Validation of a 14-drug microtitre plate that includes delamanid and bedaquiline for susceptibility testing of *M. tuberculosis.*

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Background: Universal access to drug susceptibility testing is key to ending TB. UKMYC5 is a 96-well microtitre plate designed by the *Comprehensive Resistance Prediction for Tuberculosis: an International Consortium* (CRyPTIC) which has potential to determine, at low cost, the MICs for 14 different anti-TB drugs, including several new and repurposed compounds. It is a dry-format plate and therefore easy to transport and store.

Objectives: Determine how long to incubate the plates before reading, and the optimal reading method. Establish the reproducibility of the UKMYC5 plate and compare it to established methods.

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Methods: UKMYC5 plates were tested by seven laboratories on four continents using a panel of 19 external quality assessment (EQA) strains, including H37Rv. MICs were measured from each plate by two readers using three methods (mirrored-box, microscope and Vizion™ Digital viewing system) at four different timepoints. All EQA strains were whole-genome sequenced and phenotypically characterized by MGIT960, 7H10/7H11 agar and resazurin microtitre assay.

Results: The optimum duration to incubate a plate is 14 days. The within- and between-laboratory reproducibilities for the best performing methods (mirrored-box and Vizion) were both 95% and 92%, respectively. One site was identified as requiring re-training and one drug (para-aminosalicylic acid) produced inconsistent results.

Conclusions: MICs measured using the UKMYC5 microtitre plate (i) are reproducible, (ii) compare well with the results of several established methods and (iii) correlate with the presence or absence of genetic mutations that confer resistance. This study provides the evidence that this assay can be deployed by TB reference laboratories world-wide as a diagnostic and research tool.

INTRODUCTION

The proportion of tuberculosis (TB) cases that are multi-drug resistant (MDR) is increasing worldwide. Although set against a background of a falling global incidence of TB, the net effect is that the number of MDR-TB cases continues to grow. [1] Improving the treatment success rate for MDR-TB requires each patient to receive an individual antimicrobial regimen tailored to maximize efficacy whilst minimizing toxicity; this necessitates being able to measure minimum inhibitory concentrations (MIC), to direct both the choice of drug and dose. Universal access to prompt and comprehensive drug susceptibility testing (DST) is therefore a key component of the WHO's *End TB Strategy.* [2,3] Although molecular approaches have the potential to deliver universal DST methods, they require further development work and any resulting solutions are likely to be expensive.

Liquid and solid media assays that measure MICs for TB exist, [4–9] but are time consuming and, often, expensive. Microtitre plates offer a way of testing in parallel the effectiveness of a large number of drugs at a range of concentrations on small aliquots from a single clinical isolate. Broth microdilution methods, including several using colorimetric indicators, have previously been developed that assess the MICs for a panel of compounds using a single microtitre plate. [5,10,11] A dry-format, 96-well microtitre plate assay (the Sensititre™ MYCOTBI plate; Thermo Fisher Scientific Inc., USA) containing 12 drugs has been commercially available since 2010 and early validation studies have returned promising results. [12–16] No plate-based assays, however, have so far included both new and re-purposed drugs that will be key to the successful treatment of individual MDR-TB cases in the future.

In this paper, we validate a bespoke, dry-form 96-well microtitre plate (UKMYC5) that has been designed by the global CRyPTIC (*Comprehensive Resistance Prediction*

for Tuberculosis: an International Consortium) project. Since the UKMYC5 plate, which contains 14 drugs, including two new compounds (delamanid and bedaquiline) and several re-purposed drugs, it therefore could form the basis of a new DST protocol for tailoring regimens to treat individual cases of MDR-TB. We shall assess, therefore, both the reproducibility of MIC measurements using this microtiter plate and its accuracy by comparing it to a range of established DST methods.

MATERIALS AND METHODS

Participating laboratories. Vials containing twenty external quality assessment (EQA) TB strains were distributed by the WHO Supranational Reference Laboratory at San Raffaele Scientific Institute (SRL), in Milan, Italy, to six other participating laboratories (Figure 1A). These were located in Germany (Institute of Microbiology and Laboratory Medicine, IML red GmbH, Gauting), UK (Public Health England Regional Centre for Mycobacteriology, Birmingham), India (P.D. Hinduja National Hospital and Medical Research Centre and Foundation for Medical Research, Mumbai), South Africa (Centre for Tuberculosis at the National Institute for Communicable Diseases, Johannesburg), Peru (Mycobacterial Laboratory, Cayetano Heredia University, Lima) and Vietnam (Oxford University Clinical Research Unit in Vietnam, Ho Chi Minh City).

***M. tuberculosis* strains.** Each participating laboratory received up to 31 culture vials of *Mycobacterium tuberculosis* (Figure 1B & S1, available as Supplementary data at JAC Online). One contained the H37Rv *Mycobacterium tuberculosis* reference strain ATCC 27294 (GenBank AL123456) [17] and was labelled as such. Eleven EQA strains were duplicated (one of which was also H37Rv) by the SRL prior to sending, along with one representative of eight additional EQA strains, bringing the total to 31 culture vials containing nineteen distinct strains. All vials, except the labelled H37Rv vial, were labelled CRY-1 to CRY-30, and therefore were blinded.

Preparation of replicates. Ten replicates were derived from the unblinded H37Rv ATCC27294 vial (Figure 1B), each being sub-cultured on solid media as described below before being subbed onto a UKMYC5 plate. Two replicates were created from each of the remaining thirty vials and, again following sub-culture, were inoculated onto a UKMYC5 plate. Each participating centre therefore tested up to 70 UKMYC5 plates (Figure 1C).

UKMYC5 design. The UKMYC5 plate was designed by the CRyPTIC consortium and manufactured by Thermo Fisher Scientific Inc., UK. Fourteen anti-TB drugs (rifampicin, rifabutin, isoniazid, ethambutol, levofloxacin, moxifloxacin, amikacin, kanamycin, ethionamide, clofazimine, para-aminosalicylic acid, linezolid, delamanid and bedaquiline) were included, at 5 to 8 doubling dilutions (Figure S1, Table S1). Janssen Pharmaceutica and Otsuka Pharmaceutical Co., Ltd provided, respectively, bedaquiline and delamanid pure substances. Although pyrazinamide-only plates containing lyophilised substance in different stocks were tested, poor performance due to suboptimal broth pH conditions resulted in pyrazinamide being excluded from UKMYC5.

Inoculation protocol. Laboratory scientists from all centres received training in plate inoculation and reading at the SRL. The standard operating procedure involved preparing a 0.5 McFarland suspension in saline tween with glass beads (Thermo Fisher, Scientific Inc., USA) from 20-25 day-old colonies (or no later than 14 days after visible growth) grown on Löwenstein-Jensen or 7H11 agar media after initial MGIT culture. Suspensions were diluted 100-fold with the addition of 100 µL of the suspension to 10 mL of enriched 7H9 broth. Aliquots of 100 µL of standard 1.5×10^5 CFU/mL inoculum (approximate range 5×10^4 – 5×10^5) were dispensed into wells by the semi-automated Sensititre™ Autoinoculator (Thermo Fisher, Scientific Inc., USA).

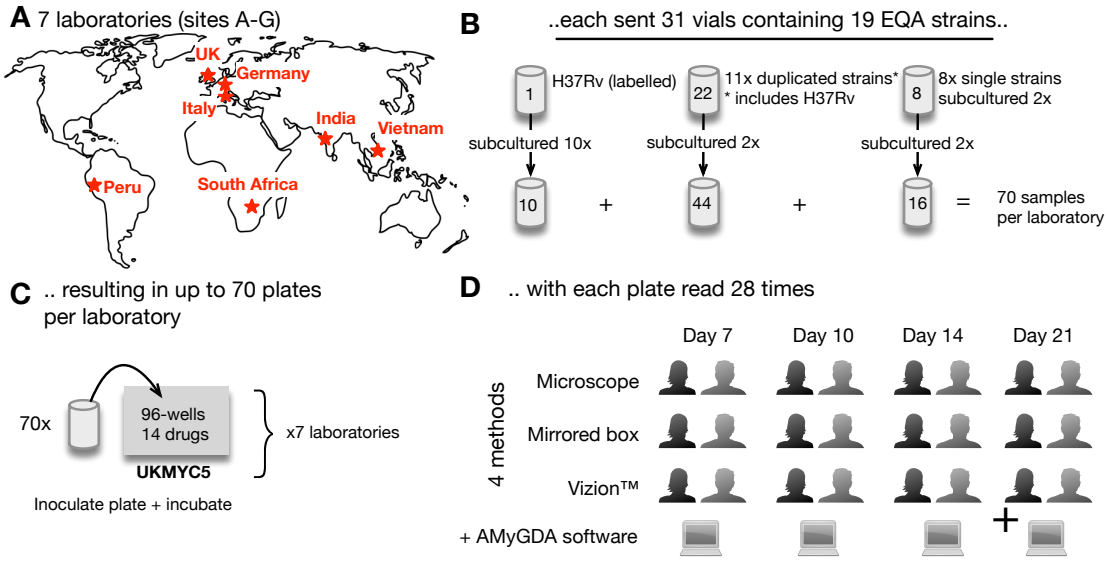

Figure 1. The study design for validating the UKMYC5 plate. (**A**) Seven laboratories (**B**) were each sent 31 vials containing 19 different WHO external quality assessment (EQA) strains. The sample of H37Rv was subcultured ten times, with all other vials sub-cultured in duplicate, making a maximum total of 70 samples. (**C**) Each was inoculated onto a UKMYC5 96-well plate. (**D**) The minimum inhibitory concentrations for each drug were independently read at 7, 10, 14 & 21 days post-inoculation by two laboratory scientists using three methods. Each plate was also photographed and the image analysed using the AMyGDA software (Figure S3).

Measurement of MICs. In each centre, two laboratory scientists independently read each microtitre plate using three different methods (Thermo Fisher Sensititre™ Vizion™ Digital MIC viewing system, a mirrored box and an inverted-light microscope) at 7, 10, 14 and 21 days post-inoculation (Figure 1D). MIC results and additional data were recorded locally onto paper and into a shared web-enabled database (Table S2). An image of each plate was captured using the Vizion™ and was stored and subsequently analysed by software, the Automated Mycobacterial Growth Detection Algorithm (AMyGDA). [18] AMyGDA analysis was performed at the University of Oxford (Figure S3).

Independent characterisation of panel strains. Each of the 19 strains used (Figure 1B) were phenotypically characterised by BACTEC™ MGIT960 (BD Lifesciences, New Jersey, USA), Middlebrook 7H10/7H11 agar dilution method (Table S3) and (with the exception of ethionamide and *para*-aminosalycilic acid) resazurin microtitre assay (REMA, Table S4) for drugs for which the WHO has endorsed critical concentrations (CCs). [19] All strains were also whole genome sequenced; genomic DNA was extracted from Löwenstein-Jensen cultures using either FastPrep24 for cell lysis and ethanol precipitation or the cetyl-trimethylammonium bromide method as described elsewhere. [20] Paired-end libraries of 101 bp were prepared using the Nextera XT DNA Sample Preparation kit (Illumina Inc., San Diego, CA, USA) and sequenced on Illumina HiSeq 2500 instruments (with the exception of one site which used NextSeq 500 instruments). A minimum genome coverage of 30x was required for SNP analysis. Variant calling in genes associated with resistance was performed by the PhyResSE web tool and the bioinformatics pipeline at the SRL [21] and the results are given in Tables S5 & S6.

Statistical analysis. Both descriptive and modeling analyses were conducted. For the latter logistic mixed-effects models were constructed since the data consist of repeated measurements. A drug on a plate is defined as readable if (i) there is acceptable growth in both the positive control wells, (ii) there is no contamination in the wells for that drug and (iii) the wells of that drug were not evaporated. Let us define two measurements as being in overall essential agreement (OEA) if the two MICs are within one doubling dilution of each other. Furthermore, the reproducibility is defined as the proportion of MICs that are within one doubling dilution of the mode. According to the International Organization for Standardization (ISO-20776-2), a clinical antimicrobial susceptibility test is required to have a reproducibility of ≥ 95 %. [22] To assess the reproducibility within a site, the mode MIC for each drug was computed for that site, pooling the results across reading methods, days, replicates

and readers. To test the reproducibility between sites, the mode was calculated only for each drug, pooling the results also across sites (besides reading methods, days, replicates and readers). We next compared UKMYC5 MICs to MICs obtained by other DST methods – this is required to be ≥ 90% according to ISO-20776-2. [22] Results were assessed in three ways: (i) the OEA between the UKMYC5 and a reference method was calculated, (ii) the categorical agreement was defined as concordant reporting of either sensitivity or resistance as defined by the critical concentration (CC) of the comparator phenotypic test (if the MIC was lower or equal to the CC the strain was defined as susceptible, otherwise it was defined as resistant). Finally, (iii) the conditional agreement was defined as resistance by the comparator method and a MIC equal to or higher than the CC on the UKMYC5, or susceptibility by the comparator method and a MIC equal to or lower than the CC plus one doubling dilution on the UKMYC5. [11]

RESULTS

The proportion of plates that can be read

The proportion of readable results (defined in the Materials and Methods) increased with elapsed time since inoculation, from 66.5% at day 7, to over 90% at day 14, to 96.8% at day 21 (Figure 2A).

For Site F the proportion of readable results was anomalously high (> 94%), regardless of reading day (Figure S4A), yet the reproducibility within the site was anomalously low, varying between 77.3%-80.2% depending on the reading day (Figure S4C). Site F also had an anomalously low between site reproducibility of only 72.2%-75.3% (Figure S4D). Logistic mixed-effect models confirmed that for Site F between reader OEA and within and between laboratory reproducibilities were all significantly lower than the other laboratories ($p<0.001$ for all comparisons; Table S7). Data from Site F was consequently excluded from subsequent analyses.

Overall within and between laboratory reproducibility.

At least 92% of all MICs read by two scientists from the same laboratory were in overall essential agreement (OEA, within a doubling dilution), regardless of reading day or method (Figure 2B). Readings within each site demonstrated at least 88% reproducibility (MIC readings within a doubling dilution of the mode, Figures 2C) for each reading-day and method, whereas between centres this was at least 85% for each reading-day and method (Figures 2D).

Selection of reading day and method.

After 7 days, only between 57.8%-66.1% of results were readable, depending on the method used, compared to over 88% at day 14, and between 95.9%-97.6% at day 21 (Figure 2A). The corresponding logistic mixed-effects model demonstrated that

significantly fewer results were readable at day 7 and 10 than at day 14, and that significantly more were readable at day 21 compared with day 14 ($p<0.001$ for all comparisons, Table S8). The proportion of results readable by inverted-light microscope or mirrored box was significantly lower than for Vizion™ ($p<0.001$ in both instances, Figure 2A, Table S8).

The OEA between two scientists examining the same plate within a laboratory increased with time (Figure 2B). For Vizion™ and mirrored box, the OEA was >95% for all reading-days, while for the inverted-light microscope it increased from 92.7% at day 7 to 96.8% at day 21 (Figure 2B). The corresponding model showed there was significantly lower OEA between readers when they used the inverted-light microscope compared to the Vizion™, or when they read the plates at day 7 compared to day 14 ($p<0.001$ for both comparisons, Table S8).

Overall, the greatest reproducibility (both within and between laboratories) was observed at day 14 (Figure 2C & 2D), with both Vizion™ and mirrored box being 95% and 92% reproducible within and between sites, respectively. For the inverted-light microscope, the corresponding reproducibilities were 92.9% and 89.9%, respectively. The logistic mixed-effects models confirmed that within and between site reproducibility for the inverted-light microscope was overall significantly lower with respect to Vizion™ ($p<0.001$ for all, Table S9). The Sensititre™ Vizion™ Digital MIC Viewing System was therefore selected for all subsequent analyses, although we note that the mirrored box also performed well.

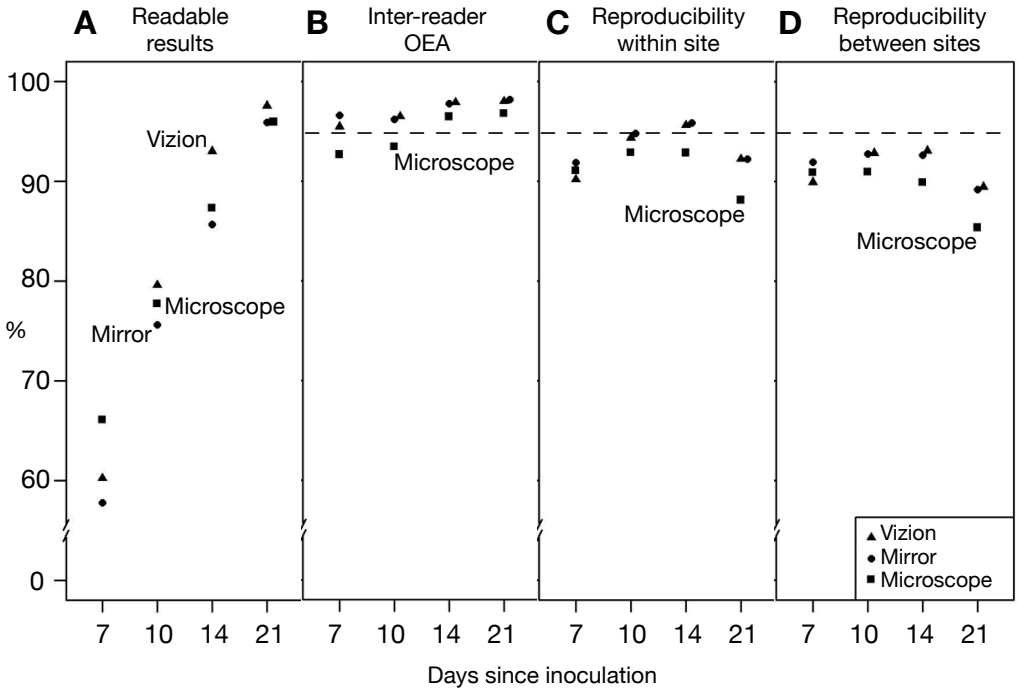

Figure 2. For each reading method, (**A**) the percentage of readable results, (**B**) the Overall Essential Agreement (OEA) between two readers, and the reproducibility (**C**) within and (**D**) between sites. The dashed line is at 95%, which is the level of reproducibility required for an antimicrobial susceptibility test device to conform to ISO-20776-2. [22]

The individual MICs measured using Vizion™ were then analyzed to establish the optimal reading-day for each drug (Figure S5). Para-aminosalicylic acid showed the lowest OEA between readers at each reading day and the lowest reproducibility both within and between sites at days 10, 14 and 21. The results of the corresponding logistic mixed-effects models (Table S10) showed that, overall, readings had a significantly lower probability of being reproducible within and between sites at days 7 and 21, than at day 14 ($p=0.001$ and $p=0.004$, respectively for day 7, and $p=0.001$ and $p<0.001$ respectively for day 21). Reproducibility was similar at days 10 and 14 ($p=0.465$ and $p=0.784$, respectively). Because fewer results were readable after 10 days, reading day 14 was preferred and was used in subsequent analyses.

MICs read using the combination of Vizion™ and UKMYC5 plate at day 14 are ≥ 95% reproducible for the same plate read by different readers or different plates, both at the same laboratory, but the reproducibility between sites (i.e. same strain, but different plate, reader and laboratory) is 92%, which is below the required threshold of 95%, although this is a very stringent test. [22]

Reproducibility was assessed also on an individual drug basis by using rifampicin as a reference standard (Figure S5 & Table S10). Although there was a statistically significant difference between many drugs, the greatest difference was seen for para-aminosalicylic acid where the reproducibilities within and between sites were significantly lower than rifampicin ($p<0.001$).

Results with AMyDGA software

MICs measured from photographs of the UKMYC5 plates using the AMyGDA software [18] were compared with those based on the Vizion™ reading method. The OEA between the two methods was above 87% for all days and increases with time since inoculation (from 87.9% at day 7, up to 93.8% at day 21, Figure 3). At day 14, the OEA between readings by AMyGDA and Vizion™ is above 90% for all drugs, except for moxifloxacin (89.3%) and para-aminosalicylic acid (73.8%), and therefore satisfies ISO-20776-2. [22] Using a logistic mixed-effect model, we found that the overall OEA at days 7, 10 and 21 is not significantly different from the overall OEA at day 14 ($p=0.143$, $p=0.479$ and $p=0.525$, respectively, Table S11). The OEA between Vizion and AMyGDA was not significantly different for all drugs at day 14 (Table S11).

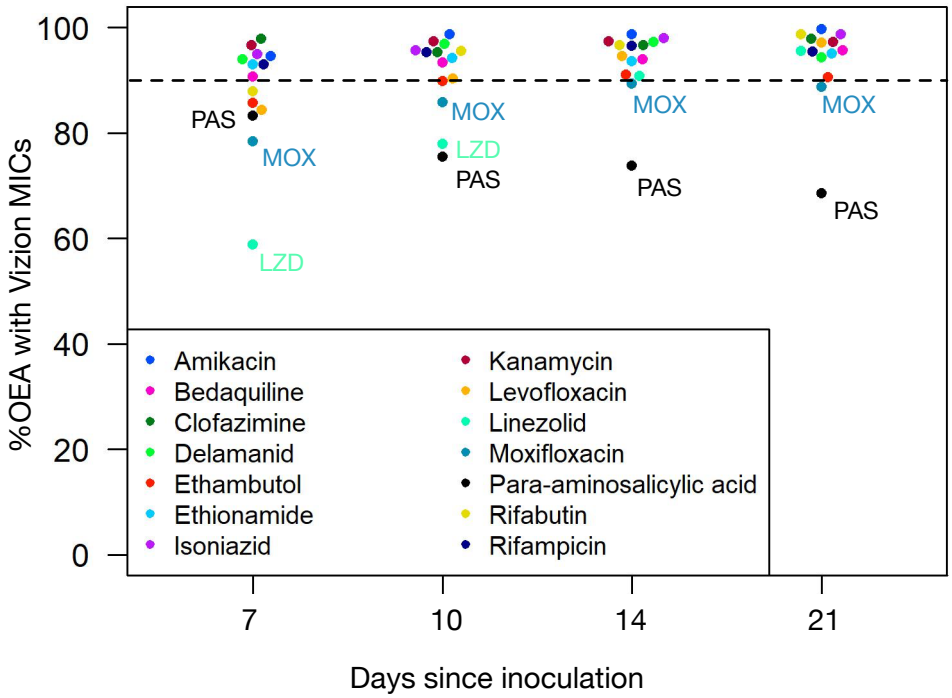

Figure 3. The proportion of Overall Essential Agreement (OEA) between MICs read by Vizion™ and determined by the AMyGDA software. The dashed line at 90% indicates the minimum threshold for an antimicrobial susceptibility test to be considered accurate by ISO-20776-2. [22]

MIC distributions for H37Rv and agreement with reference standards. MICs measured by Vizion™ at day 14 for H37Rv were compared with MICs obtained by the agar proportion method (APM) and REMA [16,23] For most of the drugs, the MIC values obtained by the APM were almost always either identical or within one doubling dilution of UKMYC5 readings, indicating a good correlation between the two methods (Figure 4). Similar results were obtained for the comparison with MICs obtained by REMA. MICs for bedaquiline, isoniazid, clofazimine, rifampicin, levofloxacin, moxifloxacin, amikacin, linezolid, ethambutol and kanamycin were

compared to results from frozen-form microtitre plates. [16] Overall essential agreement was 94.6% at day 14, dropping to 90.5% at day 21 (Figure 4 & S6), both of which are above the 90% threshold for comparison to a reference standard as specified by ISO-20776-2. [22]

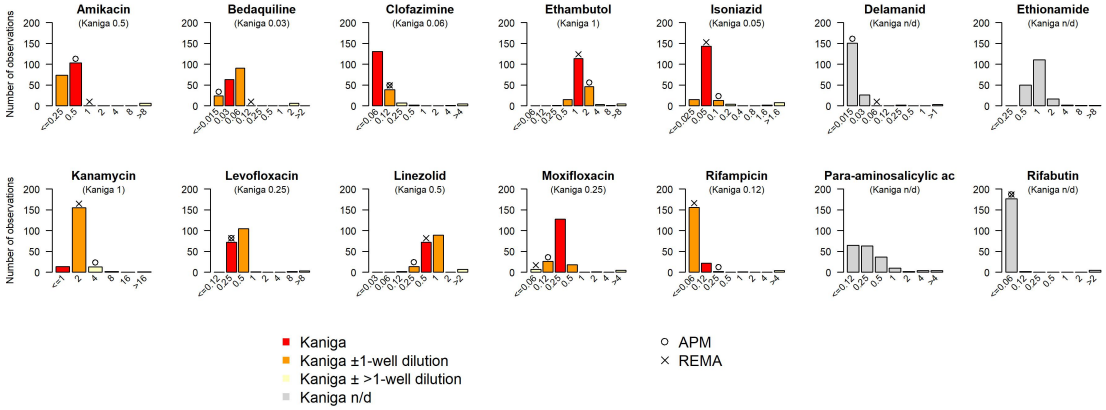

Figure 4. Minimum Inhibitory Concentration (MIC) distributions for H37Rv, as measured at day 14 by Vizion™ and compared with frozen-form microtitre assay MICs. Red bars correspond to identical frozen-form assay and UKMYC5 values; orange bars correspond to frozen-form microtitre values falling within one dilution from UKMYC5's; yellow bars correspond to frozen-form microtitre values falling beyond one dilution from UKMYC5's. Grey bars indicate that the drug is not present on the frozen plates.

For all strains, the mode of UKMYC5 MICs was calculated by drug and it was compared with the categorical results (susceptible or resistant) obtained from MGIT and APM, for drugs with established breakpoints (Figure 5) [13,19,24]. Categorical and conditional agreement between UKMYC5 MICs and those obtained from MGIT and the APM were also computed (Table S12). To infer 'sensitivity' or 'resistance' using the UKMYC5 plate, we assumed shared breakpoints with each comparator method (APM or MGIT). Discrepancies are shown in Table S13.

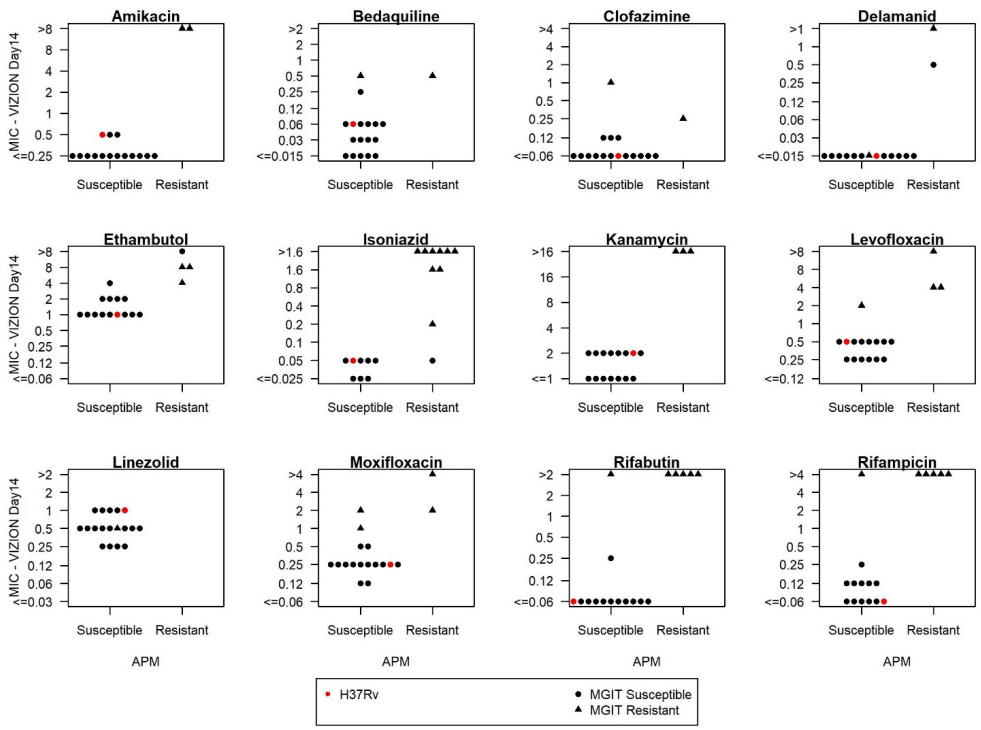

Figure 5. UKMYC5 Vizion™ MICs Day 14 versus AGAR and MGIT results for all tested strains. Filled red circles indicate the mode MIC of H37Rv ATCC 27294.

Comparison between genotype and UKMYC5 results

For all drugs, the presence of mutations in genes in which there is a high confidence that they confer resistance was associated with MICs greater than observed in wild type strains (Figure 6). [25] Mutations whose role in the drug resistance was not well established in the literature were included in the analysis. Only bedaquiline, ethambutol, linezolid and the fluoroquinolones showed some overlap in MICs between wildtype strains and strains containing mutations in genes associated with resistance. For linezolid, no difference of MICs was observed between wild type and mutant strains, but this was expected since these mutations were shown to be susceptible by MGIT and APM (Table S6), although mutations in the *rrL* gene have been reported to shift MICs. [26] As expected, mutations associated with low-level resistance, such as *inhA*_C-15T, were associated with smaller MIC increases than mutations in *katG* that is associated with high-level resistance (Figure S7). [27]

Interestingly, the EQA panel included two isolates that were resistant to delamanid according to the APM. One of these had a mutation in the *ddn* gene, and the other, in *Rv1173*. The *ddn* mutation was associated with higher MICs than the *Rv1173* mutation (Figure S7).

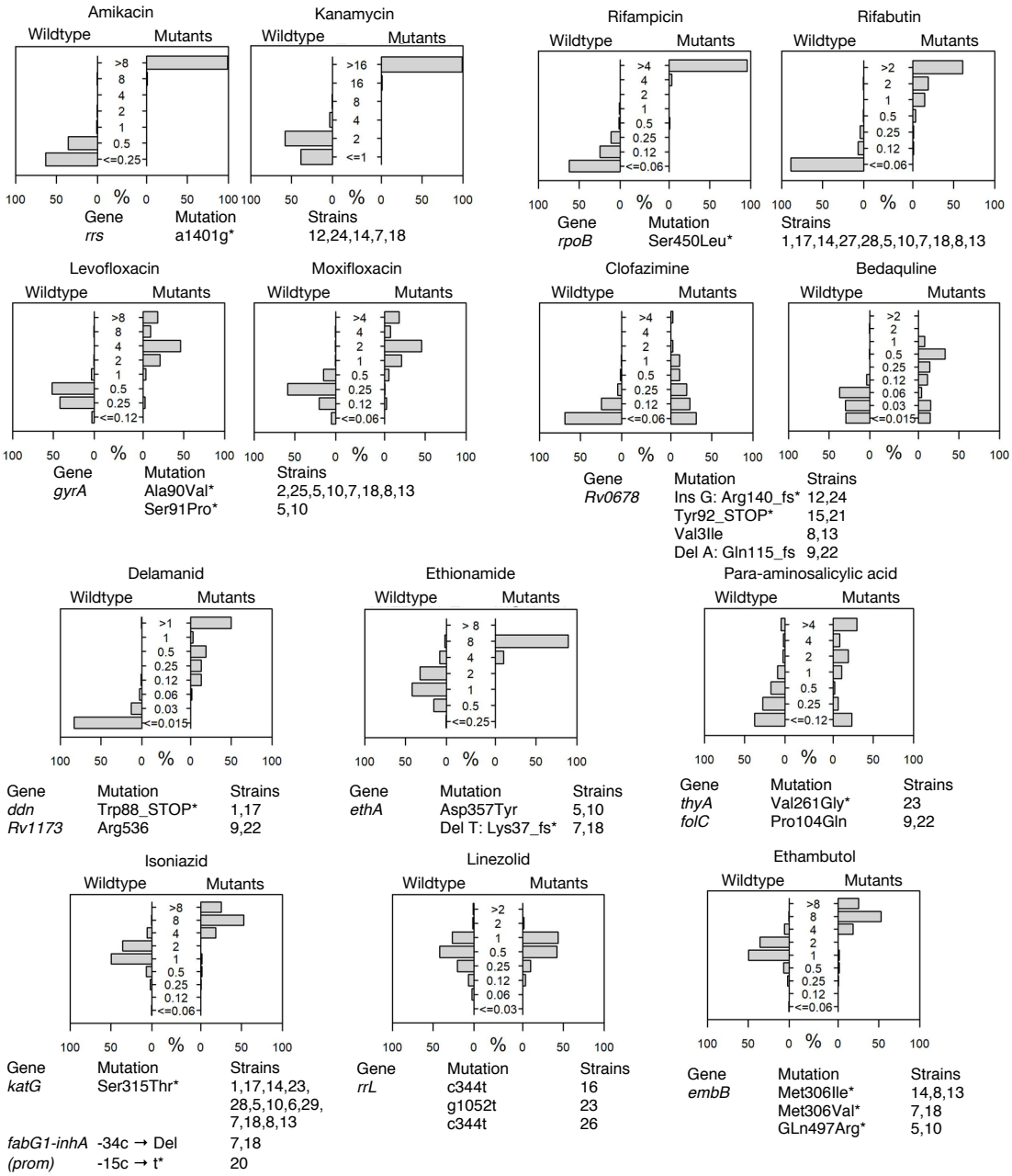

Figure 6. The MIC distributions for each drug, split by whether the resistance genes are wild-type, or contain mutations. Mutations that confer resistance with a high degree of confidence [25] are marked with an asterisk**.** Drugs that share the same resistance genes are paired.

DISCUSSION

Broth-based microdilution methods for TB DST have been proposed in the past that either directly [8] or indirectly [5,10,11] measure mycobacterial growth at a range of drug concentrations, potentially enabling the determination of MIC values. In 2011, the WHO examined whether non-commercial microdilution assays would be suitable to screen patients at risk of MDR-TB and conditionally recommended their use [28]. Subsequent studies assessed their performance [12–14] and established MIC distributions for the H37Rv reference strain, [15,16] demonstrating a good correlation with reference methods.

This validation study moves the field forward: UKMYC5 is the first microtitre plate assay to incorporate both new (delamanid, bedaquiline) and repurposed drugs (linezolid and clofazimine) in a dry-well format that is convenient to transport and store. In this paper, we have demonstrated that the measurement of MICS within and between participating laboratories is reproducible for 13 of the 14 drugs on the plate. Fourteen days after inoculation was shown to be the optimal time to read a plate and the Thermo Fisher Sensititre™ Vizion™ Digital MIC viewing system was selected as the optimal reading method. We note, however, that the mirrored box performed equally well and, since it is cheaper, is likely to be a better solution in low-income countries. The Vizion™, however, also allowed us to photograph each plate, thereby not only providing an audit trail but also enabling the use of the AMyGDA software.

By using a well characterised set of nineteen EQA strains we have been able to determine the conditional agreement with results from alternative MIC-determining DST assays. Assuming that the critical concentrations (CC) for the UKMYC5 was identical to each comparator method allowed the categorical agreement with these assays to also be assessed. In all cases, cross-comparisons demonstrated encouraging consistency. Furthermore, mutations in genes that are known to confer

resistance had a clear impact on MIC values. Taking these results together, we conclude that that the UKMYC5 plate has the potential not only to be used as a research tool but also as a clinical diagnostic assay.

The determination of MIC values for a range of drugs allows therapeutic decisions to be more nuanced since they can be guided by the degree of resistance to a drug and an understanding of the tolerability of drug doses required to overcome it. Unlike CC-based DST methods, where all errors are categorical in nature, MIC errors can be marginal, and thereby may be potentially less disruptive to treatment decisions. In addition to these advantages, the UKMYC5 plate assays 14 drugs at once, at low cost, a clear advantage over MGIT and the APM. Including both new and re-purposed drugs is a clear advantage over other microtitre plate-based quantitative DST methods.

As different drugs are available in different countries, one should be able to modify the design of the microtitre plate to reflect what drugs are locally available, or locally recommended regimens. It is therefore key that whilst the ranges of MICs is standardised, the combination of drugs included on the plate can be adjusted according to need. The challenge to regulators is therefore whether the performance of the plate can be accredited for a menu of *individual* drugs from which country-specific plates can be constructed, rather than a fixed plate layout.

Since the increasing global numbers of MDR-TB cases are a major obstacle to TB control, let alone its elimination, there is growing need for a clinical assay that can provide quantitative data on the second-line, new and repurposed drugs clinicians will have to prescribe with increasing frequency in the future. The UKMYC5 plate has the potential to guide the appropriate treatment of MDR-TB either directly, through implementation in clinical microbiology laboratories. Or indirectly, either by producing

the data necessary to characterise the effect of individual genomic mutations on drug resistance in detail or by providing detailed and timely surveillance of the prevalence of different strains by country.

Although the MICs measured using the UKMYC5 plate were shown to be reproducible between participating centres (as defined by ISO-20776-2 [22]) the readings from one laboratory were excluded from our analysis due to their relatively poor reproducibility. A retrospective analysis of the plate images recorded by Vizion™ revealed that readers at Site F had frequently mistaken sediment from the inoculum with bacterial growth, thereby allowing us to offer targetted training to the laboratory scientists involved. This process highlights the importance of storing images of all the plates as part of an audit trail. As these images can be read automatically by the AMyGDA software, we are evaluating whether incorporating the AMyGDA software within our standard operating procedure will allow us to detect significant differences in plate interpretation much earlier.

The UKMYC5 plate does have several limitations; these include the need for a pre-culture step, entailing a delay of up to six weeks before the plate is inoculated. This currently prevents the rapid turn-around of results, however, we expect this time could be reduced significantly by further development to define the optimal inoculum from MGIT culture. Given the notable advantages inherent in this microtitre plate, such further development work should clearly be pursued. The UKMYC5 plate would also benefit from a minor re-design; PAS, which performed poorly, should be excluded and the vacated wells used to expand the range of concentrations for drugs where measurements were most frequently reported at the extremes of the dilution range.

How the UKMYC5 microtitre plate could be optimally incorporated in a clinical laboratory's workflow remains to be determined, but the numerous advantages it offers suggests its adoption should be pursued with vigour. The Foundation for Innovative and New Diagnostics (FIND) have already expressed interest in pursuing endorsement by the WHO (personal communication, Claudia Denkinger). In parallel, its impact on our understanding of the effect of genomic mutations on the MICs of various drugs is likely to inform the WHO's planned assessment of WGS-based DST in 2018. [1] Whether it be through direct measurement of MICs, or predictions of MIC from genomic data, a new era of quantitative TB DST may be with us.

ACKNOWLEDGMENTS

We acknowledge the CRyPTIC members for their hard work which made this study possible, and thank the following collaborators for their crucial roles: Matteo Chiachiaretta, Marco Rossi and Giovanna Graziano from the SRL Milano for the organisation of the training sessions in Milan; Meera Gurumurthy (The Union) for the training session in Beijing; Hien Ho Van and Tien Nguyen Thanh (Oxford University Clinical Research Unit, Ho Chi Minh City) for the development and maintenance of the CRyPTIC database; Koné Kaniga (Janssen Ltd) and Yongge Liu (Otsuka Pharmaceutical) for making available bedaquiline and delamanid powder, respectively; Thermo Fisher Scientific Inc, for the manufacture of the microtitre plate assays.

Members of the CRyPTIC consortium

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FUNDING

The research was funded by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre; the CRyPTIC consortium which is funded by a Wellcome Trust/Newton Fund-MRC Collaborative Award [200205/Z/15/Z] and the Bill & Melinda Gates Foundation Trust [OPP1133541]. T.E.A.P. and D.W.C. are affiliated to the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Healthcare Associated Infections and Antimicrobial Resistance at University of Oxford in partnership with Public Health England (PHE). T.M.W. is an NIHR Academic Clinical Lecturer. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England.

TRANSPARENCY DECLARATIONS

None to declare

SUPPLEMENTARY DATA

Figures S1 to S7 and Tables S1 to S13.

REFERENCES

1. World Health Organisation. *Global Tuberculosis Report*. 2017.
2. World Health Organisation. *The End TB Strategy*. 2015.
3. Sotgiu G, Tiberi S, Centis R, *et al.* Applicability of the shorter 'Bangladesh regimen' in high multidrug-resistant tuberculosis settings. *Int J Infect Dis* 2017; **56**: 190–3.
4. Schaberg T, Reichert B, Schülin T, Lode H, Mauch H. Rapid drug susceptibility testing of Mycobacterium tuberculosis using conventional solid media. *Eur Respir J* 1995; **8**: 1688–93.
5. Franzblau SG, Witzig RS, Mclaughlin JC, *et al.* Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. *J Clin Microbiol* 1998; **36**: 362–6.
6. Goloubeva V, Lecocq M, Lassowsky P, Matthys F, Portaels F, Bastian I. Evaluation of Mycobacteria Growth Indicator tube for direct and indirect drug susceptibility testing of Mycobacterium tuberculosis from respiratory specimens in a Siberian prison hospital. *J Clin Microbiol* 2001; **39**: 1501–5.
7. Clinical and Laboratory Standards Institute. *M24 - Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes, 2nd edition*. Wayne, PA, USA; 2011.
8. Moore DAJ, Evans CAW, Gilman RH, *et al.* Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *New Eng J Med* 2006; **355**: 1539–50.
9. Rodrigues C, Jani J, Shenai S. Thakkar P, Siddiqi S, Mehta A. Drug susceptibility testing of Mycobacterium tuberculosis against second-line drugs using the Bactec MGIT 960 System. *Int J Tuberc Lung Dis* 2008; **12**: 1449–55.
10. Yajko LdM, Madej JJ, Lancaster M V, *et al.* Colorimetric Method for Determining MICs of Antimicrobial Agents for Mycobacterium tuberculosis. *J Clin Microbiol* 1995; **33**: 2324–7.
11. Leite CQ, Beretta AL, Anno IS, Telles MA. Standardization of broth microdilution method for Mycobacterium tuberculosis. *Mem Inst Oswaldo Cruz* 2000; **95**: 127–9.
12. Abuali MM, Katariwala R, LaBombardi VJ. A comparison of the Sensititre® MYCOTB panel and the agar proportion method for the susceptibility testing of Mycobacterium tuberculosis. *Eur J Clin Micro Infect Dis* 2012; **31**: 835–9.
13. Lee J, Armstrong DT, Ssengooba W, *et al.* Sensititre MYCOTB MIC plate for testing mycobacterium tuberculosis susceptibility to first-And second-Line drugs. *Antimicrob Agent Chemo* 2014; **58**: 11–8.
14. Xia H, Zheng Y, Zhao B, Van Den Hof S, Cobelens F, Zhao Y. Assessment of a 96-well plate assay of quantitative drug susceptibility testing for mycobacterium

tuberculosis complex in China. *PLoS One* 2017; **12**: 1–12.
15. Kaniga K, Cirillo DM, Hoffner S, *et al.* A Multi-Laboratory, Multi-Country Study to Determine Bedaquiline Minimal Inhibitory Concentration Quality Control Ranges for Phenotypic Drug-Susceptibility Testing. *J Clin Microbiol* 2016; **54**: JCM.01123-16.
16. Kaniga K, Cirillo DM, Hoffner S, *et al.* A Multilaboratory, Multicountry Study To Determine MIC Quality Control Ranges for Phenotypic Drug Susceptibility Testing of Selected First-Line Antituberculosis Drugs, Second-Line Injectables, Fluoroquinolones, Clofazimine, and Linezolid Land GA, ed. *J Clin Microbiol* 2016; **54**: 2963–8.
17. Kubica GP, Kim TH, Dunbar FP. Designation of Strain H37Rv as the Neotype of Mycobacterium tuberculosis. *Int J Syst Bacteriol* 1972; **22**: 99–106.
18. Fowler PW, Luíza A, Cruz G, *et al.* Automated detection of Mycobacterial growth on 96-well plates for rapid and accurate Tuberculosis drug susceptibility testing . 2017. Available at: http://dx.doi.org/10.1101/229427.
19. World Health Organization. *Updated interim critical concentrations for first-line and second-line DST*. 2012.
20. Van Soolingen D, Hermans PWM, De Haas PEW, Soll DR, Van Embden JDA. Occurrence and stability of insertion sequences in Mycobacterium tuberculosis complex strains: Evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol* 1991; **29**: 2578–86.
21. Feuerriegel S, Schleusener V, Beckert P, *et al.* PhyResSE: A web tool delineating Mycobacterium tuberculosis antibiotic resistance and lineage from whole-genome sequencing data. *J Clin Microbiol* 2015; **53**: 1908–14.
22. International Organization for Standardization. *ISO 20776-2: Clinical laboratory testing and in vitro diagnostic test systems*. 2007.
23. Schena E, Nedialkova L, Borroni E, *et al.* Delamanid susceptibility testing of Mycobacterium tuberculosis using the resazurin microtitre assay and the BACTEC™ MGIT™ 960 system. *J Antimicrob Chem* 2016; **71**: 1532–9.
24. The European Committee on Antimicrobial Susceptibility Testing. *Clinical breakpoints - bacteria (v7.1)*. 2017.
25. Miotto P, Tessema B, Tagliani E. A standardised method for interpreting the association between mutations and phenotypic drug resistance in Mycobacterium tuberculosis. *Eur Respir J* 2017.
26. Zhang S, Chen J, Cui P, *et al.* Mycobacterium tuberculosis mutations associated with reduced susceptibility to Linezolid. *Antimicrob Agents Chemother* 2016; **60**: 2542–4.
27. Brossier F, Veziris N, Jarlier V, Sougakoff W. Performance of MTBDR plus for

detecting high/low levels of Mycobacterium tuberculosis resistance to isoniazid. *Int J Tuberc Lung Dis* 2009; **13**: 260–5.
28. World Health Organization. *Noncommercial culture and drug-susceptibility testing methods for screening patients at risk for multidrug-resistant tuberculosis: Policy statement*. 2011.