MALDI TOF for Identification of Mycobacteria

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Outline

- Intro
- Comparison of instruments
- WSLH validation and findings
- Identification of Mycobacteria from primary MGIT broth
- WSLH testing algorithm using MALDI
- Successes and challenges
- APHL MALDI user's group

MALDI-TOF

- "Matrix-Assisted Laser Desorption Ionization Time-of-Flight"
- A form of mass spectrometry
- Used to analyze proteins and other macromolecules
  - For mycobacteria, total microbial proteins are analyzed
- Recent advances have allowed application to the clinical realm

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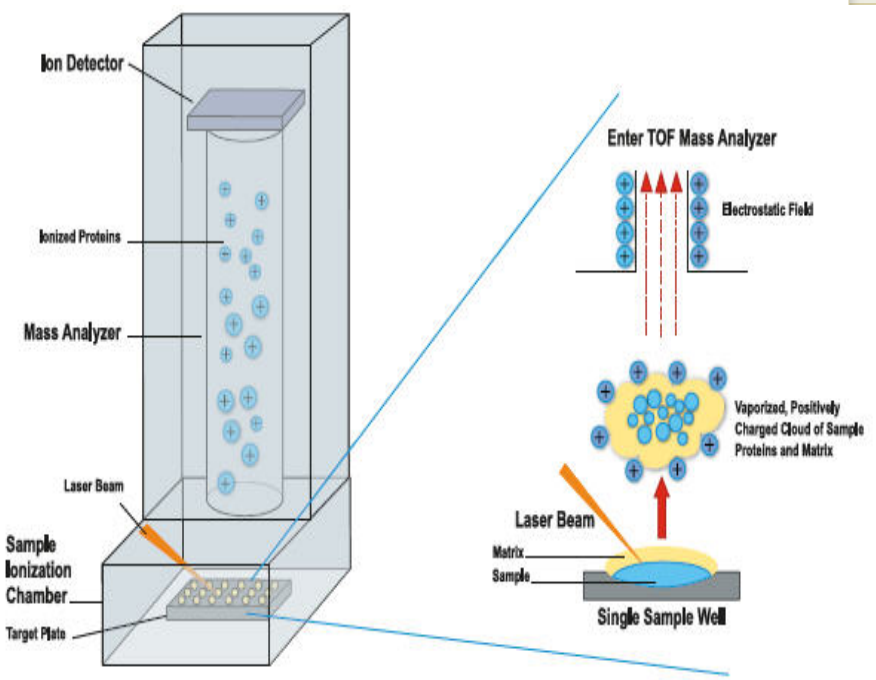
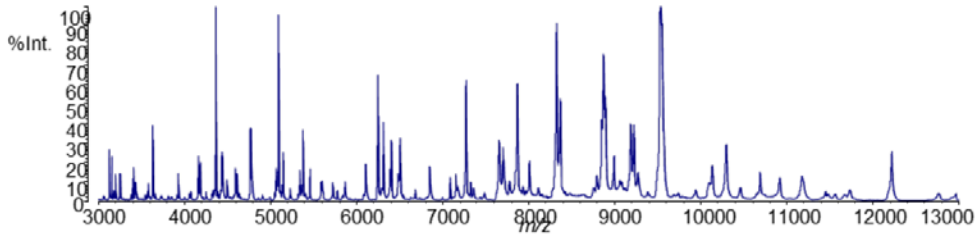
MALDI-TOF: How it works

- Charged particles are accelerated by a laser. Time of flight through mass analyzer is proportional to the ion's mass.
- Proteins and peptides are separated by increasing mass
- Particles are detected at the top of the TOF analyzer



Protein Mass Spectrum of Whole Cells

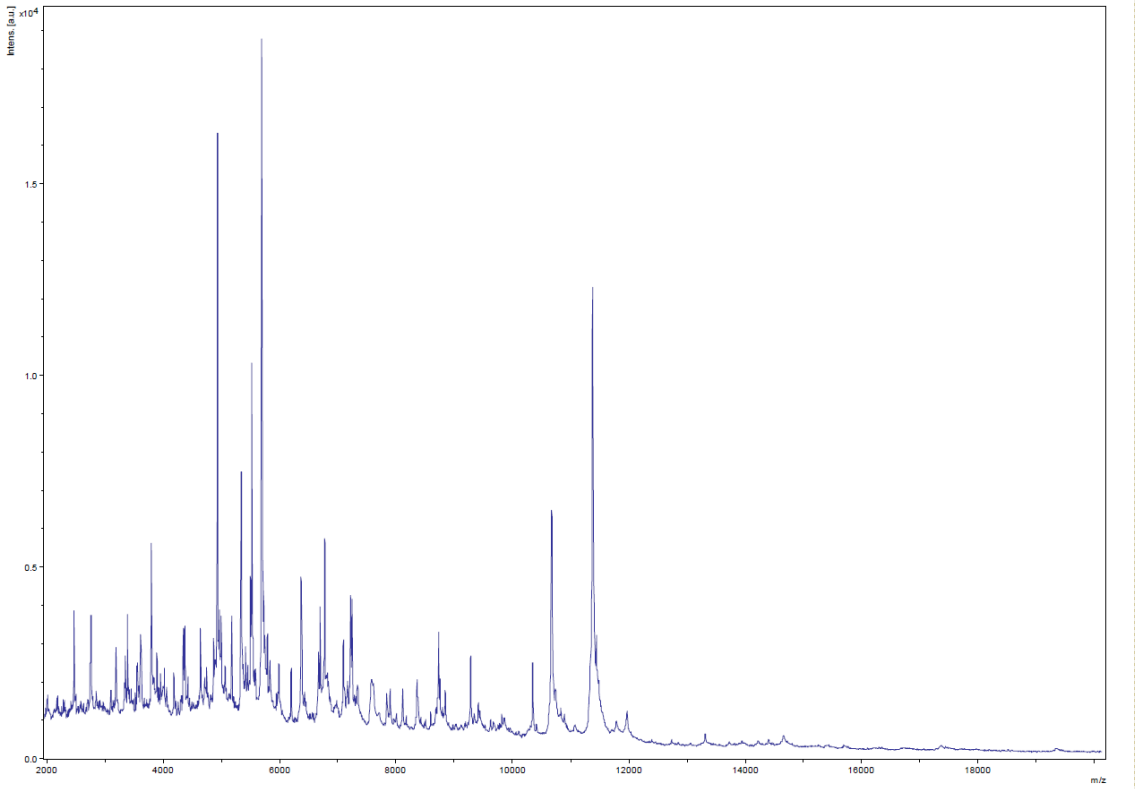


- Detection of 100-200 mass signals (peaks)
- 20-30 proteins directly match with the theoretical masses of ribosomal proteins in the sequence database
- Other peaks are mostly uncharacterized proteins but are likely post-translational modifications of ribosomal proteins

Dave Pincus, Biomerieux

MALDI-TOF: How it works

- A pattern of characteristic peaks “spectrum” is produced.
- Dedicated software analyzes mass spectra against library of stored spectra



M. tuberculosis complex

Resolution (Identification) Power

Gram-positive	Resolution Power: Low — Screening and Morphologic Tests	Medium-High — bMx Biochemical Kits*	Medium-High — TTR (h)	High	High	Higher	Highest
Enterococcus	Chromogenic media, differential media, Gram morph, catalase, bile esculin, PYR	api 20 Strep rapid ID 32 STREP VITEK 2 GP	4 - 26 4 - 4 ½ 2 - 8	16S rpoB sodA	MALDI-TOF	MLST	Full genome
Staphylococcus	Chromogenic media, differential media, Gram morph, catalase, coagulase	api Staph ID 32 STAPH VITEK 2 GP	18 - 24 22 - 26 2 - 8				
Streptococcus	Chromogenic media, differential media, hemolysis, Gram morph, catalase, bacitracin disk, optochin disk, PYR, latex test for serogroup	api 20 Strep rapid ID 32 STREP VITEK 2 GP	4 - 26 4 - 4 ½ 2 - 8				
Listeria	Chromogenic media, differential media, hemolysis, Gram morph, catalase, DALA, motility	api Listeria api 20 Strep rapid ID 32 STREP VITEK 2 GP	18 - 24 4 - 26 4 - 4 ½ 2 - 8				
Coryneforms	Gram morph, lipid requirement	api Coryne VITEK 2 ANC VITEK 2 CBC (Ind)	22 - 26 6 8				
Spore-forming bacilli	Gram morph, spore location	api 50 CHB VITEK 2 BCL (Ind)	22 - 54 14				

*Inoculum density requirement: McF 0.5 - > 6.0 depending on product

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MALDI-TOF: Comparison of two platforms

Bruker Daltonics
Biotyper Microflex

BioMerieux
Vitek MS

MALDI-TOF: Comparison of Two Platforms

	Bruker Daltonics Biotyper Microflex	**BioMerieux Vitek MS**
Instrument footprint	bench top	floor standing
Cost of Instrument	$190-200 K	$250 K (stand alone) Can be integrated by Biomerieux MYLA® to LIS
Cost of Maintenance Contract	$20 K per year (after first year)	Information not available
FDA "Cleared"	**Nov 2013**: 40 aerobic Gram negative bacteria **April 2015**: added 170 species and species groups, representing 180 clinically-relevant species of aerobic Gram positive, fastidious Gram negatives, Enterobacteriaceae, anaerobic bacteria and yeasts	**August 2013**: 193 yeast (Candida, Cryptococcus and Malassezia groups) and bacteria (Staphylococcaceae, Streptococcaceae, Enterobacteriaceae, Pseudomonadaceae, and Bacteroidaceae families)

MALDI-TOF: Comparison of Two Platforms

	Bruker Daltonics Biotyper Microflex	**BioMerieux Vitek MS**
FDA status for ID of Mycobacteria	Not FDA cleared	Not FDA cleared
Number of Mycobacteria Entries (spectra) in Library	Mycobacterium library v2.0 (2014) 313 main spectrum profiles, 128 species Mycobacterium library v3.0 (2015) 853 main spectrum profiles, 149 species[¥]	1286 spectra (2013)[€]

(€) Deol et al. 2013
(¥) Dr. Gongyi Shi, Bruker

Bruker MALDI Library v 3.0

149 species in Mycobacteria Library 3.0, new species are given in blue font

M. abscessus subsp. abscessus	M. crocinum	M. koreense	M. poriferae
M. abscessus subsp. bolletii	M. diernhoferi	M. kubicae	M. pseudoshottsii
M. africanum	M. doricum	M. kumamotonense	M. psychrotolerans
M. agri	M. duvalii	M. kyorinense	M. pulveris
M. aichiense	M. elephantis	M. lacus	M. pyrenivorans
M. algericum	M. engbaekii	M. lentiflavum	M. rhodesiae
M. alvei	M. europaeum	M. litorale	M. riyadhense
M. aromaticivorans	M. fallax	M. llatzerense	M. rufum
M. arosiense	M. farcinogenes	M. longobardum	M. rutilum
M. arupense	M. flavescens	M. mageritense	M. salmoniphilum
M. asiaticum	M. florentinum	M. malmoense	M. saskatchewanense
M. aubagnense	M. fluoranthenivorans	M. mantenii	M. scrofulaceum
M. aurum	M. fortuitum subsp. acetamidolyticum	M. marinum	M. senegalense
M. austroafricanum	M. fortuitum subsp. fortuitum	M. marseillense	M. senuense
M. avium subsp. avium	M. fragae	M. microti	M. seoulense
M. avium subsp. paratuberculosis	M. frederiksbergense	M. minnesotense	M. septicum
M. avium subsp. silvaticum	M. gadium	M. monacense	M. setense
M. bacteremicum	M. gastri	M. montefiorense	M. sherrisii
M. boenickei	M. genavense	M. moriokaense	M. shimoidei
M. bohemicum	M. gilvum	M. mucogenicum	M. shinjukuense
M. botniense	M. goodii	M. murale	M. simiae
M. bovis	M. gordonae	M. nebraskense	M. smegmatis
M. branderi	M. haemophilum	M. neoaurum	M. sp.
M. brisbanense	M. hassiacum	M. neworleansense	M. sphagni
M. brumae	M. heckeshornense	M. nonchromogenicum	M. stomatepiae
M. canariasense	M. heidelbergense	M. noviomagense	M. szulgai
M. caprae	M. heraklionense	M. novocastrense	M. terrae
M. celatum	M. hiberniae	M. obuense	M. thermoresistibile
M. chelonae	M. hodleri	M. pallens	M. tokaiense
M. chimaera	M. holsaticum	M. palustre	M. triplex
M. chitae	M. houstonense	M. parafortuitum	M. tuberculosis
M. chlorophenolicum	M. immunogenum	M. parakoreense	M. tusciae
M. chubuense	M. insubricum	M. parascrofulaceum	M. vaccae
M. colombiense	M. interjectum	M. paraseoulense	M. vanbaalenii
M. conceptionense	M. intermedium	M. parmense	M. vulnerans
M. confluentis	M. intracellulare	M. peregrinum	M. wolinskyi
M. conspicuum	M. iranicum	M. phlei	M. xenopi
M. cookii	M. kansasii	M. phocaicum	
M. cosmeticum	M. komossense	M. porcinum	

Dr. Gongyi Shi, Bruker

MALDI-TOF: Comparison of Two Platforms

Bruker Daltonics Biotyper
Scoring interpretation
2.00-3.00 = Secure genus and species identification
1.70-1.99 = Probable genus identification
0-1.699 = Unreliable identification

Analyte Name	Analyte ID	Organism (best match)	Score Value
A1 (+++)(C)	BTS	Escherichia coli	2.484
A2 (+++)(C)	BTS	Escherichia coli	2.454
A3 (++)(A)	M.avium	Mycobacterium avium	2.292
A4 (++)(A)	M.avium	Mycobacterium avium	2.232
A5 (-)(C)	14mm[redacted]	no peaks found	<0

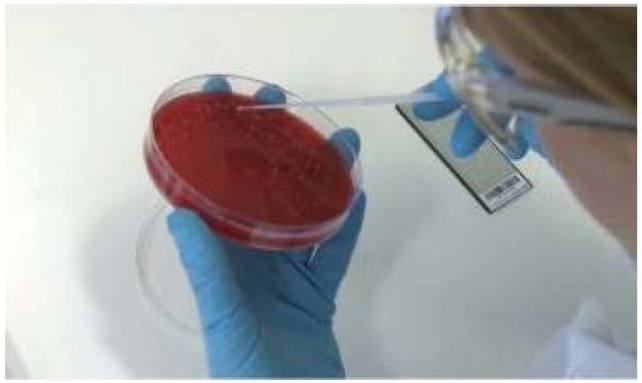
BioMerieux Vitek MS
Confidence values (%)

- Results have a strong match and are ready to report
- Results have low discrimination and require further review
- Low-quality results with no identification made

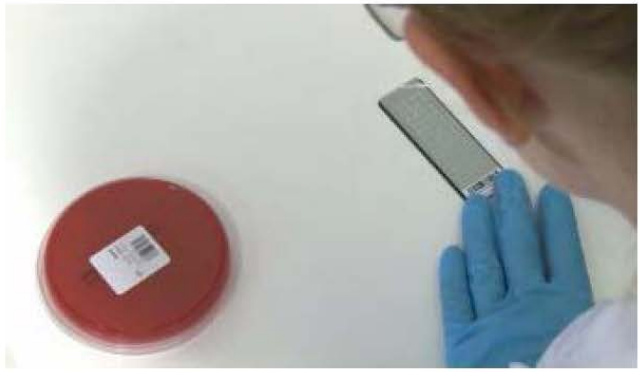
Extraction

BIOMÉRIEUX

Laboratory Workflow



1. Pick Colony



2. Smear on Target Slide

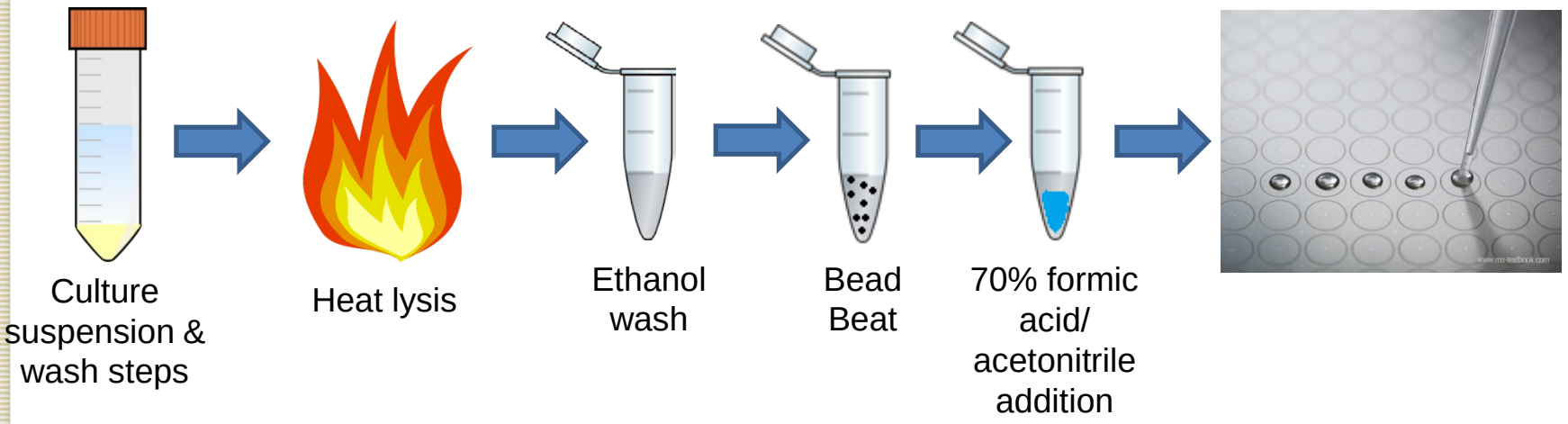
3. Add CHCA Matrix *(Formic Acid – Yeast)*

4. Load in instrument

Dave Pincus, Biomerieux

MALDI-TOF Mycobacterial Sample Preparation

- Pure growth from solid or liquid media undergoes an extraction protocol and is applied to a sample target plate and overlaid with matrix
- Necessary steps:
  - Inactivation
  - Cell disruption
  - Protein extraction



Extraction Methods

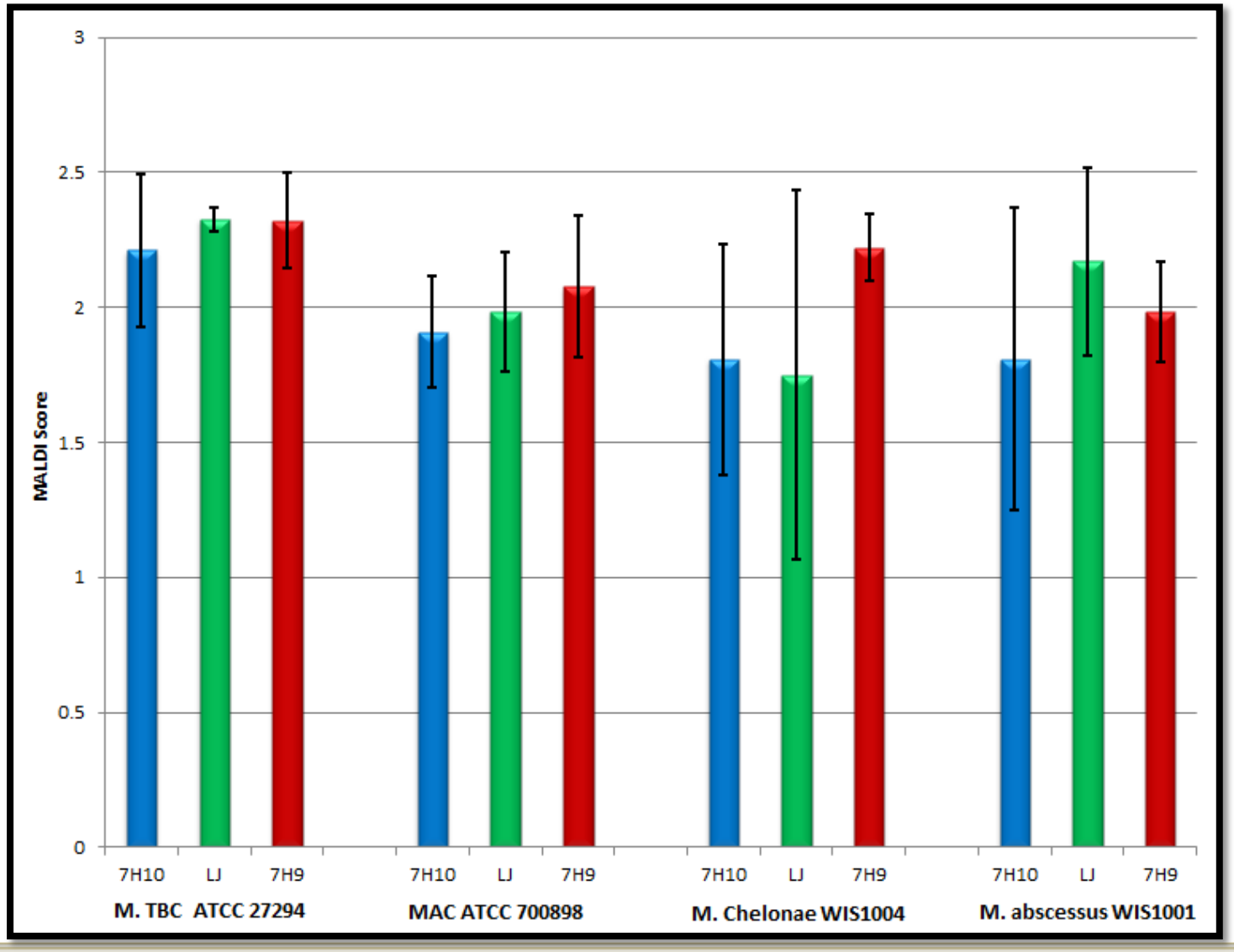
	Bruker Daltonics Biotyper Microflex	**BioMerieux Vitek MS**
Extraction method for Mycobacteria	Bruker MycoEX v3 • 30 minute heat lysis inactivation • Ethanol wash • silica bead disruption with acetonitrile/formic acid extraction	BMX Extraction • Ethanol/bead inactivation/disruption • No heat lysis • formic acid/acetonitrile extraction
Amount of time for assay: Extraction/run	2 hours/ 20 minutes	2 hours/ 20 minutes*

(*) Matthew Henson, Cincinnati Children's Hospital Medical Center

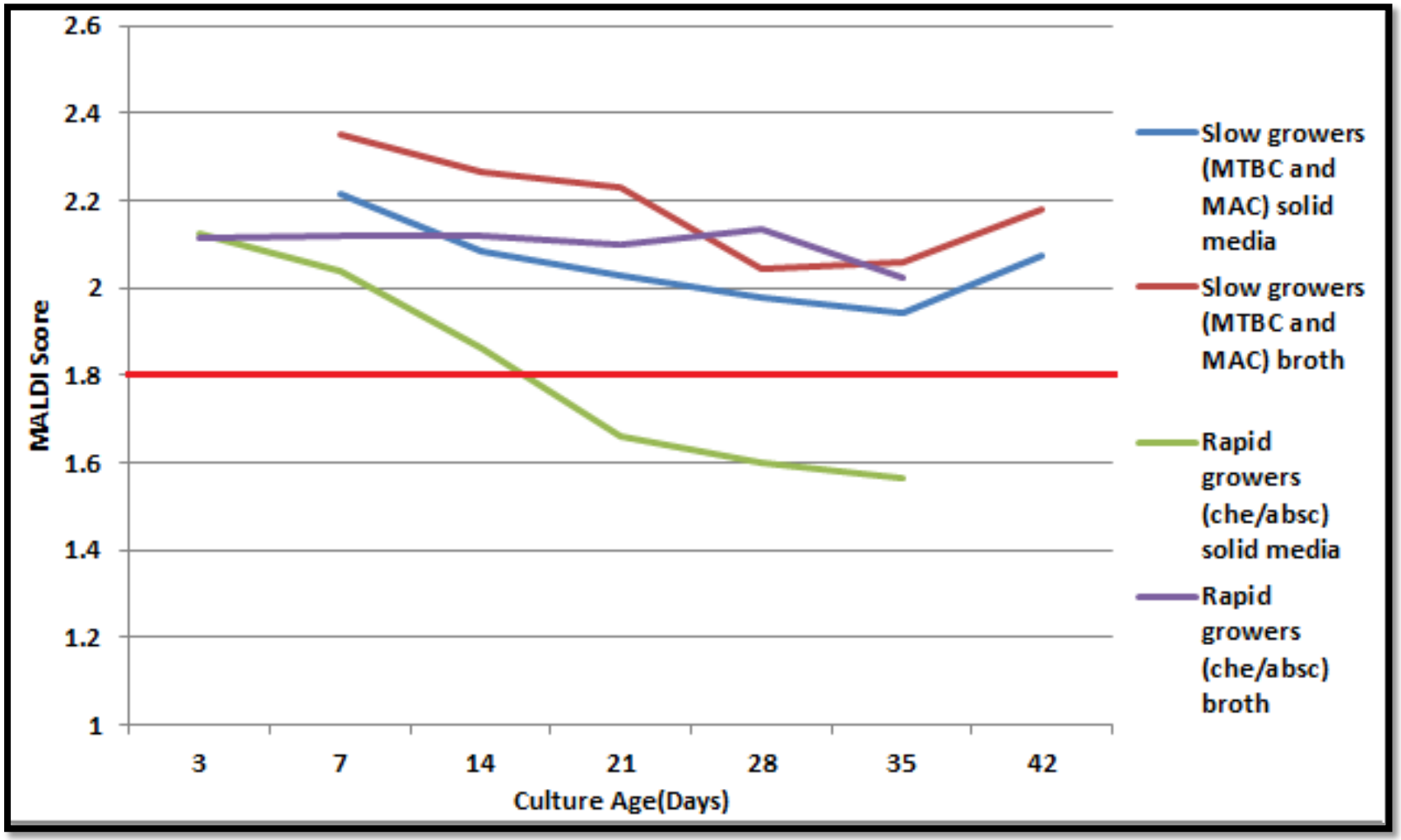
Extraction Methods

- Variations on the theme:
  - Balada-Llasat (2013), Mather (2014)
  - WSLH (unpublished, slide 37): added distilled water wash step before heat lysis for broth cultures
- For best ID, extraction method must be identical to that used to create spectral library
- Important to validate inactivation step(s) before removing specimens from BSL-3 laboratory

Effect of Media on MALDI Score



Effect of Culture Age on MALDI Score



Effect of Media and Culture Age on MALDI Score

- **Media Type**: Our validation study showed no significant difference in MALDI score between 7H10, LJ and 7H9
    - Lower scores were seen for broth cultures in some studies (Quinlan et al. 2015, Lotz et al. 2010, Balazova et al. 2014)
    - Due to lower biomass in broth cultures analyzed?
- **Culture Age**: Similar decreases in score with culture age were seen in other publications (Mather et al., 2014).
    - Mather et al. suggests that ID by MALDI should be attempted as soon as colonies of a single morphotype are visible (on solid media) to ensure the highest likelihood of finding a match in the existing database.
    - Fresh pure growth is best because that's how most of the databases were created.

Organisms Identified during Validation

Organism(s)	Number of isolates with reportable MALDI score	Percentage of isolates with reportable MALDI score	Average MALDI score
M. tuberculosis complex	20/20	100%	2.16
M. avium complex	50/50	100%	2.058
M. abscessus	10/10	100%	2.09
M. arupense	4/4	100%	2.09
M. chelonae	12/12	100%	2.04
M. fortuitum	14/14	100%	2.13
M. gordonae	14/14	100%	1.98
M. immunogenum	4/4	100%	1.98
M. kansasii	4/4	100%	2.26
M. lentiflavum	3/3	100%	1.91
M. marinum	6/6	100%	2.14
M. mucogenicum group	5/5	100%	1.94
M. neoaurum	3/3	100%	2.15
M. peregrinum	4/4	100%	2.07
M. scrofulaceum	3/3	100%	1.96
M. szulgai	4/4	100%	1.95
M. xenopi	3/3	100%	2.27

- MTBC
- MAC
- 15 species of NTM

Difficult Organisms

- MALDI cannot identify to species level within the MTB complex
- Cannot differentiate closely related species
  - *M. chimaera/intracellulare*
  - *M. mucogenicum/phocaicum*
  - *M. marinum/M. shotsii*
  - *M. kansasii/M. gastri*
- Challenging to ID to sub-species level for *M. abscessus*
  - Teng et al. (2013): cluster analysis of spectra (*abscessus* vs. *massiliense*)
  - Fangous et al. (2014): 5 discriminating MALDI peaks for three subspecies

Difficult Organisms

• *M. hiberniae*[Q] • *M. engbaekii*[Q] • *M. interjectum*[Q]	*M. conceptionense*[W] *M. genevense*[W] *M. porcinum*[W] *M. septicum*[W]

(Q) Quinlan et al. 2015
(W) WSLH validation data

Use of MALDI for Primary Positive MGIT tubes

- Need: ID directly from AFB-positive MGIT tube without need to subculture
- How much growth is needed for good MALDI ID?
- Is there something in the MGIT tubes that causes high “background” or “noise”?

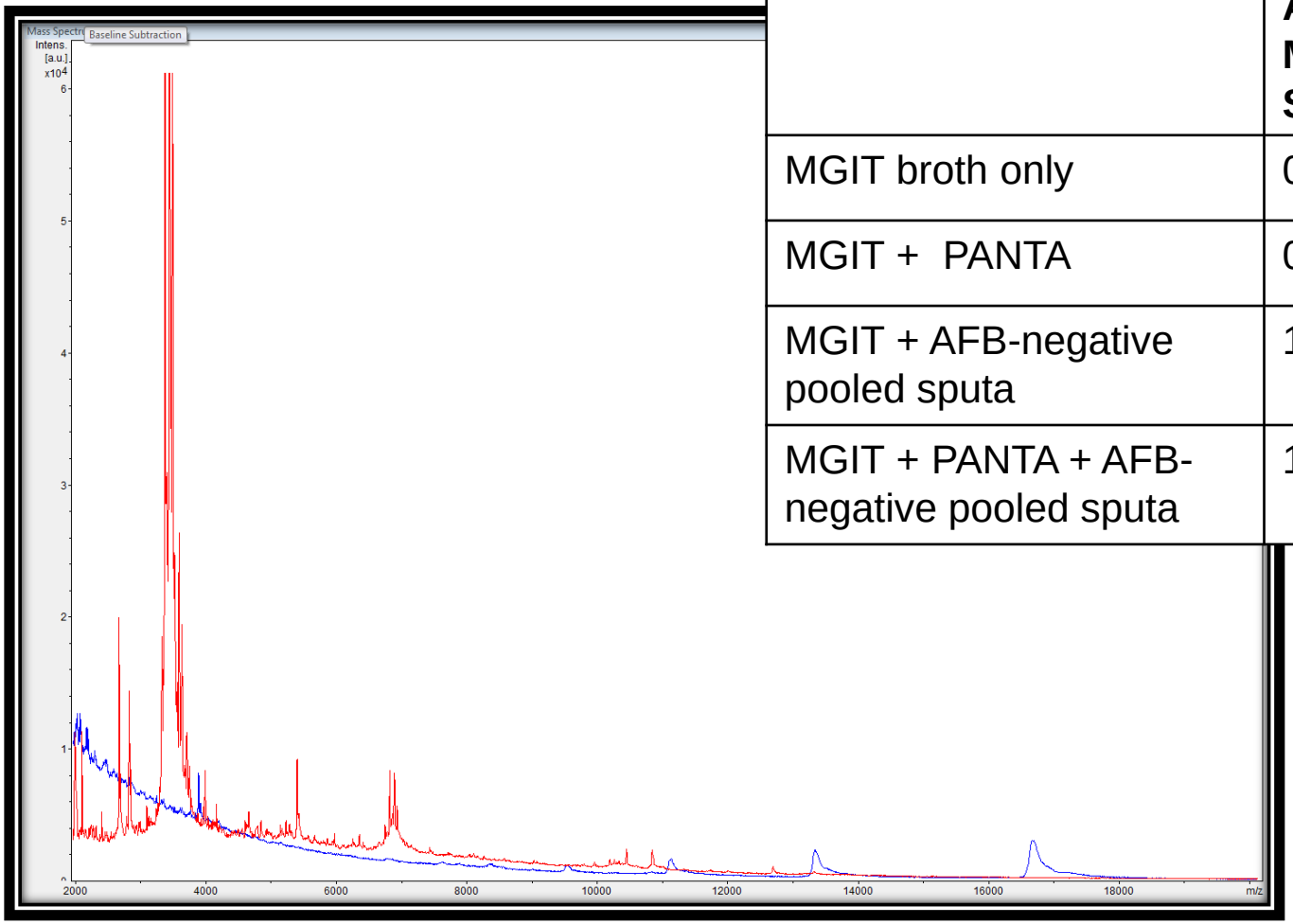
“Limit of Detection” for MALDI

	Percentage Detected		
	7H9	MGIT broth	MGIT + PANTA + AFB-negative pooled sputa
$6.00x10^8$ cells	100%	100%	66.6%
$6.00x10^7$ cells	100%	0%	0%
$3.00x10^7$ cells	12.5%	0%	0%
$6.00x10^6$ cells	0%	0%	0%

“Limit of Detection” for MALDI

- Cell concentration needed for good MALDI ID is approximately 6.00×10^7 to 6.00×10^8 cells
- This LOD is similar to that for HPLC (UV)
- Approximate concentration at which MGIT tube flags positive is 1×10^5 to 1×10^6 cells/ml (BD package insert).
- Quinlan et al. (2015) showed need for 5 days of extended incubation before adequate MALDI ID from MBBacT ALERT 3D bottles

Primary MGIT Spectral Background

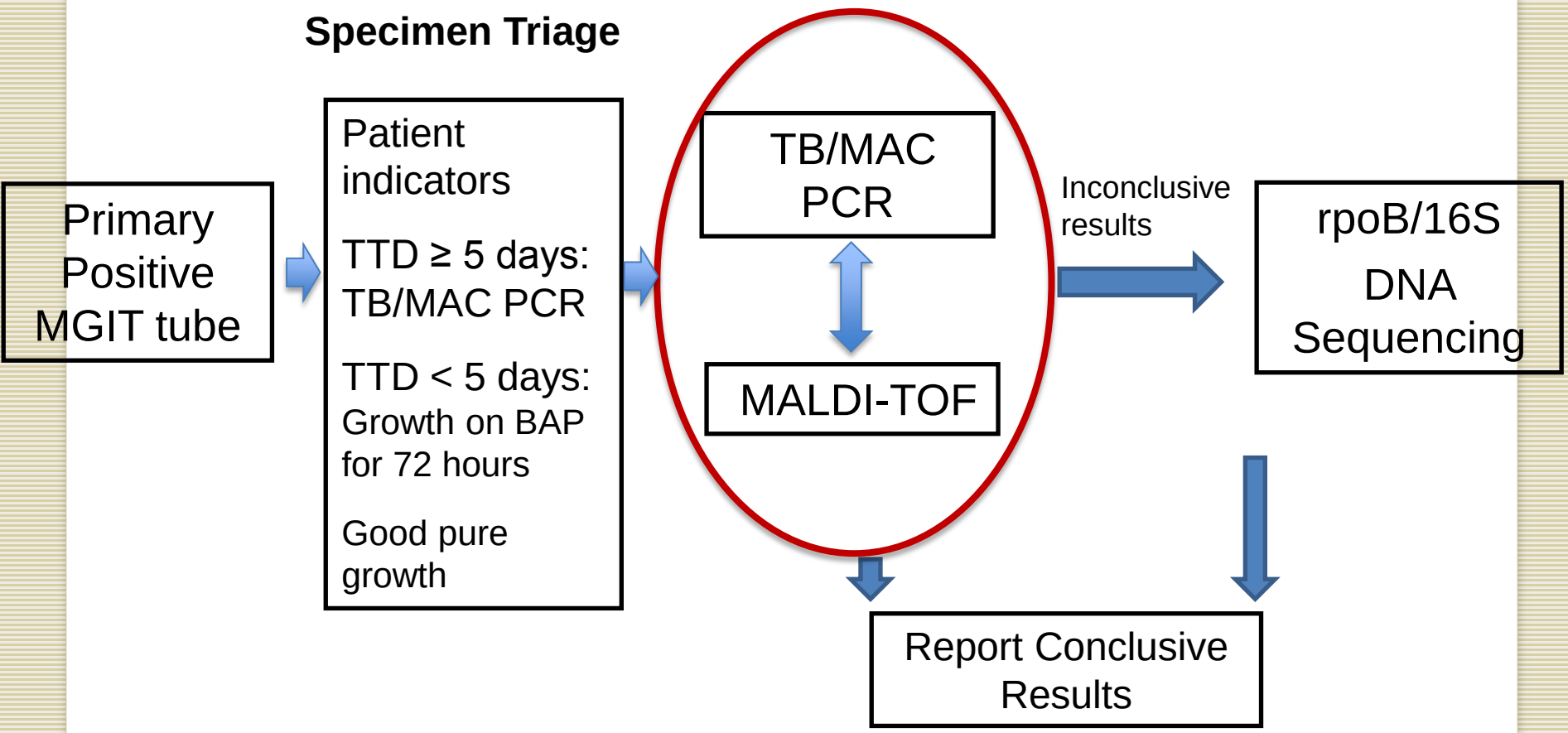


	Average MALDI Score
MGIT broth only	0
MGIT + PANTA	0 (blue)
MGIT + AFB-negative pooled sputa	1.20
MGIT + PANTA + AFB-negative pooled sputa	1.25 (red)

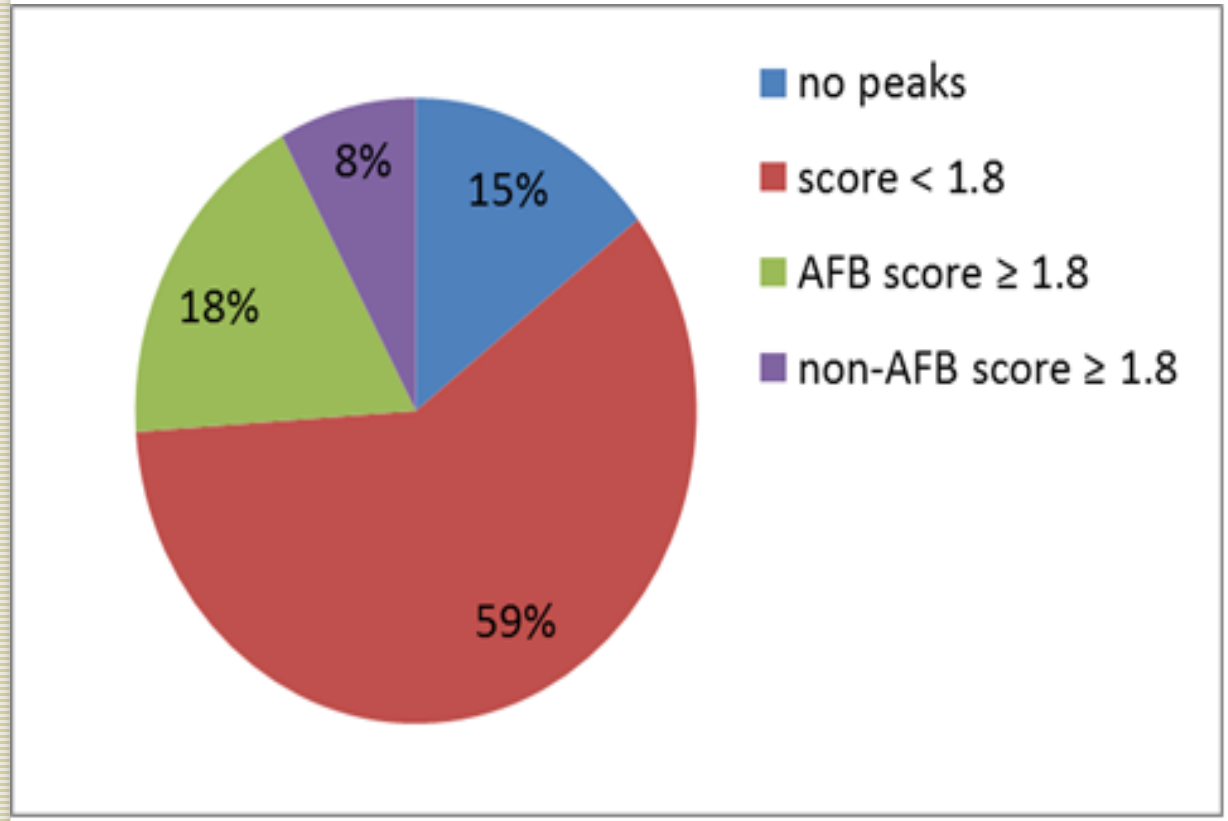
Challenges: Use of MALDI for Primary Positive MGIT tubes

- When the MGIT tube flags as positive, the cell concentration is likely too low for good MALDI ID
- Proteinaceous substances from patient inoculum may cause spectral background and low-level MALDI scores
- MGIT tubes can contain non-acid fast growth or mixed growth which can't be identified by MALDI

WSLH Algorithm for Primary Positive MGIT Tubes



MALDI ID from Primary MGIT n=152 MGIT tubes



Specimen Triage:

- MGIT tubes with TTD ≥ 5 days for which TB/MAC PCR was negative
- MGIT tubes with TTD < 5 days for which non-acid fast growth was not present after 72 hours
- Good pure growth

After-Validation use of MALDI

Media Type	Number of data points (spots)	Number of definitive ID Score ≥ 1.8	Percentage ID (%)
LJ Slant	80	67	83.7
7H10/7H11 solid media	726	508	69.9
MGIT (referred)	242	150	61.9
Blood Agar Plate	28	17	60.7
VersaTREK	38	22	57.8
7H9 broth	44	22	50
BacT/Alert broth	101	47	46.5
MGIT (primary)	303	79	26

Considerations for MALDI-TOF

Limitations

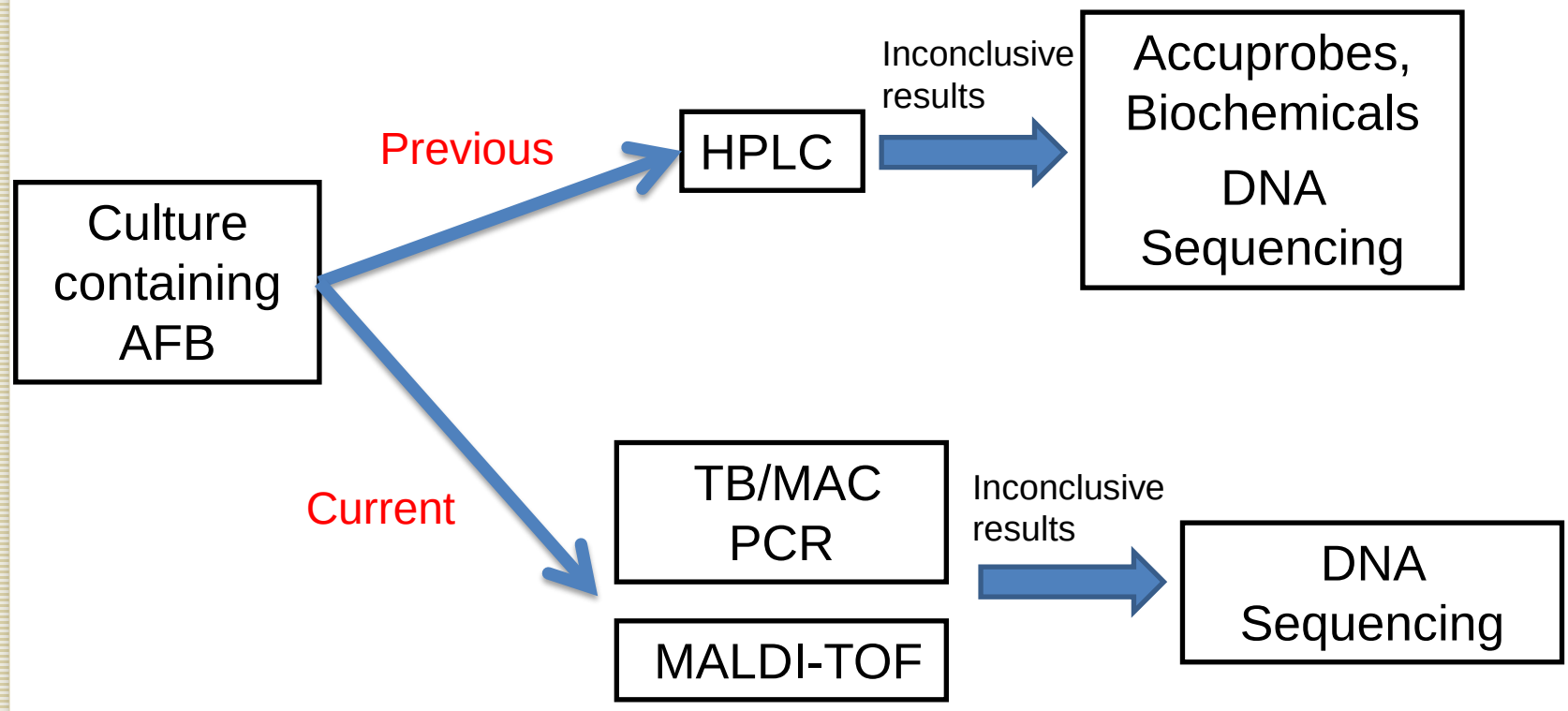
- Time-consuming extraction protocol for mycobacteria
- Cannot identify to species within the MTBC
- Need adequate biomass and pure growth for ID
  - Poor performance with primary MGIT broth
- Scores may vary depending on growth conditions
- Current database limitations
  - For best ID, extraction method must be identical to that used to create spectral library
- Initial cost investment high
- Full validation necessary

Considerations for MALDI-TOF

Advantages

- Rapid identification
- Good identification to species for rapid growers and other mycobacteria groups and complexes
- Potential sub-species ID for *M. abscessus*
- Can also be used for identification of many bacteria and fungi in the laboratory (FDA-cleared for some bacteria)
- Databases and extraction methods are quickly changing and improving

Evolution of WSLH ID Algorithm



APHL MALDI User's Group

Purpose: The *MALDI-TOF discussion group* is a forum for public health laboratorians currently using or interested in using MALDI-TOF MS for the identification of mycobacteria species to discuss questions and considerations, exchange best practices, and identify priority information needs.

Goal: Based on the content of the user's group discussions, APHL will form a workgroup to develop products that will address the priority needs. The products will assist laboratories to successfully implement MALDI-TOF for the identification of mycobacterial species.

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Poster #5

Validation of MALDI-TOF For Identification of Mycobacteria

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REVISED ABSTRACT

Background: Recent advancements in Matrix-assisted Laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS or "MALDI") have permitted its application to the field of clinical microbiology. We validated the Bruker Biotyper system for identification of mycobacteria and integrated this method as a key component of our novel testing algorithm. **Methods:** The Bruker MycoEX protein extraction protocol(version 3) was optimized through the addition of a secondary liquid media wash step, and the post-extraction viability of MTBC was determined. Variables known to affect the quality of MALDI identification scores including culture age and media type were evaluated. Most importantly, the ability of MALDI to identify acid-fast organisms directly from newly-positive MGIT broth culture was evaluated. **Results:** We analyzed almost 200 independently identified strains representing 42 species of mycobacteria and fully validated our testing algorithm for identification of *M. tuberculosis* complex (MTBC), *M. avium* complex (MAC), and 15 species of other non-tuberculous mycobacteria (NTM). MALDI could identify pure growth of mycobacteria from 7H10 plate, 7H9 broth and LJ slant with little difference in score and no difference in identification. Between $6x10^7$ to $6x10^8$ organisms were needed for adequate identification, and MALDI scores were shown to decrease as cultures age. Direct MALDI identification from positive MGIT cultures inoculated with primary patient specimens proved difficult due to low numbers of cells and high background from patient inoculum proteins. **Conclusions:** Although identification could not routinely be obtained from MGIT cultures, MALDI has the potential to yield quicker, less expensive, more automated, and more accurate results than previous identification systems if carefully integrated into an identification work flow.

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INTRODUCTION

There are over 150 species of mycobacteria, including *M. tuberculosis*. The number of characterized, clinically significant NTM has risen significantly within the last 15 years (1). Many of these newer organisms cannot be accurately identified by classical methods such as High Performance Liquid Chromatography (HPLC), biochemical reactions and Accuprobes. DNA gene sequencing, considered by some to be the gold standard, identifies most of these organisms to the species level but requires expertise, is labor intensive and expensive. With recent FDA clearance, MALDI analysis of microbial proteins is quickly becoming a standard of practice for identification of Gram-negative organisms, Gram-positive organisms, and yeast in clinical laboratories. MALDI is also being considered in many mycobacteriology laboratories due to the limitations of current identification methods. In this study we describe our evaluation of the Bruker MALDI Biotyper system for identification of mycobacteria and our incorporation of MALDI as a focal point of a new mycobacteria identification algorithm at the Wisconsin State Laboratory of Hygiene (WSLH).

METHODS

- **Extraction:** All cultures were extracted using an optimized version of the Bruker MycoEX extraction protocol featuring an additional wash step for all liquid media cultures, heat lysis, physical (silica bead beating), and chemical (formic acid/acetonitrile) extraction.
- **Viability Study:** To confirm complete inactivation of mycobacterial cells, MTBC extracts were used to inoculate 7H10 plates, blood agar plates, and 7H9 broth after completion of 30 minute heat lysis step. These cultures were incubated for 6 weeks and extracts were shown to be non-viable (data not shown).
- **Culture age/Media type study:** To investigate the effects of culture age and media type on MALDI score, four species of mycobacteria (MTBC ATCC27294, MAC ATCC 700898, *M. chelonae* WIS1004, and *M. abscessus* WIS1001) were grown on three media types (7H9 broth, 7H10 plate, and LJ slant). These cultures were extracted and analyzed in duplicate at 7 time points across 42 days of incubation (Figures 1A & 1B).
- **Species Validation:** : During our study, we validated accurate MALDI identification for *M. tuberculosis complex*, *M. avium complex* and 15 other species and groups of NTM (Table 1). Before MALDI results could be reported for each species, confirmatory testing of at least three unique patient strains was performed by Accuprobe, real-time PCR, 16S and/or *rpoβ* DNA sequencing.
- **Primary MGIT Spectral Background:** BBL MGIT broth was mixed with BBL OADC enrichment/PANTA antibiotic mixture ("PANTA") and AFB negative pooled sputa, both separately and in combination. These mixtures, along with an un-inoculated MGIT broth control, were extracted and analyzed in the absence of mycobacteria to investigate possible sources spectral background effecting MALDI-TOF analysis from primary patient MGIT cultures (Table 2 and Figure 2).
- **Limit of Detection (LOD):** To estimate the limit of detection for the Bruker MALDI Biotyper, three media types (7H9 broth, MGIT broth, and MGIT broth plus 10% PANTA and 6.25% AFB negative pooled sputa) were seeded with serial dilutions of MTBC from a 0.5 McFarland standard, with estimated concentrations ranging from $5x10^4$ to $1x10^8$ cells/ml. Each dilution was extracted and analyzed in triplicate from 6ml aliquots giving final cell counts ranging from $3x10^3$ to $6x10^6$ cells/ml (Table 3).
- **MALDI Identification from Primary Positive MGIT:** After our initial validation was complete, primary positive MGIT specimens that met the following criteria were routinely analyzed in our laboratory: 1) MGIT tubes with time to detection (TTD) ≥ 5 days for which TB and MAC real-time PCR was negative, and 2) MGIT tubes with TTD < 5 days for which non-acid fast growth on blood agar plate was not present after 72 hours of incubation (Figure 3).

ACKNOWLEDGEMENTS

This validation study would not have been possible without the hard work of the WSLH Mycobacteriology Team: Youngmi Kim, Ana Guaracao, Julie Brockman and Ali Lopez.

RESULTS

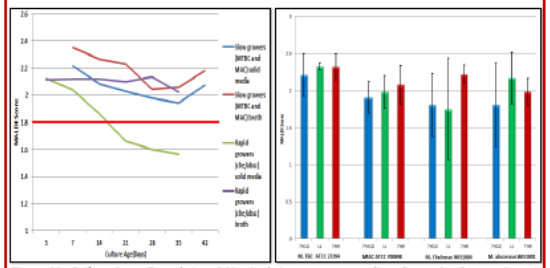


Figure 1A, Culture Age. Four strains of Mycobacteria were grown on three types of culture media and incubated for a total of 42 days. MALDI was performed on duplicate extractions from growth at 7 time points during the experiment (4 spots per species at each time point). Shown are trends of MALDI scores over time for both liquid and solid media cultures. A MALDI score of 1.8 is the validated threshold for reporting mycobacteria identifications, this benchmark is indicated by a red line. **Figure 1B, Media Comparison.** MALDI scores for each media type were combined and averaged over the 42-day period (7 time points, 28 spots total) for each of four mycobacteria strains. The black error bars depict two standard deviations from the mean. Zero misidentifications were recorded.

Organism(s)	Number of isolates with reportable MALDI score	Percentage of isolates with reportable MALDI score	Average MALDI score
M. tuberculosis complex	20/20	100%	2.16
M. avium complex	50/50	100%	2.058
M. abscessus	10/10	100%	2.09
M. arupense	4/4	100%	2.09
M. chelonae	12/12	100%	2.04
M. fortuitum	14/14	100%	2.13
M. gordonae	14/14	100%	1.98
M. immunogenum	4/4	100%	1.98
M. kansasii	4/4	100%	2.26
M. lentiflavum	3/3	100%	1.91
M. marinum	6/6	100%	2.14
M. mucogenicum group	5/5	100%	1.94
M. neoaurum	3/3	100%	2.15
M. peregrinum	4/4	100%	2.07
M. scrofulaceum	3/3	100%	1.96
M. szulgai	4/4	100%	1.95
M. xenopi	3/3	100%	2.27

Table 1, Species validation. Listed are the number and percentage of correctly identified isolates during our preliminary validation study of 146 isolates. Each identification was confirmed by an alternative method(s) including 16s/rpoβ sequencing, real-time PCR, and AccuProbe assay. We found that MALDI identifications with scores ≥ 1.8 were accurate 99% of the time. An additional 53 isolates have been analyzed and independently confirmed for 12 species NTM not yet fully validated (data not shown).

	Average MALDI Score
MGIT Broth only	0
MGIT +PANTA(10%)	0
MGIT+sputum(6.25%)	1.204333333
MGIT+PANTA(10%)/sputum(6.25%)	1.256666667

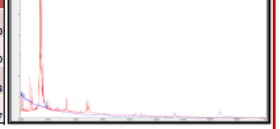
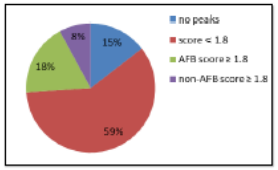


Table 2, Primary MGIT Spectral Background. Uninoculated MGIT broth was combined with AFB negative pooled sputa ("sputum") and supplement/antibiotic mixture ("PANTA") at concentrations analogous to primary specimen culturing practices. 6ml aliquots of the resulting media combinations were extracted in triplicate and analyzed in duplicate. **Figure 2, MALDI Background Spectra.** Shown are MALDI spectra exhibited by sterile MGIT broth plus 6.25% sputum (in red) and sterile MGIT broth plus 10% PANTA (in blue).

	7H9	MGIT broth	MGIT plus sputum(6.25%) /PANTA(10%)
$6.00x10^8$ cells	8/8=100%	6/6=100%	4/6=66.6%
$6.00x10^7$ cells	8/8=100%	0/6=0%	0/6=0%
$3.00x10^7$ cells	1/8=12.5%	0/6=0%	0/6=0%
$6.00x10^6$ cells	0/8=0%	0/6=0%	0/6=0%

Table 3-Limit of Detection. Shown are the number and percent of samples from three media types correctly identified by the MALDI extraction and analysis as MTBC. The cell counts are estimated from serial dilutions of a 0.5 McFarland standard of MTBC strain ATCC27294. The limit of reliable detection is estimated as $6.00x10^7$cells for 7H9 broth cultures and $6.00x10^8$ cells for MGIT cultures (with and without sputum/PANTA). These detection limits are similar to those yielded in side-by-side comparison with HPLC identification system (data not shown). The estimated cell concentration when a MGIT tube first flags positive is 1 X 10^8 to 1 X 10^9 cells/ml (3).



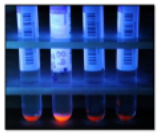


Figure 3: MALDI Identification from Primary Positive MGIT. Primary positive MGIT specimens that met the following criteria were routinely analyzed in our laboratory: 1) MGIT tubes with time to detection (TTD) ≥ 5 days for which TB and MAC real-time PCR were negative, and 2) MGIT tubes with TTD < 5 days for which non-acid fast growth on blood agar plate was not present after 72 hours of incubation. Results include 152 MGIT tubes (303 total spots) analyzed between November 2014 and April 2015. Shown are the percent of samples with no MALDI identification ("no peaks" or score < 1.8) and with definitive ID (score ≥ 1.8) of Mycobacteria ("AFB") and non-Mycobacteria ("non-AFB").

SUMMARY AND CONCLUSIONS

- **The Bruker MALDI-TOF instrument and MycoEx extraction protocol were adapted, optimized, and validated for identification of *M. tuberculosis complex*, *M. avium complex*, and 15 other species of nontuberculous mycobacteria at the Wisconsin State Laboratory of Hygiene (Table 1).**
- **Adequate identifications could be obtained with slight decreases in score over 42 days for slowly growing mycobacteria (MTBC and MAC) on solid and liquid media and rapid growers (*M. abscessus* and *M. chelonae*) in liquid culture. A significant decrease in score over time was observed in solid media cultures of rapidly growing mycobacteria (Figure 1A).**
- **Three common media types (7H9 broth, 7H10 plate, and LJ slant) could all be used for reliable identification of mycobacteria with no difference in identification and no significant difference in score (Figure 1B).**
- **Our experiments showed that neither BBL OADC enrichment/PANTA antibiotic mixture nor components of MGIT broth were detected during MALDI analysis of primary patient cultures; however, biomolecules/proteins in AFB negative pooled sputa could be detected by MALDI and significantly impacted analysis as evidenced by spectral data and numerical scores obtained (Table 2 & Figure 2).**
- **The MALDI identification system's limit of detection is approximately $6.00x10^7$cells for 7H9 broth cultures and $6.00x10^8$ cells for MGIT cultures (Table 3) which is well above the concentration of organisms present at the time a MGIT tube flags positive (3).**
- **The spectral background from patient inoculum biomolecules and a relatively high limit of detection combine to explain an inability to reliably achieve mycobacteria identifications from direct MALDI analysis of primary MGIT cultures. MALDI analysis yielded adequate identification (score ≥ 1.8) for approximately 26% of MGIT broths tested (Figure 4).**
- **WSLH implemented a testing algorithm in which new positive MGIT tubes are triaged by real-time PCR for detection of MTBC and MAC and blood agar plate for detection of non-AFB growth before MALDI is performed. This algorithm incorporates the benefits of MALDI and other molecular methods for rapid and accurate identification to species for mycobacteria.**

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2) Bruker Standard Operating Procedure Mycobacteria Extraction (MycoEX) method, Revision 3, January 2014.
3)BD BBL MGIT Mycobacteria Growth Indicator Tube 7 ml with BACTEC MGIT 960 Supplement Kit, package insert L000180JAA(01), Becton Dickinson and Company, 2013.

WSLH Extraction Method

WSLH MYCOBACTERIA EXTRACTION METHOD FOR MALDI TOF

Reagents and Supplies Needed:

1. Molecular grade water
2. 100% Ethyl Alcohol
3. 70% formic acid (prepared new each run from 100% formic acid: in a fume hood add 700ul 100% formic acid to 300 ul water in a microcentrifuge tube)
4. Zirconia/silica beads, Biospec Products, 11079105
5. Acetonitrile (1ml aliquot in microcentrifuge tube)

From broth culture (extra wash steps):

1. Concentrate each broth specimen:
   a. Vortex broth culture. Transfer 3-6 ml of broth to a 15 ml sterile conical tube for centrifugation.
   b. Centrifuge broth for 15 min at 6000 X *g*
   c. Decant as much supernatant as possible (being careful not to dislodge pellet)
2. Add 1 ml of molecular grade water, vortex, and transfer to a 1.5 ml microcentrifuge tube.
3. Centrifuge at 14000 rpm for 2 minutes and discard supernatant.
4. Add 300 ul of molecular grade water, vortex to homogenize and continue with step 6 below.

From solid media plates or slants:

5. Make suspension from growth on solid media in 300 ul of molecular grade H2O (at least a 2 McFarland works best), vortex to homogenize as necessary.
6. Heat for **30 minutes** at 90-100C degrees, cool. Centrifuge briefly to remove condensation from caps.
7. Add 900 ul of 100% EtOH, vortex briefly.
8. Centrifuge 14000 rpm for 2 minutes, Pour off supernatant.
9. Re-centrifuge pellet for 30sec at 14000 rpm and aspirate off remaining supernatant using a fine-tip transfer pipet.
10. Dry pellets by uncapping tubes and laying them in BSC for about 5 minutes.
11. Add about 20ul of zirconia/silica beads to each tube.
12. Add 10-50ul of acetonitrile and dislodge pellet using racking technique. Vortex tubes for 1 minute using multi-tube vortexer.
13. Add 10-50ul 70% formic acid (use same volume as acetonitrile), vortex 10 seconds using multi-tube vortexer.
14. Centrifuge 14000 rpm for 2 minutes
15. Spot 1ul supernatant onto the MALDI target, allow to dry. Perform in duplicate (two spots) for each specimen.
16. Overlay with 1 ul matrix, allow to dry.

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Comments or Questions??