less frequent (24), is reliably detected by pDST, including MGIT, and has not yet led to known micro-epidemics.

In our study, one (16.7%) of the isolates carrying an H445L-elusive mutation, also showed a WT minority population. RR was detected by TLA, LJ-based pDST, and Sanger sequencing, but missed by rapid molecular tests and MGIT. The mutation was detected by WGS only at low frequency. Indeed, heteroresistance may be the cause of false-susceptible results. Tests have different limits of detection for minority populations, as low as 1% for phenotypic tests, 5% for MTBDRplus, 20 to 40% for Xpert MTB/ RIF classic, 20% for Sanger sequencing (25), and 1% for WGS (26), depending on coverage depth. In addition, detection of minority populations and variants causing resistance can be challenged by preselection during primary culture isolation and multiple subcultures (27).

Surprisingly, two isolates, phenotypically RR by indirect-DST, MGIT, LJ-MIC, and, in one case, by TLA, were WT rpoB by Sanger sequencing, while WGS showed presence of the mutation P631S in ponA1. Polymorphisms in this region, which encode a protein involved in mycobacterial growth and cell wall synthesis, seem to constitute and be of advantage for growth in the presence of rifampicin and modify susceptibility to this drug (28, 29). Even if rare, the role of this mutation and other non-rpoB resistance-conferring mechanisms should be further investigated.

Of the three TLA OFX-S isolates found OFX-R on indirect-DST yet were WT by sequencing, two (paired isolates from the same patient) were borderline resistant by the indirect-DST, as they showed the same number of colonies in the drug-free and drug-containing tubes, one of them positive only with three colonies. Although these borderline results could be the cause of discordances, indirect-DST could not be repeated. For the third, no obvious explanation for the discrepancy could be found.

Laboratories in peripheral settings usually lack high-risk TB facilities with stable power supply, negative pressure, and complex equipment that is regularly maintained, which limits the implementation of phenotypic testing, especially indirect- DST (30). TLA direct DST, similarly to MODS, poses less biosafety risk, lowering requirements for moderate-risk facilities to contain biohazard potentially created by sample centrifugation and vortexing (18). In a head-to-head comparison, TLA showed to be superior to MODS in detecting resistance due to I491F mutation (12).

Our study is the first to test TLA for RMP and OFX in a high endemic setting at district level, although numbers did not suffice for full assessment of OFX performance.

A drawback pointed out for direct TLA DST is the lack of standardization of the inoculum (31), although we did not find an association of bacterial load (as determined by smear microscopy) and false DST results. Second, the direct nature of TLA testing complicates appropriate quality controls, for which fresh sputum is needed, as manipulation of strains would increase biosafety requirements. However, the use of samples spiked with avirulent strains, compatible with BSL2 hazard containment, could be considered for this purpose (32).

For 4 isolates (2.0%), discordant results could not be explained and, for this reason, were considered administrative errors. These errors are not infrequent in diagnostic laboratories (33) that handle large numbers of tests.

The TLA technique requires multiple readings of the plates, which is time-consuming. Our cumulative data suggest that the workload could be reduced by decreasing the number of readings, as readings at days 28 and 35 provided relatively low incremental yield. For future testing, reading at days 7, 14, 21, and 42 may be logistically more feasible, as well as the use of a redox indicator allowing for macroscopic reading, as suggested by recent studies (34, 35).

Our study presented some limitations. It was not possible to link on-site multiple samples collected from the same patient, so a per-patient analysis of the results was not possible. In addition, confirmatory tests were performed only on isolates showing discordances. TLA showed to correctly exclude resistance in all susceptible cases; however, our samples did not include a sufficient number of isolates resistant to OFX to fully evaluate TLA performance. In addition, molecular tests for concordant OFX tests were only partially available. In our study, OFX was considered an indicator of resistance to the class of FQs. The full performance of TLA for detection of FQ resistance, possibly including other FQs than OFX, should be assessed in settings with higher rates of FQ-R, or when TLA is used after Xpert RR diagnosis. Nevertheless, our study shows that TLA provides numerous advantages.

In settings with a high prevalence of I491F mutations, TLA could be very useful for patients with presumed RR-TB yet are Xpert MTB/RIF RS-TB. Especially in patients with INH-R and missed RR-TB, the use of an FQ-strengthened regimen, as recommended by WHO guidelines (36), would result in ethambutol and pyrazinamide as sole effective drugs, increasing the risk of developing extensively drug-resistant TB (XDR-TB) (37).

The relative gain for MTB detection by TLA when used after Xpert was limited, confirming that Xpert should be the first test for diagnosis (15, 17). However, TLA could be used for monitoring patients during treatment and also to detect amplification of RRTB in patients failing first-line treatment, especially if missed at baseline. Validation of TLA for new and repurposed drugs would be the logical next step, given the WHO recommended pDST, as TLA could replace, in the flowchart, indirect-DST in patients found to have molecular evidence of RMP and/or INH resistance. The level of phenotypic resistance to bedaquiline that is conferred by *Rv0678* mutations is, for instance, largely unknown, and TLA could save weeks over indirect pDST and at a lower biosafety level.

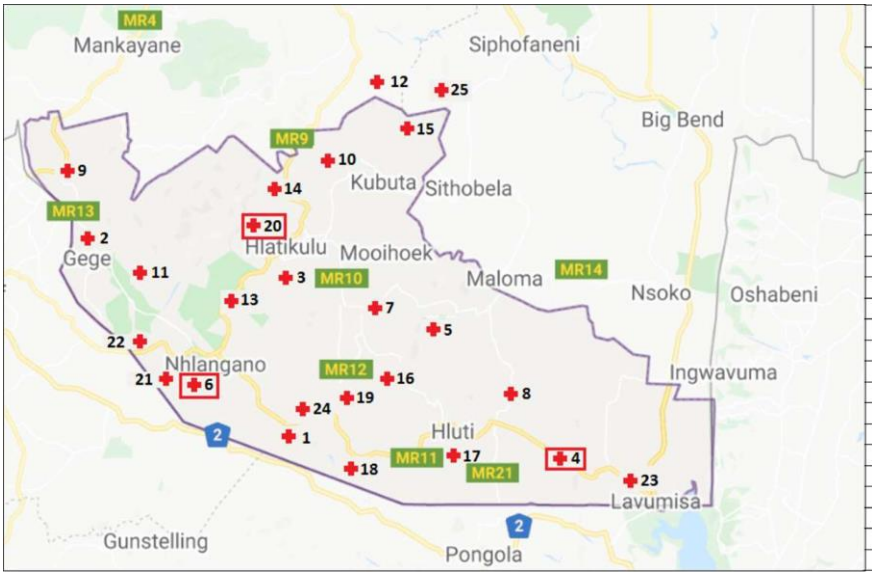
In conclusion, TLA provides a relatively rapid diagnostic approach for detecting viable TB bacilli and simultaneous susceptibility testing for RMP, showing excellent sensitivity for the detection of RR due to the I491F mutation outside the RRDR that plagues Southern Africa. In the diagnostic

algorithm of this setting, TLA could be used to test presumed RR patients yielding an RS result by Xpert MTB/RIF.

Materials and methods

Study patients and test flow. The study was conducted in the Nhlangano TB laboratory (NTBL), Eswatini, by Médecins Sans Frontières Switzerland and the Institute of Tropical Medicine (ITM), Antwerp, up to 90 km of distance (map in Fig. 3), from where samples were sent to the NTBL in cold chain (2 to 8° C) without preservatives.

Figure 3 Sample collection sites in the Shiselweni region (Google map, modified). Distance and time of driving from the site of collection to the closest of the three TB laboratories (shown by red squares)



	Site name	N of samples collected	Distance (Km)	Time (min)
1	Zombodze	372	79.2	76
2	Gege	287	31.2	48
3	Nhletjeni	71	22.8	31
4	Matsanjeni	256	0	0
5	Ntshanini	150	48.1	70
6	Nhlangano HC	855	0	0
7	New Haven	238	37.9	52
8	JCI	24	90.3	77
9	Magubheleni	516	42.0	67
10	Mfishane	43	22.6	30
11	Mashobeni	353	20.7	38
12	Moti	23	39	55
13	Dwaleni	356	16	20
14	Nkwene	76	47.3	39
15	Kaphunge GVT	17	40.6	66
16	Silele	91	38.7	53
17	Hluti	101	21.5	18
18	Olos	80	31.5	29
19	Jericho	79	35.9	45
20	Hlathikhulu	72	0	0
21	Mahlandle	87	20	25
22	Tfokotoani	264	15.4	18
23	Lavumisa	89	17.4	14
24	Mhlosheni	36	49	40
25	Kaphunga	10	40.6	66

We included all samples collected from consecutive patients older than 15 years who presented signs of presumptive TB, did not start TB treatment in the previous month, and consented to participate in this study. As per routine practice, patients were asked to produce two good-quality sputum specimens (labelled as sample A and sample B) in 50-ml sterile screw-cap containers and, when collected at the clinics, sent at the health centers, where sample A was tested by Xpert MTB/RIF following Cepheid procedures. Any patients who were Xpert MTB/RIF positive for TB on sample A, regardless of the RMP result, had sample B sent to the National TB Reference Laboratory (NTBRL) in Mbabane for routine testing with liquid culture and pDST. In this case, sample C was collected for the purpose of this study. All leftovers from sample A, sample B, and sample C, if collected, were decontaminated and tested for the study with TLA, fluorescence microscopy (FM), Xpert MTB/RIF, and LJ culture at the NTBL. For any positive TLA plate inoculated with either sample A, B, or C, the corresponding LJ isolate was sent to ITM for extended phenotypic and genotypic testing. In case the LJ culture was contaminated or remained negative, an LJ subculture from the growth of the corresponding TLA plate was shipped.

Laboratory methods at the NTBL. We performed TLA using 4-quadrant polystyrene plates prepared at the NTBL. The medium contained 7H11 agar supplemented with a broad-spectrum antibiotic mixture to suppress contamination, as previously described (15, 17). TLA plates included drug-free growth control (GC), p-nitrobenzoic acid (PNB) (500mg/ml), RMP (1.0mg/ml), and OFX (2.0mg/ml). The processed sputum sediment was resuspended with 2ml phosphate-buffered saline (PBS), and two drops were inoculated per well.

During incubation at 5% CO2, plates were read at day 5, 7 10, 13, 15, 20, 25, 30, 35, and 40 as previously described (15), with few modifications. In addition, the drug-containing wells were read on the day of GC positivity and on the next scheduled reading.

Plates were reported as positive for nontuberculous mycobacteria (NTM) in case of mycobacterial growth in the quadrant containing PNB, while no or poor growth (#3 colonies) on PNB with a positive GC compartment was considered positive for MTB (38). Any growth on PNB was tested with the MPT64 Ag test (SD Bioline).

Laboratory methods at the reference laboratory. Upon receipt of isolates at ITM, fresh subcultures were made on LJ medium, and indirect-DST was performed using the proportion method on LJ for RMP (40mg/ml) and on 7H11 for OFX (2.0mg/ml). Indirect solid DSTs were read blindly with respect to TLA.

All isolates with a discordant RMP result between any two of three tests (Xpert MTB/RIF, TLA, or indirect- DST) were further tested by MTBDRplus and had pDST done in MGIT (RMP 1.0mg/ml), and the MIC for RMP was determined on LJ (10 to 160ml/ml). All isolates showing RR on any test and 100 isolates showing RS on all tests were investigated by Sanger sequencing of the rpoB target at ITM and/or by whole-genome sequencing (WGS) performed at the Borstel Research Center (Germany) as previously described (39, 40). To constitute a composite reference standard for RMP resistance, MIC prevailed on pDST, and resistance found on any of the genotypic tests overrode any phenotypic result.

In the case of discordance between TLA and indirect-DST for OFX, isolates were investigated by LPA MTBDRsl and sequencing (target genes and/or WGS). As composite reference standard, any resistance related to a high-confidence mutation found on any of the genotypic DST overrode results showing susceptibility on another test.

Statistical analysis. For all tests conducted at the NTBL, we calculated the MTB positivity rate as the number of samples showing MTB over the total number of samples tested. The relative gain of TLA for detection of MTB over Xpert MTB/RIF was calculated as the number of samples that were TLA positive but Xpert MTB/RIF negative or inconclusive (including error, invalid, or no result) divided by the total number of samples positive on Xpert MTB/RIF. The relative gain of Xpert MTB/RIF versus TLA was calculated as well. We also calculated the median turnaround time (TAT) between sample collection and inoculation and the median time from inoculation to each test result and TAT for DST availability stratified by RMP resistance.

For all isolates received at ITM, we calculated the sensitivity and specificity (with 95% confidence intervals [CIs]) of TLA to detect resistance for RMP and specificity for OFX against the composite

reference standards. Implausible discordant results (e.g., non-RRDR mutation on WGS but detected by Xpert[41]) were considered administrative errors and excluded from the analysis.
All statistical analyses were performed with Stata 12 software (Stata Corporation, College Station, TX).

Ethics approval. The study protocol was approved by the Institutional Review Board of ITM, the Ethics Committee of the University Hospital of Antwerp, Belgium, and the Ethics Committee of Eswatini.

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CHAPTER VI

Evaluation of OMNIgene® SPUTUM and ethanol reagent for preservation of sputum prior to Xpert and culture testing in Uganda

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Abstract

Background: Xpert MTB/RIF (Xpert) and culture are the most reliable methods for tuberculosis diagnosis but are still poorly accessible in many low resource countries. We aimed to assess the effect of OMNIgene® SPUTUM (OM-S) and ethanol in preserving sputum for Xpert and OM-S for mycobacteria growth indicator tube (MGIT) testing over a period of 15 and 8 days respectively.
Methods: Sputum were collected from newly diagnosed smear-positive patients. For Xpert, pooled samples were split into 5 aliquots: 3 for Xpert on day 0, 7 and 15 days without additive and 2 with either OM-S or ethanol at day 15. For MGIT, 2 aliquots were tested without preservative and 2 with OM-S at 0 and 8 days.
Results: A total of 48 and 47 samples were included in the analysis for Xpert and culture. With Xpert, using Day 0 as reference, untreated samples stored for 7 and 15 days showed concordance of 45/46 (97.8%) and 46/48 (95.8%). For samples preserved with OM-S or ethanol for 15 days compared with untreated samples processed at day 0 or after 15 days, OM-S concordance was 46/48(95.8%) and 47/48(97.9%), while ethanol was 44/48 (91.7%) and 45/48 (93.8%). With MGIT, concordance between untreated and OM-S treated samples was 21/41(51.2%) at Day 0 and 21/44(47.7%) at day8.
Conclusions: Xpert equally detected TB in OM-S treated and untreated samples up to 15 days but showed slightly lower detection in ethanol treated samples. Among OM-S treated samples, MGIT positivity was significantly lower compared to untreated samples at both time-points.
Key words: OMNIgene®, Tuberculosis, Xpert, Culture

Introduction

Tuberculosis (TB) represents one of the most prevalent infectious diseases in the world, with an estimate of 10 million incidence cases in 2017, majority from low or middle income countries (1). In 2010, World Health Organization (WHO) endorsed Xpert MTB/RIF (Xpert) (Cepheid, Sunnyvale, CA), for simultaneous detection of TB and resistance to rifampin {Formatting Citation} and the test has been widely adopted for TB diagnosis (1). Nevertheless Xpert remains unavailable in most primary health care centres where majority of patients with presumptive TB seek care (3). Culture is the gold standard test to confirm TB, but is slow, laborious, and due to requirement for biohazardous containment, is available mainly in high level laboratories. With Xpert placed at district hospital and culture at regional hospital and national reference laboratory in many low-resource countries, sputum samples must be transported from peripheral locations for testing. In some remote setting, high temperatures and long transport make proper samples storage very challenging.
According to manufacturer's instruction, specimens to be tested on Xpert should be held at 2-8 °C for 10 days maximum or be stored at a maximum of 35°C for up to 3 days before processing (4). Even if these limitations hinder access to Xpert, studies on stability of samples prior to Xpert testing are

limited. Fixation of samples with ethanol is a low-cost and effective method of DNA preservation before PCR testing, (5); however, data on its application on samples before Xpert testing are not available. Samples for culture should be processed immediately or kept at 2-8 °C not beyond 3 days. Long sample storage before culture inoculation is known to increase contamination rate and affect mycobacterial recovery (6). Cetylpyridinium chlorite is a sample preservative widely used for sample transportation, but this reagent is not compatible with the mycobacteria growth indicator tube (MGIT) technique, commonly used for TB culture (7).

OMNIgene® SPUTUM (OM-S; DNA Genotek Inc, Ottawa, Canada) is another reagent that can be applied to samples prior to testing with both Xpert and MGIT cultures. The reagent stabilizes DNA prior to PCR testing, so that samples treated with OM-S may be stored for a maximum of 30 days at a temperature between 4 and 40 °C before Xpert testing (8). One study reported good compatibility of OM-S with Xpert in samples transported at room temperature (RT) compared to standard procedures including cold storage (9). However, this study did not systematically compare Xpert performance on OM-S with standard method for the same duration of storage.

OM-S has the ability to liquefy and decontaminate samples at the same time, offering the possibility to extend their storage until 8 days at temperatures up to 40°C prior to culture inoculation (8). However, studies investigating the effect of OM-S have shown good accuracy but mainly with Löwenstein-Jensen culture (10,11) while those using MGIT have reported contrasting results (12-15).

The objectives of this proof of concept study were to determine the effect of OM-S and ethanol when added to samples tested with Xpert after 15 days; to assess OM-S on samples tested with MGIT culture after 8 days, and to investigate the effect of delayed Xpert and MGIT culture testing beyond recommended times for untreated sputum samples.

Materials and Methods

Setting

The study was conducted at Epicentre Mbarara Research Centre, within a Regional Referral Hospital in south western Uganda. The biosafety level 3 Epicentre laboratory is quality controlled by the Supranational TB Reference Laboratory of the Tropical Medical Institute of Antwerp (Belgium).

Sample collection

Xpert and MGIT performance were investigated in Phase 1 and Phase 2 of the study among newly diagnosed smear positive (Sm+) adults.

Sm+ patients identified under routine of care were referred for informed consent and enrolment at the Epicentre Clinic, where 1 to 3 samples (A,B and C) were collected within 1-h interval, to reach total volume of at least 6 ml for the first phase and 10 ml for the second phase. Samples were pooled to obtain a homogenous bacterial load before being split in aliquots for the different testing

strategies. To verify homogeneity, smear microscopy using auramine staining according to WHO/IUATLD AFB microscopy grading (16) was performed on direct, pooled sample and on all the aliquots. Smear-negative (Sm-) pooled samples and insufficient volume samples were excluded from further evaluation. All aliquots were stored at RT between 22 and 26^{o}C in a temperature-controlled laboratory throughout the study investigation period.

Sample processing and testing

Phase 1: assessment of the effect of OM-S and ethanol on the Xpert test

Pooled samples were split into five equal aliquots: i) three additive free, with one tested on the collection day, one after 7 days and one after 15 days, and ii) two treated with either OM-S or ethanol and tested after 15 days (Fig 1A).

OM-S was added in equal volume (1:1) and at double the volume (2:1) of ethanol to achieve a 70% final concentration. Then 1 ml of the mixture was combined with 2 ml of sample reagent, mixed, and allowed to settle for 15 minutes at RT before transference of 2 ml into the Xpert cartridge for testing according to the manufacturers' protocol (4) .

Phase 2: assessment of the effect of OM-S on MGIT culture

Pooled samples were split into four equal aliquots: i) two untreated, with one tested on the collection day and another after 8 days, and ii) two aliquots added with OM-S and processed on collection day and after 8 days (Fig 1B).

Aliquots treated with OM-S were added with the reagent in a 1:1 proportion following manufacturer instructions (4), inverted vigorously and left at RT. On the scheduled day for culture inoculation, the mixture was centrifuged at 3,000xg for 20 min, the supernatant was discarded, and the sediment suspended into 1 ml of phosphate buffer before inoculation into an MGIT tube. Untreated aliquots were decontaminated with 1.5% N-acetyl L-Cysteine-Sodium hydroxide (final concentration), then centrifuged at 3,000xg for 20 min. The pellet was re-suspended with 1 ml of phosphate buffer and inoculated into MGIT. PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin) was used at double concentration according to a modified step of the BD MGIT^{TM} product insert (7). Positive cultures were checked for the presence of acid-fast bacilli (AFB) using Ziehl-Neelsen microscopy and tested on blood agar culture to exclude contamination (7). Final identification of *Mycobacterium tuberculosis* complex (MTB) was performed using MPT64 (SD Bioline) Rapid Diagnostic test. Cultures were classified as negative after 8 weeks of incubation.

Statistical analysis

A convenient sample size of 50 Sm+ TB patients was proposed for each phase of the study. Laboratory records were double entered into the Voozanoo database and analysed using STATA 12 (Texas, USA), software.

Xpert results were categorized as very low/low; medium/high, negative/not applicable (inconclusive results: either error, invalid, no result). Results were presented per stratified aliquot smear results grouped as: low (≤1+) and high (>1+) bacillary load.

To assess the effect of time alone (without preservative) on test performance, the degrees of MTB detection on Xpert were compared between day 0, day 7 and day 15 using McNemar test for matched data. To assess the effect of both preservatives, the degrees of MTB detection on Xpert were compared between aliquots treated with OM-S and ethanol at day 15, and for each method versus untreated aliquots at day 0 and day 15. Xpert results were considered discordant between aliquots if the difference exceeded one grade of positivity.

The MGIT positivity rate was stratified by smear categories: negative and low (≤1+) and high (>1+) bacillary loads. To assess the effect of time alone, untreated samples were compared at day 0 and day 8. To assess the effect of OM-S on MGIT, OM-S treated aliquots at day 8 were compared to untreated aliquots at day 0 and day 8 along with OM-S at day 0. To investigate the effect of OM-S on mycobacterial viability, treated and untreated aliquots were compared at day 0.

Finally, mean time to culture positivity and standard deviation (SD) were calculated among untreated and OM-S treated aliquots at day 0 and day 8 and compared using a paired t test.

Ethical approval: Approvals were received from Mbarara University Research Ethics Committee, the Uganda National Council for Science and Technology and ITM Ethical Review Board.

Results

Phase 1: assessment of the effect of OM-S and ethanol on the Xpert test

Between May 2016 and October 2017, the study enrolled 52 patients in phase 1. Of these, 2 submitted insufficient sample volume and were excluded. Fifteen patients (30%) provided 6ml sample, which did not require additional sample collection, 32 (64%) provided 2 samples and 3 (6.2%) provided 3 samples, for total of 88 samples. After pooling samples, 2/50 (4%) aliquots were Sm- and excluded from further analysis. Of 48 remaining samples, 10 (20.8%) were scanty positive, 14 (29.2%) had a grade of 1+, 10 (20.8%) had a grade of 2+ and 14 (29.2%) had a grade of 3+. All aliquots obtained from the same sample showed either the same grade of positivity or 1 grade level of difference, except for 5 samples (identifiers [ID] 107, 115, 140, 144, and 145) (Table 1).

Xpert detected MTB in all aliquots except 4; it produced 2 invalid results for 1+ untreated aliquots tested at day 7 (ID 109 and 144), and 2 negative results for aliquots treated with ethanol: one Sm- and 1 scanty positive (ID 115 and 120) (Table 2).

Xpert performance for untreated specimens

When we compared untreated aliquots obtained from the same sample and tested at day 0, and 15, Xpert detected MTC in all (p value=1). Except with two samples (ID 120,153) aliquots, the Xpert

grades varied within one degree of positivity (Table 3). Aliquot 120 was high at day 0 but low at all other time points. In contrast, aliquot 153 was very low at day 0 and medium at day 15.

Using day 0 as reference and excluding invalid results, 45/46 (97.8%) aliquots had results concordant with those of day 7, while 46/48 (95.8%) had results concordant with those of day 15.

Effect of OM-S and ethanol specimen treatment on Xpert performance

The results from the comparison between aliquots tested with OM-S or ethanol and versus untreated aliquots at day 0 and day 15 is shown in Table 4.

Three aliquots (ID 120,152 and 153), showed discordant Xpert results in the OM-S group. Aliquot 120 showed a lower grade of positivity with OM-S than for day 0 without treatment, while aliquot 152 and 153 had higher grade with OM-S than for untreated samples at day 0 and day 15.

OM-S aliquots had Xpert concordant results with those of untreated aliquots for 46/48 (95.8%) and 47/48 (97.9%) at day 0 and day 15, respectively.

Five aliquots (ID 152, 153,120,142 and144) showed discordances in the ethanol group. Aliquots 120, 142 and 144 with added ethanol gave lower results than for untreated aliquots at both time points, while aliquots 152 and 153 reported high Xpert results with ethanol but low or very low when untreated. Of 48 aliquots containing ethanol, 44 (91.7%) and 45 (93.8%) had concordant results for those of untreated aliquots tested at day 0 and day 15, respectively (Table 4).

Comparison of aliquots treated with OM-S and ethanol showed a concordance of 44/48 (91.7%) (Table 5). Two aliquots were positive for OM-S and negative with ethanol (ID 120,115), and two (ID 142,144), were highly positive with OM-S and exhibited low or very low results in ethanol.

All aliquots gave rifampicin-susceptible results except for ID 120, which was rifampicin resistant for untreated aliquots at day 7 and 15 and with OM-S, but rifampicin susceptible at day 0 and negative for the aliquot treated with ethanol.

Phase 2: assessment of the effect of OM-S on MGIT cultures

Of 57 patients enrolled in phase 2 between October 2016 and August 2017, 1 patient was excluded because of insufficient sample volume. Of 56 patients included, 33 (62%) provided one 10-ml sample and 23 (38%) collected 2 sputum samples, and none required a third sample. Of the 56 pooled samples, 8 were excluded because they were Sm-; the remaining 48 included 5 (10.4%) scanty positive, 18 (37.5%) 1+, 10 (20.8%) 2+, and 15 (31.2%) 3+ samples. All aliquots prepared from the same pooled sample showed either the same level of smear positivity or 1 grade level of difference, except for 4 cases (ID 206, 208, 230, and 236) (Table 6).

For sample ID 242 the untreated aliquot at day 0 was contaminated, the untreated aliquot at day 8 was not tested, and the other aliquots were smear and culture negative. One aliquot (ID 240, untreated day 8) was positive for nontuberculous mycobacteria (NTM).

As shown in Fig 2, the culture positivity across smear categories at different time points was uniformly distributed.

MGIT performance for untreated specimens

At day 0, 41/47 (87.2%) untreated aliquots had MTC culture-positive results compared to 44/46 (95.7%) at day 8.

Effect of OM-S specimen treatment on MGIT performance

Untreated and OM-S treated aliquots were compared at day 0 and day 8. Of the 41 untreated aliquots with MTC at day 0, only 18 (43.9%) treated with OM-S had MTC at day 8 (Table 7). Similarly, among 44 MTC+ untreated cultures at day 8, merely 20 (45.5%) were positive among OM-S treated aliquots on the same date (Table 7). In addition, among 21 MTC+ OM-S treated aliquots at day 0, only 11 (52.4%) were positive among OM-S treated at day 8 (Table 7).

As determinated by comparing OM-S treated aliquots at day 0 and day 8, there were 11 MTB+ cultures at both time points, 10 at day 0 and 9 at day 8 alone (Table 7).

Finally, among 41 MTB+ untreated aliquots at day 0, only 21 (51.2%) were positive among OM-S aliquots on the same date (Table 8).

Time to culture positivity for OM-S and untreated samples at different time points

At day 0, mean time to detection was 10.4 days (SD 1.1) among untreated aliquots, compared to 18.2 days (SD, 2.5) for OM-S treated aliquots, with a *P* value = 0.003. Correspondingly, it was 10.9 days (SD, 1.2) at day 8 among untreated aliquots, compared to 25.5 days (SD, 3.0) for OM-S (*P* value <0.001).

Discussion

OM-S has been proposed as sample preservative prior to testing with Xpert and culture, but so far has not been endorsed by WHO (17). This study adds more evidence of accuracy on the use of this reagent to preserve samples for delayed testing. The study also provides data on Xpert and MGIT performance on samples kept beyond the recommended 3 days at RT without preservative. Samples treated with OM-S can be stored up to 30 days at RT prior Xpert testing. Our choice to limit the delay to a maximum of 15 days was based on assumption that the benefit of this test is to provide early diagnosis and would be compromised if results are available beyond this time frame.

Overall, all aliquots gave Xpert positive results except for 4 aliquots: 2 scanty positive or Sm- treated with ethanol, which gave negative results, and 2 smear grade 1+ (untreated) and processed on day 7, which gave error codes 2008 and 5007. These errors are reported by the Cepheid as mainly related to high pressure and probe check control failure so they are due mainly to specimen handling rather than RT preservation (18). Surprisingly, the effect of long sample storage at RT without a preservative did not alter the Xpert performance over 15 days. Only 2 aliquots showed and Xpert quantitative result discordant for more than one grade. These results suggest that mycobacterial organisms in Sm+ samples may not significantly degrade by storage beyond the recommended 3 days.

There was a good concordance between aliquots added with OM-S and untreated tested at day 0 and 15. This shows that OM-S does not alter the Xpert performance on specimen stored up to 15 days at RT, compared to testing at day 0, which is considered as the best practice. It also demonstrates that the reagent did not improve MTC detection after long storage compared to

untreated samples. At the same time points, ethanol performance was lower, with 5 discordant results. However, with the exception of two aliquots that were either Sm- or scanty positive and Xpert negative, all aliquots treated with ethanol gave positive results.

In the OM-S and ethanol comparisons, all discordances (results above one grade difference) occurred in 5 samples (ID 120,142,144,152,153). Higher dilution of sediments treated with OM-S or ethanol unlikely contributed to these discordances, as all aliquots showed consistent smear grade, and in two cases the lower Xpert grade was observed in the untreated, less diluted sediments.

For ID 120 the same aliquot showed discordance with rifampicin result: Xpert positive and rifampicin susceptible at day 0 untreated but resistant for extended untreated aliquots at day 7 and 15, ethanol and OM-S both at day 15. This could be due to a clerical error from the laboratory, but other explanations cannot be excluded, such as heteroresistance or false-susceptible result due to low mycobacterial load, as reported by others (19, 20). However, this discrepancy was not further investigated.

Other studies have reported already good performance of Xpert from OM-S treated compared to untreated samples, but the samples were always processed on the same day of collection (9,12). In addition, our study showed that similar performance can be obtained beyond the recommended time with OM-S treated and untreated samples until 15 days. Although Xpert testing should be performed as soon as the sample is collected to allow rapid treatment initiation, these results are very important for remote settings where Xpert can only be tested after prolonged transport collection.

Culture positivity rate was unexpectedly lower for fresh untreated samples than for samples left untreated and processed after 8 days. The reason for these results remains unexplained, as aliquots had equivalent smear graded. MGIT performance was much lower for samples treated with OM-S than for untreated samples (50%). The poor concordance at day 0 indicates a negative effect on bacterial growth of MTB by the OM-S treatment regardless of time of exposure. Genotek has recently released a revised protocol that includes OM-S neutralization with buffer before inoculation. This procedure should be further investigated.

The negative effect of OM-S on mycobacterial recovery on MGIT has been reported in other studies (13,14). The incompatibility between the reagent and culture however has been mainly reported for MGIT system (7,13,16). One study reported poor recovery of MTB across both MGIT and LJ media (13). One study reported improved results in MGIT cultures using samples treated with OM-S for up to 3 weeks, with the only concerns being about a delay in MGIT results (15).

Other studies have reported no significant difference between untreated and OM-S treated smear positive remnant samples, with MGIT at day 8 (17,23). Although there was a difference in study design, our study used fresh samples, while FIND evaluation used sediments, this is unlikely to have caused such a difference in the results.

There was only one contaminant on untreated sample at day 0. Previous studies have shown that OM-S treated samples have lower contamination rate than untreated counterparts (10, 12–15, 23).

In our study, only one contaminant in the untreated group may not explain much about the contribution of OM-S in reducing contamination compared to standard decontamination.
Finally, we observed a substantial delay in days to positivity between untreated and OM-S treated samples at both time points. Previous studies have also noted delayed culture growth in samples treated with OM-S (13, 15,21, 22). This raises further concerns about the utility of OM-S in its current procedure and the compatibility with MGIT cultures.

Limitations

This study had few limitations. This was a proof-of-concept study, and aliquots were stored in a controlled research laboratory and not in the type of setting in which the protocols would actually be applied.
We used only known Sm+ samples and therefore we could not demonstrate the effect of the reagent in Sm- samples tested on Xpert and in MGIT liquid medium. More evaluation is needed especially among Sm-, Xpert-positive samples in high TB-HIV context.

Conclusions

In this proof-of-concept study, we have shown that there is no advantage in using OM-S reagent, or ethanol, for smear positive sputum stored at RT up to 15 days, as Xpert performance remains high even after such delays. This study brings reassuring data regarding the possibility of using Xpert on transported sputum samples without a cold chain, which is common practice in high-burden and limited-resource countries. On the other hand, this study does not support the use of OM-S for delayed culture processing, unless additional evaluation on the revised protocol give more promising results.

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Conflict of Interest: The authors declare that they have no conflict of interest. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Figure 1 A; Phase 1: assessment of the effect of OM-S and ethanol on the Xpert test

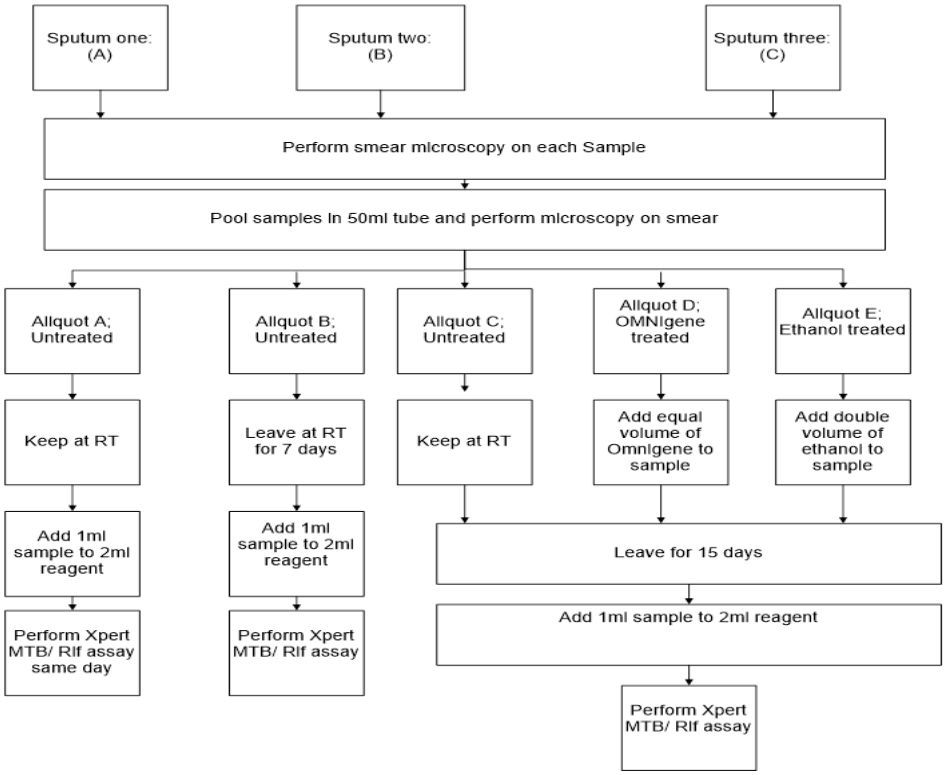
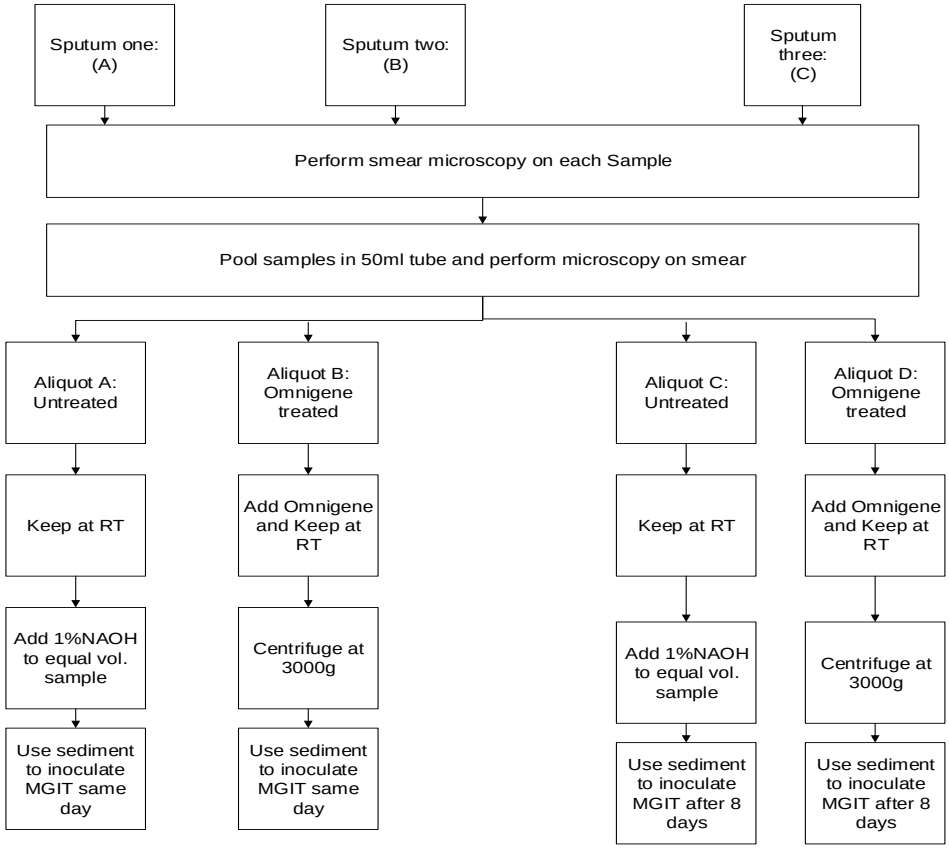
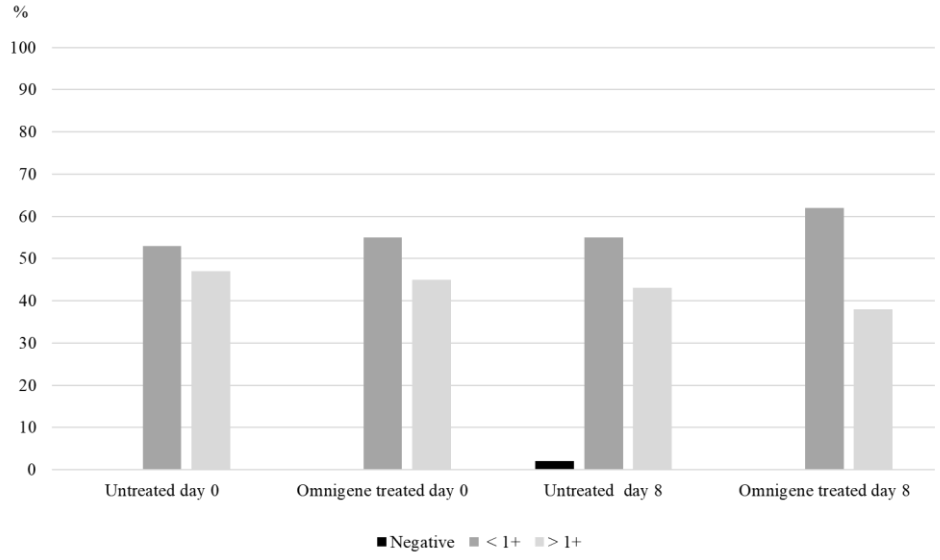


Figure 1B; Phase 2: assessment of the effect of OM-S on MGIT culture



RT: room temperature; MGIT: mycobacteria growth indicator tube

Figure 2: Culture positivity for all aliquots by smear grade



Tab 1 Individual Xpert test results

ID	Sm pool Sample	UN day 0			UN day 7			UN day 15					OM day 15		ETH day 15	
		Sm	MTBXp	RMPXp	Sm	MTBXp	RMPXp	Sm	MTBXp	RMPXp	Sm	MTBXp	RMPXp	Sm	MTBXp	RMPXp
101	3+	3+	high	S	2+	high	S	2+	medium	S	2+	medium	S	2+	high	S
103	1+	1+	low	S	1+	low	S	1+	medium	S	1+	medium	S	1+	low	S
104	1+	2+	medium	S	2+	medium	S	1+	high	S	1+	medium	S	1+	medium	S
105	1+	3AFB	low	S	5AFB	low	S	1AFB	low	S	2AFB	low	S	2AFB	low	S
106	1+	1+	low	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S
107	1+	1+	high	S	2+	high	S	3+	high	S	2+	high	S	1+	high	S
108	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
109	3AFB	1+	low	S	1+	error	n/a	1+	medium	S	1+	medium	S	1+	medium	S
110	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
111	1+	7AFB	low	S	1+	low	S	1+	low	S	1+	low	S	1+	low	S
112	2+	1+	high	S	1+	medium	S	1+	medium	S	1+	high	S	1+	medium	S
113	1+	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S
114	2+	2+	medium	S	1+	medium	S	2+	medium	S	2+	medium	S	1+	low	S
115	1AFB	2AFB	low	S	1+	low	S	1AFB	low	S	negative	low	S	negative	negative	n/a
117	1+	10AFB	medium	S	1+	medium	S	1+	medium	S	10AFB	medium	S	11AFB	medium	S
118	3+	2+	medium	S	2+	high	S	2+	high	S	2+	high	S	2+	medium	S
120	1AFB	neg	high	S	negative	low	R	negative	low	R	negative	low	R	1AFB	negative	n/a
121	3+	3+	high	S	3+	high	S	3+	medium	S	3+	high	S	3+	medium	S
122	7AFB	8AFB	medium	S	7AFB	medium	S	negative	medium	S	15AFB	medium	S	12AFB	medium	S
123	15AFB	1+	high	S	2+	high	S	2+	high	S	2+	high	S	2+	medium	S
124	1+	13AFB	medium	S	3AFB	medium	S	2AFB	medium	S	1+	medium	S	2AFB	medium	S
125	2+	15AFB	medium	S	1+	high	S	1+	high	S	12AFB	medium	S	1+	medium	S
126	1+	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	low	S
127	2+	1+	medium	S	2+	high	S	2+	high	S	2+	medium	S	2+	medium	S
128	3+	2+	medium	S	3+	medium	S	3+	medium	S	2+	medium	S	2+	medium	S
130	2+	2+	medium	S	1+	medium	S	2+	medium	S	2+	medium	S	2+	medium	S
131	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
132	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
133	3+	3+	high	S	3+	medium	S	2+	medium	S	3+	high	S	3+	medium	S
134	2AFB	3AFB	low	S	1AFB	very low	S	2AFB	very low	S	3AFB	very low	S	1AFB	very low	S
135	2+	3+	high	S	2+	high	S	2+	medium	S	2+	medium	S	2+	medium	S
136	scanty	8AFB	medium	S	3AFB	medium	S	5AFB	medium	S	5AFB	low	S	2AFB	medium	S
137	1+	2+	medium	S	1+	low	S	1+	medium	S	1+	medium	S	1+	medium	S
138	2+	3+	medium	S	2+	medium	S	3+	high	S	3+	high	S	3+	high	S
139	3+	3+	high	S	3+	high	S	3+	medium	S	3+	high	S	3+	medium	S
140	2+	3+	medium	S	1+	medium	S	3+	high	S	2+	medium	S	3+	medium	S
142	2+	1+	medium	S	2+	high	S	2+	high	S	2+	high	S	2+	very low	S
143	3AFB	1AFB	low	S	6AFB	medium	S	5AFB	medium	S	2AFB	medium	S	2AFB	low	S
144	2+	2+	high	S	1+	error	n/a	3+	high	S	3+	high	S	3+	low	S
145	3+	2+	medium	S	1+	medium	S	2+	high	S	3+	medium	S	3+	medium	S
147	1+	1+	high	S	1+	medium	S	2+	high	S	1+	high	S	1+	medium	S
149	3+	2+	medium	S	2+	high	S	1+	high	S	2+	high	S	2+	high	S
150	1+	1+	high	S	1+	medium	S	1+	medium	S	1+	medium	S	1+	medium	S
151	3+	3+	high	S	3+	high	S	3+	high	S	3+	high	S	3+	high	S
152	3+	2+	medium	S	3+	high	S	3+	low	S	3+	high	S	3+	high	S
153	scanty	2AFB	very low	S	2AFB	low	S	negative	medium	S	negative	medium	S	negative	high	S
154	scanty	2AFB	medium	S	1+	medium	S	5AFB	low	S	1+	low	S	1+	medium	S
155	1+	4AFB	medium	S	7AFB	medium	S	5AFB	low	S	1AFB	medium	S	8AFB	medium	S

MTB: *Mycobacterium tuberculosis*, AFB= Acid Fast Bacilli, UN=Untreated sample, ETH= Ethanol treated sample, sm; smear microscopy result

Table 2: Correlation between Xpert and smear grade for all samples

	≤1+					>1+				
	UND0 n %	UND7 n %	UND15 n %	OMD15 n %	ETHD15 n %	UND0 n %	UND7 n %	UND15 n %	OMD15 n %	ETHD15 n %
Very low	9 (34.6)	8 (28.6)	7	9 (34.6)	8 (28.6)	0	0	1 (4.2)	0	2 (9.1)
Med/High	17 (65)	18	17	17	18	22	20	23	24 (100)	20
Neg/Invalid	0	2 (7.1)	0	0	2 (7.1)	0	0	0	0	0
Total	26	28	24	26	28	22	20	24	24	22

UND0, UND7 and UND15: aliquot untreated tested at day 0, 7, 15 respectively; OMD15: aliquot treated with OM-S tested at day 15; ETH15: aliquot treated with ethanol tested at day 15; Med=medium; Neg= negative

Table 3: Comparison of Xpert results in untreated samples at D0, D7, and D15

		UND7						UND15						Total
UND0		Neg	Very low	Low	Med	High	N/A	Neg	Very low	Low	Med	High	N/A	
	Neg	0	0	0	0	0	0	0	0	0	0	0	0	0
	Very low	0	0	1	0	0	0	0	0	0	**1**(1)	0	0	1
	Low	0	1	4	2	0	1	0	1	3	4	0	0	8
	Med	0	0	1	15	6	0	0	0	3	10	9	0	22
	High	0	0	**1**(2)	4	11	1	0	0	**1**(2)	7	9	0	17
	Invalid	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total	0	1	7	21	17	2	0	1	7	22	18	0	48

1= ID153, 2= ID120

Table 4: Comparison of Xpert results for OM-S and Ethanol treated aliquots at different days

	UND0						UND15					Total
OMD15	Results	Neg	Very low	Low	Med	High	Neg	Very low	Low	Med	High	
	Neg	0	0	0	0	0	0	0	0	0	0	0
	Very low	0	0	1	0	0	0	1	0	0	0	1
	Low	0	0	3	2	1(1)	0	0	5	1	0	6
	Med	0	1(2)	4	15	3	0	0	1	17	5	23
	High	0	0	0	5	13	0	0	1(3)	4	13	18
	Invalid	0	0	0	0	0	0	0	0	0	0	0
	Total	0	1	8	22	17	0	1	7	22	18	48
ETHD15		**UND0**					**UND15**					
	Results	Neg	Very low	Low	Med	High	Neg	Very low	Low	Med	High	Total
	Neg	0	0	1	0	1(2)	0	0	2	0	0	2
	Very low	0	0	1	1(3)	0	0	1	0	0	1(3)	2
	Low	0	0	4	2	1(4)	0	0	2	4	1(4)	7
	Med	0	0	2	16	8	0	0	2	16	8	26
	High	0	1(2)	0	3	7	0	0	1(3)	2	8	11
	Invalid	0	0	0	0	0	0	0	0	0	0	0
	Total	0	1	8	22	17	0	1	7	22	18	48

1= ID120, 2= ID153, 3= ID152, 4=ID 142, 5=ID 144

Table 5: Comparison of Xpert results for ETHD15 vs OMD15

	OMD15					
ETHD15	Results	Neg	Very low	Low	Medium	High	Total
	Neg	0	0	2[(1)]	0	0	2
	Very low	0	1	0	0	1[(2)]	2
	Low	0	0	2	4	1[(3)]	7
	Medium	0	0	2	17	7	26
	High	0	0	0	2	9	11
	Invalid	0	0	0	0	0	0
	Total	0	1	6	23	18	48

1= ID115 and ID120, 2= ID142, 3= ID144

Table 6: Individual culture test results

Lab N°	Sm pooled sample	UN Day 0		OM Day 1		UN Day 8		OM day 8	
		Sm	Culture	Sm	Culture	Sm	Culture	Sm	Culture
202	3+	2+	MTB	2+	MTB	2+	MTB	2+	Neg
203	2+	2+	MTB	3+	MTB	2+	MTB	2+	MTB
204	3+	3+	MTB	3+	MTB	3+	MTB	3+	Neg
205	1+	1+	MTB	1+	Neg	1+	MTB	1+	Neg
206	2+	3+	MTB	3+	Neg	2+	MTB	1+	MTB
207	1+	1+	MTB	1+	MTB	1+	MTB	1+	MTB
208	3+	1+	MTB	3+	MTB	3+	MTB	3+	Neg
209	scanty	1+	MTB	1+	MTB	scanty	MTB	1+	MTB
210	3+	3+	MTB	3+	MTB	3+	MTB	3+	MTB
211	2+	3+	MTB	2+	Neg	2+	MTB	2+	MTB
214	3+	2+	MTB	2+	MTB	3+	MTB	3+	MTB
215	3+	3+	MTB	3+	MTB	3+	MTB	3+	MTB
216	2+	2+	MTB	2+	MTB	2+	MTB	2+	Neg
217	1+	2+	MTB	1+	MTB	1+	MTB	1+	Neg
218	1+	1+	MTB	1+	Neg	1+	MTB	1+	Neg
219	1+	2+	MTB	1+	MTB	1+	MTB	1+	Neg
220	2+	1+	MTB	1+	Neg	1+	MTB	1+	MTB
221	1+	1+	MTB	1+	Neg	1+	MTB	1+	Neg
223	3+	3+	MTB	3+	Neg	3+	MTB	3+	NTM
224	1+	1+	MTB	1+	MTB	1+	MTB	1+	Neg
225	1+	1+	MTB	1+	Neg	1+	MTB	1+	MTB
226	2+	2+	MTB	2+	MTB	1+	MTB	1+	Neg
227	scanty	scanty	MTB	1+	Neg	1+	MTB	1+	Neg
228	1+	1+	Neg	2+	Neg	1+	MTB	1+	MTB
229	3+	3+	Neg	3+	Neg	3+	MTB	2+	MTB
230	2+	2+	MTB	1+	MTB	3+	MTB	2+	MTB
234	1+	1+	MTB	1+	Neg	1+	MTB	1+	Neg
235	1+	1+	MTB	scanty	Neg	1+	MTB	1+	MTB
236	3+	1+	MTB	3+	Neg	3+	MTB	3+	MTB
237	3+	1+	MTB	3+	MTB	3+	MTB	3+	MTB
240	1+	1+	MTB	1+	MTB	1+	NTM	1+	Neg
241	scanty	scanty	MTB	scanty	MTB	scanty	MTB	scanty	Neg
242	scanty	Neg	Cont	Neg	Neg	Neg	MTB	Nd	Neg
243	1+	1+	MTB	2+	Neg	1+	MTB	1+	Neg
244	2+	2+	MTB	2+	Neg	2+	MTB	2+	MTB
245	2+	2+	MTB	1+	Neg	1+	MTB	1+	Neg
246	3+	3+	MTB	3+	Neg	3+	MTB	3+	Neg
248	3+	3+	MTB	3+	Neg	3+	MTB	3+	Neg
249	3+	2+	MTB	3+	MTB	3+	MTB	3+	MTB
250	scanty	scanty	MTB	scanty	MTB	1+	MTB	1+	MTB
252	1+	1+	Neg	1+	Neg	1+	MTB	1+	Neg
253	3+	3+	MTB	3+	Neg	3+	MTB	3+	Neg
254	1+	1+	MTB	1+	Neg	1+	MTB	1+	Neg
255	1+	1+	MTB	1+	Neg	1+	Neg	1+	Neg
256	3+	2+	Neg	1+	Neg	2+	MTB	1+	Neg
257	1+	scanty	Neg	scanty	Neg	scanty	MTB	scanty	Neg
260	1+	1+	MTB	1+	MTB	1+	MTB	1+	MTB

MTB= *Mycobacterium tuberculosis*, UN=Untreated sample, OM=OMNIgene treated sample, Sm= smear microscopy results, Neg=Negative, Nd=not done, Cont= contaminated

Table 7: Comparison of culture results of OMD8 with UND0, UND8 and OMD0 samples

		UND0				UND8				OMD0		
OMD8		Neg	MTB	NTM	Cont	Neg	MTB	NTM	ND	Neg	MTB	NTM
	Neg	3	22	0	1	1	23	1	1	16	10	0
	MTB	2	18	0	0	0	20	0	0	9	11	0
	NTM	0	1	0	0	0	1	0	0	1	0	0
	Total	5	41	0	1	1	44	1	1	26	21	0

Cont.: culture contaminated; Neg: culture negative; NTM: *non-tuberculous mycobacteria:* UND0 and UND88: aliquot untreated tested at day 0 and day 8; OMD0: aliquot treated with OM-S tested at day 0;

Table 8: Comparison of culture results of UND0 with UND8 and OMD0 samples

	UND8					OMD0		
UND0		Neg	MTB	NTM	ND	Neg	MTB	NTM
	Neg	0	5	0	0	5	0	0
	MTB	1	39	1	0	20	21	0
	Cont	0	0	0	1	1	0	0
	Total	1	44	1	1	26	21	0

ND: not done; Cont.: culture contaminated; Neg: culture negative; NTM: *non-tuberculous mycobacteria:* UND0 and UND8: aliquot untreated tested at day 0 and day 8; OMD0: aliquot treated with OM-S tested at day 0

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CHAPTER VII

Phenotypic and genotypic resistance to bedaquiline in multi-drug resistant tuberculosis patients from Armenia

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Abstract

Resistance to bedaquiline, core to the all-oral treatment of rifampicin resistant tuberculosis, is already on the rise. The risk factors for amplification of mutations, and their correlation with bedaquiline resistance are not fully understood, nor their association with treatment outcome. In addition, bedaquiline resistance detection is complicated by the variety of mutations in *Rv0678* (the most commonly found in clinical isolates) and their BDQ-MIC increase close to the critical concentration of 0.25 µg/ml (CC), leading to discordant results between phenotypic- and genotypic methods.

In this study, subsequent isolates from 39 Armenian patients, treated with bedaquiline in compassionate use, had bedaquiline drug susceptibility testing (DST) in MGIT, minimal inhibitory concentration (MIC) defined and whole-genome sequencing done.

Baseline high BDQ-MIC concentration (6%, n=2) was not associated with prior clofazimine exposure and was only found in patients with unfavorable treatment outcome. Baseline Rv0678 mutations were found only in two (8.7%) patients previously exposed to clofazimine, with BDQ-MIC at the CC and phenotypic bedaquiline resistant, but with favorable outcome. Mutations arising during treatment, whether alone or in combination, and irrespective of frequency and subsequent disappearance during treatment, were associated with unfavorable outcome. Most acquired mutations (88.9%) led to a BDQ-MIC increase, even if not above the CC. No mutations in *atpE* were seen, and MICs did not exceed 0.5 mg/ml.

The main risk factors for amplification of mutations and BDQ-MIC increase were poor adherence to clofazimine and bedaquiline, which was associated with acquired *Rv0678* mutations, compromising susceptibility to both drugs.

BDQ-MIC results were more informative than testing at the CC only, although lowering the CC in 7H11 to 0.125 µg/ml would improve the correlation between pDST on 7H11 and in MGIT without misclassifying WT isolates.

In conclusion, in our study previous clofazimine exposure did not significantly confer either genotypic or phenotypic baseline resistance, nor lead to unfavorable outcome. In contrast, acquired *Rv0678* mutations and increasing MICs during treatment may herald unfavorable treatment outcomes. MIC testing as well as lowering the 7H11 CC to 0.125µg/ml would improve detection of acquired BDQ resistance. The large variety of mutations complicate the development of rapid genotypic DST. Therefore the development of faster and simpler phenotypic methods is a high priority.

Introduction

Treating drug-resistant tuberculosis is difficult. Since 2013, the World Health Organization (WHO) recommends the use of Bedaquiline (BDQ) for treatment of rifampicin-resistant TB (RR-TB) (1). In 2019, Linezolid (LZD), BDQ and fluoroquinolones were classified as the three class A drugs. Since Dec 2022, the WHO recommends the use of the BPaLM (BDQ, Pretomanid, LZD and moxifloxacin) regimen because this regimen results in high cure rates (2).

Soon after its introduction, resistance to BDQ became a concern. Baseline resistance is expected to be uncommon as BDQ was only recently used at large scale (3) and spontaneous mutations conferring resistance to BDQ occur at a low rate of about 10^{-8}, comparable to rifampicin (4). Nevertheless, the 3.5% rate of acquired BDQ resistance observed in South Africa is concerning as it is much higher than the 0,1% rate of rifampicin resistance documented in patients treated with rifampicin for drug-susceptible tuberculosis (5)(6)

Diagnosing BDQ resistance is complex. The BDQ candidate resistance genes are *atpE, Rv0678, mmp5S, mmpL5, pepQ* and *Rv1979*c (7). Several studies have reported the occurrence of variants in these genes, both in people who have and have not been exposed to BDQ (3) (8)(9) (10). In BDQ exposed patients, mutations are mainly found in the *Rv0678* gene which encodes for a repressor of the mmpS5-mmpL5 efflux pump (3)(11). The association between mutations in *Rv0678* and BDQ phenotype is variable, ranging from a raised minimal inhibitory concentration (MIC) below the critical concentration (CC) (12), to phenotypic resistance with MIC above the CC, and hyper-susceptibility when an *Rv0678* mutation is present in combination with a variant in the *mmpS5* (*Rv0677c*) or *mmpL5* (*Rv0676c*) gene (13). To date, none of the *Rv0678* mutations have been statistically associated with BDQ resistance (7), resulting in a phenotype of 'unknown significance' for all reported variants. Further complicating the genotype-phenotype association is the observation that multiple *Rv0678* variants can be present in a single isolate, often as minority variants. During treatment, these mutations can either disappear or become fixed (11)(14) Mutations that emerge or are amplified during treatment have been associated with unfavourable treatment outcome (15)(16).

Clofazimine (CFZ), a widely used anti-leprosy drug (17) and class B drug for treatment of RR-TB, shares the same efflux-pump-based resistance mechanism, resulting in cross-resistance in the presence of *Rv0678* mutations (18)(19) (20). BDQ resistance has been reported in BDQ naïve patients exposed to CFZ (11). When CFZ is used together with BDQ, the risk of resistance may be increased (21), but the effect of CFZ exposure on BDQ resistance and the level of cross-resistance remain unclear (3)(22).

We aimed to investigate occurrence of BDQ resistance using data from Armenia, where Médecins sans Frontières (MSF) in collaboration with the Ministry of Health and the National Tuberculosis Centre provided compassionate use access to BDQ and LZD to patients diagnosed with TB resistant to rifampicin and isoniazid (i.e. multidrug resistant TB (MDR-TB)) plus a fluoroquinolone (FQ) and/or a second-line injectable (SLI) drug (23). At treatment initiation, we aimed to determine the prevalence of mutations in BDQ candidate resistance genes, the prevalence of phenotypic BDQ resistance, their association with CFZ exposure, and their association with treatment outcome. During treatment, we investigated the amplification of mutations in BDQ candidate resistance genes, the acquisition of phenotypic resistance, and the increases in BDQ-MIC, then the factors associated with such events and their association with treatment outcomes. Finally, we aimed to assess the agreement between two phenotypic methods (MIC on 7H10 agar and binary drug susceptibility testing on MGIT), between phenotypic and genotypic findings, and describe phenotypic cross-resistance between BDQ and CFZ.

Methods

Study population and data collection

This analysis included patients who started treatment with BDQ and LZD as part of a compassionate access program between May 2013 and April 2015.). Consenting adults (≥18 years) were eligible for the if they had MDR-TB resistant to a FQ and/or SLI, and a regimen of at least three likely effective drugs could be designed (including BDQ and LZD. Patients were included in this analysis if they had at least one *Mycobacterium tuberculosis* (MTB) isolate available for further testing.

The patient's treatment characteristics, drug exposure and treatment outcome were extracted from the on-site database. CFZ exposure was defined as ≥1 month of CFZ treatment before the start of a BDQ-containing regimen. Adherence was estimated as the proportion of prescribed doses taken, with good adherence defined as ≥80% of doses taken (24). Treatment outcomes were grouped as favorable (cure or treatment completion) or unfavorable (death due to any cause during treatment, loss to follow up, treatment failure, which included culture conversion followed by relapse) according to programmatic definitions.

Isolates were considered 'baseline' when the sample was collected before or up to 7 days after starting the BDQ and LZD-containing individualized regimen. Culture conversion was defined as the date the first of two consecutive negative cultures (from samples collected at least 30 days apart) after a positive culture was collected (26). Culture reversion during treatment was defined as two consecutive positive cultures after culture conversion without evidence of re-infection (defined by > 12 SNP difference on whole genome sequencing (WGS)). A single positive culture after conversion followed by two or more negative cultures was classified as clerical error.

Laboratory analyses

Available isolates were shipped from the National TB Reference Laboratory in Yerevan, Armenia, to the Mycobacteriology Unit at the Institute of Tropical Medicine in Antwerp, Belgium (ITM). At ITM, phenotypic drug susceptibility testing (pDST) for BDQ, MIC for BDQ and CFZ, and DNA extraction for WGS was performed. For BDQ pDST was performed in the MGIT^{960} system at a CC of 1.0 µg/ml (90). BDQ MIC was determined on 7H11 agar for a range of concentrations (0.008 µg/ml to 2.0 µg/ml) with a CC of 0.25 µg/ml. CFZ MIC was performed on 7H10 agar for concentrations ranging from 0.0313 to 2.0 µg/ml, with a CC of 1 µg/ml. An MIC increase was defined as an increase of ≥2 dilutions. MIC and DST were repeated in case of large discrepancy between BDQ pDST and MIC results, i.e. resistance on pDST but MIC ≤ 0.125 µg/ml or pDST susceptible and MIC ≥ 1.0 µ/ml. Phenotypic resistance was defined as MGIT pDST ≥1.0 µg/ml and/or BDQ-MIC above the CC (>0.25 µg/ml).

For DNA extraction, isolates were sub-cultured on Löwenstein-Jensen medium and colonies were transferred to 150 µL 0.5 M Tris-EDTA buffer. Samples were treated with lysozyme RNase A and proteinase K followed by mechanical lysis using bead beating and FastPrep-24 (MP Biomedicals, Santa Ana, USA). The Maxwell 16 Cell DNA Purification Kit (Promega, Madison, USA) was then used to purify the extracted gDNA. Fragmentation and purity were examined through gel

electrophoresis and Qubit dsDNA BR Assay Kit (Life Technologies, Carlsbad, USA). WGS was done by Illumina sequencing. Resistant variants were called by TBprofiler (27) version 4.4, with Freebayes as variant caller, to retain mutations present at a frequency ≥ 5% and detected in at least 10 reads, and a quality score of 20. For the isolates where WGS could not be performed, Sanger sequencing of *atpE* and *Rv0678* genes was done (28).

Statistical analyses

At baseline, the prevalence of phenotypic BDQ resistance was calculated as the proportion of patients with a baseline isolate classified as phenotypically resistant among all isolates with phenotypic results available. The prevalence of mutations in BDQ candidate resistance genes was calculated as the proportion of patients with genotypic BDQ resistance at baseline among all patients with a baseline WGS or Sanger result available.

During treatment, changes in phenotypic or genotypic resistance was assessed. Amplification of mutations was defined as the emergence of one or more new mutations in BDQ candidate resistance genes, regardless of its frequency or its presence or absence in subsequent isolates. Acquisition of phenotypic resistance was defined as a change in phenotype from susceptible to resistant.

MIC 'moderate increase' was defined as an increase in BDQ-MIC of ≥2 dilutions with the highest MIC below the CC.

Potential risk factors for amplification of mutations in BDQ candidate resistance genes, acquisition of phenotypic BDQ resistance or BDQ MIC moderate increase during treatment were *a priori* defined as smear positivity, presence of bilateral cavities, history of CFZ exposure, baseline CFZ-MIC above the CC and inclusion of CFZ in the regimen, number of effective drugs at baseline, amplification of resistance to any drug other than BDQ during treatment, and drug adherence. Presence of resistance to FQ or SLI drugs and the number of effective drugs in the individualized regimen initiated was defined based on a combination of WGS and phenotypic DST results. Potential risk factors for poor treatment outcome were *a priori* defined as history of CFZ exposure, baseline CFZ-MIC above the CC, baseline isolate with phenotypic BDQ resistance or presence of mutations in BDQ candidate genes, acquisition of mutations or phenotypic BDQ resistance during treatment and detection of MIC with "moderate increase" during treatment.

The agreement between BDQ phenotype classified by MIC and MGIT pDST, the agreement between phenotypic and genotypic methods was assessed using a Kappa (K) -value.

The cross resistance between CFZ and BDQ was described for baseline and follow up isolates.

Ethics statement: All patients signed informed consent for transfer of pseudo-anonymized data and biological materials. The study was approved by the Biomedical Research Ethics Committee of Armenia, the Ethical Review Board of MSF and the Institutional Review Board of ITM and Ethics Committee of Antwerp University (REF: 1026/15).

Results

Patient and treatment characteristics

Of the 62 patients, 39 (62.9%) had ≥1 culture available and could be included in the analysis. Patients included in the analysis were almost all male (95%), average age was 41 years, most (95%) were HIV negative and had smear-positive (85%) cavitary (100%) TB with bilateral (74%) lung damage. All patients had a history of second-line treatment for RR-TB, and most (74%) had been exposed to CFZ an all were BDQ-naive. Compared to 23 patients not included in the analysis, the 39 patients included were more likely to have prior CFZ exposure smear-positive TB, pulmonary cavities and bilateral pulmonary disease (Table S1).

Using data from WGS and ITM DST results, one (3%) patient was classified as MDR TB, four (10%) were also resistant to a SLI, 12 (31%) to a FQ, and 22 (56%) were resistant to FQ and SLI (Table S2)

Patients received a median of 25 months (range 2 to 35) of treatment, including a median of 6 months (range 2 to 16) of BDQ (Table S3,S4). Individualized regimens contained BDQ and LZD combined with two to six drugs selected based on prior drug exposure and DST results from prior treatment episodes. Cycloserine was used in 31 (80%) patients, a SLI in 16 (41%), CFZ in 30 (77%), PAS in 17 (44%), levofloxacin in 16 (41%), protionamide in 6 (15%), and pyrazinamide in 5 (13%) patients (Table S2).

Treatment outcomes

Of 39 patients included, 23 (59.0%) had a favorable treatment outcome: 20 (89.0%) were cured and three completed treatment. Of the 16 (41.0%) patients with an unfavorable outcome, five died, ten experienced treatment failure of which one relapsed, and one was lost to follow-up.

Overall, culture conversion occurred in 29 (74%) patients (9 of 16 patients with unfavorable outcome, 56.2%, and all patients with baseline isolates and unfavorable outcome) after a median of 3.2 months (IQR 2.1-4.1). Compared to patients with an unfavorable outcome, patients with favorable outcome were more likely to achieve culture conversion after a shorter time after initiation of the BDQ and LZD containing individualized regimen [2.9 months (IQR 2.0-4.6) vs 3.2 months (IQR 1.0-4.1)]. Prior to their unfavorable treatment outcome, of the 9 patients that experienced culture conversion, 8 patients culture reverted after a median of 9.4 months [IQR 2.2-14.9].

Results of phenotypic tests and agreement between phenotypic methods

In total 101 isolates were available for the 39 patients included: 35 were baseline and 66 follow up isolates. BDQ-MIC results were available for 85 and MGIT pDST for 84 isolates (Figure 1). Of the 84 isolates with both MIC and pDST results available, seven had discordant results. After repeat testing, four discordances were resolved (pDST MGIT susceptible and MIC between 0.6 µg/ml and 0.25 µg/ml), and three remained discordant (pDST MGIT resistant and MIC at 0.125µg/ml). Taking these repeat results into account, agreement between the two phenotypic

methods was only fair (K= 0.30) as only 11 of the 28 isolates BDQ resistant on MGIT pDST were resistant on 7H11 (MIC ≥ 0.5 μg/ml) and 50 of the 56 isolates BDQ susceptible by MGIT pDST had a MIC <0.25 μg/ml (Fig 1). If the CC in 7H11 for BDQ was lowered to 0.125 μg/ml, 72 results would have been concordant with both methods: all 28 BDQ-R in MGIT would have been also BDQ-R in 7H11, while of the 56 BDQ-S in MGIT 44 would have been BDQ-S in 7H11, with six isolates (all with MIC at 0.25μg/ml and all with mutants) reclassified as BDQ-R in 7H11 for a total 12 discordant results. The overall agreement between the two techniques, at a CC of 0.125 μg/ml for MGIT, would had been high (K= 0.68)

Based on resistance by any of the methods, 31 baseline isolates and 31 follow-up isolates were classified as phenotypically susceptible to BDQ, and 4 baseline and 30 follow-up isolates as phenotypically resistant.

Overall, 83 of the 101 isolates had a CFZ MIC result available. Of these, 44 were CFZ resistant and 39 were susceptible. The level of cross resistance between CFZ and BDQ was 57% (25 resistant to CFZ and BDQ, 1 resistant to BDQ but not CFZ and 18 resistant to CFZ but not BDQ).

Results of genotypic tests and agreement between phenotypic and genotypic methods

Of the 101 isolates, a WGS result was available for 98 isolates and Sanger sequencing for two. Two of the 34 baseline isolates with results available and 49 of the 66 follow up isolates contained at least one of the 36 different variants of interest. Almost all (86%, 31 of 36) variants were detected in the *Rv0678* gene (10 frameshifts indels and 21 SNPs), other 4 (11%) in *mmpL5* (of which 2 combined with *Rv0678* mutations), and one (3%) in *Rv1979* (in combination with a *Rv0678* mutation) (Table S5). Except for insT424, all *Rv0678* mutations were located between nucleotides 19 and 198 (Figure S1).

Only half (18/36) of these variants occurred as single mutations. The number of variants ranged from 1 in 40 of the 51 isolates (78%), to two (12%) in six isolates, and >2 in five isolates (10%).

A minority variant (<10%) occurring as the only variant present was found in one isolate (2%) vs three (6%) occurring when multiple mutations were present.

Overall, 95 isolates had phenotypic and sequencing results. Of these, 32 of the 35 (91%) isolates classified as phenotypically resistant contained at least one variant in a BDQ candidate resistance gene and 2 (6%) were wild type. Of the 60 isolates classified as phenotypically susceptible, 46 (77%) were wild type and 14 (23%) contained one or more variants. Taking a conservative approach and classifying variants in *Rv0678* of unknown significance as resistant, the agreement between phenotypic and genotypic DST was high (K=0.68).

Baseline prevalence of phenotypic resistance and/or mutations in candidate resistance genes

Of the 39 patients, 35 had phenotypic results and 34 genotypic results at baseline. Four patients were classified as phenotypically BDQ resistant, corresponding to a prevalence of 11% (95% CI 0.04, 0.25) (Table 1). Of these four patients two, resistant by MGIT and with BDQ MIC at 0.25μl/ml, had a variant in a candidate resistance gene. No other baseline isolates showed mutants, corresponding to an overall mutations prevalence at baseline of 6% (95% CI 0.01, 0.19).

Of the 35 patients, 23 had a history of CFZ exposure, of which two had a *Rv0678* variant and were also phenotypically resistant to CFZ (MIC 2.0 μg/ml) and to BDQ. Of the 12 CFZ naïve patients, none had a *Rv0678* variant all were BDQ phenotypically susceptible and two out of 11 with CFZ MIC results available were CFZ resistant (MIC 2.0 μg/ml).

Acquisition of phenotypic resistance and/or mutations in candidate resistance genes during treatment

Of the 39 patients, 8 patients culture converted after baseline, 16 patients had positive cultures at follow up, 4 did not have baseline isolates and for 10 patients the follow up positive cultures were not available for testing. One patient may have had a reinfection as there were 20 SNP differences between the baseline and month 20 strain.

Of the 24 patients included in the analysis, 8 (33%, 95% CI 0.17;0.53) acquired phenotypic resistance. Among the 23 patients with follow up and WGS data, 12 (52%, 95% CI 0.32;0.70) acquired one or more mutation (Table 2), including the 8 patients with acquired phenotypic BDQ-resistance.

In serial isolates of the same patient, the same mutation was observed in only six of 12 (50%) patients.

Both patients with a variant at baseline and phenotypic BDQ resistance (but BDQ MIC at 0.25μg/ml) described above, were positive at follow up, but only for one patient isolates were available. The patients showed an increase of BDQ-MIC of 1 dilution, and the substitution of the baseline variant (Figure S2). Both patients with phenotypic resistance (MIC at 0.5 μg/ml) at baseline but WT at follow up showed *Rv0678* mutations amplification.

Gain or loss of mutations over time were observed in eight patients (Fig S2a,b,c) and reached fixation in five of them. Replacing mutations conferred the same or higher BDQ-MIC, but resulted in a 2-dilution MIC increase, above the CC, in only one patient.

Phenotypic resistance to CFZ was acquired during treatment in 12 of the patients with follow up isolates available, 8 among those receiving CFZ and 4 not receiving CFZ. Among the total 12 patients, 10 acquired one or more mutations of interest and 8 of these also acquired phenotypic BDQ resistance, however only five reached a BDQ MIC above the CC, either at the time of the mutation amplification or later.

Two patients acquired LZD resistance conferring mutations (C154R and A2010C). In both patients, this occurred after acquisition of *Rv0678* mutation.

BDQ MIC with moderate increase

Among the 8 patients that acquired phenotypic BDQ resistance during treatment, BDQ-MIC moderate increase occurred in three patients (Table 1). Among the 5 patients that remained BDQ susceptible during the entire treatment episode, two patients experienced MIC moderate increase.

Of all five patients showing moderate MIC increase had unfavorable outcome. One remained WT throw-out treatment, while the remaining four showed amplification of mutations, either as solo mutations in Rv0678 or mix of mutants.

Factors associated with acquisition of phenotypic BDQ resistance, acquisition of variants in BDQ candidate resistance genes or MIC moderate increase during treatment

In table 2 are described the risk factors per patient, analyzed in table 3 (details per patients are provided Tables S6 and S7).

Previous exposure to CFZ as well as CFZ included in the treatment were not strongly associated to amplification of genotypic or phenotypic resistance during treatment. Low adherence to BDQ and CFZ were the factors more likely correlated to amplification of mutations, increase of BDQ-MIC and amplification of resistance with pDST .

Risk factors for treatment outcome

History of previous CFZ treatment did not affect treatment outcome, as well as high CFZ-MIC at baseline.

Of the four patients with baseline phenotypic resistance, the two patients with mutations but low BDQ-MIC had favorable outcome, while the two patients WT but with MIC above the CC failed treatment. However, as overall, baseline genotypic and phenotypic resistance were not associated with treatment outcome (Table 4).

Acquisition of mutations during treatment was highly associated with unfavorable treatment outcome. Of 12 patients that amplified mutations during treatment, only one (8%), described above as the patient with phenotypic resistance and mutation at baseline with amplified a second mutation, had a favorable outcome.

Also MIC increase was associated with treatment outcome. For a total of 10 patients MIC increased, five (50%) up to 0.5 µg/ml and 5 (50%) moderate, two of them also MGIT DST susceptible and one with amplification of mutations during treatment). All patients had unfavorable treatment outcome.

Discussion

In this cohort of patients presenting with multidrug and extensively drug-resistant TB, who were among the first to receive new drugs under compassionate use, only 59% of patients undergoing BDQ based treatment achieved a favorable treatment outcome.

Four patients (11%) showed baseline phenotypic BDQ resistance. For two of them BDQ resistance conferring genes were WT but the MIC above the CC. Both patients had unfavourable outcome, on the contrary of two patients with baseline mutations in BDQ candidate resistance genes but both exhibiting a BDQ-MIC below the CC. One patients successfully completed the treatment while the second patient was cured, despite that the initial mutation was replaced during BDQ administration by a second fixed mutation which conferred a BDQ-MIC above the CC. Baseline mutations have been associated with worse treatment outcome, even if they confer BDQ-MICs below the CC (11). Overall, our findings, although referred to limited number of patients, indicate that elevated baseline MICs, rather than baseline mutations, contribute to unfavorable outcome. However, this observation should be interpreted in the context of the study period when only a small number of patients had received BDQ, whereas baseline *Rv0678* mutations may now be

increasingly prevalent due to transmission of BDQ-resistant strains within the population.
The patients with low BDQ MIC failed treatment despite some authors (15) (16) suggest that such low-level BDQ resistance can be overcome by a strong treatment regimen that includes BDQ and LZD. However in these studies either is not clear if the baseline isolate with relatively elevated MIC showed also a mutant, or the number of WT isolates with BDQ-MIC above the CC were very limited to draw a clear conclusion.
In our study previous exposure to CFZ before initiating BDQ-containing treatment was not associated with baseline BDQ resistance (29). Among the two patients with a BDQ-MIC at 0.5 µg/ml, only one had a history of treatment with CFZ, while both patients with baseline mutations (in *Rv0678*) had received CFZ before. Previous CFZ exposure was not correlated to unfavourable treatment outcome. This observation aligns with the findings reported for the entire patient cohort described in the methods of this study (30), where previous CFZ exposure was not linked to unfavourable outcome in BDQ-based treatment.
Half of mutations in BDQ candidate resistance genes (18/36) were found as solo variant. The majority of mutations were observed in the 498bp region of the *Rv0678* efflux pump repressor, specifically between position 20 and 198. These mutations often were detected as unfixed mutations or transient minority populations, with a frequency <10%. The significance of the frequencies at which mutations were detected remains unclear. When the same mutations were identified in multiple isolates, their initial frequencies varied between 14% and 100%. This phase of heteroresistance did not consistently evolve toward fixation, and did not hinder the substitution with other mutations. We observed a multitude of mutations that emerged and disappeared during the course of treatment. Interestingly, among the 50% of mutations occurring in combination, or in case of mutation substitution, the first mutant tended to have lower frequency of heteroresistance and lower MIC than subsequent mutations. Temporary fluctuations in drug pressure can lead to the acquisition and loss of mutations conferring resistance to fluoroquinolones (31), but this mechanism is unlikely applicable to BDQ, which is characterized by a prolonged half-life. Moreover, in our study, the gain and loss of mutations were observed at any time during treatment. Nevertheless, the lack of WGS results for all culture positive samples hampers further interpretations.
With the exception of one mutation that replaced the baseline mutations in a patient who was cured, all documented amplified mutations during treatment occurred in patients with unfavorable outcomes. These mutations consistently showed an increase in BDQ-MIC, regardless of the mutant frequency and combination with other mutations, even if in only a few isolates the BDQ-MIC increased above the CC. These findings, although based on a limited number of patients, confirm previous findings demonstrating that mutations emerging during treatment are associated with an increase in BDQ-MIC, even if not always exceeding the BDQ CC, and herald further mutations and potential treatment failure (11)(15)(16).
No mutations were observed in the BDQ target *atpE* gene, while a few unfixed mutations were identified in the *mmpL5* efflux pump gene, and one unfixed mutation was detected in *Rv1979c*. These non-*Rv0678* mutations frequently co-occurred with *Rv0678* mutations and exhibited similar MICs around the CC. In contrast to other studies (13) we thus did not find evidence of

hyper susceptibility in presence of mutations in *mmpL5* (32) nor epistasis, where mutations in *mmpS5*/*mmpL5* that encode the efflux pump in presence of mutations in the transcriptional repressor *Rv0678* may re-establish BDQ susceptibility (33).

The availability of rapid molecular diagnostics tests would greatly facilitate the early identification of mutations amidst the myriad of polymorphisms that arise on BDQ-based treatment. This early detection would significantly aid in identifying patients at risk of failing BDQ based treatment. Even if not reaching the CC, a BDQ-MIC increase of 2-dilutions correlates with genotypic resistance amplification. Therefore, repeated MIC testing, unlike testing at the CC alone, would capture the majority of resistance amplification.

Our analysis of risk factors for mutation amplification and BDQ-MIC increase suggests that suboptimal adherence to BDQ and to CFZ are the only factors likely associated with the amplification of mutations in *Rv0678,* rather than the patients' clinical status or resistance to other drugs, as reported in another study (21). In our small cohort, the use of LZD combined with BDQ for patients with FQ resistant RR-TB could not prevent amplification of *Rv0678* mutations during treatment, leading to unfavourable outcomes as also reported by other authors (34). In fact, the amplification of mutations in LZD resistance correlated genes was never observed before amplification of BDQ resistance, and was found for only two patients, both with treatment failure, after amplification of mutations in the *Rv0678* gene, indicating that BDQ is a highly potent drug that exerts substantial pressure on mycobacteria. The limited resistance protecting activity may be overcome by the inclusion of pretomanid, as showed by studies on the BPaL regimen (35).

The amplification of CFZ resistance before or together with the amplification of BDQ resistance should be further investigated. In our study, the amplification of *Rv0678* mutations resulted in an increase in CFZ-MIC above the CC, which correlated with a more modest increase in BDQ-MIC. Although a newly developed high CFZ-MIC did not always correlate with BDQ resistance, the BDQ-MIC tended to increase further during treatment until above the 0.25 µg/ml CC and was correlated with unfavorable outcome. For three patients, the CFZ-MIC increased even if CFZ was not included in the treatment phase. Conversely, other studies have reported the in vitro emergence of *Rv0678* mutations following CFZ exposure which also led to BDQ resistance even in absence of BDQ exposure (11)(36). These findings suggest that (sub)exposure to either BDQ or CFZ may trigger *Rv0678* mutation amplification, compromising susceptibility to both drugs. Although simultaneous use of CFZ and BDQ is likely advantageous (37), further studies need to test whether the addition of CFZ to BDQ increases the risk of acquired BDQ resistance.

Given the knowledge gap between genotypic and phenotypic BDQ DST, phenotypic DST remains the reference method to detect BDQ resistance. However, our and other studies show that de novo mutations developed during BDQ based treatment are universally associated with an MIC increase and unfavorable outcome. In fact, such acquired resistance may be missed when phenotypic BDQ DST is only performed at the CC. Notably, the concordance between phenotypic DST in MGIT versus 7H11 was low, yet improved when the CC for 7H11 was lowered from 0.25 µg/ml to 0.125 µg/ml, which would also better correlate with the clinical breakpoint in this study. Larger studies should refine whether the CC, and CB, for 7H11 should be lowered to 0.125 µg/ml.

If the CC is lowered, all 17 MGIT BDQ-R isolates with mutations would be correctly classified resistant also on 7H11, along with six that showed MGIT BDQ-S results. None of the WT isolates would be misclassified as BDQ-R. However, even with the lower 7H11 CC, six isolates with *Rv0678* variants would still be classified as BDQ-S by both 7H11 and MGIT. Among these variants, three showed MGIT results switching between BDQ-R and BDQ-S, and MIC fluctuating around 1-fold dilution in the other isolates of the same patients carrying the same mutation, and only two showed in the last isolate available and MIC at 0.5 μg/ml and MGIT BDQ-R results. While a fluctuation around 1 fold dilution is accepted as technical error when MIC is determined, this variability can lead to discordant results when the CC is used for DST. These variable results were observed also in MGIT, which correctly classified all WT isolates, but missed the detection of some mutants as resistant.

The imperfect detection of mutations, which were predominantly observed in patients who experienced treatment failure, in five (33.3%) isolates was correlated to limited growth in the drug containing tube, ranging from 12 to 84 growth units (GU) below the 100 growth units (GU) cut off for calling resistance. Four of the five isolates showed BDQ-MIC at or 1 dilution below the CC. In contrast, susceptible isolates tend to show completely suppressed growth (0 GU). and no growth was observed in WT isolates. While these results were interpreted as susceptible, the partial grow of mycobacteria in these cases could potentially indicate an intermediate status of resistance. Therefore, it may be more appropriate to report these results as borderline, as suggested by other authors (38). Further investigation is needed to better understand the interpretation of such limited growth and its implication for interpreting MGIT based BDQ DST.

DST on 7H11 has been previously reported as misclassifying *Rv0678* mutations as BDQ-S, mainly when the MIC is close to the CC (23). Similarly, in our study the variability of results between the two media was primarily observed in the “area of technical uncertainty” around the CC for 7H11 (11). Considering these results as resistant, would lead to less discordance with MGIT pDST. However, other factors may contribute to this variability. The use of different media for subculture has been suggested as one of the reasons for selecting different variants within the bacillary population showing genetic variability, which can alter the DST results. In our study, MIC determination and DST were performed at different time points, following different numbers of subcultures prior to inoculation on different media, which may have led to different rates of loss of specific mycobacterial sub-populations during subculturing (40), which “culture bias” could also affect the gDNA preparation for WGS and the apparent loss of *Rv0678* mutants in subsequent isolates.

This study also has other limitations. Its sample size limits robust conclusions on the frequency of resistance amplification and the associated risks of poor treatment outcome. As not all patients had “baseline” isolates available, as well as isolates from other culture positive events during treatment, we cannot precisely establish the onset and frequency of mutation amplification and their evolution throughout the treatment.

Some patients had favorable outcome also in the presence of Rv0678 mutations and BDQ-MIC above the CC. The small sample size of our study allowed detecting only very strong associations and limited our ability to calculate the clinical breakpoint for BDQ accurately. However the

mutations observed in patients who experienced treatment failure often conferred resistance levels around the CC, suggesting that the clinical breakpoint may not be higher than the CC. Nonetheless, it is important to note that our analysis does not rule out that patients treated with sufficient effective drugs may still benefit from BDQ, even in the presence of *Rv0678* mutations.

Conclusion

In our study, prior exposure to CFZ did not correlate with mutations in *Rv0678* at baseline, nor with BDQ phenotypic resistance. Also, mutations in *Rv0678* at baseline were not associated with unfavorable outcome. However, during treatment, an increase in BDQ-MIC or any amplification of mutations in BDQ candidate resistance genes, regardless of their frequency, should be seen as a warning sign of resistance amplification and potential unfavorable treatment outcome. Interruptions in BDQ and CFZ drug intake were the only factors associated with the amplification of mutations and BDQ-MIC increase, accompanied by an increase in CFZ-MIC. How resistance to CFZ and BDQ occurs when the drugs are administrated in combination should be further investigated. The agreement between BDQ-DST and 7H11-MIC was suboptimal, and considering a CC of 0.125µg/ml in 7H11 may improve concordance without reducing specificity. Lastly, due to the variety of mutations observed, developing a rapid test for detecting drug resistance poses challenges, so that further implementation of BDQ MIC testing, including the development of faster and simpler methods, should be prioritized.

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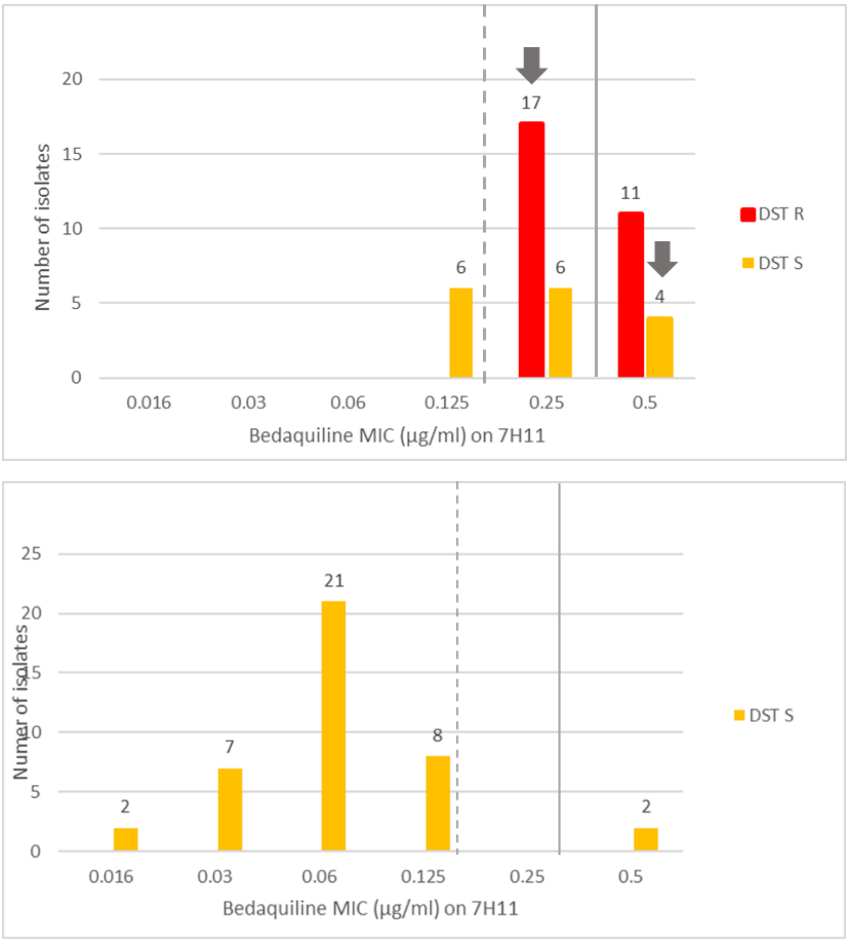
Figure 1. Bedaquiline minimal inhibitory concentration (BDQ-MIC) on Middlebrook 7H11 agar for MGIT Bedaquiline susceptible and resistant isolates, for 44 isolates with mutants and 40 wild type



Grey arrow = discordant phenotypic result; full line= 0.25µg/ml critical concentration; dotted line= tentatively lowered critical concentration for 7H11 medium (to 0.125 µg/ml); DST-R = BDQ-resistant by MGIT-DST; DST-S = BDQ-susceptible by MGIT-DST

Table 1. Phenotypic resistance and presence of variants in BDQ candidate resistance genes, acquisition of phenotypic resistance, acquisition of variants or MIC moderate increase during treatment, stratified by treatment outcome (data presented as N/total N with information available; % and 95% CI)

	All patients	Favorable treatment outcome	Unfavorable treatment outcome
	N=39	N=23	N=16
Baseline isolate*			
Mutation in Rv0678**	2/35 6% (1.5-18.6) Note: both had history of CFZ exposure	2/21 (9%)	0/14 (0%)
Phenotypic resistance by any method	4/35 11% (4.5-26.0)	2/21 10% (26.5-29.0)	2/14 14% (4.0 - 40.1)
BDQ-MIC ≥ 0.5 µg/ml	2/35 6% (1.5-18.6)	0/21 0% (0-15.4)	2/14 13% (4.0-40.1)
MGIT > 1.0 µg/ml	2/31 3% (0.7-20.7)	2/18 11% (3.1 -32.8)	0/13 0% (0-22.8)
MIC-MGIT discordance in case of any resistance***	4/4 100%	2/2 100% Note: MIC was 0.25 µg/ml for both isolates	2/2 100% Note: MIC was 0.5 µg/ml for both isolates
Follow-up isolates[$]			
Acquired mutations[&]	12/23 52% (33.0-70.7)	1/12 8% (1.5-35.4)	11/12 92% (64.6-98.5)
Acquired phenotypic resistance	8/21 38% (20.7-59.1)	0/11 0% (0-25.9)	8/10 80% (40.0 -94.3)
MIC creep (increase ≥ 2 MIC dilutions below CC)	5/21 24% (10.6-45.0)	0/11 0% (0 -25.8)	5/10 90% (23.6 – 73.3)

* Four patients did not have a baseline isolate available

** Rv0678 mutations detected at baseline were insG137 and delG198. No mutations were detected in *atpE*, *pepQ*, *mmpL5*, *mmpS5* or *1979c*

***3 initial discordances at baseline, resolved by re-preforming MIC and pDST

[$] Follow up isolate available for 24/39 patients,12/23 with favourable and 12/16 with unfavourable outcome. Denominators refer to the patients included in the analysis

[&] of the 34 unique variants acquired, 29 were in *Rv0678*, 4 in *mmpL5* (of which 2 combined with *Rv0678* variants), and 1 in *Rv1979* (in combination with a *Rv0678* mutation)

Table 2: Factors associated with acquisition of mutations in bedaquiline (BDQ) candidate resistance genes, development of phenotypic DST and MIC variations during treatment

		Acquisition of mutations in BDQ candidate resistance genes						7H11 MIC only							Acquisition of phenotypic BDQ resistance by MGIT and/or 7H11					
		Total	Yes		No		OR (95% CI)	To	Stable		Moderate Increase		Increase > CC		OR [1] (95% CI)	Total	Yes		No		OR (95% CI)
		N	N	%	N	%		N	N	%	N	%				N	N	%	N	%	
All patients		23	12		11			21	11	52	6	29	4	19		21	8	45	13	55	
History of CFZ treatment	yes	16	8	50	8	50	0.75 (0.12-4.48)	1	8	53	4	27	3	20	0.72 (0.12-4.43)	15	5	33	10	67	0.5 (0.07-3.43)
	no	7	4	57	3	43		6	3	50	1	17	2	33		6	3	50	3	50	
Baseline CFZ MIC	≤ 1	19	10	50	9	50	1.8 (0.13-23.37)	1	9	48	5	26	5	26	na	19	8	42	11	58	0.45 (0.01-12.48)
	≥2	3	2	67	1	33		1	1	0	0	0	0	0		1	0	0	1	100	
CFZ in regimen	yes	15	9	60	6	40	2.5 (0.42-14.60)	1	7	54	3	23	3	23	0.87 (0.16-4.62)	13	5	38	8	62	1.04 (0.16-6.40)
	no	8	3	38	5	62		8	4	50	2	25	2	25		8	3	38	5	62	
Number of effective drugs	<3	2	1	50	1	50	1.00 (0.05-18.08)	1	0	0	0	0	1	100	0.50 (0.25-10.1)	1	0	0	1	100	0.4 (0.01-12.01)
	≥3	21	11	50	11	50		2	11	55	3	15	6	30		21	9	43	12	57	
Smear positive	yes	17	10	59	7	41	2.14 (0.28-16.36)	1	6	42	4	29	4	29	2.49 (0.36-17.38)	16	8	50	8	50	4.0 (0.36-44.11)
	no	5	2	40	3	60		6	4	66	1	17	1	17		5	1	20	4	80	
Bilateral cavities	yes	16	10	63	6	37	4.1 (0.60-28.62)	1	6	40	4	27	5	33	8.46 (0.80-89-58)	15	8	53	7	47	14.7 (0.70-307.84)
	no	7	2	29	5	71		6	5	83	1	17	0	0		6	0	0	6	100	
Adherence to BDQ[2]	<80%	5	5	100	0	0	16.07 (0.75-343.63)	4	0	10	2	50	2	50	0.14 (0.02-1.14)	4	3	80	1	20	6.0 (0.49-73.45)
	≥80%	15	6	40	9	60		1	9	60	3	20	3	20		15	5	40	10	60	
Adherence to CFZ (month 0-6)[2]	<80%	6	6	100	0	0	18.77 (0.83-424.19)	5	0	0	3	60	2	40	0.86 (0.01-0.85)	5	4	80	1	20	12.0 (0.79-180.98)
	≥80%	10	4	40	6	60		8	6	76	1	12	1	12		8	2	25	6	75	
Adherence to other drugs (month 0-6)[2]	<80%	8	6	75	2	25	3.5 (0.50-24.27)	8	2	25	4	50	2	25	0.39 (0.07-2.18)	8	4	50	4	50	1.75 (0.27-11.15)
	≥80%	13	6	46	7	54		1	7	64	1	9	3	27		11	4	36	7	64	

1= referred to group stable; 2= Adherence is the proportion of prescribed drug taken by the patient;

Table 3: Assessment of risk factors for unfavorable treatment outcome

		Treatment outcome					
		Total patients	**Unfavorable**		**Favorable**		**OR (95% CI)**
		N	N	%	N	%	
History of CFZ exposure	yes	24	9	38	15	62	0.6 (0.18-2.53)
	no	15	7	47	8	53	
Baseline CFZ-MIC (µg/ml)	≥2.0	6	2	33	4	67	0.6 (0.09-4.01)
	≤ 1.0	28	13	36	15	64	
Baseline phenotypic BDQ resistance	yes	4	2	50	2	50	1.5 (0.19-12.78)
	no	31	12	41	19	59	
Presence of variants in BDQ candidate resistance genes at baseline	yes	2	0	0	2	100	0.8 (0.03-20.30)
	no	32	14	44	18	56	
Acquisition of variants during treatment	yes	12	11	91.7	1	8.3	110.0 (6.04-2001.43)
	no	11	1	9.1	10	90.9	
Acquisition of phenotypic resistance during treatment	yes	8	8	100	0	0	78.2 (3.30-1849.13)
	no	13	2	15.4	11	84.6	
MIC creep	yes	5	5	100	0	0	11.0 (0.08-1438.12)
	no	0	0	0	0	0	

Table S1: Characteristics of 62 patients treated with BDQ and stratified by inclusion or exclusion in analysis

	Included in the analysis	**Excluded from the analysis***
	39 (63%)	23 (37%)
Age, median in years (IQR)	41 (33-49)	40 (29-52)
Female	2 (5%)	5 (22%)
Body mass index	19.2 (17.5-21.6)	21.3 (19.3-23.9)
Diabetes mellitus	3 (8%)	2 (9%)
HIV positive**	2 (5%)	2 (9%)
Hepatitis C infected	14 (36%)	4 (18%)
Any cavities	39 (100%)	16 (70%)
Bilateral disease	29 (74%)	11 (48%)
Smear-positive	33 (85%)	6 (26%)
Drug exposure history		
CFZ	22 (62%)	4 (17%)
INJ exposed	39 (100%)	23 (100%)
FQ exposed	39 (100%)	22 (96%)
Programmatic baseline DST profile		
MDR	0 (0%)	1 (4%)
MDR+INJ^{R}	2 (5%)	5 (22%)
MDR+FQ^{r}	22 (56%)	8 (35%)
MDR+INJ^{R}+FQ^{R}	15 (39%)	9 (39%)

*Patients were excluded when no Mtb isolate was available

**HIV status missing for 1 patient excluded from the analysis

Tab S2 Efficacy of drugs included in MDR-Rx based on site DST, previous drug exposure and WGS results at start of treatment

Pt ID	Bdq mut	Bdq mic	Bdq	Lnz mut	Lnz	Cfz mut	Cfz mic	Cfz	Cs mut	Cs DST	Cs	Lfx mut	Ofx 2.0	Ofx 8.0	Lfx	Cm mut	Cm Dst	Cm	Km rrl	Km eis	Km	Amk mut	Amk Dst	Amk	PAS mut	PAS Dst	PAS	Pto mut	Pto	PZA mut	PZA Dst	PZA	EMB mut	EMB Dst	EMB	Baseline drug susceptibility
I04	MUT	0.25	S	wt	S	MUT	2.0	R	wt	S	S	MUT	R	nd	R	-	-	-	1401 A>G	WT	R	-	R	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx, Km
I05	wt	0.06	S	wt	S	wt	0.5	S	wt	S	S	MUT	R	nd	R	-	-	-	-		-	-	-	-	wt	S	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx, PAS
I07	wt	0.03	S	wt	S	-	-	-	wt	S	S	MUT	R	nd	R	-	-	-	wt	c.-10G>A	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cs, Lfx, Km
I08	wt	0.06	S	wt	S	wt	1.0	S	wt	nd	S	MUT	R	nd	R	nd	S	S	wt	c.-37G>T	R	-	S	-	-	-	-	-	-	MUT	nd	R	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx, Km, PZA
I11	wt	0.06	S	wt	S	wt	1.0	S	wt	S	S	MUT	R	nd	R	-	-	-	-		-	-	-	-	-	-	-	T	R	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx, Pto
I14	wt	0.03	S	wt	S	wt	1.0	S	-	-	-	MUT	R	nd	R	MUT	R	R	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Lfx, Cm
I13	wt	0.03	S	wt	S	wt	0.5	S	wt	nd	S	MUT	R	nd	R	-	-	-	wt	c.-37G>T	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx, Km
I17	wt	0.500	R	wt	S	wt	1.0	S	wt	nd	S	-	-	-	-	-	-	-	wt		S	-	S	-	-	-	-	wt	S	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Km, Pto
I19	wt	0.125	S	wt	S	wt	1.0	S	-	-	-	-	-	-	-	-	-	-	wt	c.-12C>T	R	-	S	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Km
L03	wt	0.06	S	wt	S	wt	1.0	S	-	-	-	MUT	R	nd	R	-	-	-	-		-	wt	R	R	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Lfx, Amk
L08	wt	0.500	R	wt	S	wt	2.0	R	wt	S	S	MUT	R	nd	R	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx
L10	wt	0.06	S	wt	S	wt	0.5	S	wt	S	S	wt	R	nd	R	-	-	-	-		-	-	-	-	wt	S	S	wt	S	wt	R	R	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx, PAS, Pto, PZA
L06	wt	0.06	S	wt	S	wt	0.5	S	-	-	-	-	-	-	-	wt	S	S	-		-	-	R	-	wt	S	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cm, PAS
L09	wt	0.06	S	wt	S	wt	1.0	S	wt	nd	S	MUT	R	nd	R	-	-	-	wt	c.-12C>T	R	-	S	-	wt	S	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Lfx, Km, PAS
L07	wt	0.06	S	wt	S	-	-	-	wt	nd	S	-	-	-	-	-	-	-	wt		S	-	S	-	wt	nd	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cs, Km, PAS
L12	wt	0.06	S	wt	S	-	-	-	wt	S	S	-	-	-	-	-	-	-	wt		S	-	S	-	wt	R	R	-	-	wt	nd	S	MUT	nd	R	Bdq, Lnz, Cs, Km, PAS, PZA , E
L24	wt	0.125	S	wt	S	-	-	-	wt	S	S	-	-	-	-	wt	nd	S	-		-	-	-	-	wt	S	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cs, Cm, PAS
L15	wt	0.06	S	wt	S	-	-	-	wt	nd	S	wt	R	nd	R	-	-	-	wt	c.-10G>A	R	-	S	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cs, Lfx, Km
L16	wt	0.125	S	wt	S	wt	1.0	S	wt	nd	S	-	-	-	-	-	-	-	-		-	-	-	-	wt	nd	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, PAS
L18	wt	0.125	S	wt	S	-	-	-	wt	nd	S	-	-	-	-	wt	nd	S	-		-	-	-	-	MUT	nd	R	-	-	-	-	-	-	-	-	Bdq, Lnz, Cs, Cm, PAS
L19	nd	nd	S	wt	S	-	-	-	nd	nd	S	-	-	-	-	nd	nd	S	-		-	-	-	-	-	-	-	nd	S	-	-	-	-	-	-	Bdq , Lnz, Cs , Cm , Pto
L21	wt	0.06	S	wt	S	wt	0.5	S	wt	nd	S	-	-	-	-	wt	nd	S	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Cm
I20	wt	0.06	S	wt	S	wt	0.5	S	-	-	-	-	-	-	-	wt	nd	S	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cm
I23	wt	0.06	S	wt	S	wt	0.5	S	wt	S	S	-	-	-	-	-	-	-	wt		S	-	S	-	wt	S	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Km, PAS
I24	MUT	0.25	S	wt	S	MUT	2.0	R	wt	S	S	-	-	-	-	-	-	-	-		-	-	-	-	wt	R	R	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, PAS
I27	wt	0.06	S	wt	S	wt	1.0	S	wt	nd	S	-	-	-	-	wt	nd	S	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Cm
L27	wt	0.03	S	wt	S	wt	1.0	S	wt	nd	S	-	-	-	-	MUT	R	R	-		-	-	-	-	wt	nd	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Cm, PAS
L11	wt	0.016	S	wt	S	-	-	-	-	-	-	-	-	-	-	-	-	-	wt		S	-	S	-	wt	S	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Km, PAS
L13	nd	0.015	S	nd	S	nd	1.0	S	nd	nd	S	nd	R	nd	R	nd	R	R	-		-	-	-	-	nd	S	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs , Lfx, Cm, PAS
L17	wt	0.03	S	wt	S	wt	1.0	S	wt	nd	S	-	-	-	-	wt	S	S	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Cm
L22	nd	nd	S	nd	S	nd	nd	S	nd	nd	S	nd	S	nd	S	-	-	-	nd		S	-	R	-	-	-	-	-	-	-	-	-	-	-	-	Bdq , Lnz, Cfz , Cs , Lfx, Km
L31	nd	0.03	S	nd	S	nd	nd	S	nd	nd	S	nd	S	nd	S	-	-	-	-		-	-	-	-	nd	nd	S	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz , Cs , Lfx, PAS
I09	wt	0.03	S	wt	S	-	-	-	-	-	-	-	-	-	-	-	-	-	wt	c.-10G>A	R	-	S	-	-	-	-	-	-	MUT	nd	R	-	-	-	Bdq, Lnz, Km, PZA
I12	nd	nd	S	nd	S	nd	nd	S	nd	nd	S	nd	R	nd	R	nd	R	R	-		-	-	-	-	nd	nd	S	nd	S	-	R	-	-	-	-	Bdq , Lnz, Cfz , Cs , Lfx, Cm, PAS, Pto
I22	wt	0.015	S	wt	S	wt	2.0	R	wt	S	S	-	-	-	-	-	-	-	-		-	-	-	-	MUT	R	R	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, PAS
I25	nd	0.06	S	nd	S	nd	nd	S	nd	S	S	-	-	-	-	nd	S	S	-		-	-	-	-	-	-	-	-	-	MUT	nd	R	-	-	-	Bdq, Lnz, Cfz , Cs, Cm, PZA
I29	wt	0.125	S	wt	S	wt	2.0	R	wt	nd	S	-	-	-	-	wt	S	S	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Cs, Cm
I30	nd	nd	S	nd	S	nd	nd	S	nd	S	S	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	nd	S	-	-	-	-	-	-	Bdq , Lnz, Cfz , Cs, Pto
I33	nd	0.06	S	nd	S	nd	1.0	S	-	-	-	-	-	-	-	-	-	-	nd		S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bdq, Lnz, Cfz, Km

S= susceptible, S= drug not tested, considered likely effective; R= resistant; Lzd=linezolid; Imp=imipenem; Lfx=levofloxacin; Cm= capreomycin; Km= kanamycin; Am= amikacin; Emb=ethambutol; PZA=pyrazinamide; CFZ= clofazimine; Pto= protionamide; Cs=cycloserine; MUT= mutation; DST=drug susceptibility testing; WGS= whole genome sequencing

Table S3 Bedaquiline minimal inhibitory concentration values (µg/ml) and mutations in bedaquiline candidate resistance genes for *Mycobacterium tuberculosis* isolates from 23 multidrug resistant tuberculosis patients with favorable outcome, by treatment phase.

	Month of treatment																															
	≤0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	Outcome
L31	0,0155 WT	0,0313 WT	+		-	-	-	-	-	-		-	-	-	-	-	-	-	-		-	C				-							Cured
I33	0,06 WT	-	-	-	-	-	-	-		-	-	-		-	-	-		-		-	-			-	-								Cured
I23	0,06 WT		0,06 WT	+	-	-	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	-								Cured
L12	0,06 WT	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		-	-	-	-	-	-									Cured
L17	0,0313 WT	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-			C	-	-	-	-	-	-	-		-		-			Cured
L11	0,0155 WT		-		-	-	-		-	-	-	-	-	0,06 Mut 18	-	-	-	-	-	-	-	-		-		-								Cured
I29	0,125 WT		-	-	-	-		-		-	-	-	-	-	-	-																	Cured
L07	0,06 WT	0,06 WT		-	-	-		-		-		-		-	-				-	-	-	-	-		-								Cured
I22	0,0155 WT	+	0,0155 WT	+	-	0.0313 WT	+	+	-	-		-	-	-	-	-	-	-	-	-	-	-	-		C		-							Cured
I30	+	+	0,06 WT		+	+	+	+		+	-	-		-	-	-		-	-	-	-	-	-		-	-								Cured
L18	0,125 WT		0,125 WT		-		-	-		-	-		-									-												Cured
I11	0,06 WT	0,06 WT	+	+	0,06 WT	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-												Cured
L13	0,0155 WT		-	-	-	-	-	-	-				-		-		-		-		-			-		-								Cured
I12	0,0313 NA	-	-		-	-		-	-	-	-	-	-		-	-	-	C		-		+	-		-									Completed
L21	0,06 WT	+	+	+	-			-	-	C	-	-				-		-	-			-		-										Cured
I19	0,125 WT	+		-	-		-	-		C	-	-	-	-	-	-		-	-	-	-		-	-										Cured
I27	0,06 WT	-	-	-	-	-	-	-	-	-		-	-		-	-			-	-														Cured
I05	0,06 WT		+		-	-	-	-	-	-	-	-	-	-			-			-	-	-	-	-		-								Cured
L16	0,125 WT	+		-	-	-	-	-	-		-		-	-	-	-		-	-															Cured
I14	0,0313 WT		+	-	-	-	-	-	-	-	-	-		-		-	-	-		-	-		-	-	-	-	-							Cured
I24	0,25 Mut 11	+	0,5 Mut 3	+				-		+	-		C	-		-		-	C			-	-											Cured
L19	+	+	+	+		0,5 Mut 15	+	C	0,5 Mut 15		-	-					-				-														Cured
I04	0,25 Mut 16	+		-	+	+	-		-	-		-		-			C	-	-		-													Cured

First column = patient ID (if in bold, amplification of mutations in BDQ candidate resistance genes occurred); Red cells = culture negative at the start of treatment; orange cells = bedaquiline (BDQ)-based treatment; purple cells= treatment without BDQ ; white cells = treatment ended or no info; culture results: + = positive but not further tested, - = negative, C = contaminated, empty cell= culture not performed; green bold font = clerical error, red font = reinfection; Pt ID red font= baseline isolates with mutations in BDQ BDQ-MIC at 0.5µg/ml; cells with black borders = month of culture conversion; NA = not available; WT = wild type sequence for BDQ candidate resistance genes; Mut x = mutation codes as described in Table 3.

Table S4: Bedaquiline minimal inhibitory concentration values (µg/ml) and mutations in bedaquiline candidate resistance genes for *Mycobacterium tuberculosis* isolates from 16 multidrug resistant tuberculosis patients with unfavorable outcome, by treatment phase.

	Month of treatment																																
	≤0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	≥32	Outcome
L10	0,06 WT		-		-	-	-	-		-			-		-								0,25 Mut 10	+	+									Relapse
I13	0,0313 WT	+	+	0.125 Mut 1		+		+		0.5 Mut 8	+	+																						Died
I20	0,06 WT	+	+	+	-		-	-	-	-				+	+		0.25 Mut 6	NA Mut 6		0,25 Mut 6				0,125 Mut 6				0,125 Mut 6			0,25 Mut 6			Failure
L09	0,06 WT	+		-	-		-	-	-	-	-			+	+		+	0,25 Mut 5	+		0,125 Mut 5	+	+	0,5 Mut 5	+								0,5 Mut 7	Failure
L15	0,06 WT	+	0,125 WT		-	-	-	-		+	+	+		+	0,5 Mut 20		+						+			0,25 Mut 12				0,25 Mut 12				0,25 Mut 12	Failure
L06	0,06 WT		+	+	+		+	+		0,125 Mut 19	+		0,25 Mut 29			+		+	+	+	+	0,25 Mut 2												Died
I07		0,0313 WT	+	+	-	-	-	-	0,25 Mut 8	-	-	-		+	+			0,25 Mut 21				0,125 Mut 8			+	0,0313 WT			0,25 Mut 8				0,25 Mut 8	0,5 Mut 8	Failure
L03	0,06 WT	+	+	0,06 WT	C	+	0,5 Mut 24	+	-		0,5 Mut 25	+	0,5 Mut 23	+	+																				Failure
I17	0,5 WT	+	+	+	+	-	-	0,5 Mut 13		+	0,25 Mut 14	+	+	+	+	+				0,25 Mut 14	NA Mut 14			+	+		+		0,5 Mut 14						Failure
L24	0,125 WT	+	+	0,125 WT		+		0,25 Mut 17	+	+	+	+					0,25 Mut 4							0,25 Mut 30										Failure
I09	0,0313 WT		+	-	-	0,125 WT																													Died
L08	0,5 WT	-		-		-	-	-	-	-	-			-	-	-	0,5 Mut 9		+		+														Failure
I08	0,06 WT	+	+	+		-		-	-	-	-	-		-		-		-	-	C	C	-		-	-										LTFU
L27	0,03 WT	+	+	-		-		-	-						+	0,0313 WT		+			0,25 Mut 26			0,25 Mut 21											Failure
L22	+	+		0,5 Mut 28	+																														Died
I25	0,06 WT	+																																	Died

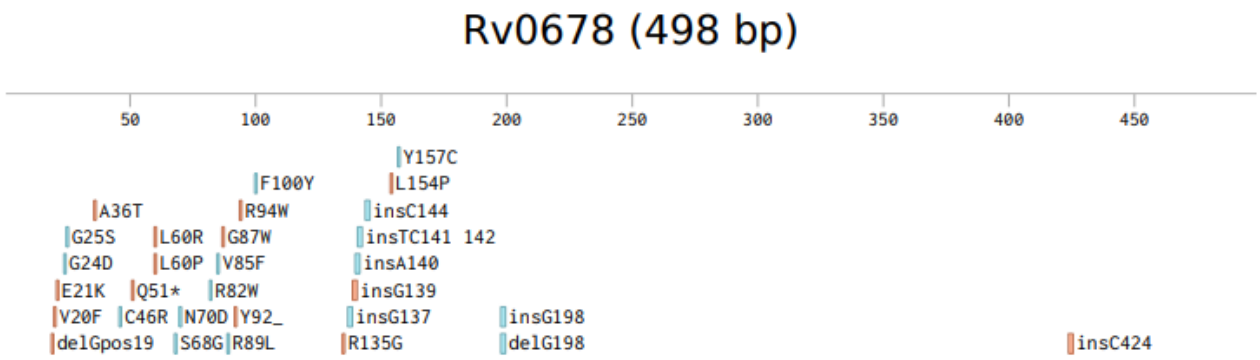
First column = patient ID (if in bold, amplification of mutations in BDQ candidate resistance genes occurred); Red cells = culture negative at the start of treatment; orange cells = bedaquiline (BDQ)-based treatment; purple cells= treatment without BDQ ; white cells = treatment ended or no info; culture results: + = positive but not further tested, - = negative, C = contaminated, empty cell= culture not performed; green bold font = clerical error, red font = reinfection; Pt ID red font= baseline isolates with BDQ-MIC at 0.5µg/ml; cells with black borders = month of culture conversion; LTFU= Lost to follow up; NA = not available; WT = wild type sequence for BDQ candidate resistance genes; Mut x = mutation codes as described in Table 3.

Table S5 Overview of bedaquiline susceptibility testing by MGIT[960], MIC on 7H11 and variants in BDQ candidate resistance genes.

Mut code	*WGS**	Nr of isolates	MGIT DST (1µg/ml)			Middlebrook 7H11 agar - MIC									*pDST* Classification
			S	R	NA	0.0008	0.0155	0.0313	0.06	0.12	0.25	0.5	1.0	2.0	na	
1	G24D (60%)	1	1							1						S
2	G25S (48%)	1	1								1					S
3	C46R (100%)	1	1									1				Discordant
4	S68G (98%)	1		1							1					Discordant
5	N70D (83-96%)	3	2	1						1	1	1				Discordant
6	R82W (98-100%)	6	3	2	1					2	2 1				1	Discordant
7	V85F (100%)	1		1								1				R
8	R89L (86-100%)	8	2	5	1					1°	3 1	2			1	Discordant
9	F100Y (100%)	1		1								1				R
10	Y157C (96%)	1	1								1					S
11	insG 137 (99%)	1		1							1					Discordant
12	insA 140 (100%)	4		4							3				1	Discordant
13	insC 141 142 °	1		1								1				R
14	insC 144 (100%)	5		2	3						1 1	1			2	Discordant
15	insG 198 (100%)	2	2									2				Discordant
16	delG 198 (94%)	1		1							1					Discordant
17	mmpL5_593C>A (24%)	1	1								1					S
18	mmpL5_Q482* (9%)	1			1				1							NA
19	R135G (78%), Q51* (29%)	1	1							1						S
20	L60P (45%), insA 140 (50%)	1		1								1				R
21	R89L (84%), insG 139 (13%)	1		1							1					Discordant
22	E21K (65%), A36T (13%), delG19 (21%), mmpL5_825C>A (8%)	1		1							1					Discordant
23	L154P (50%), insC144 (36%), delG 198 (11%)	1		1								1				R
24	L60R (11%), Y92 (13%), insC 141 142 (20%),delGpos198 (18%)	1		1								1				R
25	R94W (17%) , insC144 (42%), insC 424 (11%)	1		1								1				R
26	V20F (26%), delG19 (60%)	1		1							1					Discordant
28	delG19 (70%), delG 198 (12%)	1		1								1				R
29	G25S (50%), delG198 (31%), G87W (10%),	1	1								1					S
30	S68G (100%), Rv1979c V108A (9%)	1		1							1					Discordant
	WT	40 9	40		9		2 3	7 3	21 1	8 1		2			1	Discordant

Nr = number; MGIT[960] = automated DST in Mycobacteria Growth Indicator Tube; R = resistant; S = susceptible; MIC= minimal inhibitory concentration; Mut = mutation; WGS = whole genome sequencing (*if not specified, the mutation occurred in Rv0678); In italic mutations found as minority variants; Between brackets, frequency (or their range for mutations found in multiple isolates); NA = not applicable because pDST not available; °=tested with Sanger sequencing

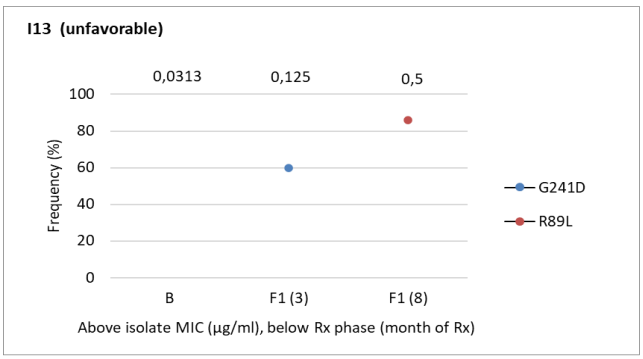
Figure S1 Mutations in the *Rv0678* gene

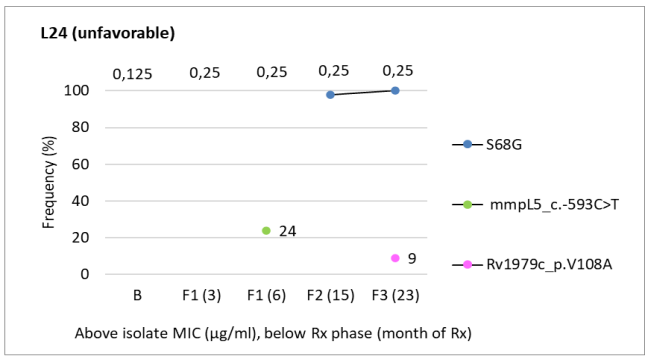


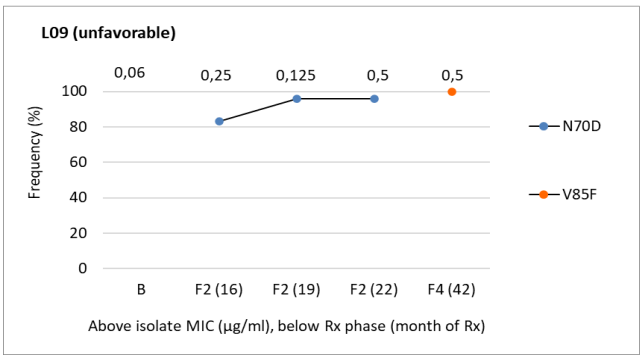
Orange: mutations found only in combination, blue: mutations found at least once alone.

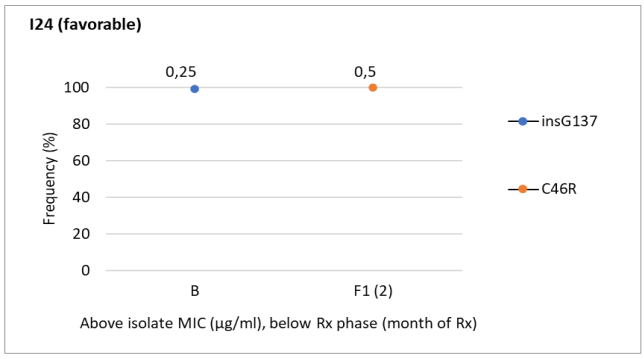
Figure S2 Frequency of transient and minority *Rv0678* mutations versus bedaquiline MIC

a) Patients with different *Rv0678* mutations during treatment

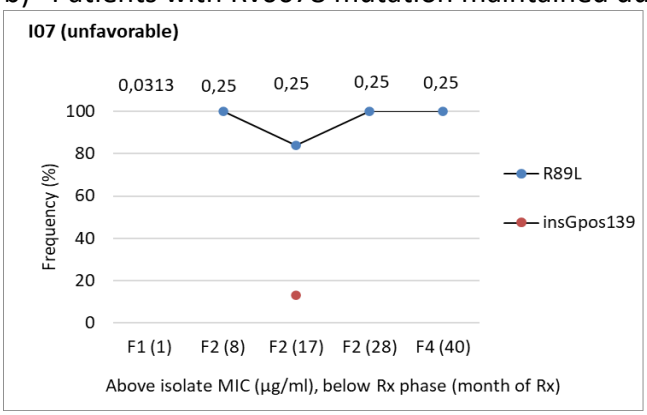


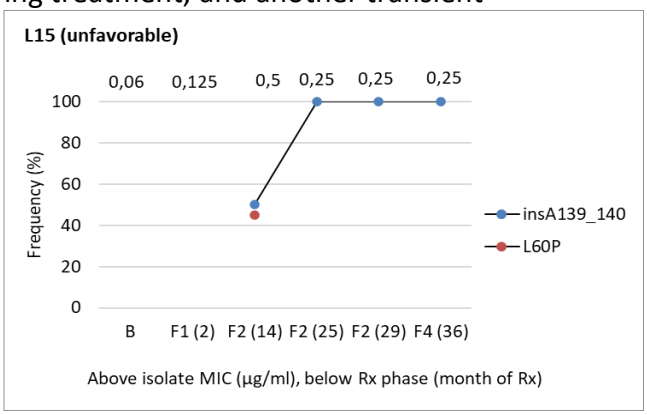




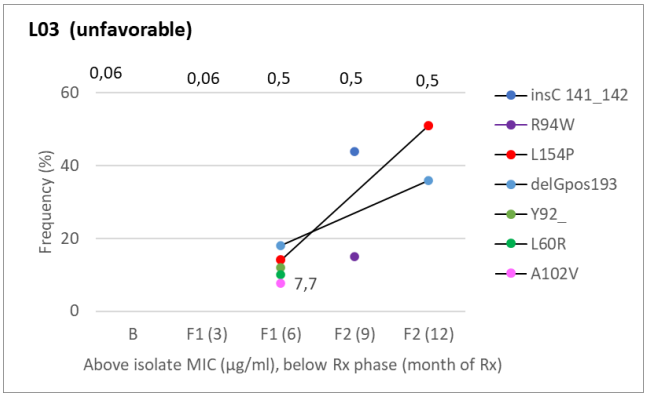


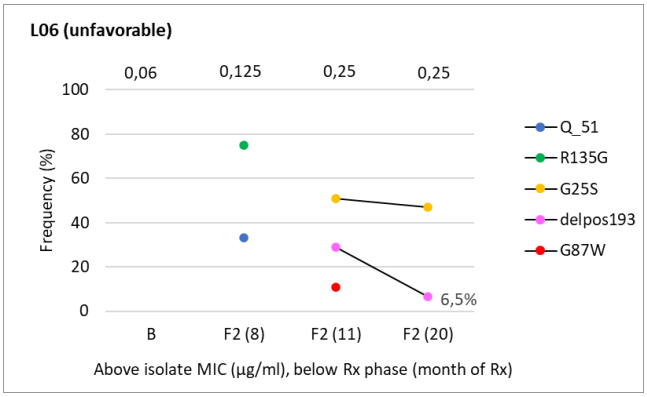
b) Patients with Rv0678 mutation maintained during treatment, and another transient





c) Patients with *Rv0678*-mut amplified as mixed





X-axis depicts only phases at which isolates were available. B= baseline; F1=BDQ-Rx phase; F2= MDR-Rx phase; F3=Post MDR-Rx phase; F4= New BDQ-Rx phase. In brackets after the phase is indicated the month of treatment. When no Rv0678-mut is reported, the isolate was WT. MIC= minimal inhibitory concentration; pink circle= isolate carrying minority variants i.e. *Rv0678*-mut detected at a frequency between 5% and 10% only by MTBSeq pipeline.

Table S6 Risk factors for amplification *of Rv0678*-mut, phenotypic BDQ resistance and MIC increase in 23 patients with favorable outcome

ID	Cfz previous exposure	Baseline Cfz MIC (µg/ml)	Cfz in MDR Rx	N effective drugs (class A,B and SLI) [1]	Smear at start of BDQ Rx	Bilateral cavities	Dot (%)			BDQ genotype	BDQ phenotype[1]	MIC only	Outcome
							Mean BDQ 6M	Mean CFZ 6 M	Mean 6M Rx except BDQ				
L17	yes	1	yes	5	scanty	Yes	100	95	94	stable	S	stable	cured
L13	yes	1	yes	5	neg	Yes	95	97	93	stable	S	stable	cured
I29	yes	2	yes	4	3+	No	na	na	na	stable	S	stable	cured
I33	no	1	yes	4	neg	No	92	94	92	stable	S	stable	cured
L12	yes	0.5	no	5	neg	Yes	na	na	na	stable	S	stable	cured
I27	no	1	yes	5	na	Yes	100	100	86	stable	S	stable	cured
L11	yes	na	no	4	3+	No	97	98	90	stable	S	stable	cured
I12	yes	1	yes	6	neg	No	84	na	77	na	S	stable	cured
I11	yes	1	yes	4	3+	No	93	92	85	stable	S-S	stable	cured
L07	yes	0.5	no	5	2+	Yes	86	na	80	stable	S-S	stable	cured
L18	no	0.5	no	4	3+	Yes	88	na	77	stable	S-S	stable	complete
I24[2]	yes	>2	yes	3	3+	No	92	90	80	amplified	R-R	na	cured
I30	no	na	yes	5	2+	Yes	99	97	95	na	na	na	cured
L31	no	na	yes	6	1+	Yes	97	95	86	na	na	na	cured
I22	no	2	yes	3	3+	Yes	100	100	98	na	na	na	cured
I23	no	0.5	yes	6	1+	No	100	100	100	na	na	na	cured
L19	no	na	no	5	2+	Yes	92	na	86	na	na	na	complete
L21	yes	0.5	yes	5	3+	Yes	100	99	98	na	na	na	cured
I19	yes	1	yes	3	1+	Yes	96	96	93	na	na	na	cured
I05	yes	0.5	yes	5	3+	Yes	95	83	83	na	na	na	cured
L16	yes	1	yes	5	scanty	Yes	100	100	94	na	na	na	cured
I14	yes	1	yes	3	1+	No	98	95	95	na	na	na	cured
I04[2]	yes	>2	yes	3	1+	Yes	61	65	60	na	na	na	complete

Table S7 Risk factors for amplification of *Rv0678*-mut ,phenotypic BDQ resistance and MIC increase in 16 patients with unfavorable outcome

ID	Cfz previous exposure	Baseline Cfz MIC	Cfz in MDR Rx	N effective drugs [1]	Smear at start of BDQ Rx	Bilateral cavities	Dot (%)			BDQ genotype	BDQ phenotype[1]	MIC only	Outcome
							mean BDQ 6M	mean CFZ 6 M	mean Rx 6M except BDQ				
I09	yes	1	no	2	scanty	No	81	na	41	stable	S-S	moderate	died
L24	yes	0.5	no	5	3+	Yes	95	67	79	amplified	S-R	moderate	failed
L09	yes	1	yes	5	2+	yes	92	94	92	amplified	S-R	>CC	failed
L06	yes	0.5	yes	5	3+	Yes	74	70	66	amplified	S-S	moderate	died
L15	yes	0.5	no	3	2+	Yes	97	na	94	amplified	S-R	>CC	failed
I07	yes	0.5	no	3	neg	Yes	83	na	84	amplified	S-R	>CC	failed
L10	yes	0.5	yes	6	neg	Yes	74	74	70	amplified	S-R	moderate	relapsed
L03	no	1	yes	3	3+	Yes	75	69	72	amplified	S-R	>CC	failed
I20	no	0.5	yes	4	3+	Yes	89	88	89	amplified	S-R	moderate	failed
I13	no	0.5	yes	4	2+	Yes	75	73	69	amplified	S-R	>CC	died
L08[3]	yes	2	yes	2	2+	Yes	72	75	74	amplified	R-R	na	failed
I17[3]	no	0.5	yes	5	1+	No	87	97	95	amplified	R-R	na	failed
L22	no	na	yes	6	1+	Yes	72	14	64	na	na	na	died
I25	no	2	yes	5	3+	Yes	90	88	89	na	na	na	died
I08	no	1	yes	4	1+	Yes	98	93	90	na	na	na	LTFU
L27	yes	1	yes	5	3+	Yes	96	93	80	na	na	na	failed

Patients with baseline isolate and negative cultures at follow up were assumed to be stable for mutation amplification and BDQ-MIC increase.

1= cumulative of MGIT DST and BDQ-MIC results; 2=Patients with baseline mutations in BDQ candidate resistance genes and baseline MGIT BDQ-DST resistance; 3=Patients with baseline BDQ-MIC at 0.5µg/ml;

BDQ = bedaquiline; Cfz = clofazimine; CFZ bold= resistant; smear in ()= sample collected after BDQ Rx start

DST = drug-susceptibility; Rx = treatment; FQ-R = multidrug-resistant and resistant to fluoroquinolone (FQ: ofloxacin, moxifloxacin or LFX); SLI-R = multidrug-resistant and resistant to second-line injectable drugs (SLI: KAN or AMK); XDR = multidrug resistant and resistant to FQ and SLI

S=susceptible; R=BDQ resistant; S-S= DST susceptible at baseline stayed susceptible at follow up; S-R= at follow up at least one DST result was R; >CC=MIC increased above the critical concentration, moderate= increase but with CC, LTFU = lost to follow up; DOT= amount of prescribed drug taken by the patient, expressed as percentage

CHAPTER VIII

Discussion

Implementation of Xpert MTB/RIF in MSF settings

The rapid detection of TB and resistance to anti-TB drugs are among the highest priorities in the fight against TB. Early detection of TB using diagnostic tests close to the patient is crucial for early treatment initiation and containment of its spread. Xpert MTB/RIF, launched in 2010, has revolutionized TB detection, replacing microscopy as initial test in many settings, enabling decentralized detection of RMP resistance in hours rather than months. However, despite these remarkable improvements, Xpert MTB/RIF is not considered a true point of care test, due to logistic constraints such as the need of a stable power supply or controlled temperature for storage of the cartridges and the equipment.

While the implementation of Xpert MTB/RIF in central laboratories is relatively straightforward, its sustained deployment in remote settings involves a considerable operational investment.

Médecins sans Frontières (MSF) was among the first organizations to implement Xpert MTB/RIF on a large scale in routine programmatic settings. In **Chapter II** we describe this experience. Xpert MTB/RIF was introduced at various facility levels across thirty-three sites, with testing strategies adapted to the specific epidemiological characteristics of each location, which represented a diverse range of TB, MDR-TB and HIV prevalence. These strategies included using Xpert MTB/RIF in parallel with microscopy for high-risk MDR-TB patients only, as first test for high-risk MDR-TB patients or as add-on test to microscopy for smear-negative presumed TB patients.

The Xpert MTB/RIF positivity rate varied significantly between sites. The relative gain of MTB detection by Xpert MTB/RIF in addition to microscopy showed considerable variation when used as add-on test, with lower rates for example observed in disadvantaged low-income settlement, where patients presented for health care with an advanced stage of disease and thus an overall higher bacterial load.

Overall, Xpert MTB/RIF showed higher relative gain in TB diagnosis (49.7%) as an add-on test relative to its use as replacement of microscopy (42.3%). Use as add-on however resulted in a substantial increase in workload, as microscopy-negative samples required additional Xpert testing, without lowering the microscopy workload. Consequently many MSF projects eventually switched to using Xpert MTB/RIF as replacement of microscopy, as also recommended later by WHO (1).

The observed relative higher gain in TB diagnosis with Xpert MTB/RIF as an add-on test versus initial test, was partially attributed to the high frequency of Xpert MTB/RIF invalid results. The release of the G4 cartridge, updated from G3, helped reducing the frequency of invalid results, which indeed decreased over time. This also suggests that staff experience matters, in contrast with the manufacturer's claim of minimal training required to implement the test.

Despite the initial description of Xpert MTB/RIF as an easy-to implement test, most sites experienced logistical challenges that demanded considerable interventions. This included replacing modules, implementing costly measures such as air conditioning, internet connectivity and stable electricity supply. Indeed, even if the diagnosis of TB was improved, the introduction of Xpert MTB/RIF in decentralized structures was not a simple "plug-and-go" process and

required substantial effort and resources. Our findings have contributed knowledge to the performance and feasibility of rapid molecular testing for MTB (-resistance) detection.
Since we completed this study on the Xpert MTB/RIF, the updated Xpert Ultra was released, as well as a Xpert MTB/XDR, which have similar logistic requirements. With Xpert XDR, rapid detection of resistance to FQs, INH and second-line injectables in decentralized settings became now possible. However, similar tests for resistance to other crucial drugs like BDQ or LZD included in currently recommended RR-TB regimens are still lacking. As a result, samples still need to be referred to higher laboratory levels, for additional DST but also to access culture isolation for treatment monitoring. In conclusion, the implementation of Xpert MTB/RIF and updated versions have significantly improved TB diagnosis and resistance detection, although logistical challenges associated with implementation in remote settings persist. Access to DST to new drugs, following the detection of MDR-TB and fluroquinolone resistance, close to sites where Xpert is implemented, is a priority.

Thin layer agar for detection of TB and resistance to anti-TB drugs
In low-resource settings, the need for a simple, inexpensive and reliable method for simultaneous detection of MTB and drug resistance testing, has led MSF to focus on improving the thin layer agar (TLA) technique. The studies presented in **chapters III, IV and V**, evaluated the performance of TLA for detection of resistance to RMP, INH and FQs, first under controlled conditions, then directly on site.
TLA is a non-commercial method applicable directly to sample sediments, without post-growth manipulation. This makes it suitable for implementation in biosafety level (BSL) 2 laboratories, which are typically located at district level, unlike regional BSL3 laboratories where standard indirect DST methods using liquid and solid media are conducted.
One of the significant challenges for mycobacterial cultures is sample preservation during shipment to limit overgrowth of contaminants without compromising the mycobacterial recovery rate. Cetylpyridinium-chloride (CPC), a commonly used preservation agent, has proven suboptimal for inoculation on agar-based media, not only reducing mycobacterial viability but also interfering with plate reading.
In **Chapter III** we present the results from a comparison of four methods to preserve and decontaminate samples prior to TLA inoculation. Leftover sediments from routine smear-positive samples after decontamination with either Petroff or NALC-NaOH/PBS were inoculated in parallel on TLA and in MGIT, while samples to which CPC had been added during transport were decontaminated on arrival with NALC-NaOH/PBS or NALC-NaOH/Difco^{BD} neutralizing buffer before inoculation on TLA and LJ.
The study identified the combination of CPC and NALC-NaOH followed by neutralization with Difco^{BD} neutralizing buffer as the best to grow MTB. Using this procedure, the TLA positivity rate reached 86.1%, close to the 88.2% obtained with MGIT applied to samples decontaminated with NALC-NaOH, considered the gold standard. Furthermore, this method improved the positivity rate for LJ, increasing from 78.7% (samples treated with CPC combined with NALC-NaOH/PBS) to 93.7% when Difco^{BD} neutralizing buffer replaced PBS, likely due to an incomplete inactivation of

the effect of chlorine and quaternary ammonium by the phospholipids present in egg-based LJ medium. Notably, the replacement of PBS with this reagent also reduced the time to positivity of TLA from 12 days to 8 days, compared to 15 days required to MGIT to become positive, so nearly half the time.

Importantly, none of the TLA plates or LJ slants were contaminated.

The ability of TLA to simultaneously detect MDR- and FQ-resistance directly from sediments was investigated in another study on smear-positive samples obtained from Georgia and shipped to ITM without preservative (**Chapter IV)**. On average, samples were processed at ITM after 12 days from collection. The results confirmed that TB isolation on TLA (95.3%) was comparable to MGIT (97.8%) for smear-positive samples. Additionally, TLA showed good sensitivity to identify resistance to RMP, INH and OFX, but less good for KAN. Specificity was at 100% for all drugs. Since 2019, second-line injectables are no longer recommended for MDR-TB treatment (2), so that further efforts to improve KAN-resistance detection may no longer be considered as a priority.

For samples that underwent longer shipment (median of 14 days) or with a lower smear grade, TLA had more invalid DST results compared to indirect MGIT. Lengthy shipments are usually correlated with loss of viability and increased contamination of the samples. However, similarly, to results from the previous study, TLA exhibited very low rates of contamination, and even when observed, the contamination only covered a limited portion of the plate, never compromising the interpretation of the drug-susceptibility profile.

While lengthy shipments in remote settings can be unavoidable, lowering the exposure time to the decontaminant, and its concentration (e.g., 1% final concentration), may help to reduce the number of invalid results, given these alkalic solutions also affect mycobacterial viability (3). This requires further testing.

One of the downsides of TLA is that results are highly operator dependent, which can affect test performance. Additionally, multiple microscopic readings can burden the laboratory with excessive work. Other authors have proposed using redox indicators on TLA applied on isolates to detect growth by visual check (4). Unpublished data from our laboratory indicates that the STC did not impact mycobacterial viability but delayed the detection time from 10 days to 18 days. However, it allowed for the detection of scantly positive plates otherwise missed, and allowed to alleviate significantly the workload in the laboratory.

Despite these challenges, TLA remains a promising method for drug resistance detection, especially in low-resources settings where access to advanced technologies may be limited.

Following the encouraging results for TLA in research-controlled conditions, its performance was evaluated in routine conditions in Nhlangano, Eswatini, in a peripheral laboratory supported by MSF in collaboration with the Ministry of Health (**Chapter V**). The study aimed to assess the ability of TLA to detect MTB and resistance to RMP, INH and OFX. Additionally, it sought to evaluate the detection of the *rpoB* I491F RR-conferring mutation, missed by Xpert MTB/RIF, Ultra, and MGIT, and highly prevalent in this setting (5)(6).

Samples collected from up to 90 km away, transported in cold chain and decontaminated with 1.5% NALC-NaOH before testing with Xpert MTB/RIF, were inoculated on TLA. All TLA positive cultures were shipped to ITM, where they were tested for susceptibility to RMP, INH and OFX on

solid medium. Despite a relatively short median delay of four days between sputum collection and processing, TLA demonstrated a lower MTB positivity rate for smear-positive samples (68.6%) compared to Xpert (90.7%) and previous studies at ITM, despite the inoculation of two drops of sediment per quadrant, versus one drop, used for plates inoculated with Georgian samples. For smear-negative samples, the positivity rate was 3.7% compared to 5.7% for Xpert. Notably, an increased time between collection and processing negatively impacted the TLA positivity rate rather than the contamination rate, which did not increase with processing delay. These results show that the general antibiotic mixture in the TLA medium can efficiently reduce overgrowth of common flora, but also confirmed that improving sample transportation conditions compatible with TLA and using a milder decontamination method upon sample reception, should be further investigated.

Interestingly, TLA showed significantly higher sensitivity in detecting RR compared to Xpert MTB/RIF, specifically for the *rpoB*_I491F mutation which caused 28.6% of all RR in the study, all correctly classified by TLA. These isolates were also INH resistant, making them MDR-TB. Additional WGS analysis evidenced that half of these isolates also had *Rv0678* mutations associated with for resistance to BDQ (7). The I491F clonal outbreak was rapidly increasing reaching 56% of RR-TB that region in 2019 (7) as a result of untreated RR strains. This lead the country recently to prioritize isolates carrying INH-resistance conferring *katG* mutations for analysis with targeted NGS by Deeplex Myc/TB. However, Deeplex requires specialized laboratory infrastructure and can be applied only on smear-positive samples or isolates. In similar epidemiological settings, where transport of samples is challenging, TLA offers a rapid and feasible solution for detecting RR in smear positive samples from the most infectious patients, addressing the limitation of Xpert MTB/RIF in detecting non-RRDR mutations such as I491F. It also allows for simultaneous exclusion of resistance to FQs while waiting for Deeplex results, in case the new generation Xpert XDR is not available. Furthermore, in contrast to Xpert, TLA can be used to detect viable mycobacteria in follow-up samples to monitor treatment success and amplification of drug resistance.

Moving forward, TLA could be used to detect resistance to new drugs like BDQ. Currently, TLA as direct and indirect method for BDQ, linezolid and delamanid MIC determination is under investigation at ITM, with encouraging results.

Based on our findings, we believe that TLA can play a role in TB diagnostic algorithms applicable in remote settings. Xpert is used for rapid detection of new TB patients and for detection of resistance to RMP, INH and FQs, while TLA would serve as a valuable diagnostic tool to detect resistance due to mutations missed by Xpert, resistance to other drugs not covered by these rapid molecular assays, and to monitor treatment success.

Cultures remain the gold standard for monitoring of treatment. However, the sensitivity of culture is lower in the context of a low bacterial load, and also the medium composition affects the recovery of mycobacteria, which may need supplementary stimuli to grow (8). One study showed how mycobacteria are better recovered in media poor glycerol but enriched in lipids (8). Another study on TLA applied to pleural fluid, showed encouraging results when resuscitation-promoting factors were added to the medium. Even if the exact mechanism is unclear, these

factors are known for their growth-stimulation effect, through hydrolase of peptidoglycan of the mycobacterial cell wall (9). One study investigate the effect of limited zinc nutrient on the mycobacterial heterogeneity in vivo, suggesting that adding zinc in the culture medium may allow for increased recovery of specific subpopulations (10). These studies and others are crucial to enhance culture positivity, and can contribute to the effort to improve mycobacterial detection and decrease invalid results on TLA for paucibacillary samples.

Optimization of sample transportation

In peripheral settings the implementation of an algorithm that includes testing at different facility levels requires an efficient sample transport system. Cepheid recommends that samples should be stored in the fridge for a maximum of 10 days or at RT up to 35°C for 2 days max (11). Samples collected for culture should be stored for up to 3 days at 2-8 °C. However, studies investigating Xpert performance on samples stored for longer periods have not been performed.

The use of a reagent that allows transport without cold chain and is compatible with Xpert and culture in MGIT would facilitate the access to diagnostic tests. OMNIgene, produced by DNA Genotek (Canada) was proposed as a promising solution to stabilize samples and prolong their storage to up to 30 days at 4-40 °C prior to Xpert testing, and 8 days at temperature ≤40°C before inoculation on MGIT culture. Our study evaluated the performance of OMNIgene applied to smear-positive samples for testing with Xpert and MGIT, in two phases. Firstly, we checked the effect of prolonged storage on Xpert performance on split samples on day 0, 7 and 15. Samples were either “fresh” (untreated) or stored in OMNIgene or ethanol, routinely used to store samples before PCR. In a second phase, aliquots were tested in MGIT either decontaminated with standard NALC-NaOH method or with OMNIgene at day 0 or at day 8. The results of the study are shown in **Chapter VI**.

The use of OMNIgene and ethanol did not impact Xpert results, which remained comparable across all testing time points. Interestingly, similar findings were observed even in samples without any preservative. This observation has to be carefully interpreted, as storage conditions in a laboratory may not accurately reflect the conditions during sample collection and transportation from peripheral centers. One drawback of preservation with OMNIgene is the requirement of a centrifugation step at x3000 for smear negative samples to obtain a concentrated pellet (12). This aspect was not evaluated in our study, which included only smear-positive samples; however, this additional step can pose challenges in settings where centrifugation equipment is not available, which should be considered when assessing the potential benefits of using the reagent under true field conditions.

The addition of OMNIgene to samples cultured in MGIT resulted in a significant decrease in viability and prolonged the time to positive results, supporting other findings (13) (14). The results on the benefit of the reagent applied to LJ medium are also unclear, with controversial results reported in the literature (15). The unclear benefit of the reagent relative to alternative preservation- and decontamination techniques, in addition to the high cost of the reagent, have hindered the widespread adoption of OMNIgene. Considering the drawbacks presented, the use of this reagent prior to TLA testing was not further evaluated.

Considering our results, we can conclude that, in peripheral settings where the storage and shipment of samples is challenging, a delay up to two weeks should not prevent Xpert testing.

Detection of Bedaquiline resistance

BDQ is a highly effective drug included in the treatment regimens for MDR-TB. While the duration of MDR-TB treatment became significantly shorter (from two years to 9-12 months), with the introduction of the non-BDQ based Shorter (Bangladesh) Treatment Regimen, BDQ has since been introduced in virtually all regimens. The combination of BDQ, pretomanid, linezolid and moxifloxacin (BPaLM) allowed even further shortening to a 6-months regimen, endorsed by the WHO in 2021(16). The approval by the US FDA in 2012 has accelerated use of BDQ over the last decade, yet access to drug-susceptibility testing (DST) seriously lags behind.

BDQ-DST results are lacking for the majority of patient starting their treatment, or results are obtained only after a considerable portion of the treatment has been completed. The lack of rapid DST is all the more concerning given worrisome reporting of BDQ resistance. Such tests are however hard to design, as mutations found in candidate genes for BDQ resistance are not clearly correlated with phenotypic BDQ resistance (17). Specifically, great diversity of mutations found in *Rv0678*, the most prevalent BDQ resistance associated mutations in clinical isolates, tend to confer only a modest increase in BDQ-MIC. Such BDQ-MIC increases, often close to the critical concentration yet still in the 'sensitive' range, lead to discrepant results between DST techniques, and isolates may also switch between 'S' and 'R' on repeat testing due to intrinsic variation in MIC results (18). Complicating the association between phenotypic and genotypic resistance further, some mutations in *Rv0678* do not confer resistance while other mutations confer hyper susceptibility.

In our study investigating BDQ resistance (**Chapter 7**), the focus was on the correlation between amplification of phenotypic resistance to BDQ and/or amplification of BDQ-associated mutations and treatment outcome, while we also compared the performance of DST for BDQ in MGIT and on 7H11 agar medium. The study included 39 patients from Armenia who were part of the very first cohorts of patients with pre-XDR and XDR-TB (the 'old' definition, including second line injectable resistance) treated with BDQ under compassionate use (prior to FDA approval).

Results revealed that previous intake of CFZ did not significantly increase the risk of BDQ resistance at the start of treatment. However low adherence to BDQ and/or CFZ could trigger the amplification of mutations in *Rv0678*, leading to loss of efficacy to both drugs. However, data on cross resistance between BDQ and CFZ are still limited (19)(16), but can have significant implications for regimens that include both drugs.

In our cohort, baseline phenotypic resistance was already present in 4 (11%) of the patients, and an elevated BDQ-MIC, even if modest, rather than baseline mutations, correlated with unfavorable treatment outcomes. This observation however must be interpreted with caution, as in the period in which these data were collected patients with MDR-TB had not previously received BDQ, and transmission of BDQ resistant MTB was unlikely, and these mutations showed an MIC value at the critical concentration.

In contrast to baseline mutations, amplification of mutations in *Rv0678* during treatment was predominantly observed in patients who failed treatment, regardless of their frequency and

stability in subsequential isolates. In most cases the amplification of mutations was concurrent with an increase in BDQ-MIC, although not always above the critical concentration. This observation suggests that either an increase in BDQ-MIC during treatment, or the detection of mutations, could predict treatment failure. However, we should consider that these study participants received individualized treatment regimens including BDQ and linezolid, while in the BPaLM regimen, pretomanid may potentially overcome BDQ resistance, because of its higher early bactericidal activity (20).

WHO has recently released a rapid communication on the use of targeted NGS for detection of resistance to new and reproposed drugs. Sensitivity for BDQ resistance detection was reported as only 68% (21) showing that this technique still cannot replace phenotypic methods.

Both genotypic and phenotypic methods have limitations in detecting BDQ resistance. For genotypic methods, difficulties do not only rely on the previously mentioned unclear correlation between *Rv0678* mutations and resistance. The use of WGS in recent years has revealed the need for example of assessing also epistatic mutations in other genes (22). In routine conditions, and mainly in resource limited settings, this approach represents a major barrier to patient management.

Phenotypic methods, however, still do not represent a simple alternative. In our study the agreement between BDQ-DST results on 7H11 and in MGIT was low, with a large number of the mutations classified as susceptible by 7H11 yet resistant in MGIT. The agreement could improve by decreasing the critical concentration for 7H11 to 0.125 μg/ml. In MGIT isolates with BDQ-MIC close to the 7H11 critical concentration on repeat testing showed switching between BDQ resistant and susceptible results. Further testing of a larger sample of BDQ-naïve and probably BDQ-resistant strain is required to justify such change.

In MGIT, for few cases the imperfect detection of mutations was observed for isolates with limited growth in the drug containing tube, below the 100 GU cut off to call resistance. The significance of this partial growth below the cut off to call for resistance has not been further investigated. If correlated with heteroresistance, this borderline result would be useful to identify isolates that may likely develop fixed BDQ resistance.

As intermediate resistance conferred by mutations in *Rv0678* can be missed by BDQ DST at one single concentration, MICs are more informative(23).

However, the recommended method to establish MIC, the EUCAST method in broth microdilution plates (BMD), is complex, requires highly skilled staff, and so far can be applied only on isolates, making it difficult to implement in remote settings, where accessibility to laboratories performing DST for BDQ is low. Either samples are added to ethanol and transported to laboratories that perform NGS, or shipped as fresh samples for phenotypic DST. To address this issue, we have initiated a validation of the direct TLA method using leftover sediment from samples received from various MSF projects. Preliminary results are not described in this thesis, yet appear promising when compared to MGIT DST and MIC results with the BMD technique.

Summary and future directions

TLA shows encouraging results to rapidly detect drug resistance in peripheral settings. However, the sensitivity for isolation of MTB drops with long transport times and in sputum with low mycobacterial load in microscopy or Xpert testing. Our study on OMNIgene reagents for sample preservation did not yield an improvement, and the question on how to improve samples preservation before testing remains open. The ongoing evaluations in ITM of TLA as direct method on sediments should integrate milder decontamination steps prior to TLA inoculation.

One of the major limitations for TLA is the high workload generated to complete the reading schedule, which requires multiple microscopy readings. STC could be used to reduce this schedule, alternating microscopic and macroscopic checks.

MSF supports several projects implementing the BPaLM regimen to treat MDR-TB. Detecting baseline BDQ and LZD resistance before starting treatment could better guide treatment decision making. Thus, further investigation into the performance of TLA for detecting BDQ resistance is warranted.

TLA could potentially increase access to rapid MIC testing at intermediate level where access to MGIT and genotypic methods remains challenging.

Currently, DST for BDQ remains the reference method, but for MGIT, the most utilized technique, results can be discordant when the BDQ MIC is close to the critical concentration. The observation of the partial mycobacterial growth, between 0 and 100 units, in the growth control tube of MGIT DST, may indicate heteroresistance. This interpretation may resolve at least part of these discordances, but requires further investigation.

CFZ remains an important drug when BPaL(M) cannot be used. Understanding the interaction between CFZ and BDQ in the amplification of mutations in, *Rv0678*, is crucial. This understanding is important, to limit the amplification of BDQ resistance and preserve the few available treatment options for TB. Further studies should focus on investigation amplification of mutations correlated to BDQ and CFZ, as well as increase of their MIC, when these drugs are used simultaneously.

Programmatic roll-out of BPaL(M) is considered a priority by WHO (24). At present baseline resistance to new drugs is still reported to be low, although resistance to BDQ is increasing (25). Further amplification of drug resistance, and its transmission, may rapidly change this scenario, mainly in settings where access to DST is limited. The implementation of new regimes represents a major improvement to the treatment of MDR-TB, although their introduction requires drug resistance surveillance and increased accessibility to DST before starting treatment (26).

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Curriculum vitae

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Professional development courses

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Oral Presentation “Discrepancies between Xpert MTB/RIF rifampicin resistant results and confirmatory tests”, 30th October, 2014 45th Union World Conference of Lungs Health, Barcelona, Spain

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