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Improving diagnostic approaches in extensively and multidrug resistant tuberculosis: new diagnostic tools and repurposing drugs against X/MDRTB

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Statement of Originality:

The work presented in this thesis is my own and is written in my own words. Everything else, including my own material that has already been published, is appropriately referenced.

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Abstract

Tuberculosis remains a major killer worldwide. Accurate, fast, and accessible diagnostics for both the disease and drug resistance remain challenges for tuberculosis (TB) control.

In four different translational projects linked by the fact they dealt with laboratory diagnosis of drug resistance, I evaluated repurposed compounds and diagnosis of tuberculosis using resources already available in most microbiology laboratories in high-income countries and rapidly expanding to low and middle-income countries. Our aim was to utilise resources already available in most microbiology laboratories in high-income countries wherever possible with the belief that these systems would subsequently expand to low and middle-income countries.

In Chapter 1, I found that the MGIT instrument missed true rifampicin resistance associated with specific *rpoB* mutations. This has now been overcome by the introduction of whole genome sequencing.

In Chapter 2, carbapenems were tested against *M. tuberculosis* using different methodologies, showing that their role in the treatment of tuberculosis remains unclear. Testing in a more physiological model of the granuloma showed similar unclear results but confirmed the excellent activity of clofazimine, a drug that until recently could not be reliably tested *in vitro*.

In Chapter 3, a MALDI-ToF MS lipidomic approach was used to identify *M. tuberculosis* and NTMs at species and some sub-species level. This approach could be easily introduced in UK microbiology laboratories today.

In Chapter 4, urine was explored as a sample for the diagnosis of pulmonary tuberculosis using a lipidomic-MALDI-ToF MS approach and a PCR-based approach aiming to detect *M. tuberculosis* trans-renal DNA.

Over the period of my PhD, I was part of the seminal projects that produced the evidence to introduce WGS for susceptibility testing and which recommended stopping routine phenotypic testing for these drugs. I was involved in the provision of phenotypic data, validation of comparative UK data, writing the standard operating procedure for DST and training technical staff to set up and read commercially available microtiter plates for slow growing mycobacteria. This included MGIT-based phenotypic test methodology which was used to pair with genotypic results. For first line drugs results were very good.

Implementation of whole genome sequencing in the UK for the diagnosis, susceptibility testing and surveillance promised to overcome some of the issues mentioned above. However, 7 years later, a reliable phenotypic drug susceptibility testing methodology is desperately needed for both new compounds and to inform on those drugs for which genomic information is unclear or sparse. New understanding of pharmacokinetics-pharmacodynamics should also be incorporated in these models.

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The biggest thanks goes to my husband Edward for all the years of emotional, personal and financial support so that I could embark on and complete this work.

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List of Abbreviations

AFB: acid fast bacilli

Amx: amoxicillin

BCCM: Belgian Co-ordinated Collections of Microorganisms

BCG: Bacillus Calmette–Guérin

BSL: biosafety level

Clav: clavulanate

CT: cycle threshold

DAT: diacyltrehalose

DHB: 2,5-dihydroxybenzoic acid

DMSO: dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DST: Drug susceptibility testing

ECCMID: European Congress of Clinical Microbiology and Infectious Diseases

ECDC: European Centre for Disease Prevention and Control

Erta: ertapenem

EtOH: etanol

FAM: Fluorescein amidite

Faro: faropenem

gDNA: genomic DNA

GSH: glutathione

HEX: Hexachloro-fluorescein

HIV: Human Immunodeficiency Virus

HPLC-MS: high-performance liquid chromatography – mass spectrometry

IFN-γ: interferon gamma

IGRA: interferon-γ release assay

IL-2Ra: interleukin 2 receptor alpha

IMRL: Irish Mycobacteria Reference Laboratory

IP-10: Inducible protein 10

LAM: lipoarabinomannan

LC-MS: Liquid chromatography–mass spectrometry

LM: lipomannan

M/XDRTB: multi-drug and extensively drug resistant tuberculosis

MALDI: Matrix-assisted laser desorption ionization

MALDI-ToF MS: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

ManLAM: mannosylated lipoarabinomannan

MDRTB: multi-drug resistant tuberculosis

Mem: meropenem

MeOH: methanol

MGIT: Mycobacteria Growth Indicator Tube

MIC: minimal inhibitory concentration

MIRU: Mycobacterial interspersed repetitive units

MMP-9: Matrix metallopeptidase 9

MS: mass spectrometry

MTB: *M. tuberculosis*

MTBC: *Mycobacterium tuberculosis* complex

NMRL: National Mycobacterium Reference Laboratory

NTM: non-tuberculous mycobacteria

OADC: Oleic-Acid, Dextrose, Catalase

PAT: polyacyltrehalose

PCR: Polymerase chain reaction

PDIM: phthiocerol dimycocerosate

PGL: Phenolic glycolipid

PI: Phosphatidyl-inositol

PIM: phosphatidyl-myo-inositol mannoside

PK/PD: pharmacokinetics/pharmacodynamics

PM: proportion method

PMBC: Peripheral blood mononuclear cells

qPCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

RR: resistance ratio

RRTB: rifampicin resistance tuberculosis

rRNA: Ribosomal ribonucleic acid

SGL: sulfoglycolipid

sIL6R: soluble interleukin 6 receptor

SL-I: sulfolipid-I

SRL: Scottish Mycobacteria Reference Laboratory

TB: tuberculosis

TDM: trehalose dimycolate

Tebi: tebipenem

TMM: trehalose monomycolate

trDNA: trans-renal DNA

TST: tuberculin skin test

UCL: University College London

UPLC: ultraperformance liquid chromatography

VNTR: variable number tandem repeat

WGS: whole genome sequencing

WHO: World Health Organization

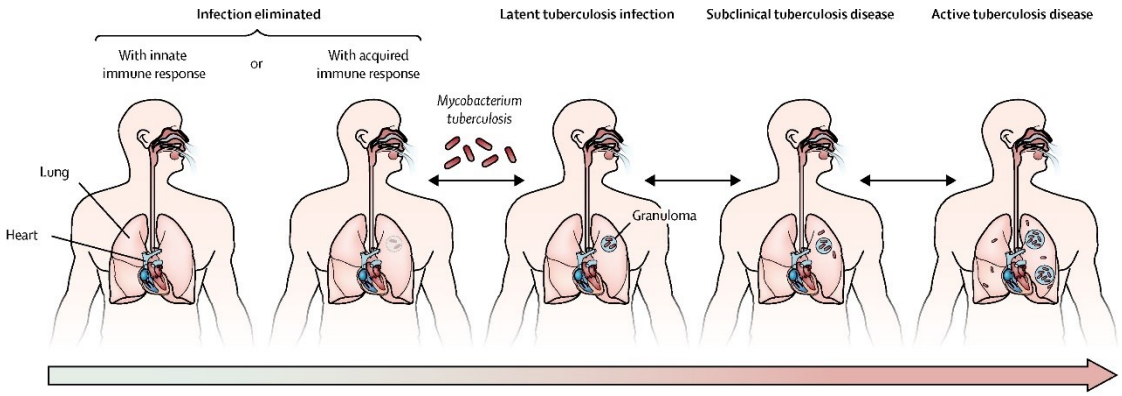
Chapter 1: Introduction and literature review

1.1 The disease

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*, characterised by the formation of granulomatous lesions, chronic inflammation and caseation (Gaby E. Pfyffer; Barbara A. Brown-Elliot; Richard J. Wallace Jr, 2003). Most infections occur due to inhalation of infectious droplet nuclei, and it can affect practically any organ and tissue in the body (Haas, 2017, Guinn and Rubin, 2017).

After inhalation, bacteria can either be eliminated by the immune system or remain viable but not reproducing (latent tuberculosis) or progress to active tuberculosis (Furin *et al.*, 2019a). A schematic representation of this can be found in Figure 1.1.

Figure 1.1 – Spectrum of tuberculosis infection and disease



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

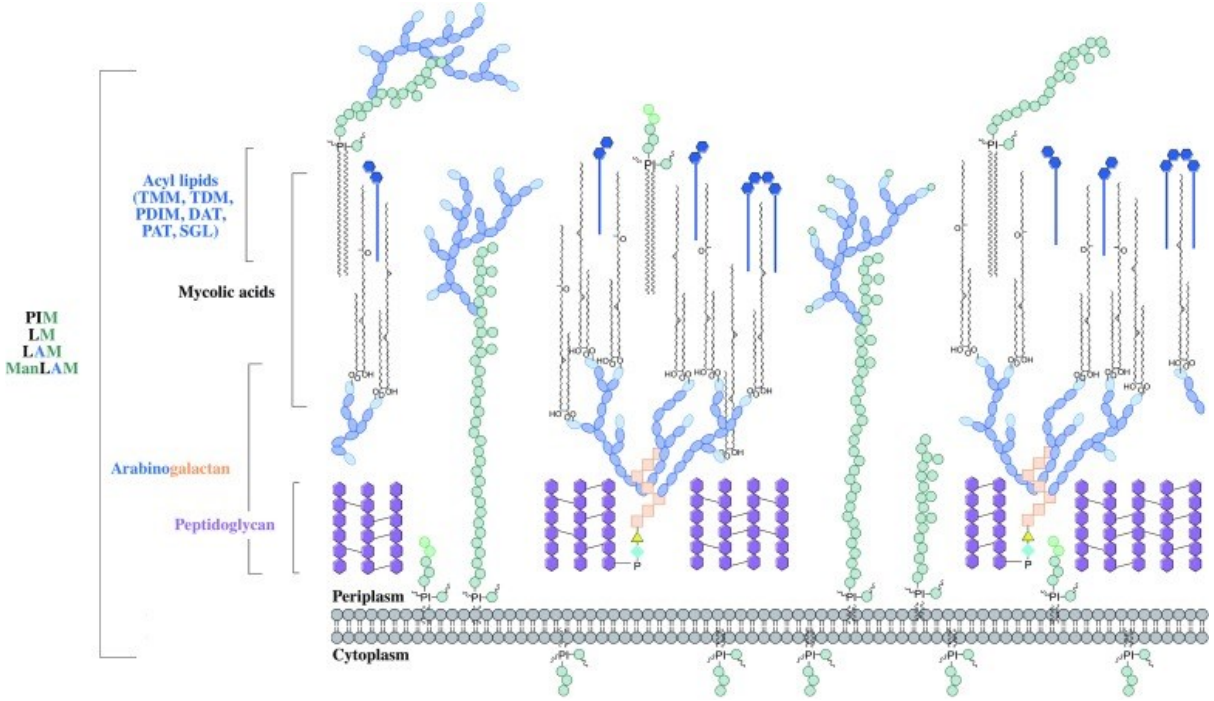
IGRA=interferon-γ release assay. TST=tuberculin skin test. Reproduced from Furin, Cox and Pai, The Lancet 2019 (Furin *et al.*, 2019a)

Tuberculosis was until 2019 the leading cause of death due to infection worldwide and is considered a global health emergency (Furin *et al.*, 2019b, World Health Organization, 2018c). Since emergence of COVID-19, most international tuberculosis control targets are off-track, with significant reduction in the diagnosis of new cases which has impacted in access to therapy, morbidity and mortality with general worsening predicted outcomes for the coming years (World Health Organization, 2021b). Control of the disease is hampered by multiple factors, including the difficulties in getting timely and accurate diagnosis, a key pillar in the WHO End tuberculosis Strategy (World Health Organization, 2018a).

1.2 *M. tuberculosis* microbiology

Mycobacterium spp. is a genus of Gram positive, aerobic, immobile, non-spore forming bacilli that has a high G+C DNA content. It also possesses a very complex, thick, multi-layered, lipid-rich (around 40% of dry weight of the cell envelope are lipids) and hydrophobic cell wall that provides its specific staining characteristics, serves as virulence factor, and mediates the interaction with the host cells and immune system, including the long-term survival inside macrophages and biofilm formation. It is a drug target as well and in recent years, it has also become the target for novel diagnostics and vaccine development. Recent studies also link the structure and thickness of the wall to antimicrobial resistance (Abrahams and Besra, 2018, Alderwick *et al.*, 2015, Dulberger *et al.*, 2020, Jankute *et al.*, 2015, Squeglia *et al.*, 2018, Wright *et al.*, 2017, Singh *et al.*, 2017, Velayati *et al.*, 2016, Kumar *et al.*, 2019, Shao *et al.*, 2020, Howard *et al.*, 2018, Sarmiento *et al.*, 2019, Bhatt *et al.*, 2007, Queiroz and Riley, 2017, Tucci *et al.*, 2020).

Figure 1.2 – The mycobacterial cell wall



A schematic representation of the mycobacterial cell wall, depicting the prominent features, including the glycolipids (PIMs, phosphatidyl-myo-inositol mannosides; LM, lipomannan; LAM, lipoarabinomannan; ManLAM, mannosylated lipoarabinomannan), peptidoglycan, arabinogalactan and mycolic acids. Intercalated into the mycolate layer are the acyl lipids (including TMM, trehalose monomycolate; TDM, trehalose dimycolate; DAT, diacyltrehalose; PAT, polyacyltrehalose; PDIM, phthiocerol dimycocerosate; SGL, sulfoglycolipid). The capsular material is not illustrated (Reproduced from Abrahams and Besra, Parasitology 2018 Vol. 145 Issue 2)

The most prominent molecules in the cell wall are peptidoglycan, arabinogalactan, mycolic acids, acyl-lipids, glycolipids. Glycolipids include phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM), lipoarabinomannan (LAM), mannosylated lipoarabinomannan (MLAM). Acyl lipids include trehalose monomycolate (TMM), trehalose dimycolate (TDM), diacyltrehalose (DAT), polyacyltrehalose (PAT), phthiocerol dimycocerosate (PDIM) and sulfoglycolipid (SGL) (Abrahams and Besra, 2018, Bhat *et al.*, 2017, Wang *et al.*, 2011).

The plasma membrane is a lipidic bilayer interacting with proteins, peptidoglycan, arabinogalactan and polysaccharides esterified with mycolic acids. The free lipids associated with the outer membrane are very complex and structurally diverse (Yokoyama *et al.*, 2018, Gonzalo *et al.*, 2021).

Some lipids are specific for *M. tuberculosis* complex while others are only found in other mycobacterial species (Laval et al., 2001, Larrouy-Maumus and Puzo, 2015a).

Phosphatidyl-inositol (PIs) and PIMs are involved in septation and permeability as well as playing a structural role. LM and LAM have a prominent role in pathogenicity and interaction with the host's cell, having an immunomodulatory role and being linked to susceptibility to β-lactam antibiotics (Fukuda *et al.*, 2013, Crick and Guan, 2016, Layre *et al.*, 2014, Pal *et al.*, 2017).

Mycolic acids are virulence factors, and they are related to cell wall permeability.

There are over a hundred and ninety *Mycobacterium* species identified (King *et al.*, 2017). A small number belong to the *M. tuberculosis* complex and the rest are collectively called Non-Tuberculous Mycobacteria (NTM) (Magee and Ward, 2015). At least 60 *Mycobacterium* species are associated with pathogenicity in humans, with *M. tuberculosis* the most prominent as human pathogen (Alcaide *et al.*, 2018a).

Mycobacterium tuberculosis complex is a group of closely related mycobacteria that cause tuberculosis in different mammals (*M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium pinnipedii*, *Mycobacterium canetti*, *Mycobacterium orygis*, *Mycobacterium caprae*, *Mycobacterium suricattae*, *Mycobacterium mungi*, and *Mycobacterium microti)* (Rodríguez-Hernández *et al.*, 2020). The most significant member of the group for humans is *M. tuberculosis*. Other members of the complex can cause disease in humans but are more commonly associated with disease in cattle and other domestic and wild animals, posing both a health risk for humans as well as a financial burden for certain economic sectors such as agriculture (Abrahams and Besra, 2018).

1.3 *M. tuberculosis* bacteriological diagnosis

Bacteriological diagnosis of tuberculosis is based on phenotypic and genotypic tests. They are summarised in Table 1.1 below. The current approach is usually a combination of both phenotypic and genotypic methodologies (El Khéchine and Drancourt, 2011, Abe, 2003, Acharya *et al.*, 2020). In recent years the WHO has emphasised the importance of employing nucleic acid amplification testing (NAAT)-based diagnostic assays on primary patient specimens such as sputa (World Health Organization, 2021d).

Table 1.1 – Common phenotypic methods for the identification of tuberculosis

Phenotypic methods:	**Smear microscopy from samples**	Auramine O	
		Auramine-rhodamine	
		Acridine orange	
		Ziehl-Neelsen	
		Kinyoun	
	Culture	Liquid	Mycobacteria Growth Indicator Tube (MGIT)
			BacT/Alert, ESP Myco
			MB Redox
			KRD "Nichi B"
			Dubos
			Kirchner
			Middlebrook 7H9
		Biphasic	Septi-Chek AFB
			Myco-Acid
			Bi-phasic
		Radiometric	BACTEC 460TB (now discontinued)
		Egg-based solid media	Ogawa
			Löwenstein-Jensen
		Agar-based solid media	Middlebrook 7H10
			Middlebrook 7H11
		Colony morphology	
		Absence of pigmentation	
		Niacin	
		Growth onT2H	
		Nitrate reduction	
		Semi-quantitative catalase	

		Negative 68º catalase	
		Tween hydrolysis	
		Tellurite reduction	
		Negative arylsulphatase 3 days	
		Pyrazinamidase	
		Urease	
		Intolerance to 5% NaCl	
		Negative iron uptake	

M. tuberculosis complex strains can be identified phenotypically by their characteristics. They form non-pigmented rough, slow growing colonies that grow best at 37°C (Vincent, 2003). Staining from microbiological liquid culture shows cording, typical of *M. tuberculosis* (El Khéchine and Drancourt, 2011).

Identification to species level and differentiation between members of the *M. tuberculosis* complex was historically based on complex biochemical tests (Vincent, 2003, Bhalla *et al.*, 2018, Abe, 2003), most of them now superseded by molecular tests.

An immunochromatographic test based on antigen MPT64 (MPB64 for *M. bovis*) is widely used, mainly paired with liquid culture. It is quick and inexpensive with high sensitivity and specificity. Its performance can be affected, though, by mutations in the *mpt64* gene (Yin *et al.*, 2013), i.e. mutations which give false negative results in a very small percentage of strains.

Some genotypic methods have been developed to be used from patient samples while others still require culture. A summary of the currently available technologies is listed below, in Table 1.2. Some tests can both diagnose tuberculosis and antimicrobial drug resistance simultaneously (MacLean *et al.*, 2020, Skenders *et al.*, 2011, Notomi *et al.*, 2015, World Health Organization, 2016).

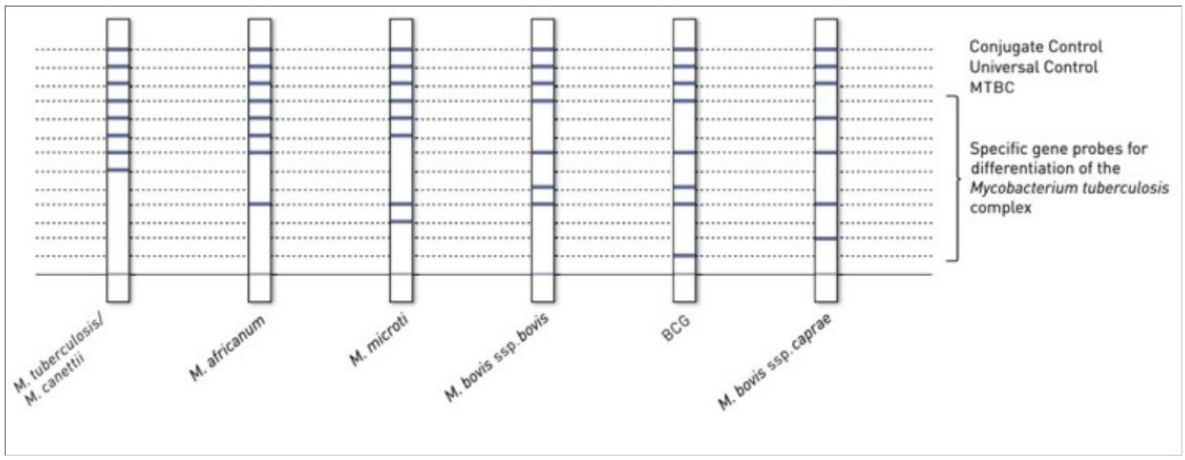
Table 1.2 – Summary of most commonly used molecular methods for the detection and identification of tuberculosis

Line probe assays:	GenoType MTBC (Hain Lifesciences-Bruker, Nehren, Germany)
	GenoType MTBDRplus
	GenoType MTBDRsl
	InnoLipa
	Nipro NTM+MDRTB II (Osaka, Japan)
Loop-mediated isothermal amplification.	TB-LAMP assay (Eiken Chemical Company, Tokyo, Japan)
	In-house LAMP assays
RT-PCR	Gene Xpert MTB/RIF (Cepheid, Sunnyvale, USA)
	Gene Xpert Ultra (Cepheid) (Ultra)
	Gene Xpert MTB XDR-TB
Point of care NAAT methods	POC GeneXpert Omni platform
Other molecular methods	EasyNAT (Ustar Biotechnologies, Hangzhou, China) cross-priming amplification
	Simultaneous amplification and testing tuberculosis (SAT-TB) (Rendu Biotechnology, Shanghai, China)
	MeltPro tuberculosis (Zeesan Biotech, Xiamen, China)
	GeneChip MDR (CapitalBio Corporation, Beijing, China)
	Truenat MTB (Molbio Diagnostics, Goa, India)
	Truenat MTB Plus (Molbio Diagnostics, Goa, India)
	Truenat MTB-Rif Dx (Molbio Diagnostics, Goa, India)
	RealTime MTB (Abbott Molecular, Abbott Park, USA)
	RealTime RIF/INH (Abbott Molecular)
	FluoroType MTB (Hain Lifescience, Nehren, Germany)
	FluoroType MTDBR (Hain Lifescience)
	Cobas MTB (Roche, Rotkreuz, Switzerland)
	Max MDRTB (BD, Franklin Lakes, USA)
	Next-generation sequencing
	Targeted Next Generation Sequencing

Line probe assays are one of the most widely used methodologies for the identification of mycobacteria as well as detection of resistance to first and selected second line drugs. DNA probes are bound to a membrane, then the target DNA is amplified, denatured, and hybridised

to the membrane-fixed probes. Substrates that change colour in the presence of binding are added, providing a pattern of bands for interpretation, Figure 1.3 (Watterson *et al.*, 1998).

Figure 1.3 – GenoType MTBC (Hain Lifesciences-Bruker, Nehren, Germany) reading aid. Reproduced from Hain Lifesciences-Bruker website https://www.hain-lifescience.de/en/products/microbiology/mycobacteria/tuberculosis/genotype-mtbc.html



Schließen

Another widely used methodology is Gene Xpert MTB/RIF (Cepheid, Sunnyvale, USA). This is a real-time PCR platform that targets the *rpoB* in *M. tuberculosis.* Its advantages over other real-time PCR platforms are related to the “laboratory in cartridge design”; samples are mixed with a solution and loaded into the cartridge and all processes occur within the cartridge, without additional operator manipulation. The result is ready in 90 minutes, once the sample is loaded into the system (Acharya *et al.*, 2020). In recent years, a new version, the Gene Xpert MTB/RIF Ultra, has been introduced to overcome technical issues associated with the previous version. The Ultra has a better limit of detection (15.6 CFU/ml of sputum for Ultra versus 112.6 CFU/ml of sputum for Xpert) with fewer false positives for rifampicin resistance. The Ultra also has improved sensitivity compared to Xpert, both overall sensitivity, 87.5% vs 81%, and sensitivity in smear-negative samples, 78.9% vs 66.1%. Specificity remained high at 98.7%. Both tests had a specificity of 98.7% (Chakravorty *et al.*, 2017). Figure 1.4.

Figure 1.4 – GeneXpert Instrument as illustrated at https://www.cepheid.com/en/systems/GeneXpert-Family-of-Systems/GeneXpert-System. System for 2, 4 and 16 cartridges.

Next generation sequencing offers a one-stop approach to diagnosis of tuberculosis as it can identify an isolate to species and sub-species level, genotype, detect antibiotic-associated mutations and provide molecular epidemiology information in one step (Acharya *et al.*, 2020, Walker *et al.*, 2015, Drobniewski *et al.*, 2015, Papaventsis *et al.*, 2017). Even though there are reports of next generation sequencing detecting *M. tuberculosis* and drug resistance from samples (Votintseva *et al.*, 2017, Brown *et al.*, 2015), in most centres it still requires prior culture. In spite of this, it provides reports 21 days earlier than routine existing methods. It is expensive but cost is falling. As with any other DNA detection method, it cannot differentiate between live and non-viable microorganisms (Acharya *et al.*, 2020). Figure 1.5 depicts the Illumina MiSeq instrument. This instrument has allowed significant reduction is costs.

Figure 1.5 – Illumina MiSeq next generation sequencing instrument reproduced from https://www.illumina.com/systems/sequencing-platforms/miseq.html

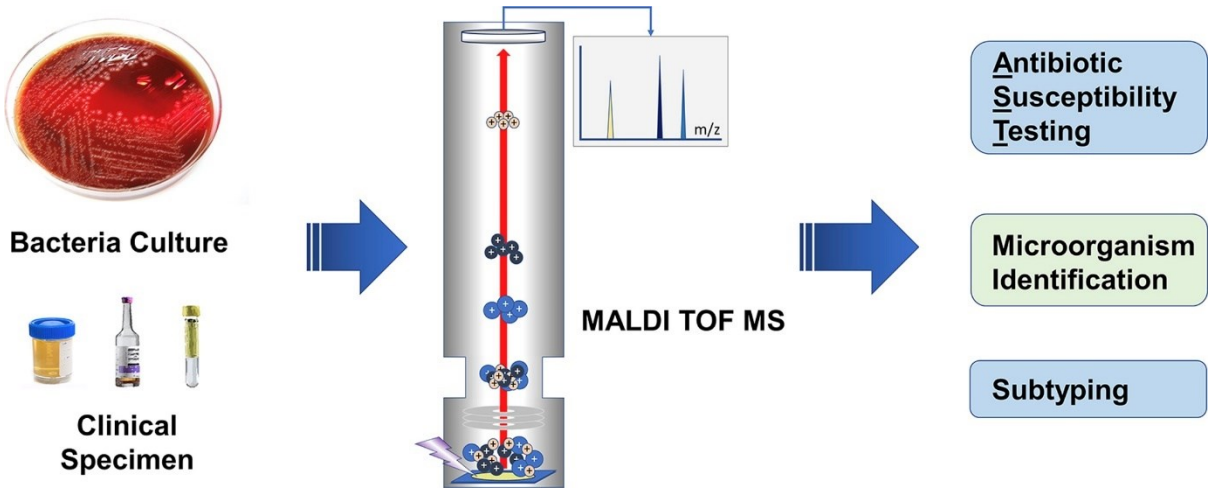
Targeted next generation sequencing offers speedier results as well as fully automated interpretation of them. It can be performed directly from samples, and it targets 18 genomic areas known to be associated with drug resistance (Jouet *et al.*, 2021, Feuerriegel *et al.*, 2021, Kambli *et al.*, 2021). Deeplex® MYC-TB represented in Figure 1.6.

Figure 1.6 – Deeplex® MYC-TB platform reproduced from https://www.deeplex.com/

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) has been the mainstay of microbiology diagnostics in high-income countries for several years and has revolutionised bacterial and fungal identification as it is fast and cheap (Dingle and Butler-Wu, 2013, Schubert and Kostrzewa, 2017, Patel, 2015, Kostrzewa, 2018, Kostrzewa *et al.*, 2019). More recently, both mycobacterial identification and limited antibiotic drug resistance testing have become available as part of the spectra repertoire in some platforms (Akyar *et al.*, 2018, Vrioni *et al.*, 2018, El Khechine *et al.*, 2011). A benchtop biotyper can be seen in Figure 1.7 and a summary figure of current use in the microbiology laboratory, in Figure 1.8.

Figure 1.7 – Bruker MALDI Biotyper®- Sirius, the next generation routine MALDI-ToF MS device for the use in routine microbiology laboratories, reproduced from https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/maldi-biotyper-sirius-system-gp-ruo.html

Figure 1.8 – Schematic use of MALDI-ToF MS in routine microbiological diagnostics, reproduced from Hou *et al.*(Hou *et al.*, 2019)



Briefly, a sample is coated with a matrix that is ionised by a laser beam. Desorption and ionisation of the sample produce protonated ions that are accelerated and subsequently separated by their mass to charge ratio (m/z). The charged molecules are detected and measured by determining the time taken to travel the length of the flight tube (time of flight).

Each analyte in the sample will produce a characteristic peak and the group of analytes in a given sample will generate a specific spectrum (Singhal *et al.*, 2015).

The current technique in use is based on protein fingerprinting which poses a problem for its broad application for mycobacteria since, in order to pick up the protein profile, samples must be treated (extraction step) (Alcaide *et al.*, 2018a). Handling and treatment of samples has to be done in biosafety level 3 facilities for *M. tuberculosis* detection.

Another bottleneck in rapid detection of *M. tuberculosis* resides in the inability to discriminate mycobacteria within the *M. tuberculosis* complex by MALDI-ToF MS based on protein profiling (El Khechine *et al.*, 2011, Zingue *et al.*, 2016, Balada-Llasat *et al.*, 2013). Members of the *M. tuberculosis* complex are characterized by more than 99.9% identity at the nucleotide level and an identical 16S rRNA sequence (Fleischmann *et al.*, 2002, Huard *et al.*, 2006, Ernst *et al.*, 2007).

The majority of *M. tuberculosis* complex infections in humans are caused by *M. tuberculosis* (90-95%), followed by *M. bovis* (1-2%) and *M. caprae* (1-2%); with *M. africanum* more prevalent in Western Africa (Bayraktar *et al.*, 2011, Wiens *et al.*, 2018, Damene *et al.*, 2020, de Jong *et al.*, 2010, Muller *et al.*, 2013, Davidson *et al.*, 2017). Despite high levels of genome similarities (Riojas *et al.*, 2018), *M. tuberculosis* complex member sub-species possess varying virulence, transmission rates, disease severity, immunogenicity, and drug susceptibility (Coscolla and Gagneux, 2010, Yanti *et al.*, 2020); *M. bovis* and *M. canettii* are both resistant to pyrazinamide, a key tuberculosis drug, as a result of genetic mutations in the *pncA* gene (Koser *et al.*, 2012, Somoskovi *et al.*, 2008). It is therefore important to differentiate between individual species rapidly and accurately, especially when advising on infection control policies and choices of treatments.

A novel approach, based on lipids from intact, heat-killed bacteria has been reported recently that would overcome, at least partially, the issues stated above (Larrouy-Maumus and Puzo, 2015b, Gonzalo et al., 2021).

Different tuberculosis lineages and mycobacterial species have different lipidomic profiles. Some resistant and susceptible strains show different lipidomic profiles. MALDI-ToF MS can be adapted to perform identification of mycobacteria and limited drug susceptibility analyses based on species-specific lipid profiles (Lahiri et al., 2016, Larrouy-Maumus and Puzo, 2015a).

1.4 Using urine for the diagnosis of pulmonary tuberculosis

Tuberculosis microbiological diagnosis relies on isolation of *M. tuberculosi*s from samples collected from the anatomical area affected by the microorganism. While certain locations are relatively easy to access, some anatomical areas require invasive procedures for the patient (Ketata *et al.*, 2015, Sharma *et al.*, 2021, Natarajan *et al.*, 2020).

There are populations of patients in whom diagnosis is particularly challenging, i.e., children (Marais, 2020), immunosuppressed individuals (Hamada *et al.*, 2021) and those with extrapulmonary tuberculosis as well of those that due to other reasons cannot undergo the procedure necessary to obtain the sample. In these groups, obtaining a sample is sometimes challenging or, if the sample can be acquired with relative ease, it tends to be paucibacillary (Peter *et al.*, 2010).

Urine can usually be obtained without invasive procedures, in relatively large quantities and with very little inconvenience for the patient. In addition, at least in the Human Immunodeficiency Virus (HIV)/tuberculosis co-infected population, detection of tuberculosis in urine has been shown to be cost effective and potentially life-saving (Reddy *et al.*, 2019). Several approaches using urine as a sample for the diagnosis of tuberculosis are being explored.

The kidney's filtration limit is between 30 and 50 kDa, so candidate molecules for tuberculosis diagnostics must be within the filtration cut-off size (Ruggiero *et al.*, 2010).

Lipids

Urine lipidic content is not very high, it is in the vicinity of 0.84 μM and 1.04 μM for men and women, respectively. Non-polar lipids should barely be detectable in healthy mammals. The

most frequently encountered ones belong to 5 classes: fatty acyls, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids. Around 836 lipid metabolites can be found in urine (Tipthara and Thongboonkerd, 2018, Yuan *et al.*, 2017, Bouatra *et al.*, 2013).

Due to the rich lipidic content of the mycobacterial wall, detection of its components in the hosts' urine represents an interesting area of research and lipidic extraction from mycobacterial culture has been described for several species. The classic Bligh and Dyer's methods, the Folch's method and, more recently, Chandramouli and Venkitasubramanian's methods have all been used successfully, with the latter giving greatest yield (Pooja Singh, 2014).

Lipid concentration and quantification methodologies from urine have been described for multiple purposes but not for mycobacteria-derived molecules, except for lipoarabinomannan discussed below (Klahr *et al.*, 1967, Jüngst *et al.*, 1984, Gross *et al.*, 1991, Yuan *et al.*, 2017, Khan and Glenton, 1996, Khoomrung *et al.*, 2013, Hounslow *et al.*, 2017, Fuller *et al.*, 2005).

Lipoarabinomannan

Lipoarabinomannan (LAM) is a 17.5 kD mycobacterial cell wall lipoglycan and virulence factor present in several mycobacteria (Guérardel *et al.*, 2003, Chan *et al.*, 1991, Nigou *et al.*, 2003, Peter *et al.*, 2010). Mannoside-capped LAMs are present in slow-growing mycobacteria and are anti-inflammatory (Nigou *et al.*, 2003). They also have immunomodulatory activity. In *Mycobacterium tuberculosis*, LAM inhibits monocyte function and controls host cell apoptosis, prolonging cell life (Maiti *et al.*, 2001).

"In macrophages for example, LAM is able to block many actions of IFN-γ, including tumor cell killing, intracellular killing of toxoplasma, and increased expression of several IFN-γ-inducible genes. It has been suggested that these actions of LAM may be related to inhibition of protein kinase C (PKC) to scavenging of cytotoxic oxygen free radicals, or to both. In T cells, LAM has been shown to suppress antigen-driven proliferation of a $CD4^+$ T cell clone. It has also been shown to inhibit the accumulation of mRNA for interleukins-2 and -3, granulocyte-

macrophage colony-stimulating factor, and the interleukin-2 receptor α-chain in Jurkat T cells stimulated with phytohemagglutinin and phorbol esters." (Knutson *et al.*, 1998).

Lipoarabinomannan is excreted in urine (Whitelaw and Sturm, 2009, Thwaites, 2014) and an assay for its detection was developed in the early 2000s (Hamasur *et al.*, 2001). It is an enzyme-linked immunosorbent assay in which monoclonal antibodies are attached to a solid phase. When in contact with the antigen, it shows as a visualised band. The assay produced by Alere (Waltham, MA) has been in use for almost two decades and has now been formally endorsed by WHO for the diagnosis of active tuberculosis in people living with HIV (World Health Organization, 2019b). The test performs better the more immunosuppressed the patient is, showing better results with lower CD4+ counts (Lawn, 2012a). In other populations, its results are not good enough to be incorporated into routine clinical practice (Peter *et al.*, 2010).

In recent years, a new version of the test developed by Fujifilm (FujiLAM) has shown a better performance when compared with the Alere assay. In the paediatric population, sensitivity of FujiLAM and AlereLAM was 42% and 50%, respectively. The specificity of FujiLAM and AlereLAM was 92% vs 66%, respectively. Both tests performed better in children older than 2 years, in children with HIV or who were malnourished, showing that the new versions of the Alere and FujiLAM, performed better when the patient's immunity is impaired. The specificity of both tests was also higher in children 2 years or older (Nicol *et al.*, 2020). However recent production problems has meant that the Fuji-LAM is not available for clinical use currently.

Trans-renal and genomic DNA

Trans-renal DNA represents acellular DNA fragments of less than 200 bp (Labugger *et al.*, 2017, Petrucci *et al.*, 2015, Green *et al.*, 2009, Cannas *et al.*, 2008). Trans-renal DNA detection was originally developed as a test for the diagnosis of certain types of malignancies and is currently used in the antenatal setting and in transplant medicine. This DNA is the

product of cells dying (Botezatu *et al.*, 2000) and it can be from the host or the pathogen (Cannas *et al.*, 2008).

The use of trans-renal DNA for the diagnosis of tuberculosis was first explored in the early 2000s. IS6110 (an insertion sequence element found in nearly all TB strains) was more often the target, but other targets have been described. The size of the targeted section varied amongst different reports from less than 40 bp to 120bp. Sensitivity and specificity for *M. tuberculosis* detection were highly variable, from 43 to 79% and 89 to 100% respectively (Fernández-Carballo *et al.*, 2019, Torrea *et al.*, 2005).

A recent report suggests sensitivity can be improved significantly by purifying the trans-renal DNA using hybridisation probes immobilised on magnetic beads. This method worked well for both HIV-positive and HIV-negative patients (Oreskovic *et al.*, 2021).

In people living with HIV, the yield of tuberculosis DNA detection in urine is higher, with some genomic platforms such as cepheid GeneXpert detecting tuberculosis in this population's urine samples (Bordelon *et al.*, 2017, Gupta-Wright *et al.*, 2018, Torrea *et al.*, 2005). In the same population, next generation sequencing of urine found 158 different tuberculosis DNA fragments varying from 19 to 44 bp (Sinkov *et al.*, 2020).

Host urine immunological biomarker

Inducible protein (IP)-10 has been found to be elevated in both blood and urine in tuberculosis patients (Petrone *et al.*, 2016). This protein is induced by gamma-interferon and is secreted by several different types of cells both immunological such as T-cells, and others such as fibroblasts and adipocytes (Hayney *et al.*, 2017). It is, however, non-specific, and it is elevated in other respiratory infections and cannot distinguish between tuberculosis and other infections (Petrone *et al.*, 2015).

Detection of 16S RNA in urine

16S-rRNA is part of the prokaryotic ribosomes. It is not present in human cells and represents a good bacterial marker of bacterial infection. Furthermore, unlike DNA that may represent dead microorganisms, the presence of 16S-rRNA shows transcription, meaning viable bacteria (Cubero *et al.*, 2013). In a study from Spain, around 50% of HIV negative patients with extrapulmonary tuberculosis had 16S-rRNA amplified in urine. Detection was high in miliary tuberculosis (90%) and genitourinary tuberculosis (89%), falling sharply when the presentation was more localised (to 45% for osteoarticular and 33% for pleural TB. For pulmonary tuberculosis, sensitivity was 17.9% (Fortún *et al.*, 2014). If sensitivity problems can be addressed, this method may represent a good option to diagnose tuberculosis and to distinguish between active and latent tuberculosis infection.

Host tuberculosis biosignature in urine

A variety of tuberculosis biosignatures have been described/proposed and these can be tuberculosis pathogen based or host derived.

Using liquid chromatography–mass spectrometry (HPLC-MS), 10 compounds were detected in urine with good correlation with the presence of active tuberculosis. Moreover, these compounds decreased after 2 months of effective anti-tuberculosis treatment, i.e., it could be used for prophylaxis determination. A combination of diacetylspermine, neopterin, sialic acid, and N-acetylhexosamine exhibited the best results in blinded validation. All these molecules represent host inflammatory metabolites (Isa *et al.*, 2018). In a follow up study, Xia *et al.* using untargeted HPLC-coupled high-resolution TOF-mass spectrometry, suggested that urinary DiAcSpm, neopterin, hydroxykynurenine, *N*-acetylhexosamine, ureidopropionic acid, sialic acid, and *m/z* 241.0903 reductions correlated with successful anti-TB treatment and sputum mycobacterial load (Xia *et al.*, 2020).

Another report using UPLC-Q Exactive MS, compared the urine metabolome of active tuberculosis patients, latent tuberculosis patients and non-tuberculosis individuals. Out of the

hundreds of metabolites found, six differential metabolites were screened for in positive and negative mode: 3-hexenoic acid, glutathione (GSH), glycochenodeoxycholate-3-sulfate, *N*-[4′-hydroxy-(*E*)-cinnamoyl]-l-aspartic acid, deoxyribose 5-phosphate and histamine. These metabolites are related to immune regulation and the urea cycle. Of these, GSH and histamine performed the best for differentiating between active and latent tuberculosis and non-tuberculosis individuals (Deng *et al.*, 2021).

Looking for proteins as tuberculosis biomarkers, Liu *et al*. found that a combination of 5 proteins (glutathione peroxidase 3, neurotrimin, poliovirus receptor, signalling lymphocytic activation molecule family 1 and hemicentin-2) could detect tuberculosis with a sensitivity of 82.7%. A three-protein combination could differentiate active from latent tuberculosis. Proteins were detected by LC-MS/MS, and they are all part of the immune/inflammatory repertoire (Liu *et al.*, 2021).

Seryl-leucine core 1 *O*-glycosylated peptide of human origin was detected using untargeted mass spectrometry and was reported to be elevated in urine of tuberculosis patients when compared to household contacts and healthy controls. This molecule was also associated with bacterial burden and inflammation, and it was also noticed to decrease during effective therapy (Fitzgerald *et al.*, 2019).

In South Africa, using a Luminex-based approach, 29 analytes were evaluated as tuberculosis biomarkers in urine. Seven biomarkers were detected as having potential for tuberculosis diagnosis, performing better for the HIV positive patients. In this sub-population, a biosignature of sIL6R, MMP-9, IL-2Ra, IFN-γ had a sensitivity of 85.7% to diagnose tuberculosis. For HIV negative patients, sIL6R and sIL-2Ra, had a sensitivity of 53.9%. All these biomarkers are part of the host inflammatory response (Eribo *et al.*, 2020).

Das *et al.* reported deregulation of the tyrosine-phenylalanine axis in tuberculosis patients by detection of metabolites in urine (i.e., norepinephrine, gentisic acid, 4-hydroxybenzoic acid,

hydroquinone, and 4-hydroxyhippuric acid using silylation), gas chromatography mass spectrometry, and standard chemometric methods (Das *et al.*, 2015).

Other methodologies using urine for tuberculosis diagnostics

A proteomics approach using SOMAscan® analysis showed that, in urine, only one SOMAmer out of 85 candidates analysed, ESXB 5557-2 was of sufficient concentration in urine to differentiate between tuberculosis and non-tuberculosis patients. However, the aptamer signals were not discriminatory enough to be diagnostically useful (Russell *et al.*, 2017).

Detection of volatile compounds in urine has also been explored for the diagnosis of tuberculosis (Dang *et al.*, 2013). In one report from Kenya, headspace volatile compounds from urine could discriminate tuberculosis from non-tuberculosis patients in an HIV negative population with 78.3% sensitivity and 69.2% specificity. This methodology performed worse in the HIV co-infected population (Sandlund *et al.*, 2018).

Detection of specific *Mycobacterium tuberculosis* Rv1681 protein in urine was successful in a small number of cases and could discriminate from other infectious diseases as well as from healthy controls (Pollock *et al.*, 2013). Rv1859 has also been investigated with good preliminary results (Kim *et al.*, 2016). A study conducted in Brazil, detected 4 specific *Mycobacterium tuberculosis* proteins in the urine of 9 patients with confirmed pulmonary tuberculosis for whom renal/urinary tract tuberculosis had been excluded. These proteins were MoaA-related protein, Ornithine carbamoyltransferase, Homoserine O-acetyltransferase and Phosphoadenosine phosphosulphate reductase (Kashino *et al.*, 2008).

1.5 Susceptibility testing

Drug susceptibility testing (DST) for tuberculosis can be done both phenotypically and genotypically. In the UK, *in vitro* testing of first line drugs was stopped, and drug susceptibility is interpreted using whole genome sequencing following a series of studies that demonstrated that genotypic prediction of resistance to first line drugs was able to accurately predict

phenotypic resistance (Casali *et al.*, 2014, Walker *et al.*, 2015). *In vitro* susceptibility is reserved for second line drugs or for those instances when mutations are not clearly interpretable (England, 2021). In other areas of the world, phenotypic drug testing remains the main approach to establish drug susceptibility (Centers for Disease Control and Prevention, 2012). A summary list of the methods for phenotypic testing can be found in Table 1.3

Table 1.3 – Drug susceptibility testing methods for mycobacteria

In vitro
Proportion method (Canetti *et al.*, 1969)
Resistance ratio (Drobniewski *et al.*, 2007)
Absolute concentration (Drobniewski *et al.*, 2007)
Microdilution (Lee *et al.*, 2014)
MODS (Minion *et al.*, 2010)
Resazurin (Coban *et al.*, 2014)
Macrodilution (Adjers-Koskela and Katila, 2003)
MGIT (Adjers-Koskela and Katila, 2003)
Ex vivo *(Turner et al., 2002)*
Animal models (Creissen *et al.*, 2021, Peña and Ho, 2016)
Genotypic
Probes (Drobniewski *et al.*, 2007)
WGS (Drobniewski *et al.*, 2007)

There are certain antibiotics for which reliable susceptibility testing is not available either genotypically or phenotypically. Clofazimine is one of these drugs. Cycloserine is in the same category as well as the two newer agents bedaquiline and delamanid (England, 2021). Within the latest BPaL and BPaLM regimes (bedaquilline, pretomanid, linezolid, with or without moxifloxacin), DST is not available for pretomanid (TB Alliance, 2022, World Health Organization, 2022, Hajikhani *et al.*, 2021).

Studies by the ECDC and WHO have shown that even in Europe where most WHO Supranational Reference Laboratories are based, most national reference laboratories are not able to reliably analyse bedaquiline, delamanid and clofazimine resistance (Farooq *et al.*, 2021).

Another significant issue related to *in vitro* testing is that it disregards host factors/role in bacterial killing. There are new methods aiming to include the host in the susceptibility testing – 2-dimensional *ex vivo* and 3-dimensional *ex vivo* models as well as animal models. A non-animal approach which is able to incorporate features of host-derived immunity into a combined approach is clearly superior, cheaper and kinder than a solely animal-based *in vivo* approach (Workman *et al.*, 2014, Andreu *et al.*, 2010).

Pharmacokinetic/pharmacodynamic aspects of TB therapy are also inadequately explored with all the current phenotypic methods as they usually involve addition of the study compound at a single time-point. There are new models that allow the regular addition of the antibiotics, more in keeping with the actual treatment of tuberculosis, when drugs are taken are regular intervals. The hollow fibre infection model is the most widely used. This dynamic model allows for the addition of compounds and nutrients at several time-points as well as the removal of metabolic waste and old drug (FiberCell Systems, 2022). In tuberculosis, it has shown good correlation with clinical outcome (Pasipanodya *et al.*, 2015, Gumbo *et al.*, 2015). There is also a similar approach that allows for daily addition of drugs nutrients and removal of metabolic products and old residual antimicrobials that also incorporates a human model of the granuloma (Bielecka and Elkington, 2018).

1.6 Current standard pharmacological treatment recommendations

Fully susceptible tuberculosis is a curable disease. Standard treatment of fully susceptible tuberculosis is 2 months of isoniazid, rifampicin, pyrazinamide and ethambutol followed by 4 months of isoniazid and rifampicin. The latter consolidation phase is extended to 10 months if there is involvement of the central nervous system (National Institute for Health and Care Excellence, 2016, World Health Organization, 2017). Recently, a shorter 4-month course of

isoniazid, rifapentine, moxifloxacin and pyrazinamide has been endorsed by the World Health Organisation (World Health Organization, 2021c, Nahid *et al.*, 2019).

For drug-resistant tuberculosis, treatment becomes more complex and, very often, longer. Isoniazid mono-resistant strains are treated with 6 months of rifampicin, levofloxacin, ethambutol and pyrazinamide. Rifampicin mono-resistant and multidrug resistant strains require a combination of all group A drugs (fluoroquinolones – either levofloxacin or moxifloxacin, linezolid and bedaquiline) plus at least one of the group B drugs (clofazimine, cycloserine or terizidone). Bedaquiline is stopped at 6 months (usually) and treatment continues with the other agents (World Health Organization, 2019a).

If only one or two group A drugs can be used, two group B agents ought to be included, clofazimine plus either cycloserine or terizidone.

If there are not sufficient drugs from groups A and B to include in the regimen, group C agents can be added. Group C drugs include ethambutol, delamanid, pyrazinamide, imipenem–cilastatin, meropenem, amikacin or streptomycin, ethionamide or prothionamide, p-aminosalicylic acid. A full listing of groups A, B and C and be found in Table 1.4 (World Health Organization, 2019a).

There are other agents that due to poorer outcomes, unclear evidence of efficacy or toxicity have been removed from the WHO anti-tuberculous drugs groups and are now listed as miscellaneous agents. These are: kanamycin, capreomycin, gatifloxacin and high-dose isoniazid, thioacetazone, clavulanic acid without imipenem-cilastatin or meropenem (World Health Organization, 2019a).

Table 1.4 – Medicines by group recommended for use in longer MDRTB regimens. Modified from WHO Consolidated Guidelines on Drug-Resistant Tuberculosis Treatment (World Health Organization, 2019a)

Group	Drugs
A	Levofloxacin or Moxifloxacin Bedaquiline

	Linezolid
B	Clofazimine Cycloserine or Terizidone
C	Ethambutol Delamanid Pyrazinamide Imipenem-cilastatin or Meropenem Amikacin or Streptomycin Ethionamide or Prothionamide p-aminosalicylic acid

A shorter regimen for MDRTB has been endorsed by WHO after recent trials suggesting non-inferiority when compared to the long regimen. It includes high-dose moxifloxacin, clofazimine, ethambutol and pyrazinamide for 40 weeks plus kanamycin, isoniazid and prothionamide during the first 16 weeks or 20-24 weeks, if the patient has a positive smear by week 16 or 20 (World Health Organization, 2019a, Nunn *et al.*, 2019, Van Deun *et al.*, 2010).

Most recently, new all oral regimens have completed trials or are being used programmatically, including the TB-PRACTEAL, six-month randomised clinical trial BPaLM; the ZeNIX study, a six-month BPaL regimen (less linezolid); NeXT trial, shorter 6 to 9 or 9 to 12 months using group A drugs. The BPaLM and BPaL regimens showed a high treatment success. A WHO rapid communication in May 2022 indicated that the evidence suggested that the six-month BPaLM in MDR or RR-TB patients, without prior exposure to these drugs, could replace the nine months or longer (>18 months) regimens in patients who are older than 15 years. BPaL alone can be used if proven fluoroquinolone resistance exists. The rapid communication also stated that the all oral nine month bedaquiline-based regimens was an improvement over the 18-month regimens for MDR/RRTB providing that there was (a) no exposure to bedaquiline and other second line drugs, (b) no fluoroquinolone resistance and (c) no extensive pulmonary or extrapulmonary disease. Otherwise continue with the individualised longer regimen (TB Alliance, 2022, World Health Organization, 2022, Development, 2022, Medicins Sans Frontieres, 2021).

1.7 Repurposing drugs – compounds of interest: carbapenems, clofazimine and nitazoxanide

Repurposing drugs against tuberculosis is a strategy that gained relevance with the emergence of drug resistance. Unlike development of a compound *de novo*, repurposing entails a shorter and cheaper process, for most drugs of interest there is a history of use for other indications and understanding of most common side effects. There is a long list of compounds currently under investigation. Some of them have direct antimicrobial effect while others are immunomodulators; some compounds exhibit both (Fatima *et al.*, 2021).

Carbapenems have been known to be effective against non-tuberculous mycobacteria since the early 1990s (Yew *et al.*, 1990), and act by inhibiting the L,D-transpeptidases (Correale *et al.*, 2013, Dubee *et al.*, 2012, Cordillot *et al.*, 2013). However, their use in treating tuberculosis was limited by their inactivation by beta-lactamases and the existence of a highly effective and cheaper oral drug regimen for drug susceptible TB. However with the advent of drug resistant tuberculosis especially M/XDRTB interest in carbapenems has increased based on their good *in vitro* and *in vivo* results (Chambers *et al.*, 2005, Coban *et al.*, 2008, Veziris *et al.*, 2011). Further studies showed that meropenem was the most stable carbapenem in the presence of the chromosomally encoded *blaC* beta-lactamase (England *et al.*, 2012) and that the addition of clavulanate, a beta-lactamase inhibitor, improved carbapenem activity (Dubee *et al.*, 2012, Mainardi *et al.*, 2011, Hugonnet *et al.*, 2009, Davies Forsman *et al.*, 2015, Kurz *et al.*, 2013, Gonzalo *et al.*, 2020).

For many years, co-amoxiclav has been considered a potentially useful anti-TB drug and classified by WHO as a miscellaneous drug (formerly group V) (World Health Organization, 2011, World Health Organization, 2019a) but there was little data to support its use (Winters *et al.*, 2015). Clavulanate is only available in the UK combined with either amoxicillin or ticarcillin, with the former the only one available orally (Gonzalo *et al.*, 2020).

Concerns that amoxicillin and meropenem may compete for binding sites and antagonise each other arose. However, the addition of amoxicillin to meropenem-clavulanate showed a synergistic effect against *M. tuberculosis* strains at concentrations easily achievable *in vivo* (Gonzalo and Drobniewski, 2012). Unfortunately, meropenem requires intravenous administration three times a day and there remains the possibility of carbapenem-resistance selection within the gut microbiota. Other carbapenems have a better administration profile: ertapenem is given intravenously once a day and faropenem and tebipenem are available for oral administration (Joint Formulary Committee, 2016, Schurek *et al.*, 2007, Hazra *et al.*, 2014, Gonzalo *et al.*, 2020).

Faropenem showed good killing activity in an *ex vivo* model of TB, using the laboratory strain H37Rv only; its activity against clinical strains using this model is still unknown (Dhar *et al.*, 2015). An additional advantage of this compound is that is stable in the presence of the MTB BlaC enzyme, which means that it would remain active against those strains that become resistant to clavulanate (Soroka *et al.*, 2015, Gonzalo *et al.*, 2020). A second oral carbapenem, tebipenem, has also shown *in vitro* activity, against a panel of clinical strains including M/XDRTB isolates (Horita *et al.*, 2014).

Ertapenem has also been showed to be active *in vitro*. However, testing is challenging since it degrades quickly at 37°C (Tiberi *et al.*, 2016, Srivastava *et al.*, 2016).

In a murine model, the combination of carbapenem-clavulanate improved mice survival but did not stop mycobacterial growth. In this model, Swiss mice infected with H37Rv were treated with the combination of a carbapenem and clavulanate and all mice survived. However, there was no difference in spleen size, number of lung lesions or colony forming units in the lung between the group treated with carbapenem plus clavulanate and those that did not receive that combination (Veziris *et al.*, 2011). While testing under hypoxic conditions at doses similar to those present within macrophages, these antibiotics showed very little activity against *M. tuberculosis* (Solapure *et al.*, 2013).

“Information regarding clinical use and outcomes in humans is starting to emerge, showing results suggestive of activity against *M. tuberculosis*. However, the contribution of the beta-lactam to the outcomes remains difficult to ascertain (Dauby *et al.*, 2011, van Rijn *et al.*, 2016). Current opinions suggest that until more evidence becomes available, these drugs should be considered companion drugs rather than effective anti-TB agents (Caminero and Scardigli, 2015, Jaganath *et al.*, 2016), particularly in light of the administration route and higher cost associated with their use (Davies Forsman *et al.*, 2015, Mainardi *et al.*, 2011). Emergence of carbapenem resistance amongst gut microbiota is also an undesirable consequence of the use of these antibiotics and it is associated not only with the use of the drug but also to the duration of the exposure, that in the case of tuberculosis, is usually prolonged (Pano Pardo *et al.*, 2014). More evidence is needed to clarify the true impact of carbapenems in both TB treatment and outcome and as well as the financial burden, complications and microbiotal ecological changes associated with their use to justify their re-classification as effective anti-tuberculosis agents” (Gonzalo *et al.*, 2020).

Clofazimine has been used since the late 1960s for the treatment of leprosy. It is a lipophilic compound that de-stabilises the mycobacterial wall by binding to phospholipids and releases reactive oxygen species. It has immunomodulatory effects mediated by improving function of a specific sub-set of memory T-cell (Fatima *et al.*, 2021, Mensa Pueyo, 2021). It is classified as a Group B drug by WHO and has gained prominence in recent years as it is part of both the all-oral combination and the short duration treatment of MDRTB (World Health Organization, 2019a, Moodley and Godec, 2016, World Health Organization, 2018b).

Nitazoxanide is a broad-spectrum antimicrobial with anti-parasitic activity as well as antiviral and anti-anaerobic bacteria. It is widely used worldwide and has shown activity against *M. tuberculosis* previously (de Carvalho *et al.*, 2009, Shigyo *et al.*, 2013, Odingo *et al.*, 2017). It exerts its antiparasitic and anti-anaerobic bacterial activity by inhibiting the action of an essential enzyme, blocking a key metabolic pathway. In viruses, it blocks the interaction

between several viral proteins (Mensa Pueyo, 2021). Its activity against *M. tuberculosis* is mediated by induction of autophagy, inhibiting a regulator of autophagy, and preventing *M. tuberculosis* proliferation in infected macrophages (Lam KKY, 2012, Hasenoehrl *et al.*, 2021), bacterial membrane potential disruption and intra-bacterial pH homeostasis(de Carvalho *et al.*, 2011) as well as amplification of MTB-stimulated RNA sensor gene expression and activity, an immunomodulatory mechanism (Ranjbar *et al.*, 2019).

Chapter 2: Materials and Methods

This chapter provides details of the methods used for the procedures described in subsequent chapters. Where methods were optimised, developed or significantly changed these are mainly described in subsequent chapters.

2.1 Strains used:

2.1.1 Strains used for Chapter 3. True rifampicin missed by MGIT:

All MTB cultures received from clinical laboratories Public Health England National Mycobacterium Reference Laboratory, Scottish Mycobacteria Reference Laboratory and Irish Mycobacteria Reference Laboratory (NMRL, SMRL and IMRL respectively) for identification and susceptibility, between July 2012 and December 2013 were included. Additionally, at the end of the study period (from 01/07/2012 until 31/12/2013) a retrospective interrogation of the NMRL mutation database was conducted of all the strains from 2010 until 2014. Strains showing the mutations observed in the prospectively collected study (D516Y, H525Y, L511P+M515I) were subcultured from the archive for analysis.

2.1.2 Strains used for Chapter 4: Repurposing of drugs: *in vitro*/*ex vivo* testing of carbapenems, clofazimine and nitazoxanide to assess role against MDR/XDR-TB

2.1.2.1 Macrodilution/Microdilution experiment:

Nineteen clinical strains with different resistance patterns to first- and second-line drugs and the reference strain H37Rv (Table 2.1) were tested against different concentrations of Faropenem and Ertapenem with and without clavulanate. Ten strains were provided by Dr Satta from University College London (UCL) and ten strains were part of my supervisor's collection and were sub-cultured from frozen aliquots in a MGIT tube containing modified 7H9 broth.

Table 2.1 – Strains tested, their susceptibility profile including meropenem. MIC: minimal inhibitory concentration; S: streptomycin; H: isoniazid; R: rifampicin; clari: clarithromycin; ethi: ethionamide; EMB: ethambutol; CAP: capreomycin; Moxi: moxifloxacin; PYR: pyrazinamide; MonoR: monoresistant; MDR: multi-drug resistant; PolyR: poly-resistant; XDR: extensively drug-resistant (Gonzalo *et al.*, 2020)

Strain	Phenotypical resistance profile	Meropenem clavulanate MIC (µg/ml)	Notes
03:013	S	32	MonoR
03:039	H	16	MonoR
04:018	H, R, clari, ethi	Failed	MDR
05:094	Fully susceptible	8	
07:116	H, ethi	4	PolyR
11:136	S, H, R	>32	MDR
11:156	S, H, R	4	MDR
11:191	H	16	MonoR
11:368	S, H, R	>32	MDR
324	Fully susceptible	8	
333	S, H, R	2	MDR
346	S, H, R	2	MDR
347	Fully susceptible	>32	
401	H, R	>32	MDR
408	S, H, R	>32	MDR
443	Fully susceptible	>32	
548	N/A*	>32	XDR
421	S, H, R, EMB, CAP, Moxi	>32	XDR
433	S, H, R, EMB, PYR, CAP, Moxi	8	XDR
H37Rv	Fully susceptible	2	Control reference strain

2.1.2.2 Microdilution experiment:

Ninety-three clinical strains with different resistance patterns to first- and second-line drugs and the reference strain H37Rv (Table 2.2) were tested against different concentrations of faropenem, ertapenem, tebipenem and meropenem with and without clavulanate. 89 strains were part of my supervisor's collection and 4 were kindly provided by Dr Satta from University College London (UCL). They were sub-cultured from frozen aliquots in a Middlebrook 7H11 plates.

Table 2.2 – Strains tested and their susceptibility profile. S: fully susceptible; RIF: rifampicin resistant; MDR: multi-drug resistant; POLYR: poly-resistant; XDR: extensively drug-resistant

Strain number	Phenotypical resistance profile
1. H37Rv	S
100. M08.14358	MDR
11. H111500010	MDR
110. RT12000326	S
113. RT12000338	S
151. M08.14399	S
175. M08.14423	S
178. M08.14426	S
32. M08.14304	MDR
38. M08.14350	XDR
4. H112080018	MDR
120. RT12000416	S
128. RT12000566	S
107. M08.14365	POLYR
118. RT12000409	S
167. M08.14415	S
37. M08.14337	MDR
8. H111840003	RIF
125. RT12000552	S
126. RT12000553	S
162. M08.14410	S
104. M08.14362	S
114. RT12000346	S
127. RT12000555	S
132. 02.292	S
144. M08.14392	S
165. M08.14413	S
18. H112140033	MDR
19. H112160033	MDR
34. M08.14310	MDR
39. M08.14354	MDR
109. RT12000324	S
112. RT12000333	S
13. H111620002	MDR
136. 03.013	S
149. M08.14397	S
15. H111860011	MDR
152. M08.14400	S
154. M08.14402	S
169. M08.14417	S
184. M08.14432	S

31. M08.14303	XDR
42. M08.14377	MDR
6. H112080012	S
105. M08.14363	S
111. RT12000328	S
131. 11.368	MDR
160. M08.14408	S
163. M08.14411	S
192. M08.14440	S
7. H110860461	S
9. H111980010	POLYR
10. H111540004	MDR
12. H111620021	MDR
164. M08.14412	S
174. M08.14422	S
20. H111040027	MDR
26. M08.14486	XDR
29. M08.14471	S
3. H111900041	S
55. M08.14309	S
98. M08.14353	S
108. M08.14366	XDR
130. 05.177	S
189. M08.14437	S
2. H111900039	S
24. H112990114	MDR
27. M08.14493	XDR
33. M08.14306	XDR
103. M08.14361	S
115. RT12000347	S
121. RT12000443	S
159. M08.14407	S
16. H111740353	MDR
166. M08.14414	S
177. M08.14425	S
186. M08.14434	S
191. M08.14439	S
21. H111880072	XDR
25. M08.14361	XDR
28. M08.14543	XDR
97. M08.14352	S

2.1.2.3 Three-dimensional model of the granuloma experiment:

Mycobacterium tuberculosis H37Rv was transformed using the Lux operon, making it fluorescent (MTB lux) (Andreu *et al.*, 2010). This strain was a gift from Dr Brian Robertson, Imperial College London.

2.1.3 Strains used for Chapter 5: MALDI-ToF MS for the identification of mycobacteria

2.1.3.1 Experiment 1: identification of *M. tuberculosis* vs NTM

275 isolates were included comprising 252 clinical *Mycobacterium tuberculosis* isolates, 2 H37Rv reference isolates and 21 Non-Tuberculous Mycobacterial (NTM) reference strains. All *M. tuberculosis* cultures were originally isolated from sputa of patients with pulmonary *M. tuberculosis* infections.

2.1.3.2 Experiment 2: identification of NTM

Reference bacterial strains. Reference strains were obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM, Antwerp, Belgium) and from European Centres for Disease Prevention and Control (ECDC). Table 2.3.

Table 2.3 – *Mycobacterium* reference strains. BCCM: Belgian Co-ordinated Collections of Microorganisms; ECDC: European Centres for Disease Prevention and Control

Strains (n=29)	**Complex**	**Source**	
M. abscessus	*M. abscessus* complex	BCCM	C04975
M. avium	*M. avium* complex	BCCM	C01788
M. bohemicum		ECDC	
M. celatum		ECDC	
M. chelonae			17T9705
M. chimaera	*M. avium* complex	BCCM	130444
M. chimaera	*M. avium* complex	BCCM	2015-01546
M. fortuitum	*M. fortuitum* complex	BCCM	52847
M. fortuitum	*M. fortuitum* complex	ECDC	
M. gordonae		BCCM	C02565
M. interjectum		ECDC	
M. intermedium		ECDC	
M. intracellulare	*M. avium* complex	ECDC	

M. intracellulare	*M. avium* complex	BCCM	120584
M. kansasii		BCCM	960032
M. marinum		ECDC	
M. nonchromogenicum		ECDC	
M. peregrinum	*M. fortuitum* complex	BCCM	970462
M. peregrinum	*M. fortuitum* complex	ECDC	
M. scrofulaceum		BCCM	C01934
M. scrofulaceum		ECDC	
M. shimoidei		ECDC	
M. simiae		BCCM	C02428
M. simiae		ECDC	
M. smegmatis		BCCM	M010336
M. szulgai		BCCM	M003303
M. ulcerans		BCCM	C05150
M. xenopi		ECDC	
M. xenopi		BCCM	C05984

The lyophilized BCCM strains were re-suspended in 10 µL of Middlebrook 7H9 (Sigma Aldrich). ECDC strains were obtained frozen in 7H9 plus 10% glycerol. All isolates were then incubated in 5 mL of Middlebrook 7H9 with OADC (oleic acid-albumin-dextrose-catalase) for 2-4 weeks to obtain 1 mL biomass of each strain. The strain was then frozen at -80ºC. Subsequent subcultures were prepared by inoculating 100 µL of strain on selected media and incubated at 37ºC for up to 5 weeks.

***M. abscessus* complex clinical isolates.** 32 *M. abscessus* complex clinical isolates, originally isolated from the lungs of cystic fibrosis patients in the UK, were grown from frozen stocks in Middlebrook 7H9 liquid media with OADC at 37ºC for 2 weeks before analyzing with MALDI-ToF MS. Genomic information was available for these strains and had been sub-speciated as *abscessus, bolletii*, and *massiliense.*

2.1.3.3 Experiment 3: identification of sub-species with the *M. tuberculosis* complex

A total of 100 mycobacterial isolates from the Scottish Mycobacteria Reference Laboratory were cultured from frozen aliquots into Middlebrook 7H9 broth supplemented with Oleic-Acid, Dextrose, Catalase (OACD) without glycerol (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK) and incubated for 3-6 weeks at 37ºC. The isolates were comprised of 79 *M.*

tuberculosis, 3 *M. africanum*, 12 *M. bovis* BCG, and 6 *M. bovis*. Strains were identified using a combination of microscopic commercial molecular line probe assay identification (Hain, Nehren, Germany) and whole genomic sequencing (Walker *et al.*, 2015, Drobniewski *et al.*, 2015).

2.2 Antimicrobial drug susceptibility testing

2.2.1 For Chapter 3: True rifampicin resistance missed by MGIT

Susceptibility testing was performed as follows:

1. Resistance ratio (RR): briefly, for every new batch of in-house prepared medium, 3 known strains plus *M. tuberculosis* H37Rv were used to determine the "modal" susceptibility pattern against which the clinical strains were tested. Three different concentrations of rifampicin were tested (5, 10 and 20 mg/L) and results, read at 14, 21 and 28 days, were reported as susceptible, borderline, resistant or highly resistant after the following calculation:

   RR= Test MIC/Modal control MIC (Inderlied, 2005)

2. Proportion method on Middlebrook 7H10 (PM): critical concentration of rifampicin 1 mg/L. Critical proportion 1%. Plates were read at 14, 21 and 28 days. The valid read was the one obtained when the growth control plate showed more than 50 colonies (Canetti *et al.*, 1969).
3. MGIT 960 (Becton Dickinson Microbiology Systems, Sparks, Md.) susceptibility testing according to manufacturer's instructions (Palomino *et al.*, 1999).
4. MIC determination by microdilution (TREK Diagnostic Systems, Cleveland, OH) Concentrations tested were 0.12-16 mg/L (Abuali *et al.*, 2012).

Mutations conferring resistance to rifampicin were detected using line probe assays (HAIN GenoType MTB DR plus (Hain Lifescience GmbH, Nehren, Germany) and by *rpoB* sequencing using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA, USA).

Performance of the genotypic analysis was blinded to results of the phenotypic MGIT-based analysis.

Variable Number Tandem Repeat (VNTR) mini-satellite analysis was performed on all strains as part of routine epidemiological surveillance (Brown *et al.*, 2009).

2.2.2 For Chapter 4: Testing carbapenems against MDR/XDRTB

2.2.2.1 Experiment 1: Microdilution

Ninety-three clinical strains and the laboratory strain, H37Rv (see above), were tested against ertapenem, faropenem, tebipenem and meropenem and their combination with amoxicillin and clavulanic acid using microdilution. Methodology is described in more detail in Chapter 4.

2.2.2.1 Experiment 2: Macrodilution/Microdilution

19 clinical strains and the reference strain *M. tuberculosis* H37Rv were sub-cultured from frozen aliquots and tested against selected carbapenems using microdilution in microtiter plates and microdilution in the BACTEC/MGIT system. For details, please see Chapter 4.

2.2.2.1 Experiment 3: Testing antibiotics in a 3-dimensional model of the granuloma

Peripheral blood mononuclear cells (PMBCs) from 2 healthy donors were isolated and infected with the bacteria MTB-lux bacteria at multiplicity of infection of 10 to 1.

Infected cells were re-suspended in complete RPMI medium, mixed with sterile alginate-collagen a 1 x 10^6 cells per mL as described in Chapter 4. Microspheres were prepared and injected as described in Chapter 4. *M. tuberculosis* luminescence was monitored using GloMax® 20/20 Luminometer. Antimicrobials were added 24 h later. The antimicrobials and concentrations tested can be found in Chapter 4.

2.3 MALDI-ToF for identification of Mycobacteria

A detailed description of the method can be found in Chapter 5. Briefly, all isolates were sub-cultured from frozen stocks and incubated for until the optimal growth of bacteria was obtained. 100 µL of bacterial suspension were placed into 1.5 mL Eppendorf tubes and heat killed before leaving the BSL3 laboratory.

MALDI sample preparation. The heat killed MTB pellet was washed. The re-suspended pellet was placed on the MALDI plate and mixed with matrix.

Instrument and data analysis. Samples were analyzed in the positive and negative ion modes.

Spectra were visually analysed and identified blindly by two different operators, following the processed summarized in Chapter 5.

2.4 Urine for the diagnosis of tuberculosis

2.4.1 Detection of lipids in urine by MALDI-ToF MS

2.4.1.1 Pilot experiment

A detailed description of the methodology can be found in Chapter 5. Briefly, urine belonging to known tuberculosis patients and healthy controls were included.

An Applied Biosystems 4800 MALDI-ToF/TOF™Analyzer (Applied Biosystems/MDS SCIEX MDS Sciex, Concord, Ontario, Canada) was used for analysis.

2.4.1.2 Experiment 2: Detection of lipid from spiked urine

Lipids were detected in urine by spiking and by optimizing 6 different solvents for lipid extraction with each different solvent targeting lipids with different polarity in a limited sub-set of samples. Detailed methodology is described in Chapter 6.

Urine from a healthy control was spiked with purified mycobacterial lipids (*Mycobacterium tuberculosis* H37Rv total lipids, bei Resources, American Type Culture Collection, USA) at different concentrations (6mg/mL, 2µg/mL and 0.5ng/mL). Briefly, lyophilised lipids were resuspended in 0.6 mL of chloroform and 2.4 mL of urine from a healthy individual was added. Serial dilutions were obtained by adding 0.6 mL of the previous concentration to 2.4 mL of urine (1/5 dilutions).

2.4.2 Detection of trans-renal DNA for the diagnosis of tuberculosis

2.4.2.1 Experiment 1: Tuberculosis DNA detection from gDNA spiked urine samples

A detailed description of the methodology can be found in Chapter 6. Briefly, 87 urine samples were be collected from healthy volunteers, aliquoted and spiked with different concentrations of *M. tuberculosis* gDNA.

Phenol/Chloroform/Isoamyl Alcohol and commercially available kits (Urine DNA Isolation Maxi Kit (Slurry Format), Norgen Biotek, Canada; cfPure® Cell Free Nucleic Acid Extraction kit, BioChain, Newark, USA) were be used to extract the gDNA. *M. tuberculosis* related signal was detected using real-time PCR.

2.4.2.2 Experiment 2: TB DNA detection from patient's urine samples

87 urine samples from healthy controls and 42 microbiologically confirmed tuberculosis patients were prospectively collected. Healthy controls were collected in the UK, Russia, and Lithuania. Known tuberculosis patient's samples were collected in Lithuania and Russia and blindly tested for tuberculosis at source and in the UK.

DNA extraction was done using the phenol-chloroform method and commercially available kits. The methodology is described in detail in Chapter 6.

Once extracted, the DNA was amplified using real-time PCR.

Chapter 3: Assessing current methodology for detection of drug resistance in MTB

3.1 Introduction

Current phenotypic drug resistance assays may mislead clinicians in their therapeutic decisions for M/XDRTB patients since *in vitro* testing may not accurately factor in host factors affecting results (e.g. the intracellular environment with its pH changes essential for pyrazinamide activity) or methodological problems influencing accuracy (e.g. degradation of compounds during media preparation or incubation) (McDermott W and Tompsett R, 1954, Salfinger and Heifets, 1988). It is not unusual to find differences or discrepancies between different *in vitro* methods (e.g., due to differences in protein binding, or degradation caused by heating when preparing solid media) and between *in vitro* results and results from animal models.

In recent years, reports have highlighted the lack of consistency in the quality of drug susceptibility testing for *Mycobacterium tuberculosis* and an awareness of problems with current gold standard microbiological approaches. Efforts have been made to harmonise and improve performance, through the development of laboratory networks using external quality assessment, training and mentoring with observable improvements in Europe (Nikolayevskyy *et al.*, 2016).

Data from a multinational collaborative project to assess reproducibility and accuracy of current methodologies to test thionamides against *M. tuberculosis* complex was performed and found significant discrepancies. Thionamides are a family of second line drugs commonly used in the treatment of MDRTB. Results were summarised and presented in a poster at the ECCMID conference in 2016. The abstract of the poster presentation can be found in Appendix I.

Even for rifampicin, the backbone of anti-tuberculosis treatment, phenotypic susceptibility testing is far from perfect. Culture using the Mycobacteria Growth Indicator Tube (MGIT) is one of the gold standard methodologies for phenotypic susceptibility testing endorsed by the World Health Organisation (World Health Organization, 2015). Its introduction improved turn-around times compared to culture on solid media, and it has been adopted by tuberculosis laboratories worldwide.

Despite the advantages of MGIT-based DST, a key study identified that the MGIT approach misidentified a proportion of strains resistant on solid media and genotypic testing as rifampicin sensitive (Rigouts *et al.*, 2013). After this original report it transpired that this was not just an *in vitro* laboratory problem but that clinical failure followed when patients carrying strains with this pheno/genotype were treated with standard rifampicin-based regimens (Van Deun *et al.*, 2013, Van Deun *et al.*, 2015, Ocheretina *et al.*, 2014).

For other drugs for which susceptibility is not so well established (including novel and repurposed drugs), results are even more difficult to evaluate.

3.2 Methods

The different methodologies were described in Chapter 2.

3.3 Results and Discussion

Data from a collaborative project with the tuberculosis reference laboratories in Ireland, Scotland, England and Wales were collated and analysed. The project evaluated the performance of MGIT, a WHO-endorsed commercial susceptibility testing method, in comparison with *rpoB* gene sequencing for the detection of specific mutations associated with resistance to rifampicin and which had been missed by the MGIT method but not by standard egg-based solid culture medium DST.

In the study period, there were 7234 isolates of *M. tuberculosis* identified in the UK and Ireland. 346 first line DST analyses were performed at IMRL (9 sent to NMRL for second line drug

testing), 422 at the SMRL (3 were sent to NMRL for further testing), 3959 positive tuberculosis cultures had first line drug susceptibility testing performed at the NMRL, 2557 in other English centres (38 strains sent to the NMRL for second line testing), 323 isolates were re-tested as per protocol.

17 isolates presented the previously reported phenotype. Eight isolates were resistant and 8 were highly resistant to rifampicin on resistance ratio (RR) testing. 1 of 17 isolates was borderline resistant by RR. This isolate had an MIC to RIF of 4 mg/L and was also resistant by the PM using 7H10 medium. Of the resistant strains, 5 had an MIC of 1 mg/L, one of 2 mg/L and 2 of 4 mg/L. Of the highly resistant strains, 2 showed an MIC of 1 mg/L, 2 of 2 mg/L, 2 of 4 mg/L and 2 of 8 mg/L.

All 17 isolates had mutations in the *rpoB* gene, detected by the Hain line probe assay (see Chapter 2 for methodology), by absence of the wild type bands and/or the presence of mutant bands. D516Y was the mutation most frequently found. It was present in 13 isolates. H526L was found only once and the combination of L511P and M515I mutations was present in 3 isolates.

From the VNTR analysis, two isolates belonged to the Beijing lineage whereas 5 were of the Euro American lineage and 10 were of the CAS lineage. There were 3 pairs of isolates belonging to the same patients, but otherwise no clusters of cases were defined by VNTR. Table 3.1.

Table 3.1 – Rifampicin susceptibility testing by different methodologies. RR: resistance ration; LJ:Lowenstein Jensen Medium; PP: proportion method; MGIT: Mycobacteria growth indicator tube; 7H10: Middlebrook 7H10 medium; MIC: minimum inhibitory concentration; VNTR: variable number tandem repeat; HR: highly resistant; N/G: no growth; S: susceptible; R: resistant; BL: borderline; MUT: mutation present (Gonzalo *et al.*, 2017)

Strain number	Origin	RR LJ	PP 7H10 Week 2	PP 7H10 Week 3	PP 7H10 Week 4	MGIT	MIC mg/L	*rpoB*	Sequencing	VNTR	Lineage
H122160005	Ireland	HR	N/G	S	S	S	2 (R)	MUT	D516Y	42235 2642517333 25242336_	CAS
H123220062	London	HR	N/G	R	R	S	4 (R)	MUT	L511P/M515I	32234 2642516332 _41423384	CAS
H123720020	London	HR	N/G	R	R	S	8 (R)	MUT	L511P/M515I	32234 2642516332 _4142338_	CAS
H124160071	London	HR	S	S	R	S	2 (R)	MUT	D516Y	42235 2642515333 332423474	CAS
H124780123	London	BL	N/G	N/G	R	S	4 (R)	MUT	L511P+M515I	41435 2332517333 455443382	Beijing
H125140093	Ireland	R	N/G	S	CX	S	2 (R)	MUT	D516Y	-2235 2642-17333 2524233-3	CAS
H130660137	West Midlands	R	N/G	S	S	S	1 (S)	MUT	D516Y	32333 2332415324 4324434-3	EuroAm
H134020126	London	HR	S	S	R	S	1 (S)	MUT	D516Y	32333 2232615323 123423352	EuroAm

H134060565	London	R	S	S	S	S	4 (R)	MUT	D516Y	42235 2542517333 412423362	CAS
H134020139	London	HR	N/G	R	R	S	8 (R)	MUT	H526L	42235 2642516333 4724233_4	CAS
H134280018	London	R	S	S	R	S	4 (R)	MUT	D516Y	42235 2542517333 412423362	CAS
H134560426	SMRL	HR	S	S	R	S	4 (R)	MUT	D516Y	42234 2442519332 442423374	CAS
H134560425	SMRL	R	N/G	S	S	S	1 (S)	MUT	D516Y	41535 2322517333 455443482	Beijing
H134760022	West Midlands	R	S	S	R	S	1 (S)	MUT	D516Y	32333 2332515324 432443483	EuroAm
H134760024	West Midlands	R	N/G	S	S	S	1 (S)	MUT	D516Y	32333 2332515324 432443483	CAS
H134880264	London	HR	S	S	R	S	1 (S)	MUT	D516Y	32333 2232615323 123423352	EuroAm
H134980257	London	R	S	S	R	S	1 (S)	MUT	D516Y	32333 2232615323 12342335_	EuroAm

When retrospectively screening the NMRL strain collection for the genotype containing the stated mutations, 10 new isolates were found: 4 isolates containing the D516Y mutation were resistant to RIF in the MGIT. However, six isolates were susceptible in the MGIT system but were resistant or highly resistant when tested using the RR method. Table 3.2.

Table 3.2 – Further strains containing the mutations D516Y and H526L (from 2010-2014). RR: resistance ration; LJ:Lowenstein Jensen Medium; MGIT: Mycobacteria growth indicator tube; VNTR: variable number tandem repeat; HR: highly resistant;; S: susceptible; R: resistant (Gonzalo *et al.*, 2017)

NMRL number	RR LJ	MGIT	Sequencing	VNTR	Lineage
H104140086	R	S	D516Y	32333 2532515323 246443353	EuroAm
H111620002	R	S	D516Y	42235 2632515333 432423375	CAS
H112760022	HR	S	D516Y	42235 1542517343 242423384	CAS
H113240102	HR	R	D516Y	42435 2232517334 4564433-2	Beijing
H114700009	HR	S	H526L	42235 2642518331 44242337-	CAS
H121260031	R	S	D516Y	32532 -722511324 332443383	EuroAm
H130140042	HR	R	D516Y	76466 2422622321 245323271	EAI
H134780024	R	R	D516Y	42235 2542517333 412423362	CAS
H133900529	HR	S	D516Y	32333 2232615323 123423352	EuroAm
H140680262	HR	R	D516Y	31423 1232515323 233423382	EuroAm

Of the 27 isolates containing the above mutations only 4 were identified as resistant by the MGIT system.

Rifampicin-resistant mutations associated with failure of detection by the MGIT system were present in the UK and Ireland at the time of the study. The false susceptibility result in the MGIT system was associated only with these specific *rpoB* mutations and genotypic methodologies could aid in identifying these strains and a WGS or similar approach would also be immune to this error. The prevalence of strains behaving this way was low in the British Isles.

The results of this chapter were published. The published manuscript can be found in Publications section (Gonzalo *et al.*, 2017).

Chapter 4: Repurposing of drugs: *in vitro*/*ex vivo* testing of carbapenems, clofazimine and nitazoxanide to assess role against MDR/XDR-TB

4.1 Introduction

The rationale to explore these compounds of interest has been summarised above (see Chapter 1). The aim of this work was to assess carbapenems with a better administration profile than meropenem, namely ertapenem, faropenem and tebipenem against M/XDRTB and the presence of a synergistic effect with amoxicillin both *in vitro* and *ex vivo* as well as to evaluate these carbapenems, clofazimine and nitazoxanide in a more physiological model of TB.

4.2 Methods

4.2.1 Experiment 1 - Microdilution

Ninety-three clinical strains and the laboratory strain, H37Rv (see above), were tested against ertapenem, faropenem, tebipenem and meropenem and their combination with amoxicillin and clavulanic acid using microdilution. Antibiotic stock solution preparation was as follows: actual weight of powdered drug was calculated using the equation below.

$$Volume = \frac{Actual\ Weight x Potency}{Concentration}$$

Powders were diluted in DMSO, except clavulanic acid and amoxicillin that were diluted in distilled water. Solutions were sterilised using a membrane filter with a pore size of 0.20 µm. The first 10% to 15% of the filtered solution was discarded. Small volumes (500 µL approximately) of the sterile stock solutions were dispensed into sterile polypropylene or polyethylene vials appropriate for low-temperature storage, carefully sealed, and stored for up to 6 months at -20 ºC.

Working solution concentrations were obtained using the equation below:

C1xV1=C2xV2

From frozen aliquots, the seed lot was generated by culturing it on Middlebrook 7H11 medium. The plates were read twice a week. When growth was detected (more than 50 colony forming units), the susceptibility test was promptly set up, always within 7 days.

Susceptibility was performed in microtiter plates in a final volume of 0.1 mL. The full protocol can be found below.

Preparation of plates

30 µL of 7H9 supplemented with OADC were dispensed in wells A, B, C, D, E, F, G, H 1 to 8. 30 µL of faropenem solution were added to rows A and B and serial dilutions were performed. 30 µL of ertapenem solution were dispensed in rows C and D and serial dilutions were done. 30 µL of meropenem solution were added to rows E and F and serial dilutions were performed. 30 µL of tebipenem solution were added to rows G and H and serial dilutions were performed. 10 µL of clavulanate were to each well A, B, C, D, E, F, G, H 1 to 8. 10 µL of amoxicillin were added to each well B, D, F and H 1 to 8. 10 µL of plain 7H9 supplemented with OADC was added to each well A, C, E and G 1 to 8. 50 µL of plain 7H9 supplemented with OADC was dispensed to each well H11 and H12. Plates were covered and taken to BSL3 for inoculation.

Preparation of inoculum

Colonies from Middlebrook 7H11 plates were suspended in 7H9 supplemented with OADC. Glass beads were added. The tube was shaken until bacterial clumps were broken. The suspension was matched to a McFarland of 1 and left to rest for 20 min. 100 µL were transferred to 11 mL of 7H9 supplemented with OADC and vortex for 20 seconds. 50 µL of bacterial suspension were inoculated in each well A, B, C, D, E, F 1 to 8. The plate was sealed, double-bagged, placed in a plastic container and incubated at 37° 5% CO_2 and read weekly

until 28 days. Plates were deemed ready for interpretation when there was visible growth in the growth control wells (H11 and H12).

The final concentrations tested can be found in Table 4.1.

Table 4.1 – Concentrations of ertapenem, faropenem, meropenem and tebipenem tested in combination with amoxicillin and clavulanate (in mg/L). F: faropenem; C: clavulanate; A: amoxicillin; M: meropenem; E: ertapenem; T: tebipenem

F/C	0.125+2.5	0.25+2.5	0.5+2.5	1+2.5	2+2.5	4+2.5	8+2.5	16+2.5
F/C/A	0.125+2.5 +2	0.25+2.5+ 2	0.5+2.5+2	1+2.5+2	2+2.5+2	4+2.5+2	8+2.5+2	16+2.5+2
E/C	0.25+2.5	0.5+2.5	1+2.5	2+2.5	4+2.5	8+2.5	16+2.5	32+2.5
E/C/A	0.25+2.5+ 2	0.5+2.5+2	1+2.5+2	2+2.5+2	4+2.5+2	8+2.5+2	16+2.5+2	32+2.5+2
M/C	0.25+2.5	0.5+2.5	1+2.5	2+2.5	4+2.5	8+2.5	16+2.5	32+2.5
M/C/A	0.25+2.5+ 2	0.5+2.5+2	1+2.5+2	2+2.5+2	4+2.5+2	8+2.5+2	16+2.5+2	32+2.5+2
T/C	0.25+2.5	0.5+2.5	1+2.5	2+2.5	4+2.5	8+2.5	16+2.5	32+2.5
T/C/A	0.25+2.5+ 2	0.5+2.5+2	1+2.5+2	2+2.5+2	4+2.5+2	8+2.5+2	16+2.5+2	32+2.5+2

The Minimal Inhibitory Concentration (MIC), which is the first well with no visible growth, was recorded for each strain.

4.2.2 Experiment 2 – Macro/Microdilution

From frozen aliquots, isolates were subcultured in a MGIT tube containing modified 7H9 broth. When visible growth was detected, growth was decanted into 1.5 mL Eppendorff tubes and sent to UCL for MGIT testing, as previously described (Springer *et al.*, 2009). Briefly, the methodology for MIC testing was as follows. MGIT method was used as previously described by Krunner *et al.* (Kruuner *et al.*, 2006). Serial dilutions of the selected carbapenems were added to the MGIT tube. The tubes were then inoculated with the strains of *M. tuberculosis*. A growth control tube was inoculated with 1/100 the inoculum of the test isolate. The MIC is the concentration with less than 100 growth units in the tube when the growth control has reached the cut-off by which the instrument flags it as positive (400 growth units). All tubes other than the growth control one exhibiting 100 or more growth units are considered positive. That means that the concentration held in that tube has failed to halt the growth of *M. tuberculosis.*

The same complete set was set up in microdilution plates, as described for experiment 1 at Imperial College London, as part of a collaborative effort. The antibiotics and concentrations can be found in Table 4.2.

Table 4.2 – Concentrations of ertapenem and faropenem tested in combination with clavulanate (in mg/L)

	Ertapenem	**Ertapenem Clavulanate**	**Faropenem**	**Faropenem Clavulanate**
BACTEC960 MGIT system	16-8-4-2	16-8-4-2 (clavulanate 2.5)	8-4-2-1	8-4-2-1 (clavulanate 2.5)
Microdilution	0.25-0.5-1-2-4-8-16-32	0.25-0.5-1-2-4-8-16-32 (clavulanate 2.5)	0.125-0.25-0.5-1-2-4-8-16	0.125-0.25-0.5-1-2-4-8-16 (clavulanate 2.5)

4.2.3 Experiment 3 – Testing antibiotics in a 3-dimensional model of the granuloma

For testing of compounds in the 3-D *ex vivo* model, infected cells were re-suspended in complete RPMI medium, mixed with sterile alginate-collagen a 1 x 10^6 cells per mL and injected into the Electrostatic Bead Generator (Nisco, Zurich, Switzerland) to form microspheres via a Harvard syringe driver. After generation, microspheres were equally distributed into 2 mL Eppendorfs (microsphere volume 0.4ml), immersed in 1mL of complete RPMI and incubated at 37°C 5% CO_2. *M. tuberculosis* luminescence was monitored using GloMax® 20/20 Luminometer (Bielecka *et al.*, 2017). Antimicrobials were added 24 h later. The antimicrobials and concentrations tested can be found in Table 4.3.

All drugs were dissolved in DMSO except clavulanate that was dissolved in distilled water. Stock solutions were filter-sterilised and frozen at -80ºC in 500 µL aliquots.

Experiments were carried out in triplicates.

Bacterial luminescence was monitored for up to 21-28 days.

Table 4.3 – Concentrations of ertapenem, faropenem and tebipenem tested in combination with clavulanate (in mg/L), nitaxozanide and clofazimine in the 3-D *ex vivo* model

Meropenem	2	8	32
Faropenem	2	8	32
Tebipenem	2	8	64
Nitaxozanide	8	64	
Clofazimine	2	32	
Clavulanic acid	2.5	2.5	2.5

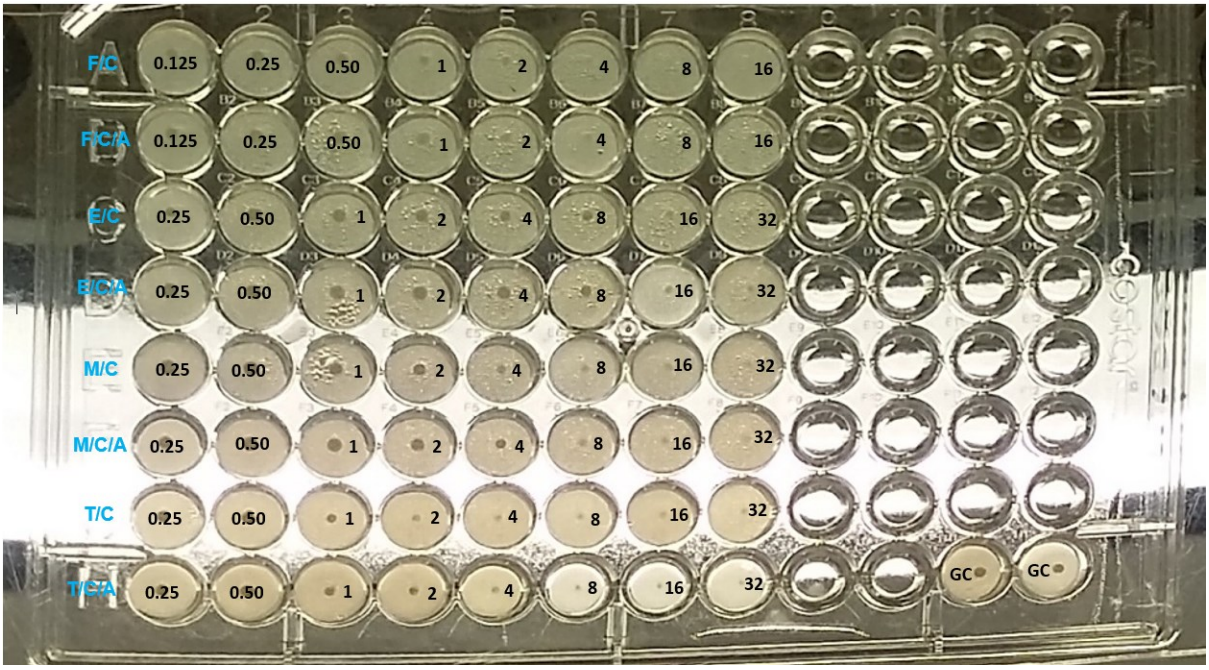
4.3 Results and Discussion

We conducted phenotypic MIC testing initially using two approaches (micro and macro-dilutions). Both methods present advantages and disadvantages. The macrodilution approach used here is based on the MGIT system that offers automated reading, is widely used worldwide, and relatively easy to adapt. The clear disadvantage for MIC determination using this approach is the large number of tubes required which makes it rather expensive when compared to microdilution. In addition to this, it also involved handling relatively large volumes of positive cultures with the biohazard associated to it. Microdilution, on the other hand, manages much smaller volumes, the cost is less, and it can be easily adapted to accommodate new compounds. The plates have to be read visually which may be difficult as the fact that the plates need to be sealed for safety reasons could generate visual distortions. The latter method does not require any additional instruments, as the MGIT-based macrodilution approach.

4.3.1 Carbapenem testing by microdilution

Ninety-three clinical strains and the laboratory strain, H37Rv were assessed by a microdilution methodology against ertapenem, faropenem, tebipenem and meropenem and their combination with amoxicillin and clavulanic acid using a microtiter plate format.

Figure 4.1 – Microtiter plate ready to be read. Concentrations of ertapenem, faropenem, meropenem and tebipenem tested in combination with amoxicillin and clavulanate (in mg/L). F: faropenem; C: clavulanate; A: amoxicillin; M: meropenem; E: ertapenem; T: tebipenem. The MIC are those of carbapenems. Amoxicillin was tested at 2 mg/L fixed dose. Clavulanate was tested at 2.5 mg/L fixed dose



The Minimal Inhibitory Concentration (MIC), which is the first well with no visible growth, was recorded for each strain.

A readable susceptibility profile was obtained for 82 strains (87,2%). In Figure 4.1, there is plate with appropriate growth to be read. The rest failed to grow in the microtiter plates. A full set of results can be found in Table 4.4 and MIC50 and MIC90 can be found in Table 4.5.

Table 4.4 – Full set of susceptibility results. The MICs refer to the carbapenem. Faro: faropenem; clav: clavulanate; Erta: ertapenem; Amx: amoxicillin; Mem: meropenem; Tebi: tebipenem.

Strain number	Faro+Clav	Faro+clav+Amx	Erta+clav	Erta+clav+Amx	Mem+Clav	Mem+clav+Amx	Tebi+clav	Tebi+clav+Amx
1. H37Rv	<0.125	<0.125	0.5	1	>32	1	<0.25	0.5
100. M08.14358	<0.125	<0.125	32	>32	8	2	4	4
11. H111500010	<0.125	<0.125	0.5	1	<0.25	<0.25	<0.25	<0.25
110. RT12000326	<0.125	1	16	16	0.5	4	1	1
113. RT12000338	<0.125	<0.125	16	0.5	4	2	1	1
151. M08.14399	<0.125	<0.125	32	32	0.5	8	<0.25	<0.25
175. M08.14423	<0.125	<0.125	>32	>32	8	2	1	16
178. M08.14426	<0.125	<0.125	8	8	4	2	0.5	<0.25
32. M08.14304	<0.125	<0.125	4	<0.25	2	0.5	<0.25	<0.25
38. M08.14350	<0.125	0.125	<0.25	<0.25	2	<0.25	0.25	<0.25
4. H112080018	<0.125	<0.125	16	>32	8	2	<0.25	<0.25
120. RT12000416	0.125	0.5	2	2	<0.25	<0.25	<0.25	<0.25
128. RT12000566	0.125	0.125	16	2	2	2	0.5	0.5
107. M08.14365	0.25	0.5	0.5	1	1	0.25	0.25	0.25
118. RT12000409	0.25	1	2	32	16	2	0.5	0.5
167. M08.14415	0.25	<0.125	16	8	2	4	1	<0.25
37. M08.14337	0.25	<0.125	2	1	8	8	<0.25	<0.25
8. H111840003	0.25	<0.125	16	4	4	4	8	8
125. RT12000552	0.5	0.5	>32	>32	2	2	0.5	0.5
126. RT12000553	0.5	0.25	8	8	4	1	0.5	0.5
162. M08.14410	0.5	1	8	8	8	4	<0.25	<0.25
104. M08.14362	1	1	16	8	2	2	<0.25	<0.25
114. RT12000346	1	0.5	4	16	2	2	<0.25	<0.25
127. RT12000555	1	<0.125	8	4	<0.25	<0.25	<0.25	<0.25

132. 02.292	1	0.5	8	16	0.5	0.5	2	1
144. M08.14392	1	1	32	32	16	8	<0.25	<0.25
165. M08.14413	1	0.5	>32	>32	8	8	<0.25	<0.25
18. H112140033	1	0.5	<0.25	<0.25	16	32	4	4
19. H112160033	1	1	8	16	16	16	16	16
34. M08.14310	1	4	32	16	16	8	2	1
39. M08.14354	1	2	16	16	8	4	1	0.5
109. RT12000324	2	0.5	8	16	8	8	1	1
112. RT12000333	2	0.5	8	4	4	2	<0.25	<0.25
13. H111620002	2	<0.125	32	32	8	4	8	8
136. 03.013	2	0.25	4	4	0.5	0.5	0.5	1
149. M08.14397	2	1	>32	>32	32	8	0.5	0.25
15. H111860011	2	3	4	8	1	1	4	4
152. M08.14400	2	8	>32	>32	32	32	1	1
154. M08.14402	2	2	>32	>32	>32	>32	32	32
169. M08.14417	2	1	>32	32	4	8	<0.25	<0.25
184. M08.14432	2	1	>32	32	32	16	4	4
31. M08.14303	2	2	16	8	4	1	0.5	0.5
42. M08.14377	2	2	32	32	16	8	<0.25	<0.25
6. H112080012	2	2	16	16	8	1	<0.25	0.5
105. M08.14363	4	8	16	16	8	4	8	4
111. RT12000328	4	1	8	8	4	4	1	2
131. 11.368	4	4	32	32	32	8	16	2
160. M08.14408	4	1	>32	>32	16	8	16	0.5
163. M08.14411	4	8	>32	>32	>32	>32	8	8
192. M08.14440	4	4	>32	>32	32	32	4	4
7. H110860461	4	4	32	32	8	8	8	4
9. H111980010	4	2	16	16	1	1	4	0.25

10. H111540004	8	8	>32	>32	32	8	32	4
12. H111620021	8	8	>32	>32	8	4	8	2
164. M08.14412	8	2	32	32	32	4	<0.25	<0.25
174. M08.14422	8	8	>32	>32	>32	8	1	1
20. H111040027	8	8	>32	>32	1	0.25	16	1
26. M08.14486	8	8	32	32	>32	32	32	32
29. M08.14471	8	8	>32	>32	>32	>32	16	32
3. H111900041	8	16	>32	>32	4	0.5	2	0.5
55. M08.14309	8	<0.125	8	16	<0.25	<0.25	<0.25	<0.25
98. M08.14353	8	8	>32	>32	>32	>32	32	>32
108. M08.14366	16	16	32	32	32	32	32	32
130. 05.177	16	4	32	32	32	16	4	2
189. M08.14437	16	16	>32	>32	>32	32	4	4
2. H111900039	16	16	32	32	8	1	<0.25	0.5
24. H112990114	16	8	32	32	16	16	>32	>32
27. M08.14493	16	16	32	32	2	0.5	8	1
33. M08.14306	16	8	>32	>32	16	16	16	16
103. M08.14361	>16	4	>32	>32	8	8	5	2
115. RT12000347	>16	>16	>32	>32	>32	>32	>32	>32
121. RT12000443	>16	>16	>32	>32	>32	>32	>32	>32
159. M08.14407	>16	>16	>32	>32	>32	>32	>32	>32
16. H111740353	>16	>16	>32	>32	8	4	4	4
166. M08.14414	>16	>16	>32	>32	>32	>32	>32	>32
177. M08.14425	>16	>16	>32	>32	>32	>32	>32	>32
186. M08.14434	>16	>16	>32	>32	>32	>32	>32	>32
191. M08.14439	>16	>16	>32	>32	>32	>32	>32	>32
21. H111880072	>16	>16	>32	>32	2	2	8	2
25. M08.14361	>16	>16	32	32	>32	>32	>32	>32

28. M08.14543	>16	>16	32	8	>32	>32	32	4
97. M08.14352	>16	>16	>32	>32	>32	>32	>32	>32

Table 4.5 – MIC 50 and MIC 90 in mg/L for all carbapenems tested. Faro: faropenem; clav: clavulanate; Erta: ertapenem; Amx: amoxicillin; Mem: meropenem; Tebi: tebipenem.

	Faro+Clav	Faro+clav+Amx	Erta+clav	Erta+clav+Amx	Mem+Clav	Mem+clav+Amx	Tebi+clav	Tebi+clav+Amx
MIC50	2	2	32	32	8	4	2	1
MIC90	>16	>16	>32	>32	>32	>32	>32	>32
Mode MIC	2	<0.125	>32	>32	>32	8	<0.25	<0.25

The MIC50 for Faropenem-clavulanate with and without amoxicillin was 2 mg/L while MIC90 was >16 mg/L. Sixty-two isolates showed an MIC of 8 or less, falling into the susceptibility category, and sixty-five were susceptible to faropenem-clavulanate-amoxicillin.

Ertapenem-clavulanate showed MIC50 of 32 mg/L that did not change with the addition of amoxicillin. MIC90 was >32. Twelve strains had an MIC of 4 mg/L or less, which is the cut-off based on PK/PD models for current dosing of 1g once a day. If dosing is changed to 2g twice a day, the cut off is 16 mg/L in which case thirty-four strains would be considered susceptible. With the addition of amoxicillin, 14/82 strains show an MIC of 4 mg/L or less and thirty-four strains of 16 mg/L or less.

Meropenem-clavulanate, the model drug, showed an MIC50 of 8 mg/L and decreased to 4 mg/L after amoxicillin was added. MIC90 was >32 mg/L. Forty-six had an MIC of 8 mg/L or less, the susceptibility cut off. With the addition of amoxicillin, fifty-seven strains were susceptible having MICs of 8 mg/L or less. A decrease in at least 2-fold dilution is expected when synergy is present, a rise in MIC would suggest antagonism and no changes or reduction in less than 2-fold dilution would mean additive effect (Chou and Talalay, 1984, Odds, 2003, Gonzalo and Drobniewski, 2012).

Tebipenem-clavulanate had an MIC50 of 2 mg/L that decreased to 1mg/L after the addition of amoxicillin. MIC90 was >32mg/L. Table 4.5. Sixty strains showed an MIC of 8 or less, which would be considered susceptible. This number grew to 64 when adding amoxicillin.

4.3.2 Carbapenem testing by macrodilution/microdilution

Results (Table 4.6) were obtained for the nineteen clinical strains and the reference strain H37Rv.

Table 4.6 – MIC obtained for all drugs tested in mg/L using MGIT-based macrodilution and microdilution in microtiter plates. Concentrations are expressed in mg/L. Clavulanate was used at a fixed concentration of 2.5 mg/L. Faro= faropenem, Erta=ertapenem, clav=clavulanate, microdil=microdilution, MGIT=Mycobacterium Growth Incubator Tube, LJ=Lowenstein Jensen. Green shading: concentrations achievable *in vivo*; blue shading: concentrations above those achievable *in vivo* with current dosing recommendations.

Strain number (n=20)	Microdil Faro	MGIT Faro	Microdil Faro+clav	MGIT Faro+clav	Microdil Erta	MGIT Erta	Microdil Erta+clav	MGIT Erta+clav
11:156	2	8	4	2	>32	>16	>32	>16
11:368	>16	>8	>16	>8	>32	>16	>32	>16
07:116	4	4	4	1	>32	16	>32	16
11:191	8	>8	8	>8	>32	>16	>32	>16
11:136	8	8	8	4	>32	>16	>32	>16
03:013	8	8	8	4	>32	>16	>32	>16
05:094	4	4	16	4	>32	>16	>32	>16
03:39	16	8	4	4	>32	>16	>32	>16
H37Rv	2	>8	4	>8	8	>16	16	>16
04:18	0.5	4	<0.125	4	>32	>16	>32	>16
324	2	>8	0.5	>8	8	>16	16	>16
333	2	>8	0.5	>8	8	>16	4	>16
346	1	8	0.5	8	4	>16	16	>16
347	>16	>8	>16	8	>32	>16	>32	>16
401	>16	>8	>16	>8	>32	>16	>32	>16
408	>16	8	>16	8	>32	>16	>32	>16
443	>16	>8	>16	>8	>32	>16	>32	>16
548	>16	>8	>16	>8	>32	>16	>32	>16
421	>16	>8	>16	>8	>32	>16	>32	>16
433	0.25	>8	<0.125	>8	2	>16	1	>16

The faropenem MIC50 was 2 mg/L in microdilution and 8 mg/L in the microdilution approach. When clavulanate was added, MIC50 was 4 mg/L by both methods. Ertapenem MIC50 was >16 mg/L and > 32 mg/L by macro and microdilution respectively. Half of the strains showed some susceptibility to faropenem and the addition of AMC further reduced the MIC level in six isolates. Resistance or susceptibility to faropenem was associated with resistance or

susceptibility to meropenem (p = 0.04) but not to resistance profiles to other drugs, including M/XDRTB status (Gonzalo *et al.*, 2020).

MIC90 for both drugs by both methods were higher than the highest concentrations tested. The addition of clavulanate did not change this.

4.3.3 Three-D *ex vivo* model

A full set of luminescence results is available in Appendix II.

Clofazimine showed good killing activity at both concentrations tested and was the only drug of the panel that killed bacteria at the lowest concentration tested. At 2 mg/L, in both experiments, it showed good activity from the beginning, achieving 1 log reduction in luminescence by day 20, not changing after that time point (only experiment 2). Figures 4.2 and 4.3.

Figure 4.2 – Experiment 1 – donor 1. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; clof: clofazimine

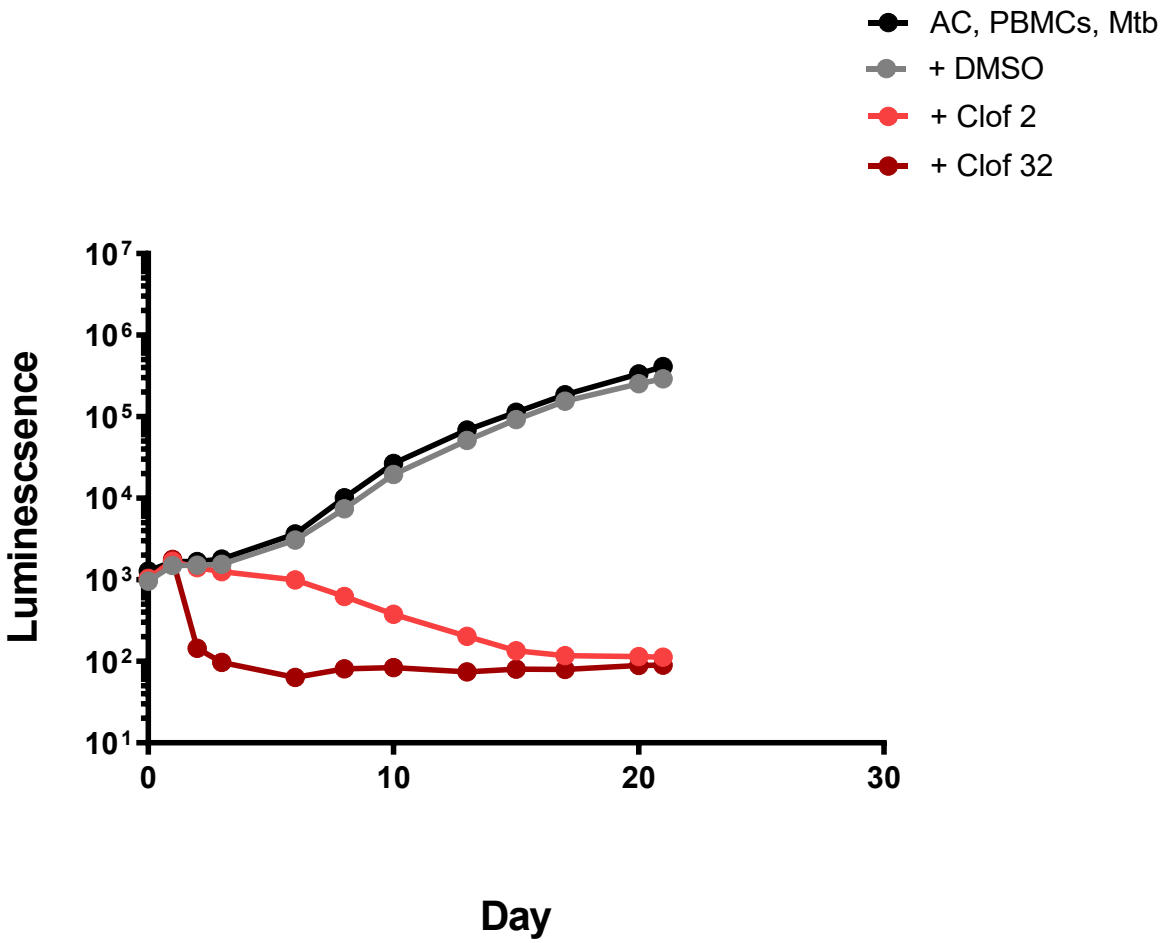
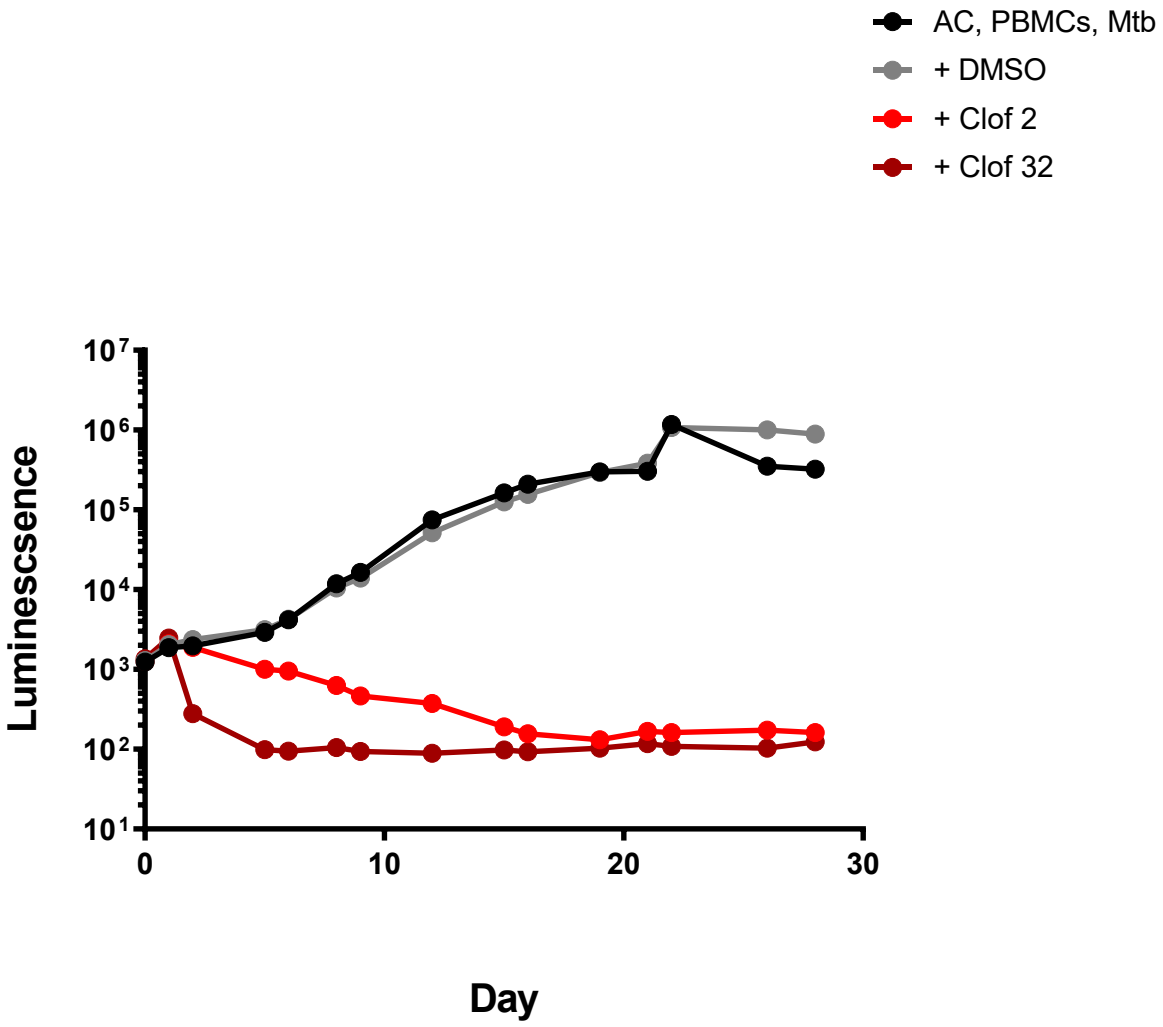


Figure 4.3 – Experiment 2 – donor 2. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; clof: clofazimine



Nitaxozanide was effective only at the highest concentration tested, showing no activity at 8 mg/L. Figures 4.4 and 4.5.

Figure 4.4 – Experiment 1 – donor 1. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Nita: nitazoxanide

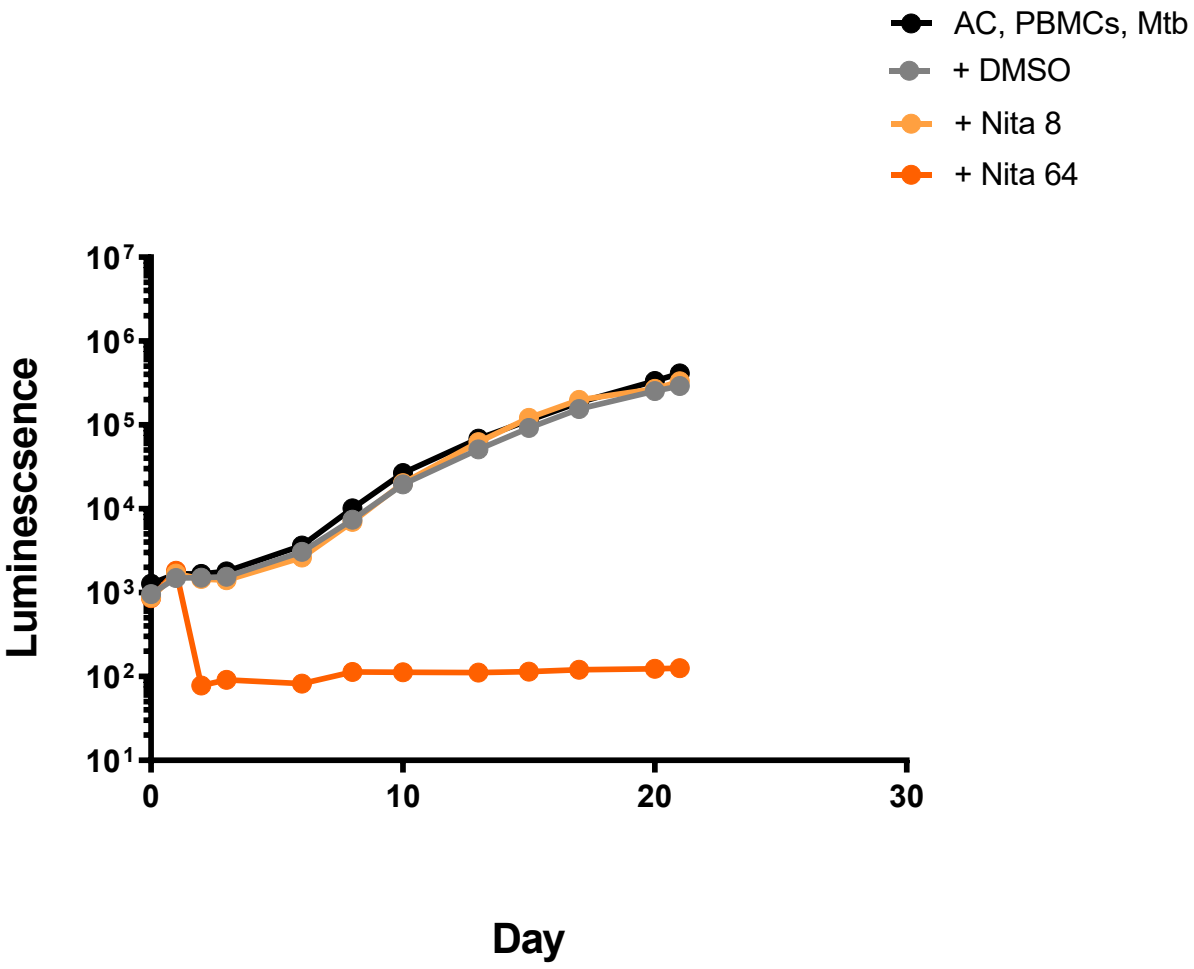
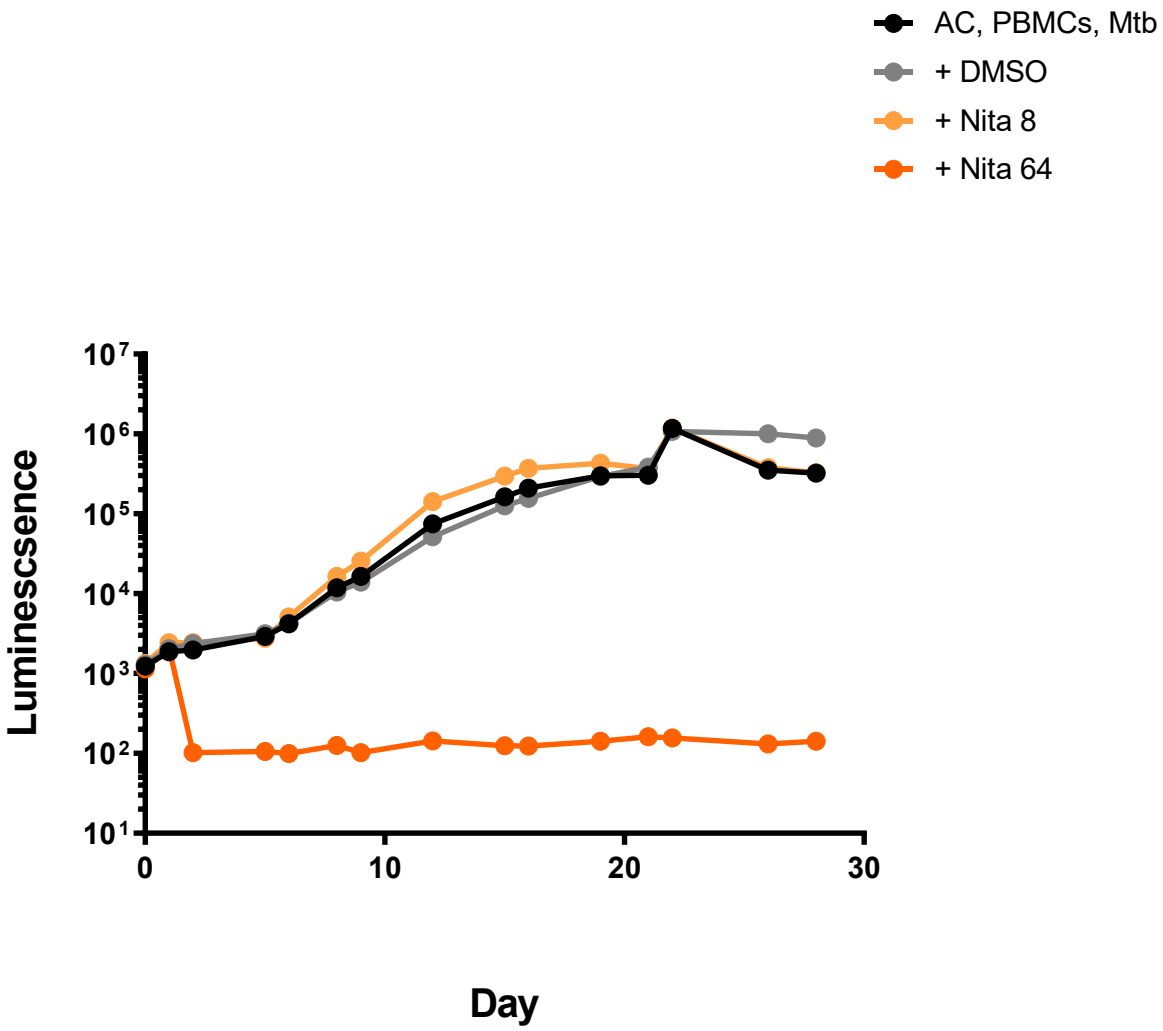


Figure 4.5 – Experiment 2 – donor 2. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Nita: nitazoxanide



Carbapenems showed some modest initial activity that was lost at around day 10 of incubation.

Meropenem, the model drug for the carbapenem group, showed good inhibition at a concentration of 32 mg/L up to the 10th day of incubation. Bacterial growth increased after day 10 suggesting bacteriostatic effect and/or degradation of the drug in the medium. The addition of clavulanic acid did not produce any differences in luminescence. Figures 4.6 and 4.7.

Figure 4.6 – Experiment 1 – donor 1. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Mero: meropenem; Clav: clavulanate

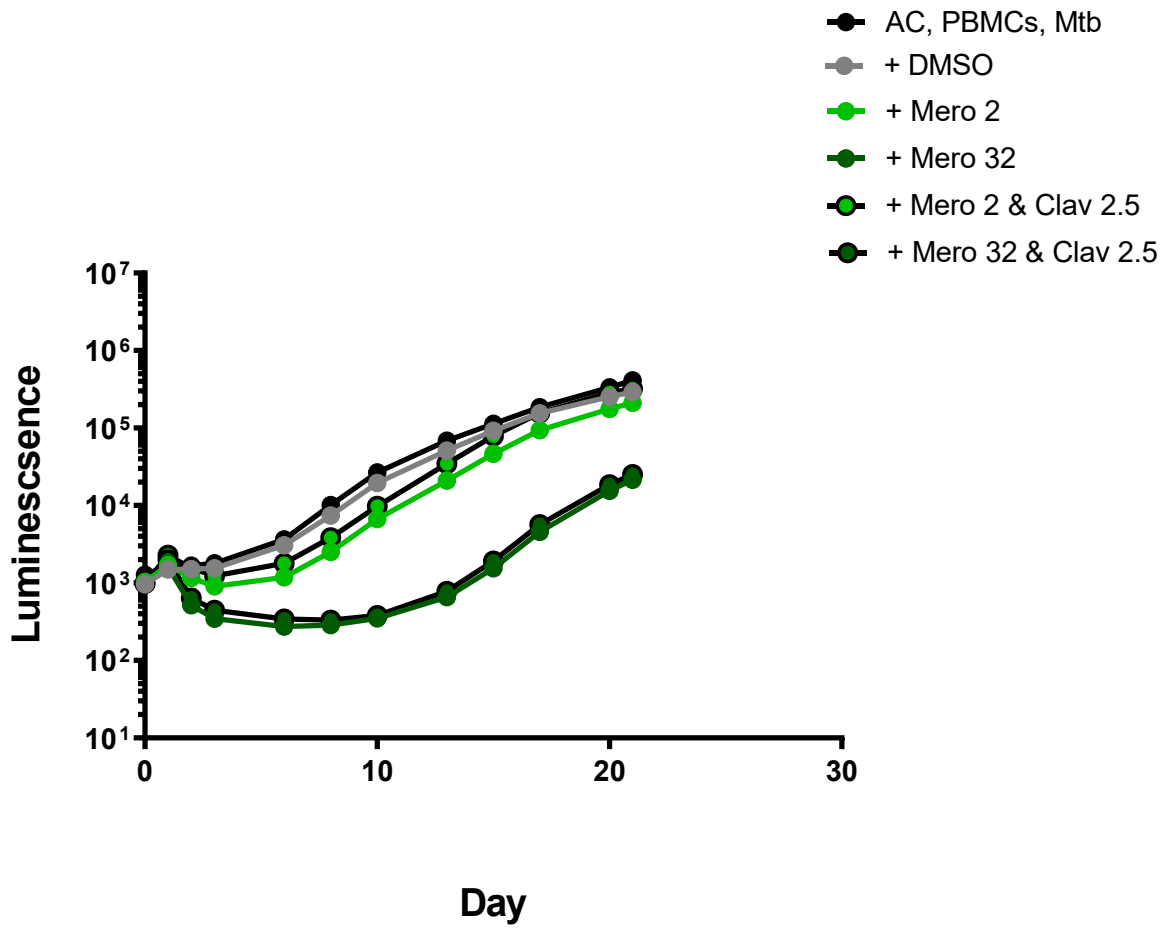
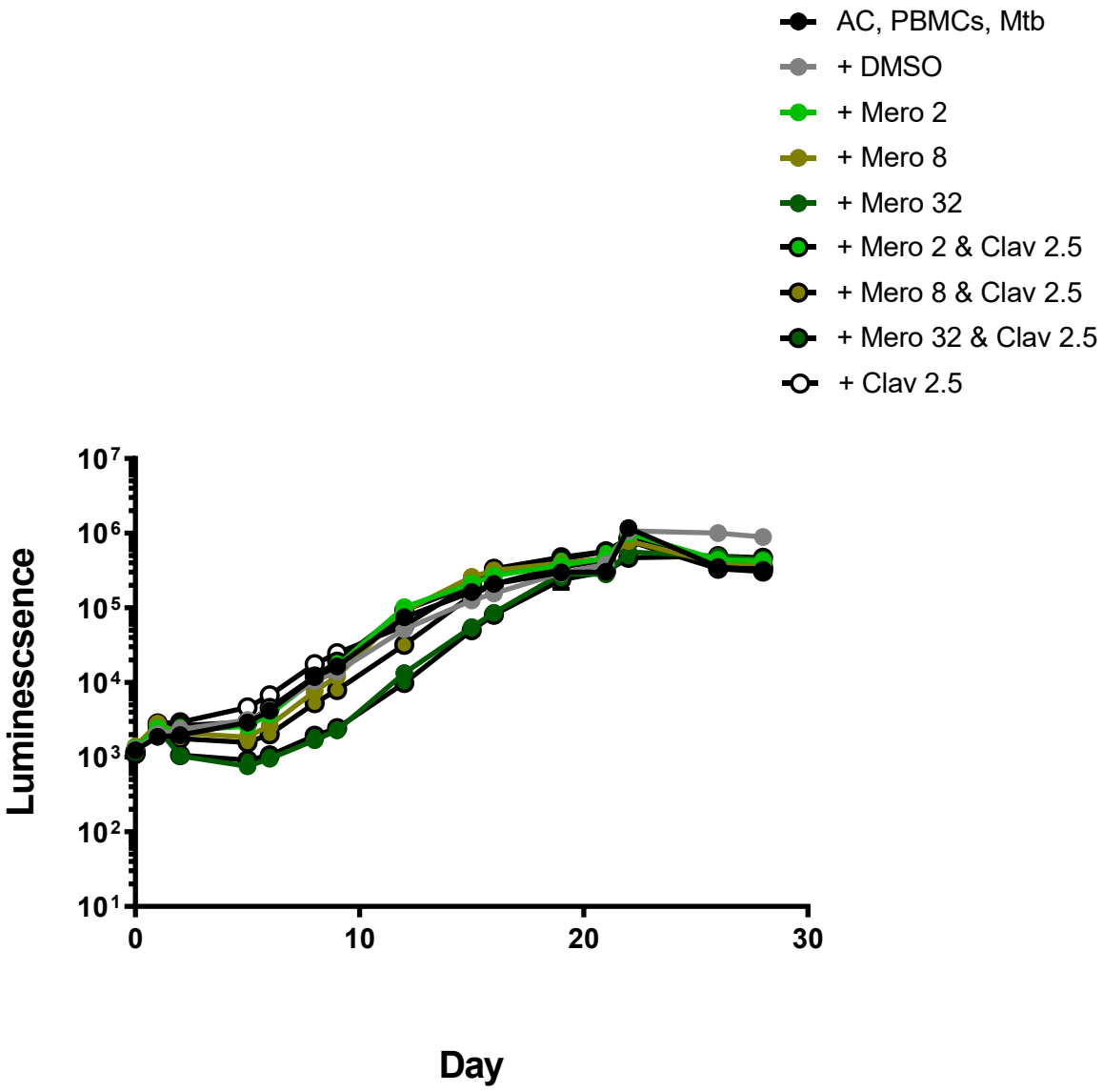


Figure 4.7 – Experiment 2 – donor 2. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Mero: meropenem; Clav: clavulanate



Faropenem showed a similar result except that luminescence only started to increase later, around day 15. Addition of clavulanate did not improve killing effect. Figure 4.8 and 4.9.

Figure 4.8 – Experiment 1 – donor 1. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Faro: faropenem; Clav: clavulanate

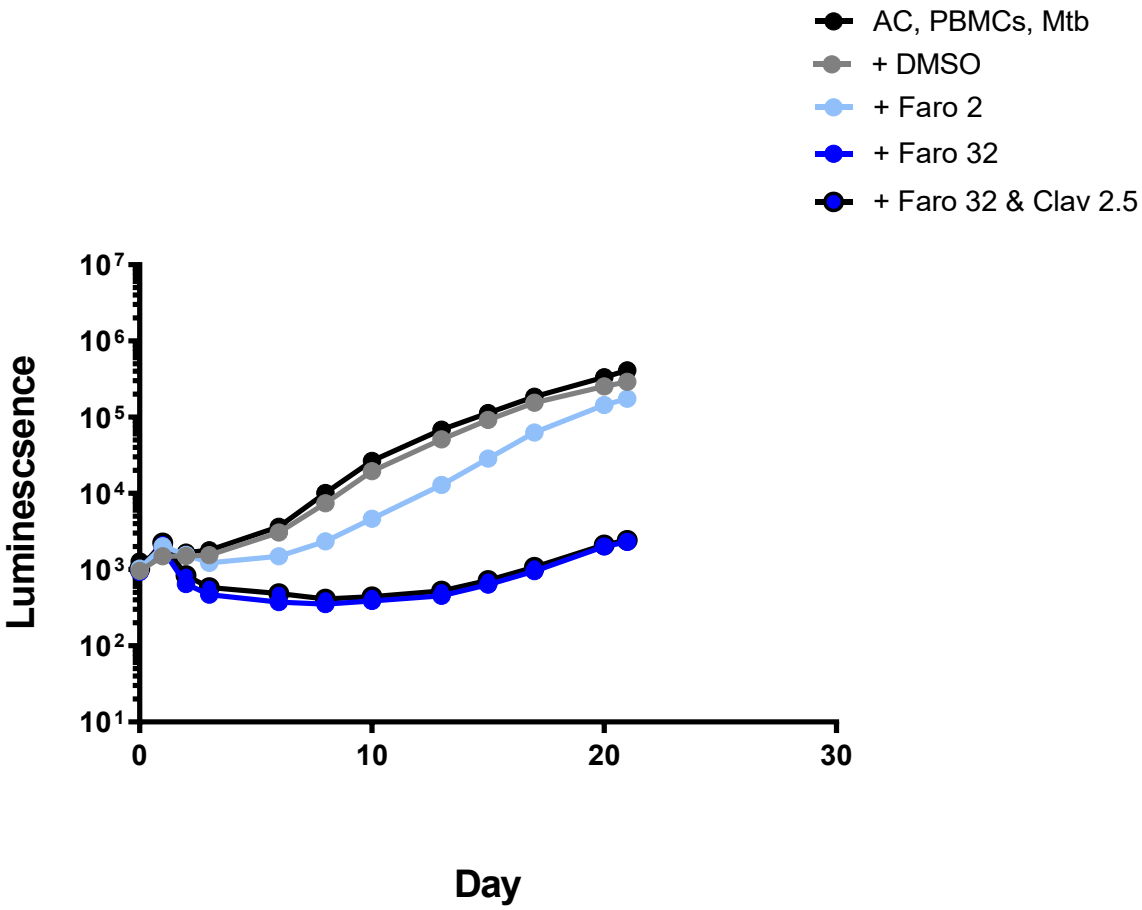
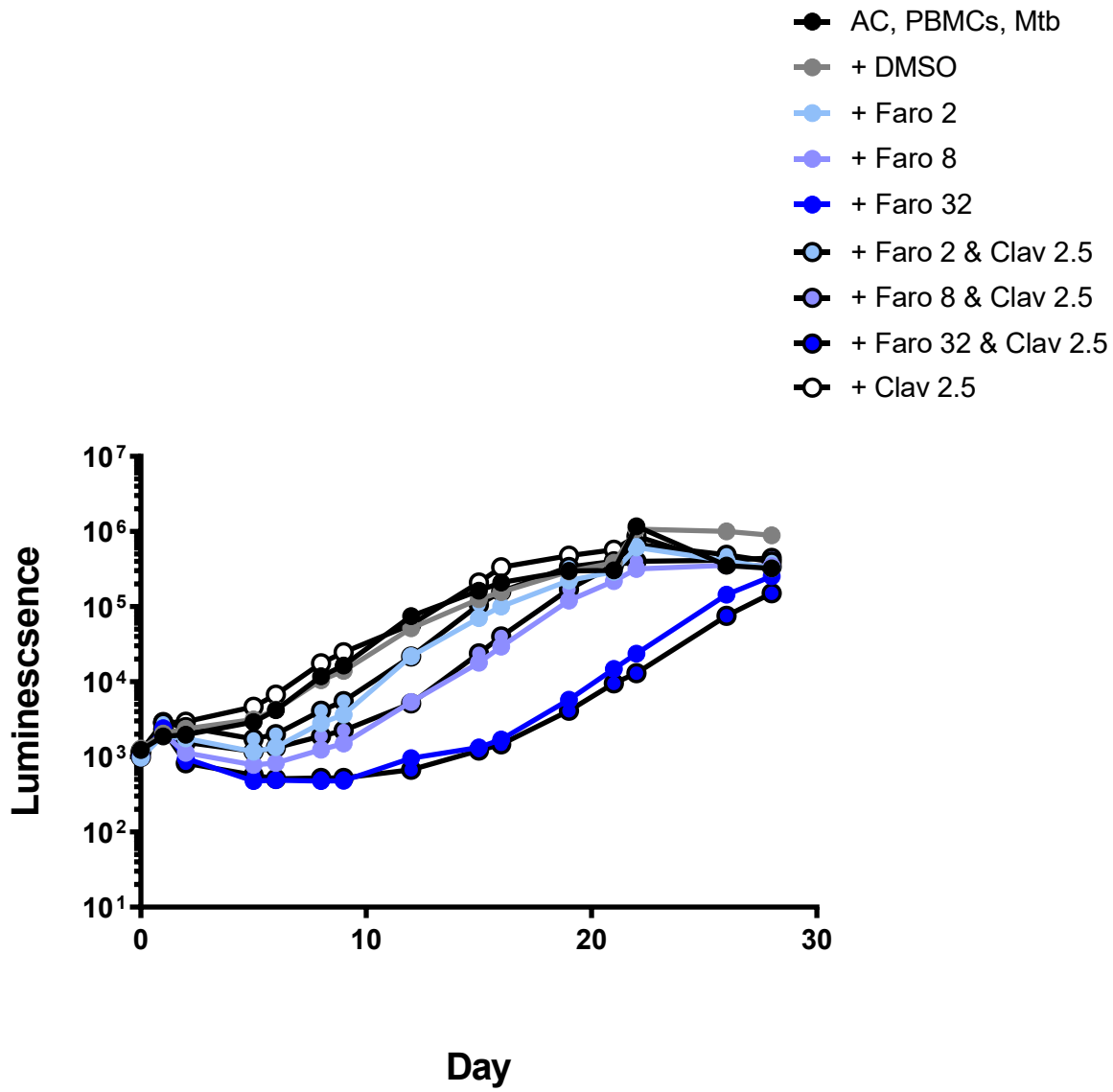


Figure 4.9 – Experiment 2 – donor 2. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Faro: faropenem; Clav: clavulanate



Tebipenem was active at 32 mg/L. The luminescence curve remained stable after 10 days, suggesting it is more stable or active than the other two carbapenems in this model. Addition of clavulanate did not increase killing activity. Figures 4.10 and 4.11.

Figure 4.10 – Experiment 1 – donor 1. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Tebi: tebipenem; Clav: clavulanate

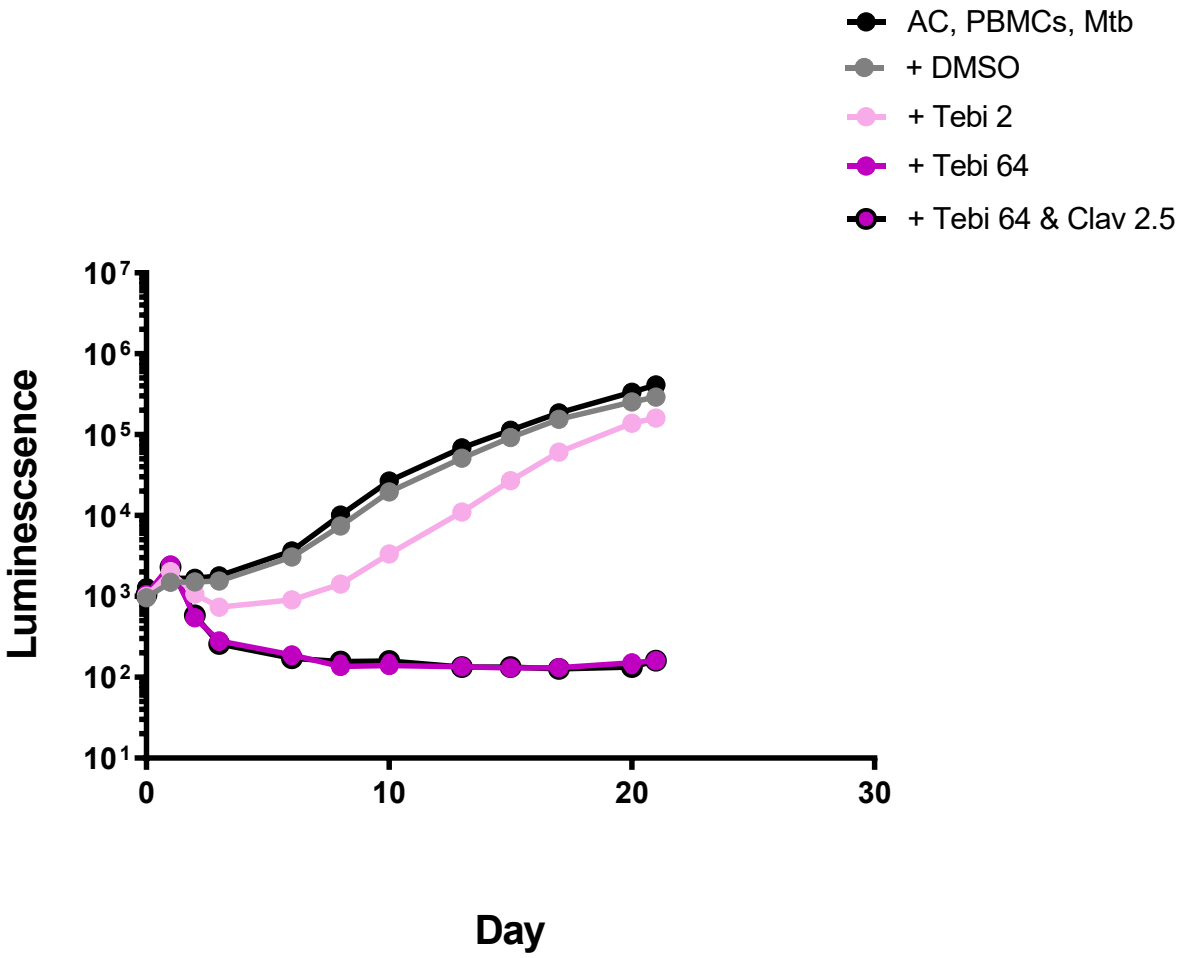
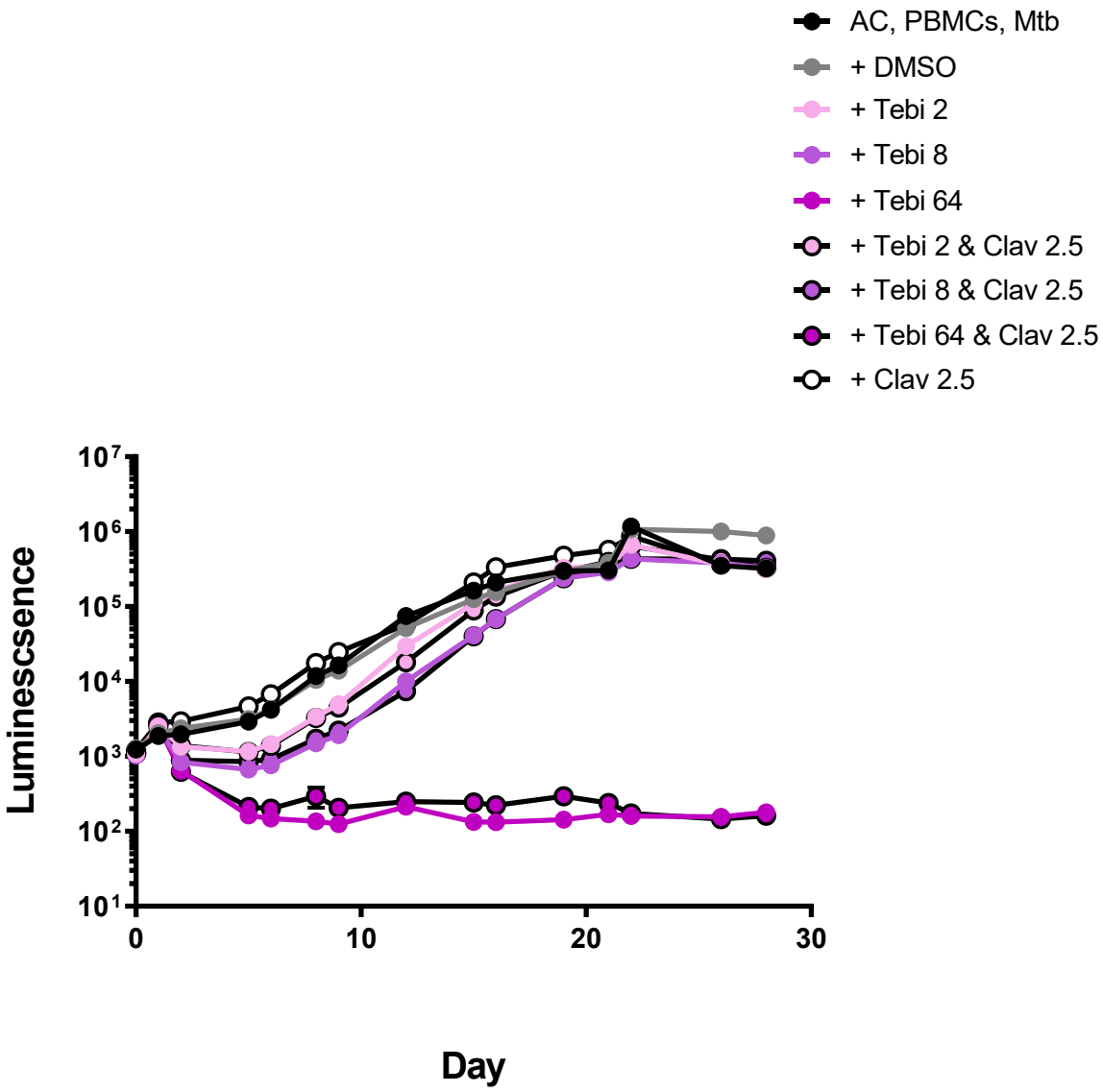


Figure 4.11 – Experiment 2 – donor 2. AC: alginate-collagen; PMBCs: Peripheral blood mononuclear cells; Mtb: *M. tuberculosis*; DMSO: dimethyl sulfoxide; Tebi: tebipenem; Clav: clavulanate



There were differences in MICs obtained in the three experiments. Generally, the microdilution method produced lower MICs, i.e., it tended to show drugs were more active. This may be associated with technical issues regarding incubation conditions, i.e. less availability of oxygen but this is not completely clear since previous reports suggest that gradual depletion of oxygen halts exponential growth of mycobacteria (Dick *et al.*, 1998) but more recent reports found that microaerophilic conditions speed up growth (Ghodbane *et al.*, 2014).

Ertapenem consistently showed limited activity in experiments 1 and 2, with only a few isolates showing susceptibility only in the microdilution model. This lack of activity is likely to be an artefact associated with the reported phenomenon of ertapenem degradation *in vitro* (Srivastava *et al.*, 2016). Given the slow replication of *M. tuberculosis*, this leads to a challenging situation in testing where the antibiotic is possibly degrading before killing or inhibiting bacterial growth. Some authors have suggested the daily addition of antibiotics to this experimental set up (Watt *et al.*, 1992) but this would hamper the evaluation of the dose tested and it would increase the risk of contamination as well as posing a repeated risk for the operator when working with M/XDRTB. The addition of amoxicillin-clavulanate did not translate into significant improvements in susceptibility. Although, ertapenem has been reported as useful in the treatment of tuberculosis previously, as part of combination therapy its role remains unclear (Veziris *et al.*, 2011, Tiberi *et al.*, 2016). Previous animal studies reported the ertapenem MIC at 4 mg/L (Veziris *et al.*, 2011, Gonzalo *et al.*, 2020).

Stability is not a problem for faropenem as it is thermo-stable at 37 degrees (Viaene *et al.*, 2002). Sixty-two out of 82 in experiment 1 (75.6 %) and 10 isolates out of 20 (50 %) in experiment 2, exhibited different degrees of susceptibility to faropenem and the addition of amoxicillin-clavulanate further reduced the MIC in 3 and 7 clinical isolates respectively. This is in line with previous experiments with other carbapenems, in particular meropenem (Gonzalo and Drobniewski, 2012). However, most of the MICs in experiment 2 were very close to 8 mg/L, which suggests we are reaching the limit of effectiveness. The current breakpoint for Gram positive bacteria is 2 mg/L and 8 mg/L for Gram negative microorganisms (EUCAST, 2019, Gonzalo *et al.*, 2020).

Faropenem did show some limited activity (MIC of 4 mg/L or lower) in strains completely resistant to meropenem (MIC of 32 mg/L or higher). However, the five isolates fully susceptible to first line antituberculous drugs were completely resistant at the highest concentration of faropenem used. This highlights a major issue of unpredictability and explains its limited use in clinical practice as no susceptibility pattern to traditional drugs can

predict susceptibility to faropenem and susceptibility testing is not routinely available. In addition, further studies are still needed to assess which antibiotic level is actually achievable in the blood (and in the lung parenchyma) after the oral administration of faropenem as reaching the concentrations tested in these experiments may be challenging (Gonzalo *et al.*, 2020).

In the three-dimensional model of the granuloma, tebipenem was the most effective carbapenem tested, but none of them showed activity at concentrations achievable *in vivo* (Eckburg *et al.*, 2019, Gill *et al.*, 2010, Mouton and van den Anker, 1995).

Carbapenems' activity against mycobacteria has been reported extensively in the last two decades (Sotgiu *et al.*, 2016, Gonzalo *et al.*, 2020). However, conflicting *in vitro* results, different methodologies and different drug choice have contributed to the lack of clarity regarding which carbapenem is the best for use in human tuberculosis disease and what method, if any, is the best for testing (Gonzalo *et al.*, 2020, Guo *et al.*, 2019, van Rijn *et al.*, 2019, Gonzalo and Drobniewski, 2012). Clinical outcome evidence is limited and difficult to interpret as tuberculosis therapy of MDR and XDR-TB involves combination of several drugs. Currently, no well-powered control trial exists (Sotgiu *et al.*, 2016).

Analysis of antibiotic efficacy in 3-D granuloma model complements findings in more standard systems. This approach could be used instead of animal models to test new or repurposed compounds. The *M. tuberculosis* killing efficacy of the compounds tested was dose dependent in the 3-D model.

The role of carbapenems remains unclear and this work can offer limited clarification.

"Although carbapenems are promising agents, this work has highlighted some of the limitations of their use. Information regarding clinical use and outcomes in humans is starting to emerge, showing results suggestive of activity against *M. tuberculosis*. However, the contribution of the beta-lactam to the outcomes remains difficult to ascertain. Current

opinions suggest that until more evidence becomes available, these drugs should be considered companion drugs rather than effective anti-TB agents, particularly in light of the administration route, higher cost associated to their use and emergence of carbapenem resistance amongst gut microbiota (Woerther *et al.*, 2018).

More evidence is needed to clarify the true impact of carbapenems in both tuberculosis treatment and outcome and as well as the financial burden, complications and microbiota ecological changes associated with their use to justify their re-classification as effective anti-TB agents" (Gonzalo *et al.*, 2020).

Carbapenems remain reserve drugs for the treatment of tuberculosis and, after more than 10 years of use, their role remains unclear. *In vitro* testing is not readily available and different methodologies have shown very different results.

Ertapenem remains challenging to test as it degrades quickly at 37 degrees. Pharmacokinetic/pharmacodynamic models such as the hollow fibre model would allow to overcome the issue of degradation of the drug as well as to test for multiple compounds simultaneously (Peloquin and Davies, 2021). Elkington *et al*. have also developed a model based on the three-dimensional granuloma that allows for multiple-drug testing as well as daily addition of antimicrobials (Bielecka and Elkington, 2018). However, daily manipulation of cultures and replenishing of drugs would pose a significant hazard when dealing with MDR/XDR tuberculosis strains.

Carbapenems showed modest *in vitro* activity using microdilution and microdilution methods. The susceptibility is strain specific and cannot be assumed a priori as it is not associated with M/XDRTB status.

Carbapenems are not active against *M. tuberculosis* if the current EUCAST cut-off for Gram positive microorganisms is followed. However, using PK/PD criteria, there could be some activity for meropenem, faropenem and tebipenem. The killing efficacy of the compounds

tested was dose dependent. Tebipenem was most efficient in killing MTB. Addition of Clavulanate (2.5 mg/L) did not increase the killing efficacy of the antibiotics, except for meropenem and to a lesser degree for faropenem in the macrodilution experiment.

As amoxicillin and clavulanic acid were both tested at a fixed dose, it is not possible to draw any conclusions about how higher doses would impact the carbapenems MICs against mycobacteria. The rationale to take this approach rather than to set up a full three-way checkerboard experiment was related to the fact that the dose of clavulanate cannot be increased in clinical practice due to potential liver toxicity. Local biosafety regulations also limited the total volume of live mycobacteria in the incubator at any given time which led to designing the experiment in such a way to prioritise the evaluation of the compounds of interest, i.e., carbapenems.

More research is needed to clarify the true role of these antimicrobials in the treatment of *M. tuberculosis* though the data presented above suggests some scepticism is warranted in considering their value. The recent introduction of novel inhibitors/carbapenems, such as ralebactam and varbobactam, could be explored.

Clofazimine was the most effective antibiotic tested (supporting its current use as a key agent for M/XDRTB patients). It inhibited the growth of *Mycobacterium tuberculosis* at as low as 2 mg/L in the 3-D bioelectrospray model. Nitazoxanide requires further work to establish whether the lack of activity is a true drug-related phenomenon, or it is an artefact related to extensive protein binding.

The microdilution experiment was accepted as a poster presentation at the 20th ECCMID (published as abstract - Appendix IV) and was published in Antibiotics (Gonzalo and Drobniewski, 2022).. The macro/microdilution experiment results were published in BMC Microbiology (Gonzalo *et al.*, 2020). The 3-D model experiment results have been submitted for publication and are awaiting a final decision and results were presented as a poster in the 32nd ECCMID in Lisbon, 2022. Appendix V.

Chapter 5: MALDI-ToF for identification of Mycobacteria

5.1 Introduction

A simple procedure able to identify mycobacteria quickly and cheaply using standard commercially available MALDI-ToF MS instruments in clinical microbiology laboratories without the need of extensive work in a BSL3 containment facility would be a significant asset. This would result in shorter turn-around-times, enabling more timely clinical decision making at low cost (Gonzalo *et al.*, 2021).

The change to a centralized next generation sequencing based system in the UK led to a more rapid genotypic based system of MTB identification and first line drug susceptibility of TB cultures. However as both identification and DST are performed simultaneously (using the same genomic sequence) culture identification which can (and was) performed using a molecular identification system (e.g., Hain Lifesciences) in one day has ironically become delayed until the complete Identification and DST analysis has been completed (at about 7-13 days). In low antibiotic resistance countries (e.g. UK, USA, EU/EEA states), the critical question for healthcare systems is usually whether an individual has TB or not as this will determine (1) individually appropriate therapy; (2) the need for respiratory isolation and (3) need for public health action to prevent disease transmission. Where a potential TB diagnosis exists, medical staff have to play safe even if they believe that the mycobacterial culture is likely to be an NTM. A rapid and inexpensive alternative to molecular identification of grown cultures, such as the MALDI-ToF MS system, used in nearly all clinical microbiological laboratories would be time-saving and cost-saving by reducing the need for inappropriate hospitalization/isolation.

The aim of this study was to establish (1) a methodology to differentiate *M. tuberculosis* isolates from the non-tuberculous mycobacteria (NTM) reliably by generating NTM and *M. tuberculosis* species-specific profiles, and (2) then identify *M. tuberculosis* complex sub-

species based on species-specific lipid profiling using lipid fingerprints in MALDI-ToF MS on intact bacteria.

5.2 Materials and methods

The strains used in the experiments listed below are listed in detail in Chapter 2.

5.2.1: MALDI-ToF for identification of Mycobacteria

5.2.1.1: Experiment 1: Identification of *M. tuberculosis* vs non-tuberculous mycobacteria

Analysis of the mass spectra was conducted blindly (Gonzalo *et al.*, 2021).

All isolates were sub-cultured from frozen stocks in 7H9 Middlebrook medium without glycerol, supplemented with 10% OADC and incubated for 2-6 weeks at 37°C. Once the optimal growth of bacteria was obtained 100 µL of bacterial suspension were placed into 1.5 mL Eppendorf tubes and heat killed at 95 ºC for 30 minutes before leaving the BSL3 laboratory.

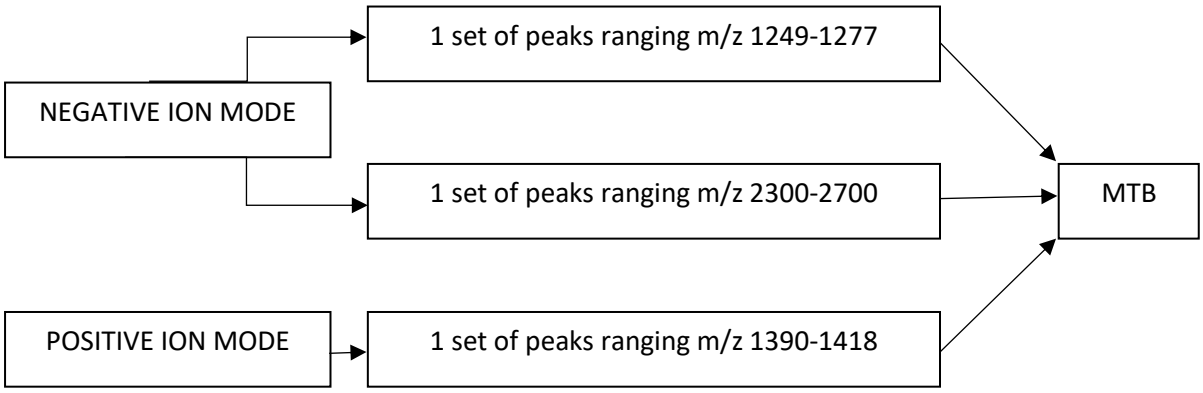
MALDI sample preparation. The heat killed mycobacterial pellet was washed four times with 200 µL distilled water. Then 0.4 µL of the re-suspended pellet was placed on the MALDI plate and mixed with 0.8 µL of 10mg DHB matrix diluted in solvent A (CHCl3:MeOH 98:2) and with 0.6 µL of 10mg DHB matrix diluted in solvent B (50% Ethanol), C (25% Ethanol), and D (50% Methanol).

Instrument and data analysis. An Applied Biosystems 4800 MALDI-ToF/TOF™Analyzer (Applied Biosystems/MDS SCIEX MDS Sciex, Concord, Ontario, Canada) was used. Samples were analyzed in the positive and negative ion modes operating at 20kV and were set to acquire mass spectral peaks with mass/charge ratio (m/z) from 400 to 4000 mass. Mass spectrometry data were analyzed using Data Explorer® Software version 4.9 from Applied Biosystems.

Spectra were visually analysed and identified as *M. tuberculosis*/NTM blindly by two different

operators using the flowchart represented in Figure 5.1.

Figure 5.1 – Flowchart for species allocation MTBC/NON-TB



5.2.1.2: Experiment 2: Identification of non-tuberculous mycobacteria

Sample preparation. Three variables were selected for initial evaluation of NTMs strains on MALDI-ToF MS.

1. Phase of culture growth
2. Growth media. Three media were used for mycobacterial growth
    a. Middlebrook 7H9 (Sigma Aldrich) with OADC – liquid medium
    b. Middlebrook 7H11 (Sigma Aldrich) with OADC – solid medium
    c. Kirchner (Sigma Aldrich) – liquid medium
3. Matrix composition: 10 mg of 2,5-dihydroxybenzoic acid (DHB) diluted in four types of solvents to check the best performance:
    a. A - in chloroform:methanol (95:5) (C/M)
    b. B - in 50% Ethanol (EtOH)
    c. C - in 25% Ethanol (EtOH)
    d. D - in 50% Methanol (MeOH)

About 0.5 mL - 1 mL of liquid mycobacterial biomass from the bottom of the tube was pipetted into 1mL Eppendorf tube and heat-killed in a water bath at 95ºC for 30 min in containment

level 3 laboratory. The samples were then transported into containment level 2 laboratory where all further work was performed. The liquid mycobacterial biomass was centrifuged at 15,000 x g for 5 min and the supernatant was discarded. The bacterial pellet was washed 4 times with 200 µL of ultra-purified water. After each washing, the pellet was centrifuged, and the supernatant was removed. After the last washing around 50 µL of mycobacterial biomass was left, ready for further testing. When further testing was differed, pellets were frozen at -80ºC.

The mixtures were dried at room temperature. Samples were analyzed on Applied Biosystems 4800 MALDI TOF/TOF™Analyzer. (Applied Biosystems/MDS SCIEX MDS Sciex, Concord, Ontario, Canada)

Reproducibility. To check the technical reproducibility and the robustness of the approach three samples were prepared from a single culture of ten ECDC strains. Additionally, thirty-two *M. abscessus* clinical isolates were tested blindly in duplicates/triplicates to confirm the results.

5.2.1.3: Experiment 3: Identification of sub-species within *M. tuberculosis* complex

Sample preparation. Once the optimal growth of bacteria was obtained, 100 µL of bacterial suspension were placed into 1.5 mL Eppendorf tubes and heat-killed at 95 ºC for 30 minutes before leaving the BSL3 containment area. The heat killed MTBC pellets were washed four times with 200 µL of double distilled water. Then, 0.4 µL of the re-suspended pellet was pipetted onto the MALDI matrix plate and mixed with 0.8 µL of the MALDI matrix. The matrix used consisted of a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (super-DHB, Sigma-Aldrich) at a concentration of 10 mg/mL in chloroform:methanol 9:1.

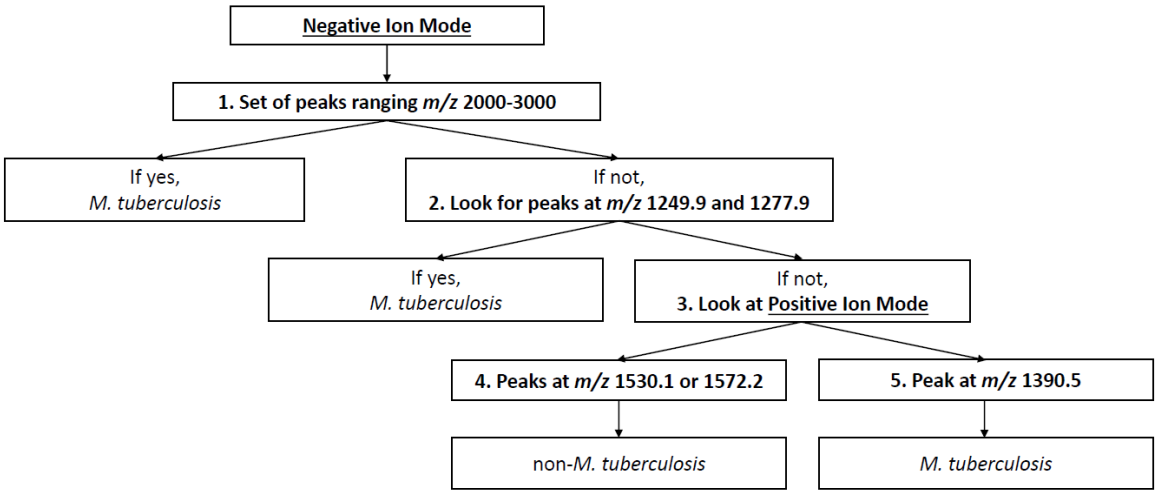
Mass spectrometry analysis. MS analyses were performed on a MALDI Biotyper sirius® system (Bruker Daltonics, Germany) and on Applied Biosystems 4800 MALDI

TOF/TOF™Analyzer (Applied Biosystems/MDS SCIEX MDS Sciex, Concord, Ontario, Canada). The mass spectra were scanned in the range of *m*/*z* 1,100 to 2,200. The mass profiles were acquired using FlexControl 3.4 software (Bruker Daltonics, Germany). The spectra were recorded in the linear positive-ion mode (laser intensity 95%, ion source 1 = 10.00 kV, ion source 2 = 8.98 kV, lens = 3.00 kV, detector voltage = 2652 V, pulsed ion extraction = 150 ns). Each spectrum corresponded to ion accumulation of 2,000 to 5,000 laser shots randomly distributed on the spot. The spectra obtained were processed with default parameters using FlexAnalysis v.3.4 software (Bruker Daltonics, Germany).

Statistical analysis. Analysis of the mass spectra of the clinical isolates was conducted by randomizing the selection of the cultures to be analyzed and by having two operators blinded to the reference culture results. Sensitivity, specificity and 95% Confidence Interval were calculated as described in the literature (Mercaldo *et al.*, 2007, Zhou and Qin, 2005, Griner *et al.*, 1981, Qin *et al.*, 2006).

Spectra were visually analysed and identified as *M. tuberculosis/other M. tuberculosis* complex member blindly by two different operators following the algorithm represented in Figure 5.2.

Figure 5.2 – Flowchart for species allocation MTB/Other MTBC member



5.3 Results and discussion

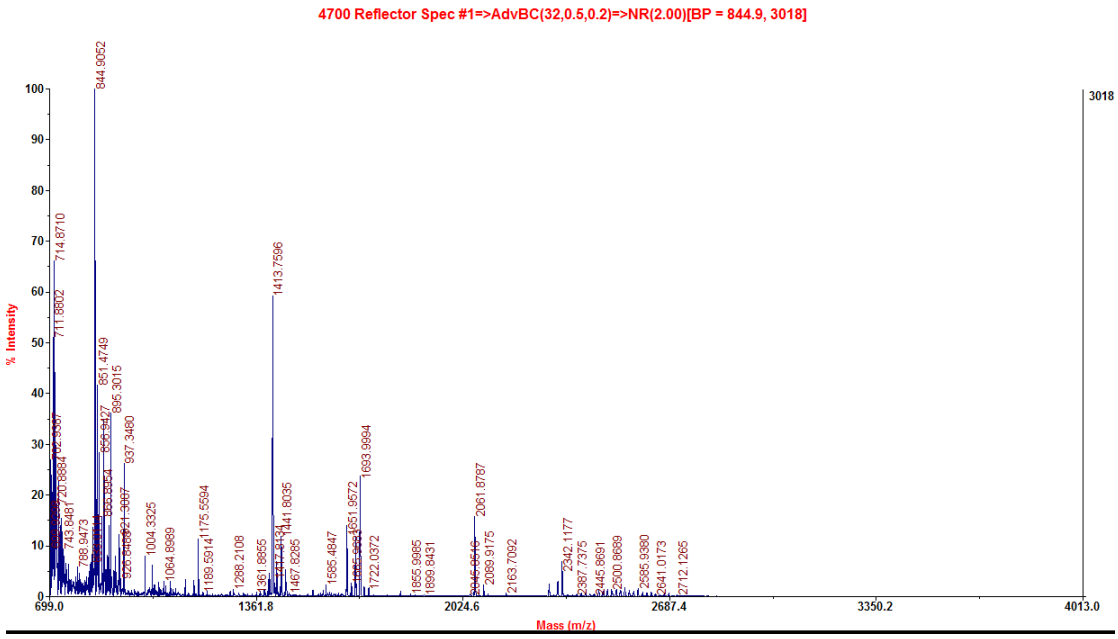
5.3.1 Experiment 1: Identification of *M. tuberculosis* vs non-tuberculous mycobacteria

The study examined tuberculosis isolates' lipidomic profile by MALDI-ToF mass spectrometry. A total of 273 isolates were tested (244 Mtb isolates and 29 NTM isolates) (see Chapter 2). The mass spectra were acquired in both positive and negative mode.

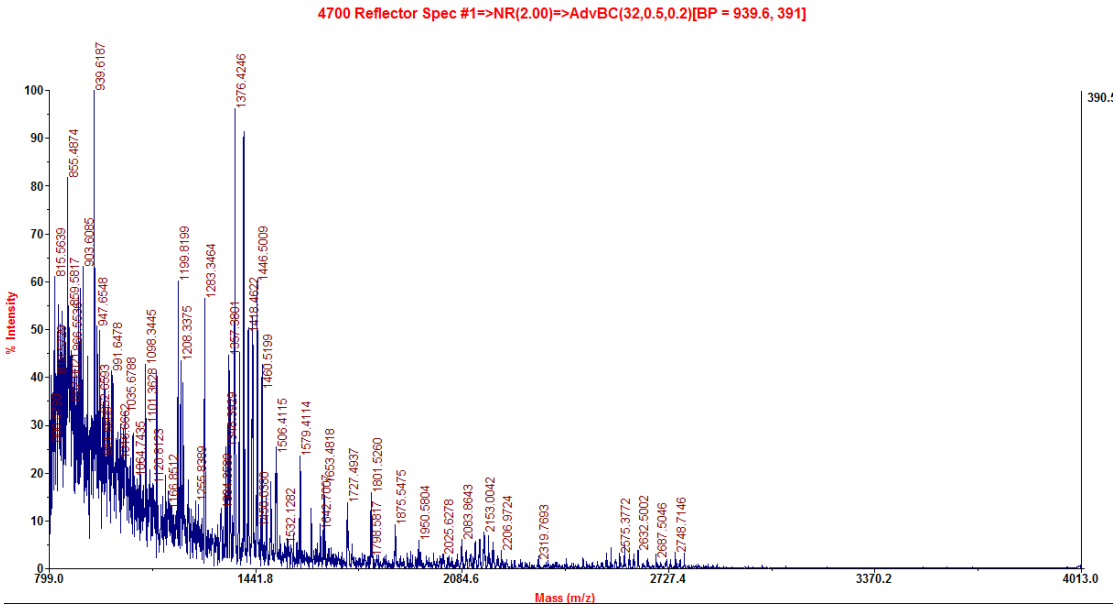
Examples of spectra for both *M. tuberculosis* and NTM in negative and positive ion modes can be found in Figures 5.3 A-D

Figure 5.3 – Spectra for *M. tuberculosis* and NTM in negative and positive ion modes

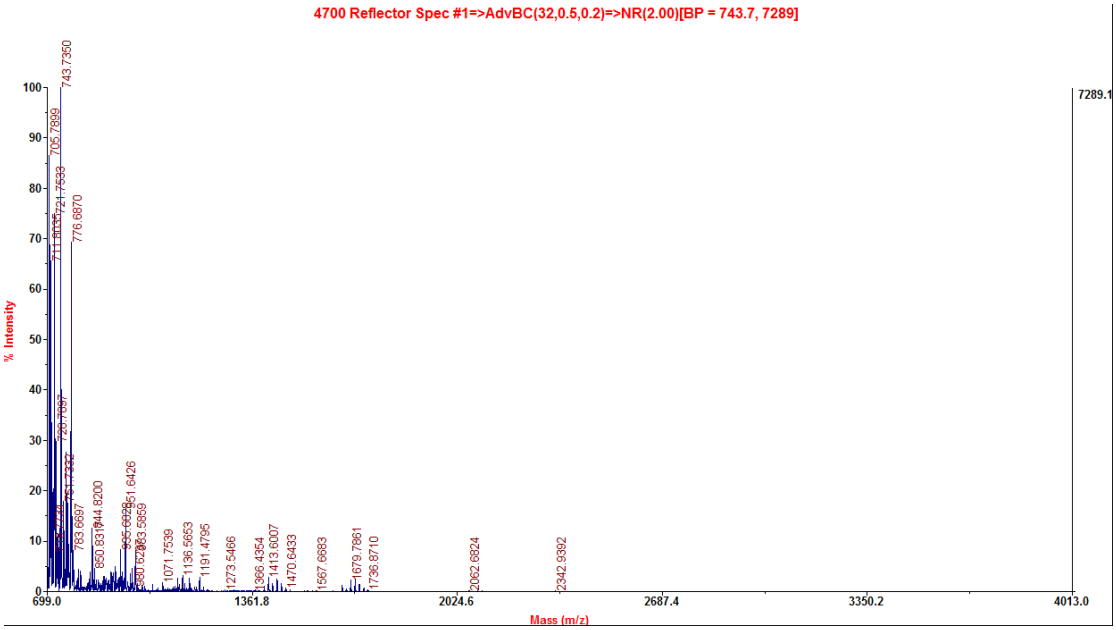
A – *M. tuberculosis* Negative ion mode



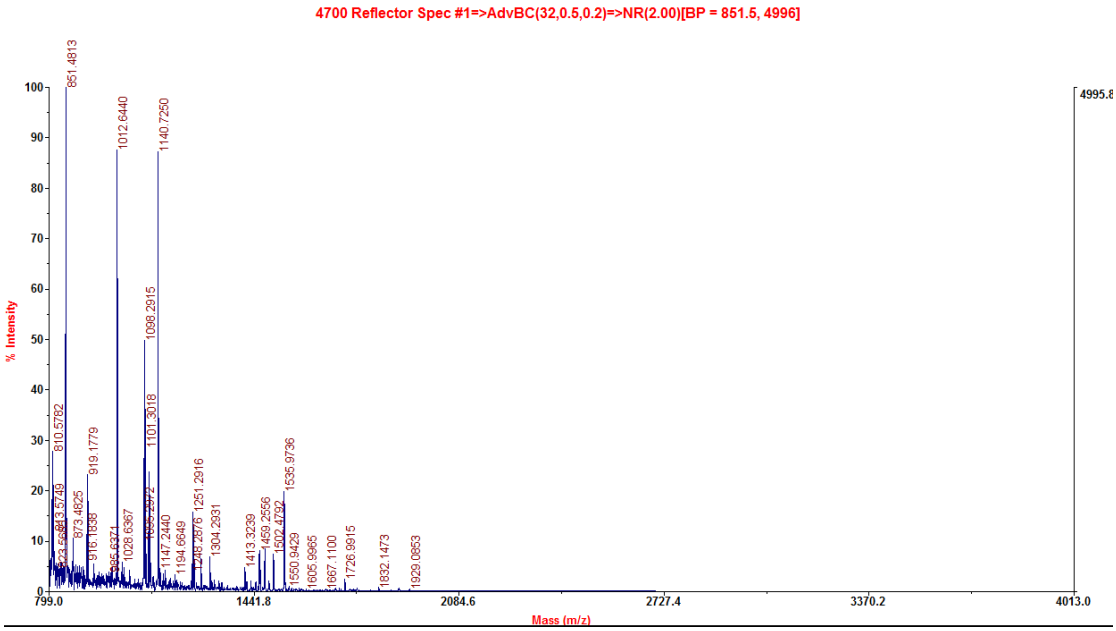
B – *M. tuberculosis* positive ion mode



C – NTM negative ion mode



D – NTM – positive ion mode



“Analysis of the mass spectra was conducted blindly. A good mycobacterial mass spectral signal (Resolution > 300 and Signal-to-Noise > 5) in negative mode was generated for 235 out of 273 isolates; 38 isolates (14%) could not be assigned to either the *M. tuberculosis* or NTM group and 9 isolates (3%) were misidentified. Good signals (Resolution > 300 and Signal-to-Noise > 5) for most of the NTMs were generated in positive and negative ion modes” (Gonzalo *et al.*, 2021).

“The 226 isolates of 235 which were correctly assigned were based on the negative mode signature for *M. tuberculosis* and the positive mode signatures for NTMs. All data were compared to molecular identification and reference strains designation from the ECDC” (Gonzalo *et al.*, 2021). Identification of *M. tuberculosis* was achieved by the presence of SL-I, which is a negatively charged lipid uniquely found in *M. tuberculosis* (Layre *et al.*, 2011, Goren, 1970). Within the NTM group (29 clinical isolates), indentification was performed in the postive ion mode by the ionization of species-specifc lipids fingerprint, such as glycopeptidolipids, C-mycosides, polar glycopeptidolipids, glycerol monomycolate and phenolic glycolipids (Brennan and Goren, 1979, Aspinall *et al.*, 1991, Aspinall *et al.*, 1992, Torrelles *et al.*, 2002,

Gautier *et al.*, 1992, Lopez Marin *et al.*, 1991, Lopez-Marin *et al.*, 1994, Brennan and Nikaido, 1995, Schorey and Sweet, 2008, Riviere and Puzo, 1991, Fournie *et al.*, 1987a, Fournie *et al.*, 1987b), associated to each of the species exhibiting distinct and unique signatures or "chemical barcoding", allowing clear discrimination of the NTMs.

Nine (3%) isolates gave discrepant results compared to molecular DNA-derived identification and/or other biochemical analysis which had given the identification by the ECDC European Reference Laboratory network; 7/211 *M. tuberculosis* isolates were wrongly identified as NTMs and 2/24 NTMs, *M. nonchromogenicum* and *M. smegmatis*, were assigned as *M. tuberculosis* by MALDI-ToF. The 38 uninterpretable isolates (14%; 38/273) were tested twice from the same heat killed washed pellets. The results remained the same, i.e. uninterpretable. Of those 38 isolates, 33 were *M. tuberculosis* and 5 NTM isolates. Regarding the 5 NTM isolates, *M. abscessus*, *M. mucogenicum*, *M. phlei*, *M. shimoidei*, the quality of the spectra were poor, associated with a low signal-to-noise (Signal-to-Noise <5) precluding a convincing assignment of the data generated. For the 33 *M. tuberculosis* isolates, distributed across Beijing, Ghana-like 1, Latin American Mediterranean and clade A groups, as our method relies mainly on the presence of SL-I, failure to assign these as *M. tuberculosis* could be explained by the low abundance of SL-I in those strains, i.e. below the limit of detection. It has been reported in the literature that some Mtb strains within the same Mtb lineages can have low or no detectable SL-I (Krishnan *et al.*, 2011, Gonzalo *et al.*, 2020).

The sensitivity and specificity of the MALDI-ToF excluding uninterpretable results was 96.7% (204/211) with a 95% Confidence Interval of [93.3%-98.5%] and 91.7% (22/24) with a 95% Confidence Interval of [73.0%-98.9%] respectively (Table 5.1).

Table 5.1 – Sensitivity and Specificity of the lipid fingerprint-based MALDI-ToF method. WGS: whole genome sequencing.

Mycobacterial identification (n=235)		**WGS**		**Sensitivity (%)**	**Specificity (%)**
		Positive	Negative	96.7	91.7
MALDI-ToF	Positive	204	2		
	Negative	7	22		

5.3.2 Experiment 2: Identification of non-tuberculous mycobacteria

Twenty-nine NTM reference strains (including 22 species) and 32 *M. abscessus* complex clinical isolates were analyzed by MALDI-ToF MS.

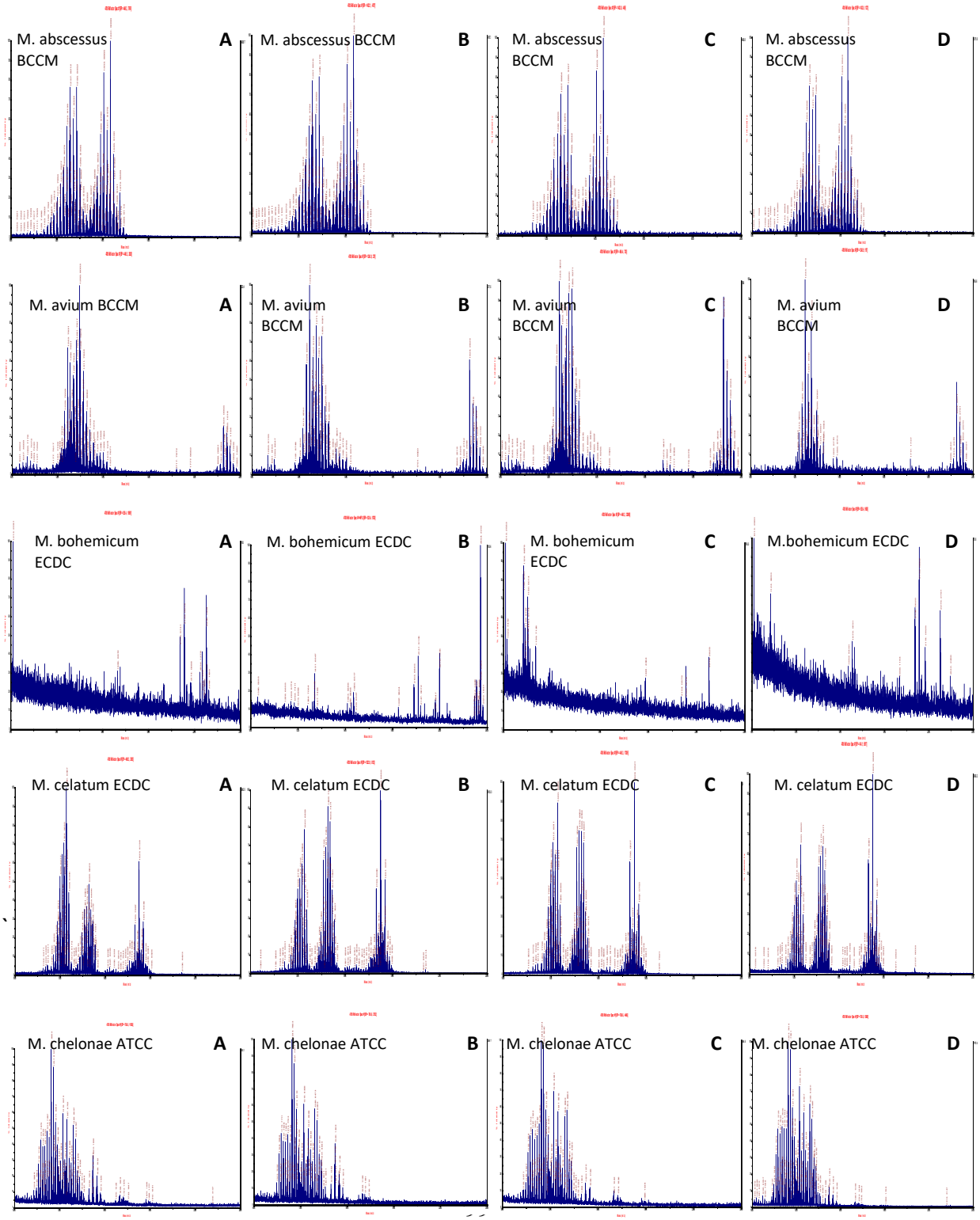
Nineteen out of twenty-two selected NTMs species generated a species-specific mass spectral profile allowing visual identification at species level.

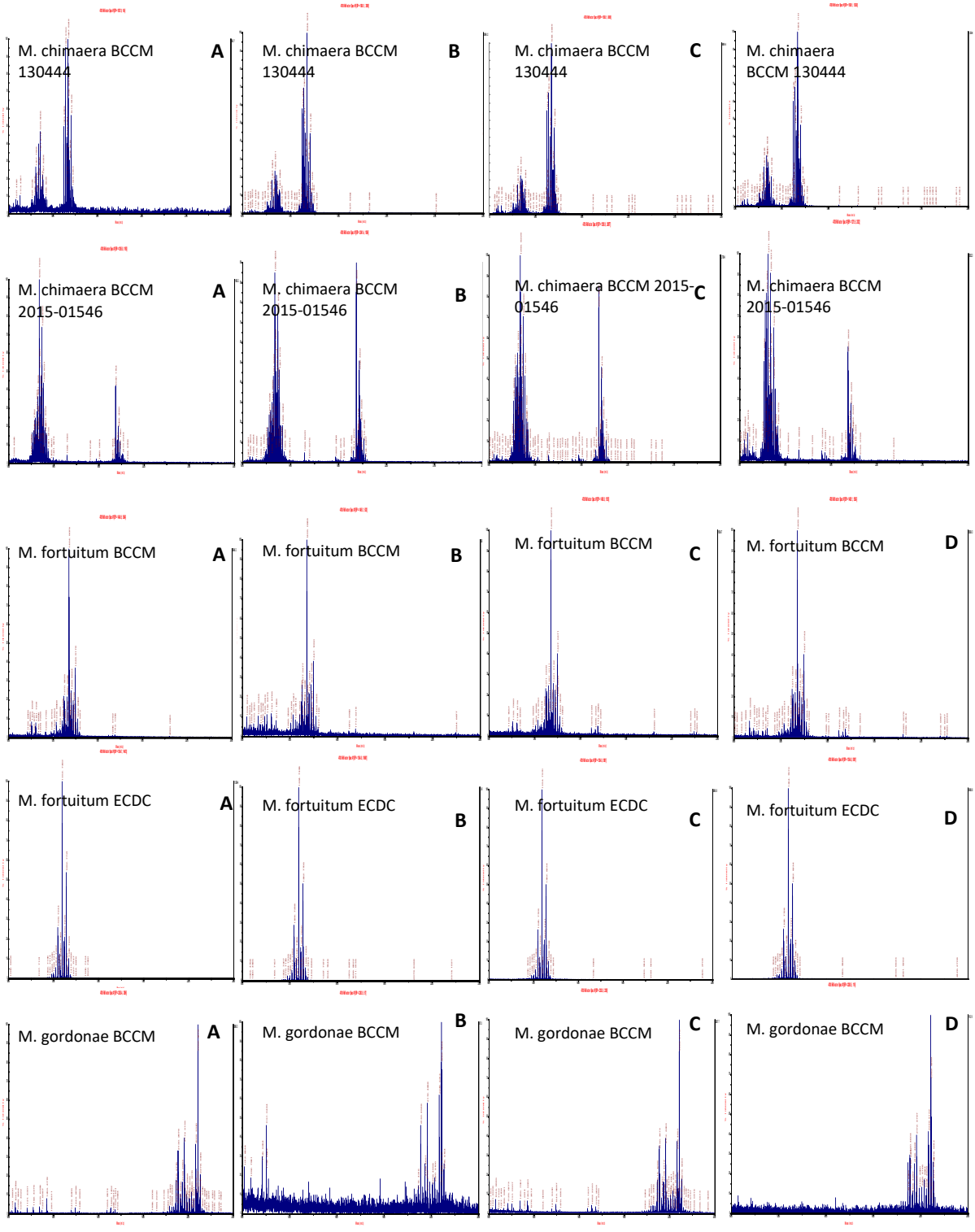
Logarithmic vs stationary phase of growth. There was a significantly better mass spectral signal for stationary rather than logarithmic phase of growth. Our samples were kept in the incubator till the relevant growth was reached.

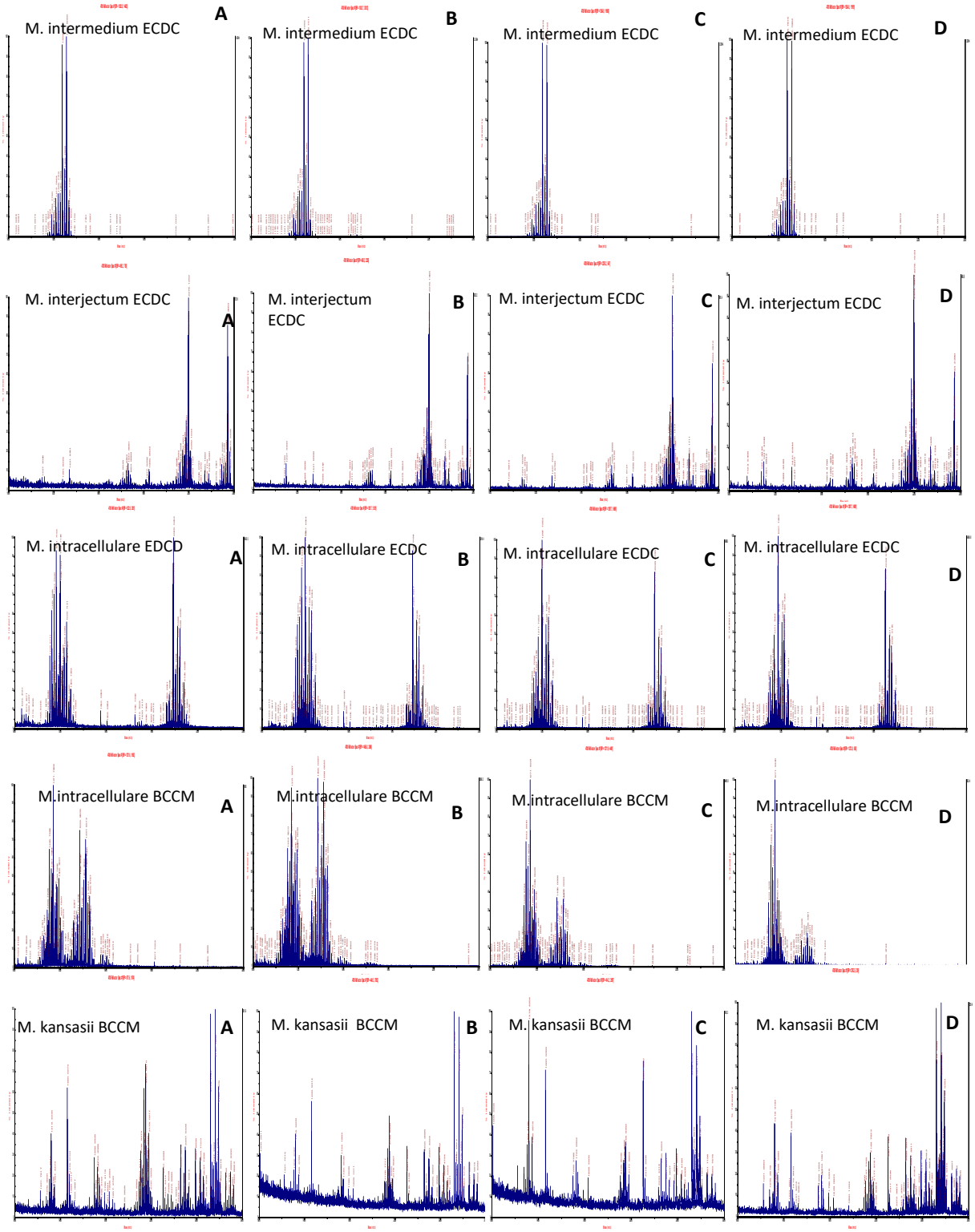
First, 10 ECDC reference strains were grown on Middlebrook 7H9 media with OADC and three copies were prepared from a single culture to check the reproducibility of the lipid signature. All isolates were tested on MALDI-ToF MS with matrixes solubilized in solvent A and B. The reproducibility was high with both solvents. The quality of the spectra was either equal or better with matrix diluted in solvent B (50% EtOH) than A (C/M 95:5).

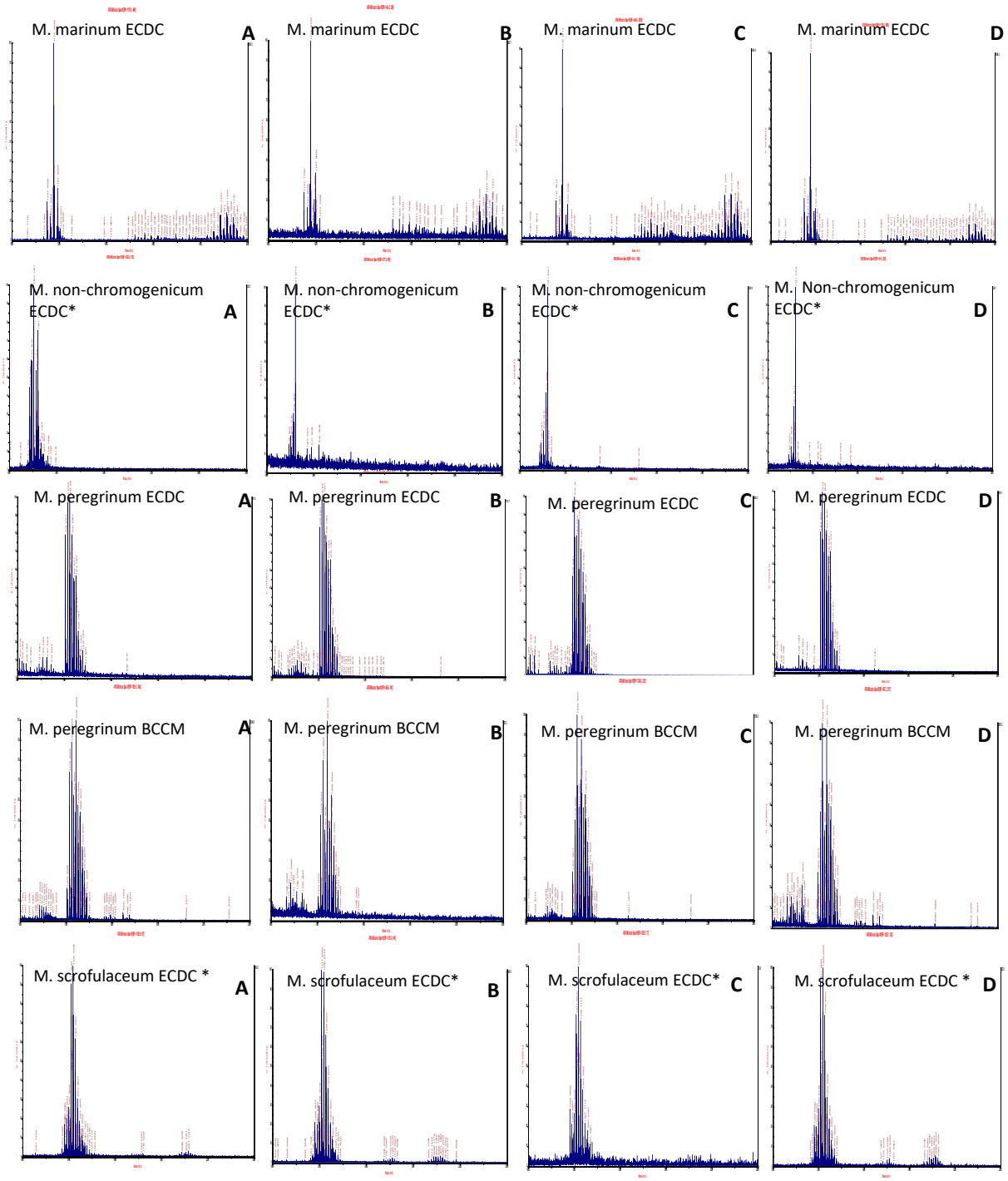
Two additional solvents were compared, solvent C (25% EtOH) and solvent D (50% MeOH) All results were comparable but the best quality was obtained with DHB matrix diluted in solvent B and C and analyzed on MALDI-ToF in positive ion mode. Figure 5.4. A poor mass spectral signature was generated by *M. bohemicum*, *M. non-chromogenicum* (except isolate growing on Kirchner media) and *M. simiae.*

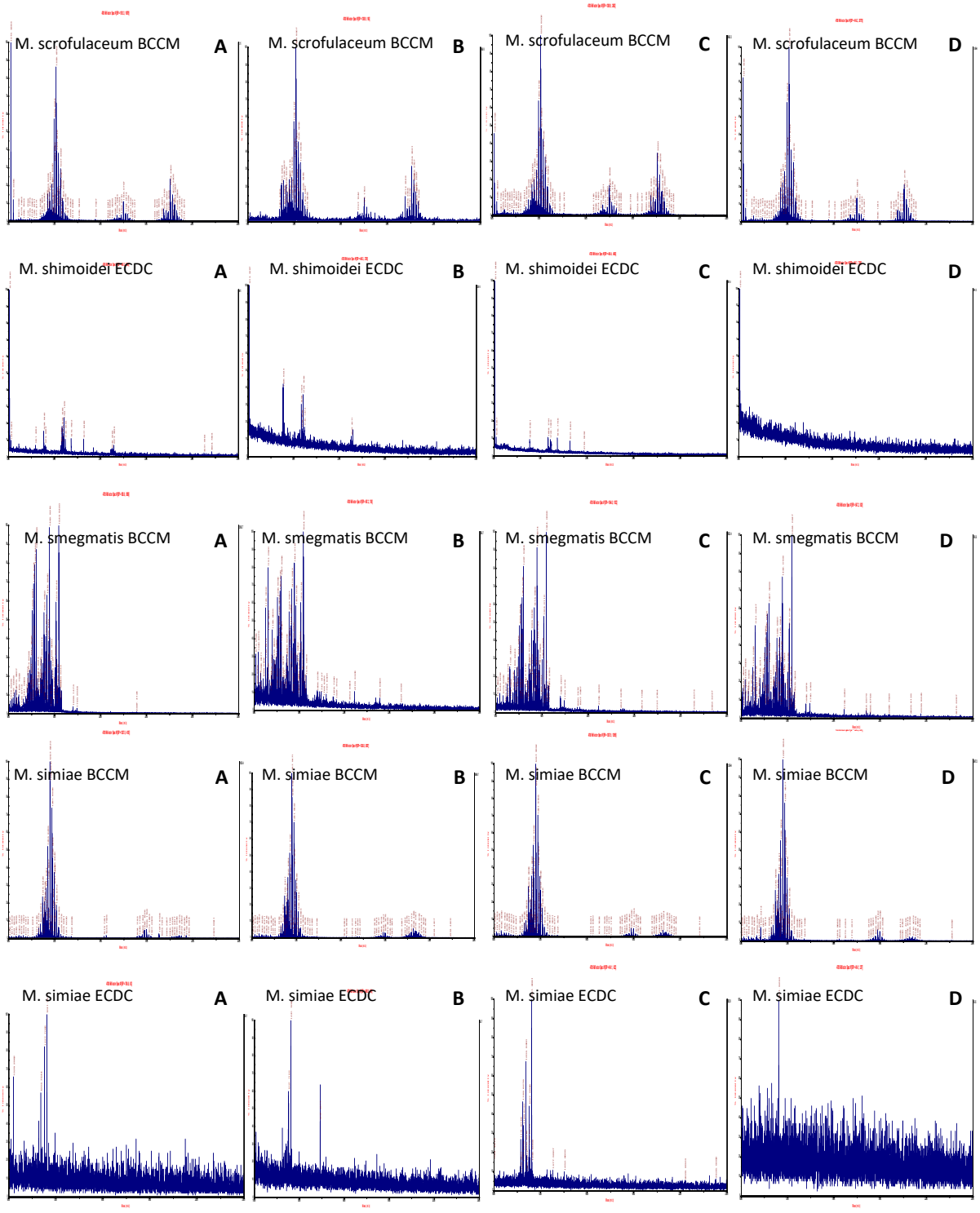
Figure 5.4 – 29 NTM reference strains. MALDI-TOF mass spectra comparison of DHB matrix (10mg/mL) solubilized in four different solvents A (CHCl3:MeOH 95:5), B (50 % ethanol), C (25% ethanol), and D (50% methanol). * *M. xenopi* ECDC - 7H9 medium (OACD) logarithmic phase . ** *M. xenopi* ECDC – Kirchner medium stationary phase

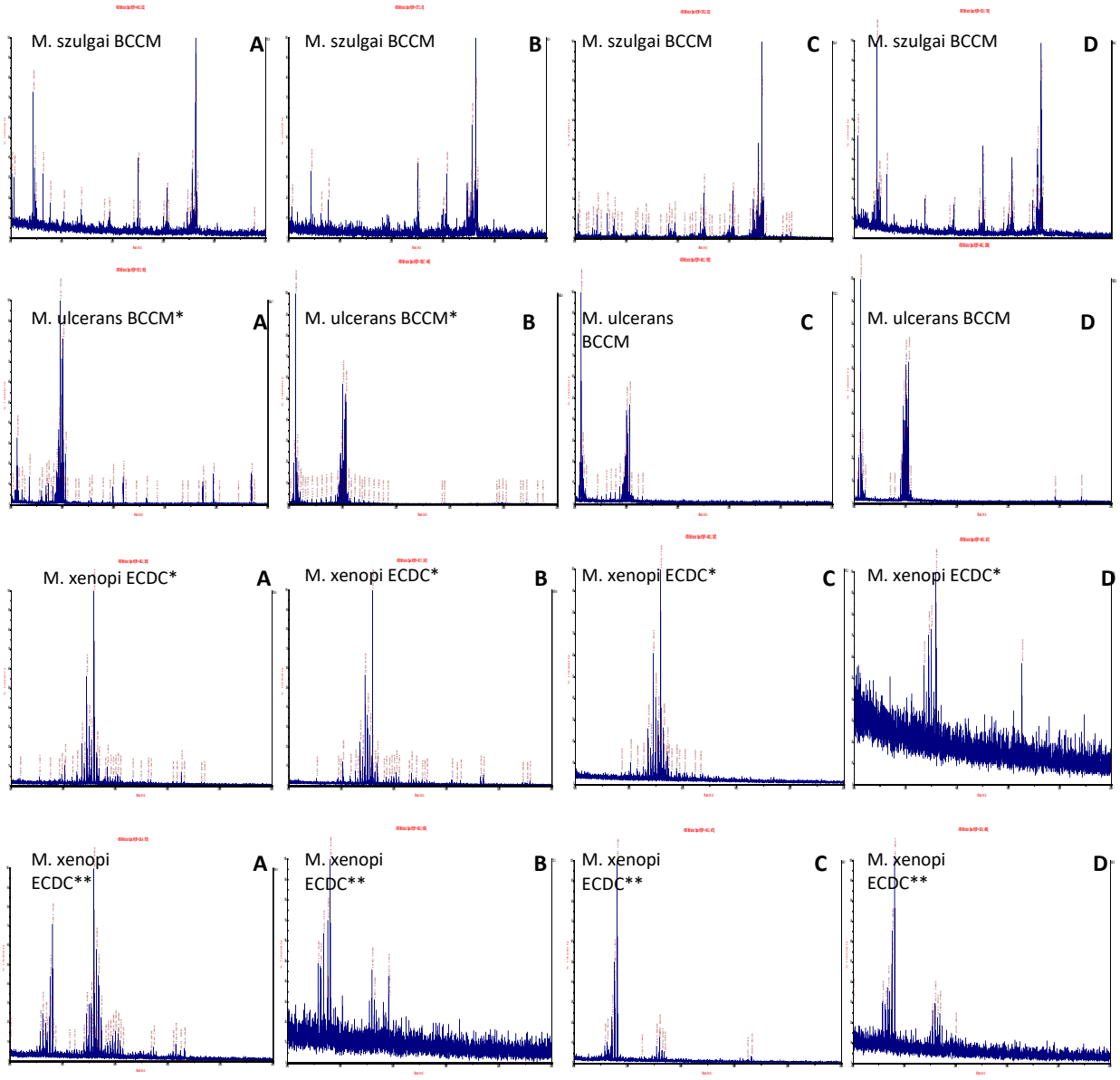



M. chimaera BCCM 130444
A
M. chimaera BCCM 130444
B
M. chimaera BCCM 130444
C
M. chimaera BCCM 130444
M. chimaera BCCM 2015-01546
A
M. chimaera BCCM 2015-01546
B
M. chimaera BCCM 2015-01546
C
M. chimaera BCCM 2015-01546
M. fortuitum BCCM
A
M. fortuitum BCCM
B
M. fortuitum BCCM
C
M. fortuitum BCCM
D
M. fortuitum ECDC
A
M. fortuitum ECDC
B
M. fortuitum ECDC
C
M. fortuitum ECDC
D
M. gordonae BCCM
A
M. gordonae BCCM
B
M. gordonae BCCM
C
M. gordonae BCCM
D


M. intermedium ECDC
A
M. intermedium ECDC
B
M. intermedium ECDC
C
M. intermedium ECDC
D
M. interjectum ECDC
A
M. interjectum ECDC
B
M. interjectum ECDC
C
M. interjectum ECDC
D
M. intracellulare EDCD
A
M. intracellulare ECDC
B
M. intracellulare ECDC
C
M. intracellulare ECDC
D
M.intracellulare BCCM
A
M.intracellulare BCCM
B
M.intracellulare BCCM
C
M.intracellulare BCCM
D
M. kansasii BCCM
A
M. kansasii BCCM
B
M. kansasii BCCM
C
M. kansasii BCCM
D


M. marinum ECDC A
M. marinum ECDC B
M. marinum ECDC C
M. marinum ECDC D
M. non-chromogenicum ECDC* A
M. non-chromogenicum ECDC* B
M. non-chromogenicum ECDC* C
M. Non-chromogenicum ECDC* D
M. peregrinum ECDC A
M. peregrinum ECDC B
M. peregrinum ECDC C
M. peregrinum ECDC D
M. peregrinum BCCM A
M. peregrinum BCCM B
M. peregrinum BCCM C
M. peregrinum BCCM D
M. scrofulaceum ECDC * A
M. scrofulaceum ECDC* B
M. scrofulaceum ECDC* C
M. scrofulaceum ECDC * D


M. scrofulaceum BCCM
A
M. scrofulaceum BCCM
B
M. scrofulaceum BCCM
C
M. scrofulaceum BCCM
D
M. shimoidei ECDC
A
M. shimoidei ECDC
B
M. shimoidei ECDC
C
M. shimoidei ECDC
D
M. smegmatis BCCM
A
M. smegmatis BCCM
B
M. smegmatis BCCM
C
M. smegmatis BCCM
D
M. simiae BCCM
A
M. simiae BCCM
B
M. simiae BCCM
C
M. simiae BCCM
D
M. simiae ECDC
A
M. simiae ECDC
B
M. simiae ECDC
C
M. simiae ECDC
D



***M. abscessus* complex**. The 32 clinical isolates were pipetted on the MALDI-ToF target slide (*M. abscessus*, 24; *M. bolletii*, 3; *M. massiliense*, 5). The analysis generated a strong signal with DHB matrix diluted in solvent A (Chloroform/Ethanol 95:5) (Figure 5.5), B (50% Ethanol) (Figure 5.6), and C (25% EtOH) (Figure 5.7).

Figure 5.5 – Positive ion MALDI-ToF MS spectra (m/z 1000-2600) of *M. abscessus* (A), *M. masiliense* (B), *M. bolletii* (C). The matrix DHB was diluted at 10mg/mL in solvent A (C/M 95 :5).

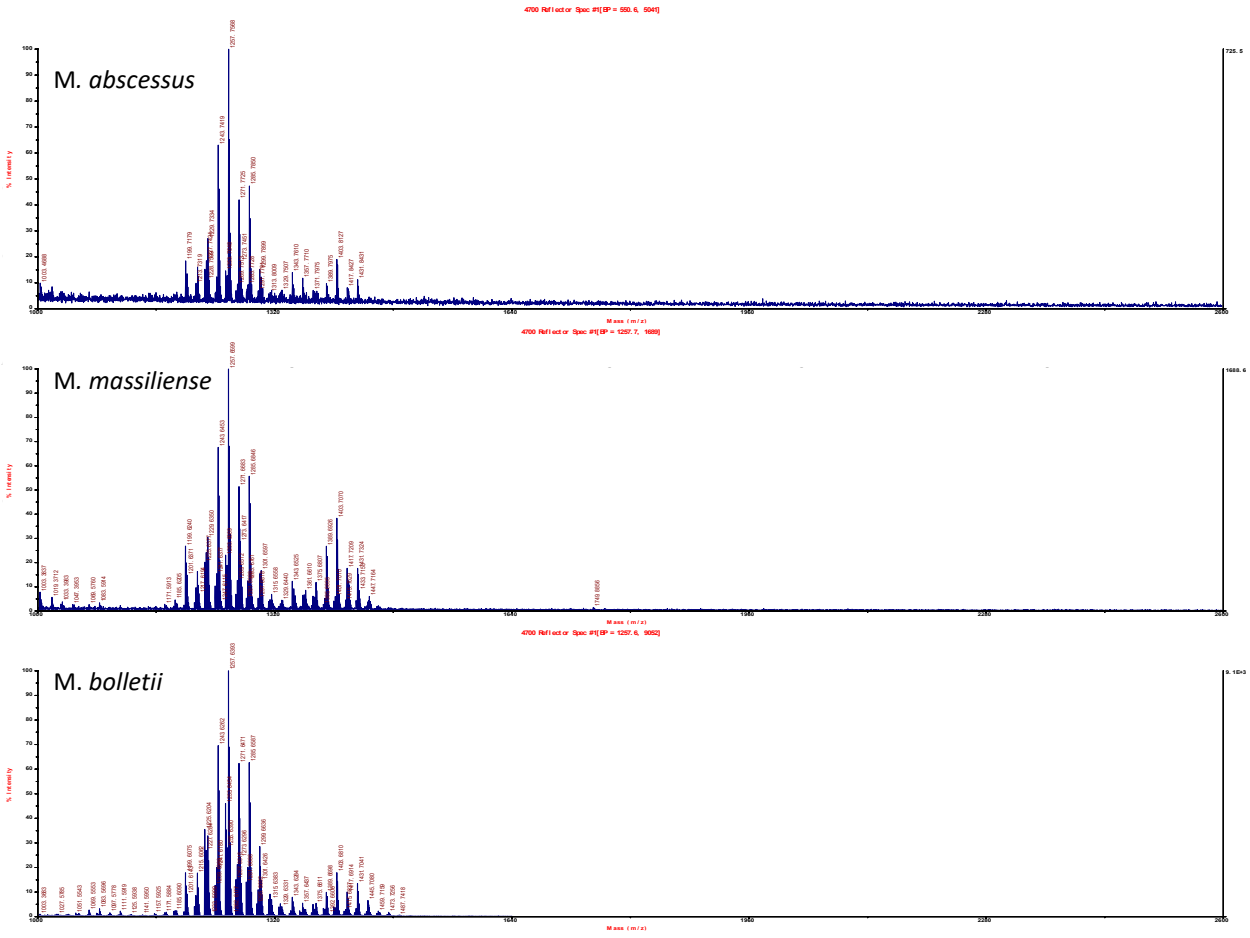


Figure 5.6 – Positive ion MALDI-ToF MS spectra (m/z 1000-2600) of *M. abscessus* (A), *M. masiliense* (B), *M. bolletii* (C). The matrix DHB was diluted at 10mg/mL in solvent B (50% EtOH)

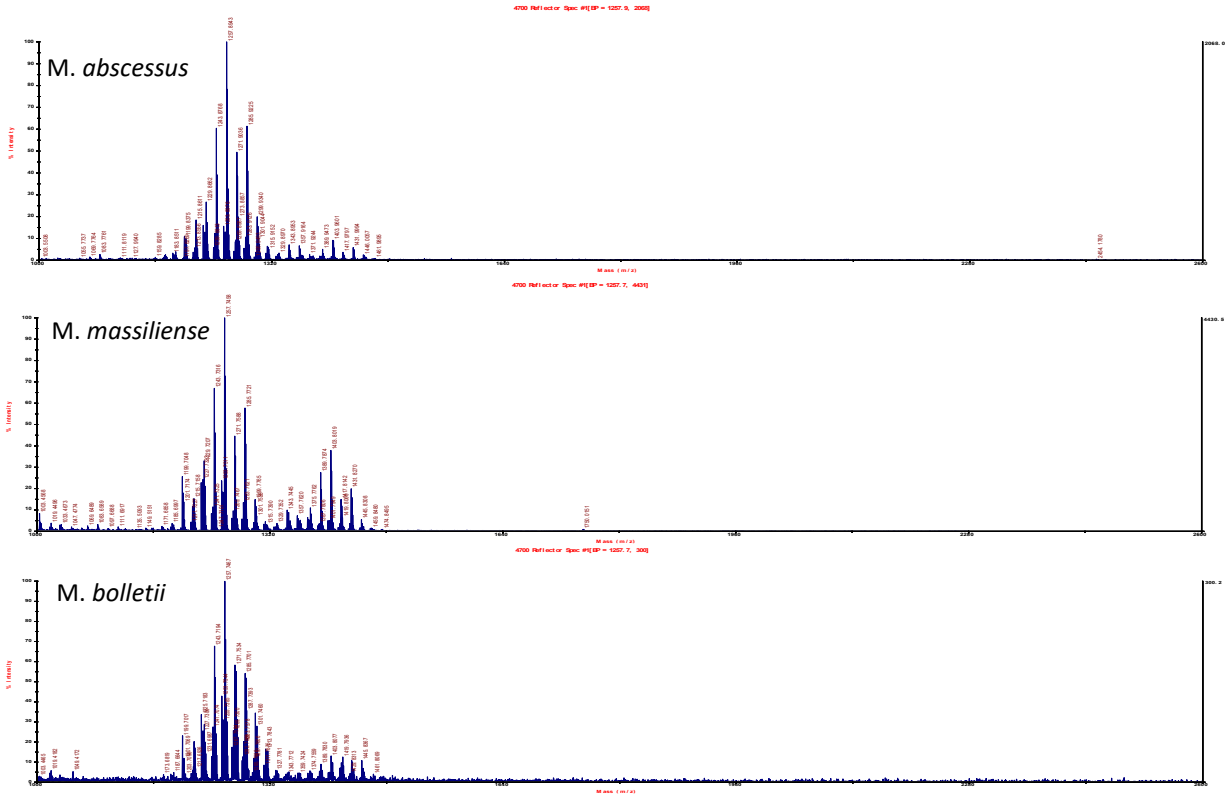
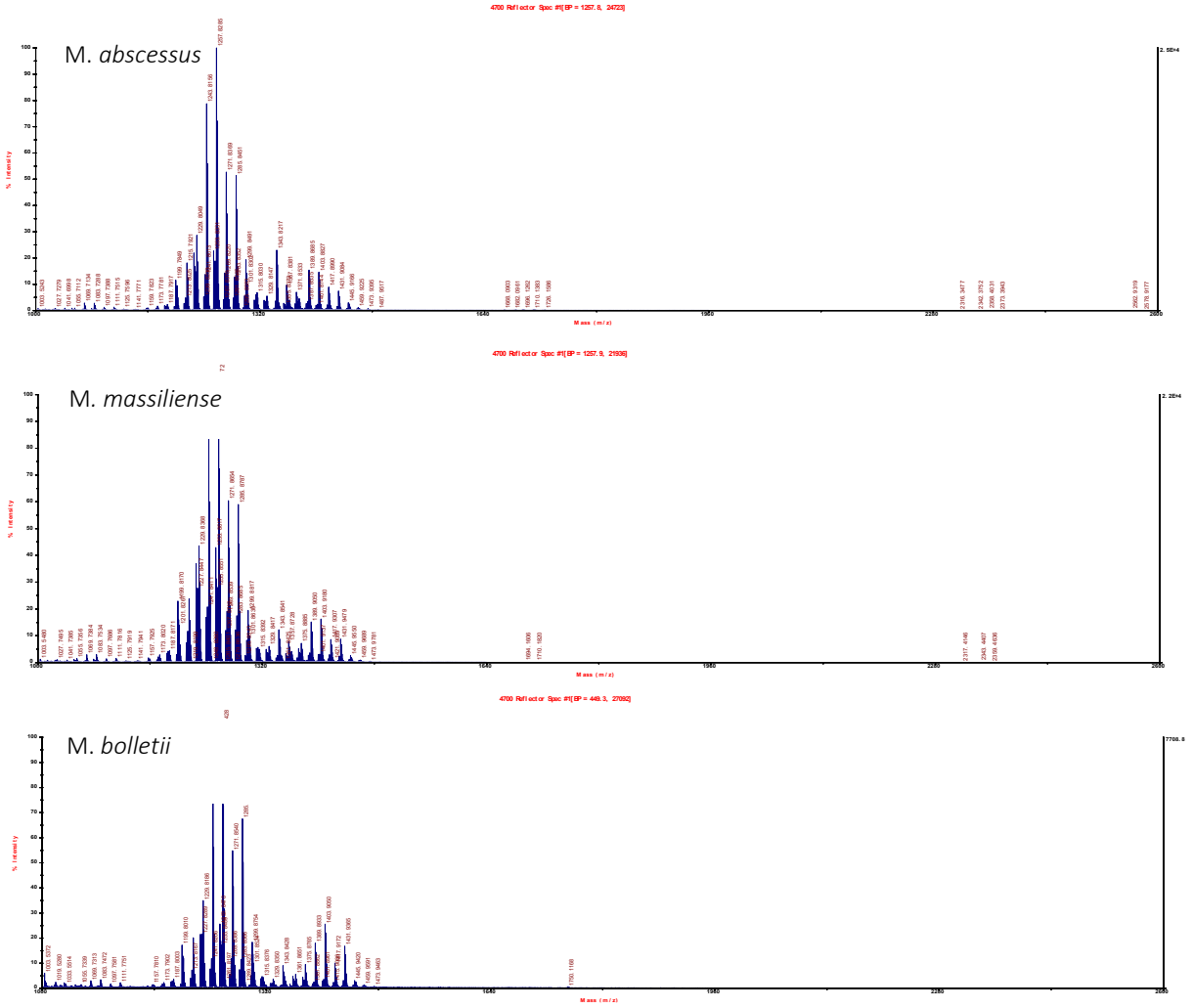


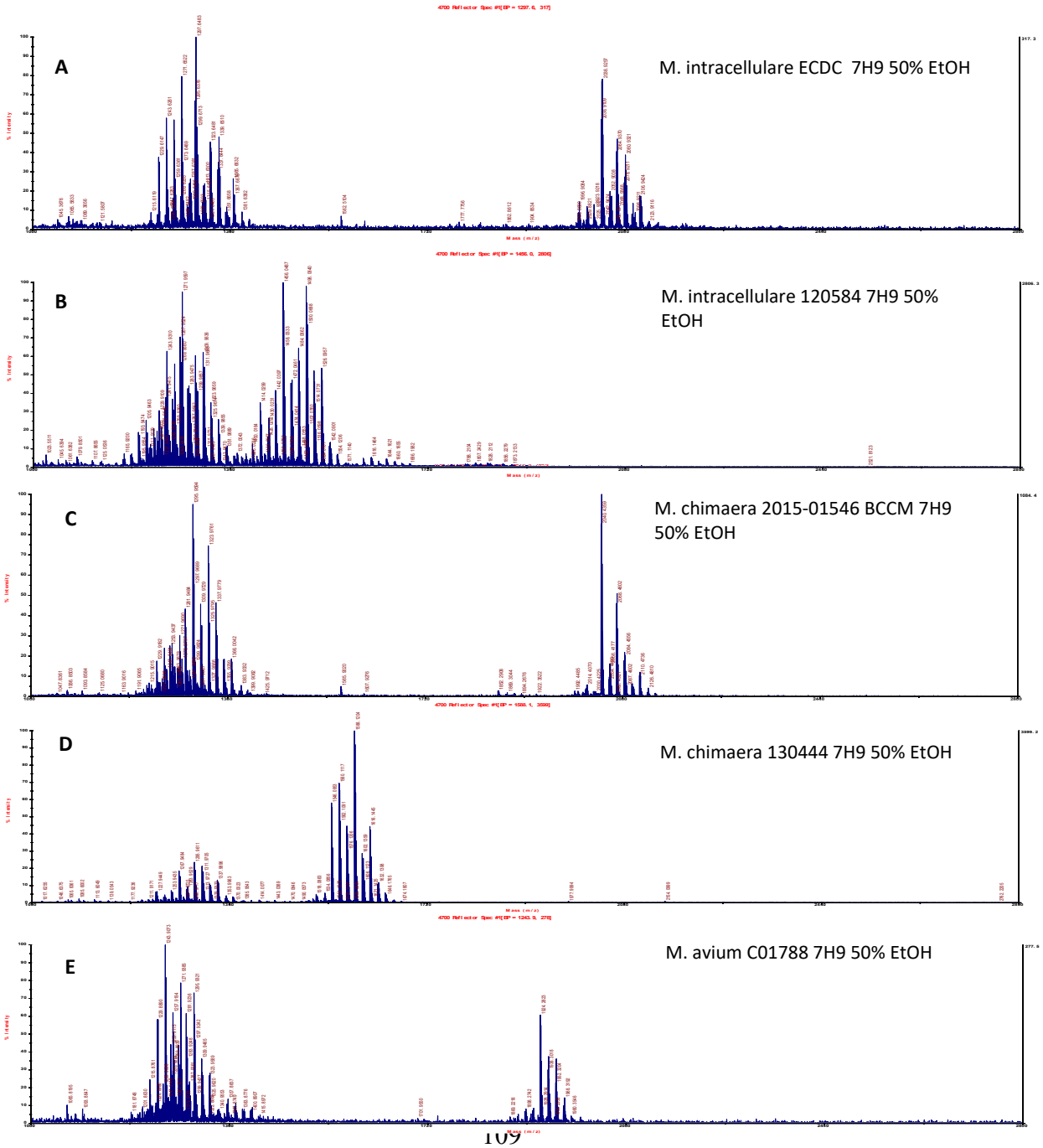
Figure 5.7 – Positive ion MALDI-ToF MS spectra (m/z 1000-2600) of *M. abscessus* (A), *M. masiliense* (B), *M. bolletii* (C). The matrix DHB was diluted at 10mg/mL in solvent C (25% EtOH).



M. *avium* complex comprises several species, three of which are the most clinically relevant, *M. avium, M. intracellulare*, and *M. chimaera*. Commonly used diagnostic methods can easily distinguish between *M*. *avium* and *M*. *intracellulare.* However, *M*. *chimaera* is very difficult to differentiate from *M. intracellulare*. Visual analysis of the MALDI-ToF MS mass spectra of the MAC reference strains showed characteristic peaks for all of the subspecies (Figure 5.8). In addition, a set of peaks present in *M*. *intracellulare* from 1443 m/z to 1569 m/z with the most intense peaks at 1471 m/z, 1499 m/z and 1513 m/z were absent in both strains of *M*. *chimaera* isolates. The two *M*. *chimaera* isolates were visually different: the *M*. *chimaera* 20215-01546 had additional peaks at 2040-2068-2096 m/z and the *M*. *chimaera* 130444 at 1546-1560-1588-1602-1616 m/z. They could potentially be specific peaks allowing differentiation from *M*. *intracellulare*.

Figure 5.8 – MAC strains lipid mass spectra on MALDI-TOF MS A) *M. intracellulare* sensu stricto form ECDC reference strains collection. This strain was WGS to confirm the ID. B) *M. intracellulare* 120584 from BCCM (Antwerp) reference strains collection. This strain originally is from Bangladesh. The ID was only confirmed by 16s rRNA C) *M. chimaera* 2015-01546 reference strain from BCCM (Antwerp) collection and D) *M. chimaera* 130444 reference strain from BCCM (Antwerp) collection. Those strains are both African origin and were 16s rRNA sequenced (awaiting further id) E) *M. avium* C01788 reference strain from BCCM (Antwerp) serotype 16, the origin unknown.

All strains were culture on 7H9 medium with OADC supplement and tested on DHB matrix diluted in 50% EtOH.



Culture media. The mass spectral differences from mycobacteria grown on different media (Middlebrook 7H11, Middlebrook 7H9, and Kirchner) were very minor and the quality of peaks depended on species rather than the media. However, among the media used the best quality of biomass was generated by Middlebrook 7H11 agar. **Matrices.** 2,5-DiHydroxyBenzoic acid solubilized in solvents A, B, C or D. For all strains, matrix solvents B and C performed better than A and D.

5.3.3 Experiment 3: identification of sub-species within *Mycobacterium tuberculosis* complex

The study examined *M. tuberculosis* complex isolates' lipidomic profile by MALDI-ToF mass spectrometry. Ten isolates, 8 *M. tuberculosis* isolates and 2 *M. bovis* BCG, could not be recovered for growth and so, a total of 90 out of 100 isolates were tested. The mass spectra were acquired in the positive ion mode. A combination of molecular line probe assay identification (Hain, Nehren, Germany) and whole genome sequencing was used to identify the reference cultures at the SMRL.

The *M. tuberculosis* mass spectrum was dominated by the set of peaks at *m*/*z* 1376.3 to *m*/*z* 1502.4 assigned to sodium cationized glycerol monomycolates, [GroMM + Na]+ (Layre *et al.*, 2009). The latter lipids are present in other *M. tuberculosis* complex species. In the other *M. tuberculosis* complex strains (*M. africanum*, *M. bovis* BCG, *M. bovis*, *M. caprae*, *M. pinnipedii*), in addition to GroMM, a set of prominent peaks at *m*/*z* 1488.1, *m*/*z* 1530.1 and *m*/*z* 1572.1 was found and assigned to sodium cationized phenolic glycolipids [PGL + Na]+ in which the phenolphthiocerol dimycocerosate aglycone is glycosylated by the 2-0-Me-α-L-Rhap (Daffe *et al.*, 1988, Vercellone and Puzo, 1989).

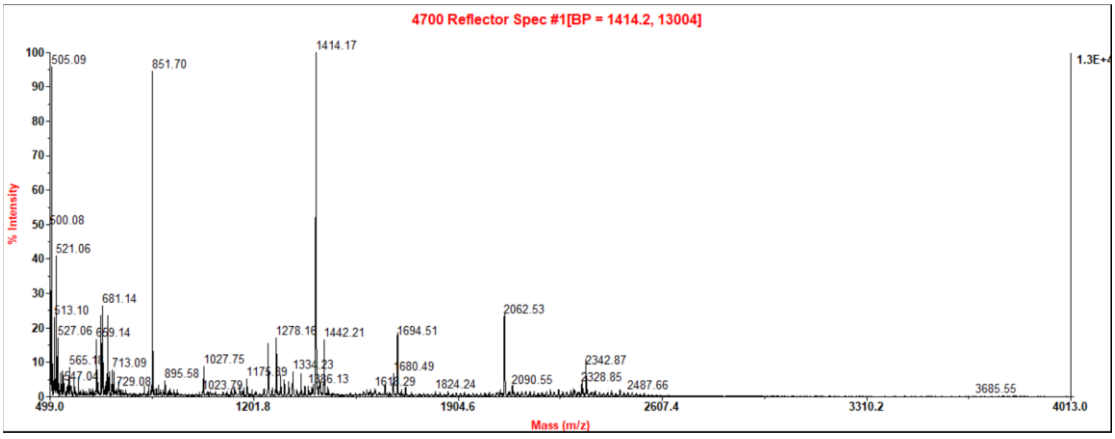
A good mycobacterial mass spectral signal (Resolution > 200 and Signal-to-Noise > 5) in the positive ion mode was generated for the 90 isolates; 4 isolates could not be assigned to either the *M. tuberculosis* or to other members of the *M. tuberculosis* complex group due to a poor

crysalisation on the MALDI-ToF MS target plate possibly due to contamination by non-ionic detergent arising from the culture medium. Out of 86 isolates that generated good spectra, 85 were correctly assigned, based on the positive mode signature mentioned above.

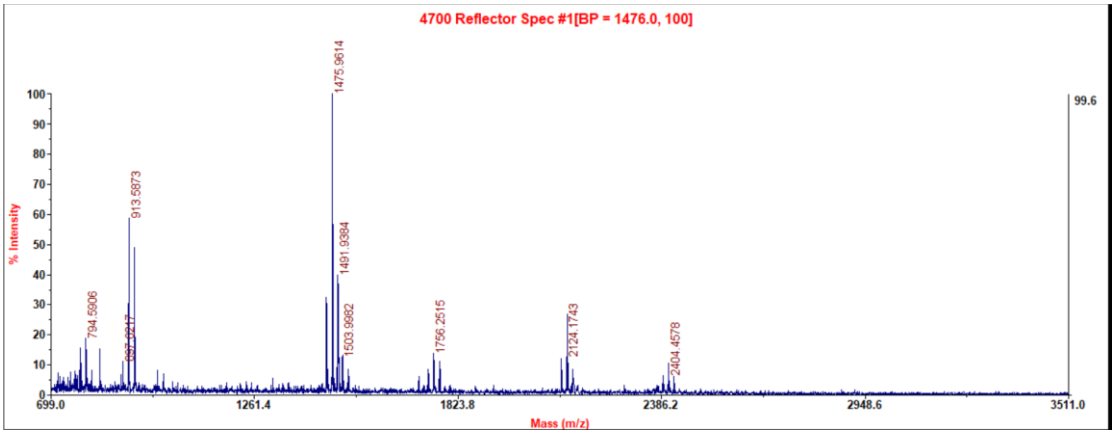
Excluding the 4 uninterpretable results, the sensitivity and specificity of the MALDI-ToF MS, were 100% (95% CI 94.72%-100%) and 94.44% (95% CI 72.71%-99.86%) respectively. Spectra obtained in negative and positive ion mode for M. tuberculosis complex subspecies using the Applied Biosystems 4800 MALDI TOF/TOF™Analyzer can be found in Figure 5.9 A-D. Spectra obtained using the biotyper can be found in Figure 5.10.

Figure 5.9 – Spectra obtained in negative and positive ion mode using the Applied Biosystems 4800 MALDI TOF/TOF™Analyzer

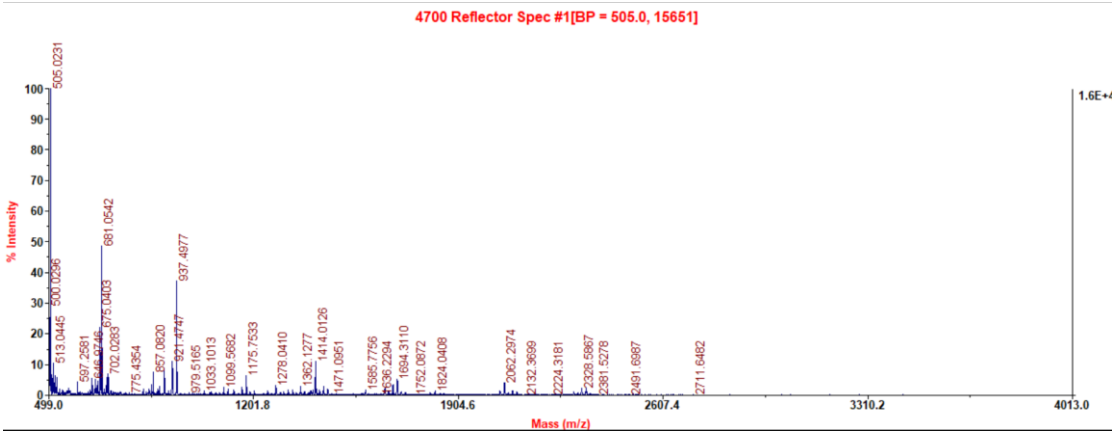
A – *M. tuberculosis* negative ion mode



B – *M. tuberculosis* positive ion mode



C – Other *M. tuberculosis* complex members negative ion mode



D – Other M. tuberculosis complex members positive ion mode

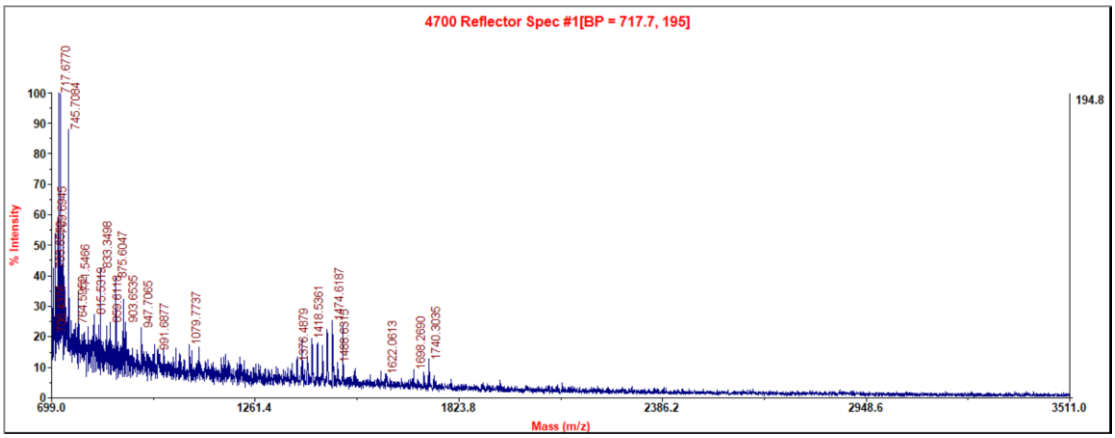
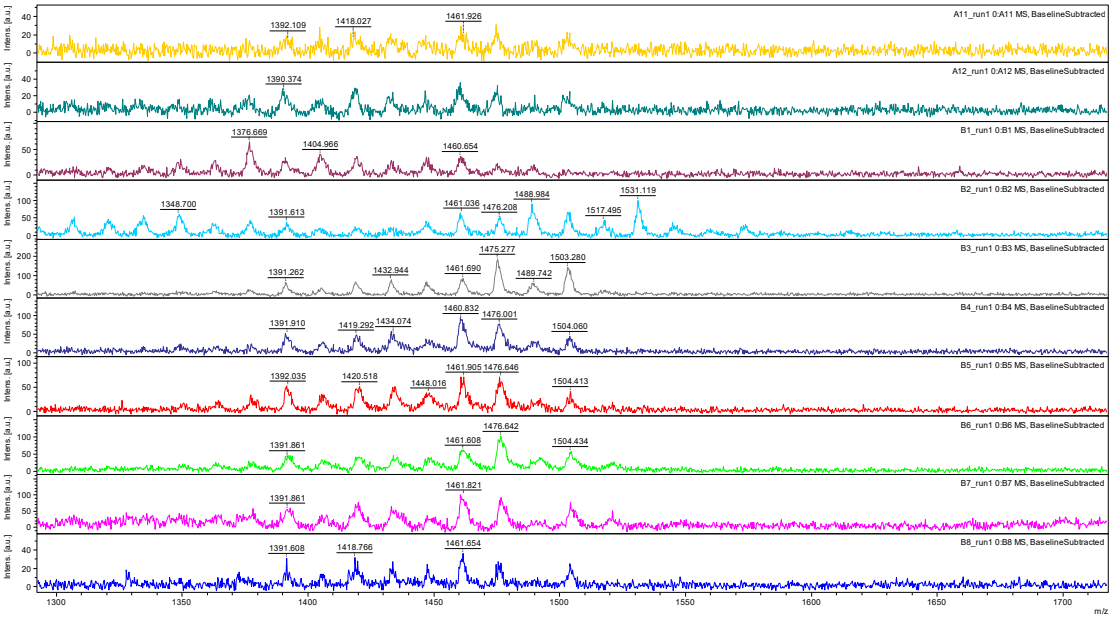


Figure 5.10 – Spectra obtained with the Bruker biotyper. All MTB Sensu Strictu except B2, that is a different member of MTBC



Compared to current MALDI-ToF proteomic approaches which requires several extraction steps and incubations for mycobacterial disruption to have access to intracellular proteins (Murugaiyan *et al.*, 2018a, Mediavilla-Gradolph *et al.*, 2015, Cao *et al.*, 2018, Shitikov *et al.*, 2012, Rodriguez-Sanchez *et al.*, 2016), the lipidomic approach, allows identification of surface exposed species-specific lipids, in a simpler two-steps process. This makes workflow in the clinical laboratory very straightforward.

Using the lipidomic approach, MALDI-ToF MS generated high-quality interpretable spectra for 86% of the cultures tested. Excellent results were obtained for differentiation between *M. tuberculosis* and NTM (235/273), with a sensitivity (96.7%; 204/211) very similar to the quickest proteomic-based approaches on MALDI-ToF MS, correct species/complex level identification was 91.7% with an additional 4.4% to genus level (Rotcheewaphan *et al.*, 2019), but with the advantage of a single processing step after heat inactivation. This method also generated a high-quality interpretable spectrum in 95% (86/90) of instances by MALDI-ToF MS for the subspeciation within members of the *M. tuberculosis* complex.

The samples were also heat-treated pre-analysis rendering the *M. tuberculosis* cultures safe for analysis.

Phosphatidylinositol mannosides (PIMs), sulfolipids (SL-I) and NTMs species specific lipids were easily recognized visually using this method, allowing for genus(Rahlwes *et al.*, 2019) and *M. tuberculosis* complex identification respectively. When the spectra were poor quality, non- genus level identification was still possible. Failure to produce a good quality signal might be due to poor sample quality. The overall sensitivity and specificity for isolates for which a spectrum was identifiable was 96.7% (204/211) and 91.7% (22/24), respectively. The sensitivity and specificity for isolates for which a spectrum was identifiable in the *M. tuberculosis* complex subspecies experiment was 98.5% (68/69) and 100% (17/17), respectively. These are higher than those found when using a protein based approach (Cao *et al.*, 2018, Huang *et al.*, 2018, Alcaide *et al.*, 2018b, Quinlan *et al.*, 2015a, Rodriguez-Temporal *et al.*, 2018), suggesting that the lipid based approach is a very promising tool for the identification of mycobacteria the routine application of the MALDI-ToF MS, especially when using the Bruker Sirius which is the next generation of clinical microbiology systems operating both in positive and negative ion modes (Gonzalo *et al.*, 2021).

However, even though the method was sufficient to discriminate *M. tuberculosis* from *M. canettii* (data not shown) and *M. bovis* BCG, *M*. bovis and *M. africanum* to some extent, it could not achieve discrimination of *M. bovis* BCG and *M.* bovis. This is perhaps not surprising as the vaccine strain is derived from *M. bovis*.

Overall the good discrimination between *M. tuberculosis* and *M. bovis* is probably explained by the evolution of *Mycobacterium tuberculosis* complex i.e. the genetic deletions or rearrangement of genomic regions encoding enzymes involved in the synthesis of surface exposed lipids and glycolipids (Ernst *et al.*, 2007, Supply and Brosch, 2017, Orgeur and Brosch, 2018, Brites and Gagneux, 2017, Bottai *et al.*, 2014, Malaga *et al.*, 2008). Studies have shown that for example, the monoglycosylated PGL formed by *M. bovis* differs from the

triglycosylated PGL synthesized by *M. tuberculosis* (PGL-tb) due to two genetic defects associated with a frameshift mutation within the gene Rv2958c, encoding a glycosyltransferase involved in the transfer of the second rhamnosyl residue of the PGL-tb, and a deletion of a region that encompasses two genes *rv1511* and *rv1512.* These two genes, encode a GDP-D-mannose 4,6-dehydratase and a GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase/reductase, required for the formation of activated L-fucose (Malaga *et al.*, 2008, Perez *et al.*, 2004). Interestingly, such frameshifts have been reported in some *M. africanum* strains (RD7-, RD8-, and RD10-), *M. microti* and *M. pinnipedii* leading to PGLs that are all monoglycosylated mycoside B-like substances (Malaga *et al.*, 2008). Based on the literature(Malaga *et al.*, 2008), regarding the discrepant strain annotated *M. tuberculosis* instead of *M. africanum* (ID 17582E), we can hypothesise that the discrepant *M. africanum* strain does not harbour the frameshift mutation in the *rv2958*c ortholog, which in turn does not lead to mycoside B-like PGL and therefore harbours a similar MS profile as *M. tuberculosis*. In addition, this study does not present results obtained for clinical isolates of *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium orygis* and *Mycobacterium pinnipedii* which are extremely rare in humans. Taken together, the lipid profiles generated fit with the known genetic evolution of the *M. tuberculosis* complex which can be classified into 3 groups: Group I: *M. canettii*, Group II: *M. tuberculsosis* and Group III: *M. africanum*, *M. bovis*, *M. microti*, *M. pinnipedii*, *M. caprae* and *M. bovis* BCG.

In addition to its potential for medical use the methodology is likely to be of value to the veterinary sector, i.e., proof of animal tuberculosis is critical especially if it can be correctly distinguished from vaccine strains (Bacanelli *et al.*, 2019, Filia *et al.*, 2016, Bernitz *et al.*, 2021).

For NTMs, similar issues were found in closely related species. *M. chimaera* and *M. intracellulare* have been very difficult to differentiate in the past by several methodologies, including MALDI-ToF MS based on protein profiles (Lecorche *et al.*, 2018, Pranada *et al.*, 2017). So much so, that it is possible that many of the isolates previously identified as *M.*

intracellulare were in fact *M. chimaera* (unpublished data – personal communication based on observation at NMRL). Here, the method was able to identify *M. chimaera* specific peaks. The inconsistencies in *M. avium* subspecies profiles might be associated to geographical location of origin or horizontal transfer of genes involved in evolution of subspecies creating diverse lipid structures (Ravva *et al.*, 2017, Krzywinska *et al.*, 2004, Uchiya *et al.*, 2017).

M. kansasii had also presented challenges in the past, possibly due to the fact that there are now known to be at least 7 genotypes circulating and not all of them are robustly represented in available databases. Human disease, however, is mainly caused by genotypes I and II (Samli and Ilki, 2016, Girard *et al.*, 2016, Murugaiyan *et al.*, 2018b). The method presented here had less problems with reliable identification of *M. kansasii* and *M. avium* complex. Nevertheless, the lipidomics approach could not separate *M. abscessus* complex subspecies, *absessus, bolletii*, and *massiliense*. This is probably due to the high degree of genetic similarity conferring very limited or no lipidic differences (Yoshida *et al.*, 2018).

M. bohemicum, *M. non-chromogenicum* (except the isolate growing on Kirchner media) and *M*. *simiae* did not grow.

A disadvantage of MALDI-ToF MS that has been highlighted before and was also encountered in NTMs here is that bacteria incubated for longer periods generate better spectra (Buckwalter *et al.*, 2016, Quinlan *et al.*, 2015b). In this study, the best spectra were obtained in stationary growth phase, which means prolonged incubation times, particularly for slow growing mycobacteria. This can be partially overcome by culturing in liquid medium. In this study, all media performed comparably well. Of the two broths used in this study, 7H9 was of particular interest as it is available as part of the commercially and globally available MGIT system (Mycobacterium Growth Indicator Tube, Becton-Dickinson Microbiology Systems, Cockeysville, MD), used in diagnostics laboratories for culture and drug susceptibility testing of mycobacterial species. BacT/Alert® (BioMérieux, Durham, NC), another automated platform for bacterial detection, also uses 7H9 for its system (Quinlan *et al.*, 2015b).

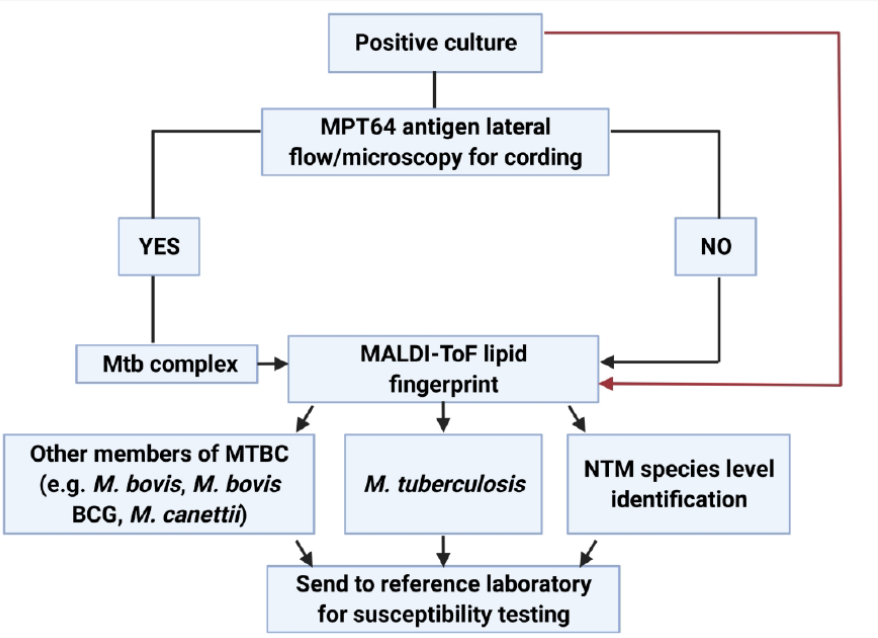
Newer systems such as the Bruker Sirius will become the next generation of clinical microbiology systems operating in positive and negative ion mode. Currently, most clinical laboratories have systems which operate in positive ion mode only. Where this is the case, a combination of inexpensive rapid antigen testing (MPT64) combined with lipidomic MALDI-ToF MS analysis would provide definitive tuberculosis identification (*M. tuberculosis* or other members of the *M. tuberculosis* complex and likely NTM species) rapidly and cheaply in all clinical laboratories and provide species level identification for some NTMs which can have immediate management implications in certain populations, i.e. cystic fibrosis, bronchiectasis, cardiothoracic surgery, immunosuppressed individuals (Furukawa and Flume, 2018, McShane *et al.*, 2013, Johnson and Odell, 2014, Campins Marti *et al.*, 2019, Henkle and Winthrop, 2015).

The lipidomics approach, using the identification of surface exposed species-specific lipids, can discriminate between NTM and some subspecies within the *M. tuberculosis* complex, unlike the current proteomic-based approaches (Murugaiyan *et al.*, 2018a, Mediavilla-Gradolph *et al.*, 2015, Cao *et al.*, 2018, Shitikov *et al.*, 2012, Rodriguez-Sanchez *et al.*, 2016) using a straightforward two-step preparation process.

The MALDI-ToF system is widely available and has a low consumable cost (El-Bouri *et al.*, 2012, Ge *et al.*, 2017, Samli and Ilki, 2016).

A flowchart exploring how this method could be implemented in the laboratory can be found in Figure 5.11.

Figure 5.11 – Proposed flowchart for the implementation of MALDI-ToF MS lipidomic based approach in the routine clinical microbiology laboratory. With modern clinical MALDI-ToF MS systems delivering positive ion mode (e.g., Bruker Biotyper Sirius), an inexpensive, rapid, direct pathway from mycobacterial culture to identification of MTBC sub-species is possible (red arrow). An optional second step that consists of rapid tuberculosis antigen test can still be implemented acting as additional confirmation of *M. tuberculosis* complex. Such workflow is based on the presence and absence of characteristic peaks obtained from the lipid fingerprint by routine MALDI-ToF mass spectrometry in the positive ion mode.



Part of the results presented in this chapter has been published (Gonzalo *et al.*, 2021). A copy of the manuscript can be found in Publications section.

Chapter 6: Urine for the diagnosis of extra-renal tuberculosis using PCR and lipidomics

6.1 Introduction

A fast, reliable, and inexpensive method for the diagnosis of tuberculosis particularly for those patients with extra-pulmonary tuberculosis, children, or populations likely to have paucibacillary infections is desperately needed. If this can be done using equipment already widely available, or as a simple point-of care test it would mean a great step forward in tuberculosis diagnostics.

The aim of the work presented in this chapter was to try to establish a methodology to diagnose pulmonary tuberculosis using urine sample and MALDI-ToF MS, technology already available in many microbiology laboratories worldwide.

The use of PCR for the detection of *M. tuberculosis* trans-renal DNA was also explored.

6.2 Methods

6.2.1 Detection of lipids in urine by MALDI-ToF MS

6.2.1.1 Pilot experiment

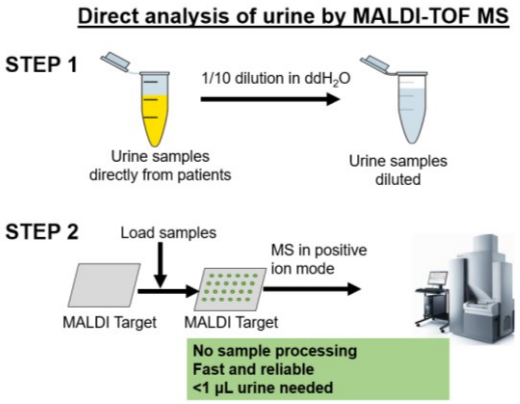
Urine belonging to 97 known tuberculosis patients and 87 healthy controls were included. Samples were processed neat and in 1:10 dilution.

0.4 µL of the sample was placed on the MALDI-ToF MS plate and mixed with 0.8 µL of 10mg DHB matrix diluted in CHCl3:MeOH 98:2.

An Applied Biosystems 4800 MALDI-ToF/TOF™Analyzer (Applied Biosystems/MDS SCIEX MDS Sciex, Concord, Ontario, Canada) was used. Samples were analyzed in the positive and negative ion modes operating at 20kV and were set to acquire mass spectral peaks with

mass/charge ratio (m/z) from 400 to 4000 mass. Mass spectrometry data were analyzed using Data Explorer® Software version 4.9 from Applied Biosystems. A schematic summary of the process can be found in Figure 6.1

Figure 6.1 – Direct analysis of urine by MALDI-ToF MS



6.2.1.2 Experiment 2 – Detection of lipid from spiked urine

In order to improve sensitivity, 6 different solvents were used for lipid extraction from urine detailed in Table 6.1 (Tipthara and Thongboonkerd, 2016), with each different solvent targeting lipids with different polarity in a limited sub-set of the samples mentioned above. Solvents increasing detection of amphipathic lipids were selected (hexane/isopropanol 3 :2 v/v, chloroform and chloroform/methanol 2 :1 v/v).

Table 6.1 – Solvents used for lipid extraction from urine

Protocol	Description	Solvents used
I	C:M 1:1	Chlorophorm/Methanol 1:1 v/v
II	C:M 2:1	Chlorophorm/Methanol 2:1 v/v
III	Hex:IPA 3:1	Hexane/Isopropanolol 3:1 v/v
IV	$CHCl_3$	Chloroform
V	Ether	Diethyl ether
VI	Hexane	Hexane

Urine from a healthy control was spiked with purified mycobacterial lipids (*Mycobacterium tuberculosis* Strain H37Rv total lipids, bei Resources, American Type Culture Collection, USA)

at different concentrations (6mg/mL, 2µg/mL and 0.5ng/mL). Lyophilised lipids were resuspended in 0.6 mL of chloroform and 2.4 mL of urine from a healthy individual was added. Serial dilutions were obtained by adding 0.6 mL of the previous concentration to 2.4 mL of urine (1/5 dilutions).

Samples were then centrifuged at 1,000g and room temperature (RT; set at 25°C) for 5min, the clear supernatant was collected and processed for lipid extraction using the 3 selected lipid extraction protocols and 2 mL of spiked sample for 6 mL of solvent.

6.2.2 Detection of trans-renal DNA for the diagnosis of tuberculosis

6.2.2.1 Experiment 1 – Tuberculosis DNA detection from gDNA spiked urine samples

87 urine samples were be collected from healthy volunteers (recruitment poster available on Appendix III) and treated with 25 mM EDTA. Samples were divided in 5 mL and 30 mL aliquots and spiked with different concentrations of *M. tuberculosis* genomic DNA (gDNA) (10,000-1 pg/µL) to determine the detection limit.

gDNA was prepared as follows. *M. tuberculosis* clinical isolates were cultured from frozen aliquots on Middlebrook 7H11 agar plate supplemented with the 10% OADC (**Oleic Albumin Dextrose Catalase**) and incubated for 4-6 weeks at 37 ºC. Colonies were harvested and aliquoted with 300 µL of TE buffer (10 mM Tris at pH 8.0, 1 mM EDTA) and inactivated by heating at 95 ºC for 30 minutes in containment level 3 (CL3) laboratory. The inactivated cultures were taken into the containment level 2 (BSL2) laboratory for further processing. After lysis by vortexing for 3 minutes with 0.1mm glass beads, gDNA) was purified using a DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) and quantified using NanoDrop One/One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, UK). Table 6.2 shows the final concentration of DNA in spiked samples.

Table 6.2 – Concentrations of gDNA for spiking

	Sample	Spiked H37Rv gDNA	Volume	Extraction method	Final concentration
A1	urine	1 pg/µL	200 µL	chloroform	10 pg/µL
A2	urine	1 pg/µL	200 µL	chloroform	10 pg/µL
B1	urine	0.1 pg/µL	200 µL	chloroform	1 pg/µL
B2	urine	0.1 pg/µL	200 µL	chloroform	1 pg/µL
C1	urine	0.01 pg/µL	200 µL	chloroform	0.1 pg/µL
C2	urine	0.01 pg/µL	200 µL	chloroform	0.1 pg/µL
D1	urine	0.001 pg/µL	200 µL	chloroform	0.01 pg/µL
D2	urine	0.001 pg/µL	200 µL	chloroform	0.01 pg/µL

DNA concentration in spiked urine was done using 2-butanol method, as follows. Equal volume of sec-butanol was added to the spiked urine sample and mixed well by vortexing, then centrifuged for 5 minutes at 1200 × g (2500 rpm), at room temperature. The upper phase containing sec-butanol was discarded. The process was repeated. After that, the lower, aqueous phase was extracted with 25:24:1 phenol/chloroform/isoamyl alcohol and ethanol precipitate. Residual butanol was removed by mixing diethyl ether with an equal volume of water or TE buffer (pH 8) in a polypropylene tube, vortexed vigorously for 10 seconds and let the phases to separate. The top (ether) phase was collected.

Phenol/Chloroform/Isoamyl Alcohol and commercially available kits (Urine DNA Isolation Maxi Kit (Slurry Format), Norgen Biotek, Canada; cfPure® Cell Free Nucleic Acid Extraction kit, BioChain, Newark, USA) were be used to extract the gDNA. M. tuberculosis related signal was detected using PCR.

Real time PCR was performed using iCycler iQ™ Multi-Colour Real Time PCR Detection System (Bio-Rad, California, USA). Each PCR assay is prepared in a 10 µl final volume containing 2x SensiMix II Probe (Bioline Reagents Ltd, London, UK). The primers and probes sequence were the same as used by Barletta and colleagues (Table 6.3) (Barletta *et al.*, 2014). The thermal profile used incorporated: activation of polymerase for 10 minutes at 95 ºC,

followed by 45 amplification cycles of 10 seconds at 95 for denaturation, 60 ºC for 60 seconds for annealing and extension and 4 ºC for hold. The standard curve was prepared using the pure gDNA of H37Rv in concentration 10,000 to 0.001 pq/µL.

Table 6.3 – Primers and Probes used in Real-Time PCR reaction (Barletta *et al.,* 2014)

Name	**Sequence 5' → 3'**	**Final Concentration (nM)**	**Nucleotide Location**	**Sense**	**Band size on agarose gel (bp)**
IS*6110*-probe	TCTCAGTACACATCGATCCGGT	100	1014	+	~120
IS*6110*-forward	AGACGTTATCCACCATAC	400	986	+	
IS*6110*-reverse	AGTGCATTGTCATAGGAG	400	1108	-	
ERV3- probe	CGAACCTGCACCATCAAGTCA	100	972	+	-
ERV3-forward	CCCAAGATAATTTCACACTAA	400	938	+	
ERV3-reverse	GAGCAATACAGAATTTTCCA	400	1042	-	

A PCR reaction was considered positive if IS*6110* or *IS6110* and human endogenous retrovirus group 3 gene (*ERV3*) signals was observed. Reactions only amplifying *ERV3* were considered negative. The product generated during each cycle was directly proportional to the amount of template that was initially present at the start of the PCR. A signal was produced once a fluorescent signal was detected above the threshold cycle (Ct). The real-time PCR products were also analyzed by 1.5% (wt/vol) agarose gel electrophoresis and were visualized using Benchtop UV Transilluminator (BioDoc-iT™ Imaging System). A positive band for IS*6110* at ~120 bp was observed against 1 kB plus DNA ladder (Invitrogen).

6.2.2.2 Experiment 2 – Tuberculosis DNA detection from patient's urine samples

Urine samples from healthy controls and microbiologically confirmed tuberculosis patients were prospectively collected. Healthy controls were collected in the UK, Russia, and

Lithuania. Known tuberculosis patient's samples were collected in Lithuania and Russia and blindly tested at source and in the UK.

Butanol concentration was not performed in these samples as it showed to concentrate contaminants and inhibitors in experiment 1.

DNA extraction was done using the phenol-chloroform method in 400 µL of sample in London and 25 mL of sample in Russia and Lithuania. This discrepancy in volume was associated to local health and safety regulations that would not allow the use of organic solvents in large amounts. For Norgen Urine DNA Maxi, 50 mL of sample were used, as per manufacturer's instructions. BioChain CF DNA extraction kit was validated for 500 µL of sample.

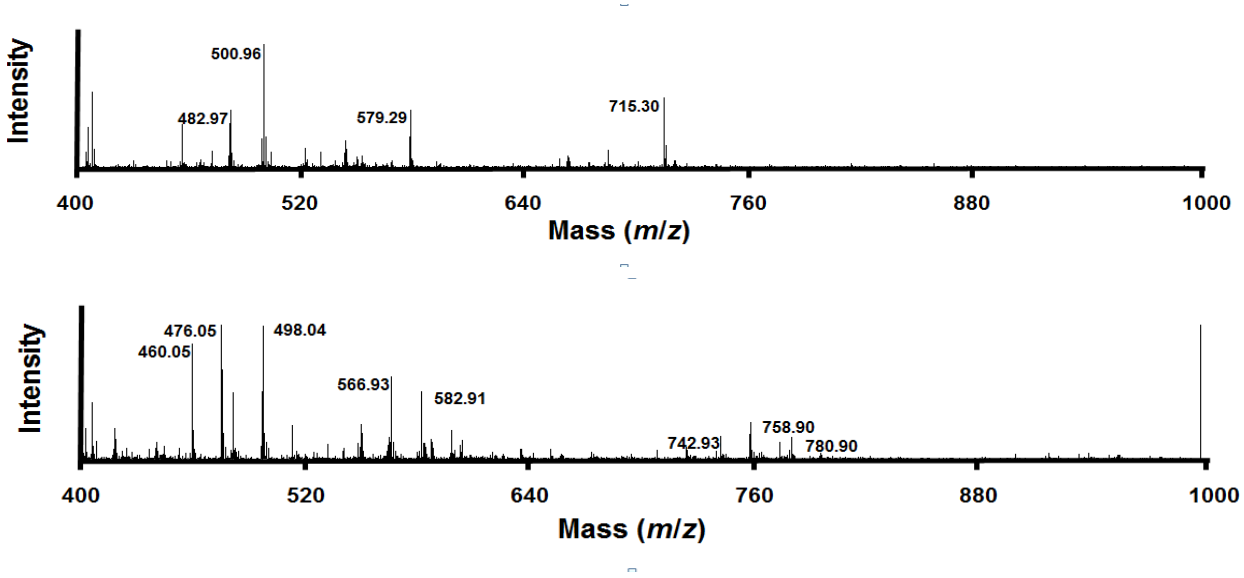
Once extracted, the DNA was amplified using real-time PCR. The selected primer was a 120 base pairs region of IS6110, as described above for experiment 1.(Barletta *et al.*, 2014, Bharj, 2017)

6.3 Results and discussion

6.3.1 Pilot study

Good spectra were obtained from all samples. In Figure 6.2, examples of spectra from healthy volunteers and known tuberculosis patients are shown.

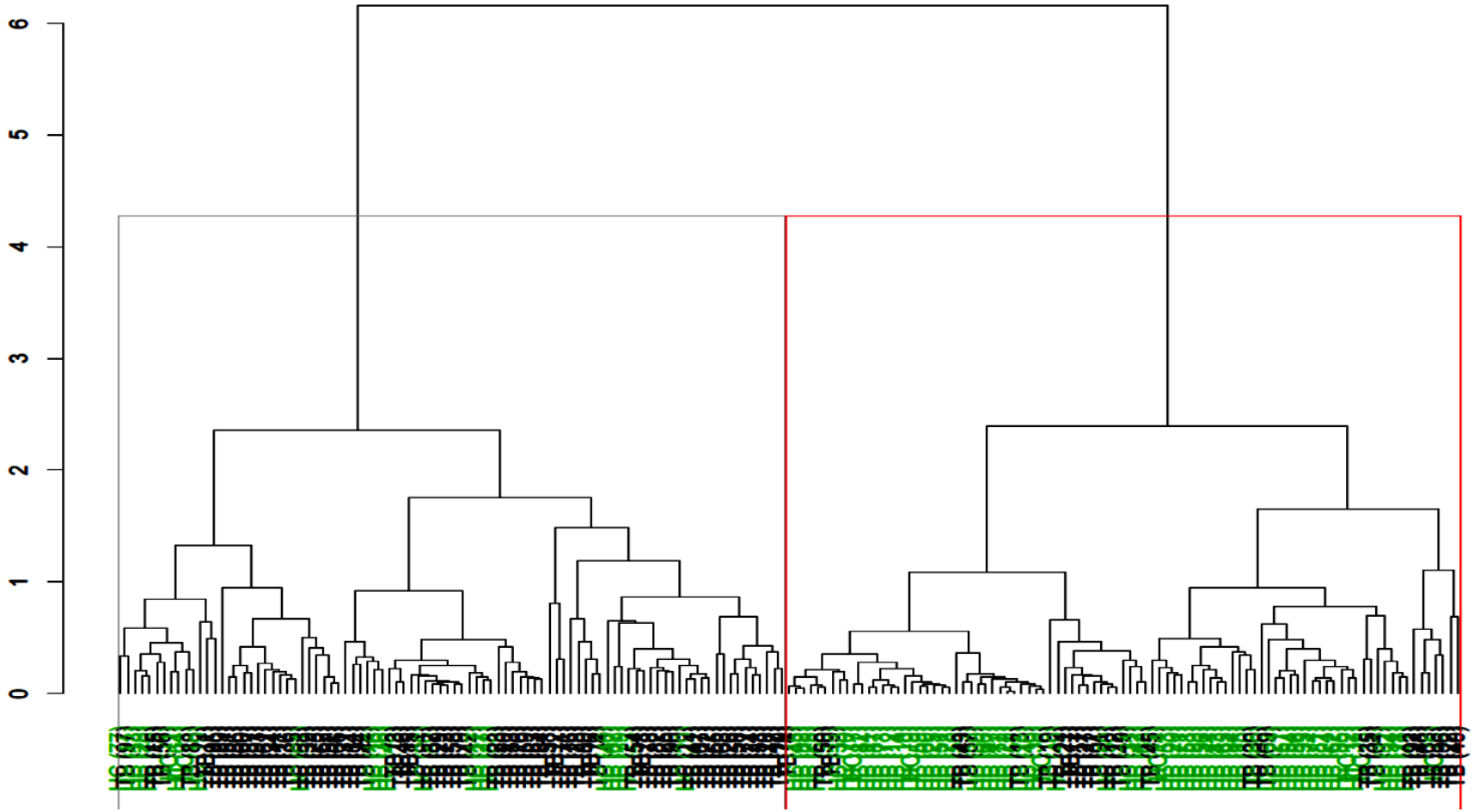
Figure 6.2 – Spectra obtain from healthy volunteers (top spectrum) and tuberculosis patients (bottom spectrum)



Results were forwarded to the mathematics department for blinded analysis to distinguish tuberculosis patients from healthy controls. Cluster analysis is represented in Figure 6.3.

The algorithm correctly identified 71/97 known tuberculosis patients and 66/87 healthy controls, with a sensitivity of 73% and a specificity of 76%, positive predictive value of 77% and negative predictive value of 71%.

Figure 6.3 – Clusters obtained by algorithm - tuberculosis patients (Black) and healthy controls (Green)

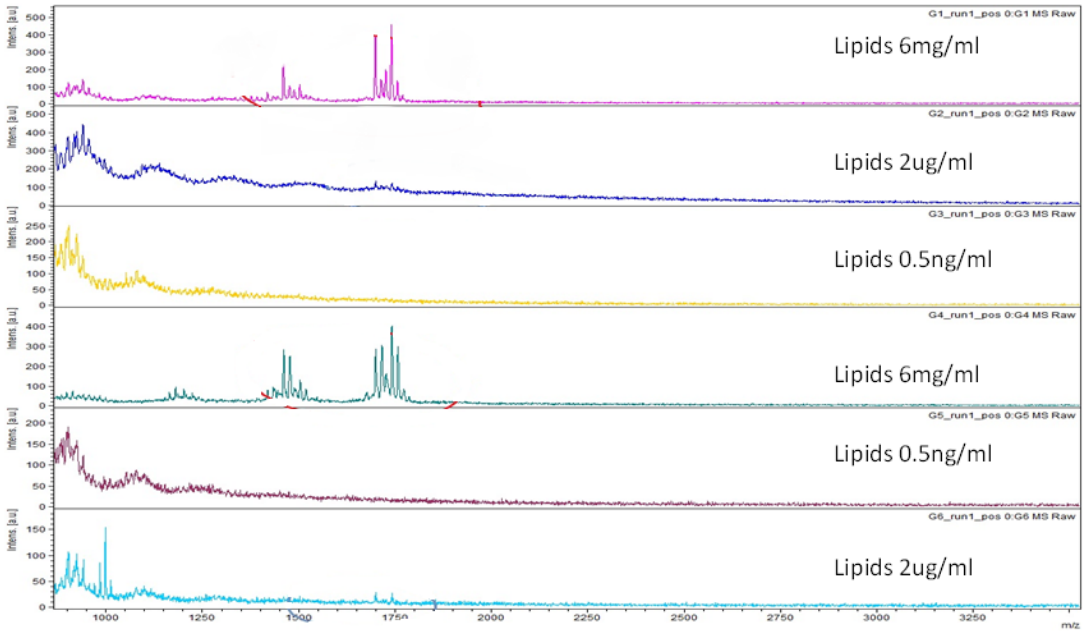


6.3.2 Lipid-Spiked urine

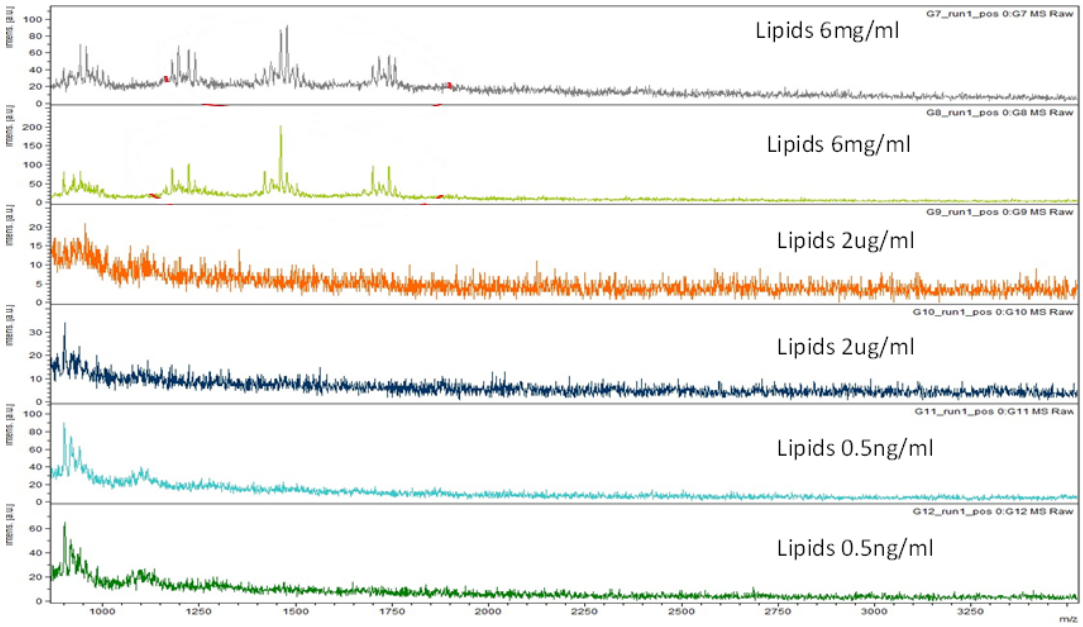
Urine was spiked with different concentrations of purified *M. tuberculosis* lipids and extracted using three different solvents targeting amphipathic compounds. All the 3 methods, chloroform/methanol 2:1 v/v, hexane/isopropanolol 3:2 v/v and chloroform, could detect the highest concentration of lipids (6mg/mL). Only hexane could detect lipids at a low concentration of 2 µg/mL. Lipid's concentration of 0.5 ng/mL was not detectable by any extraction method. Figure 6.4 A-C.

Figure 6.4 – MALDI-ToF MS from spiked urine sample using 3 different solvents for extraction

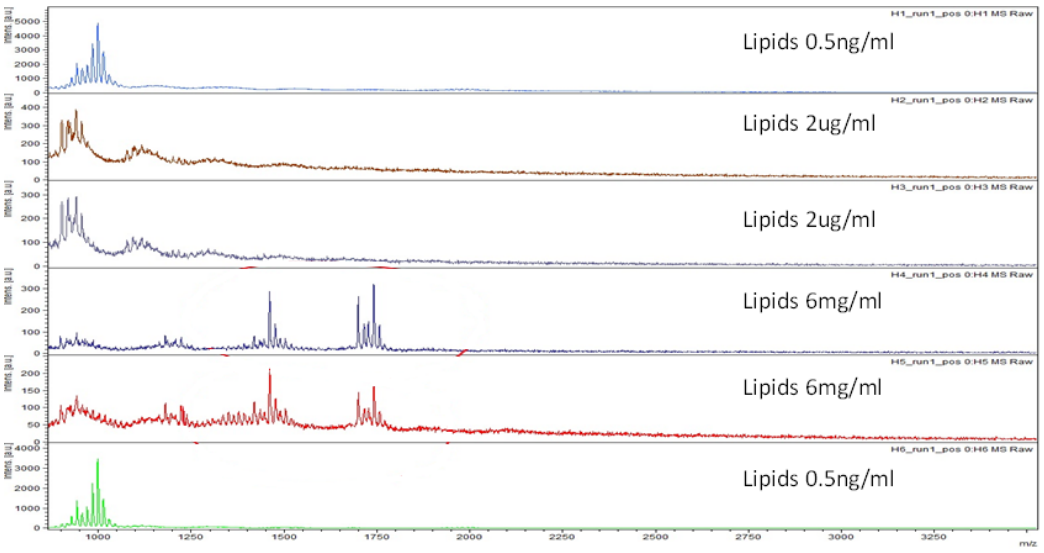
A – Total tuberculosis lipids extraction using Hexane/isopropanol



B – Total tuberculosis lipids extraction using chloroform



C – Total tuberculosis lipids extraction using chloroform/methanol



6.3.3 gDNA-spiked urine

The *IS6110* fragments were detected in all spiked urine samples and qPCR could detect as little as 0.001pg/µl of gDNA with 27-30 cycles (equivalent to 100 bacilli according to (Barletta *et al.*, 2014) qPCR results can be found in Figure 6.5. Agarose gel for the same sample set can be found in Figure 6.6.

Figures 6.5 – qPCR for spiked urines in duplicates

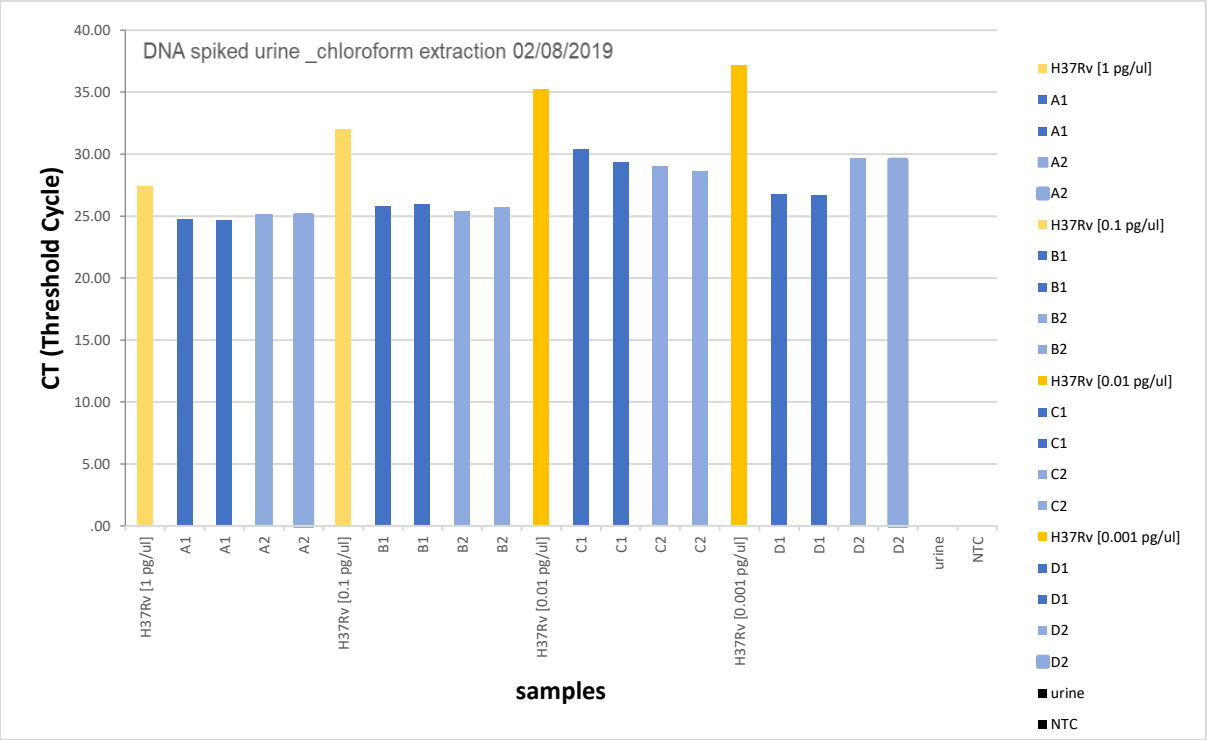
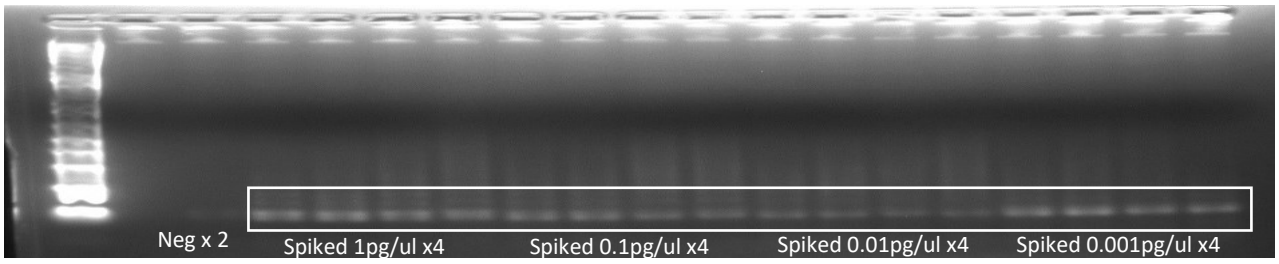


Figure 6.6 – 1.5 % agarose gel, spiked urine. A band can be seen for 120 bp segment of IS6110 (white box)



Results for clinical samples were less successful using all extraction methods.

In the Lithuanian cohort, using phenol-chloroform for extraction, qPCR was positive for 3 out of 13 samples belonging to known tuberculosis patients. When using the Norgen kit, validated

for larger volumes, all 10 samples belonging to known tuberculosis patients were positive. However, all CT values were above 30. When using the BioChain kit, 8/10 samples were positive in duplicate. 2 samples were positive in one copy and negative in the other.

In the Russian cohort of tuberculosis patients and healthy controls, the Norgen Kit allowed detection of all positives. The Russian cohort of samples contained information about smear microscopy. Out of the 19 samples from known positive patients, 14 had a positive result. 5 healthy controls (from the same population, i.e., from the same region in Russia) were all positive as well at least in one of the copies. These results are summarised in the Tables 6.4 to 6.6 below.

Table 6.4 – Lithuanian cohort – phenol chloroform extraction. ID: sample identification; CT: cycle threshold; N/A: no amplification; FAM: Fluorescein amidite; HEX: Hexachloro-fluorescein

Sample	ID	Volume (mL)	DNA concentration	Bacterial load	Chloroform extraction	Final dilution (µL)	Volume in PCR (µL)	CT	
								FAM	HEX
spiked urine	PC	25	1ng/ul		x	150	1	20.54	N/A
negative control	NC	25			x	150	1	N/A	39.5
TB urine	VT029	25		2+	x	150	1	39.01	34.55
TB urine	VT059	25		3+	x	150	1	37.73	36.89
spiked urine	PC	25	2ng/ul		x	150	1	18.2	N/A
negative control	NC	25			x	150	1	N/A	35.46
TB urine	VT013	25		2+	x	150	1	N/A	37.04
TB urine	VT013	25		2+	x	150	1	N/A	35.36
TB urine	VT014	25		3+	x	150	1	33.59	28.09
TB urine	VT014	25		3+	x	150	1	34.59	27.45
TB urine	VT044	25		2+	x	150	1	N/A	31.58
TB urine	VT044	25		2+	x	150	1	N/A	31.62
TB urine	VT045	25		3+	x	150	1	3.9	38.72
TB urine	VT045	25		3+	x	150	1	N/A	38.47
spiked urine	PC	25	1ng/ul		x	150	1	30.35	N/A
negative control	NC	25			x	150	1	N/A	N/A

TB urine	VT052	25		3+	x	150	1	N/A	N/A
TB urine	VT052	25		3+	x	150	1	N/A	N/A
TB urine	VT054	25		2+	x	150	1	N/A	N/A
TB urine	VT054	25		2+	x	150	1	N/A	N/A
TB urine	VT048	25		2+	x	150	1	N/A	N/A
TB urine	VT048	25		2+	x	150	1	N/A	N/A
TB urine	VT037	25		1+	x	150	1	N/A	N/A
TB urine	VT037	25		1+	x	150	1	N/A	N/A
spiked urine	PC	25	1ng/ul		x	150	1	19.59	36.08
negative control	NC	25			x	150	1	N/A	32.44
TB urine	VT052	25		3+	x	150	1	N/A	32.52
TB urine	VT052	25		3+	x	150	1	N/A	31.73
TB urine	VT054	25		2+	x	150	1	35.55	35.72
TB urine	VT054	25		2+	x	150	1	36.88	35.78
TB urine	VT048	25		2+	x	150	1	38.11	30.64
TB urine	VT048	25		2+	x	150	1	35.6	30.51
TB urine	VT037	25		1+	x	150	1	N/A	N/A
TB urine	VT037	25		1+	x	150	1	N/A	36.19

Table 6.5 – Lithuanian cohort – commercial kits. A: NORGEN; B: BioChain C: run controls.

A – Extraction using NORGEN Kit. Ct: cycle threshold.

	Name	**Volume mL**	**Ct**
S1	LTHU1	25	36.72
S1	LTHU1	25	34.86
S2	LTHU2	25	35.91
S2	LTHU2	25	33.81
S3	LTHU3	25	34.93
S3	LTHU3	25	36.1
S4	LTHU4	25	35.71
S4	LTHU4	25	34.27
S5	LTHU5	25	35.93
S5	LTHU5	25	36.05
S6	LTHU6	25	33.83
S6	LTHU6	25	34.11
S7	LTHU7	25	35.8
S7	LTHU7	25	35.59
S8	LTHU8	25	31.17
S8	LTHU8	25	31.9
S9	LTHU9	25	34.64
S9	LTHU9	25	35.62

S10	LTHU10	25	32.01
S10	LTHU10	25	31.82

B – Extraction using BioChain Kit. Ct: cycle threshold.

	Name	Volume mL	Ct
S1	LTHU1	0.5	35.74
S1	LTHU1	0.5	35.98
S2	LTHU2	0.5	35.79
S2	LTHU2	0.5	36.17
S3	LTHU3	0.5	38.09
S3	LTHU3	0.5	36.39
S4	LTHU4	0.5	31.34
S4	LTHU4	0.5	37.73
S5	LTHU5	0.5	35.74
S5	LTHU5	0.5	35.99
S6	LTHU6	0.5	36.95
S6	LTHU6	0.5	37.18
S7	LTHU7	0.5	30.07
S7	LTHU7	0.5	39.79
S8	LTHU8	0.5	36.85
S8	LTHU8	0.5	N/A
S9	LTHU9	0.5	N/A
S9	LTHU9	0.5	36.49
S10	LTHU10	0.5	33.93
S10	LTHU10	0.5	35.6

C – Run controls. NEG HC: negative healthy control; POS SUR; positive spiked urine; UR: urine; N/A: no amplification; Neg Ctrl: negative control; NTC: no template control; Ct: cycle threshold; Pos Ctr: positive control.

	Name	Volume mL	Ct
NEG HC	NEG HC	0.5	N/A
NEG HC	NEG HC	0.5	30.52
POS SUR	Spiked UR	0.5	37.17
POS SUR	Spiked UR	0.5	37.11
Neg Ctrl	Urine HC		N/A
Neg Ctrl	Urine HC		N/A
NTC	NTC		N/A
NTC	NTC		N/A
Pos Ctr	Pos Ctr		12.66
Pos Ctr	Pos Ctr		12.64

Table 6.6 – Russian cohort – Biochain kit. AFB: acid fast bacilli. Ct: cycle threshold. N/A: no amplication.

BioChain					
		Smear for AFB (at the start of treatment)	**Name**	**Volume mL**	**Ct**
A01	RTB50	1+	TB50	0.5	N/A
A02	RTB50			0.5	38.54
A03	RTB62	neg	TB62	0.5	37.42
A04	RTB62			0.5	35.01
A05	RTB74	1+	TB74	0.5	N/A
A06	RTB74			0.5	N/A
A07	std			0.5	7.76
A08	std			0.5	8.45
A09	DNA 0.1pg/ul		POS CTR	0.5	31.83
A10	DNA 0.1pg/ul			0.5	32.70
B01	RTB51	1+	TB51	0.5	33.00
B02	RTB51			0.5	N/A
B03	RTB63	neg	TB63	0.5	N/A
B04	RTB63			0.5	37.08
B05	RTB75	1+	TB75	0.5	N/A
B06	RTB75			0.5	N/A
B07	std			0.5	17.30
B08	std			0.5	17.42
C01	RTB52	neg	TB52	0.5	33.45
C02	RTB52			0.5	32.87
C03	RTB65	neg	TB65	0.5	N/A
C04	RTB65			0.5	36.57
C05	RTB76	neg	TB76	0.5	N/A
C06	RTB76			0.5	N/A
C07	std			0.5	21.37
C08	std			0.5	20.58
D01	RTB53	scanty	TB53	0.5	N/A
D02	RTB53			0.5	34.87
D03	RTB66	neg	TB66	0.5	38.47
D04	RTB66			0.5	36.79
D05	RCH77		HC77	0.5	N/A
D06	RCH77			0.5	37.67
D07	std			0.5	25.89
D08	std			0.5	26.16
E01	RTB54	neg	TB54	0.5	37.73
E02	RTB54			0.5	N/A
E03	RTB67	1+	TB67	0.5	N/A
E04	RTB67			0.5	37.63

E05	RCH78		HC78	0.5	36.72
E06	RCH78			0.5	N/A
E07	std			0.5	29.61
E08	std			0.5	29.50
F01	RTB55		TB55	0.5	36.21
F02	RTB55			0.5	34.20
F03	RTB71	neg	TB71	0.5	33.98
F04	RTB71			0.5	N/A
F05	RCH79		HC79	0.5	36.59
F06	RCH79			0.5	38.51
F07	std			0.5	32.82
F08	std			0.5	33.36
G01	RTB60	scanty	TB60	0.5	N/A
G02	RTB60			0.5	38.16
G03	RTB72	neg	TB72	0.5	N/A
G04	RTB72			0.5	N/A
G05	RCH80		HC80	0.5	38.70
G06	RCH80			0.5	N/A
G07	std			0.5	36.58
G08	std			0.5	36.18
H01	RTB61	neg	TB61	0.5	34.84
H02	RTB61			0.5	N/A
H03	RTB73	1+	TB73	0.5	38.11
H04	RTB73			0.5	38.68
H05	RCH81		HC81	0.5	N/A
H06	RCH81			0.5	38.81
H07	NTC		NTC		N/A
H08	NTC				N/A

The pilot lipidomics study presented here provided some degree of differentiation between known tuberculosis patients and healthy controls albeit not good enough for urine to be recommended for the routine clinical diagnosis of tuberculosis employing this approach.

It is not clear whether the difference in spectra picked up by the algorithm comes from bacterial components present in urine or if it represents the presence of molecules associated to the host's response to the disease. Further spiking experiments with purified lipids may add information on this matter. The preliminary spiking experiment done with whole bacterial extract showed that the current MALDI-ToF MS instruments in use clinically can only detect relatively large concentrations of lipids in urine. In general lipids are present in small amounts

in the urine of healthy mammals.(Ruggiero *et al.*, 2010, Tipthara and Thongboonkerd, 2018, Yuan *et al.*, 2017, Bouatra *et al.*, 2013). There is more optimisation to be done for this method.

Detection of trans-renal DNA correctly identified tuberculosis patients. However, CT values were very often above 30. This may be related to the fact that the target is present in very small amounts, or the primers are binding to other DNA present in the sample, either from other bacteria commonly found in urine (sub clinical, colonisation with increasing age) or trDNA from other bacterial species. So that we minimised this potential problem, the target used was IS6110, a highly specific *M. tuberculosis* insertion sequence/element (Cannas *et al.*, 2008). Unlike previous reports, bacterial load stratified by smear microscopy positivity was not associated with a higher chance of a positive PCR result (Marangu *et al.*, 2015).

The experiments presented here exhibited several problems. The most striking is the positivity of 5/5 healthy controls in the Russian experiment using the BioChain kit. It is unclear why this occurred. Contamination is a possibility in the laboratory or at source, albeit not very likely as all negative controls were appropriately negative for that run. Another option is that these patients had tuberculosis (either active or latent) as they were from a region with high incidence of tuberculosis, so trDNA was present in their urine (Meshkov *et al.*, 2019).

Previous reports on trDNA for the diagnosis of tuberculosis used shorter sequences than the one used in this experiment. However, Cannas *et al.* used semi-nested PCR with the first reaction amplifying a region of very similar length to the one used here, 129 bp and 123 bp respectively (Cannas *et al.*, 2008).

Concentration of urine was not as good as approach as expected as it concentrated substances that acted as inhibitors of the PCR reaction (Khan *et al.*, 1991). There were reports of concentration working for the better using GeneXpert in HIV population (the more advanced the HIV, the better the Cepheid performed) (Peter *et al.*, 2012). A study from the late 1990s reported the same. Interestingly, in this cohort 25/80 healthy controls had a positive PPD (Aceti *et al.*, 1999). Even in the HIV negative population, undetected renal involvement was detected

in over 25% of the cases (Gopinath and Singh, 2009). In other reports, sensitivity of GeneXpert in HIV negative population for the diagnosis of tuberculosis using urine was 3.8% (Shenai *et al.*, 2013).

The fact that urine as a sample performs better, either for detection of DNA or LAM, in the HIV positive population with advanced disease suggests that there is either dissemination of tuberculosis with renal involvement or substances that should not be there are filtered due to HIV associated nephropathy (Lawn *et al.*, 2012b, Lawn, 2012a).

Until these issues are clarified, detection of trDNA and detection of lipids by MALDI-ToF MS cannot be used reliably in a clinical setting for the diagnosis of TB. The numbers tested were too small to draw final conclusions about the sensitivity, specificity, positive and negative predictive values for the PCR. Due to the significant technical issues encountered, it is unlikely that these methods can be easily applied in a clinical microbiology laboratory without changes to improve performance.

Chapter 7: Conclusion

Some of the problems associated with drug susceptibility testing discussed at the start of my PhD programme and this thesis, i.e., true rifampicin resistance missed by the MGIT system, have been overcome by the use of molecular DNA based testing and more particularly by the move to the use of whole genome sequencing (WGS) for *M. tuberculosis* identification and antimicrobial resistance testing in the UK and a small number of mainly high-income countries elsewhere. In the UK this has led to the use of WGS for the identification of all *M. tuberculosis* strains and identification of resistance by the identification of key mutations associated with resistance in target genes. In practice a relatively small number of genes are evaluated for drug resistance with the remaining sequence used primarily to map person-to person transmission for tuberculosis (i.e., for public health control). The latter use replaced VNTR-MIRU and other techniques due to the improved resolution provided by a WGS approach (Wyllie *et al.*, 2018).

However, the use of WGS in the UK and elsewhere for clinical AMR is largely a targeted one focusing on key genes known or highly suspected of being associated with drug resistance. Other mutations either have no effect on the MIC of the antibiotic inhibiting the strain, or the mutational effect is unknown. The WHO Drug Resistance Mutation catalogue encapsulated this by listing mutations with varying certainty of effect on resistance. This offers commercial e.g., DEEPLEX or non-commercial users a basis for interpretation.

Three UK studies largely established the value and ability of WGS to replace phenotypic microbiological susceptibility testing to first line drugs in the UK: Pankhurst *et al,* and two studies by Walker *et al.* (Walker *et al.*, 2015, Allix-Béguec *et al.*, 2018, Pankhurst *et al.*, 2016).

In the first, a comparison of blinded in silico analysis of genotype mutation was made against clinical TB strains which had already had phenotypic sensitivity performed.

Pankhurst *et al.* led a prospective study of analysis at 3 laboratory sites comparing results produced (including turnaround time and cost) with the Reference Laboratory analysis.

Finally a global consortium CRyPTIC developed a robust catalogue of mutations associated with first line resistance in parallel with that conducted elsewhere leading ultimately to the WHO catalogue (World Health Organization, 2021a).

Since the UK has switched to an all mutational analysis for first line drugs performance has been very good but a number of issues have been revealed-for example, turnaround time is slower when conducted operationally than when conducted by research staff (7-13 days); tuberculosis identification comes with the drug resistance so it is now delayed compared to prior molecular identification tests and this can result in longer periods of isolation, delays in contact tracing or inappropriate therapy. New mutations in these genes need phenotypic analysis to characterise the mutational effect (if any). So, we are slightly locked in a tautological argument—why do we need WGS analysis? We need WGS because phenoptypic analysis is unreliable and slow. How do we know WGS identified mutations do/do not have a physiological effect? Because of the excellent phenotypic testing which informs us of the effect. With the exception of fluoroquinolones and aminoglycosides, mutation analysis is still not reliable enough for all drugs especially the newer ones e.g., bedaquiline, delamanid, pretomanid. Even for well-established second line drugs e.g. cycloserine, clofazimine, linezolid, testing is not always straightforward as recent assessments have shown (Farooq *et al.*, 2021).

I was part of the seminal projects that produced the evidence to introduce WGS for susceptibility testing and which recommended stopping routine phenotypic testing for these drugs (Allix-Béguec *et al.*, 2018, Pankhurst *et al.*, 2016) (copy of the article can be found in the Publications section). I was involved in the provision of phenotypic data, validation of comparative UK data, writing the standard operating procedure for DST and training technical staff to set up and read commercially available microtiter plates for slow growing mycobacteria. This included MGIT-based phenotypic test methodology which was used to pair with genotypic results. This gave me the opportunity to explore the technical issues and advantages of this methodological approach. For first line drugs, as reported, results were very good.

However, for second line drugs and new agents, the need for high quality phenotypic microbiology remains and, without a good phenotypic methodology or clinical correlates, genomic information remains difficult to interpret. It is also expensive and requires extensive infrastructure and bioinformatics support (although new technology such as the Oxford Nanopore Minion may reduce the need for complex bioinformatics). Similarly commercial systems such as DEEPLEX have become relatively bioinformatic-free for clinical microbiological use.

The other critical issue mentioned earlier and associated with current routinely available phenotypic tests of susceptibility is that they fail to incorporate antibiotic PK/PD information and host-related response. The latter can be overcome by animal models or, with less cruelty and cost, cheaper and more physiological assays, can be developed and utilised. I focused on one such assay: the 3-dimensional model used in Chapter 4.

The PK/PD aspects of DST remain difficult to incorporate as most models in the literature involve adding antibiotics to the ongoing culture medium, an approach that has several problems when dealing with *M. tuberculosis*. The potential risk of systematic exposure of the operator to M/XDRTB is the most salient one as well as technical issues around drug stability, protein binding etc. In reality every model will be artificial to a degree and the key question is how far from reality can a model system be and still give clinically useful results. For example, drugs are tested singly usually but always given in combination. No drug should be used clinically without ensuring that it is at least not inhibitory when used with other drugs in the regimen.

The microdilution method was relatively easy to set up, cheap and adaptable to new or repurposed compounds and offered a good first step in evaluating compounds either alone or in combination.

As was mentioned earlier, in low AMR resistance countries like the UK, the key clinical information is whether the mycobacteria seen or cultured from patient samples is *M. tuberculosis* or NTM. This information when timely stops inappropriate and costly isolation and other unnecessary public health action. If *M. tuberculosis*, then rapid knowledge of rifampicin and isoniazid resistance is arguably the only further information needed quickly. As the UK and many other high-income countries employ a system where cultures are submitted to a regional or national centre with the delays indicated, we sought to explore methodologies appropriate to general clinical microbiological laboratories for mycobacterial identification.

The current UK WGS strategy produces a delay in identification to species level, when compared to previously available molecular methodologies such as line probe assays. This delay is not insignificant (around a week) but can be partially compensated for by detection of cording in microscopy of cultures or the use of immunochromatographic assay for the

detection of MPT64 antigen. However, these simple tests are not widely used in UK clinical laboratories.

We managed to develop an effective approach for the identification of *M. tuberculosis* and selected subspecies of the *M. tuberculosis* complex using equipment already available in the routine clinical microbiology laboratory. The lipidomic based approach presented here could be implemented rapidly, saving time in the identification of key mycobacteria.

Urine for the diagnosis of pulmonary tuberculosis failed to deliver results that would allow us to translate the method to routine use based on the current approaches we explored. There are some technical issues to address to improve performance before urine can be recommended for routine use as a diagnostic sample, except in patients with HIV and low CD4+ count for whom LAM detection in urine has been endorsed by WHO. Enhanced LAM based assays and host proteomic and metabolomic approaches are increasingly showing promise.

Over the next decade we anticipate the development and implementation of a suite of diagnostics, some focused on high-throughput laboratory approaches, others to a point-of-care approach with a tailoring dependent on the populations served, the prevalence of TB and drug resistance and the health economic position of a given region or country.

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Original article

True rifampicin resistance missed by the MGIT: prevalence of this pheno/genotype in the UK and Ireland after 18 month surveillance

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ABSTRACT

Objectives: To characterize rifampicin-resistant strains missed by the Mycobacteria Growth Indicator Tube (MGIT) 960 system but not by egg-based media in the UK and Ireland and to ascertain their prevalence.

Methods: All strains sent for second-line susceptibility testing were prospectively collected. Drug Susceptibility Testing was performed by Resistance Ratio (RR), Proportion Method (PM), MGIT 960 and MIC determination by microdilution. Rifampicin-resistance-conferring mutations were detected with line probe assays and sequencing. At the end of the study period, retrospective archived strains from 2010 to 2014 showing key mutations were analysed phenotypically and genotypically.

Results: Seventeen of 7234 prospective isolates were included. All of them were susceptible by MGIT. One was borderline by RR (MIC to rifampicin of 4 mg/L) and was resistant by PM. Eight were resistant and eight were highly resistant on RR. These 16 isolates had MICs between 1 and 8 mg/L on microdilution. With PM, 16/17 were susceptible to rifampicin. 17/17 had mutations in the *rpoB* gene. D516Y was the mutation most frequently found (13/17). Retrospectively, ten additional strains with key genotypes were found in our collection: 6/10 were susceptible in the MGIT and resistant in RR. Of the 27 studied strains, the MGIT only detected resistance in four.

Conclusions: Rifampicin resistance is missed by the MGIT system. In the UK and Ireland the prevalence of these strains is low. The introduction of routine molecular testing would detect false susceptibility. Further research is needed to ascertain the role of these strains in clinical failure and their prevalence in other settings. **X. Gonzalo, Clin Microbiol Infect 2017;23:260**

Introduction

Rifampicin is the backbone of anti-tuberculosis treatment and resistance in *Mycobacterium tuberculosis* is associated with increased mortality [1]. Accurately determining susceptibility is of paramount importance, not only to establish the treatment regimen and duration but also to improve cure rates and survival [2,3].

Currently, a range of genotypic and phenotypic susceptibility methods exist to detect rifampicin resistance. Even though molecular detection of rifampicin resistance is recommended, not all laboratories in the UK and Ireland use them on all *M. tuberculosis* cases or have them among their locally available methods. In the UK National Mycobacterium Reference Laboratory (NMRL), molecular detection of rifampicin resistance is available but it is not performed universally; it is done on request or upon detection of either risk factors for drug resistance or phenotypic resistance. At the Scottish Mycobacteria Reference Laboratory (SMRL) direct molecular testing for rifampicin and usually isoniazid is carried out on specimens that are *M. tuberculosis* complex quantitative PCR first-film-positive respiratory specimens and on request [4]. Similarly

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Prediction of Susceptibility to First-Line Tuberculosis Drugs by DNA Sequencing

The CRyPTIC Consortium and the 100,000 Genomes Project

ABSTRACT

BACKGROUND

The World Health Organization recommends drug-susceptibility testing of *Mycobacterium tuberculosis* complex for all patients with tuberculosis to guide treatment decisions and improve outcomes. Whether DNA sequencing can be used to accurately predict profiles of susceptibility to first-line antituberculosis drugs has not been clear.

METHODS

We obtained whole-genome sequences and associated phenotypes of resistance or susceptibility to the first-line antituberculosis drugs isoniazid, rifampin, ethambutol, and pyrazinamide for isolates from 16 countries across six continents. For each isolate, mutations associated with drug resistance and drug susceptibility were identified across nine genes, and individual phenotypes were predicted unless mutations of unknown association were also present. To identify how whole-genome sequencing might direct first-line drug therapy, complete susceptibility profiles were predicted. These profiles were predicted to be susceptible to all four drugs (i.e., pansusceptible) if they were predicted to be susceptible to isoniazid and to the other drugs or if they contained mutations of unknown association in genes that affect susceptibility to the other drugs. We simulated the way in which the negative predictive value changed with the prevalence of drug resistance.

RESULTS

A total of 10,209 isolates were analyzed. The largest proportion of phenotypes was predicted for rifampin (9660 [95.4%] of 10,130) and the smallest was predicted for ethambutol (8794 [89.8%] of 9794). Resistance to isoniazid, rifampin, ethambutol, and pyrazinamide was correctly predicted with 97.1%, 97.5%, 94.6%, and 91.3% sensitivity, respectively, and susceptibility to these drugs was correctly predicted with 99.0%, 98.8%, 93.6%, and 96.8% specificity. Of the 7516 isolates with complete phenotypic drug-susceptibility profiles, 5865 (78.0%) had complete genotypic predictions, among which 5250 profiles (89.5%) were correctly predicted. Among the 4037 phenotypic profiles that were predicted to be pansusceptible, 3952 (97.9%) were correctly predicted.

CONCLUSIONS

Genotypic predictions of the susceptibility of *M. tuberculosis* to first-line drugs were found to be correlated with phenotypic susceptibility to these drugs. (Funded by the Bill and Melinda Gates Foundation and others.)

The members of the writing group (Timothy M. Walker, D.Phil., A. Sarah Walker, Ph.D., and Tim E.A. Peto, D.Phil.) assume responsibility for the overall content and integrity of this article. The authors' full names and academic degrees are listed in the Appendix. The authors' affiliations are listed in the Supplementary Appendix, available at NEJM.org. Address reprint requests to Dr. Timothy Walker at the Department of Microbiology, Level 7, John Radcliffe Hospital, Headley Way, Headington, Oxford, OX3 9DU, United Kingdom, or at timothy.walker@ndm.ox.ac.uk.

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RESEARCH ARTICLE Open Access

Ertapenem and Faropenem against *Mycobacterium tuberculosis*: in vitro testing and comparison by macro and microdilution

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Abstract

Background: Interest in carbapenems has been rising in the last few years due to the emergence of drug resistant tuberculosis. Ertapenem (ETP), given once a day parenteral, and faropenem (FAR), oral, have a better administration profile than meropenem (MEM), imipenem (IPM) and doripenem (DOR). The addition of amoxicillin-clavulanate (AMC) inhibits the hydrolysis by the carbapenemase present in *Mycobacterium tuberculosis* (MTB).
The aim of this study was to determine the in vitro activity of ETP and FAR against susceptible and resistant clinical MTB strains by two widely use methodologies, the BACTEC960 MGIT and microdilution.

Results: 19 clinical isolates with different susceptibility profiles and H37Rv were included. Minimal inhibitory concentration (MIC) testing was performed using two methods of different concentrations of ETP and FAR with and without AMC.
MIC50 was 2 and 8 for FAR with and without AMC by both methods. MIC90 was > 16 and > 8 by microdilution and MGIT respectively and did not change after AMC addition.
18/20 samples were resistant to the highest concentration of ETP, with and without AMC. Half of the samples had some susceptibility to FAR; addition of AMC further reduced the MIC level in seven isolates.
10/20 isolates showed susceptibility to FAR and the addition of AMC further reduced the MIC in 7 isolates. However, most of the MICs were near the limit of effectiveness (8 μg/mL).
Resistance to FAR was associated with resistance to MEM ($p = 0.04$) but not to resistance profiles of other drugs, including M/XDR status.

Conclusions: The lack of ETP activity may be associated with its degradation, independent of carbapenemase, during incubation.
No susceptibility pattern to traditional drugs can predict susceptibility to FAR and susceptibility testing is not routinely available. PK/PD studies are needed as reaching the concentrations tested in these experiments may be challenging.
This work highlighted some of the limitations of carbapenem use. More evidence is needed to clarify their true impact
(Continued on next page)

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Original article

Performance of lipid fingerprint-based MALDI-ToF for the diagnosis of mycobacterial infections

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ABSTRACT

Objectives: Bacterial diagnosis of mycobacteria is often challenging because of the variability of the sensitivity and specificity of the assay used, and it can be expensive to perform accurately. Although matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has become the workhorse of clinical laboratories, the current MALDI methodology (which is based on cytosolic protein profiling) for mycobacteria is still challenging due to the number of steps involved (up to seven) and potential biosafety concerns. Knowing that mycobacteria produce surface-exposed species-specific lipids, we here hypothesized that the detection of those molecules could offer a rapid, reproducible and robust method for mycobacterial identification.

Methods: We evaluated the performance of an alternative methodology based on characterized species-specific lipid profiling of intact bacteria, without any sample preparation, by MALDI MS; it uses MALDI-time-of-flight (ToF) MS combined with a specific matrix (super-2,5-dihydroxybenzoic acid solubilized in an apolar solvent system) to analyse lipids of intact heat-inactivated mycobacteria. Cultured mycobacteria are heat-inactivated and loaded directly onto the MALDI target followed by addition of the matrix. Acquisition of the data is done in both positive and negative ion modes. Blinded studies were performed using 273 mycobacterial strains comprising both the *Mycobacterium tuberculosis* (Mtb) complex and non-tuberculous mycobacteria (NTMs) subcultured in Middlebrook 7H9 media supplemented with 10% OADC (oleic acid/dextrose/catalase) growth supplement and incubated for up to 2 weeks at 37°C.

Results: The method we have developed is fast (<10 mins) and highly sensitive (<1000 bacteria required); 96.7% of the Mtb complex strains (204/211) were correctly assigned as MTB complex and 91.7% (22/24) NTMs species were correctly assigned based only on intact bacteria species-specific lipid profiling by MALDI-ToF MS.

Conclusions: Intact bacterial lipid profiling provides a biosafe and unique route for rapid and accurate mycobacterial identification. **Ximena Gonzalo, Clin Microbiol Infect 2021;27:912.e1–912.e5**

Introduction

Tuberculosis is one of the biggest infectious disease killers in the world, remaining in the top ten causes of mortality worldwide [1]. Control of the disease is hampered by multiple factors, including the difficulties in getting a timely and accurate diagnosis, a key pillar in the WHO End TB Strategy [2]. In the last few decades, matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) has been implemented in the general bacteriology laboratory, revolutionizing bacterial identification; it has also been useful in

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EXPERT REVIEW OF RESPIRATORY MEDICINE
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Urine biomarkers of pulmonary tuberculosis

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INTRODUCTION Sputum-based tuberculosis diagnosis does not address the needs of certain categories of patients. Active development of a noninvasive urine-based diagnosis could provide an alternative approach. We reviewed publications covering more than 30 urine biomarkers proposed as significant for TB diagnosis. Analytical approaches were heterogeneous in design and methods; few studies on diagnostic outcome prediction described a formal specificity and sensitivity analysis.

AREAS COVERED This review describes studies of non-sputum diagnostic approaches of pulmonary TB based on urine using specific TB biomarkers. The search was performed until December 2021, using terms [Tuberculosis] + [urine] + [biomarkers] in PubMed and Cochrane databases. Publications concerning LAM urine diagnostics were excluded as they have been described elsewhere.

EXPERT OPINION Microbiological culture of sputum is considered to be the 'gold standard' diagnostic for pulmonary TB but the methodology is slow due to the slow growth of the TB bacteria. Urine provides a large volume of sample. Investigators have evaluated urine for either TB pathogen biomarkers or host biomarkers with some success as the review demonstrates. Detection sensitivity remains a significant problem. In future, combination of host and pathogen biomarkers could increase the sensitivity and specificity of TB diagnosis.

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1. Introduction

Tuberculosis continues to be one of the most significant causes of morbidity and mortality globally; at the time of writing approximately 6 million people have died of COVID-19 disease but almost 1.3 million die of TB each year. Moreover, the global number of TB deaths increased between 2019 and 2020, caused by reduced access to essential TB diagnostic and treatment services during the COVID-19 pandemic, as per the WHO Global Tuberculosis Report 2021 [1].

Despite the fact that TB is one of the oldest diseases known to humanity, current methods for the diagnosis of pulmonary TB are imperfect and are based primarily on using sputum (or broncho-alveolar) samples. Microbiological culture or molecular methods based largely on DNA amplification technologies applied to *M. tuberculosis* DNA, have revolutionized diagnosis [2]. Nevertheless, sputum collection is not always possible in some categories of patients, such as children. This has led to alternative detection strategies based on host-responses as well as microbial pathogen detection including application of metabolomics analyses for a number of different biomarkers in various patients' samples. The biomarker

Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study

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Summary

Background Slow and cumbersome laboratory diagnostics for *Mycobacterium tuberculosis* complex (MTBC) risk delayed treatment and poor patient outcomes. Whole-genome sequencing (WGS) could potentially provide a rapid and comprehensive diagnostic solution. In this prospective study, we compare real-time WGS with routine MTBC diagnostic workflows.

Methods We compared sequencing mycobacteria from all newly positive liquid cultures with routine laboratory diagnostic workflows across eight laboratories in Europe and North America for diagnostic accuracy, processing times, and cost between Sept 6, 2013, and April 14, 2014. We sequenced specimens once using local Illumina MiSeq platforms and processed data centrally using a semi-automated bioinformatics pipeline. We identified species or complex using gene presence or absence, predicted drug susceptibilities from resistance-conferring mutations identified from reference-mapped MTBC genomes, and calculated genetic distance to previously sequenced UK MTBC isolates to detect outbreaks. WGS data processing and analysis was done by staff masked to routine reference laboratory and clinical results. We also did a microcosting analysis to assess the financial viability of WGS-based diagnostics.

Findings Compared with routine results, WGS predicted species with 93% (95% CI 90–96; 322 of 345 specimens; 356 mycobacteria specimens submitted) accuracy and drug susceptibility also with 93% (91–95; 628 of 672 specimens; 168 MTBC specimens identified) accuracy, with one sequencing attempt. WGS linked 15 (16% [95% CI 10–26]) of 91 UK patients to an outbreak. WGS diagnosed a case of multidrug-resistant tuberculosis before routine diagnosis was completed and discovered a new multidrug-resistant tuberculosis cluster. Full WGS diagnostics could be generated in a median of 9 days (IQR 6–10), a median of 21 days (IQR 14–32) faster than final reference laboratory reports were produced (median of 31 days [IQR 21–44]), at a cost of £481 per culture-positive specimen, whereas routine diagnosis costs £518, equating to a WGS-based diagnostic cost that is 7% cheaper annually than are present diagnostic workflows.

Interpretation We have shown that WGS has a scalable, rapid turnaround, and is a financially feasible method for full MTBC diagnostics. Continued improvements to mycobacterial processing, bioinformatics, and analysis will improve the accuracy, speed, and scope of WGS-based diagnosis.

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Introduction

In 2013, WHO estimated that *Mycobacterium tuberculosis* complex (MTBC) caused 9 million new active infections and 1·5 million deaths worldwide.[1] Non-tuberculous mycobacteria also cause considerable morbidity and mortality.[2] Protracted MTBC diagnosis and phenotypic drug susceptibility testing (DST) due to slow growth in culture contribute to reported treatment initiation delays of 8–80 days from first contact with health services, risking poor clinical outcomes and transmission control.[3–6] Although genotypic assays such as the Cepheid Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) and Hain line-probe (Hain Lifescience, Nehren, Germany) assays can rapidly (less than a day) identify mycobacterial species and mutations conferring MTBC drug resistance independent of culture, they do not detect all resistance-conferring mutations and are typically still used after microbial culture.[3–6] Besides identifying species and doing DST, high-income countries also genotype MTBC using mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) for outbreak detection.

Findings from retrospective studies[5,8–10] show the potential for whole-genome sequencing (WGS) to predict drug susceptibility and simultaneously track outbreaks with high resolution. WGS could replace the entire MTBC diagnostic workflow from Mycobacteria Growth Indicator Tubes (MGITs; BACTEC MGIT; Becton

antibiotics

Article

Are the Newer Carbapenems of Any Value against Tuberculosis

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Abstract: Our aim was to assess whether newer carbapenems with a better administration profile than meropenem (ertapenem, faropenem and tebipenem) were more effective against *Mycobacterium tuberculosis* including M/XDRTB and determine if there was a synergistic/antagonistic effect with amoxicillin or clavulanate (inhibitor of beta-lactamases that MTB possesses) in vitro. Whilst meropenem is given three times a day intravenously, ertapenem, though given parenterally, is given once a day, faropenem and tebipenem are given orally. Eighty-two clinical drug-sensitive and -resistant MTB strains and a laboratory strain, H37Rv, were assessed by a microdilution methodology against ertapenem, faropenem, tebipenem and meropenem with and without amoxicillin or clavulanic acid. Ertapenem showed a limited activity. The addition of amoxicillin and clavulanate did not translate into significant improvements in susceptibility. Sixty-two isolates (75.6%) exhibited susceptibility to faropenem; the addition of amoxicillin and clavulanate further reduced the MIC in some isolates. Faropenem showed a limited activity (MIC of 8 mg/L or lower) in 21 strains completely resistant to meropenem (MIC of 16 mg/L or higher). Fifteen of the meropenem-resistant strains were susceptible to tebipenem. Carbapenems' activity has been reported extensively. However, there remains uncertainty as to which of them is most active against TB and what the testing methodology should be.

Keywords: carbapenems; microdilution; antibiotic resistance; meropenem; faropenem; tebopenem; ertapenem

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1. Introduction

Carbapenems were discovered to be active against non-tuberculous mycobacteria in the last decade of the 20th century [1]. They inhibit the L,D-transpeptidases present in *Mycobacterium* spp. [2–4].

For the treatment of tuberculosis (TB), however, the interest in these drugs grew later, with the advent of multidrug and extensively drug-resistant tuberculosis (M/XDRTB). *Mycobacterium tuberculosis* (MTB) possesses a class C beta-lactamase that can inactivate carbapenems. Since the early 2000s, reports of good in vitro and in vivo results have emerged [5–7]. Of all the drugs in this class, meropenem proved to be the most stable against the chromosomally encoded *blaC* beta-lactamase [8]. The addition of clavulanic acid improves carbapenem activity probably by inhibiting beta-lactamase [3,9–13].

Co-amoxiclav has been included in the WHO Group C (former group V) antituberculous drugs for many years, despite the paucity of data to support its use [14–16]. Clavulanate (the beta-lactamase inhibitor in co-amoxiclav) can be administered orally. However, it is not available in the UK alone, but only in combination with antibiotics such as amoxicillin [13].

Concerns about pharmacological antagonism between amoxicillin and meropenem by means of completion for the binding sites have arisen. However, the addition of amoxicillin to meropenem and clavulanate shows a synergistic effect against *M. tuberculosis* strains at concentrations easily achievable in vivo [17].

Meropenem is given three times a day intravenously, and its use could drive emergence of resistance in the gut microbiota. Ertapenem, even though still requiring par-

Appendices

Appendix I: Thionamide Testing: issues regarding quality of results and its implications for the diagnostic laboratory – Poster presentation 26th ECCMID, Amsterdam, 2016

Imperial College London

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Thionamide Testing: issues regarding quality of results and its implications for the diagnostic laboratory

Introduction and Purpose

Thionamides are second line anti TB agents widely used in the management of MDR and XDRTB.[1] They are particularly valuable in CNS infection as they cross the blood-brain barrier.[2] Resistance emerges quickly and cross resistance between ethionamide (ETH) and prothionamide (PTH) is believed to be complete.[3] However, when testing *in vitro* using current WHO recommeded critical concentrations,[4] this is not always observed. As part of international efforts to standardise second line testing, an assessment of accuracy of current methods was performed.

Methods

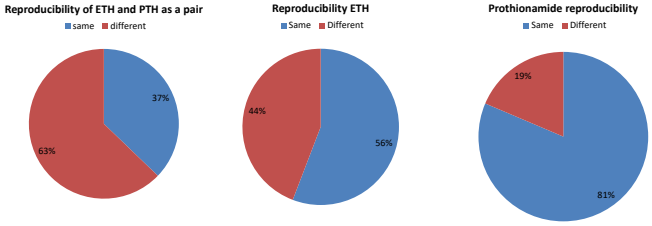
Second line susceptibilities were set up in the MGIT 960® (BD Diagnostics, Sparks, MD) at the National Mycobacterium Reference Laboratory, London.[5] Ethionamide was purchased from Sigma-Aldrich. Prothionamide was kindly provided by Fatol. All those that showed discrepant results were repeated. A structured questionnaire was distributed amongst Tuberculosis laboratories in Europe to determine drug concentrations and methodologies used.

Results

Between 01/07/2012 and 31/12/2013, 308 second and third line susceptibility tests were performed as part of the routine workflow. 43 (13.96%) had discrepant results for ETH and PTH and were repeated following current standard operating procedures. Reproducibility as a pair was 37%, ETH 56% and PTH 81%. The most frequent pattern was ETH resistant/PTH susceptible.

12 laboratories answered the questionnaire. 8 laboratories tested ETH; 2 tested PTH and 2 tested both (Table 1). Solvents used to dissolve the drugs varied as well. DMSO, ethylene-glycol and methanol were used.

	Ethionamide							Prothionamide						
Lab code	SM–PM	CC	LM	CC	Solvent	Filter sterilize	Stock concentration	SM–PM	CC	LM	CC	Solvent	Filter sterilize	Stock concentration
1								LJ	40 mg/L	MGIT	2.5 mg/L	DMSO	No	2100 mg/L
2	7H10	5 mg/L			Ethylene-Glycol	No								
3			MGIT	5 mg/L	Methanol	No	420 mg/L							
4										MGIT	2.5 mg/L	DMSO	Yes	700 mg/L
5	LJ	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown							
6	LJ	20 mg/L	MGIT	5 mg/L	Ethylene-Glycol	No	600 mg/L							
7			MGIT	5 mg/L	Methanol	No	415 mg/L							
8			MGIT	5 mg/L	DMSO	No	420 mg/L							
9			MGIT	5 mg/L	DMSO	No	420 mg/L							
10	7H11	10 mg/L	MGIT	5 mg/L	DMSO	No	10000 mg/L	7H10	2 mg/L			DMSO	No	10000 mg/L
11	LJ	40 mg/L			Methanol	Yes	1000 mg/L							
12			MGIT	5 mg/L	Ethylene-Glycol	Yes	420 mg/L			MGIT	2.5 mg/L	Ethylene-Glycol	Yes	210 mg/L



Conclusions

PTH results are more reproducible than those of ETH. This may be partially explained by the well-known solubility issues related to these compounds that are more apparent for ETH since the stock solutions for the latter are more concentrated.

Testing in liquid medium had already been reported to have less sensitivity than solid media.[6] Ethionamide MGIT testing does not correlate very well with the proportion method (PM) in LJ which suggests that the current critical concentrations (CC) are not appropriate.[7] When compared with results using 7H10 media , discrepancies were also frequent.[8,9]

The current critical concentration is very close to the ecological cut-off. It has been suggested that in those circumstances, the introduction of the intermediate category may compensate for methodological variations.[9] However, it is always difficult to interpret such a category in the clinical setting.

Even though these two drugs are reported to have 100% cross resistance, it is not unusual to see one result as (S) while the other is resistant (R). Reproducibility of each drug is problematic.

Laboratories only using MGIT and/or testing only ETH may be overcalling drug resistance.

Some of these errors may be overcome using molecular (inhA).

Significant differences in methodologies were found accross the centres. This may affect the final real concentration of drug in the stock solutions compromising the accuracy of the assays. Quality control programmes are needed.

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SEVENTH FRAMEWORK PROGRAMME

TB PAN NET European network for study and clinical management of TB drug resistance

Public Health England

Queen Mary University of London

26th ECCMID Amsterdam, Netherlands 9 – 12 April 2016 ESCMID

Appendix II: Luminescence measured at different incubation days for all drugs

		Luminescence																																																	
		Control			DMSO			Clofazimine 2 mg/L			Clofazimine 32 mg/L			Meropenem 2 mg/L			Meropenem 32 mg/L			Nitazoxanide 8 mg/L			Nitazoxanide 64 mg/L			Faropenem 2 mg/L			Faropenem 32 mg/L			Tebipenem 2 mg/L			Tebipenem 64 mg/L			Meroropenem 2 + Clavulanate 2.5 mg/L			Meropenem 32+ Clavulanate 2.5 mg/L			Faropenem 32 + Clavulanate 2.5 mg/L			Tebipenem 64 +Clav 2.5 mg/L			DMSO duplicate	
Experiment 1	Incubation day 0	1180	1313	1345	1059	946	882	991	955	1163	1122	1021	934	1014	978	1094	1012	894	975	913	878	833	850	913	825	1067	1026	990	908	930	952	1000	1040	984	1031	1036	1073	1019	1003	942	945	943	1082	940	1045	1068	940	1087	1124	979	947
	1	1552	1740	1726	1622	1465	1407	1597	1642	1810	1796	1746	1789	1735	1577	1814	1657	1544	1670	1591	1866	1606	1675	1791	2008	2179	2027	1889	1719	1909	1849	2013	2181	1882	2229	2439	2673	2311	2288	2275	1920	1878	2198	2179	2291	2279	2069	2362	2499	2257	2256
	2	1582	1735	1689	1557	1513	1444	1321	1392	1516	154	139	140	1205	1049	1226	561	480	505	1461	1544	1373	92	69	74	1705	1514	1463	626	673	642	1082	1153	960	477	597	598	1526	1572	1529	586	693	642	896	814	812	591	605	555	2436	2460
	3	1669	1842	1890	1688	1492	1469	1211	1192	1370	107	77	106	945	883	887	357	351	335	1470	1414	1321	94	96	85	1292	1219	1174	450	480	478	772	758	667	260	283	300	1263	1287	1151	423	481	427	627	537	586	244	242	285	2543	2535
	6	3112	3767	4031	3543	2843	2816	947	954	1083	78	54	59	1243	1022	1273	284	275	266	2214	2975	2702	86	81	80	1549	1514	1435	348	394	384	938	946	828	154	209	202	1787	1891	1721	308	337	385	465	477	520	176	180	161	4777	4604

		8	8592	10658	11108	8620	6716	6858	629	593	652	85	73	85	2634	2096	2819	335	259	263	5638	8039	7318	119	102	118	2608	2381	2058	351	353	352	1420	1563	1259	123	122	162	4029	4027	3454	306	319	373	373	422	439	157	134	177	10191	10242
		10	22790	27412	30068	22735	17439	18372	358	390	382	80	92	79	7370	5227	7436	380	306	362	16307	23272	20927	114	107	117	5023	4641	4307	418	395	347	3334	3759	2926	129	131	158	10494	10144	8664	356	412	377	424	464	436	150	134	193	22930	24000
		13	60856	70694	73692	57762	46461	48560	182	197	230	88	61	73	22838	16831	23244	709	620	649	52220	69596	64593	103	117	113	14159	12735	11772	449	393	513	11235	12299	9735	149	135	119	35387	35682	32820	736	805	793	565	483	529	126	130	146	63012	70380
		15	104524	114771	119471	100142	87893	89439	136	124	144	85	79	76	49933	38996	50316	1716	1408	1517	110914	128093	126837	119	111	113	31246	28438	26137	588	624	687	26978	29744	24380	117	129	144	77336	80601	78010	1798	1950	1997	745	714	702	122	153	126	123725	140321
		17	174331	188505	194934	162035	152275	148708	113	114	125	77	81	81	101803	81838	97465	5340	4035	4360	187592	204175	206825	113	118	131	70556	62712	56276	914	936	1007	60287	64817	56504	143	115	137	141810	159409	166929	5378	5963	5649	1090	1098	1052	110	119	151	226404	258184
		20	3190	3475	3353	2719	2424	2430	120	123	100	98	72	96	1910	1587	1798	180	137	146	2716	2744	2632	124	124	124	1577	1480	1290	1863	2093	2048	1390	1447	1301	152	154	148	2524	2814	3251	166	193	195	2237	2036	2086	121	129	151	4025	4338

Experiment	Incubation day																										
			58	26	63	48	90	85							12	08	55	36	30	65	15	63	07				38
		21	401059	421930	415149	316264	277615	278794	131	115	92	99	84	86	225789	193834	212783	25501	18921	20213	354002	332774	309971	121	138	118	191639
Experiment 2	Incubation day	0	1295	1230	1174	1269	1408	1182	1191	1214	1360	1304	1474	1284	1361	1259	1406	982	1173	1364	1172	1595	1239	1065	1206	1161	1009
		1	1903	1841	1890	2048	2244	1838	2035	1930	2310	2299	2552	2553	2364	2471	2443	2058	2501	2781	2106	2945	2208	1955	2232	2126	1958
		2	2078	1913	1935	2366	2563	2152	1769	1823	2097	217	363	256	2192	2237	2395	981	992	1136	2309	2919	2073	81	116	109	1796
		5	3193	2767	2801	3216	3478	2763	924	923	1157	99	100	98	2441	2686	2688	682	741	836	2665	3109	2497	120	101	97	1229
		6	4635	3792	4094	4276	4786	3702	864	872	1128	102	90	92	3589	3926	3754	816	927	1110	4664	6484	4152	90	96	113	1402
		8	132	110	112	107	115	90	575	560	752	93	126	96	106	115	110	13	16	20	139	227	127	129	115	134	27

Experiment	Incubation day																										
			20	62				15	10	79				77	38	29	27	92	46							03	60
		21	177460	156497	1868	2572	2461	160911	166565	153006	155	166	159	280118	318709	366099	22419	26285	27003	2554	2242	2492	143	176	161	469859	487936
Experiment 2	Incubation day	0	893	1037	964	1051	1083	1013	1042	1206	1064	1124	1009	1117	1310	1036	977	1263	1125	992	1158	1314	1045	1151	1147	1240	1171
		1	1720	2110	2122	2518	2577	2475	2394	2699	2295	2714	2575	2823	2997	2431	2345	3053	2757	2559	2766	3163	2679	2796	2744	2807	2883
		2	1525	1987	845	947	1071	1386	1264	1389	594	642	696	2517	2920	2504	932	1164	1108	2110	2498	2850	603	643	633	3320	3278
		5	1074	1227	419	486	525	1204	1108	1174	155	150	186	2872	3473	2553	875	968	900	1455	1751	1975	206	208	222	4757	4428
		6	1176	1449	471	472	532	1485	1338	1562	154	144	148	4300	5437	4008	952	1196	1016	1698	1933	2371	208	185	207	6315	5892
		8	25	31	455	469	508	34	31	34	122	142	143	114	145	101	18	21	18	35	39	49	198	214	473	146	136

			70	05	35	50	44	99							77	46	11	85	27	33	86	73	83				80	11	32				84	66	72				62	92	91	07	92	31	06	78	11				13	42
		9	18119	15224	16169	13946	15763	12046	441	461	496	89	95	98	15797	17464	16875	1917	2152	2803	22275	35656	19121	110	111	85	3804	3161	3971	458	441	540	5338	4550	5054	114	125	135	17331	23235	16497	2241	2863	2235	4627	5306	6592	217	172	229	19688	18517
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		21	319228	288706	303251	360476	413945	383147	190	144	169	101	133	118	424799	438618	510596	232834	268463	344448	318557	433035	352556	130	185	171	306254	262786	303864	13406	14401	16496	359141	322003	369485	175	178	155	533076	583706	583504	230236	364180	324894	334782	394545	471382	214	254	250	692692	672283
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		28	340497	313917	315011	860120	929943	881065	155	171	158	117	125	129	373924	367456	538092	345065	414291	567093	280029	412736	284048	152	126	147	334904	302973	333156	239856	265843	255524	300933	311028	330734	186	167	184	290704	342459	285962	452315	488599	468646	368193	389082	431743	121	165	197	766606	742154

Appendix III: Slide for healthy donors to collect urine samples

We need your urine!

- If you were born and have lived all your life in any of the countries listed below (**low TB incidence countries**), we want your urine!
- Albania; American Samoa , Andorra, Anguilla, Antigua and Barbuda, Argentina, Aruba, Australia Austria, Bahamas, Bahrain, Barbados, Belgium, Belize, Bermuda, Bonaire, Saint Eustatius and Saba, British Virgin Islands, Bulgaria Europe, Canada, Cayman Islands, Chile, Colombia, Comoros, Cook Islands, Costa Rica, Croatia, Cuba, Curacao, Cyprus, Czech Republic, Denmark, Dominica, Egypt, Estonia, Finland, France, French Polynesia, Germany, Greece, Grenada, Hungary, Iceland, Iran, Ireland, Israel, Italy, Jamaica, Japan, Jordan, Kuwait, Lebanon, Luxembourg, Malta, Mauritius, Mexico Monaco, Montenegro, Montserrat, Netherlands, New Caledonia, New Zealand, Niue, Norway, Oman, Poland, Portugal, Puerto Rico, Qatar, Saint Kitts and Nevis, Saint Lucia, Saint Vincent and the Grenadines, Samoa, San Marino, Saudi Arabia, Serbia, Seychelles, Saint Maarten (Dutch part), Slovakia, Slovenia, Spain, Suriname, Sweden, Switzerland, Syrian Arab Republic, The Former Yugoslav Republic of Macedonia, Tokelau, Tonga, Trinidad and Tobago, Tunisia, Turkey, Turks and Caicos Islands, US Virgin Islands, United Arab Emirates, United Kingdom, United States of America, Uruguay, Venezuela, Wallis and Futuna Islands, West Bank and Gaza Strip,
- Short holidays (1-6 weeks) in countries outside of this list are fine too, but otherwise please do not volunteer.
- **Do Not Volunteer if you:**
  - have had tuberculosis or a family member has had tuberculosis
  - have a UTI, or are on any antibiotics or prescribed medication
  - are from a country not on the WHO list
- Collection pots available in the toilets outside the MDLs during practical classes tomorrow
- Please leave a small, anonymous donation – a few mls is fine!

Appendix IV: Repurposing drugs against M/XDRTB. ECCMID abstract

Abstract 6597

Drugs repurposing: *in vitro* testing of licensed drugs to assess role against MDR/XDR-TB

Ximena Gonzalo*[1], Francis Drobniewski[1,2]

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Background: Carbapenems inhibit mycobacterial L,D-transpeptidases but they are inactivated by beta-lactamases. The addition of clavulanate improves carbapenem activity.

Clavulanate is available in the UK combined with amoxicillin whose addition to meropenem-clavulanate was synergistic against *Mycobacterium tuberculosis* (MTB). Unfortunately, meropenem requires IV administration 3 times a day. Ertapenem, faropenem and tebipenem have a better administration profiles. Faropenem showed good killing activity *ex vivo*. Tebipenem and ertapenem showed activity against multi-drug resistant (MDR) and extensively drug resistant (XDR) MTB isolates.

Materials/methods: Susceptibility was performed in microtiter plates in a final volume of 0.1 mL of Middlebrook-7H9. 8 concentrations of carbapenems (faropenem: 0.064 - 16 mg/L; ertapenem, meropenem and tebipenem: 0.125 – 32 mg/L) were tested combined with fixed concentrations of clavulanate (2.5 mg/L) and amoxicillin (2mg/L).

The plates were incubated at 37° CO_2 and read every 7 days. Susceptibility was interpreted when visible growth was seen in the growth control (GC) well.

Results: 82 fully susceptible, MDR and XDR MTB isolates were tested.

Faropenem/clavulanate minimal Inhibitory Concentration (MIC) 50 was 2 mg/L and the MIC90 was >16 mg/L. The addition of amoxicillin did not change these values. Meropenem/clavulanate MIC50 was 8 mg/L while the addition of amoxicillin produced a value of 4 mg/L; MIC90 was >32 mg/L.Tebipenem/clavulanate MIC50 was 2 mg/L and 1 mg/L after the addition of amoxicillin. Ertapenem showed MIC50 of 32 mg/L and MIC90 >32 mg/L with no changes after the addition of amoxicillin.

Conclusions: Meropenem, tebipenem and faropenem showed modest activity against some MTB strains. Resistant pattern was not associated with resistance to other drugs so being fully susceptible, MDR or XDR could not predict susceptibility to these drugs. Synergy, if present, was strain specific rather than a drug-combination phenomenon. Ertapenem testing remains a challenge as it degrades quickly.

Since these drugs are increasingly used, further research is required to determine ecological cut-offs, breakpoints and the best methodology for testing.

Information regarding clinical use and outcomes in humans is emerging, showing results suggestive of activity against MTB. However, the contribution of these beta-lactams to the outcome remains uncertain.

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Appendix V: Testing selected compounds against *M. tuberculosis* in 3-D model of the granuloma. ECCMID abstract

Imperial College London

UNIVERSITY OF Southampton

Testing three carbapenems, clofazimine and nitazoxanide in a novel *ex vivo* model of human tuberculosis

Ximena Gonzalo, Magdalena Bielecka, Liku Tezera, Paul Elkington, Francis Drobniewski

Background

TB is a major killer, representing the main infectious cause of mortality in the world until 2020.

Re-purposing drugs to use against multi-drug resistant (MDR-TB) and extensively-drug resistant tuberculosis (XDR-TB) has become a strategy in the search of active compounds against the most resistant forms of the disease.

Most of the information about these compounds comes from in vitro studies that are devoid of host cells, or animal models that do not replicate the role of human cells in both the pathogenicity and the response to antimicrobial therapy.

Objectives

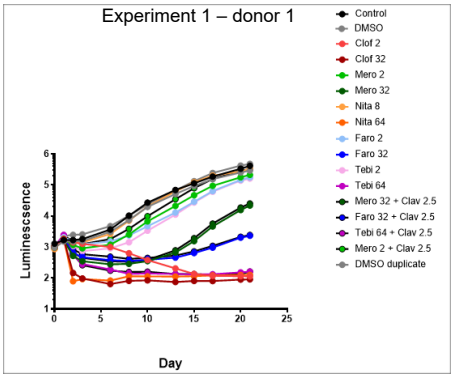
The aim of this work was to test the activity of three carbapenems, clofazimine and nitazoxanide in a novel more physiological 3-D bioelectrospray model of the granuloma.

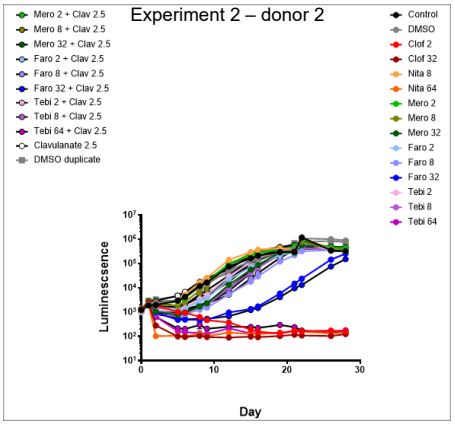
Methods

PBMCs collected from two healthy donors were isolated and infected with M. tuberculosis H37Rv lux. Microspheres were generated with the infected cells, the antimicrobial compounds were added the next day and bacterial luminescence was monitored for up to 21-28 days.

Results

Clofazimine showed good killing activity at both concentrations tested and was the only drug of the panel that killed bacteria at the lowest concentration tested. Nitazoxanide was effective only at concentrations not achievable with current dosing recommendations. Carbapenems showed some modest initial activity that was lost at around day 10 of incubation. Addition of clavulanate did not increase killing activity for any carbapenem.





Conclusions

The M. tuberculosis killing efficacy of the compounds tested was dose-dependent. Clofazimine was the most effective antibiotic tested. It inhibited the growth of M. tuberculosis at 2 mg/L in the 3-D bioelectrospray model. Nitazoxanide requires further work to establish whether the lack of activity is a true drug-related phenomenon or it is an artefact related to extensive protein binding. Carbapenems role remains unclear and this work can offer limited clarification. Of the ones tested here, tebipenem was the most efficient in killing MTB, albeit at a high concentration. The addition of clavulanate did not increase their killing efficacy.

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