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Original Article

Middlebrook 7h11 Reduces Invalid Results and Turnaround Time of Phenotypic Drug-Susceptibility Testing of *M. tuberculosis*

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Abstract

Background: Phenotypic drug-susceptibility testing (pDST), which relies on growth inhibition in the drug-containing media, remains a challenge for fastidious Mycobacterium tuberculosis complex (MTBc) isolates due to insufficient growth on the growth controls (GC). Middlebrook 7H11 (M7H11) medium contains casein hydrolysate, which may favor the growth of such strains. **Method:** In this study, we tested whether M7H11 reduces invalid results due to insufficient growth on the GCs and the turnaround time (TAT) of pDST for MTBc compared to Middlebrook 7H10 (M7H10) without affecting the accuracy of the pDST results and how it differs between rifampicin- and isoniazid-susceptible non multi-drug resistant (non-MDR), MDR and MDR with additional resistance to fluoroquinolones (Pre-XDR) MTBc isolates. We compared the proportions of invalid pDST results due to lack of growth on the GCs, TATs of valid parallel drug-susceptibility testings as an indicator of speed of MTBc growth, and colony-forming unit (CFU) count on the most diluted GC of the parallel pDSTs after equal incubation periods as an indicator of growth abundance on M7H11 and M7H10. We also analyzed the agreement between the pDST results of the same drug or drugs in the same drug class, tested in parallel on both media. **Results:** For MDR and pre-XDR isolates, relative to M7H10, M7H11 significantly reduced the occurrence of invalid pDST results due to insufficient growth on the GCs (odds ratio [OR] = ∞ [95% confidence interval (CI) 1.9–∞], P = 0.004 for MDR, OR = ∞ [95% CI 3.3–∞], P = 0.0001 for pre-XDR) and the TAT of pDSTs (OR = 17 [95% CI 2.6–710.4], P = 0.0001 for MDR, OR = 9.3 [95% CI 4.0–26.5], $P < 0.0001$ for pre-XDR). The growth abundance of MTBc on M7H11 was significantly higher compared to M7H10 (17 CFU on M7H10 vs. 28 on M7H11), irrespective of drug-resistance profiles. The agreement between the pDST results between the two media was high (Cohen's $k > 0.98$). **Conclusion:** Our study findings suggest that M7H11 is preferred over M7H10 for pDSTs of MTBc isolates.

Keywords: Mycobacterium Tuberculosis, Drug-susceptibility testing, proportion method

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Introduction

The prompt and accurate diagnosis of drug resistance (DR) is key to assign an effective treatment regimen, which minimizes further development and transmission of drug-resistant tuberculosis (TB). Due to the slow-growing nature of the *Mycobacterium tuberculosis* complex (MTBC), as well as the cost and the demand for the sophisticated infrastructure of conventional phenotypic drug-susceptibility testing (pDST), rapid genotypic DST represents the most convenient option to obtain drug-susceptibility data for the clinical management of the patients. However, for the majority of anti-TB drugs, culture-based pDST remains the reference standard, also for resistance conferred by mutations outside the "hotspot"

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regions targeted by World Health Organization-endorsed rapid molecular tests, such as GeneXpert MTB/RIF (Cepheid, USA), Genotype MTBDR*plus* and MTBDR*sl* (Hain Life Sciences, Germany).[1-4] More importantly, rapid molecular tests are not yet available for novel anti-TB drugs, for which a knowledge gap remains on the correlation of resistance-conferring mutations and MICs, and the clinical breakpoint is not yet known.[5] Therefore, pDST remains important for accurate diagnosis of DR.

The indirect proportion method is the most commonly used pDST method to determine the drug susceptibility of MTBc starting from a pure culture. This method compares the colony-forming unit (CFU) count of a known inoculum on a drug-free medium known as the growth control (GC) versus the CFU count on the drug-containing media containing the critical concentration (CC) of a drug.[6] Lowenstein-Jensen (LJ), Middlebrook 7H10 (M7H10), and Middlebrook 7H11 (M7H11) are commonly used solid media for this method.[7,8] Agar-based, M7H11, and M7H10 have advantages over egg-based LJ medium as growth appears earlier and it is easy to visualize colonies on transparent agar-based media.[7] M7H11 is considered an improved version of M7H10 due to the presence of casein hydrolysate in M7H11; which provides nitrogen, vitamins, and amino acids and is reported to favor the growth of fastidious, drug-resistant MTBc that grow poorly on M7H10.[7,9]

The most complex treatment decisions pertain to patients whose strain is already resistant to rifampicin. Such strains have mutations in the essential *rpoB* gene that encodes the β sub-unit of RNA polymerase in MTBc, resulting in variable degrees of growth defects.[10] Since pDST methods measure growth inhibition in drug-containing media, such mutations may lead to higher proportions of failed pDSTs due to insufficient growth on the GCs, or result in false susceptibility if the GC yields a valid result, but the lower fitness strain requires longer incubation to grow in the presence of rifampicin.[11] We, therefore, analyzed if M7H11 could reduce the occurrence of invalid results due to insufficient growth on the GCs, and increase growth speed, and growth abundance compared to M7H10 without affecting the accuracy of the pDST results. We also assessed whether results differed between rifampicin and isoniazid susceptible nonmulti-drug resistant (non-MDR), MDR, and MDR with additional resistance to fluoroquinolones (Pre-XDR) MTBc isolates.

Methods

Sample size and inclusion criteria

A total of 401 MTBc isolates, originating from Afghanistan, Armenia, Belarus, Georgia, India, Kenya, Kyrgyzstan, Mozambique, Myanmar, and Ukraine, with known resistance profiles for rifampicin, isoniazid, and fluoroquinolones, were included in this study. Based on resistance/susceptibility to rifampicin, isoniazid, and fluoroquinolones, we categorized these isolates into three DR groups: Non-MDR (23 isolates susceptible to rifampicin, isoniazid, and fluoroquinolones), MDR (102 isolates resistant to rifampicin, isoniazid but susceptible to fluoroquinolones) and Pre-XDR (276 MDR with additional resistance to fluoroquinolones). The study design and the workflow are summarized in Figure 1.

Media and antibiotics

M7H10 and M7H11 media were prepared as per the manufacturer's recommendations[12] and stored at 2–8°C for 6 months maximum. Stock solutions were prepared for ofloxacin (Sigma-Aldrich, O8757) and levofloxacin (Sigma–Aldrich, 28,266) at 10000 mg/L in 0.1 N sterile NaOH, and for linezolid (Sigma–Aldrich, PZ0014) at 10000 mg/L in dimethyl sulfoxide ([Sigma–Aldrich, D5879]). All stock solutions were stored in aliquots at below −18°C for 12 months maximum.

Phenotypic drug-susceptibility testing

pDSTs for linezolid (1 μg/ml) on both M7H11 and M7H10, levofloxacin (1 μg/ml) on M7H10, and ofloxacin (2 μg/ml) on M7H11 were performed in parallel, by the same operator on the same day using the same bacterial suspension, using the proportion method. Bacterial colonies were scraped from fresh MTBc cultures on LJ slants, not older than 2 weeks after the first colonies were visible, and thoroughly homogenized in sterile water with glass beads. The density of the suspension was visually adjusted to McFarland 1. An inoculum of 10^{-1} of McFarland 1 was used for the drug-containing tubes and the least diluted GC1, while the most diluted GC2 was inoculated with a 10^{-3} dilution. CFUs were enumerated after 4 weeks of incubation at 34°C–38°C with 5%–10% CO_2, using the quantitative scale as shown in Table 1. If both GC1 and GC2 had sufficient growth at this point, i.e. GC1 ≥1+ (51–100 CFUs) and ≤1+ GC2 >3 CFUs, CFU counts were recorded accordingly, and pDST results were interpreted. An isolate was considered resistant to the drug tested if the drug-containing tube had equal or more growth than the GC2. If GC1 and/or GC2 had insufficient CFU counts at 4 weeks, tubes were incubated at 37°C for two more weeks. Any test that had insufficient CFU counts on GC1 and/or GC2 after 6 weeks of incubation or that had more than 1+ growth on GC2 was considered invalid.

Targeted deep sequencing (*gyrA* and *gyrB* for fluoroquinolones, *rplC,* and *rrl* for linezolid) was performed using the

Table 1: Quantitative scale used for the growth of *Mycobacterium tuberculosis* on solid media

CFU count	Recording method
0-50	The exact number of colonies
51-100	1+
101-200	2+
>201	3+
Confluent growth	4+

CFU: Colony-forming unit

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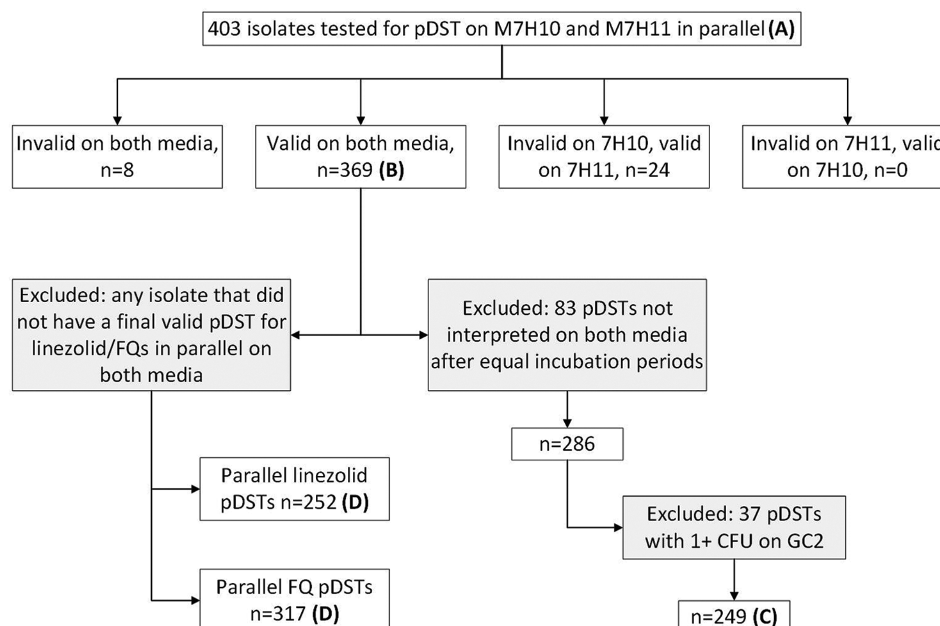

Figure 1: Flow diagram for the use of isolates for different analyses. A: Used to compare the proportion of invalid results for the pDSTs performed in parallel on M7H10 and M7H11, B: Used to compare the growth speed between the two media, C: Used to compare growth abundance on GC2 between M7H10 and M7H11, D: Used to analyze the agreement between pDSTs between the two media, CFU: Colony-forming units, FQ: Fluoroquinolones, GC2: The most diluted growth control, pDST: Phenotypic drug-susceptibility testing

Deeplex-MycTB assay (Genoscreen, Lille, France) described elsewhere[13] on any isolate that had discrepant pDST results between the two media.

Quality control

The pan-susceptible MTBc strain H37Rv (ATCC 27294, BCCM/ITM 2008-03715) and reference strains for each drug (BCCM/ITM 102197 for levofloxacin and ofloxacin, and 130318 for linezolid) were included as quality control strains for each new batch of drug stock solutions and media.

Statistical analysis

Statistical data analysis was performed using Stata/SE 17.0 software (Stata Corp, USA). The Exact McNemar's test was used to compare paired categorical data, such as the occurrence of an invalid result and the turnaround time (TAT) of the pDSTs between the two media. The TAT of the pDSTs was used as an indicator of the growth speed and all isolates with a valid pDST result on both media in parallel were included in this data set. CFU count on the GC2 of a sub-set of these isolates, whose pDST results were interpreted after equal incubation time (e.g., pDSTs that were interpreted after 4 weeks of incubation on both media) and with countable colonies (0–50 colonies) on the GC2 of both media was compared as an indicator of growth abundance using Wilcoxon Matched-Pairs signed Ranks test. The occurrence of invalid pDST results due to insufficient growth on the GCs, TAT, and growth abundance between the two media were compared irrespective of the DR profile (overall) and also separately for the three DR groups. The difference in proportion or mean difference was calculated with a 95% confidence interval (CI) and P value, which was considered statistically significant at <0.05. Cohen's Kappa coefficient was used to analyze the extent of agreement between pDST results for the same drug/drugs in the same drug class on M7H11 and M7H10.

Results

Percentage of invalid results

Of 401 parallel pDSTs, 32 (7.9%) were invalid at least on one medium: 8 (2.0%) were invalid on both media, 24 (6.0%) were invalid only on M7H10, and none was invalid only on M7H11. All 32 pDSTs were invalid due to less than three CFUs on the GC2 after 6 weeks of incubation.

Overall, there was a statistically significant reduction [odds ratio (OR) = ∞ (95% CI 6.01–∞), P < 0.0001, Table 2] of invalid pDST results due to lack of growth on the GC2 on M7H11 compared to M7H10 [Figure 1]. When stratified by the DR profile, in both MDR [OR = ∞ (95% CI 1.9–∞), P = 0.004, Table 2] and Pre-XDR groups [OR = ∞ (95% CI 3.3–∞), P = 0.0001, Table 2], the occurrence of invalid pDST results due to lack of growth on the GC2 was significantly higher on M7H10 but not in the non-MDR group [OR = ∞ (95% CI 0.025–∞), P = 1.0, Table 2].

Growth speed

A total of 369 isolates had a valid pDST result in parallel on both media, using the same bacterial suspension. Overall, there was a significant reduction of the TATs of the pDSTs

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Table 2: Occurrence of initially invalid phenotypic drug-susceptibility testing results due to <3 colony-forming unit on the most diluted growth control (most diluted growth control (10^{-3}))

Overall	M7H11			Non-MDR	M7H11			MDR	M7H11			Pre-XDR	M7H11		
	Valid	Invalid	Total		Valid	Invalid	Total		Valid	Invalid	Total		Valid	Invalid	Total
M7H10				M7H10				M7H10				M7H10			
Valid	369	0	370	Valid	21	0	21	Valid	91	0	91	Valid	257	0	257
Invalid	24	8	32	Invalid	1	1	2	Invalid	9	2	11	Invalid	14	5	19
Total	393	8	401	Total	22	1	23	Total	100	2	102	Total	271	5	276
OR=∞ (95% CI 6.01–∞)				OR=∞ (95% CI 0.025–∞)				OR=∞ (95% CI 1.9–∞)				OR=∞ (95% CI 3.3–∞)			
McNemar's P<0.0001				McNemar's P=1				McNemar's P=0.004				McNemar's P=0.0001			

GC2: Most diluted growth control (10^{-3}), MDR: Multi-drug resistant, XDR: Extensively drug resistant. OR: Odds ratio, CI: Confidence interval, M7H10: Middlebrook 7H10, M7H11: Middlebrook 7H11

on M7H11 compared to M7H10 (OR = 10.8 [95% CI 5.0–27.9), P < 0.0001); thus, the growth speed of MTBc was significantly higher on M7H11 [Table 3]. When stratified by the DR profile, the TATs of the pDSTs on M7H11 were significantly lower compared to M7H10 in the MDR (OR = 17 [95% CI 2.6–710.4], P = 0.0001) and Pre-XDR groups (OR = 9.3 [95% CI 4.0–26.5], P < 0.0001) but not in the non-MDR group (OR = ∞ [95% CI 0.41–∞], P = 0.25) [Table 3].

Growth abundance

Of 401 pDSTs inoculated on both media using the same bacterial suspension, 286 (71.3%) were interpreted after equal incubation periods. From these, we excluded 37 pDSTs with 1+ growth on the GC2 of both/any pDSTs and compared the CFU count on GC2 of the remaining 249. On M7H11, growth abundance was significantly higher (Wilcoxon signed–rank test P < 0.0001) with a median of 28 CFUs (interquartile range [IQR] 27) compared to 17 CFUs (IQR 23) on M7H10 [Figure 2]. The difference was significant across all DR groups (Wilcoxon signed–rank test P = 0.03 [Non-MDR], P = 0.004 [MDR], P < 0.0001 [Pre-XDR]) [Table 4].

Agreement between the phenotypic drug-susceptibility testing results

A total of 252 isolates had valid pDST results for linezolid in parallel on both media. All but one pDST results were concordant (99.6% agreement, Cohen's k: 0.98). One isolate was resistant to linezolid on M7H10 but susceptible on M7H11. Targeted deep sequencing of this isolate did not detect any drug-resistance conferring mutations in the *rplC* and *rrl* genes. A total of 317 isolates had valid pDST results for levofloxacin (1 µg/ml) on M7H10 and ofloxacin (2 µg/ml) on M7H11 in parallel. All pDST results for levofloxacin and ofloxacin had an excellent agreement of 100%.

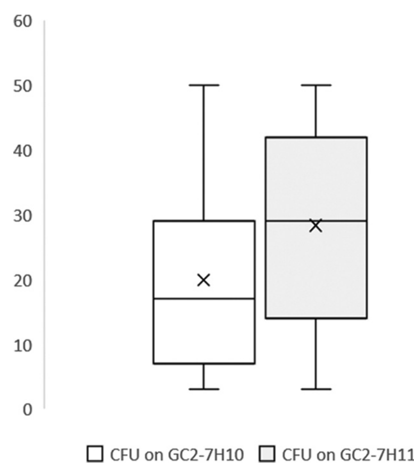

Figure 2: Distribution of the CFU count on GC2 of 249 phenotypic drug-susceptibility tests inoculated on M7H11 and M7H10 agar in parallel using the same bacterial suspension. X = Mean of the plotted CFU counts. CFU: Colony-forming units, GC2: Growth control 2

Discussion

In this retrospective study, we tested if M7H11 reduces the occurrence of invalid results due to lack of growth on the GCs, and increases the growth speed and abundance of MTBc isolates compared to M7H10. Our results showed that, for non-MDR-TB isolates, the differences between the proportions of invalid pDST results due to lack of growth on the GCs or TATs between the two media were not significant. However, for MDR and pre-XDR isolates, compared to M7H10, the M7H11 medium not only significantly lowered the occurrence of invalid results due to lack of growth on the GCs, thus reducing the need to repeat the tests but also significantly reduced the TAT of the pDST results, (~19% of pDSTs had a net gain of 2 weeks in TAT) without affecting the accuracy of the pDST results. Any decrease in TAT of pDSTs is precious for the clinical management of the patients. Even though some researchers have suggested that the addition of casein hydrolysate makes no difference to the growth of MTBc,[14] our study results suggest casein hydrolysate improves the growth of MTBc, especially for isolates with DR-conferring mutations, which are known to affect *in vitro* fitness to different degrees.[15]

This study has limitations. While most rifampicin-resistance conferring mutations carry a significant fitness cost, we did not study the impact of casein hydrolysate on the growth of different *rpoB* mutants and different lineages of MTBc. In addition, in this study, we did not study if M7H11 provides an advantage for the growth of MTBc already exposed to anti-TB drugs, as suggested by Joloba *et al*.[16] Another limitation is the comparison

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Table 3: Turnaround time of the parallel phenotypic drug-susceptibility testing with a valid result

Overall	M7H11			Non-MDR	M7H11			MDR	M7H11			Pre-XDR	M7H11		
	4 weeks	6 weeks	Total		4 weeks	6 weeks	Total		4 weeks	6 weeks	Total		4 weeks	6 weeks	Total
M7H10				M7H10				M7H10				M7H10			
4 weeks	265	7	272	4 weeks	15	0	15	4 weeks	66	1	67	4 weeks	184	6	190
6 weeks	76	21	97	6 weeks	3	3	6	6 weeks	17	7	24	6 weeks	56	11	67
Total	341	28	369	Total	18	3	21	Total	83	8	91	Total	240	17	257
OR=10.8 (95% CI 5.0–27.9)				OR=∞ (95% CI 0.41–∞)				OR=17 (95% CI 2.6–710.4)				OR=9.3 (95% CI 4.0–26.5)			
McNemar's *P*=0.0001				McNemar's *P*=0.25				McNemar's *P*=0.0001				McNemar's *P*<0.0001			

MDR: Multi-drug resistant, XDR: Extensively drug-resistant, OR: Odds ratio, CI: Confidence interval, M7H10: Middlebrook 7H10, M7H11: Middlebrook 7H11

Table 4: Median colony-forming units on most diluted growth control (10^{-3}) of parallel phenotypic drug susceptibility testings interpreted after equal incubation periods

	Median CFU count on GC2		Wilcoxon signed-rank (*P*)
	M7H10	M7H11	
Overall	17 (IQR=23)	28 (IQR=27)	<0.0001
Non-MDR	17 (IQR=22)	30 (IQR=27)	0.03
MDR	17 (IQR=24)	28 (IQR=27.5)	0.004
Pre-XDR	17 (IQR=24)	28 (IQR=27)	<0.0001

CFU: Colony-forming unit, GC2: Most diluted growth control (10^{-3}), MDR: Multi-drug resistant, XDR: Extensively drug resistant, M7H10: Middlebrook 7H10, M7H11: Middlebrook 7H11

of levofloxacin (1 μg/ml) on M7H10 with ofloxacin (2 μg/ml) pDST results on M7H11. Even though in our data set, there was a perfect agreement between the results, these two drugs have different levels of *in vitro* activity against MTBc.

Finally, for non-MDR isolates, M7H11 showed no statistically significant advantages over M7H10 to reduce the proportion of invalid pDSTs due to lack of growth on the GCs or the TAT of the pDSTs. However, this should be generalized with caution as the sample size of non-MDR isolates included in this analysis was smaller compared to the other two DR groups, and it might have impacted the power of statistical analyses performed for non-MDR isolates. Moreover, we did not find a detrimental effect of using M7H11 for non-MDR isolates. Patients infected with RR-TB often require additional DR testing for new/repurposed anti-TB drugs for which genotypic drug-susceptibility testing is not yet well established; based on our study findings, we recommend M7H11 medium over M7H10, for pDSTs of not only MDR or pre-XDR but also for non-MDR isolates.

Ethical statement

Ethics approval was not required for this laboratory-based study, as anonymized stored clinical isolates were used.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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