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MALDI-TOF for the Identification and Drug Sensitivity Testing of *Mycobacteria*

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PHE, 2017/05/12

OD-Communicable and infectious diseases

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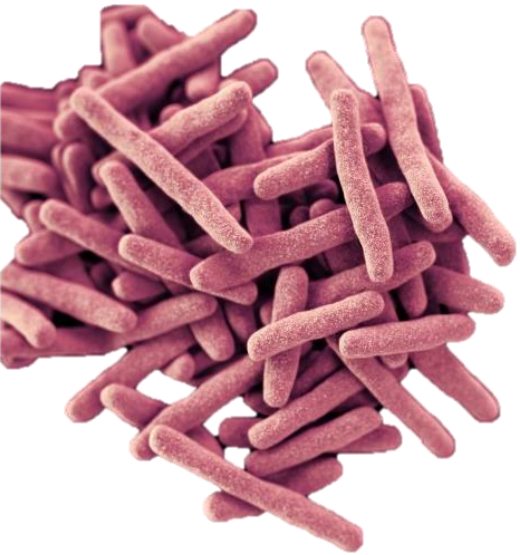
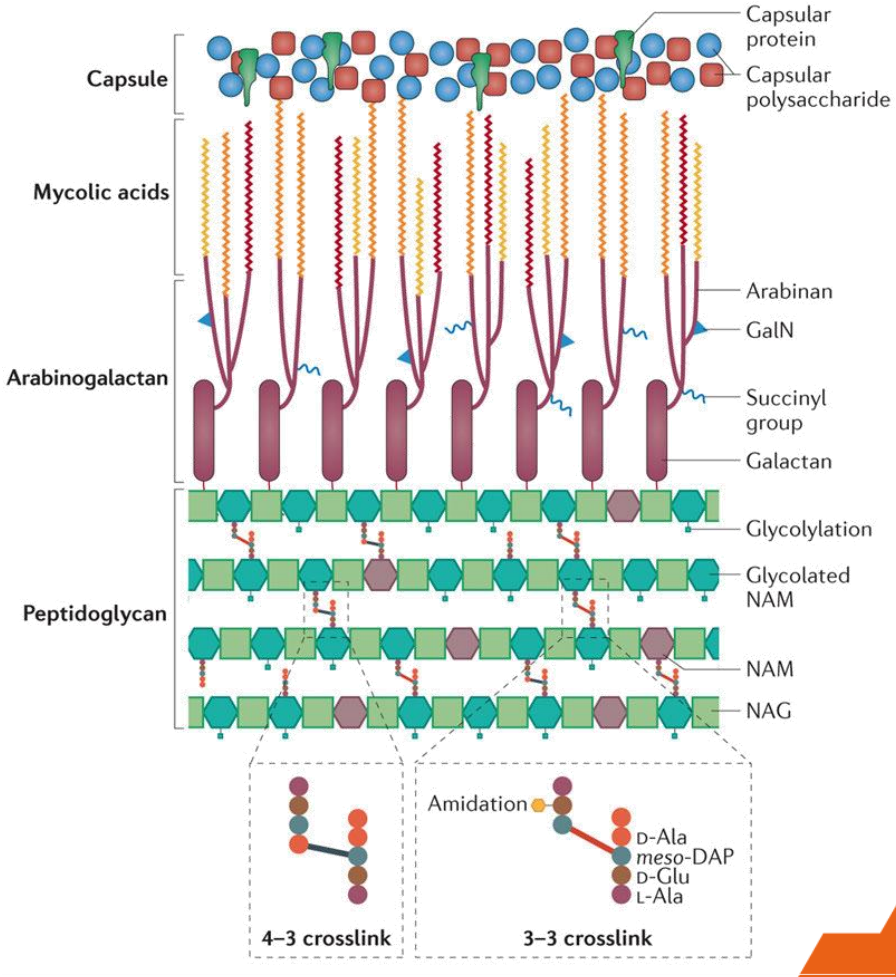
Mycobacteria

- Gram-positive, aerobic, acid-fast bacteria
- Actinobacteria
- Slow growing
- Unusual thick, hydrophobic cell wall





Kieser KJ et al. Nature Reviews Microbiology 12, 550–562 (2014)

Mycobacteria

- ***Mycobacterium tuberculosis* complex:** causative agent of Tuberculosis

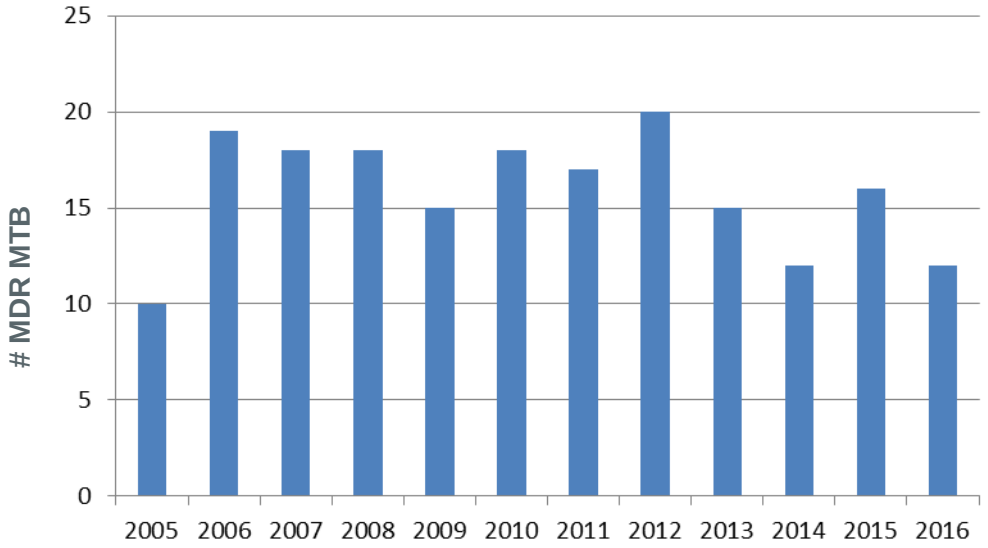
M.tuberculosis
M.africanum
M.canetti
M.bovis
M.caprae
M.microti
M.pinnipedii

DNA homology >99,95 %

- 3 PCRs targeting specific deletions
- Genotype MTBC (Hain)

Situation in Belgium:

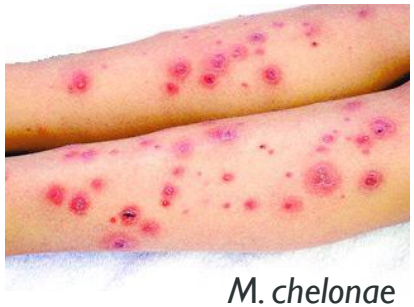
- MTB Incidence of **8.8/100k** inhabitants
- MDR-TB (2%) almost all associated with Eastern European patients



Mycobacteria

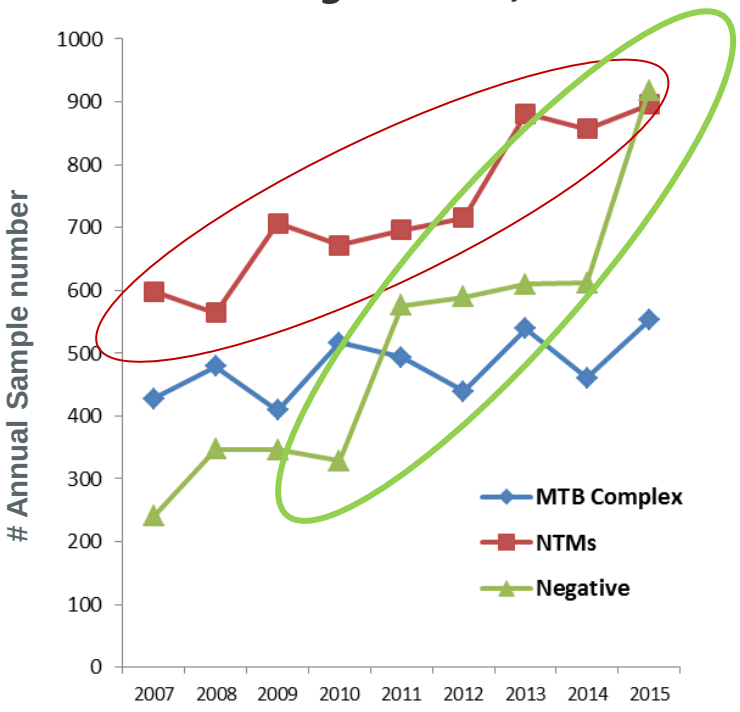
- **Nontuberculous (atypical) mycobacteria: >150 species**

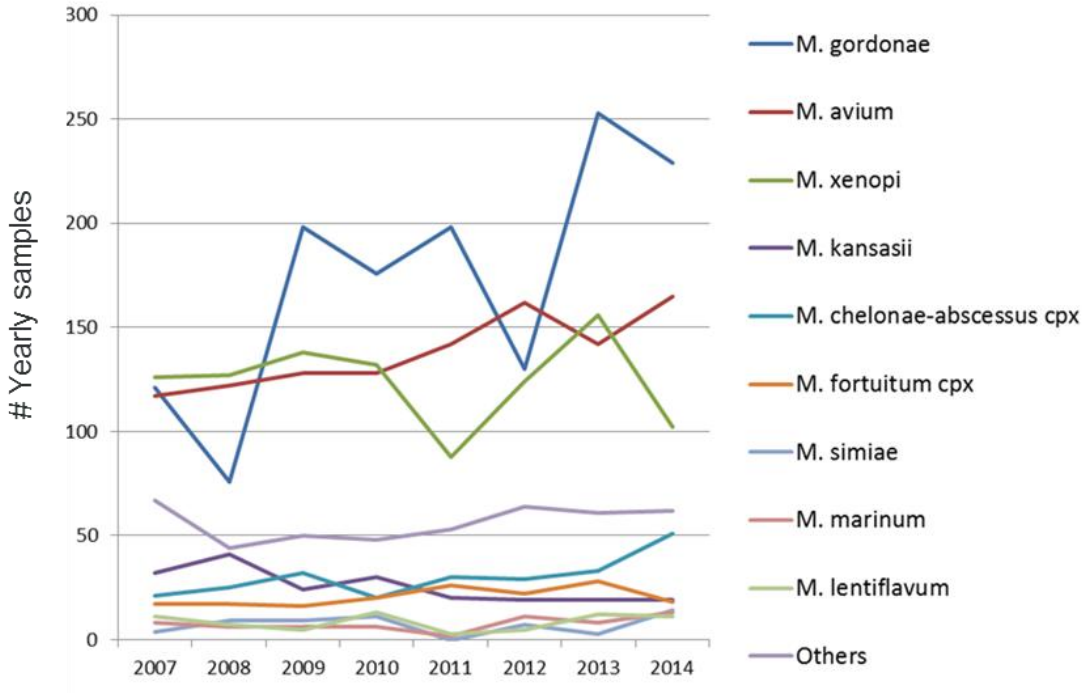
M. Marinum
M. Abscessus
M. Avium - Intracellulare complex
M. Kansasii



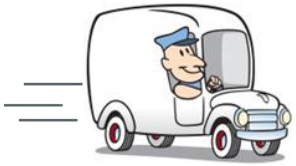
M. chelonae

Situation in Belgian NRC , 2015:





A day in the life of a reference lab…



DNA extract | **Culture** | **Human sample**

Species identification

Heat inactivation

- Consecutive PCR
- Sequencing
- Commercial kits

→ **Genotyping**

- MIRU-VNTR

- Growth characteristics

- GeneXpert
- IGRA

→ **Replace all by NGS ?**

Drug Sensitivity Testing

- Drug-resistance mutations

- MTB complex: Bactec MGIT™
- NTM: Sensititre™

- GeneXpert

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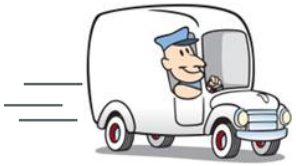

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MALDI-TOF

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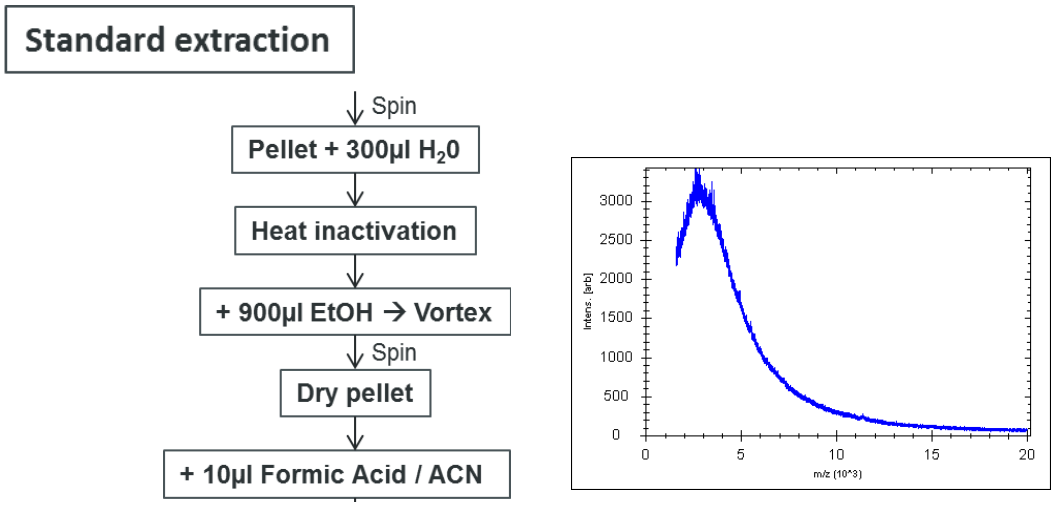
MALDI-TOF in Mycobacteriology

- Still not widely used: 3/15 Belgian BSL3 labs
- MALDI-TOF typically not available in BSL3 environment

> **Heat inactivation** required
>
> **Cell disruption** required



➢ 2015-2016:

Many home-brew protocols published

[1]**O'Conner (2016)** J Clin Microbiol. 54:495-6; [2] **Mareković (2016)** Chemotherapy 61: 167-170; [3] **Kehrman (2016)** Diagn Microbiol Infect Dis. 84:43-7. [4] **Kodana (2016)** J Infect Chemother. 22:32-5; [5] **Rodríguez-Sánchez (2015)** J Clin Microbiol. 53:2737-40; [6] **Quinlan (2015)** J Clin Pathol.68:229-35; [7] **Wilen (2015)** J Clin Microbiol. 53:2308-15; [8] **Tudo (2015)** Eur J Clin Microbiol Infect Dis. 34:1527-32.

Optimizing MALDI protocol

Standard extraction

Spin

Pellet + 300µl H_2O

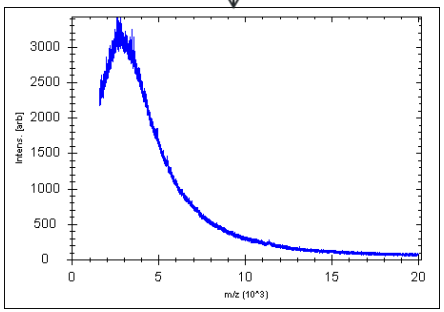
Heat inactivation

+ 900µl EtOH → Vortex

Spin

Dry pellet

+ 10µl Formic Acid / ACN



Bead Beater

Spin

Pellet + 300µl H_2O

Heat inactivation

+ 900µl EtOH / beads

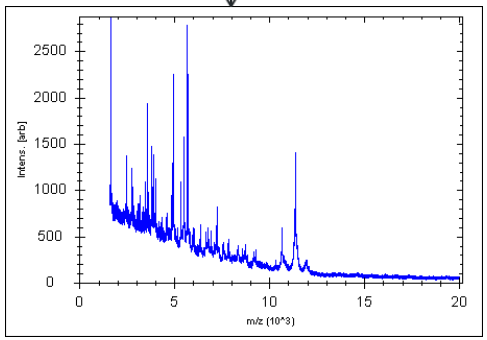
1 minute bead beating

Spin

Dry pellet

+ 10µl 70% Formic acid → Incubate T_R

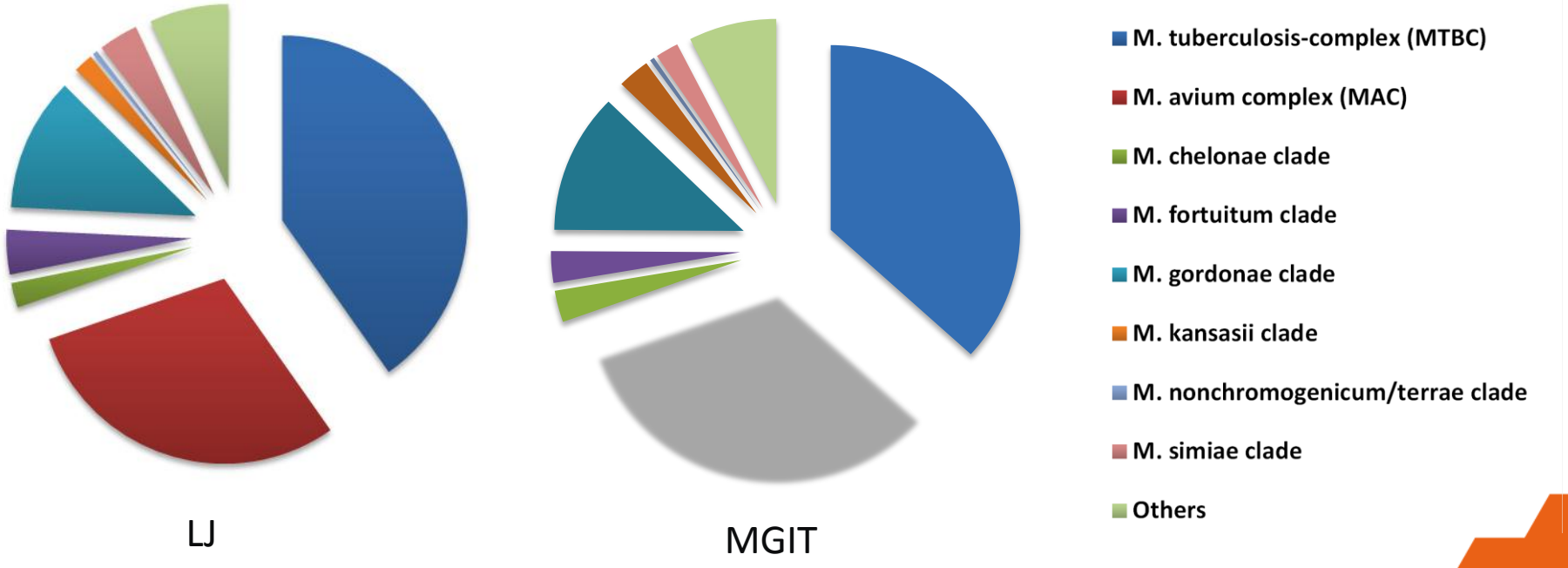
+ 10µl ACN → Vortex



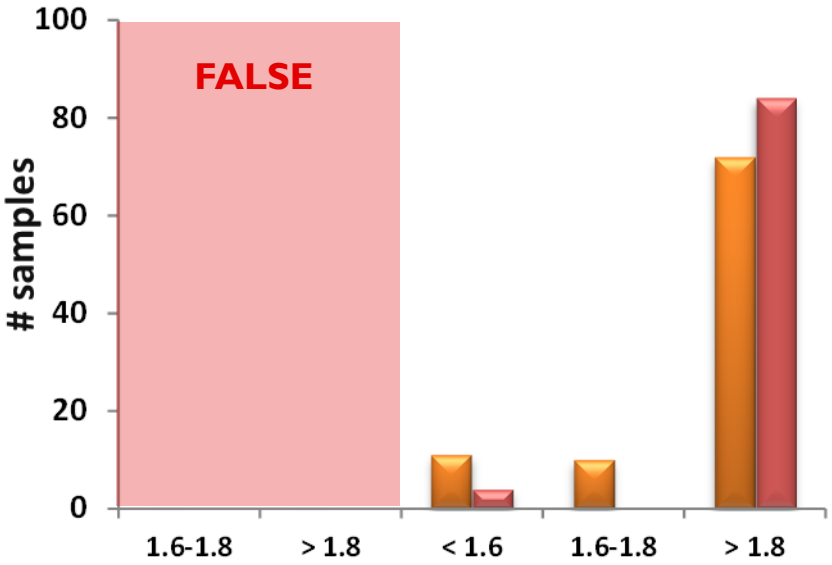
- Cell wall disruption
- Biomass: visible cell pellet needed

Species Identification: Validation study

- 31 species
- 248 MGIT, 231 Lowenstein Jensen cultures
- 30 'No Mycobacteria' cultures
- Bruker BioTyper Microflex (Mycobacteria database 4.0, Biotyper Compass®)



Species Identification: *M. tuberculosis* complex

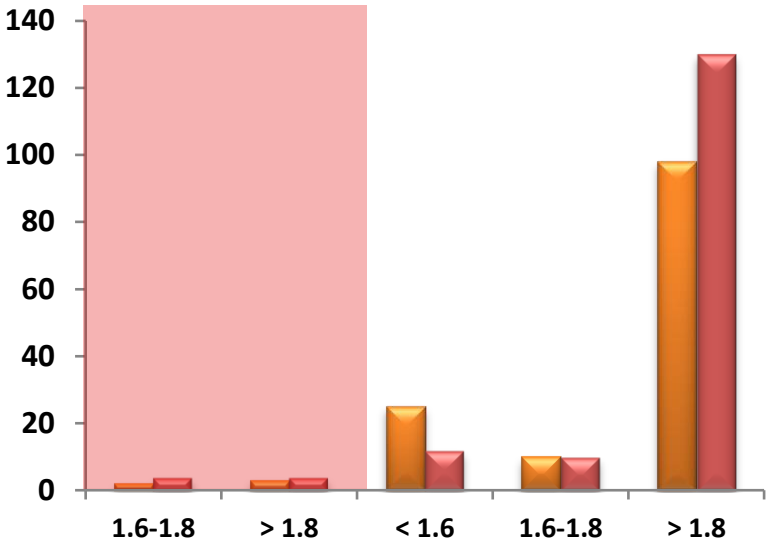


- No false positives for MTBC samples
- 96% /100% intra- and interreproducibility
- 100% inter- and intrarepeatibility

Database v4.0, threshold at 1.6:

- **91.7% strains** had a concordance with gold standard ID method
- No distinction possible within MTB Complex

Species Identification: nontuberculous mycobacteria



- No NTM samples were classified as MTBC
- 12/13 MisIDs were from same clade and flagged as 'Matching Hints'

  Typical ex: M. fortuitum ⇔M. peregrinum

Database v4.0, threshold at 1.6:

- **82.3% strains** had a concordance with gold standard method
- Extraction remains issue

THAT WAS 2016…

2017: Recent Developments

- Peak discrimination allows differentiation of Chimaera and Intracellulare (Timke et al., 2017)

- Consortium with 18 EU partners to streamline extraction protocol and validate MALDI-TOF ID of NTMs from liquid cultures

Inside BSL3:

- Centrifuge: 14,000 rpm for 5 minutes.
- Discard the supernatant
- Add 300 µl H_2O (Point 5.1 in the MycoEX protocol)
- Mix by pipetting up and down (gently!)
- **Heat for 30 min. at 95°C.**
- Take out of BSL3, store at 4°C or continue extraction

- Pipette 900 µL EtOH into the Eppendorf Safe-Lock Tube and mix using a vortex mixer.
- Centrifuge for 2 min at maximum speed (≥ 13,000 rpm) and decant supernatant.
- Centrifuge again and carefully remove residual liquid using a pipette.
- Dry the pellet at room temperature (a few minutes should be sufficient).

Outside BSL3:

- Add 50µl Acetonitrile and 40 mg of 0.5 mm glass beads to the pellet (a spatula tip, as shown in Bruker MycoEX protocol)
- Vortex at max speed for 5s
- Sonicate for 15 min (bath type sonicator with only one mode)
- Add 50µl Formic Acid, vortex for 10s and centrifuge 2 minutes at max speed
- Pipette 1µl of supernatant onto the MALDI plate (3 spots for each strain). Allow to dry
- Overlay with 1µl of HCCA matrix
- Collect spectra and analyse using MALDI-TOF and Biotyper-software

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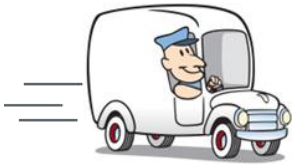
Sonication Bath

A day in the life of a reference lab…



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MALDI-TOF

Drug Sensitivity Testing

- Drug-resistance mutations

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- NTM: Sensititre™

- GeneXpert

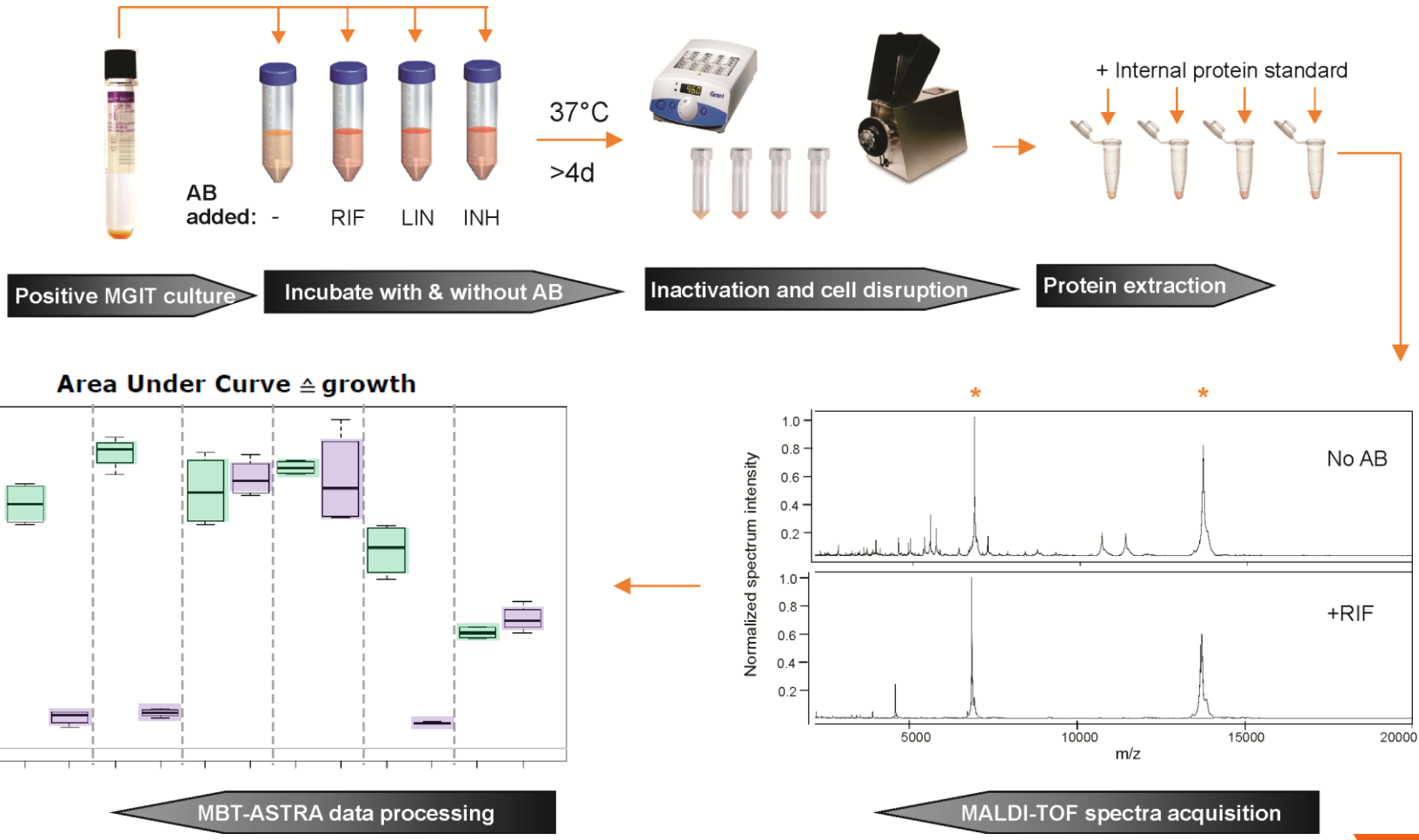
Mycobacterial drug sensitivity testing

- Current phenotypic methods, starting from positively flagged MGIT tubes :
  - *M. tuberculosis* complex: Automated Bactec MGIT™ cultures based on measuring oxygen consumption
  - Nontuberculous Mycobacteria:
    - RAPMYCO / SLOWMYCO Sensititre® plates
    - Agar proportion method

- Requires specific hardware
- TAT of 5-21 days from positive cultures

MALDI Biotyper-Antibiotic Susceptibility Test Rapid Assay

- MBT-ASTRA™: Semi-quantitative assessment of bacterial biomass by creating MALDI-TOF spectra in presence of an internal standard [1,2]



[1]Lange C (2014) J. Clin. Microbiol. 52:4155-4162
[2]Sparbier K (2016) Methods. S1046-2023:30008-1.

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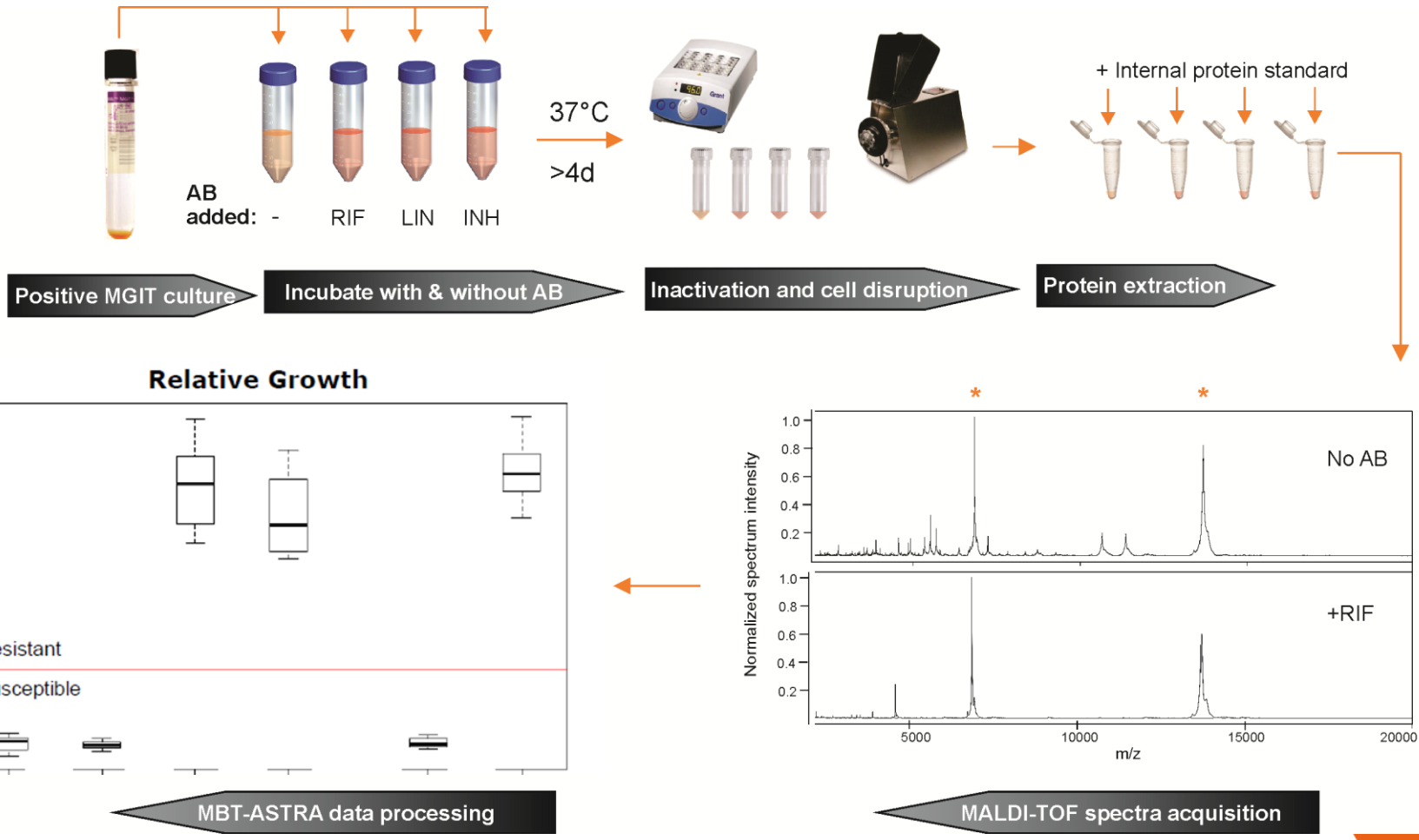
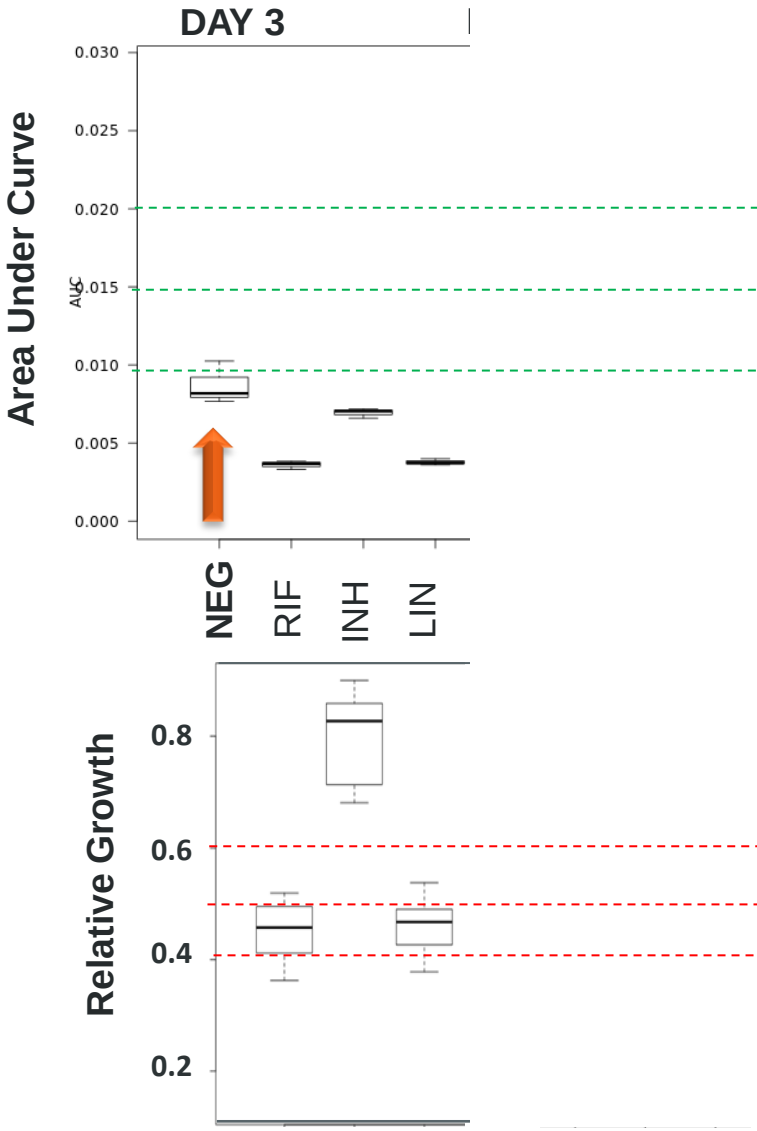


[1]Lange C (2014) J. Clin. Microbiol. 52:4155-4162
[2]Sparbier K (2016) Methods. S1046-2023:30008-1.

Applied to *M. tuberculosis* H37rv



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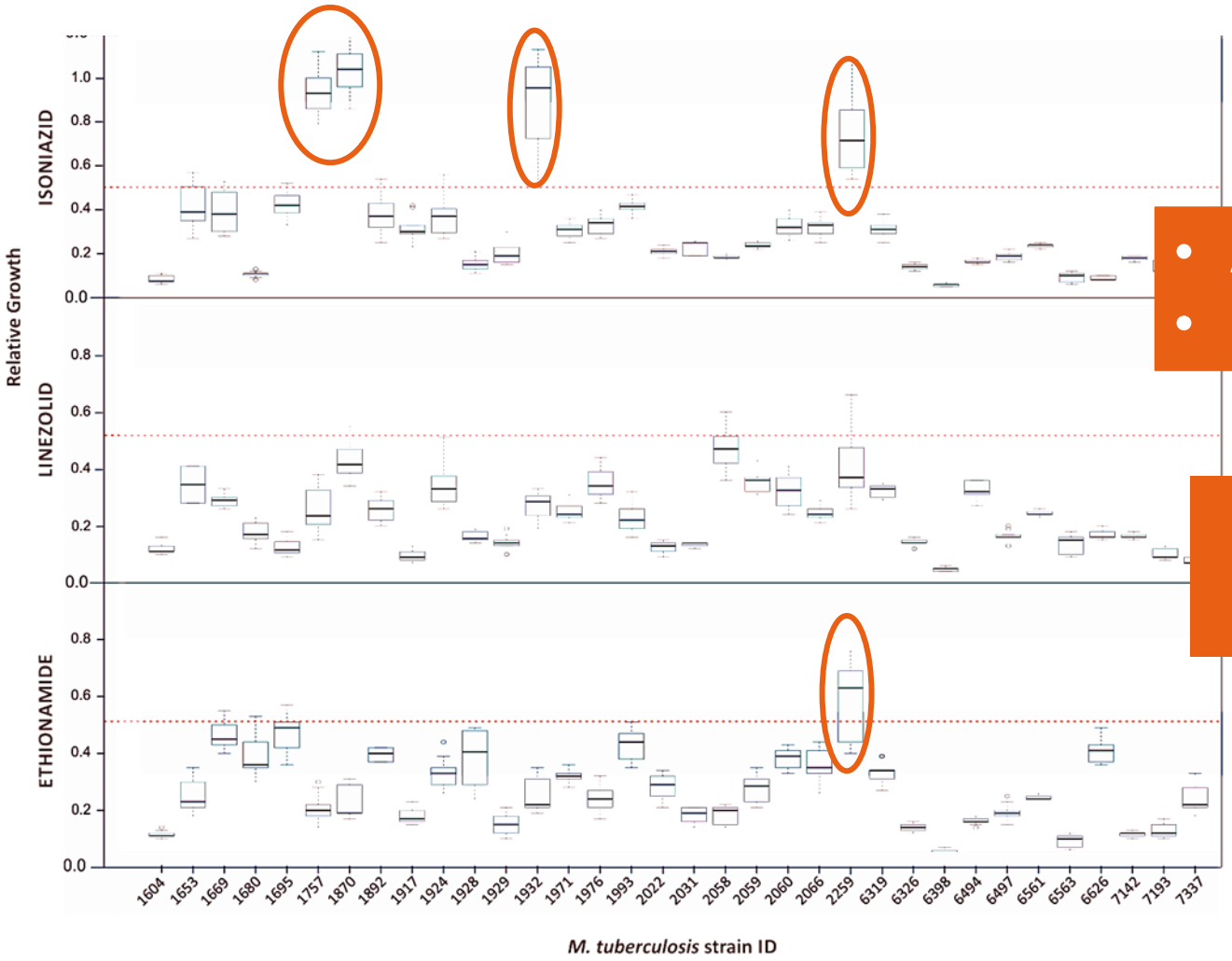
Parameter determination required

Parameter determination – *M. tuberculosis*

- Set-up:
  - ➢72 Mycobacterial strains (44 MTB) strain with known ABR profile
  - ➢Positively flagged MGIT cultures diluted ½ in 7H9 + OADC +/- antibiotics
  - ➢Six antibiotics from different classes
    - Rifampicin (1 µg/ml)
    - Isoniazid (0.1 µg/ml)
    - Linezolid (1 µg/ml)
    - Ethambutol (5 µg/ml)
    - Rifabutin (2 µg/ml)
    - Clarithromycin (8 µg/ml)
  - ➢Incubated (non-shaking) at 37 ° C
  - ➢4 ml samples at 4-5d, consecutive sampling at 2-3d interval
  - ➢Extraction in presence of internal standard (Brüker, 1:50 vol/vol)

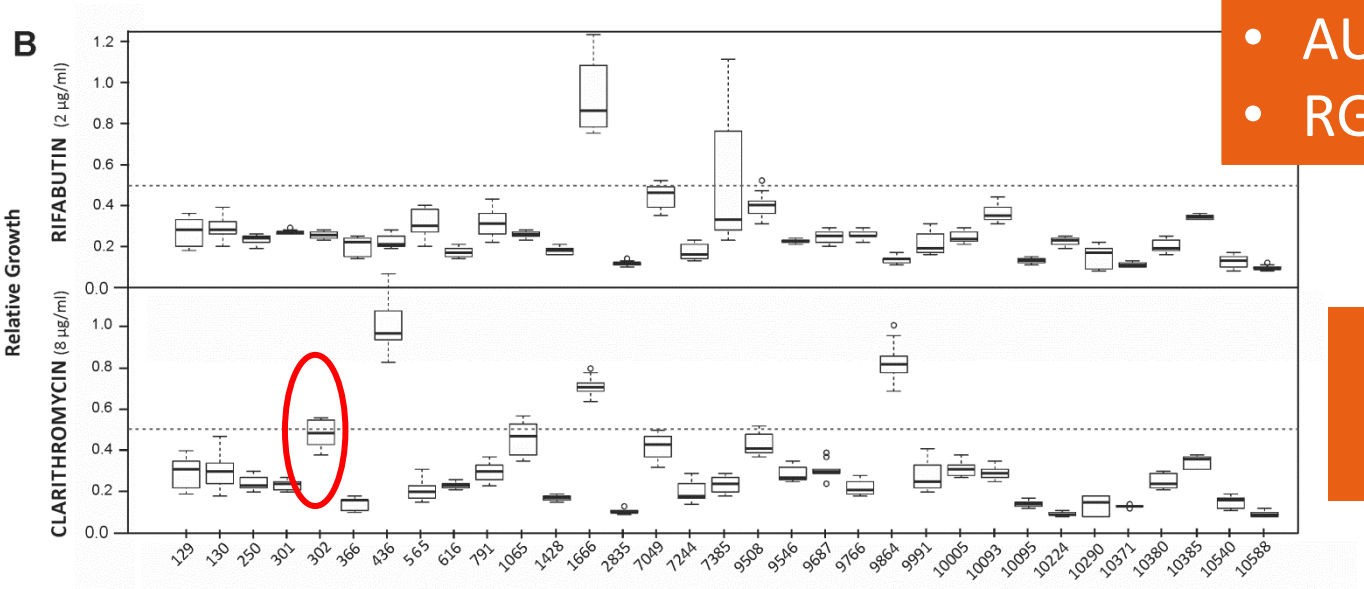
MTB complex



- AUC > **0.015**
- RG < **0.5**

100% Concordance with Bactec MGIT™ results

NTMs



- AUC **> 0.015**
- RG **< 0.5**

65/66 Concordance with Sensititre™ results

Combined species identification & DST

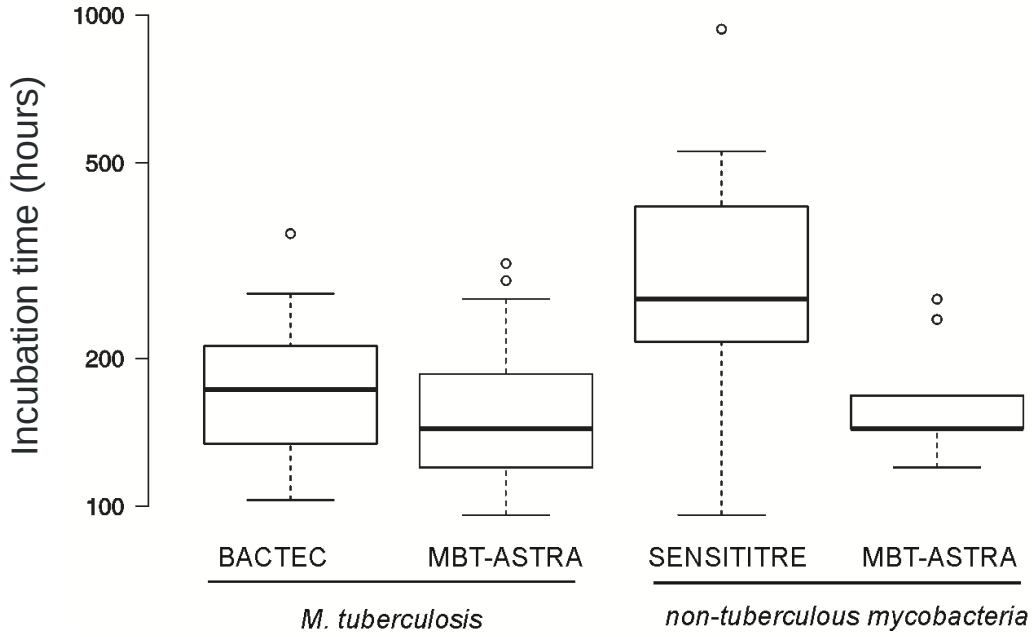
ID	Species	Score
1604	MTB complex	1.88
1653	MTB complex	2.257
1669	MTB complex	2.345
1680	MTB complex	2.295
1695	MTB complex	2.041
1757	MTB complex	2.251
1870	MTB complex	2.282
1892	MTB complex	2.12
1917	MTB complex	1.679
1924	MTB complex	2.162
1928	MTB complex	2.401
1929	MTB complex	2.382
1932	MTB complex	2.263
1971	MTB complex	2.241
1976	MTB complex	2.124
1993	MTB complex	1.834
2022	MTB complex	1.88
2031	MTB complex	2.103
2058	MTB complex	2.316
2059	MTB complex	2.171
2060	MTB complex	2.311
2259	MTB complex	2.197
6319	MTB complex	2.383
6326	MTB complex	2.269
6398	MTB complex	2.475
6494	MTB complex	2.298
6497	MTB complex	2.31
6563	MTB complex	2.113
6626	MTB complex	2.361
7142	MTB complex	2.308
7193	MTB complex	2.321
7337	MTB complex	2.353

ID	Species	Score
129	M. chimaera/intracellulare group	1.513
130	Mycobacterium avium	2.121
250	M. chimaera/intracellulare group	1.723
301	Mycobacterium avium	1.741
302	Mycobacterium avium	2.118
366	Mycobacterium avium	2.182
436	M. chimaera/intracellulare group	2.11
565	M. xenopi	1.935
616	M. xenopi	1.960
791	M. xenopi	2.043
1065	M. xenopi	1.902
1428	M. xenopi	2.007
7244	M. chimaera/intracellulare group	1.849
7385	Mycobacterium avium	2.000
9508	Mycobacterium avium	1.891
9546	Mycobacterium avium	2.259
9687	Mycobacterium avium	2.251
9766	M. chimaera/intracellulare group	1.92
9864	M. fortuitum	2.353
9991	Mycobacterium avium	2.179
10005	Mycobacterium avium	2.054
10093	M. simiae	1.764
10095	M. chimaera/intracellulare group	2.175
10224	M. abscessus	2.131
10290	M. chimaera/intracellulare group	2.028
10371	M. chimaera/intracellulare group	1.81
10380	Mycobacterium avium	2.234
10385	Mycobacterium avium	1.825
10540	Mycobacterium marseillense	1.958
10588	Mycobacterium avium	2.202

- Control spectra scored against Mycobacterial Library v4.0:
  - 95.3% score > 1.8
  - 1.4% score < 1.6

The 2 internal standard peaks do not hinder correct identifications

Time to result

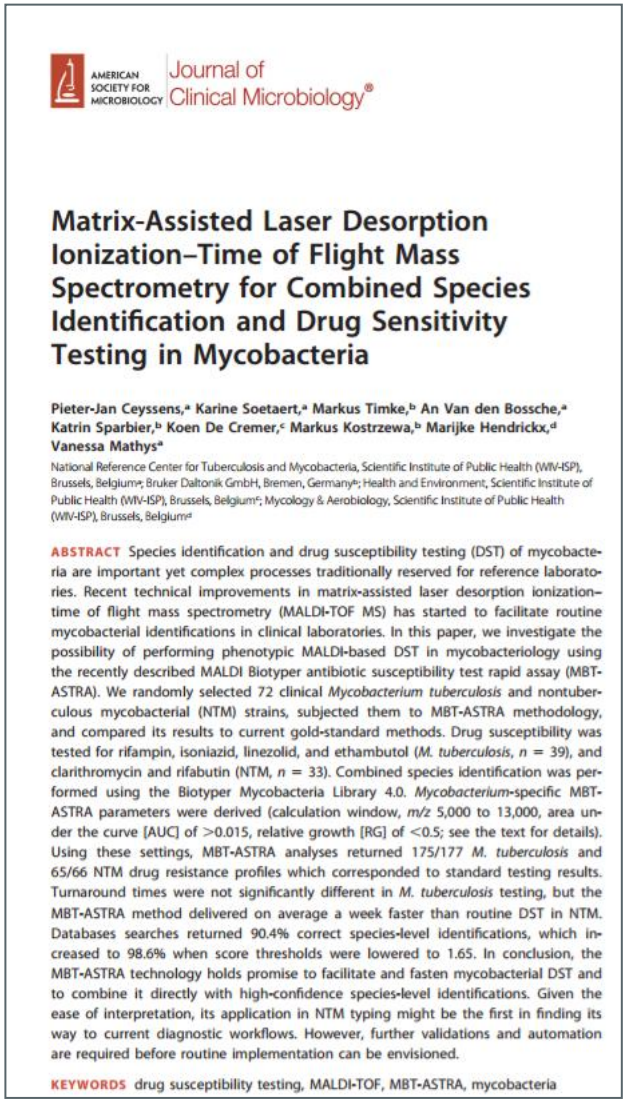


Experiment was not designed to investigate / minimize incubation time

(e.g., not daily check of biomass/AUC, standard incubation using 7H9, etc.)

- Minimal growth required (measured in AUC), and this is not automatically checked
- Few 'breakpoint concentrations' are known for NTMs

Want to read more?



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Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Combined Species Identification and Drug Sensitivity Testing in Mycobacteria

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National Reference Center for Tuberculosis and Mycobacteria, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium[a]; Bruker Daltonik GmbH, Bremen, Germany[b]; Health and Environment, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium[c]; Mycology & Aerobiology, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium[d]

ABSTRACT Species identification and drug susceptibility testing (DST) of mycobacteria are important yet complex processes traditionally reserved for reference laboratories. Recent technical improvements in matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has started to facilitate routine mycobacterial identifications in clinical laboratories. In this paper, we investigate the possibility of performing phenotypic MALDI-based DST in mycobacteriology using the recently described MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). We randomly selected 72 clinical *Mycobacterium tuberculosis* and nontuberculous mycobacterial (NTM) strains, subjected them to MBT-ASTRA methodology, and compared its results to current gold-standard methods. Drug susceptibility was tested for rifampin, isoniazid, linezolid, and ethambutol (*M. tuberculosis*, $n = 39$), and clarithromycin and rifabutin (NTM, $n = 33$). Combined species identification was performed using the Biotyper Mycobacteria Library 4.0. *Mycobacterium*-specific MBT-ASTRA parameters were derived (calculation window, m/z 5,000 to 13,000, area under the curve [AUC] of >0.015, relative growth [RG] of <0.5; see the text for details). Using these settings, MBT-ASTRA analyses returned 175/177 *M. tuberculosis* and 65/66 NTM drug resistance profiles which corresponded to standard testing results. Turnaround times were not significantly different in *M. tuberculosis* testing, but the MBT-ASTRA method delivered on average a week faster than routine DST in NTM. Databases searches returned 90.4% correct species-level identifications, which increased to 98.6% when score thresholds were lowered to 1.65. In conclusion, the MBT-ASTRA technology holds promise to facilitate and fasten mycobacterial DST and to combine it directly with high-confidence species-level identifications. Given the ease of interpretation, its application in NTM typing might be the first in finding its way to current diagnostic workflows. However, further validations and automation are required before routine implementation can be envisioned.

KEYWORDS drug susceptibility testing, MALDI-TOF, MBT-ASTRA, mycobacteria

Journal of Clinical Microbiology 2017 Feb; 55(2): 624-634.

Take home messages: MALDI-TOF in Mycobacteriology

IDENTIFICATION

- Strong improvements in the last years
- Very reliable distinction between MTB and NTM species if score thresholds are lowered from ≥2.0 for routine bacterial identifications, to ≥1.8 for mycobacteria
- Optimized cell disruption step is crucial
- Visible pellet is required as minimal amount of biomass
- Ongoing EQA for NTMs in MGITs, contact mbelen.rodriguez@iisgm.com if interest in participation

DRUG RES.

- **MBT-ASTRA™**
  - 98-100% concordance with established DST methods, using specific parameters
  - Direct combination with species identification possible
  - Arguably first application in NTMs with difficult read-out of broth microdilution
  - Potential for more cost-effective and rapid drug sensitivity screen in Mycobacteria, but further validations & automation required

Thanks to :

Christophe Lange
Markus Timke
Katrin Sparbier
Markus Kostrzewa

Karine Soetaert
Vanessa Mathys
An Van den Bossche
Kristien De Greef

You