Reduced turn-around time for *Mycobacterium tuberculosis* drug susceptibility testing with a proportional agar microplate assay

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Abstract

Multidrug-resistant tuberculosis is a major issue worldwide; however, accessibility to drug susceptibility testing (DST) is still limited in developing countries, owing to high costs and complexity. We developed a proportion method on 12-well microplates for DST. The assay reduced the time to results to <12 days and <10 days when bacterial growth was checked with the naked eye or a microscope, respectively. Comparison with the Canetti–Grosset method showed that the results of the two assays almost overlapped (kappa index 0.98 (95% CI 0.91–1.00) for isoniazid, rifampicin, streptomycin; and kappa index 0.92 (95% CI 0.85–0.99) for ethambutol). The sequencing of genes involved in drug resistance showed similar level of phenotype–genotype agreement between techniques. Finally, measurement of the MICs of rifampicin and ethambutol suggests that the currently used critical ethambutol concentration should be revised, and that the current molecular drug susceptibility tests for rifampicin need to be re-evaluated, as *in vitro* rifampicin-sensitive isolates could harbour drug resistance-associated mutation(s).

Keywords: Drug resistance, drug susceptibility testing, *M. tuberculosis*, MIC, proportional microplate assay, sequencing
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Introduction

Multidrug-resistant tuberculosis (MDR-TB), i.e. tuberculosis (TB) resistant to at least isoniazid and rifampicin, the two essential first-line anti-TB drugs [1]), is a major challenge. However, in the countries with the highest MDR-TB burden, less than one-fifth of patients with MDR-TB are detected, owing to the lack of diagnostic capacity [2], leading to treatments without prior drug susceptibility testing (DST) [3–5]. Sputum conversion is achieved in only 33–38% of retreatment cases in such settings [5,6], which is a much lower rate than that achieved with DST-based treatments (38% vs. 79%) [5].

Liquid medium-based DST with automated culture systems such as BACTEC 460 TB and BACTEC MGIT 960 [7] is efficient and fast, but expensive. Other disadvantages are invisible contaminations, overgrowth of atypical mycobacteria, inability to check the colony morphology and to determine the MIC, and the need for multiple tubes or bottles [8]. GeneXpert, a fast molecular-based test, was endorsed by the WHO for the identification of suspected MDR-TB cases [9]. However, the high cost hampers its routine use in developing countries [9]. Moreover, the suspected MDR-TB cases detected by GeneXpert still need to be confirmed with a reference standard technique [10], such as proportional DST on Löwenstein–Jensen medium [11–13], which takes 6–8 weeks [14].

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The proportional method on M7H10 agar developed by the National Committee for Clinical Laboratory Standards is the reference standard drug susceptibility test for mycobacteria in the USA [15,16]. It is also used in The Netherlands and elsewhere, but on microplates instead of in culture tubes or bottles, providing a low-cost, high-throughput, fast and easy-to-read test with high sensitivity, specificity, and reproducibility [8,17]. Recent studies have shown that some drug-resistant *Mycobacterium tuberculosis* isolates do not grow or grow very slowly on M7H10 medium, leading to false drug-sensitive results. Therefore, it was recommended to replace M7H10 with M7H11 agar, which is supplemented with a pancreatic digest of casein, to facilitate the growth of fastidious *M. tuberculosis* cultures [18,19].

Here, we describe a proportional method for DST in 12-well microplates with M7H11 medium supplemented with OADC and low-magnification microscopic monitoring of bacterial growth to reduce turn-around time. We compared the results with those obtained with the conventional Canetti–Grosset proportional method. We also sequenced the genes involved in drug resistance to validate the DST profiles, and measured MICs in *M. tuberculosis* isolates with conflicting DST and sequencing results.

Materials and methods

Ethics statement

The National Institute of Hygiene and Epidemiology (NIHE) ethics review committee, Vietnam, approved the study protocol.

M. tuberculosis isolates

Two hundred and twelve *M. tuberculosis* isolates were randomly selected at the National Lung Hospital (NLH) in Hanoi, which has the highest rates of MDR-TB.

DST

The sensitivities of all *M. tuberculosis* isolates to the four drugs used as first-line TB treatment in Vietnam during the study period (isoniazid, rifampicin, streptomycin, and ethambutol) were tested with the Canetti–Grosset proportional technique, as previously described [14], at the NHL, and also with the new proportional microplate technique (described below) at the TB Laboratory, NIHE, Vietnam. All experiments were performed in biosafety level 3 laboratories. The critical inhibitory drug concentrations (CIDCs) recommended by the WHO were used (Table S1). Both methods determine the 'growth proportion' of *M. tuberculosis* colonies in drug-containing medium relative to that in drug-free medium, with the same inoculum. An isolate was considered to be drug sensitive when no growth or a growth proportion of <1% was observed, and drug resistant when the growth proportion was $\geq$1%.

Preparation of DST microplates

The M7H11 agar (Difco, Maryland, USA) was prepared according to the manufacturer's instructions, and enriched with 10% OADC (Difco). Freshly prepared anti-TB drugs (Sigma; INH: India, RMP, STM, EMB: China) at 100 × CIDC were added (final concentration of 1 × CIDC), or not added, to the agar, which was then distributed at 3 mL/well in 12-well microplates (Thermo Scientific Nunc), as described in Fig. S1. Plates were labelled, kept at 4°C, and used within 1 month.

M. tuberculosis inoculation in DST microplates

Bacterial colonies from fresh cultures (4 weeks) were homogenized by vortexing in 15-mL Falcon tubes containing water and glass beads. Larger particles were allowed to settle. The supernatants were then transferred to new glass tubes, and bacterial stock solutions (10^8 CFU/mL) were prepared with McFarland standard No. 2 for adjustment. Bacterial solutions of 10^5 CFU/mL and 10^3 CFU/mL were prepared by making serial ten-fold dilutions from the stock solutions in water. Fifty microlitres of 10^5 CFU/mL bacterial solution was added to each well of the DST microplate, except for control 2, where 50 µL of 10^3 CFU/mL bacterial solution was inoculated. Three sides of the microplate were sealed with tape, and plates were incubated at 37°C in an incubator. Culture contamination and *M. tuberculosis* growth were monitored by eye and under a microscope at low resolution (10×) from day 1 to day 14, and recorded on a follow-up form.

DST microplate reading

Results were read by naked eye or microscopy. The minimum number of colonies in control 2 had to be at least ten for interpretation of the assay. Isolates were considered to be sensitive when, in drug-containing wells, no colonies or fewer colonies than in control 2 were observed. Isolates were considered to be resistant if the number of colonies in drug-containing wells was higher than or equal to the number of colonies in control 2 (Fig. 1).

Sequencing of drug resistance genes

PCR amplification and DNA sequencing. The genetic elements of resistance to rifampicin (a 1148-bp fragment in *rpo*B containing the hotspot region), isoniazid (the entire sequences of *kat*G and *inh*A, and the *inh*A promoter), streptomycin (the entire sequences of *rps*L and *rrs*) and ethambutol (a 1312-bp fragment in *emb*B containing the hotspot region) were PCR amplified and sequenced. The primers and amplified regions are shown in Table S2 [20–25]. The PCR conditions (with HotStarTag) were

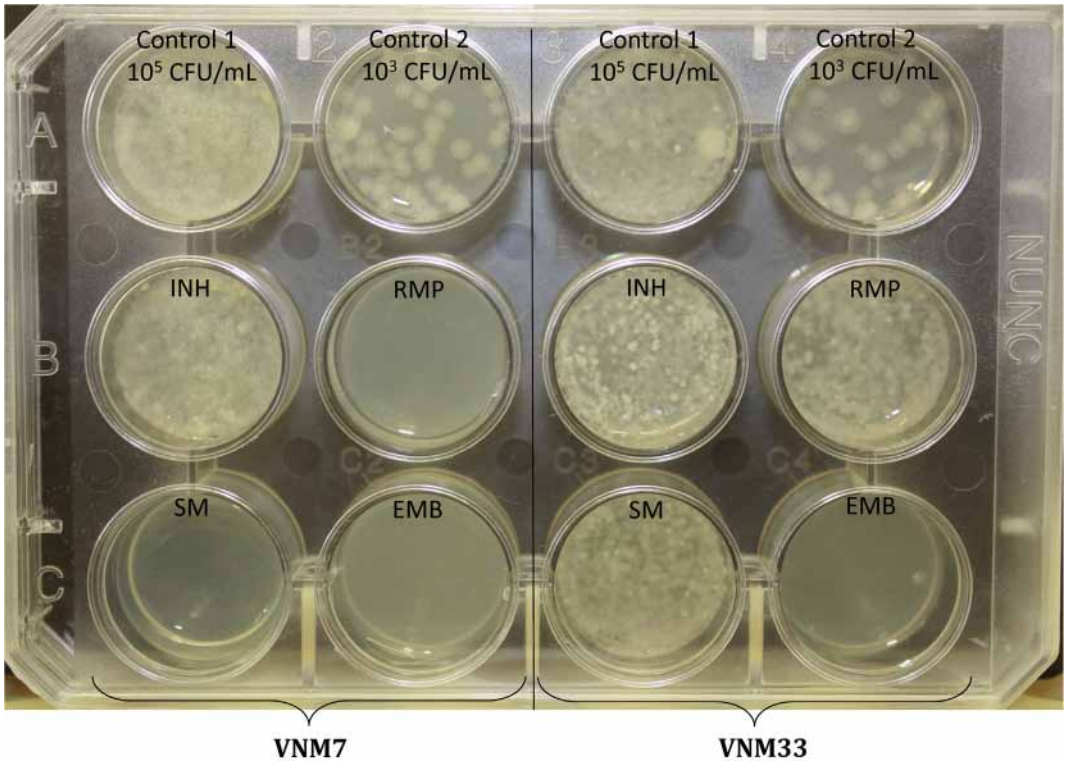

FIG. 1. Reading of results in a 12-well microplate drug susceptibility testing assay. VNM7: *Mycobacterium tuberculosis* isolate resistant to isoniazid (INH) (left half of the 12-well plate); VNM33: *M. tuberculosis* isolate resistant to INH, rifampicin (RMP), and streptomycin (SM) (right half of the 12-well plate). Photograph taken at day 11 of culture.

as follows: 15 min of Taq activation at 95°C, 35 cycles of 95°C for 1 min, 58–62°C (Table S2) for 1.5 min, and 72°C for 2 min, and 72°C for 5 min. PCR products were sequenced by Eurofins MWG Operon or 1st BASE.

Sequence analysis

Each sequence was analysed with Bioedit and ClustalW softwares. Point mutations were identified by comparison with the sequence of the *M. tuberculosis* H37Rv reference strains in GenBank (NC 000962).

Determination of rifampicin and ethambutol MICs

MICs were determined by use of the M7H11 microplate assay with 0.031, 0.062, 0.124, 0.248, 0.496, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/L rifampicin, and 2, 3, 4, 5, 6, 7 and 7.5 mg/L ethambutol. The highest concentrations used for MIC testing were the CIDCs recommended by the WHO.

Data analysis

Data were analysed with STATA 12 (Statacorp LP, TX, USA). Sensitivity, specificity and Cohen's kappa coefficient with two-sided 95% CIs were calculated for the proportional microplate DST assay relative to the Canetti–Grosset method. A kappa coefficient of 0.81–0.99 was considered to indicate almost perfect between-test agreement, and a kappa value of 1.0 was considered to indicate perfect agreement [26].

Results

As four *M. tuberculosis* isolates were contaminated by fungi during subculture and DST, only 208 isolates were analysed.

DST

DST results obtained with the Canetti–Grosset and the 12-well microplate methods showed between-test discrepancies in only eight *M. tuberculosis* isolates, with only one drug susceptibility difference in most of the cases (Table S3 and 2 × 2 table in Table S4). The sensitivity, specificity and kappa coefficients for detection of multidrug resistance and resistance to the four drugs showed almost perfect agreement between methods (Table 1).

Time to results with the proportional microplate DST assay

Visual growth monitoring of the first 140 *M. tuberculosis* isolates from day 1 to day 14 indicated that DST results could be read at

TABLE 1. Sensitivity, specificity and kappa coefficients for the comparison of the proportional 12-well microplate and Canetti–Grosset drug susceptibility testing assays

Drug resistance	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Kappa coefficient (± SE)
RMP	100 (94.5–100)	98.6 (95.0–99.8)	0.98 (0.91–1.00)
INH	99.0 (94.7–100)	99.0 (94.8–100)	0.98 (0.91–1.00)
SM	100 (96.8–100)	98.9 (94.2–100)	0.98 (0.91–1.00)
EMB	96.4 (81.7–99.9)	98.3 (95.2–99.7)	0.92 (0.85–0.99)
MDR	100 (93.8–100)	98.7 (95.3–99.8)	0.98 (0.91–1.00)

EMB, ethambutol; INH, isoniazid; MDR, multidrug resistant; RMP, rifampicin; SE, standard error.

days 5–6 for 108 isolates (77.1%) and at days 7–11 for the remaining 32 isolates (22.9%). The mean time to results was 6.3 days. By microscopic observation (10×), DST results could be read at days 4–5 for 115 isolates (82.1%) and at days 6–9 for 25 isolates (17.9%). The mean time to results was 4.7 days (Table 2). Fig. 2 shows the microscopic follow-up of *M. tuberculosis* growth in a control well from day 1 to day 12.

Sequencing of drug resistance genes

To validate the DST profiles, the main genes associated with resistance to the four tested anti-TB drugs were sequenced in the eight isolates with discordant DST results between techniques and in 115 randomly selected isolates.

Overall, previously described drug resistance-associated mutations were found at: codons 511, 516, 518, 522, 526, 531, 533 and 572 of *rpo*B (rifampicin resistance); at codon 315 of *kat*G, at codon 21 of *inh*A, and at position −15 (C-T) of the *inh*A promoter (isoniazid resistance); at codons 43 and 88 of *rps*L, and at positions 514, 517, 878, 1001 and 1401 of *rrs* (streptomycin resistance); and at codons 306, 330, 354, 406 and 497 of *emb*B (ethambutol resistance). Synonymous mutations and mutations previously reported as not being associated with drug resistance were also identified (Table S5) [24,25,27,28].

The frequencies of drug resistance-associated mutations among the resistant and sensitive isolates identified with the two DST assays are shown in Table 3. The level of agreement between phenotypic susceptibility and genotypic determinants was similar for the two techniques.

Gene sequencing of the eight isolates with discordant DST results showed that, among these, both methods correctly detected three of the six resistant isolates with mutations (Table 4). For the ones without mutations, we did not consider which assay results were more precise, because the known molecular determinants targeted by sequencing cannot detect 100% of the drug resistant isolates.

TABLE 2. Time to results when bacterial growth is monitored in the proportional 12-well microplate drug susceptibility testing assay by eye and under a low-magnification microscope (140 *Mycobacterium tuberculosis* isolates)

	No. of isolates (%)	
Time (days)	By eye	By microscopy
4	—	93 (66.4)
5	49 (35.0)	22 (15.7)
6	59 (42.1)	9 (6.4)
7	7 (5.0)	6 (4.3)
8	5 (3.6)	6 (4.3)
9	8 (5.7)	4 (2.9)
10	8 (5.7)	
11	4 (2.9)	

Determination of the MICs for rifampicin and ethambutol in isolates with phenotype–genotype discrepancies

Because of the significant number of isolates with phenotype–genotype discrepancies concerning susceptibility to rifampicin (seven of 74 isolates) and ethambutol (13 of 98 isolates), their MICs for rifampicin and ethambutol were determined. In three of the seven rifampicin-sensitive isolates with a rifampicin resistance-associated mutation, and in all three control rifampicin-sensitive isolates without detectable mutations, the MIC for rifampicin was 0.248 mg/L. The other four rifampicin-sensitive isolates with mutations had an MIC for rifampicin between 0.6 mg/L and 0.9 mg/L (Table 5).

The MICs for ethambutol in 12 randomly selected ethambutol-sensitive isolates without *emb*B mutations (controls) were $\leq$2 mg/L. Conversely, among the 13 ethambutol-sensitive isolates with an ethambutol resistance-associated mutation, 11 had an MIC for ethambutol of 6 mg/L, and two had an MIC for ethambutol of 5 mg/L (Table 6).

Discussion

Simplification and time reduction for *M. tuberculosis* DST with the proportional microplate assay

This proportional microplate assay is faster and simpler than the currently used Canetti–Grosset method. Indeed, the M7H11 medium promotes *M. tuberculosis* growth. Moreover, microplates allow the microscopic detection of bacterial colonies before they can be seen with the naked eye, further decreasing the time to results. Previous studies have shown that *M. tuberculosis* can be detected on M7H11 from sputum samples as early as 7 days [29–31]. A meta-analysis of the results obtained with thin-layer agar and microscopic-observation drug susceptibility assays, in which patients'

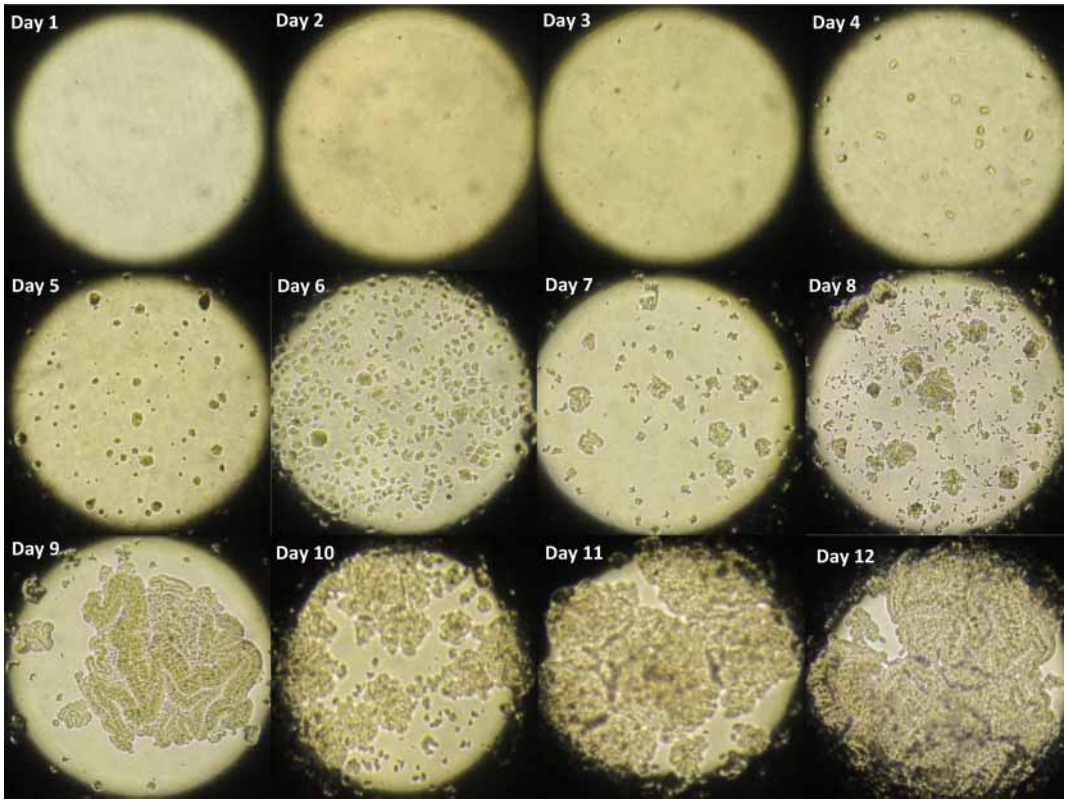

FIG. 2. *Mycpbacterium tuberculosis* growth on a control well of the 12-well microplate from day 1 to day 12 (control well: 10^5 CFU/mL bacterial solution and no drug), monitored with a low-magnification (10×) microscope.

specimens are inoculated directly on drug-free and drug-containing medium for DST, showed that the mean turn-around times for the two assays were 11.1 days (95% CI 10.1–12.0) and 9.9 days (95% CI 4.1–15.8), respectively [32]. With our indirect proportional microplate assay and a low-magnification microscope, primary results could be read at day 4 (or day 6 with the naked eye) and the complete results at day 9 (or day 11–12 by eye).

A 12-well plate can be used to test four anti-TB drugs in two *M. tuberculosis* isolates, whereas 12 culture tubes are needed for testing the same number of drugs and isolates with the Canetti–Grosset technique. Therefore, inoculation, growth monitoring and result reading are much easier and less labour-intensive and time-consuming. Moreover, the M7H11 medium without fresh eggs is much simpler to prepare than Löwenstein–Jensen medium. On the basis of our experience, with

TABLE 3. Frequency of mutations associated with resistance to antituberculosis drugs in the drug-resistant and drug-sensitive *Mycobacterium tuberculosis* isolates characterized with each drug susceptibility testing (DST) technique and with both techniques

	Mutation frequency, no. (%)			
M. tuberculosis	**Drug resistance (1)**	**Canetti–Grosset (2)**	**Microplate DST (3)**	**Both techniques (4)**
Drug-resistant isolates	RMP	44/46 (95.6)	47/49 (96)	44/46 (95.6)
	INH	73/77 (94.8)	73/77 (94.8)	73/76 (96)
	SM	62/70 (88.6)	62/71 (87.3)	62/70 (88.6)
	EMB	22/24 (91.7)	20/22 (91)	19/21 (90.5)
Drug-sensitive isolates	RMP	10/77 (13)	7/74 (9.5)	7/74 (9.5)
	INH	2/46 (4.4)	2/46 (4.4)	2/45 (4.4)
	SM	0/53 (0)	0/52 (0)	0/52 (0)
	EMB	11/99 (11.1)	13/101 (12.9)	14/10/98 (10.2)

Columns (2), (3) and (4) shows the number of isolates with mutations that confer resistance to the drug indicated in column (1) relative to the total number of isolates that are resistant/sensitive to that drug (determined with each DST techniques and with both techniques); the corresponding mutation frequency is in parentheses.
EMB, ethambutol; INH, isoniazid; RMP, rifampicin; SM, streptomycin.

TABLE 4. Mutations in genes involved in drug resistance found in the *Mycobacterium tuberculosis* isolates with discrepant drug susceptibility testing results by the proportional Canetti–Grosset and 12-well microplate techniques

Isolates	Drug resistance profile		Drug resistance-associated mutations in the studied genes					
	Canetti–Grosset	Microplate DST	*rpo*B	*inh*A/its promoter	*kat*G	*rps*L	*rrs*	*emb*B
VNM48	RHSE	RHS	Asp526Val	ND	Ser315Thr	Lys88Arg	ND	Met306Val
VNM53	Sensitive	H	ND	ND	ND	ND	ND	ND
VNM56	HS	RHS	Leu533Pro	ND	Ser315Thr	Lys88Arg	ND	ND
VNM151	H	RH	Leu533Pro	ND	Ser315Thr	ND	ND	Gln497Arg
VNM152	H	Sensitive	ND	ND	ND	ND	ND	ND
VNM165	RH	RHSE	Ser531Leu	ND	Ser315Thr	ND	ND	Asp354Ala
VNM180	HE	H	Leu533Pro	ND	Ser315Thr	ND	ND	Met306Val
VNM182	HE	H	ND	ND	Ser315Thr	ND	ND	Met306Val

E, ethambutol; H, isoniazid; R, rifampicin; S, streptomycin; ND, not detected.

the same amount of time and labour, the number of tests performed can be increased by at least four-fold.

Validation of proportional microplate DST for *M. tuberculosis*

For detection of multidrug resistance and resistance to isoniazid, rifampicin, and streptomycin, the sensitivity of proportional microplate DST ranged between 99.0% (95% CI 94.7–100%) and 100.0% (95% CI 96.8–100%) and the specificity ranged between 98.6% (95% CI 95.0–99.8%) and 99.0% (95% CI 94.8–100%), as compared with the proportional Canetti–Grosset technique. Similarly, the kappa coefficients between tests was 0.98 (95% CI 0.91–1.00), indicating almost perfect agreement between the techniques. Previous studies also showed that all assessments of rifampicin and isoniazid resistance with the thin-layer agar assays yielded 100% accuracy, showing a promising diagnostic technique. However, the WHO expert group agreed that more evidence is needed before the use of these assays can be recommended [32].

For ethambutol, the specificity was as high as for the other drugs (98.3%; 95% CI 95.2–99.7%), whereas the sensitivity was lower (96.4%; 95% CI 81.7–99.9%), and consequently also the kappa index was lower (0.92; 95% CI 0.83–0.97). DST results for ethambutol are frequently less reproducible [33]. Thus, a lower kappa index does not mean that the microplate assay is less precise than the Canetti–Grosset technique. Ängeby *et al.* showed that the low reproducibility for ethambutol is due to the use of non-optimal critical drug concentrations that cannot discriminate between wild-type and drug-resistant isolates [33].

Sequencing showed similar phenotype–genotype agreement for the two techniques. This suggests that the precision of the proportional microplate assay is comparable to that of the proportional Canetti–Grosset technique.

The need for adjustment of the critical concentration for ethambutol susceptibility testing

On the basis of the WHO recommended critical concentrations, we missed 11 (33%) and 13 (39%) ethambutol-resistant mutants with the Canetti–Grosset technique and the microplate assay, respectively. All of the missed isolates had significantly higher MICs than those of the non-mutants (5–6 mg/L vs. $\leq$2 mg/L). These data strongly suggest that the critical

TABLE 5. Measurement by microplate assay of the MIC for rifampicin (RMP) in rifampicin-sensitive isolates that harbour or do not harbour rifampicin resistance-associated mutation(s) in *rpo*B

No.	Isolate	RMP susceptibility		RMP resistance-associated mutation(s) in *rpo*B	MIC (mg/L)
		Canetti–Grosset	Microplate assay		
1	VNM148	S	S	Leu511Pro	0.248
2	VNM162	S	S	Leu533Pro	0.248
3	VNM168	S	S	Leu533Pro	0.6
4	VNM192	S	S	Asp516Tyr	0.248
5	VNM193	S	S	Leu511Pro	0.7
6	VNM194	S	S	Leu533Pro	0.7
7	VNM208	S	S	Leu533Pro	0.9
8	VNM137	S	S	ND	0.248
9	VNM166	S	S	ND	0.248
10	VNM182	S	S	ND	0.248

ND, not detected; S, sensitive.

TABLE 6. Measurement by microplate assay of the MIC for ethambutol (EMB, China) in ethambutol-sensitive isolates harbouring or not harbouring ethambutol resistance-associated mutation(s) in *emb*B

No.	Isolate	EMB susceptibility		EMB resistance-associated mutation(s) in *emb*B	MIC (mg/L)
		Canetti–Grosset	Microplate assay		
1	VNM32	S	S	Gln497Arg	6.0
2	VNM69	S	S	Gln497Arg	6.0
3	VNM57	S	S	Met306Ile	6.0
4	VNM72	S	S	Met306Ile	6.0
5	VNM76	S	S	Met306Ile	6.0
6	VNM163	S	S	Gln497Arg	5.0
7	VNM164	S	S	Phe330Leu	6.0
8	VNM179	S	S	Gly406Asp	5.0
9	VNM188	S	S	Gly406Asp	6.0
10	VNM151	S	S	Gln497Arg	6.0
11	VNM48	R	S	Met306Val	6.0
12	VNM180	R	S	Met306Val	6.0
13	VNM182	R	S	Met306Val	6.0
14	VNM137	S	S	ND	≤2.0
15	VNM138	S	S	ND	≤2.0
16	VNM152	S	S	ND	≤2.0
17	VNM153	S	S	ND	≤2.0
18	VNM51	S	S	ND	≤2.0
19	VNM36	S	S	ND	≤2.0
20	VNM107	S	S	ND	≤2.0
21	VNM178	S	S	ND	≤2.0
22	VNM56	S	S	ND	≤2.0
23	VNM85	S	S	ND	≤2.0
24	VNM185	S	S	ND	≤2.0
25	VNM96	S	S	ND	≤2.0

ND, not detected; R, resistant; S, sensitive.

ethambutol concentration should be revised, especially because the currently used cut-offs are not based on clinical evidence [33].

Rifampicin-sensitive isolates carrying rifampicin resistance-associated mutations

All of the isolates that carried rifampicin resistance-associated mutations but were phenotypically rifampicin-sensitive had MICs lower than the critical concentration recommended by the WHO. Three of them had MICs as low as those of the non-mutants (0.248 mg/L). This suggests that, for these isolates, molecular-based DST could lead to false-positive results. A recent study also indicated that not only the presence but also the nature of *rpo*B mutations must be assessed for accurate diagnosis of rifampicin resistance in *M. tuberculosis* [34]. Moreover, all of the mutations found in susceptible isolates in our study have been previously reported to be associated with low rifampicin resistance [27,35–39]. This observation supports the need to re-evaluate the current molecular-based DST assays.

Conclusions

Our proportional microplate assay for *M. tuberculosis* DST is as accurate as the proportional Canetti–Grosset technique, but easier to perform. It can provide DST results as rapidly as automated liquid culture systems [7], but is much more affordable. It also allows calculation of the growth proportion and observation of the colony morphology, thus reducing the contamination problems of liquid cultures. Therefore, it could facilitate the diagnosis of drug-resistant TB in developing countries for better control of drug-resistant TB. However, the critical ethambutol concentration may need to be adjusted to improve the precision of ethambutol susceptibility testing.

Transparency declaration

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.08.024.

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