



Riffing on the Rifamycins



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Reducing the critical concentration of rifampin in Mycobacteria Growth Indicator Tube (MGIT™)-based susceptibility testing of *Mycobacterium tuberculosis*

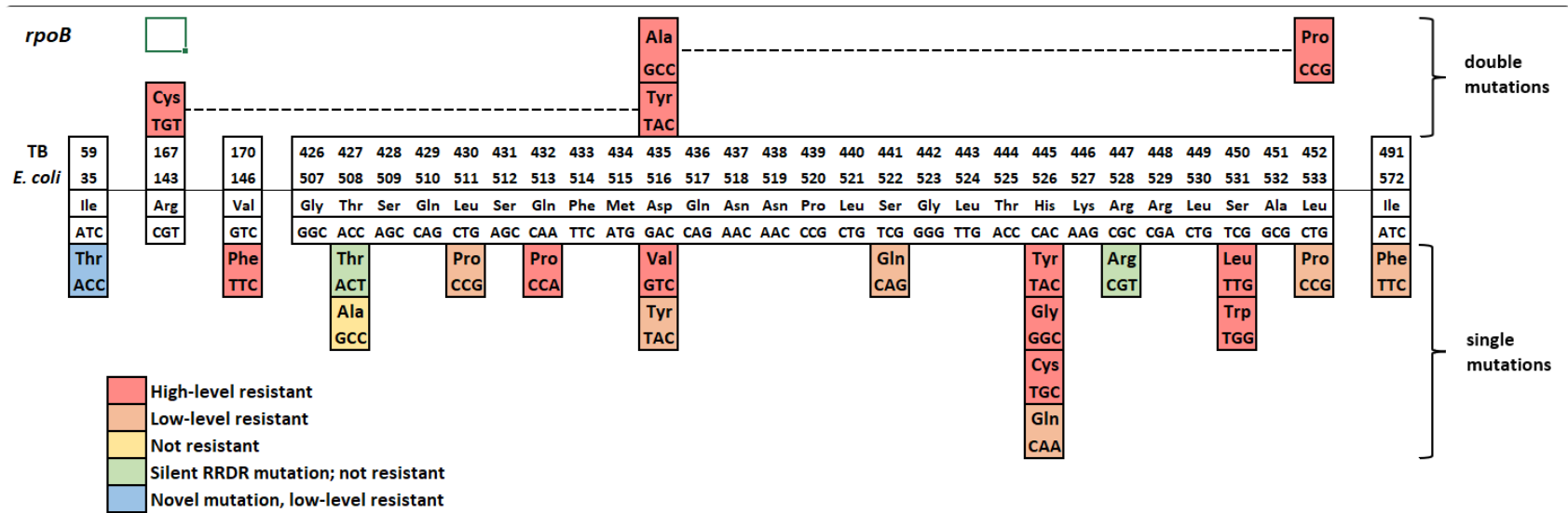
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Background

- Rifampin is the most commonly used member of the rifamycin drug class.
- Potent bactericidal activity.
- Critical component of treatment regimen for infections with rifampin susceptible *Mycobacterium tuberculosis* complex (MTBC).
- Rapid and reliable detection of rifampin resistance is crucial for the diagnosis and treatment of drug-resistant and multidrug-resistant (MDR) tuberculosis.





RIF resistance in MTBC is mainly caused by the presence of specific mutations in the 81 bp rifampin resistance determining region (RRDR) of the *rpoB* gene.

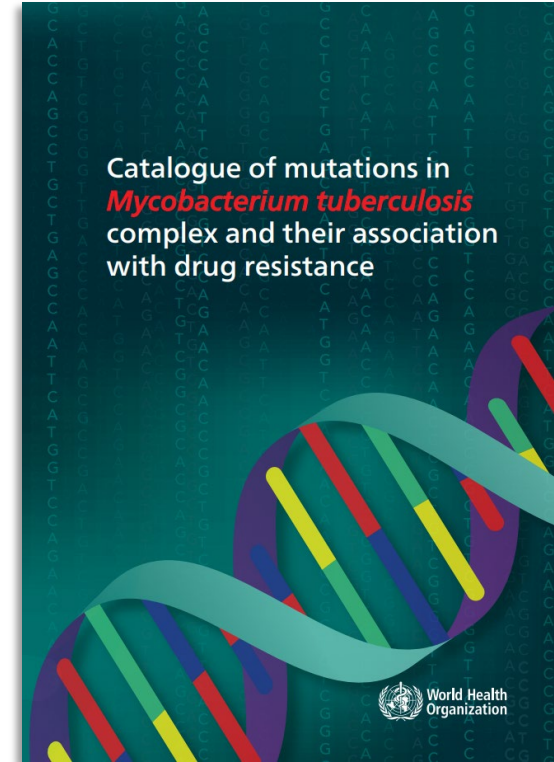
Low-level rifampin resistance

- Some *rpoB* mutations present in the RRDR region do not confer resistance to rifampin at the concentration recommended for testing (1 ug/ml), resulting in discrepant results between genotype and phenotype.
- Phenotypically susceptible by MGIT™ or agar proportion (AP) testing.
- Elevated MIC compared to susceptible MTBC strains carrying WT *rpoB*.
- Associated with poor patient outcomes if regimen includes rifampin.

Which mutations are low-level rifampin resistance mutations in *M. tuberculosis* complex?

Mutations classified as low-level or borderline by WHO:

Asp 435Tyr
Leu452Pro
His445Leu
Leu430Pro
Ile491Phe
His445Asn
His445Ser



Number of *rpoB* mutations in MTBC isolates tested at Wadsworth Center

Year	2016	2017	2018	2019	2020	2021	2022	2023 (Jan to April)
Total # of <i>rpoB</i> mutations	20	47	37	67	18	16	56	23
Single low-level mutations	3 (15%)	5 (10.6%)	8 (21.6%)	19 (28%)	0 (0%)	6 (37.5%)	7 (12.5%)	3 (13%)
# of isolates with 2 <i>rpoB</i> mutations	1	2	1	11	1	0	3	1



Drug susceptibility for MTBC has traditionally relied on the testing of anti-TB agents at a single, critical concentration particular to each drug.

Critical concentration = the lowest concentration of an anti-TB agent that will inhibit *in vitro* the growth of 99% of phenotypically wild type strains of MTBC.

Recent evidence shows that critical concentrations used for phenotypic methods to detect rifampin resistance may incorrectly classify strains with certain mutations as susceptible.

Reducing the critical concentration from 1µg/ml to 0.5µg/ml will help address the differences between phenotypic and molecular methods to detect rifampin resistance and improves the accuracy of DST. As a result, patients with tuberculosis will receive a more accurate diagnosis.



Technical report on critical concentrations for drug susceptibility testing of isoniazid and the rifamycins (rifampicin, rifabutin and rifapentine).
Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO

Technical Report on critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis.
Geneva: World Health Organization; 2018 (WHO/CDS/TB/2018.5). Licence: CC BY-NC-SA 3.0 IGO



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Validate new rifampin critical concentration in MGIT susceptibility testing

- **Purpose:** Validate the use of rifampin at a critical concentration (CC) of 0.5µg/ml to replace the 1.0µg/ml CC used in our routine panel for susceptibility testing of MTBC isolates using the BACTEC[™] MGIT[™] system.
- **Pan-susceptible (pan-s) control strain** *M. tuberculosis* H37Ra (ATCC 25177) was tested against rifampin at a CC of 0.5µg/ml and 1.0 µg/ml on multiple days.
- **A collection of 71 retrospective clinical strains** were tested at both CC and results were compared, together with MIC results and WGS susceptibility predictions.
- **Reproducibility:** A subset of the retrospective strains was tested in duplicate or triplicate.





- Reconstitute BACTEC™ MGIT™ 960 SIRE kit rifampin lyophilized drug vial with 8ml of sterile distilled/deionized water to make a stock solution of 41.5µg/ml.



- Prepare 2 BACTEC™ MGIT™ tubes by adding 0.8ml of BACTEC™ MGIT™ SIRE Supplement to each tube. One tube will be the growth control and the other is the drug tube.

- Aseptically pipet 100µl of rifampin stock solution (41.5µg/ml into the drug tube). Final concentration of rifampin in MGIT™ tube = 0.5µg/ml.

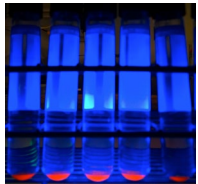


- Follow manufacturer's instructions to dilute organism in saline and inoculate drug tube and growth control tube.

- Enter drug set into BACTEC™ MGIT™ instrument as "undefined drug".

- Instrument reads the set until the growth control tube has 400 growth units.

- Growth units of drug tube <100 is susceptible, ≥100 is resistant.



Pan-S MTBC isolates tested with 0.5 µg/ml rifampin CC

- Tested 25 pan-S MTBC isolates from lineages 1 through 7.
- Selected isolates for which WGS showed no mutations in *rpoB* and with MIC results of $\leq 0.06 - 0.25$ µg/ml by broth microdilution assay (CLSI interprets an MIC ≤ 1 µg/ml as susceptible).
- All 25 isolates were susceptible in the BACTEC™ MGIT™ assay at rifampin CC of 0.5 µg/ml.

MTBC isolates highly resistant to rifampin tested with 0.5 µg/mL rifampin CC

- 20 retrospective MTBC clinical isolates with *rpoB* mutations conferring high-level resistance to rifampin.
- 15 isolates with Ser450Leu mutation, 4 with His445Tyr mutation, 1 with His445Gly mutation in *rpoB*.
- Belong to lineages 2 and 4.
- Rifampin MIC for all high-level resistant strains was >16 µg/mL.
- All strains were resistant at 1.0 and 0.5µg/mL by MGIT™ assay.



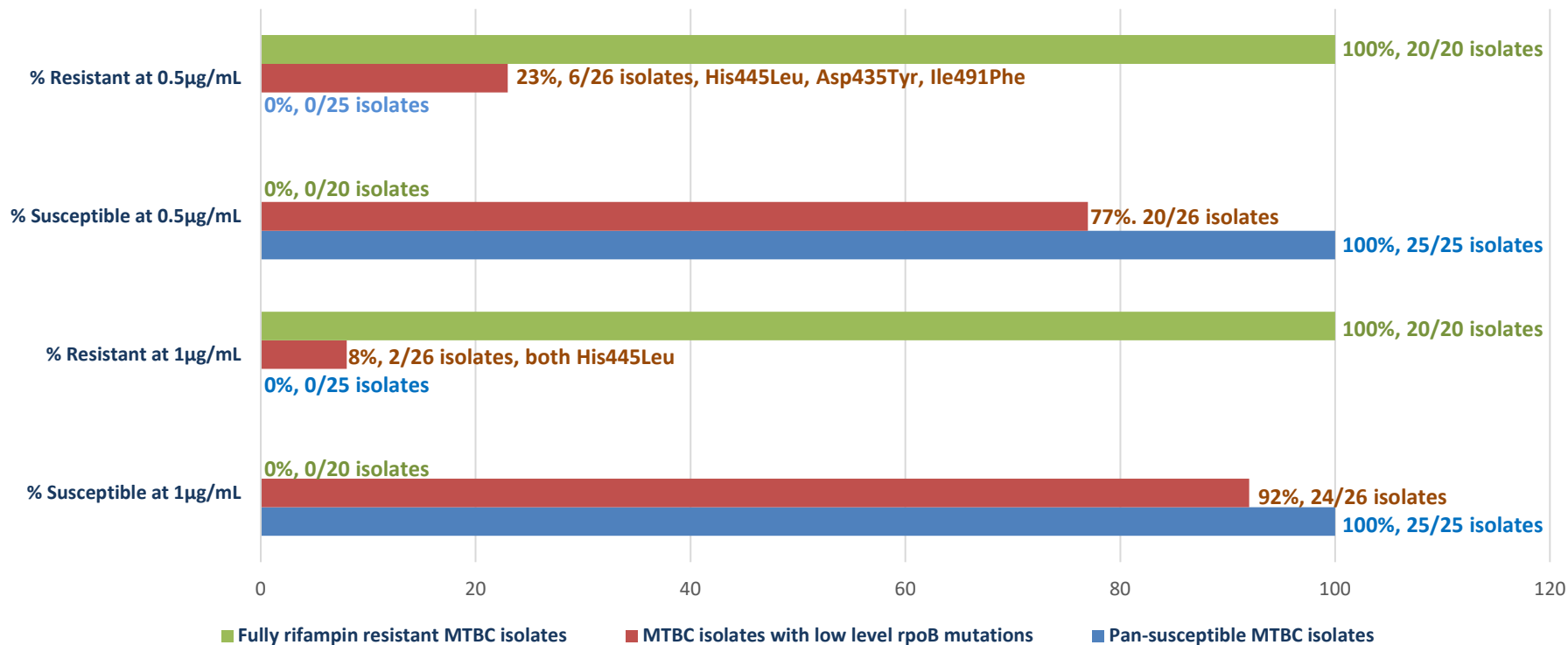
Low level resistant MTBC isolates tested with 0.5 µg/mL and 1.0 µg/mL rifampin CC

Low level <i>rpoB</i> mutation	Number tested	Lineage	MIC range µg/mL
Leu430Pro	7	L4	<=0.12 - >16
	2	L2 Beijing	2 - 4
Asp435Tyr	3	L2 Beijing	8 - 16
	3	L1	2 - 8
Leu452Pro	3	L2 Beijing	1 - 4
Ile491Phe	2	L4	1 - 2
	1	L2 Beijing	1
His445Leu	3	L4	0.5 - 8
His445Asn	1	L1	0.5
	1	L4	0.5

- 26 retrospective MTBC clinical isolates with *rpoB* mutations conferring low-level rifampin resistance.
- Isolates carrying 6 of the 7 WHO-designated low-level resistance *rpoB* mutations.



Susceptibility to Rifampin at 0.5 µg/mL and 1.0 µg/mL CC



Reproducibility for MTBC isolates at 1.0 $\mu\text{g/mL}$ and 0.5 $\mu\text{g/mL}$

- A subset of each category was tested in duplicate or triplicate at both critical concentrations on multiple days:
 - 11 pan-s MTBC strains, belonging to lineages 1, 2, 4, 5 and 6
 - 11 high level resistant strains (lineages 2 and 4)
 - 6 low level resistant strains
- Pan-susceptible H37Ra strain was tested 21 times in both concentrations on multiple days
- 100% agreement for all categories at both critical concentrations.



Conclusions about the validation for the use of 0.5 µg/mL rifampin CC

- All the high-level rifampin resistant MTBC isolates were resistant at the new critical concentration of 0.5 µg/mL.
- All pan-susceptible MTBC isolates were still susceptible when the critical concentration was decreased to 0.5 µg/mL.
- Detection of rifampin resistance at 0.5 µg/mL CC was increased by 14% among the low-level resistant MTBC isolates, compared to 1 µg/mL.
- We implemented the change of rifampin critical concentration to the MGIT™ rifampin assay in September 2022.



Could the rifampin critical concentration be lowered below 0.5 µg/mL?

- 77% of low-level rifampin resistant MTBC isolates in our study were still mischaracterized as susceptible to rifampin at this critical concentration.
- Therefore, we will likely continue to miss a significant number of these strains if we continue to use the 0.5 µg/mL CC.
- More investigation is needed.



Recent studies (1)

- WHO: Evaluated 16 studies on rifampin susceptibility testing in MGIT™.
 - 116 (74%) of 156 isolates with low-level *rpoB* mutations were susceptible to rifampin at 1.0 µg/mL CC.
 - 83 isolates (53%) were susceptible to rifampin at 0.5 µg/mL CC.
 - 55 isolates (35%) were susceptible to rifampin at 0.25 µg/mL CC.

Recent studies (2)

53 MTBC strains tested

- 5 resistant strains (all with Ser450Leu) – 100% resistant at all cc's
- 35 isolates with low level mutations:
 - 80% resistant at 0.125 µg/mL CC
 - 31.4% resistant at 0.25 µg/mL CC
 - 14.3% resistant at 0.5 µg/mL CC
 - 5.7% resistant at 1.0 µg/mL CC (2/35 isolates)
- 13 wild-type (pan-S) isolates:
 - 84.6% susceptible at 0.125 µg/mL CC. 2/13 pan-susceptible strains were resistant at 0.125 µg/mL CC
 - 100% susceptible at 1.0, 0.25 and 0.5 µg/mL CC.

Extended MGIT incubation time at all critical concentrations

- 0.5 µg/mL CC with extended incubation: 65.7% of low level strains were resistant
- 0.25 and 0.125 µg/mL CC with extended incubation – started to see resistance in wild-type strains.

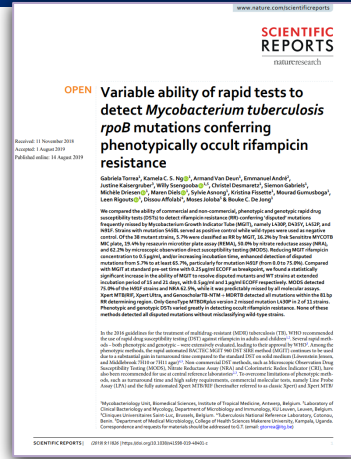
Recommended lowering the critical concentration to 0.5 µg/mL CC in combination with extended MGIT incubation time.

Torrea G, Ng KCS, Van Deun A, André E, Kaisergruber J, Ssengooba W, Desmaretz C, Gabriels S, Driesen M, Diels M, Asnong S, Fissette K, Gumusboga M, Rigouts L, Affolabi D, Joloba M, De Jong BC. Variable ability of rapid tests to detect *Mycobacterium tuberculosis* *rpoB* mutations conferring phenotypically occult rifampicin resistance. *Sci Rep.* 2019 Aug 14;9(1):11826. doi: 10.1038/s41598-019-48401-z. PMID: 31413308; PMCID: PMC6694172.



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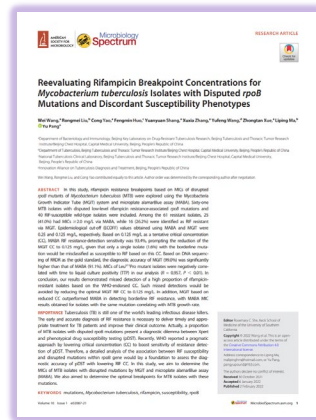


Recent studies (3)

- 303 MTBC isolates were tested in MGIT™ system at 0.5 µg/mL and 1.0 µg/mL of rifampin:
 - 181 pan-susceptible isolates.
 - 122 isolates with *rpoB* mutations, including 32 with single low-level rifampin resistance mutations.
 - Only 2 low-level rifampin resistant isolates that were susceptible at 1.0 µg/mL CC were phenotypically resistant at 0.5 µg/mL CC.
 - One pan-susceptible strain was classified as rifampin resistant at 0.5 µg/mL CC
- Concluded that changing the rifampin critical concentration from 1.0 to 0.5µg/mL did not significantly increase the sensitivity of the MGIT DST assay.



Recent studies (4)



- 101 MTBC isolates
 - 61 strains with low-level rifampin resistance *rpoB* mutations.
 - 40 pan-susceptible strains with wild-type *rpoB*.
 - Used a range from 0.125 µg/mL to 1 µg/mL for rifampin CC in the MGIT™ system.
 - All 40 of pan-s strains were still correctly called susceptible at 0.125 µg/mL CC.
 - 60 of the 61 low-level strains were correctly called resistant at this CC.
- Concluded that rifampin CC should be dropped to 0.125 µg/mL for MGIT™ testing.

Wang W, Liu R, Yao C, Huo F, Shang Y, Zhang X, Wang Y, Xue Z, Ma L, Pang Y. Reevaluating Rifampicin Breakpoint Concentrations for Mycobacterium tuberculosis Isolates with Disputed *rpoB* Mutations and Discordant Susceptibility Phenotypes. Microbiol Spectr. 2022 Feb 23;10(1):e0208721. doi: 10.1128/spectrum.02087-21. Epub 2022 Feb 2. PMID: 35107324; PMCID: PMC8809345.



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Testing MTBC isolates at 0.25 µg/mL and 0.125 µg/mL rifampin CC

- Rifampin from BACTEC™ MGIT™ SIRE kit was further diluted:
 - Stock solution at 20.75 µg/mL was used in MGIT™ tubes to give a final critical concentration of 0.25 µg/mL.
 - Stock solution at 10.375µg/mL was used in MGIT™ tubes to give a final critical concentration of 0.125 µg/mL.
- Manufacturer instructions were followed for testing using these lower concentrations.
- The 26 MTBC isolates with low-level rifampin resistance *rpoB* mutations and 23 of the pan-s MTBC isolates were tested at 0.25 and 0.125 µg/ml.



Picture from BD.com

Testing reproducibility with H37Ra control strain at 0.25 µg/mL and 0.125 µg/mL CC

Date Set Up	Date Positive	MGIT™ Rifampin				
		Control Growth Units	0.25µg/mL		0.125µg/mL	
			Growth (Y/N)	Units	Growth (Y/N)	Units
5/18/2022	5/28/2022	400	N	0	N	0
5/23/2022	5/31/2022	400	N	0	N	0
6/1/2022	6/8/2022	400	N	0	N	0
7/19/2022	7/28/2022	400	N	0	N	0
7/27/2022	8/6/2022	400	N	0	N	0
8/4/2022	8/12/2022	400	N	0	N	0
8/10/2022	8/18/2022	400	N	0	N	0
8/24/2022	9/1/2022	400	N	0	N	0
9/1/2022	9/10/2022	400	N	0	N	0
9/9/2022	9/17/2022	400	N	0	N	0
9/15/2022	9/24/2022	400	N	0	N	0
9/21/2022	9/30/2022	400	N	0	N	0
9/28/2022	10/12/2011	400	N	0	N	0
11/29/2022	12/7/2022	400	N	0	N	0
12/7/2022	12/14/2022	400	N	0	N	0
1/30/2023	2/6/2023	400	N	0	N	0
2/22/2023	3/5/2023	400	N	0	N	0
3/7/2023	3/18/2023	400	N	0	N	0
4/13/2023	4/25/2023	400	N	0	N	0
5/3/2023	5/15/2023	400	N	0	N	0
5/9/2023	5/17/2023	400	N	0	N	0

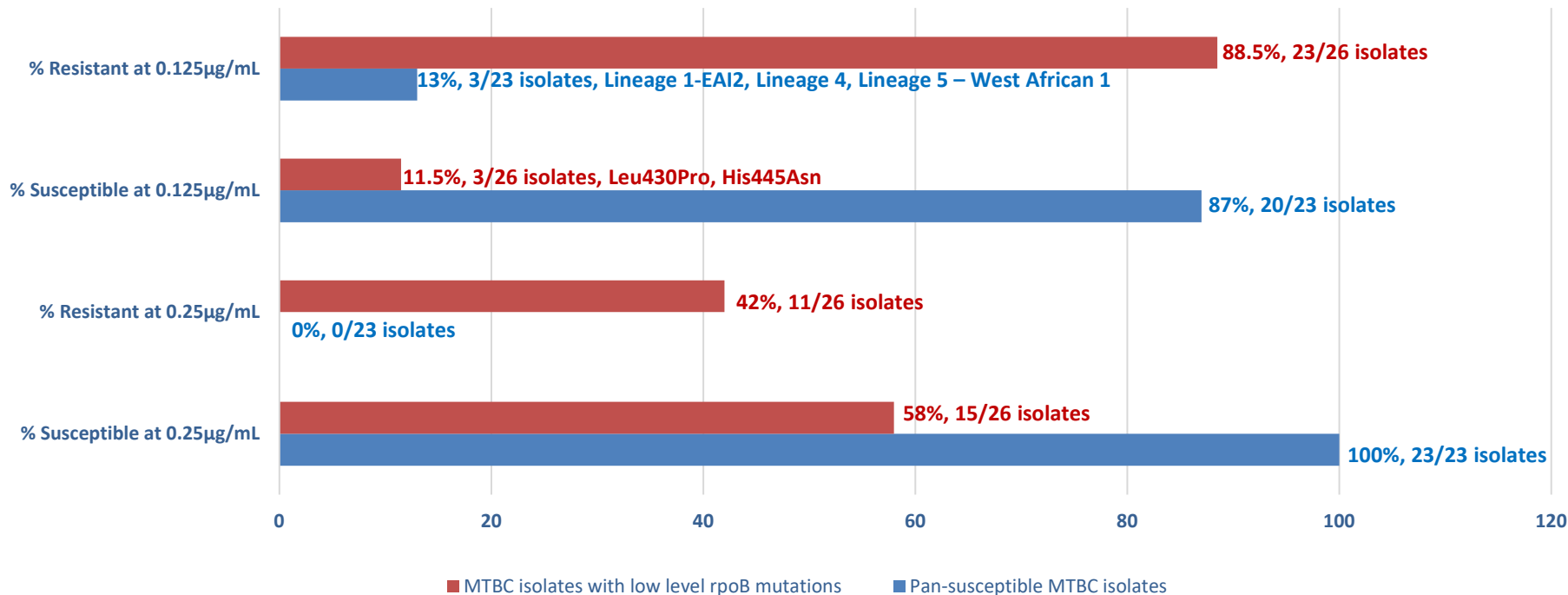
- H37Ra control strain tested at 0.25 µg/mL and 0.125 µg/mL rifampin CC on 21 different days.
- 100% susceptible.



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Rifampin susceptibility results at 0.25 µg/mL and 0.125 µg/mL CC



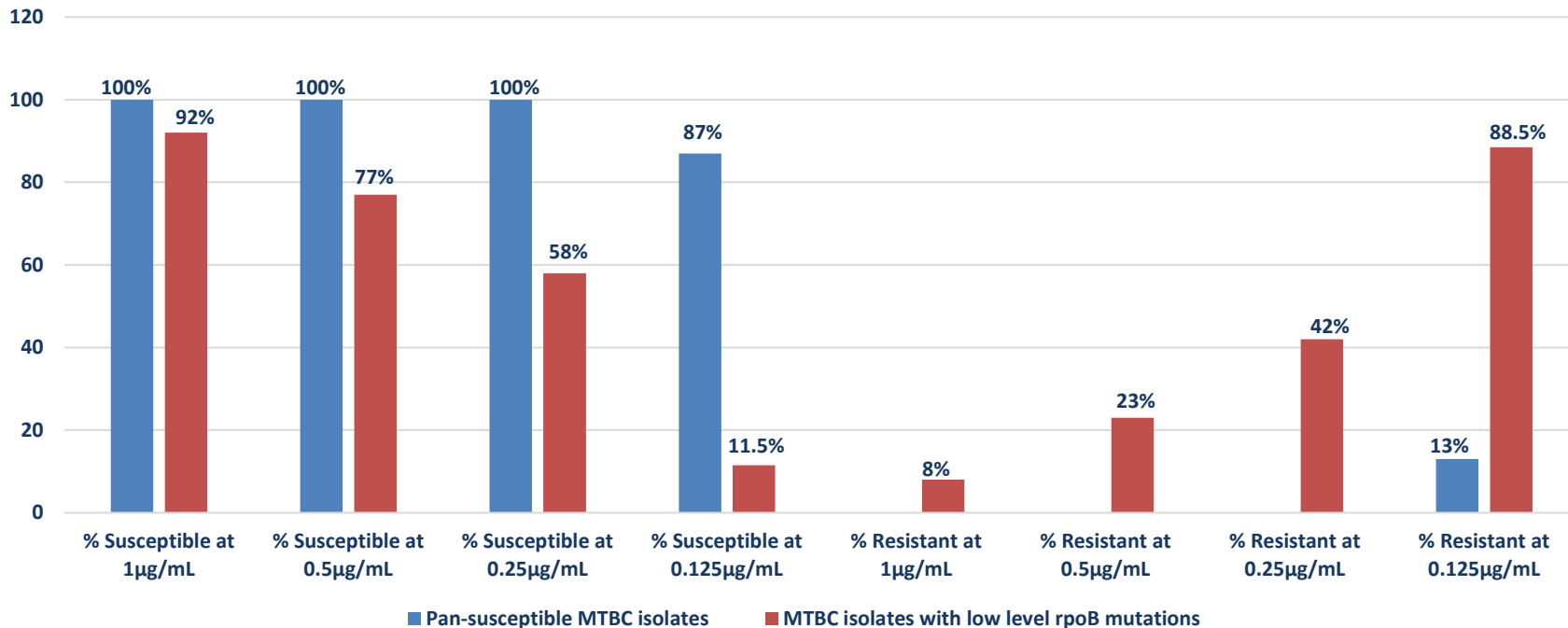
Testing reproducibility with low-level rifampin resistant MTBC isolates at 0.25 µg/mL rifampin CC

IDR#	<i>rpoB</i> mutation present	Lineage	Date Set Up	Date Positive	MGIT growth units at 0.25µg/mL
IDR1700013003	Ile491Phe	L4-X3	6/1/2022	6/13/2022	400
			6/1/2022	6/14/2022	400
			5/5/2023	5/17/2023	400
IDR1900012037	His445Leu	L4 Euro-American	1/20/2022	2/1/2022	400
			1/20/2022	2/1/2022	400
			4/14/2022	4/26/2022	400
			4/14/2022	4/26/2022	400
IDR1900029234	Leu430Pro	L2 -Beijing	12/30/2021	1/7/2022	0
			12/30/2021	1/8/2022	0
IDR2200049133	Asp435Gly	L2 -Beijing	12/7/2022	12/12/2022	0
			12/7/2022	12/12/2022	0
IDR1900031541	Leu430Pro	L4-X3	8/11/2021	8/19/2021	400
			8/11/2021	8/20/2021	0
IDR2100031507	Asp435Tyr	L2 -Beijing	7/15/2021	7/25/2021	400
			7/15/2021	7/26/2021	400
			7/15/2021	7/26/2021	400
			9/23/2021	10/1/2022	372
IDR2200042285	Leu430Pro	L4-LAM4	9/21/2022	10/4/2022	233
			10/3/2022	10/14/2022	400

- 7 isolates with low level mutations tested multiple times on different days
- 6 isolates showed 100% agreement
- 1 isolate was resistant once and susceptible once at 0.25 µg/mL rifampin CC.



Overall summary of rifampin susceptibility testing results



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Conclusion (1)

- Our results demonstrate that a high proportion of low-level rifampin resistant MTBC isolates will still not be detected phenotypically using MGIT™ DST at the new WHO-endorsed critical concentration of 0.5 µg/mL .
- Rifampin critical concentration of 0.25 µg/mL might be a possible alternative.
- The highest number of low-level rifampin resistant isolates detected was obtained with a critical concentration of 0.125µg/mL. However, some pan-s strains with wild-type *rpoB*, mostly belonging to ancient lineages, exhibited growth at this concentration.



Conclusion (2)

- More testing needs to be done, especially for the lower rifampin critical concentrations, before implementation, if appropriate.
- Extending MGIT™ DST incubation time might be helpful
- Combination of genotypic and phenotypic DST testing is advisable to detect low-level rifampin resistance.

Miotto P, Cabibbe AM, Borroni E, Degano M, Cirillo DM. Role of Disputed Mutations in the *rpoB* Gene in Interpretation of Automated Liquid MGIT Culture Results for Rifampin Susceptibility Testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2018 Apr 25;56(5):e01599-17. doi: 10.1128/JCM.01599-17. PMID: 29540456; PMCID: PMC5925711.



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THANK YOU!!



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