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Breathing Science is Life.®

Mycobacterial Lab

Reeti Khare, PhD
Associate Professor

NTM Lecture Series

for Providers

April 25-26, 2024

Conflicts of Interest

- Lab contract research: INSMED, Spero Therapeutics, RedHill Biopharma, AN2 Therapeutics, Paratek Pharmaceuticals, Mannkind Corporation
- Lab research grants: Illumina
- Lab reagents received: BioMerieux

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Learning objectives

- Understand smear and culture methods for NTM
- Evaluate methods of NTM identification
- Review methods for NTM susceptibility testing

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Classification and taxonomy

Non-tuberculous mycobacteria

Growth on solid media within 7 days

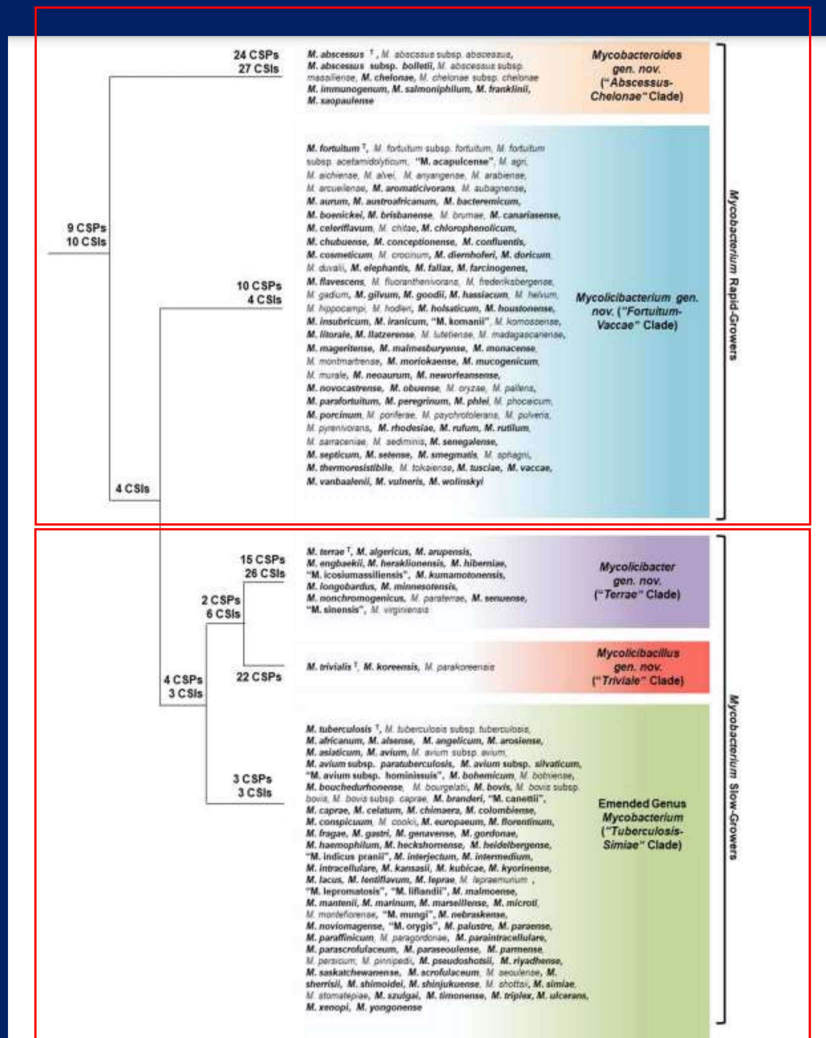
Rapidly growing mycobacteria (RGM)

Growth on solid media after 7 days

Slowly growing mycobacteria (SGM)

Runyoun classification:

1) Nonchromogens
2) Photochromogen
3) Scotochromogen



Gupta et al. Phylogenomics and Comparative Genomic Studies Robustly Support Division of the Genus Mycobacterium into an Emended Genus Mycobacterium and Four Novel Genera. Front Microbiol, 2018

Slowly growing mycobacteria (SGM)

Mycolicibacter

M. terrae [T], **M. algericus, M. arupensis, M. engbaekii, M. heraklionensis, M. hiberniae, "M. icosiumassiliensis", M. kumamotonensis, M. longobardus, M. minnesotensis, M. nonchromogenicus,** M. paraterrae, **M. senuense, "M. sinensis",** M. virginiensis

Mycolicibacillus sp.

M. trivialis [T], **M. koreensis,** M. parakoreensis

Mycobacterium sp.

M. tuberculosis [T], M. tuberculosis subsp. tuberculosis, **M. africanum, M. alsense, M. angelicum, M. arosiense, M. asiaticum, M. avium,** M. avium subsp. avium, **M. avium subsp. paratuberculosis, M. avium subsp. silvaticum, "M. avium subsp. hominissuis", M. bohemicum,** M. botniense, **M. bouchedurhonense,** M. bourgelatii, **M. bovis,** M. bovis subsp. bovis, M. bovis subsp. caprae, **M. branderi, "M. canettii", M. caprae, M. celatum, M. chimaera, M. colombiense, M. conspicuum,** M. cookii, **M. europaeum, M. florentinum, M. fragae, M. gastri, M. genavense, M. gordonae, M. haemophilum, M. heckshornense, M. heidelbergense, "M. indicus pranii", M. interjectum, M. intermedium, M. intracellulare, M. kansasii, M. kubicae, M. kyorinense, M. lacus, M. lentiflavum, M. leprae,** M. lepraemurium **, "M. lepromatosis", "M. liflandii", M. malmoense, M. mantenii, M. marinum, M. marseillense, M. microti,** M. montefiorense, **"M. mungi", M. nebraskense, M. noviomagense, "M. orygis", M. palustre, M. paraense, M. paraffinicum,** M. paragordonae, **M. paraintracellulare, M. parascrofulaceum, M. paraseoulense, M. parmense,** M. persicum, M. pinnipedii, **M. pseudoshottsii, M. riyadhense, M. saskatchewanense, M. scrofulaceum,** M. seoulense, **M. sherrisii, M. shimoidei, M. shinjukuense,** M. shottsii, **M. simiae,** M. stomatepiae, **M. szulgai, M. timonense, M. triplex, M. ulcerans, M. xenopi, M. yongonense, M. vulneris**

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Rapidly growing mycobacteria (RGM)

Mycobacteroides sp.

M. abscessus T, M. abscssus subsp. abscessus, **M. abscessus subsp. bolletii,** M. abscessus subsp. massiliense, **M. chelonae,** M. chelonae subsp. chelonae **M. immunogenum, M. salmoniphilum, M. franklinii, M. saopaulense**

Mycolicibacterium sp.

M. fortuitum T, M. fortuitum subsp. fortuitum, M. fortuitum subsp. acetamidolyticum, **"M. acapulcense"**, M. agri, M. aichiense, M. alvei, M. anyangense, M. arabiense, M. arcueilense, **M. aromaticivorans**, M. aubagnense, **M. aurum, M. austroafricanum, M. bacteremicum, M. boenickei, M. brisbanense**, M. brumae, **M. canariasense, M. celeriflavum**, M. chitae, **M. chlorophenolicum, M. chubuense, M. conceptionense, M. confluentis, M. cosmeticum**, M. crocinum, **M. diernhoferi, M. doricum**, M. duvalii, **M. elephantis, M. fallax, M. farcinogenes, M. flavescens**, M. fluoranthenivorans, M. frederiksbergense, M. gadium, **M. gilvum, M. goodii, M. hassiacum**, M. helvum, M. hippocampi, M. hodleri, **M. holsaticum, M. houstonense, M. insubricum, M. iranicum, "M. komanii"**, M. komossense, **M. litorale, M. llatzerense**, M. lutetiense, M. madagascariense, **M. mageritense, M. malmesburyense, M. monacense**, M. montmartrense, **M. moriokaense, M. mucogenicum**, M. murale, **M. neoaurum, M. neworleansense, M. novocastrense, M. obuense**, M. oryzae, M. pallens, **M. parafortuitum, M. peregrinum, M. phlei**, M. phocaicum, **M. porcinum**, M. poriferae, M. psychrotolerans, M. pulveris, M. pyrenivorans, **M. rhodesiae, M. rufum, M. rutilum**, M. sarraceniae, M. sediminis, **M. senegalense, M. septicum, M. setense, M. smegmatis**, M. sphagni, **M. thermoresistibile**, M. tokaiense, **M. tusciae, M. vaccae, M. vanbaalenii, M. vulneris, M. wolinskyi**

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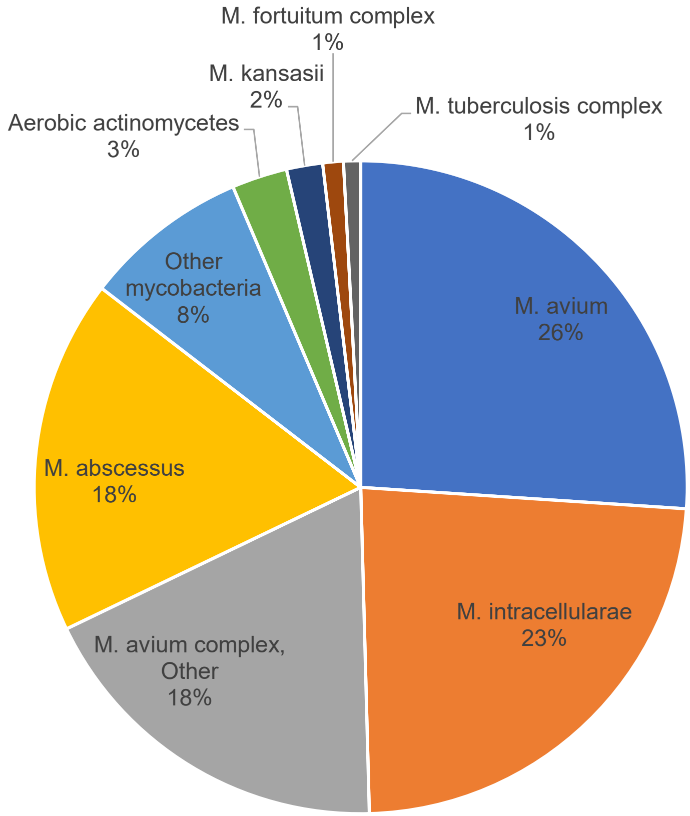
Most common organisms seen in our lab



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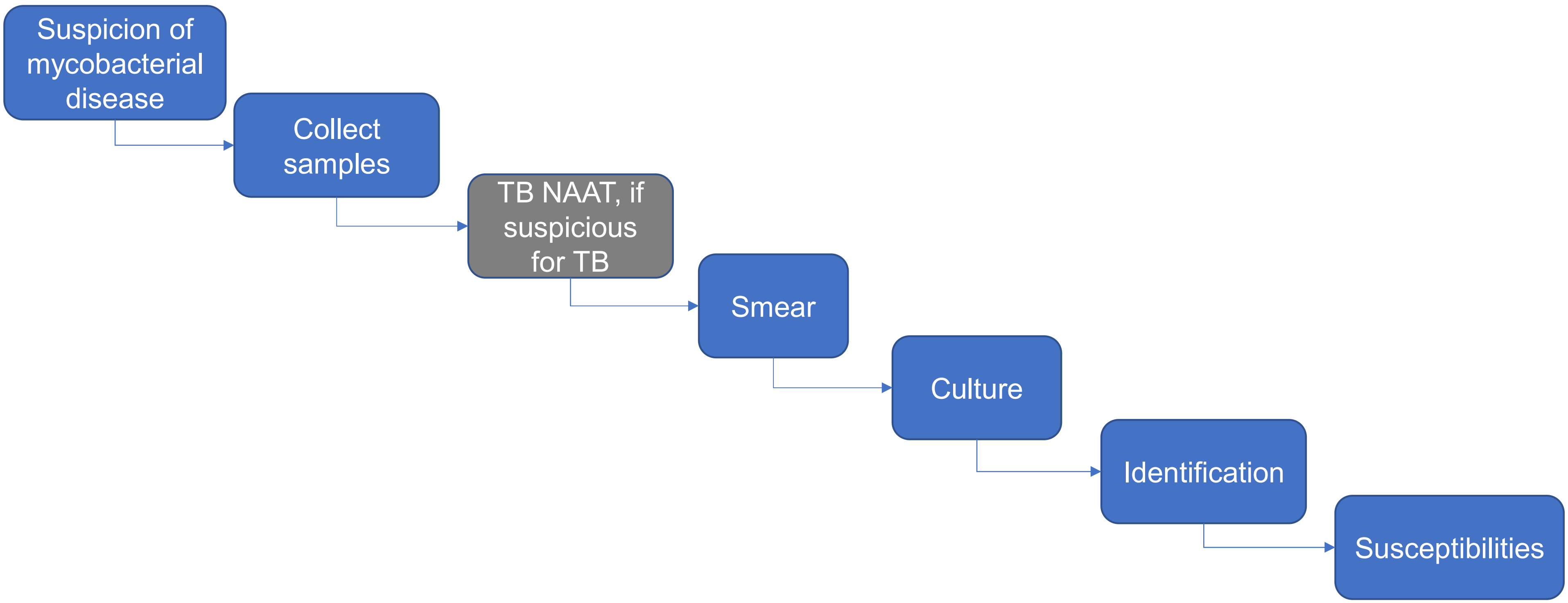
General algorithm for testing mycobacteria in the lab



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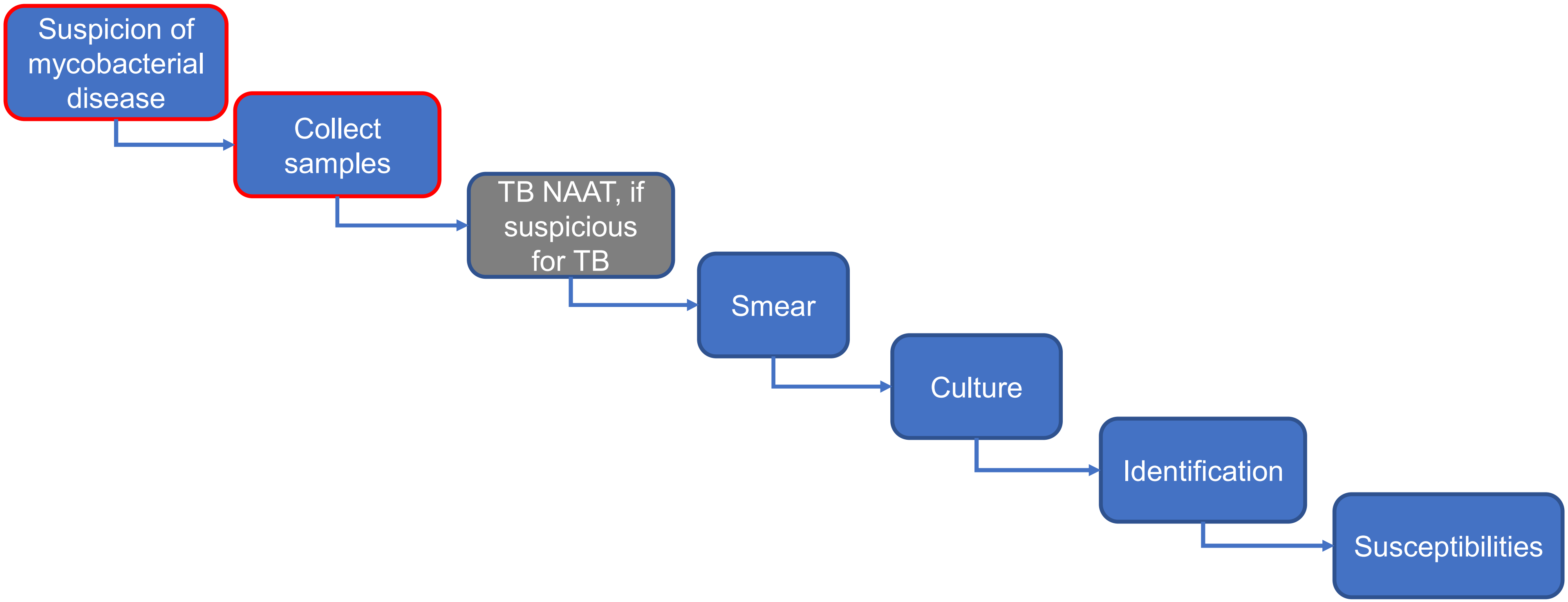

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Lab tests to detect mycobacteria

- NTM exist naturally in the environment, especially water
- NTM can be part of the normal flora[1]
  - Oral flora (likely transient) in 26-33% of people[2]
  - Presence of NTM without progressive disease
- Detection ≠ infection

[1] Thornton et al. The respiratory microbiome and nontuberculous mycobacteria: an emerging concern in human health. European Respiratory Review 2021 30: 200299

[2] Wali et al: The presence of atypical mycobacteria in the mouthwashes of normal subjects: role of tap water and oral hygiene. Ann Thorac Med 2008; 3: pp. 5-

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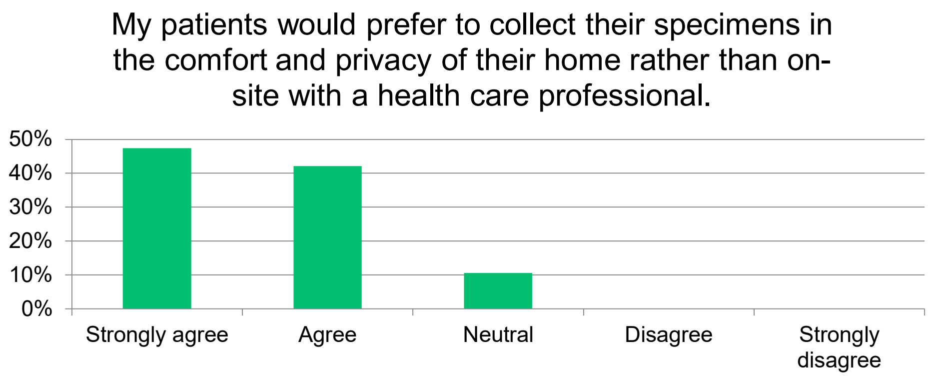
Specimen collection

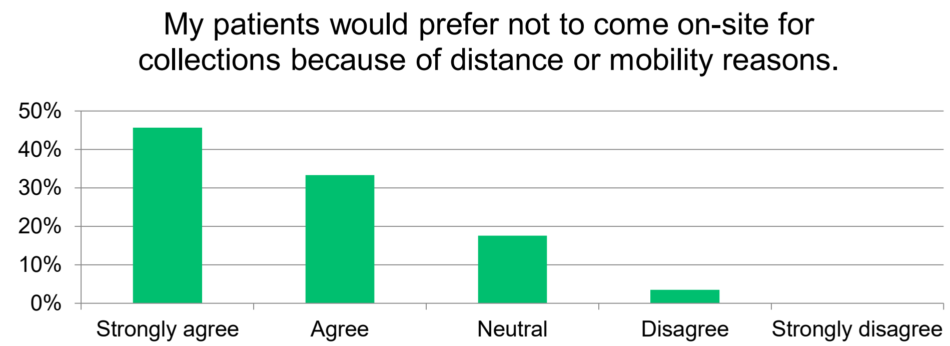
- Collect a “good” sputum (no saliva)
  - Recommended: 5-10 ml[1]
  - Expectorated or induced
- Multiple sputum specimens
  - Positivity over time[2]
  - ≥ 2 sputum cultures positive is more likely to be clinically significant (as high as 98% agreement for clinically significant MAC)[3]
- BALs/Bronchial washings may be more sensitive than sputum
- Home sputum collection

[1]Warren et al. A minimum 5.0 ml of sputum improves the sensitivity of acid-fast smear for Mycobacterium tuberculosis, Am J Respir Crit Care Med, 2000.
[2]van Ingen. Microbiological Diagnosis of Nontuberculous Mycobacterial Pulmonary Disease. Clinics in Chest Medicine, 2015-03-01, Volume 36, Issue 1, Pages 43-54
[3]Daley et al. Treatment of Nontuberculous Mycobacterial Pulmonary Disease: An Official ATS/ERS/ESCMID/IDSA Clinical Practice Guideline. Clinical Infectious Diseases, 2020

Home sputum collection

- Allows patients to collect at home
- Allows direct access to a full-service mycobacteriology lab





[1] Khare et al. Evaluation of Home Collected Sputum for Increased Access to High Quality Laboratory Testing of Nontuberculous Mycobacteria. In progress, World Bronchiectasis Conference, NY, 2023

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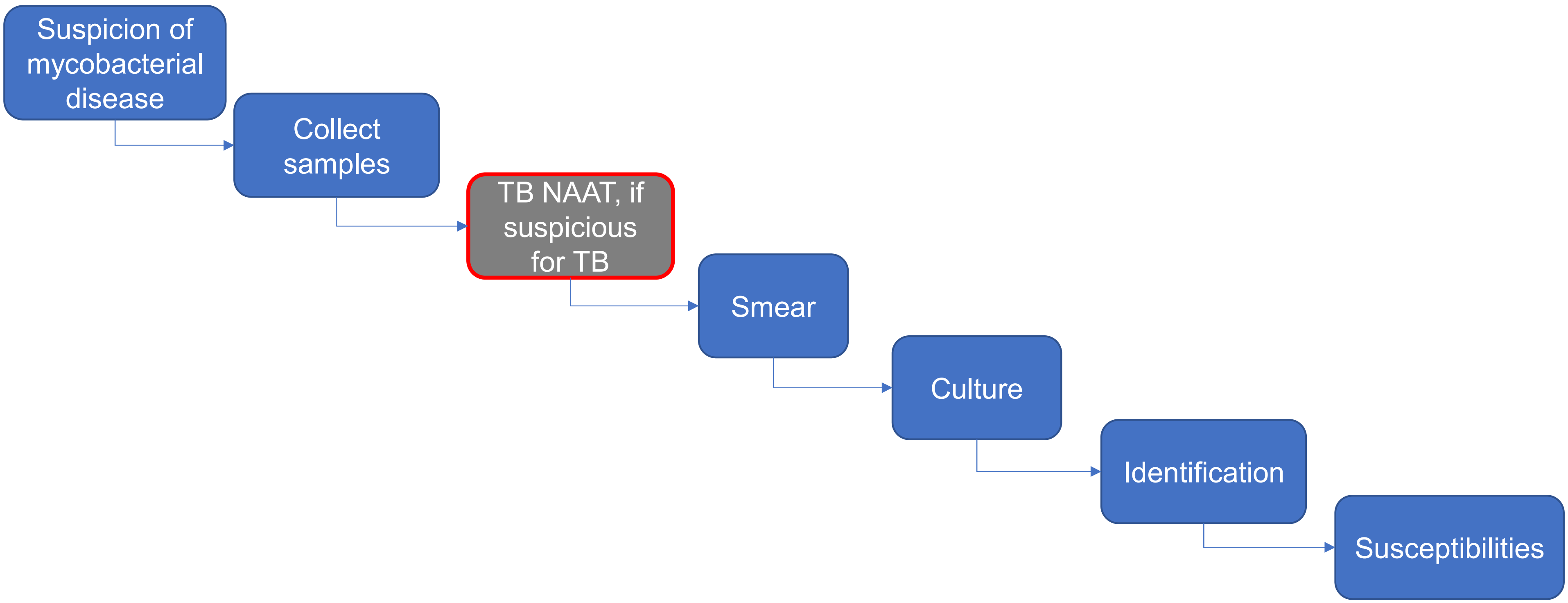
General algorithm for testing mycobacteria in the lab



TB-NAAT assays from specimen

- NAAT: nucleic acid amplification test
- Lab developed tests
- Cepheid Xpert MTB/Rif
  - Real-time PCR
  - Report and rifampin resistance

https://www.cepheid.com/en_US/tests/Critical-Infectious-Diseases/Xpert-MTB-RIF

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MTB		Xpert MTB/RIF	
Source	Population	Sensitivity (%)	Specificity (%)
Sputum/ Pulmonary	Adult	85 (81 for HIV+)	98
	Children	65 (72 for HIV+)	99
Gastric aspirate	Children	73	98-99
Pleural fluid	Adults	50	99
Peritoneal fluid	Adults	59	97
Cerebrospinal fluid	Adults	70	97
Synovial fluid	Adults	97	94
Lymph node aspirate	Adults	89	86
Lymph node biopsy	Adults	82	79
Urine	Adults	85	97

Rifampin		Xpert MTB/RIF	
Source	Population	Sensitivity (%)	Specificity (%)
Pulmonary	Adults	96	98
Extrapulmonary	Adults	96	99

[1]Module 3: Diagnosis - Rapid diagnostics for tuberculosis detection. World Health Organization, Geneva, 2020
[2]Rowlinson, Musser, Khare. *Mycobacterium tuberculosis* Complex, Manual of Clinical Microbiology, 13th ed., in review, 2022

What about NTM-NAAT for direct detection from sputum?

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What about an NTM-NAAT?

- Change in management?
  - Not like TB, which is always considered a pathogen!
  - A positive result is not necessarily useful (can represent contamination)
  - A negative result is not necessarily useful (false negatives)
- Greater diversity that is needed to be detected
- No assays easily available in the US

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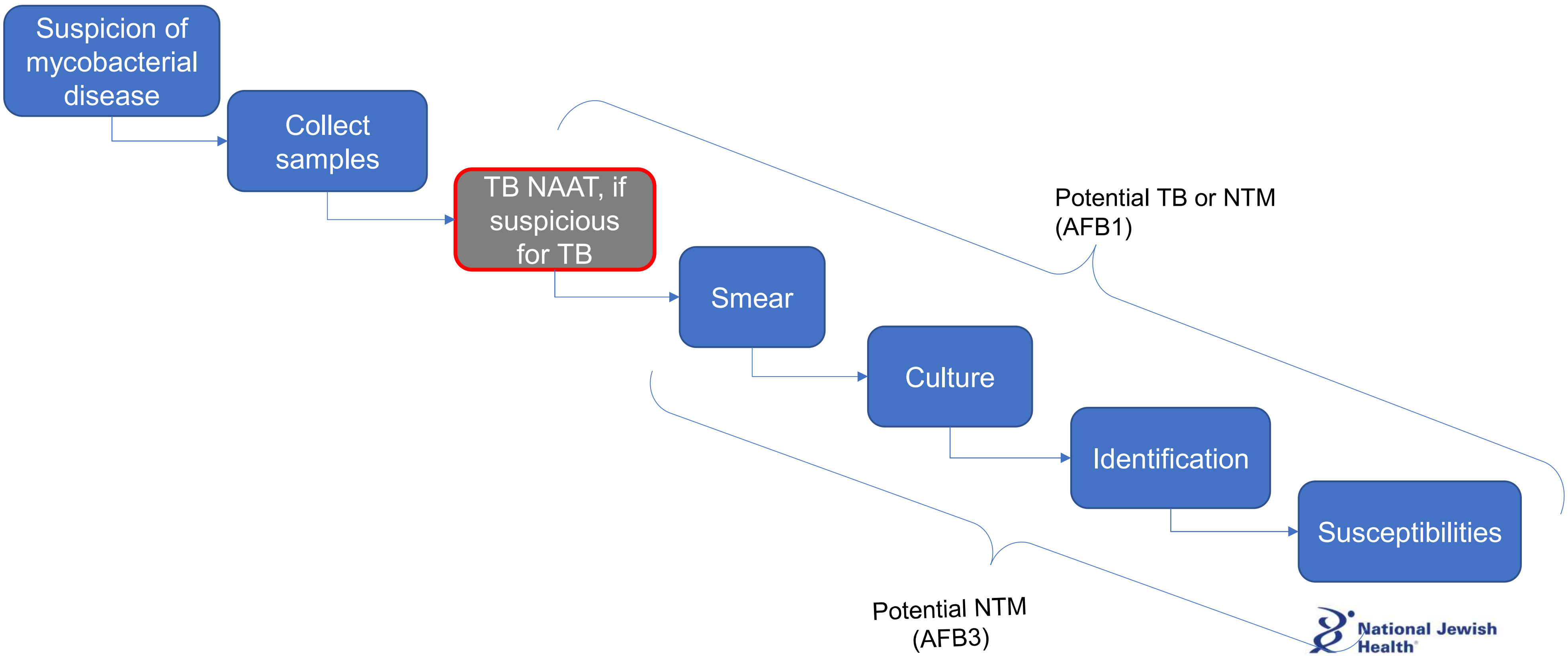
General algorithm for testing mycobacteria in the lab



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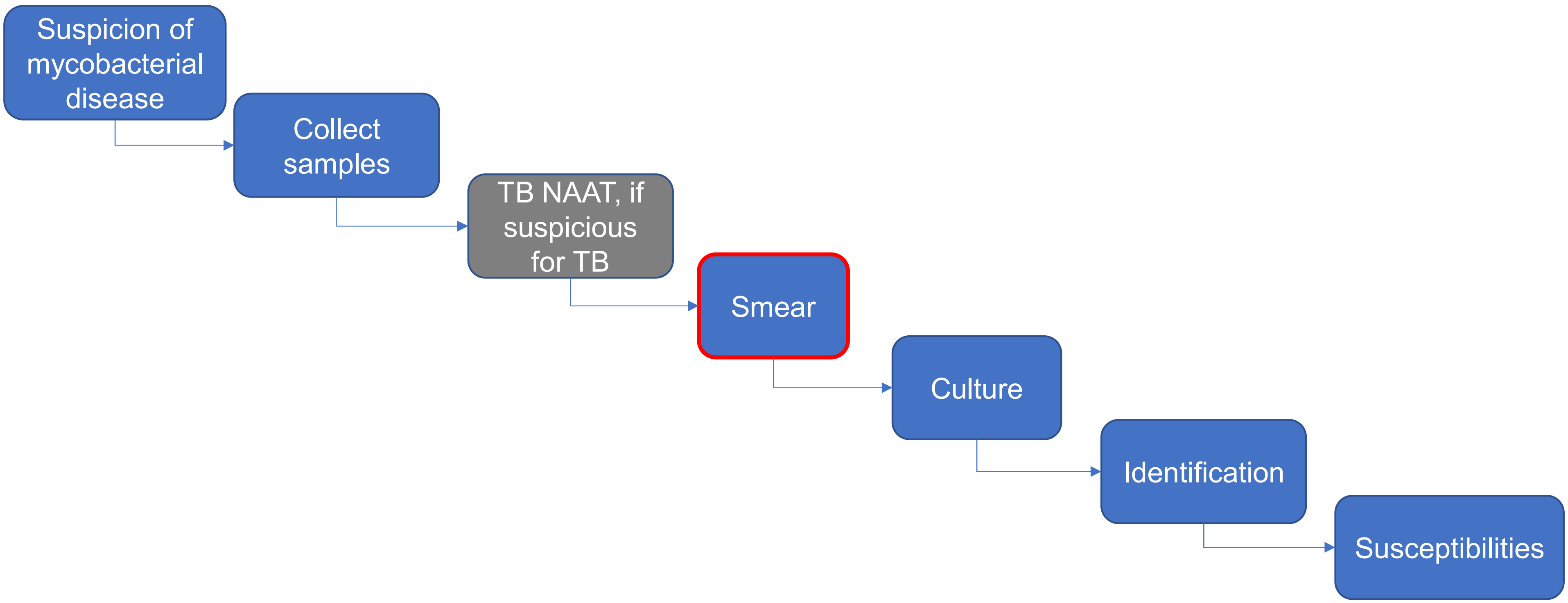
General algorithm for testing mycobacteria in the lab



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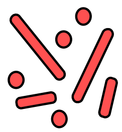
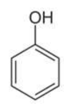
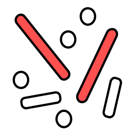
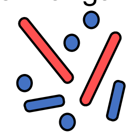
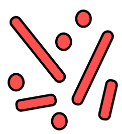
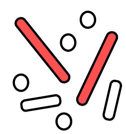
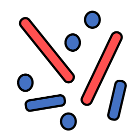
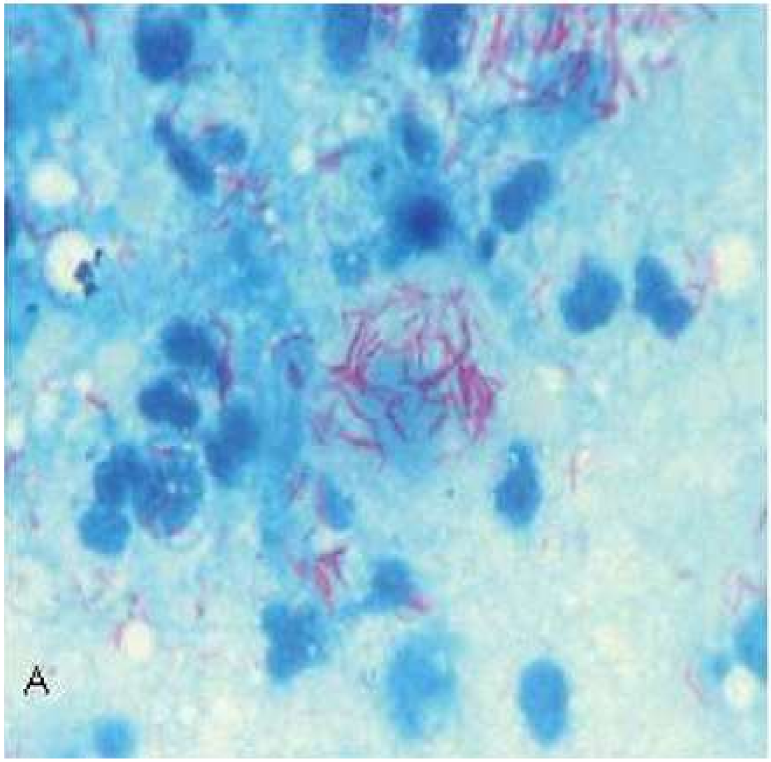
	Primary Stain	Mordant	Decolorizer	Counterstain
Auramine rhodamine	 Auramine-rhodamine		 0.5% acid-alcohol	 Potassium permanganate

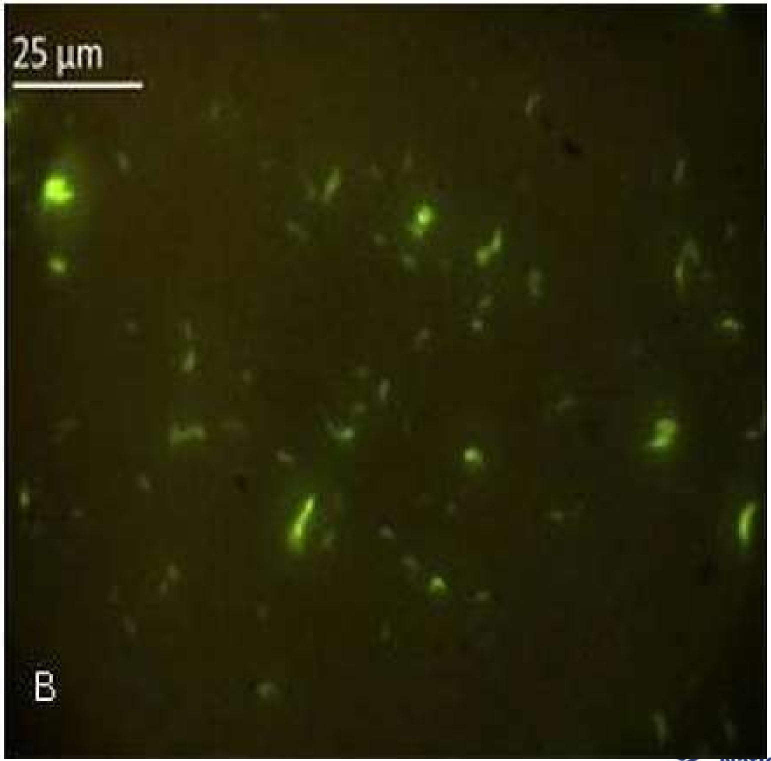
	Primary Stain	Mordant	Decolorizer	Counterstain
Auramine rhodamine	 Auramine-rhodamine		 0.5% acid-alcohol	 Potassium permanganate
Kinyoun	 Carbolfuchsin	 Phenol	 3% acid-alcohol	 Methylene blue
Ziehl Neelsen	 Carbolfuchsin	 Heat	 3% acid-alcohol	 Methylene blue

	Primary Stain	Mordant	Decolorizer	Counterstain
Auramine rhodamine	Auramine-rhodamine		0.5% acid-alcohol	Potassium permanganate
Kinyoun	Carbolfuchsin	Phenol	3% acid-alcohol	Methylene blue
Ziehl Neelsen	Carbolfuchsin	Heat	3% acid-alcohol	Methylene blue
Gram stain	Crystal violet	Iodine	Alcohol	Safranin

Ziehl-Neelsen

Auramine





O'Shea, Wilson. https://www.semanticscholar.org/paper/Tuberculosis-and-the-military.-O%E2%80%99Shea-Wilson/6a18505cd7cce1cbde4df1409085862d8a7cd376

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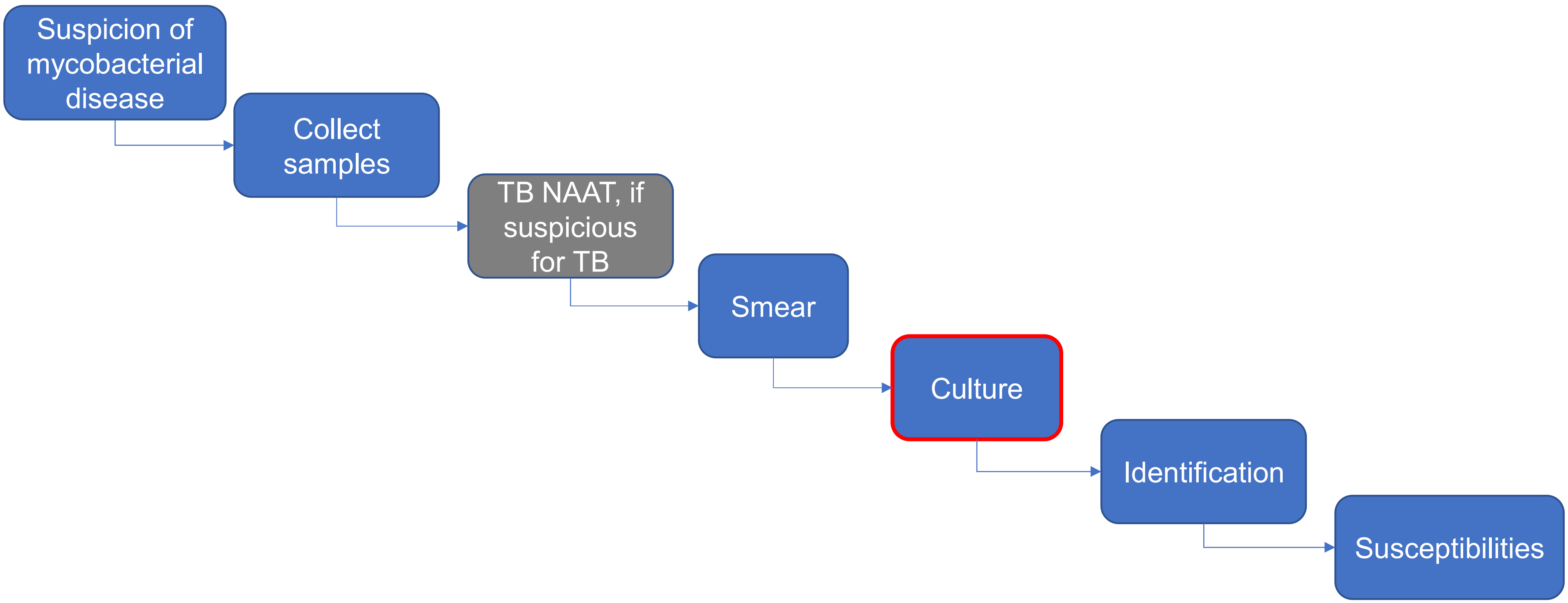
AFB Smears

- Ziehl Neelsen: sensitivity = 20-70%; need ~10^4-10^5 bacilli/ml
- Auramine-rhodamine smears: ~5-10% more sensitive
- Turnaround time: 24 hours

Somoskovi et al. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2925666/#R2
Cattamanchi et al. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2754584/
Singh, Parija. https://www.ncbi.nlm.nih.gov/pubmed/10772577
Azadi et al. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5897959/
Ghiasi et al. https://link.springer.com/article/10.1007/s40475-015-0043-1

General algorithm for testing mycobacteria in the lab



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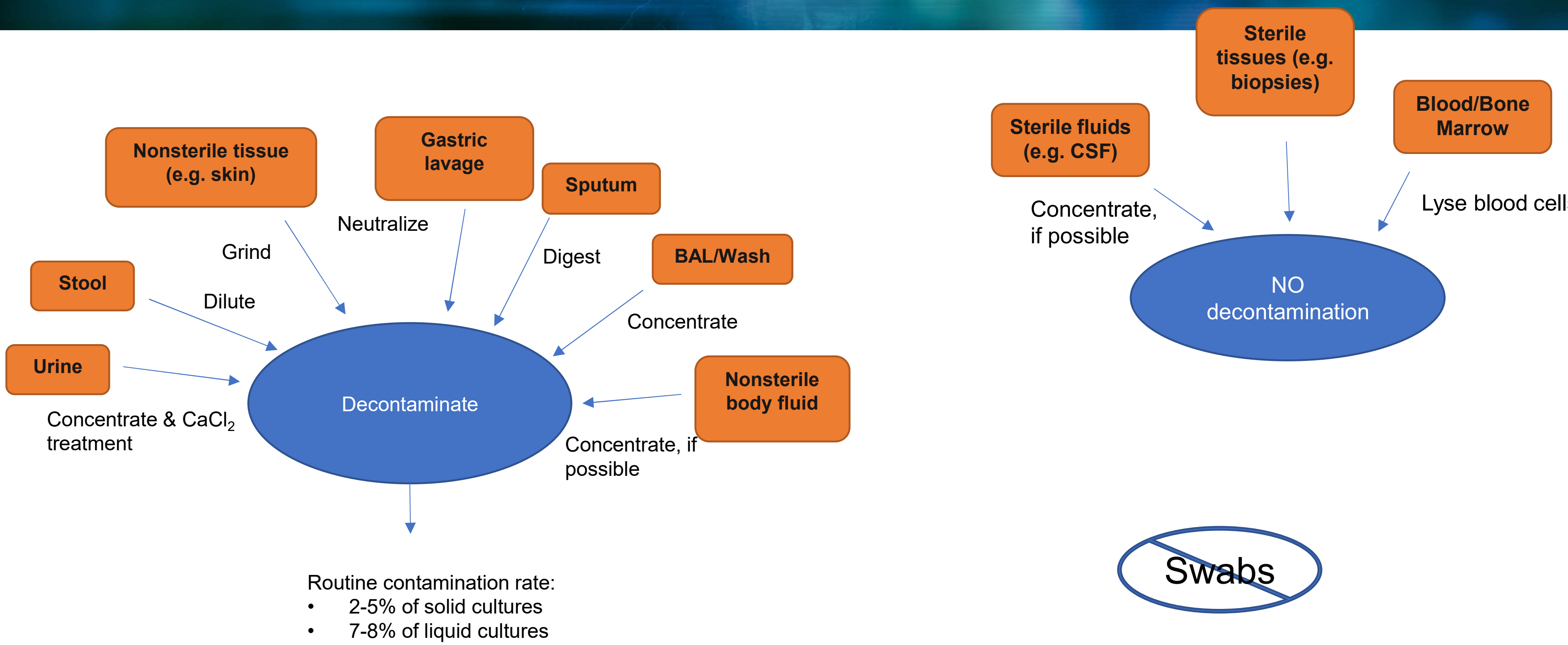
Sample Processing

Sample Processing



2016. 7.2 General Mycobacterial Procedures, p.7.2.2.1-7.2.2.5. Leber AL *Clinical Microbiology Procedures Handbook,* 4th Edition. ASM Press, Washington, DC.

Culture techniques

- Liquid cultures
  - Supplements
    - Sugars (dextrose or glucose)
    - Oleic acid
    - Catalase
  - Antibiotic cocktails
  - More sensitive than solid cultures.
  - More rapid growth compared to solid culture

VersaTREK Myco

https://www.fishersci.com/shop/products/versatrek-myco-media-6/Y711142

MGIT tubes

Positive MGIT

Positive MGIT under UV

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Culture techniques

- Solid culture
  - Lowenstein Jensen agar
    - contains egg and malachite green
  - Middlebrook agar
    - Contains casein hydrolysate (for MDR TB)

LJ

https://www.fishersci.ca/shop/products/lowenstein-jensen-medium-lj/p-4523753

Middlebrook 7H10 or 7H11

https://www.fishersci.ca/shop/products/remel-middlebrook-7h11-agar/r01605

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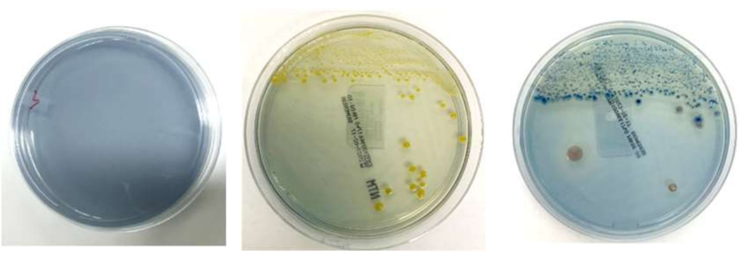
Newer media

- NTM Elite agar (BioMerieux, previously known as RGM medium)
  - Selective media, but also some differentiation
  - Reduced presence of contamination from ~30% to 3%
  - Addition of this media may increase overall sensitivity of culture (from 91% to 100%)
  - Picks up unusual NTM (e.g. *M. llatzerense*)



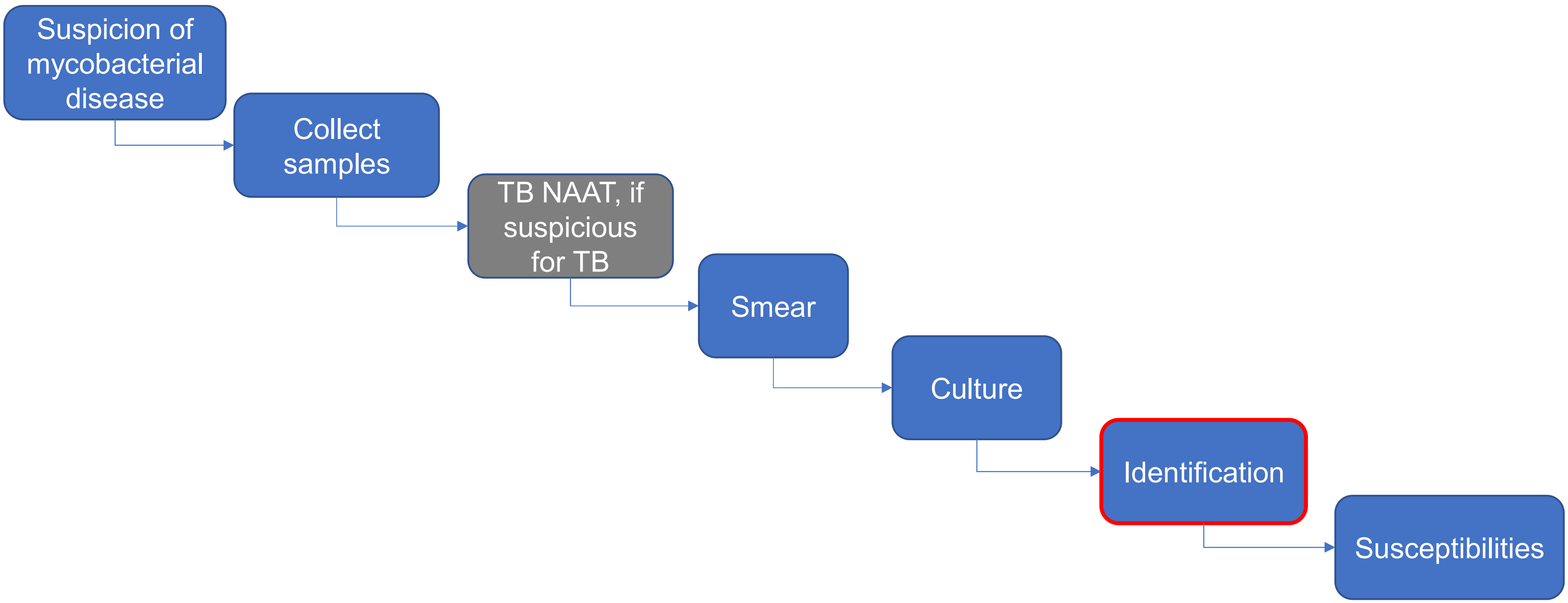
Media type	Sensitivity
MGIT broth	77.5% (69/89)
7H11/7H11S biplates	76.4% (68/89)
NTM Elite	57.3% (51/89)
LJ media	29.2% (26/89)

Caldwell M, Tisdale J, Khare R. Improved recovery of nontuberculous mycobacteria in culture with adjunctive use of a selective agar. J Clin Microbiol. 2024 Mar
Broncano-Lavado A et al. Clinical Evaluation of Nontuberculous Mycobacteria (NTM) Elite Agar, a New Medium for the Isolation of NTM: a Multicenter Study. J Clin Microbiol. 2023 Apr 20

Special conditions for fastidious bugs

- Culture incubated for 6-8 weeks
- Temperatures
  - 35-37^{o}C: routine
  - 42^{o}C: *M. xenopi*
  - 30-32^{o}C: *M. marinum, M. haemophilum, M. ulcerans*
- Supplements:
  - Hemin, ferric ammonium citrate – *M. haemophilum*
  - Egg yolk – *M. ulcerans*
  - Mycobactin J – *M. genavense, M. avium* subsp. *paratuberculosis*
- Media
  - NTM Elite – *M. llatzerense*

General algorithm for testing mycobacteria in the lab



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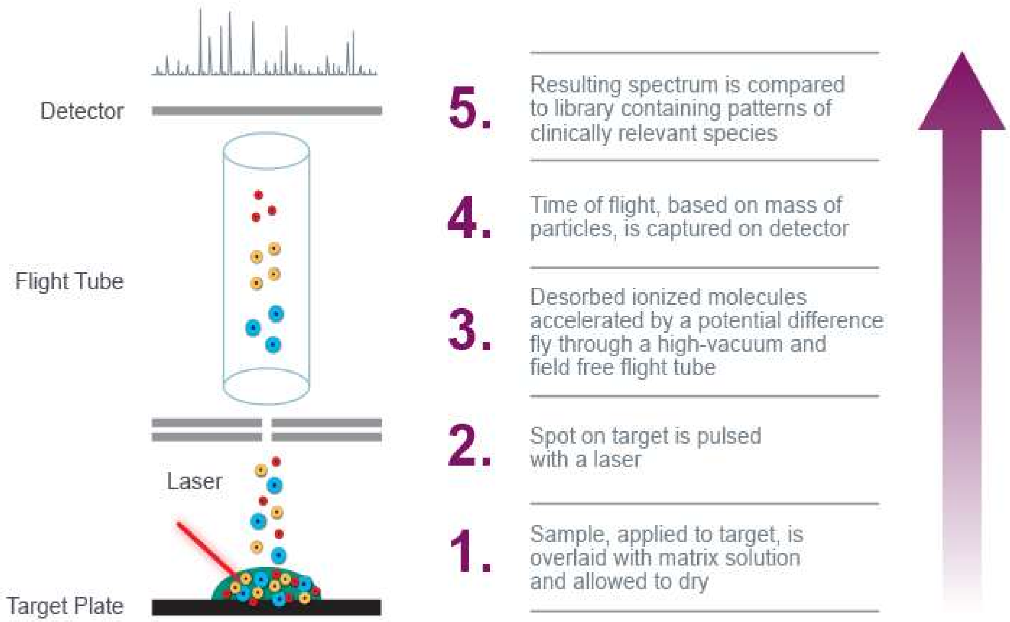
Identification techniques

- Gross morphology – Not very useful
- MALDI-TOF mass spectrometry
- PCR-based line probes
- Sequencing

MALDI-TOF Mass Spectrometry



https://www.beckmancoulter.com/products/microbiology/maldi-tof-mass-spectrometry

Vitek

Bruker

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Why differentiate within complexes and subspecies?

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RGM Antibiogram

Group	Subgroup	Organism	n	CLR	AMK	TOB	CIP	MXF	FOX	IPM	LZD	SXT	DOX
Mycobacteroides		*M. abscessus* subsp. *abscessus*	1344	61	93	1	0	0	30	19	8	0	0
Mycobacteroides		*M. abscessus* subsp. *bolletii*	93	61	92	2	0	0	19	15	14	0	0
Mycobacteroides		*M. abscessus* subsp. *massiliense*	754	95	82	0	1	1	28	12	9	0	0
Mycobacteroides		*M. chelonae*	526	96	90	87	8	5	1	10	37	1	14
Mycobacteroides		*M. immunogenum*	29	97	97	3	21	3	0	3	24	3	0
Mycolicibacterium	*M. fortuitum* complex	*M. fortuitum*	564	21	100	2	97	97	26	92	47	69	38
Mycolicibacterium	*M. fortuitum* complex	*M. peregrinum*	46	89	100	33	98	98	74	83	85	76	9
Mycolicibacterium	*M. fortuitum* complex	*M. porcinum*	93	32	94	42	99	100	73	82	73	63	1
Mycolicibacterium	*M. fortuitum* complex	*M. senegalense*	69	96	100	26	97	99	70	97	70	61	93
Mycolicibacterium	*M. mucogenicum* complex	*M. mucogenicum*	163	98	99	40	78	93	93	95	93	97	47
Mycolicibacterium	*M. mucogenicum* complex	*M. phocaicum*	85	98	100	24	67	87	93	100	91	100	19
Mycolicibacterium		*M. goodii*	21	5	100	90	100	100	24	95	95	100	95
Mycolicibacterium		*M. mageritense*	30	7	83	0	97	97	90	100	80	67	7
Mycolicibacterium		*M. neoaurum*	29	62	100	86	100	100	93	97	100	100	100

100% – 80% – 60% – 40% – 20% – 0%

Hunkins, Calado, Eddy, Daley, Khare. In vitro Susceptibility Patterns for Rapidly Growing Nontuberculous Mycobacteria in the United States, DMID, 2022

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SGM Antibiogram

Group	Organism	AMK n	AMK %S	CLR n	CLR %S	LZD n	LZD %S	MXF n	MXF %S	CIP n	CIP %S	RFB n	RFB %S	RIF n	RIF %S	DOX n	DOX %S	MIN n	MIN %S	SXT n	SXT %S
M. avium complex	*M. avium*	3412	65	3404	94	[illegible]	6	[illegible]	27	[illegible]	-	3416	-	3447	-	1603	-	1218	-	1605	-
M. avium complex	*M. bouchedurhonense*	47	91	46	96	[illegible]	31	[illegible]	39	46	-	47	-	46	-	30	-	28	-	30	-
M. avium complex	*M. colombiense*	32	91	32	100	[illegible]	22	[illegible]	41	32	-	32	-	31	-	19	-	14	-	19	-
M. avium complex	*M. intracellulare* subsp. *chimaera*	1322	81	1312	98	[illegible]	2	[illegible]	4	[illegible]	-	1315	-	1323	-	603	-	453	-	603	-
M. avium complex	*M. intracellulare* subsp. *intracellulare*	2916	71	2897	96	[illegible]	2	[illegible]	6	[illegible]	-	2914	-	2919	-	1311	-	948	-	1307	-
M. avium complex	*M. intracellulare* subsp. *yongonense*	69	64	68	96	[illegible]	11	[illegible]	20	66	-	68	-	68	-	12	-	9	-	12	-
M. avium complex	*M. marseillense*	80	82	79	100	[illegible]	9	[illegible]	15	78	-	80	-	80	-	45	-	36	-	45	-
M. avium complex	*M. timonense*	27	89	27	100	[illegible]	19	[illegible]	22	27	-	27	-	27	-	11	-	8	-	11	-
	M. arupense	158	49	157	97	155	9	[illegible]	[illegible]	157	3	158	97	159	18	85	1	70	3	85	31
	M. asiaticum	54	93	54	100	54	11	54	65	54	2	54	59	55	4	25	0	14	-	25	40
	M. europaeum	18	94	17	100	17	24	17	18	17	0	18	100	18	50	11	-	10	-	11	-
	M. gordonae	144	94	145	99	143	63	144	59	144	15	145	93	145	23	144	14	144	17	144	62
	M. haemophilum	48	100	49	100	48	100	48	100	50	82	50	100	49	100	0	-	0	-	0	-
	M. interjectum	46	87	45	100	45	62	46	61	46	2	46	96	46	59	23	4	17	0	23	61
	M. kansasii	196	90	242	98	214	80	230	65	201	7	192	98	214	63	188	1	189	1	190	48
	M. kubicae	31	94	31	90	31	42	31	71	31	10	31	90	31	19	16	19	10	-	16	50
	M. lentiflavum	184	82	185	100	184	16	184	32	182	15	186	98	185	32	78	5	56	2	79	39
	M. malmoense	24	83	23	96	23	70	23	74	23	30	24	88	23	30	8	-	5	-	8	-
	M. marinum	207	99	208	99	205	96	207	57	207	27	60	100	61	57	208	34	208	37	208	77
	M. nebraskense	38	97	37	100	38	71	37	70	37	46	37	95	38	84	13	-	9	-	13	-
	M. paraense	27	85	27	93	27	70	27	26	27	11	27	85	27	52	15	0	13	-	15	60
	M. paraffinicum	83	89	82	98	82	29	82	33	82	9	83	84	83	61	33	6	30	3	33	33
	M. paragordonae	19	100	20	95	18	44	20	70	20	10	19	84	19	37	19	5	19	0	19	58
	M. parascrofulaceum	39	92	39	95	39	46	39	33	39	0	39	97	39	72	22	0	12	-	22	14
	M. scrofulaceum	29	97	29	86	29	34	29	48	29	7	29	100	29	45	11	-	6	-	11	-
	M. shimoidei	19	95	21	90	16	94	21	90	21	81	17	100	21	24	2	-	2	-	2	-
	M. simiae	156	74	156	82	155	2	155	13	155	1	155	37	156	3	58	0	44	0	59	8
	M. stomatepiae	18	78	18	94	18	33	18	33	18	0	18	83	18	0	7	-	3	-	7	-
	M. szulgai	22	95	26	100	23	65	24	25	26	4	25	76	24	38	21	0	21	5	21	43
	M. terrae	45	56	45	96	44	7	45	40	46	15	46	93	48	12	28	0	17	0	28	43
	M. triplex	34	71	34	100	34	50	34	29	34	3	34	100	34	47	16	0	13	-	16	38
	M. xenopi	62	92	65	98	65	89	68	82	64	41	60	98	62	39	58	5	58	3	58	67

100%
80%
60%
40%
20%
0%

Calado, Nguyen, Hunkins, Daley, Khare. In vitro Susceptibility Patterns for Slowly Growing Nontuberculous Mycobacteria in the USA from 2018-2022, JAC,

SGM Antibiogram

Group	Organism	AMK n	AMK %S	CLR n	CLR %S	LZD n	LZD %S	MXF n	MXF %S	CIP n	CIP %S	RFB n	RFB %S	RIF n	RIF %S	DOX n	DOX %S	MIN n	MIN %S	SXT n	SXT %S
M. avium complex	*M. avium*	3412	65	3404	94	3377	6	3375	27	3364	-	3416	-	3447	-	1[illegible]03	-	1218	-	1605	-
M. avium complex	*M. bouchedurhonense*	47	91	46	96	45	31	46	39	46	-	47	-	46	-	[illegible]0	-	28	-	30	-
M. avium complex	*M. colombiense*	32	91	32	100	32	22	32	41	32	-	32	-	31	-	[illegible]9	-	14	-	19	-
M. avium complex	*M. intracellulare* subsp. *chimaera*	1322	81	1312	98	1299	2	1299	4	1298	-	1315	-	1323	-	[illegible]03	-	453	-	603	-
M. avium complex	*M. intracellulare* subsp. *intracellulare*	2916	71	2897	96	2878	2	2877	6	2873	-	2914	-	2919	-	1[illegible]11	-	948	-	1307	-
M. avium complex	*M. intracellulare* subsp. *yongonense*	69	64	68	96	66	11	66	20	66	-	68	-	68	-	[illegible]2	-	9	-	12	-
M. avium complex	*M. marseillense*	80	82	79	100	79	9	78	15	78	-	80	-	80	-	[illegible]5	-	36	-	45	-
M. avium complex	*M. timonense*	27	89	27	100	27	19	27	22	27	-	27	-	27	-	[illegible]1	-	8	-	11	-
	M. arupense	158	49	157	97	155	9	157	48	157	3	158	97	159	18	85	1	70	3	85	31
	M. asiaticum	54	93	54	100	54	11	54	65	54	2	54	59	55	4	25	0	14	-	25	40
	M. europaeum	18	94	17	100	17	24	17	18	17	0	18	100	18	50	11	-	10	-	11	-
	M. gordonae	144	94	145	99	143	63	144	59	144	15	145	93	145	23	144	14	144	17	144	62
	M. haemophilum	48	100	49	100	48	100	48	100	50	82	50	100	49	100	0	-	0	-	0	-
	M. interjectum	46	87	45	100	45	62	46	61	46	2	46	96	46	59	23	4	17	0	23	61
	M. kansasii	196	90	242	98	214	80	230	65	201	7	192	98	214	63	188	1	189	1	190	48
	M. kubicae	31	94	31	90	31	42	31	71	31	10	31	90	31	19	16	19	10	-	16	50
	M. lentiflavum	184	82	185	100	184	16	184	32	182	15	186	98	185	32	78	5	56	2	79	39
	M. malmoense	24	83	23	96	23	70	23	74	23	30	24	88	23	30	8	-	5	-	8	-
	M. marinum	207	99	208	99	205	96	207	57	207	27	60	100	61	57	208	34	208	37	208	77
	M. nebraskense	38	97	37	100	38	71	37	70	37	46	37	95	38	84	13	-	9	-	13	-
	M. paraense	27	85	27	93	27	70	27	26	27	11	27	85	27	52	15	0	13	-	15	60
	M. paraffinicum	83	89	82	98	82	29	82	33	82	9	83	84	83	61	33	6	30	3	33	33
	M. paragordonae	19	100	20	95	18	44	20	70	20	10	19	84	19	37	19	5	19	0	19	58
	M. parascrofulaceum	39	92	39	95	39	46	39	33	39	0	39	97	39	72	22	0	12	-	22	14
	M. scrofulaceum	29	97	29	86	29	34	29	48	29	7	29	100	29	45	11	-	6	-	11	-
	M. shimoidei	19	95	21	90	16	94	21	90	21	81	17	100	21	24	2	-	2	-	2	-
	M. simiae	156	74	156	82	155	2	155	13	155	1	155	37	156	3	58	0	44	0	59	8
	M. stomatepiae	18	78	18	94	18	33	18	33	18	0	18	83	18	0	7	-	3	-	7	-
	M. szulgai	22	95	26	100	23	65	24	25	26	4	25	76	24	38	21	0	21	5	21	43
	M. terrae	45	56	45	96	44	7	45	40	46	15	46	93	48	12	28	0	17	0	28	43
	M. triplex	34	71	34	100	34	50	34	29	34	3	34	100	34	47	16	0	13	-	16	38
	M. xenopi	62	92	65	98	65	89	68	82	64	41	60	98	62	39	58	5	58	3	58	67

100%
80%
60%
40%
20%
0%

EMB

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Calado, Nguyen, Hunkins, Daley, Khare. In vitro Susceptibility Patterns for Slowly Growing Nontuberculous Mycobacteria in the USA from 2018-2022, JAC,

Rifampin and Ethambutol

M62, 1st ed.

Table 3. Antimycobacterial Agents and Breakpoints for Testing MAC

QC recommendations (see Table 12 for acceptable QC ranges):

Routine QC strain:

- *M. marinum* ATCC®* 927

Supplemental QC strain:

- *Staphylococcus aureus* ATCC® 29213

General Comments

(1) Although ethambutol, rifampin, and rifabutin are useful clinically, breakpoints for determining susceptibility and resistance have not been established, and previous studies show that there is no correlation between *in vitro* MIC results and clinical response in patients with MAC.[1,2] Streptomycin may be used in place of amikacin. However, because no studies correlating streptomycin MIC values and clinical response have been performed, if the drug is tested, only the MIC value should be reported.

M62, 1st ed. Performance Standards for Susceptibility Testing of Mycobacteria, *Nocardia* spp. and other Aerobic Actinomycetes. CLSI

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Rifampin and Ethambutol

AMERICAN SOCIETY FOR MICROBIOLOGY Antimicrobial Agents and Chemotherapy®

CLINICAL THERAPEUT

In Vitro MIC Values of Rifampin and Ethambutol and Treatment Outcome in *Mycobacterium avium* Complex Lung Disease

Byoung Soo Kwon,[a] Mi-Na Kim,[b] Heungsup Sung,[b] Younsuck Koh,[a] Woo-Sung Kim,[a] Jin-Woo Song,[a] Yeon-Mok Oh,[a] Sang-Do Lee,[a] Sei Won Lee,[a] Jae-Seung Lee,[a] Chae-Man Lim,[a] Chang-Min Choi,[a] Jin-Won Huh,[a] Sang-Bum Hong,[a] Sojung Park,[c] Tae Sun Shim,[a] Yong Pil Chong,[d] Kyung-Wook Jo[a]

2018

"These findings suggest that the *in vitro* MICs of rifampin and ethambutol may be related to treatment outcome in MAC-LD."

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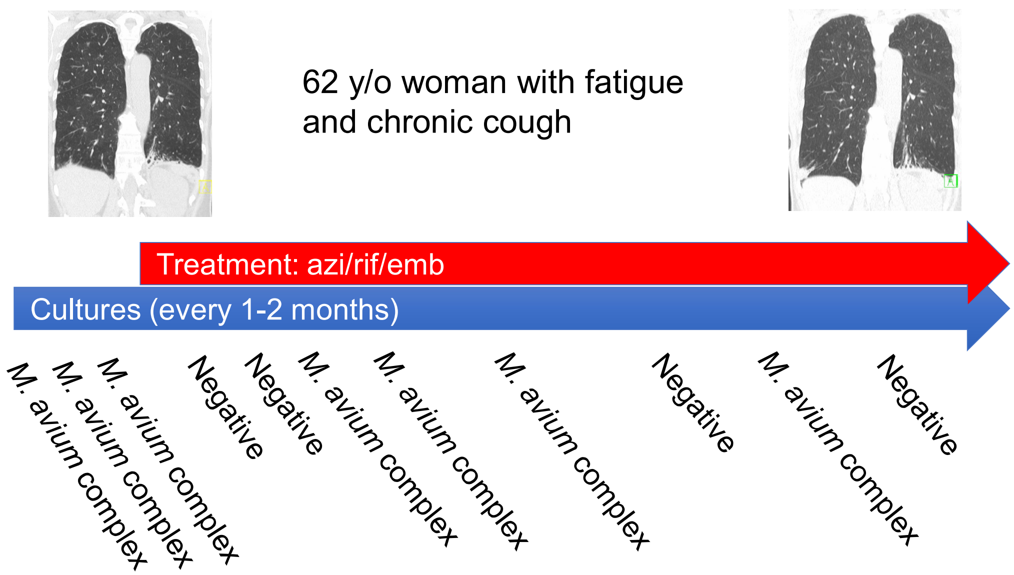
Why differentiate within complexes and subspecies?

- Different treatment patterns
- Different diseases and risk factors (*M. avium* subsp. *paratuberculosis*)
- Is prolonged positivity due to re-infection with a similar species?

Mixed Infection/Re-infection with Different MAC Species



Modified from slide created and contributed by Dr. Chuck Daley

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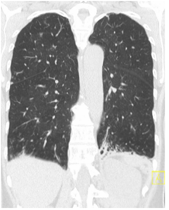
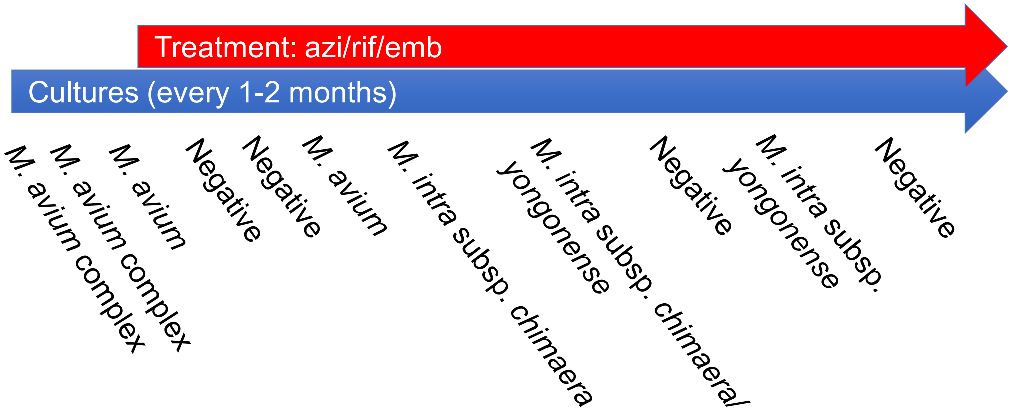
Mixed Infection/Re-infection with Different MAC Species



62 y/o woman with fatigue and chronic cough



Slide created and contributed by Dr. Chuck Daley

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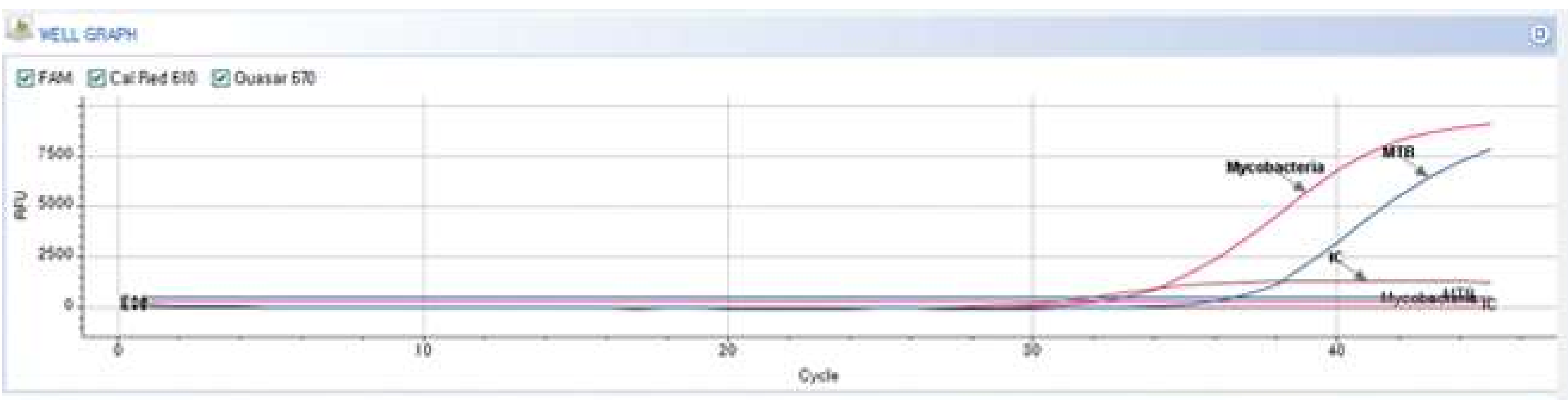
Real-time PCR

- E.g. Anyplex MTB/NTM Real-time Detection (Seegene, Korea)
- Limitations: only a few targets because of fluorescence overlap



https://www.seegene.com/assays/anyplex_mtb_ntm_realtime_detection

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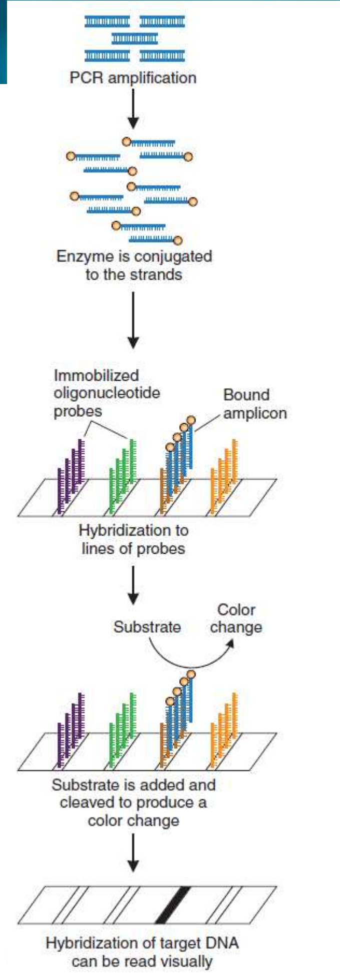
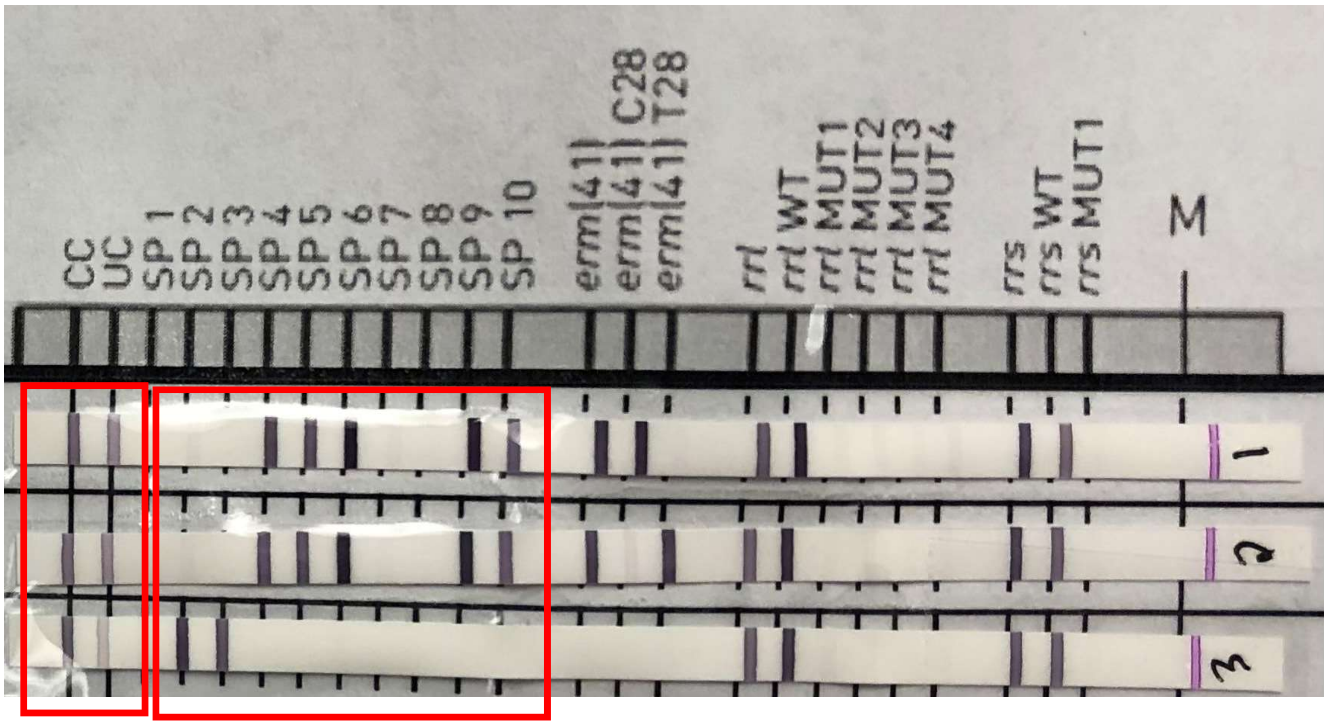
Line probe assays (LPAs)

- Advantages: better accuracy, better speciation/subspeciation, can simultaneously detect drug resistance markers.
- Limitations: Not available in the US ☹; also costly. The line probe is labor-intensive and subjective.
- How do they work?
  - Step 1: multiplex-PCR
  - Step 2: Amplicons are bound onto a membrane containing capture probes for identification and drug resistance genes
  - Step 3: Pattern of binding is read



Khare. Guide to clinical and Diagnostic Virology, 2019

LPA results



M. abscessus subsp. *abscessus*

M. abscessus subsp. *abscessus*

M. intracellulare subsp. *chimaera*

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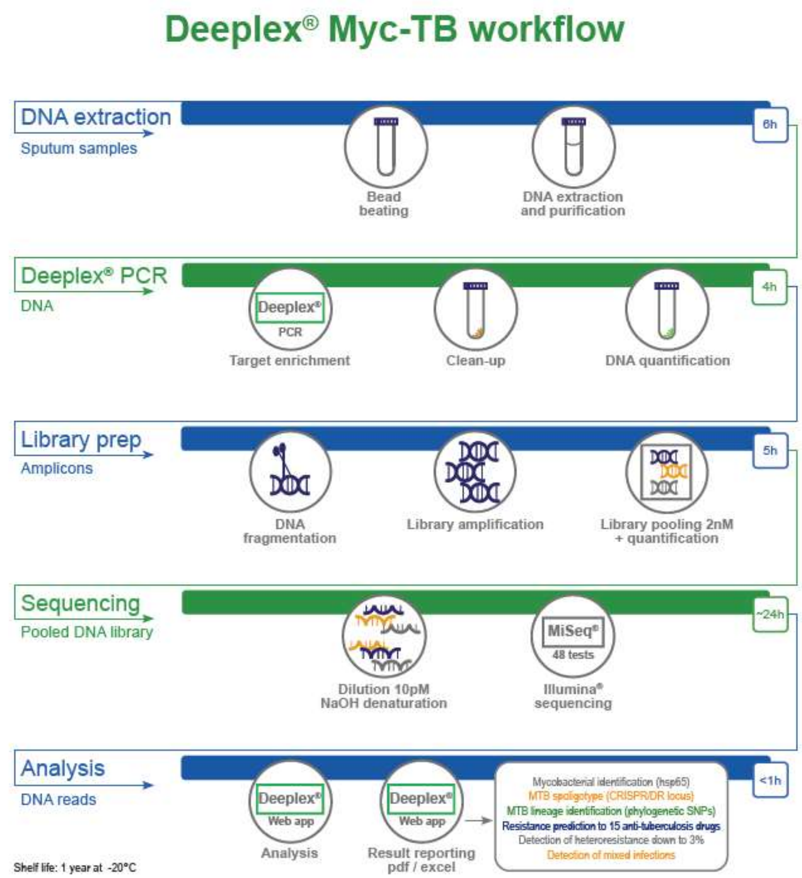
Deeplex Myc-TB

- Targeted NGS for the hsp65 gene
- Advantages: >140 NTM
- Limitations: cost, technical expertise needed



Deeplex Myc-TB, Technical Note, Deeplex, 2020

Name of Assay	Method	ID for MTB C	ID of NTM	# of NTM species or subspecies identified	Manufacturer
Intended for samples (e.g. sputum) only					
GenoType CMdirect VER 1.0[74]	NAAT (Line probe)	Y	Y	20	Hain Lifescience, Germany
AdvanSure TB/NTM real time PCR[75]	NAAT (Real-time PCR)	Y	Y	0; (generic NTM detection)	LG Life Science, Korea
NTM from sputum and isolates					
MolecuTech Real MTB-ID[76]	NAAT (Real-time PCR)	Y	Y	6	YD Diagnostics, Korea
MolecuTech TB-Tag Two[76]	NAAT (nested, conventional PCR)	Y	Y	8	YD Diagnostics, Korea
MolecuTech REBA Myco-ID[76]	NAAT (Reverse Blot Hybridization)	Y	Y	19	YD Diagnostics, Korea
MolecuTech Real MTB-ID[76]	NAAT (Real-time PCR)	Y	Y	6	YD Diagnostics, Korea
Genoscholar NTM+MDRTB II (Previously called NTM+MDRTB Detection Kit 2)[77]	NAAT (Line probe)	Y	Y	3	Nipro, Japan
Anyplex MTB/NTM Real-time PCR[78]	NAAT (Real-time PCR)	Y	Y	0; generic NTM detection	Seegene, Korea
PowerChek MTB/NTM Real time PCR Kit[79]	NAAT (Real-time PCR)	Y	Y		Kogene Biotech, Korea
Deeplex Myc-TB[80]	Targeted NGS	Y	Y	>100	Genoscreen, France
TB/NTM PCR[81]	NAAT (Conventional PCR, 2 reactions)	Y	Y	0; generic NTM detection	Biocore, Korea
TB/NTM PCR (one tube)[82]	NAAT (Conventional PCR, single reaction)	Y	Y	0; generic NTM detection	Biocore, Korea
TB/NTM Real Time PCR[83]	NAAT (Real-time PCR)	Y	Y	0; generic NTM detection	Biocore, Korea

Name of Assay	Method	ID for MTB C	ID of NTM	# of NTM species or subspecies identified	Manufacturer
NTM from isolates only					
GenoType NTM-DR VER 1.0[74,85]	NAAT (Line probe)	N	Y	7	Hain Lifescience, Germany
GenoType Mycobacterium AS[74]	NAAT (Line probe)	N	Y	19	Hain Lifescience, Germany
GenoType Mycobacterium CM VER 2.0[74]	NAAT (Line probe)	Y	Y	20	Hain Lifescience, Germany
FluoroType Mycobacteria VER 1.0[86]	NAAT (Asymmetrical FRET PCR)	N	Y	32	Hain Lifescience, Germany
MolecuTech MTB-ID V3[76]	NAAT (Nested, conventional PCR)	Y	Y	11	YD Diagnostics, Korea
Speed-Oligo Mycobacteria[87]	NAAT (Line probe)	Y	Y	14	Vircell, Spain
INNO-LiPA Mycobacteria v2[88]	NAAT (Line probe)	Y	Y	16	Fujirebio, Japan

Khare, Elliot. Culture, Identification and Antimicrobial Susceptibility Testing of Nontuberculous Mycobacteria. Clinics in Chest Medicine, In preparation, 2023

Sanger Sequencing

- Step 1: amplify a target gene
  - Most common gene for identification
    - *rpoB* – encodes subunit of bacterial RNA polymerase[1]
    - 16S –rDNA encoding the 30S ribosomal rRNA
    - 16S-23S ITS – intergenic spacer region
    - *hsp65* – encodes a bacterial heat shock protein
- Step 2: sequence the amplified gene
- Step 3: compare the sequence to a database

https://www.thermofisher.com/us/en/home/life-science/sequencing/sanger-sequencing/sanger-sequencing-technology-accessories/3730-series-genetic-analyzers/jcr:content/MainParsys/image_2014.img.full.high.jpg/1678310442624.jpg

Simmon et al. Simultaneous Sequence Analysis of the 16S rRNA and *rpoB* Genes by Use of RipSeq Software To Identify *Mycobacterium* Species. JCM, 2010

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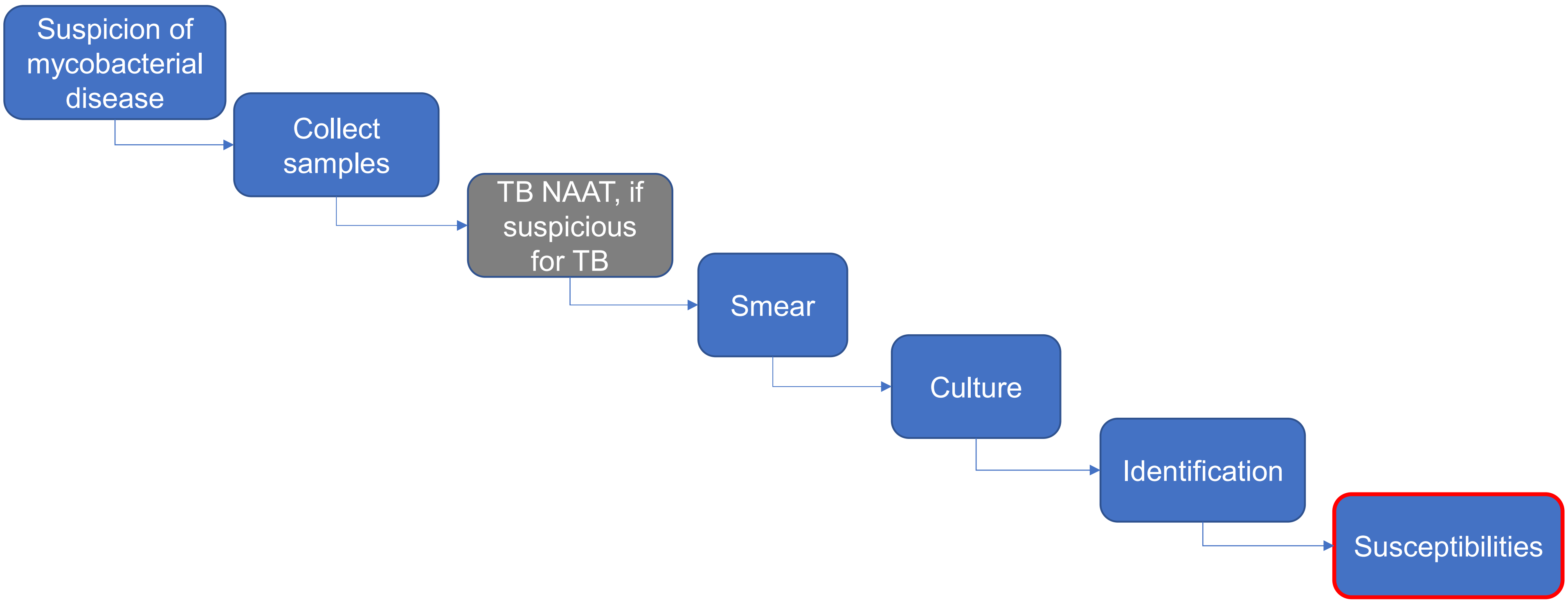
General algorithm for testing mycobacteria in the lab



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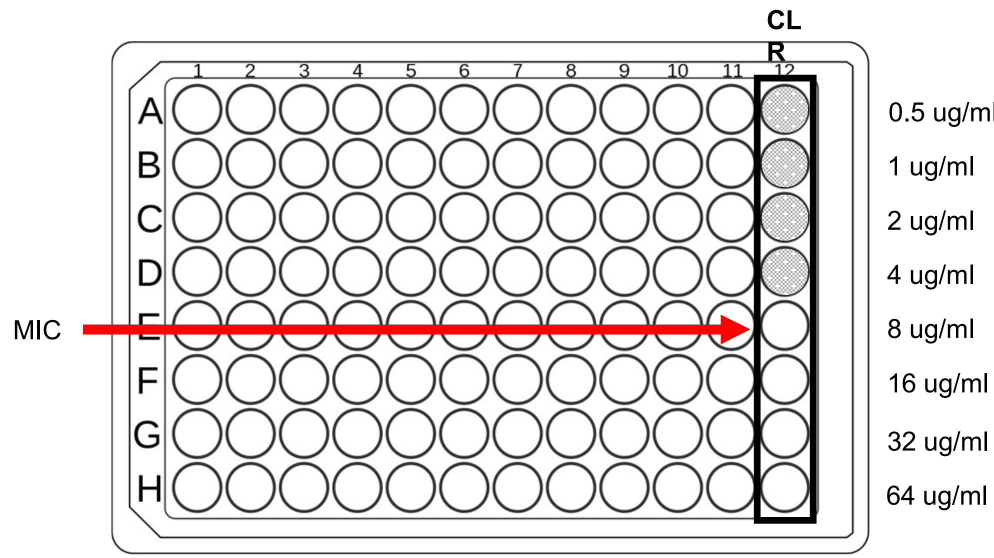
Antibiotic susceptibility testing (AST)

- Broth microdilution testing
- No agar proportion (TB) or Etest methods have been approved by CLSI
- Read manually (subjective, labor intensive)



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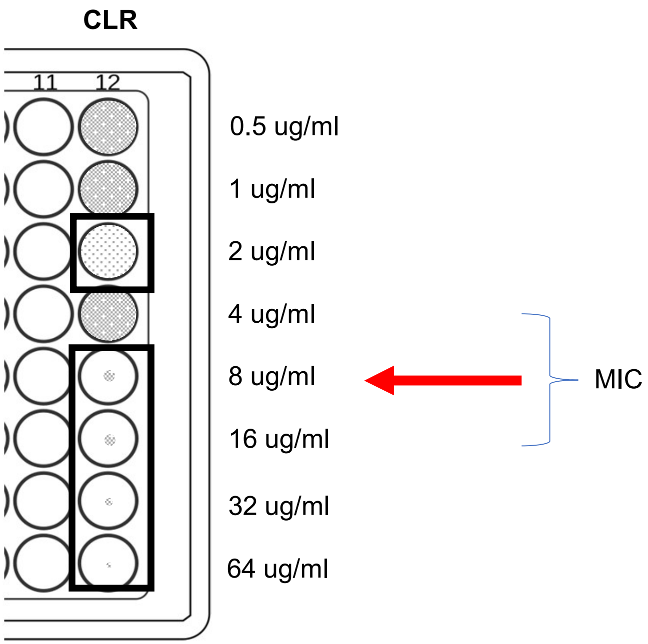
Antibiotic susceptibility testing (AST)

- Read manually (subjective, labor intensive)
- Technically challenging
  - Uneven growth
  - trailing endpoints
  - clumping, buttons vs. turbidity; Trimethoprim/sulfamethoxazole is read at 80% inhibition



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AST Interpretation

- Minimum Inhibitory concentration = MIC value
- Interpretation
    - S, I, R = Susceptible, Intermediate, Resistant
    - Defined by CLSI M62, 2018 (updated M24S, 2023)

MAC

Antimicrobial Agents	MIC, µg/mL		
	S	I	R
First Line			
Clarithromycin	≤8	16	≥32
Amikacin (IV)	≤16	32	≥64
Amikacin (liposomal, inhaled)	≤64	–	≥128
Second Line			
Moxifloxacin	≤1	2	≥4
Linezolid	≤8	16	≥32

Rapid growingly mycobacteria

Antimicrobial Agent	MIC, µg/mL		
	S	I	R
Amikacin (IV)	≤16	32	≥64
Cefoxitin	≤16	32–64	≥128
Ciprofloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Doxycycline	≤1	2–4	≥8

CLSI Breakpoints for NTM

- Amikacin has different formulations and breakpoints for MAC

Rapid growers

Antimicrobial	MIC (µg/mL)		
	Susceptible	Intermediate	Resistant
Amikacin	≤16	32	≥64
Cefoxitin	16	32-64	≥128
Clarithromycin	≤4	8	≥16
Ciprofloxacin	≤1	2	≥4
Doxycycline	≤1	2-4	≥8
Imipenem	≤4	8-16	≥32
Linezolid	≤8	16	≥32
Minocycline	≤1	2-4	≥8
Meropenem	≤4	8-16	≥32
Moxifloxacin	≤1	2	≥4
Linezolid	≤8	16	≥32
Tigecycline	None, report MIC only		
Tobramycin	≤2	4	≥8
Trimethoprim/ sulfamethoxazole	≤2/38	-	≥4/76

Slow growers (MAC)

Antimicrobial	MIC (µg/mL)		
	Susceptible	Intermediate	Resistant
Amikacin (Intravenous)	≤16	32	≥64
Amikacin (Inhaled, liposomal)	≤16	32	≥128
Clarithromycin	≤8	16	≥32
Ciprofloxacin	≤1	2	≥4
Doxycycline	≤1	2-4	≥8
Linezolid	≤8	16	≥32
Minocycline	≤1	2-4	≥8
Moxifloxacin	≤1	2	≥4
Rifabutin	≤2	-	≥4
Rifampin	≤1	-	≥2
Trimethoprim/ sulfamethoxazole	≤2/38	-	≥4/76

Khare, Elliot. Culture, Identification and Antimicrobial Susceptibility Testing of Nontuberculous Mycobacteria. Clinics in Chest Medicine, In preparation, 2023

CLSI Breakpoints for NTM

- Clarithromycin is the class drug for macrolides

Rapid growers

Antimicrobial	MIC (µg/mL)		
	Susceptible	Intermediate	Resistant
Amikacin	≤16	32	≥64
Cefoxitin	16	32-64	≥128
Clarithromycin	≤4	8	≥16
Ciprofloxacin	≤1	2	≥4
Doxycycline	≤1	2-4	≥8
Imipenem	≤4	8-16	≥32
Linezolid	≤8	16	≥32
Minocycline	≤1	2-4	≥8
Meropenem	≤4	8-16	≥32
Moxifloxacin	≤1	2	≥4
Linezolid	≤8	16	≥32
Tigecycline	None, report MIC only		
Tobramycin	≤2	4	≥8
Trimethoprim/ sulfamethoxazole	≤2/38	-	≥4/76

Slow growers

Antimicrobial	MIC (µg/mL)		
	Susceptible	Intermediate	Resistant
Amikacin (Intravenous)	≤16	32	≥64
Amikacin (Inhaled, liposomal)	≤16	32	≥128
Clarithromycin	≤8	16	≥32
Ciprofloxacin	≤1	2	≥4
Doxycycline	≤1	2-4	≥8
Linezolid	≤8	16	≥32
Minocycline	≤1	2-4	≥8
Moxifloxacin	≤1	2	≥4
Rifabutin	≤2	-	≥4
Rifampin	≤1	-	≥2
Trimethoprim/ sulfamethoxazole	≤2/38	-	≥4/76

Khare, Elliot. Culture, Identification and Antimicrobial Susceptibility Testing of Nontuberculous Mycobacteria. Clinics in Chest Medicine, In preparation, 2023

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Turnaround time

- Slow growers:
  - read at 7-14 days
  - Fastidious species at 3-4 weeks
  - *M. genavense*: Requires acid pH and mycobactin J supplementation; At least 6 weeks of incubation; long incubation time may cause antimicrobial degradation
- Rapid growers:
  - Most drugs read at 3-5 days
  - Clarithromycin: read at 14 days (because of inducible resistance)
- Other factors
  - Insufficient growth? Needs subculture
  - Mixed culture? Needs re-isolation 😱

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Molecular AST for NTM

- **erms**: functional erythromycin ribosomal methylase causes inducible macrolide resistance

Group	Organism	Gene	% resistant
MTBC	*M. tuberculosis, M. africanum, M. microti, M. bovis*	*erm*(37)	100%
M. abscessus	*M. abscessus* subsp. *abscessus*	*erm*(41); A point mutation (T28C) can inactivate the methylase and prevent inducible resistance	70%
	M. abscessus subsp. *bolletii*		100%
	M. abscessus subsp. *massiliense*	Deletions in *erm*(41) result in a truncated, nonfunctional erm	0%
M. fortuitum complex	*M. fortuitum*	*erm*(39)	84%
	M. peregrinum		31%
	M. porcinum		90%
	M. septicum		86%
	M. senegalense	Nonfunctional *erm*(39)?	0%
Other RGM	*M. smegmatis, M. goodii*	*erm*(38)	?
	M. chelonae, M. iranicum? M. obuense?	*erm*(55)^{p} (plasmid)	Rare
	M. chelonae	*erm*(55)^{C} (chromosomal), *erm* (55)^{T} (transposon)	Rare
	M. mageritense, M. wolinskyi	*erm*(40)	

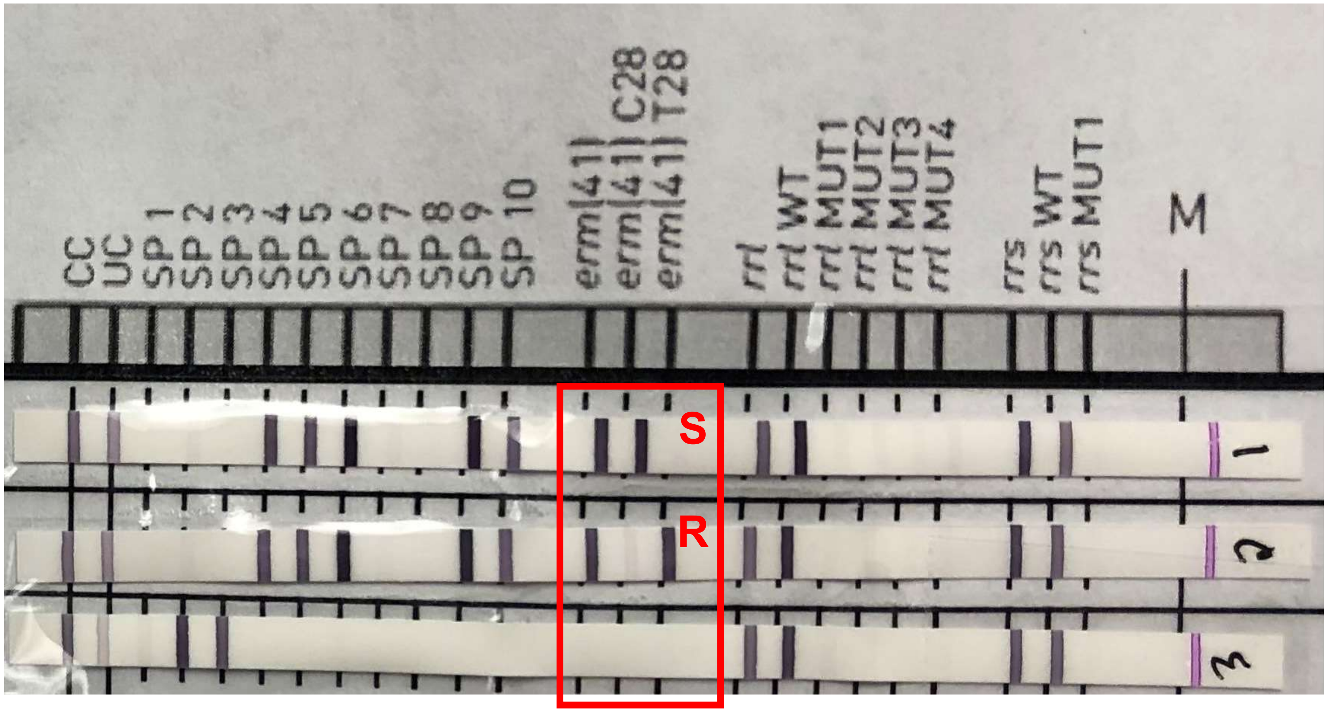
Hunkins, Calado, Eddy, Daley, Khare. In vitro Susceptibility Patterns for Rapidly Growing Nontuberculous Mycobacteria in the United States, DMID, 2022, Kim et al. Species Distribution and Macrolide Susceptibility of Mycobacterium fortuitum Complex Clinical Isolates, AAC, 2019, Nash et al. Molecular basis of intrinsic macrolide resistance in clinical isolates of Mycobacterium fortuitum, JAC, 2004, Toft Madson et al. Methyltransferase Erm(37) Slips on rRNA to Confer Atypical Resistance in Mycobacterium tuberculosis, JBC 2005
Elliott et al. Emergence of Inducible Macrolide Resistance in Mycobacterium chelonae Due to Broad-Host-Range Plasmid and Chromosomal Variants of the Novel 23S rRNA Methylase Gene, erm(55), JCM 2023
Madsen et al. Mycobacterium smegmatis Erm(38) Is a Reluctant Dimethyltransferase AAC, 2005
Brown-Elliott et al. Utility of Sequencing the erm(41) Gene in Isolates of Mycobacterium abscessus subsp. abscessus with Low and Intermediate Clarithromycin MICs, JCM 2015

Molecular Methods of AST

- Pros:
  - Faster! (hours-days instead of 6-8 weeks of culture based AST)
  - Some well characterized mutations that correlate well with phenotypic AST
    - ***rrl***: Mutations in the 23S rRNA (A2058G or A2059G) peptidyl transferase represents acquired (constitutive) macrolide resistance
    - ***rrs***: specific mutations in the 16S ribosomal RNA represent constitutive aminoglycoside resistance

Common NTM	Present or not
M. abscessus subsp. *abscessus*	*rrl, rrs*
M. abscessus subsp. *bolletii*	*rrl, rrs*
M. abscessus subsp. *massiliense*	*rrl, rrs*
M. avium sensu stricto	*rrl, rrs*
M. intracellulare subsp. *chimaera*	*rrl, rrs*
M. avium complex	*rrl, rrs*
M. chelonae	*rrl, rrs*
M. kansasii	N/A
M. fortuitum	N/A

Line probe assays



M. abscessus subsp. *abscessus*

M. abscessus subsp. *abscessus*

M. intracellulare subsp. *chimaera*

New drugs with potential activity against mycobacteria

	Drug class	RGM	SGM	MTBC
Omadacycline	Tetracycline	Potential	Modest	
Eravacycline	Tetracycline	Potential	Potential	
Fobrepodacin	Amino-benzimidazole	Modest	Potential	
Epetraborole	Benzoxaborole	Potential[a]	Potential	
Bedaquiline	Diarylquinolone	Potential	Potential	Yes
Pretomanid	Nitroimidazole	No	No, except *M. kansasii*	Yes
Delaminid	Nitroimidazole	No	Potential	
Tedizolid	Oxazolidinone	Potential	Potential	
Imipenem+Ceftaroline Ceftazidime+imipenem Ceftazidime+ceftaroline	Dual beta-lactam therapy	Potential[b]	Unknown	

a. Sullivan et al. Efficacy of epetraborole against *Mycobacterium abscessus* is increased with norvaline, PLOS pathog 2021
b. Kumar et al. Management of Mycobacterium avium complex and Mycobacterium abscessus pulmonary disease: therapeutic advances and emerging treatments, Eur Resp Review 2022

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In Summary…

- Updates in taxonomy/nomenclature
- Review of smear and culture
- Evaluated identification techniques
- Antimicrobial susceptibility testing
  - Phenotypic testing
  - Genotypic testing
- Guidelines

Thank you!

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