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ISBN 978-92-4-004741-9 (electronic version)
ISBN 978-92-4-004742-6 (print version)

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Acknowledgements

The development of this document was led by Nazir Ismail with input from Alexei Korobitsyn, Matteo Zignol and Karin Weyer, under the overall guidance of Tereza Kasaeva (WHO Global TB Programme), based on an analytical report on the use of broth microdilution plates prepared by Claudio Köser (University of Cambridge, United Kingdom of Great Britain and Northern Ireland), and consensus at a Technical Expert Group meeting convened by WHO on 25 February 2020, in Geneva, Switzerland.

Members of the WHO Technical Expert Consultation Group

Lynette Berkeley (US Food and Drug Administration, United States of America), Daniela Cirillo (San Raffaele Scientific Institute, Italy), Derrick Crook (University of Oxford, United Kingdom of Great Britain and Northern Ireland), Kelly Dooley (Johns Hopkins University School of Medicine, United States of America), Gerry Davies (Liverpool School of Tropical Medicine, United Kingdom of Great Britain and Northern Ireland), Bernard Fourie (University of Pretoria, South Africa), Philip Fowler (University of Oxford, United Kingdom of Great Britain and Northern Ireland), Christopher Gilpin (International Organization for Migration, Switzerland), Patricia Hall (Centers for Disease Control and Prevention, United States of America), Rumina Hasan (Aga Khan University, Pakistan), Marguerite Massinga Loembé (Africa Centres for Disease Control and Prevention, Gabon), Charles Peloquin (University of Florida, United States of America), Max Salfinger (University of South Florida, United States of America), Thomas Schön (Linköping University, Sweden), Thomas Shinnick (Independent consultant, United States of America), Sabira Tahseen (National Reference Laboratory, Pakistan), Gabriela Torrea (Prince Leopold Institute of Tropical Medicine, Belgium), and Armand Van Deun (Prince Leopold Institute of Tropical Medicine, Belgium).

Members of the WHO External Review Group

Derek Armstrong (Johns Hopkins University, United States of America), Rossella Baldan (FIND, Switzerland), Emmanuelle Cambau (National Reference Center for Mycobacteria and Antimycobacterial Resistance, France), Peter Keller (University of Bern, Switzerland), Scott Heysell (University of Virginia, United States of America), Florian Maurer (Research Center Borstel, Germany), Shaheed Omar (National Institute of Communicable Diseases, South Africa), Nikki Parrish (Johns Hopkins University, United States of America), James Posey (Centers for Disease Control and Prevention, United States of America), Juliano Timm (TB Alliance, United States of America), Nancy Wengenack (Mayo Clinic, United States of America), and Jim Werngren (Public Health Agency of Sweden, Sweden).

Observers

Toby Hampshire (Thermo Fisher Scientific, United Kingdom of Great Britain and Northern Ireland), Koné Kaniga (Johnson & Johnson, United States of America), Mischka Moodley (Otsuka Novel Products GmbH, Germany) and Brigit Quinn (Becton Dickinson, United States of America).

Technical resource person

Claudio Köser.

Major data contributors

Daniela Cirillo, Alamdar Hussain (National Reference Laboratory, Pakistan), Faisal Masood (National Reference Laboratory, Pakistan), Andrea Spitaleri (San Raffaele Scientific Institute, Italy), and Sabira Tahseen.

Acknowledgement of financial support

This work was commissioned by and funded in part by WHO. The United States Agency for International Development and the Federal Government of Germany covered a proportion of Claudio Köser's salary for this report as part of his work for the European Laboratory Initiative, led by Soudeh Ehsani (WHO Regional Office for Europe, Denmark). Claudio Köser is research associate at Wolfson College and visiting scientist at the Department of Genetics, University of Cambridge. Moreover, we thank Gunnar Kahlmeter and Erika Matuschek (European Committee on Antimicrobial Susceptibility Testing Development Laboratory, Sweden) for their advice and the European Society of Clinical Microbiology and Infectious Diseases for support provided through an observership.

Declaration and management of conflict of interest

All the contributors completed a WHO Declaration of Interest form. All stated declarations of interest were evaluated by members of the Steering Group for the existence of any possible financial conflict of interest which might warrant exclusion from membership of the Technical Expert Consultation Group or from the discussions as part of the consensus process. Intellectual conflict of interest was not considered for exclusion from membership of the Group, as broader expertise on DST methods for MTBC was considered as criteria for the selection. In addition, the diversity and representation in the Groups was large enough to balance and overcome any potential intellectual conflict of interest. During the consensus development process and the meeting, any emergence of intellectual conflict of interest was monitored by the Chair and there was no perceived intellectual conflict of interest identified during the meeting.

Abbreviations

7H9 = Middlebrook 7H9
AMK = amikacin
ATU = area of technical uncertainty
BDQ = bedaquiline
BMD = broth microdilution
CAP = capreomycin
CB = clinical breakpoint (as defined by WHO rather than EUCAST)
CC = critical concentration
CFZ = clofazimine
CI = exact binomial confidence interval
CLA = clavulanic acid
CLSI = Clinical & Laboratory Standards Institute
CP = critical proportion
DCS = D-cycloserine
DLM = delamanid
DMSO = dimethyl sulfoxide
DST = drug-susceptibility testing
ECOFF = epidemiological cut-off value
EMB = ethambutol
ETO = ethionamide
EUCAST = European Committee on Antimicrobial Susceptibility Testing
FQ = fluoroquinolone
GC = growth control
gDST = genotypic drug susceptibility testing
gWT = genotypically wild-type
HLR = high-level resistance/resistant
H37Rv = *Mycobacterium tuberculosis* H37Rv ATCC 27294
IMP-CLN = imipenem-cilastatin
IFUs = instructions for use
INH = isoniazid
ISO = International Organization for Standardization
LFX = levofloxacin
LLR = low-level resistance/resistant
LoF = loss-of-function
LZD = linezolid
KAN = kanamycin
MDR = multidrug-resistant
MFX = moxifloxacin
MGIT = BACTEC™ Mycobacterial Growth Indicator Tube™
MIC = minimum inhibitory concentration
MPM = meropenem
MTBC = *Mycobacterium tuberculosis* complex
MYCOTB(I) = Sensititre™ MYCOTB or Sensititre™ MYCOTBI
OADC = oleic acid albumin dextrose catalase

OFX = ofloxacin
PAS = *para*-aminosalicylic acid
pATU = potential area of technical uncertainty
pCB = potential clinical breakpoint
pDST = phenotypic drug susceptibility testing
pECOFF = potential epidemiological cut-off value
pQC = potential quality control
PMD = pretomanid
PTO = prothionamide
pWT = phenotypically wild-type
PZA = pyrazinamide
QC = quality control
R = resistance/resistant
RFB = rifabutin
RIF = rifampicin
RR = rifampicin resistant
RRDR = rifampicin resistance-determining region
RPT = rifapentine
S = susceptible/susceptibility
SOP = standard operating procedure
STR = streptomycin
TB = tuberculosis
TEG = Technical Expert Group
TFS = Thermo Fisher Scientific
TZD = terizidone
WHO = World Health Organization
XDR = extensively drug-resistant

1 Rationale for an optimized broth microdilution plate

Throughout the past decade, treatment outcomes for multidrug-resistant and rifampicin resistant tuberculosis (MDR/RR-TB) have improved globally.[1] Nevertheless, the provision of comprehensive drug susceptibility testing (DST) remains insufficient in many countries.[2] Indeed, only 61% of bacteriologically confirmed pulmonary TB cases were tested for rifampicin (RIF) resistance in 2019 and 71% of the notified MDR/RR-TB patients were tested for resistance to fluoroquinolones (FQs), compared with a target of 100%, set out in the End TB Strategy.[2]

Owing to the inherently slow growth rate of the *Mycobacterium tuberculosis* complex (MTBC), as well as to the cost and infrastructure requirements of conventional phenotypic DST (pDST), the increased adoption of rapid genotypic DST (gDST) represents the most appropriate option to close the diagnostic gap for the aforementioned medicines and the new and repurposed drugs.[3-5] To this end, the World Health Organization (WHO) has endorsed a number of gDST assays and more are currently being evaluated.[2] In some cases, however, these assays only rule in resistance as they have a limited sensitivity and do not cover some drugs (e.g. bedaquiline [BDQ] and linezolid [LZD]). Current targeted next-generation sequencing assays interrogate a larger number of resistance genes, with the exception of delamanid (DLM) and pretomanid (PMD) that require up to seven resistance genes spanning more than 8,400 bp to be covered.[6-8] Yet, even if all relevant resistance genes could be analysed cost effectively and directly from clinical samples, the interpretation of novel mutations would remain a challenge, particularly if the effect of a mutation depends on the genetic background (e.g. *Rv0678* mutations, can only confer BDQ and clofazimine [CFZ] cross-resistance if the efflux pump, encoded by *mmpL5-mmpS5*, is functional).[9-12] Therefore, even countries that have introduced routine whole genome sequencing cannot eliminate pDST completely.[13,14]

Most laboratories that use WHO-endorsed pDST methods rely on the proportion method with one of three solid media (Löwenstein-Jensen [LJ] or Middlebrook 7H10/7H11) or the liquid Middlebrook 7H9 medium supplemented with oleic acid albumin dextrose catalase (7H9-OADC) using the commercial macrodilution BACTEC™ Mycobacterial Growth Indicator Tube™ (MGIT) system by Becton Dickinson.[15] By contrast, pyrazinamide (PZA) testing using MGIT relies on a modified 7H9-OADC medium with polyoxyethylene stearate and pH 5.9.[15-18] These methods typically require a separate slant or tube for each concentration and/or drug tested, although in some cases the same growth control (GC) can be used for multiple concentrations or drugs (e.g. using the same MGIT carrier set). To minimise costs, this has meant that usually only the critical concentration (CC) and, if applicable, the clinical breakpoint (CB) is tested and that DST is carried out sequentially (e.g. second-line DST is usually only carried out once resistance to a priority drug is detected, causing delays).[19,20]

For most bacterial pathogens, the cheapest methods to obtain semi-quantitative pDST results for multiple drugs simultaneously is disk diffusion testing or gradient strip assays.[20,21] Yet, because of the following issues, neither approach is likely suitable for pDST for MTBC. First, although an antibiotic gradient can be established on solid medium for MTBC, it is not clear whether the edges of inhibition zones can be easily defined and it is unlikely that low-frequency heteroresistant populations close to the critical proportion (CP) of 1% (10% in the case of pyrazinamide [PZA]) can be identified reliably.[22-25] Second, past studies that evaluated the Etest used a high inoculum (McFarland 4) to shorten the incubation period, which may be challenging to obtain from some positive MGIT cultures and may result in inoculum effects for some antibiotics.[22,26-28]

An alternative approach is to use broth microdilution (BMD) testing in a 96-well microplate format (Table 1), which offers several potential advantages:

1. The quality of testing could be monitored more effectively by using an on-scale quality control (QC) strain, as is the case for most major bacterial pathogens (Table 2).[29,30]
2. Depending on how drugs are arranged on the plate and the number of concentrations included per drug, approximately 12 drugs can be tested simultaneously, which would simplify workflows in laboratories.
3. For the majority of TB drugs, some resistance mechanisms result in minimum inhibitory concentration (MIC) distributions that overlap with the MIC distribution of susceptible strains.[31-46] As a result, the CC, which corresponds to the epidemiological cut-off value (ECOFF), intersects the MIC distribution of these mechanisms.[46] This manifests in a poor reproducibility of categorical pDST (i.e. even if the same strain is tested multiple times in the same laboratory under controlled conditions, it will variably test susceptible and resistant at the CC because of the inherent technical variability of pDST).[45,47-51] Five measures can be taken to decrease the misclassification of resistant strains as susceptible (i.e. very major errors) due to this phenomenon[52]:
    a. The optimal solution would be to eliminate or, at least, to minimize the degree of overlap between distributions by reducing the technical variability of MIC testing as much as possible.[53-57]
    b. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has introduced so-called areas of technical uncertainty (ATUs), which typically encompass one dilution (i.e. the concentration of the breakpoint in question).[58-61] In the example in Figure 1, an MIC result of ≤0.5 mg/L would be reported as susceptible, whereas MICs of >1 mg/L would be resistant. By contrast, an MIC result of 1 mg/L, which corresponds to the ATU, would be "uncertain" as the strain in question could not be unequivocally classified as either susceptible or resistant based on the single MIC result because of the overlap in MIC distributions (i.e. this applies to the borderline resistance mechanism but not high-level resistance [HLR] mechanism).[45] Although the prevalence of borderline resistance mechanisms in a particular setting can give an indication of which of these possibilities is more likely, other experimental results are needed to resolve this situation conclusively. For example, if the molecular basis of the borderline resistance mechanism is known and is detected, the strain could be reported as resistant (i.e. a composite reference standard is used).[45,51] The Clinical & Laboratory Standards Institute (CLSI) has endorsed an "inconclusive" category for ethambutol (EMB) for the Sensititre™ MYCOTB plate by Thermo Fisher Scientific (TFS), which essentially corresponds to an ATU.[i,35,62,63] Although ATUs have not been endorsed by WHO to date, they may be needed for several agents and could be easily implemented with a BMD plate.
    c. Testing additional concentrations (e.g. 0.75 mg/L in the example in Figure 1) may theoretically minimise the overlap between the distributions.[39] However, this would not conform to the requirements by the International Organization for Standardization (ISO).[64]

[i] N. Wengenack, personal communication.

d. Adopting interpretative reading, whereby the results of two antibiotics that share at least one resistance mechanisms are analysed together, may be helpful (e.g. BDQ and CFZ to minimise very major errors associated with *Rv0678* mutations).[39,65,66]
e. A surrogate drug could be tested. For example, EUCAST recommends pefloxacin as a surrogate for FQ resistance in *Salmonella enterica* and kanamycin (KAN) as a surrogate for amikacin (AMK) resistance for *Staphylococcus aureus*, even though neither agent is used clinically for these organisms.[67-69] The risk of this approach is that strains with exceptional resistance mechanisms may exist, for which testing a surrogate drug increases the likelihood of false-susceptible results compared with testing the agent in question (e.g. as discussed for *gyrB* E501D in Section 2.4).

4. Because the ECOFF is set to encompass approximately 99% of phenotypically wild-type (pWT) strains, the positive predictive value of pDST will be poor in settings with a true rate of resistance that is close to 1% (i.e. because, on average, 1% of susceptible strains would be misclassified as resistant based on the inevitable technical variation).[45] To some extent, MIC testing would enable for such random false resistance results to be identified (e.g. for drugs to which the dominant resistance mechanisms confer large MIC increases, MICs just one concentration above the ECOFF might represent such errors, whereas this is less likely for strains with MIC two concentrations above the ECOFF).[47,70]
5. At present, treatment outcomes are usually correlated with categorical pDST results because only the CC is tested, as was the case in the recent meta-analysis that informed the MDR/RR-TB treatment guidelines by WHO.[71] This precludes an analysis of the impact of the level of resistance. Depending on how many concentrations above the CC are included on a BMD plate, it could be investigated whether modest MIC increases may be treatable with either the standard or increased exposure of a drug (i.e. to provide evidence whether a breakpoint above the ECOFF can be set, which WHO refers to as CBs).[15]
6. A comprehensively validated BMD plate would be an alternative to MGIT, resulting in greater competition between commercial pDST providers, thereby potentially reducing costs and the risk of stockouts of reagents by a dominant provider.

Figure 1. ATU for hypothetical MIC distributions of susceptible and resistant strains.

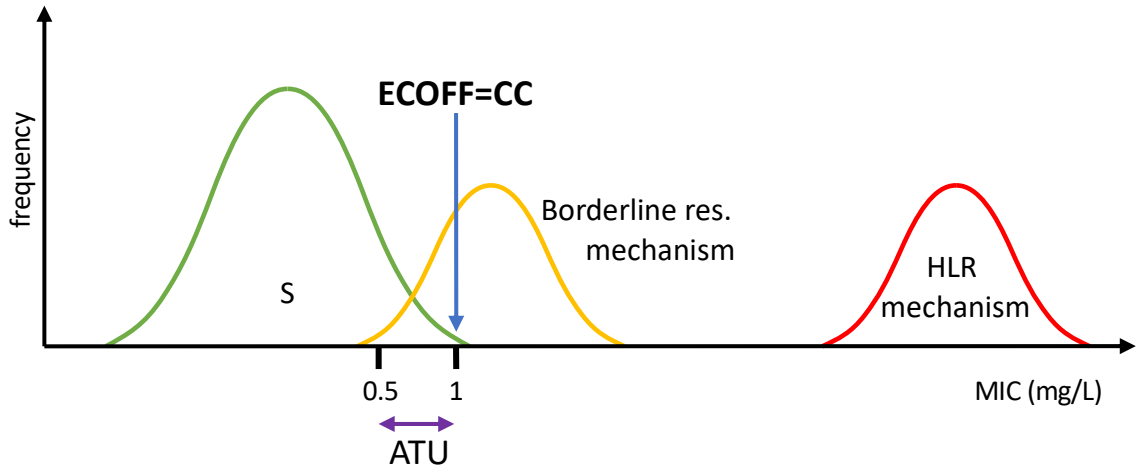

Table 1. Overview of six commercial or research BMD plates by TFS compared with EUCAST reference method.

		EUCAST[72]	Janssen[73-77]	Sweden[78,79]	MYCOTB(I)[62,63]	UKMYC5[44,80]	UKMYC6[44]	Potential need for ATU[g]	Priority for inclusion on optimized BMD plate	Consensus decision
Plate type	**Dry**		x[d]		x	x	x			x
	Frozen		x[d]	x						
	Liquid[a]	x								
Medium	**7H9**	x	x	x	x	x	x			x
	% OADC	10	10	10	10	10	10			10
	% glycerol	0.2	0.5	0.5	0.5	0.5	0.5			0.5[i]
	% casitone		(0.2)[d]	0.2						[j]
Inoculum	**McFarland**	0.5	1	0.5	0.5	0.5	0.5			0.5
	Final dilu-	1 in	1 in	1 in	1 in	1 in	1 in			1 in 111[k]
	tion[b]	200	98	111	111	111	111			
Atmosphere	**5–7% CO_2**		(x)[e]		(x)[e]	(x)[e]	(x)[e]			ambient air
Number of GCs	**100% positive**	6	1	2	2	2	2			1
	1% positive	6								1
	Negative	6	1							1
Number of drugs		NA[a]	12	12	13	14	13			12
1st line	**RIF**		x	x	x	x	x	x[45,46]	very high	x
	INH		x	x	x	x	x	x[38,43]	very high	x
	EMB		x	x	x	x	x	x[32,33,35,38, 43,44]	high	x
	PZA								very high but not possible with 7H9	
Group A	**LFX or**		x	x		x	x	x	very high[h]	x
	MFX		x	x	x	x	x		use LFX as surrogate	
	BDQ		x			x	x	x	very high	x
	LZD		x			x	x	x[36,39,42]	very high	x
Group B	**CFZ**		x			x	x	x[36,39]	very high	x
	DCS or			x	x			x[34]	high	desirable
	TZD								use DCS as surrogate	
Group C	**DLM**					x	x		very high	x
	IMP-CLN or								low	
	MPM								see IMP-CLN	
	AMK		x	x	x	x	x	x	medium but use KAN as surrogate	
	STR			x	x			x[31,38,40,43]	low	
	ETO or			x	x	x	x	x[38,43,44]	high	x
	PTO			x					use ETO as surrogate	
	PAS				x	x			low and testing not reproducible[80]	
Other	**PMD**								very high but method not established	x
	RPT								use RIF as surrogate	
	RFB				x	x	x	x[46]	very low	

OFX[c]		x	x	x			x	equivalent to LFX[h]	
KAN[c]		x	x	x	x	x	x	surrogate for AMK	x
CAP[c]		x	x				x[36]	not needed	

[a] Manually prepared, non-commercial reference method.[81] The choice of drugs and layout can be customized.

[b] Includes the final dilution step in antibiotic if applicable (e.g. for EUCAST reference method). There are several open questions regarding the corresponding CFU/mL targets and ranges, which is why the final inocula are expressed relative to the initial McFarland standards that are used to prepare them. In light of an average colony count of $8.5*10^4$ CFU/mL obtained during the initial development of the EUCAST reference method, it not clear why EUCAST chose $5*10^4$ CFU/mL as the target with a corresponding range of $2.5*10^4$–$2.5*10^5$ CFU/mL.[28,72] The target of $5*10^5$ CFU/mL in the instructions for use (IFUs) for MYCOTB(I) by TFS, which is also endorsed by CLSI, is identical to the upper end of the range of $5*10^4$–$5*10^5$ CFU/mL set by TFS, which is not sound.[62,63,82] Targets or ranges have not been published for the remaining plates.

[c] No longer recommended for clinical use.[83]

[d] A dry and frozen variant of this plate design exist.

[e] Only in frozen variant uses casitone.

[f] The IFUs by TFS state that the MYCOTB(I) plates should be used with ambient air.[62,63] However, the CLSI endorsed this method with CO_2.[82] Therefore, it is likely that at least some of the testing for the UKMYC5 and UKMYC6 was also done with CO_2. The frozen surveillance plates by Janssen were kept in a plastic bag, which may have protected them from CO_2 where it was used (K. Kaniga, personal communication).

[g] Unless otherwise referenced, the rationale for the ATU can be found in Section 2.4.

[h] Testing OFX at concentration x is equivalent to testing LFX at x/2, given that OFX consists of equal amounts of the active L-isomer of OFX (i.e. LFX) and the largely inactive D-isomer, as reflected in past CCs of OFX.[36]

[l] It is not clear why TFS included 0.5% glycerol for its plates compared with 0.2% that TFS recommends when preparing 7H9 from dehydrated medium.[84] However, there is currently no reason to change the 0.5%.

[j] Unlike 7H10, 7H11 contains casitone, which facilitates the growth of fastidious strains.[85] Whether and to what extent this is also the case for 7H9 broth is not known. Therefore, there is currently no reason to include casitone in the TFS medium used for dry plates.

[k] In the absence of conclusive evidence about whether one of the different inocula used for BMD testing to date offers significant advantages, there is no pressing need to change the current inoculum concentration recommended by TFS (see Section 4).

Table 2. Overview of current *Mycobacterium tuberculosis* H37Rv ATCC 27294 QC ranges and ECOFFs/CCs for BMD plates.

Drug	QC range (mg/L)					ECOFFs/CCs (mg/L)			
	dry format[a]		frozen format[a]			dry format[a]			frozen format[a]
	TFS[62,63]	CLSI[35]	CLSI[35]	Janssen (tier-2)[73,74]	Janssen (tier-3)[77]	CLSI[35]	CRyPTIC[44]	Janssen[75]	Janssen[75,77]
RIF	≤0.125[b]–0.5	≤0.125[b]	≤0.06[b,d]–0.25	≤0.06[b,d]–0.25	≤0.06[b,d]–0.5	0.5[e]	0.5		
INH	≤0.03[b]–0.5[c]	≤0.03[b]–0.125	≤0.03[b]–0.125	≤0.03[b]–0.125	≤0.03[b]–0.125	0.125	0.1		
EMB	≤0.5[b]–2	≤0.5[b]–2	≤0.25[b]–2	≤0.25[b]–2	0.5–4	2 (4)[f]	4		
LFX			≤0.125[b]–1	≤0.125[b]–1	0.25–1		1		1
BDQ			0.016–0.06	0.016–0.06	0.016–0.125		0.25	0.125	0.125
LZD			0.25–2	0.25–2	0.25–2		1		2
CFZ			≤0.06[b,d]–0.25	≤0.06[b,d]–0.25	0.03–0.25		0.25		0.5
DCS	4–16								
DLM							0.125		
KAN	≤0.6[b]–5	1.2–5	0.25–2	0.25–2	0.5–4		4		4
ETO	0.6–5	0.6–2.5					4		

[a] Table 1 shows which BMD plates used a dry or frozen format.

[b] Lowest concentration on plate, resulting in a truncated QC range, which precludes the reliable detection of shifts towards lower MIC.[29]

[c] Higher than CC of 0.125 mg/L set by CLSI.

[d] Shown as 0.03–0.25 mg/L by CLSI and in the publications in question, even though the lowest concentration tested was 0.06 mg/L.

[e] The current CC of 1 mg/L is due to be lowered in the upcoming guidelines (N. Wengenack, personal communication).[45]

[f] 4 mg/L essentially serves as an ATU (N. Wengenack, personal communication).

2 Design considerations

2.1 Main limitations of MYCOTB(I) plate

The MYCOTB plate has been available commercially since 2010.[86] It contains 12 first- and second-line TB drugs in a lyophilized 96-well microtiter plate (Table 1), but this selection of drugs is no longer optimal in light of the most recent updates to WHO treatment guidelines, particularly the change to the definition of extensively drug-resistant (XDR) TB.[3,83,87] The inoculum for this medium has to be prepared from a solid medium culture. In Europe, this plate is called MYCOTBI and is CE-marked for *in vitro* diagnostic use. Yet, this approval rests on a self-certification process rather than independent evaluation by a strict regulatory authority. In fact, the IFUs of MYCOTBI state that the "[p]erformance of the procedure has not been established at this point" and do not include any breakpoints for the interpretation.[62,63]

Reliable QC is not possible for at least half of the drugs on the MYCOTB(I) plate given that the relevant MIC distributions of *M. tuberculosis* H37Rv ATCC 27294 (H37Rv) are truncated at the lower end, which precludes the comprehensive assessment of shifts towards lower MICs (Table 2).[29] Moreover, the QC ranges endorsed by CLSI differ from the QC ranges provided in the IFUs for some drugs.[35,62,63] Notably, 0.5 mg/L, the upper end of the QC range for isoniazid (INH) in the IFUs, is two concentrations higher than the CLSI CC of 0.125 mg/L, which CLSI considers to be the upper end of the QC range (i.e. based on the IFUs, an MIC result in the resistant range would be acceptable, despite the fact that H37Rv is pan-susceptible).

It is not clear whether these differences in the QC ranges are caused by the two key differences between the CLSI standard operating procedure (SOP) for the MYCOTB(I) plate and the official IFUs by TFS.[62,63,82] First, CLSI recommends a sedimentation step after vortexing, after which the supernatant is transferred to a new tube before the inoculum is measured and adjusted.[82] This minimizes carryover of bacterial clumps, which are a known problem for DST for MTBC.[15] By contrast, the IFUs state that the inoculum should be measured and adjusted immediately after vortexing without prior sedimentation and transfer to a new tube.[62,63] Second, when the MYCOTB plate was originally developed, 5–7% CO_2 was used for MTBC growth, which is still recommended by CLSI.[82] By contrast, the official IFUs do not recommend the use of CO_2.[62,63] The precise reason for this change is not clear and likely represents a mistake by TFS when the IFUs were prepared (i.e. the assay was not revalidated using ambient air).[ii] As a result, many American laboratories use CO_2 whereas most laboratories globally follow the IFUs and use ambient air.

The use of CO_2 could affect MICs through different mechanisms. CO_2 is known to facilitate the growth of some MTBC strains, particularly highly drug-resistant ones.[iii] Moreover, CO_2 lowers the pH of media, which reduces the activity of some antibiotic classes against other bacteria and, consequently, increases the MIC (e.g. for aminoglycosides, azoles and quinolones).[88-94] It is also possible for CO_2 and the resulting lower pH to have no effect (e.g. for RIF) or to even lower the MICs for some agents by potentiating the action of the agent (e.g. tetracyclines and penicillins), which underlines the importance of including a full QC range for MIC testing.[93,94] Whether and to what extent CO_2 alters MICs for MTBC is not known. However, is known that the pH affects MTBC MICs to BDQ, CFZ, D-cycloserine (DCS), and PZA.[26,27,95] Based on this indirect evidence, it is possible that the technical

[ii] Killian Scott (TFS), personal communication.
[iii] Nikki Parrish, personal communication.

variability for at least some of the anti-TB agents was increased due to this lack of standardization. Therefore, the current practice of pooling MICs from what are effectively two different methods may not be appropriate as systematic differences may not only have affected the QC range but also the resulting CCs. For example, one study with CO_2[86] and two studies with ambient air[96,97] are cited in support of the CCs that CLSI set in 2018 for RIF, INH, and EMB.[35] Similarly, WHO did not consider the potential effect of CO_2 in its past reviews of the CCs for 7H10/7H11 and has made the use of CO_2 optional for these media whereas it is mandated by CLSI.[15,36,46,82]

Additional methodological and design limitations are outlined in Section 3.

2.2 Overview of other BMD plates

At least five additional BMD plates have been manufactured by TFS for MTBC research to date (Table 1). Custom-made frozen plates were used in the Janssen BDQ-resistance surveillance studies related to its BDQ trials and by Swedish researchers for therapeutic drug monitoring, respectively.[77,78] Moreover, a dry version of the BMD plate used in the Janssen studies exists.[75,76] The CRyPTIC Consortium developed UKMYC5 as a variant of the dry MYCOTB(I) plate, which it refined in a subsequent version, UKMYC6.[44,80] Between 12 and 14 drugs were included on these plates. Finally, EUCAST recently endorsed a non-commercial BMD format as the reference MIC method for MTBC, for which it will set QC ranges, QC targets, and breakpoints in the future.[28,72,81,98] The medium and associated drug dilutions for this method must be freshly prepared prior to testing. This means that the choice of drugs can be customized (i.e. only the arrangement of the controls and wells with water is fixed) but also that this method is too labour-intensive for routine pDST, particularly for drugs that require dimethyl sulfoxide (DMSO) as the solvent for the stock solution and subsequent dilutions.[57] All plates use 7H9 with 10% OADC but the percentage of glycerol used differ and the frozen TFS plates additionally contain casitone.[iv]

2.3 Upper limit of drugs on optimized BMD plate

QC ranges for other bacterial pathogens typically encompass between 3–4 dilutions, which is in line with the QC data generated for BMD testing using H37Rv (Table 2).[35,99,100] Assuming that the upper end of the QC range corresponds to the CC for all drugs, this means that a minimum of 5–6 concentrations are needed to ensure that the QC range is on-scale (i.e. 4–5 concentrations, including the concentration below the lower end of the QC range and one concentration above the CC). By contrast, the lower end of the pWT distribution does not need to be covered, should it be lower than the lower end of the QC range given that biological variation within the pWT distribution can usually not be distinguished from technical variability, at least based on a single MIC result, unless genotypic information can be marshalled (e.g. to identify hyper-susceptibility to BDQ and CFZ due to loss-of-function [LoF] mutations in *mmpL5-mmpS5*).[10,12,55]

The Technical Expert Group (TEG) agreed that for most of the WHO-approved drugs, stratifying the level of resistance is currently only of scientific interest (i.e. the inclusion of additional concentrations above the CC should not come at the expense of excluding other drugs). By contrast, clinically actionable results have already been recognized by WHO or may be endorsed in the future for the following drugs:

[iv] Thomas Campbell (TFS), personal communication.

1. At least two concentrations above the CC are needed for levofloxacin (LFX), which is proposed as a surrogate for moxifloxacin (MFX) (see Section 2.4), to accommodate the equivalent concentration of the MFX CB.[15]
2. INH resistance is currently stratified genotypically but no corresponding CB has been set.[46] If one were endorsed to distinguish strains with only *inhA* promoter mutations from strains with higher MICs, the potential CB (pCB) would likely have to be set three concentrations above the CC (i.e. at 1 mg/L).[46,101,102] Additional concentrations would be needed if strains with only the *katG* S315T mutation were also deemed to benefit from high-dose INH treatment.
3. Higher doses of RIF are currently being evaluated given that 600 mg daily represents the minimal effective dose, which was originally chosen because of the high cost of RIF and fear of dose-related adverse events.[103] A higher dose may render strains with only modest MIC increases due to borderline *rpoB* mutations treatable. It is unlikely that sufficient evidence to prove this hypothesis conclusively will become available soon, but the inclusion of 3–4 concentrations above the ECOFF would facilitate the routine data collection to investigate this question.[46,104]
4. A pCB one concentration above the ECOFF is likely needed if KAN is used as the surrogate for AMK resistance (see Section 2.4).

Taken together, it is unlikely that more than 12 drugs can be accommodated on a plate (i.e. typically one drug per column, whereby the three GCs [Figure 2] would be included in columns with drugs that require fewer than eight wells). The configuration of one drug per column is also important to simplify reading of plates.

Figure 2. Tentative layout of an optimized BMD plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative GC	1% pos. GC	100% pos. GC	LFX 8	BDQ 1	CFZ 1	RIF 2	INH 2	PMD 2	DLM 0.25	KAN 16	ETO 16
B	EMB 8	DCS 64	LZD 4	LFX 4	BDQ 0.5	CFZ 0.5	RIF 1	INH 1	PMD 1	DLM 0.125	KAN 8	ETO 8
C	EMB 4	DCS 32	LZD 2	LFX 2	BDQ 0.25	CFZ 0.25	RIF 0.5	INH 0.5	PMD 0.5	DLM 0.06	KAN 4	ETO 4
D	EMB 2	DCS 16	LZD 1	LFX 1	BDQ 0.125	CFZ 0.125	RIF 0.25	INH 0.25	PMD 0.25	DLM 0.03	KAN 2	ETO 2
E	EMB 1	DCS 8	LZD 0.5	LFX 0.5	BDQ 0.06	CFZ 0.06	RIF −0.125−	INH 0.125	PMD 0.125	DLM 0.016	KAN *1*	ETO 1
F	EMB −0.5−	DCS 4	LZD 0.25	LFX 0.25	BDQ 0.03	CFZ *0.03*	RIF 0.06	INH 0.06	PMD 0.06	DLM *0.008*	KAN −0.5−	ETO 0.5
G	EMB *0.25*	DCS −2−	LZD 0.125	LFX *0.125*	BDQ 0.016	CFZ 0.016	RIF *0.03*	INH −*0.03*−	PMD 0.03	DLM 0.004	KAN 0.25	ETO −*0.25*−
H	EMB 0.125	DCS 1	LZD *0.06*	LFX 0.06	BDQ *0.008*	CFZ 0.008	RIF 0.016	INH 0.016	PMD 0.016	DLM 0.002	KAN 0.125	ETO 0.125
Drug type	1st line	B	A	A	A	B	1st line	1st line	other	C	C	C

GC
pCB
alternative pECOFFs
pECOFF
lower end of pQC range

−lowest MYCOTB(I) conc.−
lowest UKMYC6 conc.

2.4 Drug priorities for inclusion on optimized BMD plate

Table 1 provides a summary of the prioritization of drugs based on the latest treatment guidelines by WHO.[83] Further details or unusual properties of the different drugs are listed here.

Rifamycins

Borderline resistance mutations confer MICs that span the ECOFF of RIF. Therefore, they cannot be reliably confirmed by testing the CC, which has prompted WHO to reaffirm the expert rule that any mutation, with the exception of synonymous mutations, in the rifampicin resistance determining region (RRDR) and *rpoB* I491F should be assumed to confer RIF resistance (i.e. even if they have never been described before).[13,46,105] WHO has acknowledged that exceptions may exist (e.g. *rpoB* T427A), which would have to be excluded from this rule.[13,45,46] Therefore, capacity for MIC testing with an on-scale QC result is needed to classify potential neutral RRDR mutations with confidence. This is not possible with the MYCOTB(I) plate.[29] Should a higher dose of RIF be endorsed in the future, additional concentrations above the ECOFF may be needed (see Section 2.3).

The relative MIC increases conferred by *rpoB* mutations are smaller for rifabutin (RFB) than RIF (at least for the concentration ranges tested to date that yielded on-scale results for both agents). Whether this difference can be exploited clinically remains to be seen (i.e. CLSI has not provided a rationale for setting its CC several concentrations above the ECOFF).[46] Because of this uncertainty and that RFB is not endorsed by WHO, RFB has not been prioritized.

WHO has recently endorsed a 4-month regimen with rifapentine (RPT), INH, PZA, and MFX, which means that RPT will become a first-line drug and MFX will function as a first- and second-line drug.[106,107] Based on the limited studies that tested strains against both RIF and RPT, it appears that the relative MIC increases conferred by *rpoB* mutations for RPT are more similar to RIF than RFB.[108,109] Until data emerge that RPT is a better surrogate for RIF resistance or that some *rpoB* mutation are treatable with RPT but not RIF, gDST and pDST results for RIF can be extrapolated to RPT, as occurred in the trial of the 4-month RPT regimen and is currently recommended by WHO.[46,106]

Isoniazid

WHO considers pDST for INH to be important as it is a widely used drug for preventative therapy, first-line therapy, as well as the standardized shorter MDR/RR-TB regimen.[110,111] The only uncertainty from the point of view of designing a plate is whether a CB will be endorsed for this drug (see Section 2.3).

Ethambutol

EMB is considered the least important first-line drug.[112] Nevertheless, it is still widely used for first line treatment and is included in the standardized shorter MDR/RR-TB regimen. MIC results are more useful than categorical pDST results to classify resistance mutations accurately given the dominant EMB resistance mechanisms confer only modest MIC increases resulting in a significant overlap with the pWT MIC distribution, unless secondary mutations are present that increase the MIC further.[32,33,35,38,43,44]

Pyrazinamide

PZA is widely used and included in first- and second line regimens. Given the large spectrum of resistance mutations for PZA, its inclusion on a plate would be highly desirable.[41] Yet, owing to the requirement for polyoxyethylene stearate and a lower pH, this is not possible using the standard 7H9 medium for BMD testing.[16,113,114]

Fluoroquinolones

WHO currently recommends testing the FQ that is used clinically, which means that laboratories have to test up to three concentrations (i.e. the CCs for LFX, the CC for MFX, and the CB for MFX, although some laboratories have adopted different practices, such as testing the LFX CC and MFX CB only).[15] The analysis of 631 strains tested at the San Raffaele Scientific Institute with either the UKMYC5 or UKMYC6 plate, for which genomes were available, suggested that LFX is the suitable surrogate FQ for pDST (Table 3).

The primary goal of pDST for FQs is to distinguish susceptible strains from resistant strains with either low-level resistance (LLR) or HLR mutations (i.e. *gyrA* A90V and D94G are the most frequent mutations of the respective groups).[36,115] Based on the UKMYC data (Table 3), the rate of misclassification of *gyrA* A90V as susceptible was significantly smaller at the potential ECOFF (pECOFF) for LFX than the MFX pECOFF (i.e. 6% [95% CI 2–14] vs. 41% [95% CI 30–52]).[v] This means that MIC testing for LFX is more likely to give a definitive result as fewer strains fall into the potential ATU (pATU) of 1 mg/L than the equivalent MFX pATU of 0.5 mg/L. The main driver for this difference was that the MFX MIC distributions for lineage 3 strains were lower by approximately one doubling dilution compared with lineages 1, 2, and 4, which is in line with an analysis of the wider CRyPTIC data.[10] This applied to the gWT, *gyrA* A90V, and *gyrA* D94G MIC distributions, which was most evident when comparing their modes. This did not appear to be the case for LFX, although a lineage effect may exist that was obscured by how the LFX MIC distributions were divided by the 2-fold dilution scheme employed for testing (i.e. testing at additional intermediate concentrations might reveal a difference).

When WHO set a CB to stratify LLR from HLR mutations for MFX in 2018, it was already clear that there is considerable overlap between both populations, which was also the case with the UKMYC data (i.e. the modes for *gyrA* A90V spanned 0.5–1 mg/L compared with 2 mg/L for *gyrA* D94G on 7H10).[36] To maximize the benefit of high-dose MFX, WHO set the CB two concentrations above the MFX CC for 7H10 and MGIT (at one concentration above the CC, approximately half of *gyrA* A90V mutants would be misclassified as HLR simply due to the technical variation in testing). The TEG was aware that this would increase the likelihood of misclassifying HLR strains as LLR but deemed this trade-off acceptable provided that upfront gDST was done to detect HLR mutations.[36] Based on the UKMYC data (Table 3), testing the LFX pCB of 4 mg/L as the surrogate for the MFX pCB of 2 mg/L would reduce this concern given there was a trend towards a lower rate of misclassification of HLR mutants as LLR (i.e. 50% [95% CI 40–59] vs. 63% [95% CI 53–72]).

In the recently published catalogue of resistance mutations, WHO endorsed an expert rule whereby any mutations that confers resistance to LFX at its ECOFF should also be assumed to confer resistance

[v] The pECOFF was defined by approximating the pWT MIC distribution using genotypically wild-type (gWT) strains that lacked 10 *gyrA* mutations (G88A, G88C, D89N, A90V, S91P, D94A, D94G, D94H, D94N, and D94Y), any non-synonymous mutations at *gyrB* codons 497-502, *gyrB* A504V, *rpoB* V170F, any mutations at *rpoB* codons 426-452 (except synonymous ones), or *rpoB* I491F.

to MFX and vice versa.[13,14] Yet, on rare occasions, testing LFX as the surrogate for MFX may be problematic for mutations that confer MFX resistance but have little to no effect on LFX (i.e. are at greater risk of either being missed completely or misclassified as LLR instead of HLR if only LFX is tested). This might be the case for some rare *gyrB* mutations, such as E501D, although it should be noted that this mutation is unlikely to be missed in practice given that it is inferred by the Hain GenoType MTBDR*sl* v2 line probe assay.[10,36,116-118] Other examples may exist, but they are likely too uncommon to justify including MFX on a plate instead of another antibiotic.

Table 3. Comparison of LFX and MFX MICs for key resistance mechanisms.

			UKMYC MIC (mg/L)																
			LFX								MFX								
			≤0.125	0.25	0.5	1	2	4	8	>8	≤0.06	0.125	0.25	0.5	1	2	4	>4	total
gWT	Lineage 3	count	8	**51**	26	3	0	2			57	26	3	3	1				90
		% sum (left to right)	8.9	65.6	94.4	97.8	97.8	100.0			63.3	92.2	95.6	98.9	100.0				
	Lineage 1, 2 & 4	count	4	144	**180**	14	2			1	15	117	**164**	46	2			1	345
		% sum (left to right)	1.2	42.9	95.1	99.1	99.7			100.0	4.3	38.3	85.8	99.1	99.7			100.0	
gyrA A90V	Lineage 3	count				2	**22**	14		1		1	2	**25**	10			1	39
		% sum (right to left)				5.1	61.5	97.4	97.4	100.0		2.6	7.7	71.8	97.4			100.0	
	Lineage 1, 2 & 4	count			1	2	**17**	**17**	3	2			1	8	**16**	11	6		42
		% sum (right to left)			2.4	7.1	47.6	88.1	95.2	100.0			2.4	21.4	59.5	85.7	100.0		
gyrA D94G	Lineage 3	count				3	1	**31**	28	4				5	19	**35**	8		67
		% sum (right to left)				4.5	6.0	52.2	94.0	100.0				7.5	35.8	88.1	100.0		
	Lineage 1, 2 & 4	count					2	**20**	**20**	6				1	2	10	**24**	11	48
		% sum (right to left)					4.2	45.8	87.5	100.0				2.1	6.3	27.1	77.1	100.0	

Concentrations in **bold** correspond to the modes of an MIC distribution, where these could be defined.
The green lines denote the pECOFFs.
The red line denotes the pCB to stratify LLR and HLR for MFX (i.e. the LFX pCB would not be relevant for LFX).

Bedaquiline and clofazimine

Cross-resistance between BDQ and CFZ is not complete (i.e. *atpE* mutations only confer resistance to BDQ, whereas *Rv1979c* appears to only confer resistance to CFZ, although more recent findings have called the role of *Rv1979c* into question).[9,119] Given the controversy surrounding *Rv1979c* and that other mechanisms may exist that only confer resistance to CFZ, BDQ and CFZ are both needed on a plate. The inclusion of both agents would enable interpretative reading for *Rv0678* (see Section 1).

Linezolid

According to latest WHO classification, LZD is one of the most important agents (group A) for the treatment of MDR/RR-TB and, consequently, resistance to LZD is one of the criteria for the new definition of XDR-TB.[87] Moreover, it is part of the PMD-BDQ-LZD regimen that is WHO-approved for use under operational research conditions.[83]

D-cycloserine and terizidone

In light of the recent reclassification of DCS and its variant terizidone (TZD) to group B drugs for MDR/RR-TB treatment and their side effects profile, the inclusion of DCS on a plate would be desirable, particularly as no WHO-endorsed CC currently exists for any medium.[36,83] Some researchers believe that DCS cannot be tested in liquid media but it is not clear whether this is a general phenomenon or applies only to some liquid media (e.g. those containing alanine).[95] The limited published DCS results for the current MYCOTB(I) plate are contradictory. Nakatani *et al*. were unable to clearly differentiate susceptible strains from alanine racemase mutants, whereas this was possible with MGIT.[120] By contrast, both methods worked well for this mechanism in a study by Evangelopoulos *et al*.[121] Deshpande *et al*. proposed a pECOFF of 64 mg/L, but this decision was likely biased by the inclusion of too many RIF-resistant strains that might have harboured alanine dehydrogenase mutations that only confer modest MIC increases.[34,122] A pilot study that investigates the reproducibility of DCS with

H37Rv and a set of mutants with known resistance mechanisms would provide insights into these open questions.

Nitroimidazoles

Although few studies have tested the same strains against both DLM and PMD, it appears that cross-resistance between DLM and PMD is not complete, which means that both nitroimidazoles need to be included.[6,123] This is particularly important in light of the recent observation that lineage 1 strains have intrinsically elevated PMD MICs compared to the other major MTBC lineages, which does not appear to be the case for DLM.[124]

Carbapenems

Little MIC testing has been carried out for either imipenem-cilastatin (IMP-CLN) or meropenem (MPM) with clavulanic acid (CLA) to date.[125,126] No pECOFF exists for any medium and the only known resistance mechanisms to date involve *blaC* and the yet unannotated *crfA* gene.[127-132] Therefore, it is not clear how to best carry out pDST (e.g. which fixed concentration of CLA to test, whether a single carbapenem is sufficient and, if so, which one provides the best resolution between susceptible and resistant strains). Given these uncertainties and the fact that carbapenems have limited use compared with other agents, this class of drugs is not currently a priority for inclusion on a plate.

Amikacin

KAN is no longer recommended for the treatment of MDR/RR-TB and the use of AMK is minimized in the latest WHO guidelines.[83] Nevertheless, AMK plays an important role in constructing regimens, particularly when all-oral regimens are not an option.[133] Given the toxicity of AMK, pDST capacity is needed to complement results from upfront gDST assays.

The results of an analysis of 1,706 strains tested at the San Raffaele Scientific Institute with either the UKMYC5 or UKMYC6 plate, for which genomes were available, are shown in Table 4. These data suggest that KAN should be adopted as a surrogate for AMK pDST as it offers the following advantages:

1. Testing KAN would minimize the misclassification of *eis* c-14t and *rrs* c1402t as susceptible, which have recently been recognized as AMK resistance mutations by WHO in accordance with the interpretation of the WHO-endorsed Cepheid Xpert MTB/XDR.[13,134] The reason for this is that both mutations conferred a more marked increase to KAN, resulting in MIC increases above the pECOFF for KAN, whereas the modes for both mutations corresponded to the pECOFF for AMK of 1 mg/L.[vi] A pATU spanning 0.5–1 mg/L of AMK would not be an option as it would result in too many genuinely susceptible strains being classified as "uncertain". This could be minimized by narrowing the pATU to just 1 mg/L, but this would result in more very major errors. By contrast, KAN MICs of >8 mg/L could be used to classify a strain as AMK resistant, whereas 8 mg/L could be designated as a pATU to alert clinicians that this could be due to *eis* c-37t, g-10a or c-12t that are not currently considered to be clinically relevant. Additional testing could be carried out (e.g. by sequencing DNA extracted from the 4 mg/L well to amplify a minority *eis* mutant subpopulation that was below the limit of detection when gDST was carried out from the primary sample) or the pDST result could be reported as AMK resistant to err on the side of caution.

[vi] The pECOFFs were defined by approximating the pWT MIC distribution using gWT strains that lacked mutations 100 bp upstream of *eis* or in three positions of *rrs* (1401, 1402, and 1484).

2. Testing KAN would likely maximize the chance of identifying strains, for which *eis* mutations are not valid markers of resistance because of epistasis.[12] Specifically, 39% (95% CI, 14–68) of *eis* c-14t mutants in this dataset harboured LoF mutations in the *eis* coding region, which means the promoter mutation cannot confer resistance. Because the MIC distribution for these LoF mutants was truncated at the lower end for both drugs, it was not clear whether these were hyper-susceptible to KAN and AMK. Nevertheless, it is likely that testing KAN would better distinguish *eis* promoter mutants with functional *eis* from those in a LoF background (i.e. the KAN MICs for LoF mutants were ≤2 mg/L vs. ≥8 mg/L in a wild-type background compared with ≤0.25 mg/L and ≥0.5 mg/L for AMK).
3. Should one or more of the remaining *eis* promoter mutations be found to be clinically relevant for AMK, KAN would also be the preferred drug to test (i.e. the KAN breakpoint could simply be lowered to the pECOFF of 4 mg/L and, if necessary, a pATU could be set at 4 mg/L).

Adopting KAN as the surrogate for AMK would have the following risks:

1. It could be misinterpreted as a signal that KAN can be used clinically. This could be minimized by converting the KAN MIC into a categorical pDST for AMK and only reporting that to clinicians, in accordance with the precedents from other pathogens (see Section 1).
2. Should clinical outcome data demonstrate that *eis* c-14t and *rrs* c1402t are not clinically relevant for AMK, the KAN breakpoint would have to be raised to at least 16 mg/L (i.e. AMK would become the better drug to test as fewer concentrations above the AMK pECOFF would be needed).

These findings are in line with existing data from 7H10, although the degree of overlap between different mutations differs slightly for the latter medium.[36]

Table 4. Comparison of KAN and AMK MICs for key resistance mechanisms.

		UKMYC MIC (mg/L)													
		KAN						AMK							
		≤1	2	4	8	16	>16	≤0.25	0.5	1	2	4	8	>8	total
gWT	count	455	**753**	297	14	2	1	844	618	55	2	2		1	1,522
	% sum (left to right)	29.9	79.4	98.9	99.8	99.9	100.0	55.5	96.1	99.7	99.8	99.9		100.0	100.0
eis c-14t & LoF	count	4	1					5							5
eis c-14t	count				1	**6**	1		2	**3**	3				8
rrs c1402t	count					**1**				**1**					1
eis c-37t	count				**2**	1		**2**	1						3
eis g-10a	count		1		**4**	3		2	**3**	**3**					8
eis c-12t	count		1	18	**21**	2	1	6	**33**	3	1				43
rrs a1401g	count	3	1		1	2	109	3	1					112	116

Concentrations in **bold** correspond to the modes of an MIC distribution, where these could be defined.
The green lines denote the pECOFFs.
The red line denotes the pCB for use as the surrogate for AMK resistance
The concentrations in grey denote the pATUs.

Streptomycin

Given that streptomycin (STR) is only recommended when AMK is not available or AMK is not an option because of resistance, and rates of resistance to STR are very high in many high-burden MDR/RR-TB settings, STR is not a priority drug.[83]

Thioamides

It has been known since the 1960s that the considerable overlap between the MIC distributions of susceptible and resistant strains presents a major challenge for pDST of ethionamide (ETO).[38,43,44,135] The underlying resistance mechanisms also confer elevated MICs to prothionamide (PTO), which means that cross-resistance between both analogs is thought to be complete and that only one thioamide needs to be tested.[136] A recent study suggests that *Rv0565c* may confer larger relative MIC increases for PTO than ETO.[137] Yet, this remains to be confirmed and no systematic data exist whether this may also be the case for the remaining resistance mechanisms (i.e. whether testing PTO instead of ETO or vice versa might reduce the degree of overlap between MIC distributions and, consequently, the need for ATUs). In the absence of such data, ETO is the preferred choice as more MIC testing has been carried out with this analog (Table 1).

Para-aminosalicylic acid

The reproducibility of *para*-aminosalicylic acid (PAS) in UKMYC5 was poor, which is why it was not included in UKMYC6 (Table 1).[80] To date, it has not been explored whether adjusting the reading instructions could overcome this problem, which is caused by trailing endpoints linked to the bacteriostatic activity of PAS.[138] Therefore, PAS cannot be included on a plate.

2.5 Tentative layout of an optimized BMD plate

Figure 2 shows a tentative plate layout that was prepared based on the following principles:

1. The control wells were placed in the upper left-hand corner of the plate to minimize the possibility of an accidental inoculation of these controls if plates are loaded manually. The disadvantage of this decision is that it increases the risk of invalidating the entire plate if one of these controls is affected by evaporation.
2. Each column has a single agent to minimize interpretation errors. Whenever possible, drugs were ordered according to their WHO grouping and shared resistance mechanisms (e.g. DLM and PMD).
3. Given that EMB is the least important first-line drug and ETO is the lowest ranking group C drug that is suitable for BMD testing, these were placed in the outer columns (i.e. if evaporation were to prevent the interpretation for these drugs, this would be less important than for other drugs).[83,112]
4. No comprehensive and systematic evaluation of pECOFF for BMD testing has been carried out to date. However, noting differences between the proposals to date (Table 2) and the wider body of knowledge, the following were important considerations:
    a. As outlined in Section 2.4, the LFX pECOFF would be used to define resistance to LFX and LLR to MFX, whereas the pCB would only be relevant as a surrogate for HLR to MFX.
    b. When modelling the pWT distribution for BDQ and CFZ, the CRyPTIC Consortium and Janssen did not exclude *Rv0678* mutants.[44,77] Given that some, but not all, *Rv0678* mutations result in the overexpression of the *mmpL5-mmpS5* efflux pump and, provided that the pump is active, phenotypically non-wild type MICs that overlap with the upper end of the pWT distribution, this may have resulted in an overestimate of

the 99th percentile of the pWT distribution.[12,36,139] Janssen compensated for this potential confounder by selecting the 97.5th percentile as the ECOFF for BDQ (i.e. 0.125 mg/L).[75,77] By contrast, CRyPTIC Consortium advocated an ECOFF of 0.25 mg/L, despite considering strains with MICs of 0.25 mg/L as resistant and 0.125 mg/L as borderline resistant when classifying *Rv0678* mutants (i.e. effectively using an ECOFF of 0.125 mg/L with an ATU at that concentration).[44,140] A consensus is also lacking for CFZ for similar reasons. More work is needed to resolve these questions.

   c. 2 mg/L has been proposed as the pECOFF for LZD for the frozen BMD plate but 1 mg/L appears to be more appropriate for the dry plate.[44,77] The QC range for this drug will have to be analysed in more detail as H37Rv appears to be more resistant than clinical strains, which is unusual (i.e. the mode of its distribution is 0.5–1 mg/L compared with 0.25–0.5 mg/L for clinical strains).[44]
   d. As mentioned in Section 2.4, the DCS pECOFF proposed by Deshpande *et al*. of 64 mg/L is likely too high, but it is not clear whether 32 mg/L is the actual ECOFF.[120-122]
   e. Even though the pWT MIC distribution of DLM was severely truncated on the UKMYC plates, the proposed pECOFF of 0.125 mg/L appears to be too high given a mode of ≤0.008 mg/L for the pWT distribution.[36,44] To define the lower end of the pWT MIC distribution and set a QC range/target, concentrations down to at least 0.002 mg/L need to be included.[44] Once the lower end of the QC range has been defined, the concentration range may have to be optimized.
   f. The pECOFF of 4 mg/L for KAN is included for information only. As outlined in Section 2.4, the pCB at 8 mg/L would be used as the surrogate for AMK resistance.

5. To date, PMD has only been tested systematically in MGIT.[124] The suggested concentration range is merely meant as a starting point to evaluate whether and to what extent MICs are systematically shifted in a lyophilized format compared with MGIT.[vii] In this context, the implications of lineage 1 effect for PMD will have to be considered.[124]
6. In accordance with the rationale from Section 2.3, several concentrations above the pECOFFs were assigned to RIF and INH.
7. Two or three concentrations above the most likely pECOFFs for BDQ and CFZ were allocated to facilitate the interpretation for *Rv0678* mutations.

[vii] J. Timm, personal communication.

3 Methodological considerations

3.1 Measures to ensure high-quality BMD testing

The TEG agreed on the following measures to maximize the quality of BMD testing:

1. A plate with lyophilized rather than frozen antimicrobials should be developed to avoid the need for a cold chain and minimize the probability of contamination.
2. The precise effect of the shape of wells on trailing endpoints has not been evaluated systematically for MTBC.[141] But because reading times can be shortened using U-bottom-shaped wells compared with flat ones, these are recommended in line with the current TFS plates and the EUCAST reference method. The plates should be made of polystyrene to ensure that BDQ and CFZ[viii] can be tested accurately and their surface should be untreated.[27,56] The effect of changing the supplier of plates must be monitored by manufacturers as even different types of polystyrene may affect the MIC of some drugs.[142]
3. The plates should be covered with a semi-permeable seal to improve biosafety and minimize evaporation.
4. One negative GC and two positive GCs, with a 100% and 1% inoculum respectively, are needed (see Section 3.2 for the rationale for the 1% GC). Highlighting these three wells visually (e.g. by circling them with different colours) would minimize pipetting errors if the plate is inoculated manually. Similarly, showing the relevant drug abbreviations for each plate column may minimize reading errors. Finally, labelling the breakpoints on the plates would focus the attention on growth at these thresholds, although this may introduce reading bias.
5. The 7H9-OADC must not contain malachite green as resistance mutations in five of the seven known nitroimidazole resistance genes confer hyper-susceptibility to this decontamination agent.[6] Moreover, the medium must not include alanine as it interferes with the antimicrobial activity of DCS.[95,143] Tween 80 must not be used in the medium as it is known to effect the MIC and leads to carbon flux rerouting in MTBC.[27,56,144] The current practice of using glycerol should be maintained, although an improved understanding of the effect of not including pyruvate on the growth of *Mycobacterium africanum* and animal-adapted MTBC members is needed.[145]
6. Some antibiotics require that DMSO must be used to prepare the stock solution and the subsequent dilutions to ensure that they do not precipitate (e.g. for BDQ and CFZ).[57,146,147] The number of pipetting steps may also be relevant for some drugs given that tips are not available in polysterene.[57,142]
7. Concentrations that comply with the ISO requirements (i.e. two-fold dilution series based on 1 mg/L) should be used and on-scale QC testing with H37Rv must be possible to define a QC range and QC target.[21,29,30,64,100]
8. BMD testing is more prone to fluctuations of the inoculum compared with the proportion method as MICs are usually measured at full inhibition (see Section 3.2). Therefore, the IFUs by TFS for this step must be revised to minimize this source of variation. For example, the use of a suitably calibrated nephelometer to prepare the 0.5 McFarland suspension, which is used to prepare the final inoculum (Table 1), should be mandatory rather than optional and a colony count should ideally be carried out for every strain to ensure that the inoculum was prepared accurately (or at least regularly for QC purposes).[62,63] To this end, a sound inoculum target and range must be established against which results can be compared (this is not currently possible as discussed in footnote b of Table 1). Moreover, the current recommendation by TFS to measure the inoculum immediately after vortexing without a prior

[viii] Leen Rigouts (Prince Leopold Institute of Tropical Medicine), personal communication.

sedimentation step is not suitable for MTBC. The resulting aerosols not only represent an unacceptable biohazard and risk for cross-contamination but also may result in an inappropriately low inoculum because large clumps would contribute to the McFarland reading but would settle during the subsequent sedimentation step.[62,63,148] Instead, a sedimentation step must be included after the vortexing, followed by a transfer of the supernatant to a new tube, which should be used for the measurement and adjustment of the inoculum, as recommended by CLSI and EUCAST for BMD testing.[72,82] More broadly, the IFUs should be reviewed to identify additional measures to improve biosafety, where relevant.[148]

9. Unless a protocol is developed to enable reliable testing from freshly positive, actively growing MGIT cultures, the routine clinical value of BMD testing will be limited (i.e. to avoid the need for a lengthy subculture on a solid medium). In fact, starting from MGIT would also be preferable as clumping is reduced.
10. Requiring the use of CO_2, as recommended by CLSI, would represent a significant barrier to uptake to some countries because incubators are not compatible with CO_2, as well as the cost and the lack of a stable CO_2 supply.[82] BMD testing should, therefore, be validated and carried at ambient air. This may result in a proportion of strains not showing sufficient growth to be read at day 21 but false-susceptible results would be limited because of the 1% GC. Any laboratory using the plate with CO_2 (i.e. contrary to the updated IFUs) would be doing so at its own risk.
11. Plates should be checked on day 2 or 3 post-inoculation for contamination with fast-growing organisms to avoid unnecessary delays.
12. Details regarding plate reading are covered in Section 3.2.

3.2 Plate reading

WHO currently defines the MIC as "the lowest concentration of an antimicrobial agent that prevents growth of more than 99% [of] a microorganism in a solid medium or broth dilution susceptibility test."[15] Thus, the MIC for MTBC relies on the CP of 1% for all drugs, with the exception of PZA, for which 10% is used.[149] This contrasts with most other bacterial pathogens, for which the MIC is defined as the lowest concentration that inhibits all visible growth by the naked eye (hereafter referred to as the "visual CP"), although some expectations exist to accommodate trailing endpoints.[82,138] Notably, all variants of the TFS plates (Table 1), the EUCAST reference method for MTBC, and the vast majority of reference methods for other pathogens rely on the latter approach for MIC testing.[138] This strategy was used by EUCAST and TFS because an accurate comparison of the degree of growth with the 1% GC is not possible with the naked eye in a BMD format, even if dyes are added at the end of the incubation. Moreover, the addition of dyes should be avoided for safety reasons as this requires manipulation of an open positive plate whereas the current TFS plate is sealed throughout the entire period. In addition, because dyes measure metabolic activity, as opposed to standard growth, dyes do not necessarily yield comparable results with visual growth.

The 1% (or 10% for PZA) CPs serve two main functions in the TB field. First, they improve the reproducibility of testing. For example, MGIT results are invalid if the 1% (or 10%) GC reaches 400 growth units in fewer than 4 days to exclude contamination or over-inoculation (particularly when starting from positive MGIT cultures, for which the inoculum is not measured).[17,18] Results are also invalid if this growth threshold is not reached by the 1% GC within 13 days (or 21 days by the 10% GC for PZA) to control for insufficiently large inocula or poor growth due to other factors.[17,18] Second, the CPs represent a cut-off below which the frequency of resistant bacilli is traditionally regarded not be clinically relevant.[150,151] Yet, the TEG acknowledged that, at least for the newer anti-TB agents (e.g.

BDQ, CFZ, FQs, and PMD), there is no clinical evidence that samples with a resistant subpopulation of 0.01% or 0.1% typically respond to the antibiotic in question whereas those at 1% usually do not (although a higher subpopulation is more likely to be significant *a priori*). Instead, 1% was simply chosen because it is the current standard for most drugs. The only theoretical criterion for selecting a CP is that it has to be greater than the mutation frequency of MTBC to a clinically effective drug or else all samples would test resistant, provided that a sufficiently large inoculum is tested.[152] Indeed, 1% is approximately 250 times higher than the percentage of bacilli of pWT strains that are resistant at the INH CC of 0.2 mg/L on LJ.[153] This also means that even if 1% were the optimal CP for one drug, this would not necessarily be the case for a drug that has a significantly different mutation frequency or plays a different role in the regimen (e.g. a core vs. a companion drug).[112] In other words, using the same CP of 1% for different drugs is largely arbitrary. This is underlined by the fact that WHO had originally recommended a CP of 10% for nine of 12 drugs, which was later changed to 1%, largely due to the efforts of the US Centers for Disease Control and Prevention to simplify and bring greater standardization to pDST of MTBC (e.g. rather than testing an ETO CC of 20 mg/L with a 10% CP, 40 mg/L is now used with a 1% CP).[15,151,154] The only reason why 10% is still the CP for PZA is to accommodate for poorer growth at a lower pH, which is also why a longer protocol of 21 days instead of 13 days is used for MGIT.[ix,17,18]

Despite the arbitrary nature of the 1% CP, the TEG agreed that BMD testing needed to have a limit of detection of approximately 1% for resistant subpopulations. To achieve this, the procedure for reading the MIC from the EUCAST reference method was endorsed.[72] The use of a nephelometer and the requirement for growth of the 1% GC within 21 days should minimize variation of the inoculum and ensure that the MIC is only read when sufficient growth is achieved to detect low-frequency heteroresistance. Moreover, it was agreed that the MIC should be read on the earliest timepoint at which the 1% and 100% GCs are positive, but the negative GC shows no growth. Specifically, day 7, 14 and 21 were suggested, as opposed to the combination of day 10 and 21 currently recommended by TFS given that day 10 may cause problems if reading is not possible on weekends.[x,28,62,63] The reason for selecting the earliest timepoint for reading was to minimize the difference between the 1% CP and the visual CP, which may affect the MIC. For example, if both GCs were positive at day 7, resulting in an MIC in the susceptible range, but the sample had a high-level resistant subpopulation that only became visible at day 21, the MIC would increase simply because the visual CP at day 21 would end up being considerably lower than 1%. The TEG noted that even if both GCs are positive at day 7, some samples might have a higher MIC by BMD testing compared with the 1% proportion method if they have a resistant subpopulation above the visual CP but below the 1% CP. However, there was agreement that this scenario would likely be rare because the frequency corresponding to the visual CP at day 7 is unlikely to be much lower than 1%, which could be tested using artificial mixtures of resistant and susceptible strains.[155,156]

In summary, TEG was not aware of any convincing clinical evidence that 1% is the optimal CP. Nevertheless, the aforementioned measures should minimize the proportion of samples with MIC shifts caused by the difference between the 1% and visual CPs. This is important given that pDST for MTBC is carried out from heterogenous cultures rather than a limited number of colonies.[157]

[ix] Richard Pfeltz (BD), personal communication.

[x] Some TEG members noted that the reading at day 7 would not be helpful given that the proportion of strains that show sufficient growth would be too small.

4 Conclusion

The key finding of this analysis was that, with the exception of PZA, all key anti-TB agents can be arranged on a single BMD plate whilst meeting necessary QC requirements.[29] However, it was also clear that several open questions remain even for the plates manufactured by TFS. Chief amongst them are:

1. The pECOFFs and pCBs mentioned in this report (Figure 2) have not been reviewed independently and are not WHO-endorsed. Instead, they were merely meant to inform the tentative plate design and a comprehensive review of existing MICs is needed. Furthermore, the proposed changes to the inoculum preparation and MIC reading, particularly the use of a 1% positive GC, may affect both the breakpoints and QC range/targets (i.e. the revised plates have to be validated based on new experimental data generated with the updated SOPs).
2. The WHO plate design is not designed to fit the specification of any specific regulatory agency. Manufacturers would need to review the relevant requirements. In the USA, guidance from CLSI and FDA will be relevant, whereas in the European Union calibration against the EUCAST reference method and additional reproducibility testing would be needed to meet CE-IVDR requirements.[81]
3. PMD has not been tested at all in a lyophilized format.
4. Satisfactory QC ranges/targets for H37Rv have not been established for the dry format, which means that the reproducibility for key drugs is not understood (Table 2).[44] In this context, special attention should be paid to the possibility of trailing endpoints, which may require adjustments to the reading instructions (e.g. as is the case for LZD for other pathogens).[82,138]
5. The preparation of the inoculum must be improved to reduce the risk of aerosols.
6. The inoculum concentration can affect MICs for MTBC (e.g. for BDQ and PZA).[26-28] Yet, it is not known which, if any, of the previously used inocula (e.g. the MYCOTB(I) inoculum is almost twice as concentrated as the EUCAST inoculum [Table 1]) minimizes the technical variability and, crucially, the degree of overlap between the MIC distribution of susceptible strains and key resistance mechanisms. Ideally, this should be explored systematically by comparing different inocula using mutations that are known to confer only modest MIC increases (e.g. *rpoB* I491F with RIF and *Rv0678* M146T with BDQ).[39,46] Until relevant data are available, there is no clear reason to change the current 1 in 111 dilution step from a McFarland 0.5 that TFS currently recommends, but a sound end target and range must be set for this inoculum that can be used for QC purposes.
7. The optimal combination of reading days has to be determined (currently the IFUs recommend day 10 and 21 but the effect of including day 7 and 14 should also be explored).[62,63] In this context, the consequence of including the 1% GC will be important. For example, given that the TEG recommended ambient air instead of CO_2, a proportion of strains may yield sufficient growth with the 100% GC but not the 1% GC at day 21 and, therefore, a definitive MIC could not be read. However, should the strain in question show visible growth above the breakpoint, it could still be reported as resistant for that antibiotic (with a disclaimer to explain why an MIC endpoint cannot be provided).
8. No SOP exists for initiating BMD testing from positive MGIT cultures.

Given the pressing diagnostic, surveillance and research needs for pDST, WHO would like to stress that the global demand for a well-designed, validated and WHO-endorsed BMD plate is high.[5] To this end, global collaboration between stakeholders, ranging from assay and drug developers on the one hand and academia and regulators on the other, is urgently needed.[52,158] This report provides a consensus

statement on the principal technical and methodological characteristics of the BMD plate to ensure a solution that can be produced by all interested manufacturers and would meet public health needs while also meeting key requirements for future WHO endorsement for clinical use.

Finally, TEG concluded that, once designed in line with present consensus statement and consecutively validated, BMD testing could be endorsed by WHO based on a comprehensive synthesis and assessment of all available evidence, in line with GRADE requirements, proving the ability of this method to effectively detect clinically significant resistance to the most relevant anti-TB agents.

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For further information, please contact:
World Health Organization
20, Avenue Appia CH-1211 Geneva 27 Switzerland
Global TB Programme
Web site: https://www.who.int/teams/global-tuberculosis-programme/overview