



Early View

Task force report

Epidemiological cutoff values for a 96-well broth microdilution plate for high-throughput research antibiotic susceptibility testing of *M. tuberculosis*

The CRyPTIC Consortium

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Epidemiological cutoff values for a 96-well broth microdilution plate for high- throughput research antibiotic susceptibility testing of *M. tuberculosis*

The CRyPTIC Consortium¹

Abstract

Drug susceptibility testing of *M. tuberculosis* is rooted in a binary susceptible/resistant paradigm. Whilst there are considerable advantages in measuring the minimum inhibitory concentrations (MICs) of a panel of drugs for an isolate it is necessary to measure the epidemiological cutoff values (ECOFF/ECVs) to permit comparison with qualitative data. Here we present ECOFF/ECVs for 13 anti-TB compounds, including bedaquiline and delamanid, derived from 20,637 clinical isolates collected by 14 laboratories based in 11 countries on five continents. Each isolate was incubated for 14 days on a dry 96-well broth microdilution plate and then read. Resistance to most of the drugs due to prior exposure is expected and the MIC distributions for many of the compounds are complex and therefore a phenotypically wild-type population could not be defined. Since a majority of samples also underwent genetic sequencing, we defined a genotypically wild-type population and measured the MIC of the 99th percentile by direct measurement and via fitting a Gaussian using interval regression. The proposed ECOFF/ECV values were then validated by comparing to the MIC distributions of high-confidence genetic variants that confer resistance and to qualitative drug susceptibility tests obtained via Mycobacterial Growth Indicator Tube and the Microscopic-Observation Drug-Susceptibility assay. These ECOFF/ECV values will inform and encourage the more widespread adoption of broth

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microdilution – this is a cheap culture-based method that tests the susceptibility of 12-14 antibiotics on a single 96-well plate and so could help personalise the treatment of tuberculosis.

INTRODUCTION

Mycobacterium tuberculosis kills more people worldwide than any other single pathogen, SARS-CoV-2 excepted (1). Despite its impact on global health, antibiotic susceptibility testing (AST) for *M. tuberculosis*, has lagged behind other bacterial diseases due to its slow growth rate, difficulty in culturing and its low prevalence in high-income countries. The consequence is that most patients in the world receive empiric, or semi-empiric, treatment, which reduces the chance of treatment success and risks the amplification of resistance where too few effective drugs are prescribed.

The dramatic reduction in genetic sequencing costs has enabled genetics-based AST where the genome of a pathogen is sequenced and then examined for known variants that confer resistance to specific antibiotics. *M. tuberculosis* is well-suited to this approach (2–10), and several public health bodies have adopted whole genome sequencing as their standard AST method (11). Although PCR platforms can deliver universal antibiotic susceptibility testing in its narrowly defined sense (12), genome sequencing is the only approach that can realistically deliver comprehensive AST in settings where phenotyping remains too expensive and too infrastructure dependent, and comprehensive AST is the only way to optimise treatment regimens and outcomes.

The Comprehensive Research Prediction for Tuberculosis: an International Consortium (CRyPTIC) research project has collected 20,637 clinical *M. tuberculosis* samples from across the world. The primary aim of the project is to identify mutations in the *M. tuberculosis* genome that confer phenotypic resistance to a wide range of antibiotics. The CRyPTIC project measured minimum inhibitory concentrations (MIC) of each drug to permit quantitative analyses, associating mutations with MIC values with a view to using genome sequencing data to personalise drug regimens and doses. From the start the CRyPTIC project has taken a data-driven approach whereby all analyses are algorithmic, hence the allocation of a sample to subgroup requires little or no expert, and hence subjective, intervention. This has the virtue of ensuring the results are reproducible.

The most practical and affordable means of determining MICs at scale was to use a pre-prepared 96-well 7H9 broth microdilution plate based on the Thermo Fischer Sensititre MYCOTB MIC plate (13–18), but including the new or repurposed antibiotics that feature in current WHO

guidance (19). The CRyPTIC project designed a variant of the MYCOTB plate, called UKMYC5, that contains fourteen antibiotics, including bedaquiline, delamanid, clofazimine and linezolid but not pyrazinamide (Fig. 1A). Based on a multi-laboratory study that examined the inter- and intra-laboratory reproducibility of the UKMYC5 plate and determined the optimum reading methods and incubation period (17), CRyPTIC subsequently modified the design by removing para-aminosalicylic acid and extending/changing the concentration of certain drugs, leading to the 13-drug UKMYC6 plate (Fig. 1B).

In this paper we propose epidemiological cut-off values (ECVs or ECOFFs) for the UKMYC series of plates to enable subsequent research on this dataset. The ECOFF is the highest MIC observed within a phenotypically wild-type population, usually defined as the MIC which encompasses 99% of that population (20), and allows interpretation of an MIC value as 'susceptible or 'resistant' – crucial to the decision on whether to prescribe a drug. The standard approach requires uncensored MICs and assumes that the phenotypic wild-type population can be readily identified, either because the population has been minimally exposed to the drug, or because the MIC distribution is strongly bimodal. These conditions are not universally met in our dataset and we shall therefore identify a *genotypically* wild-type population from which we can either measure the ECOFF/ECV directly or via a Gaussian fitted using interval regression, a statistical technique that can fit to censored data. We have made Python code publicly available that enables anyone to reproduce most of the figures and tables in a web browser window (21). Although ECOFF/ECVs have been proposed for the MYCOTB microdilution plate using 385 strains from South Africa (22), we are here able to draw upon a far larger and more geographically diverse *M. tuberculosis* dataset.

RESULTS

AST was performed on 20,637 isolates to 13 anti-TB drugs using either the UKMYC6 (12,672, 61%) or the UKMYC5 (7,965, 39%) plate design (Table 1, Fig. 1A & B). These data were generated in fourteen CRyPTIC laboratories based in eleven countries on five continents (Fig. 1C, Table S1). The isolates themselves were collected from 27 countries, with 19 countries contributing ten or more, and 15 countries contributing 100 or more isolates (Table S2). Due to differences between the laboratories, it was not possible to collect clinical outcome data for the samples. Quality control processes detected that one laboratory developed a problem inoculating the plates – these plates were removed – and that another laboratory never managed to inoculate successfully: all their plates were excluded. Excluded these left 17,054 plates.

Of these 12,362 also had their whole genome sequenced (Methods, Table 1), allowing us to infer species and lineage information using SNP-IT (23). All isolates belonged to the *Mycobacterium tuberculosis* complex (MBTC), with the majority (12,348, 99.9%) confirmed as *M. tuberculosis* (Table S3), of which the majority belonged to either Lineage 2 (35%) or 4 (50%, Table S4) with the expected geographic distribution (Table S5, Fig. S1) (24).

Table 1. The number of isolates collected, split by the two microtitre plate designs used. An asterisk indicates that this is the average number of plates across all drugs. This table can be reproduced (21).

	Total	UKMYC6	UKMYC5
Isolates collected	20,637	12,672	7,965
Readable plates	17,054	10,010	7,044
Readable plates with images	15,138	9,272	5,866
Readable plates with genetics	12,362	6,019	6,343
Readable plates with genetics and images	10,938	5,552	5,386
Readable plates with images and passing quality assurance	*11,801	*6,896	*4,904
Readable plates with genetics, images and passing quality assurance	*8,553	*4,027	*4,526
Readable plates with genetic, images, passing quality assurance and genotypically wild-type	*3,328	*1,606	*1,722

As expected, the MIC histograms differ between drugs (Fig. 2, S5, S6); the MICs for some compounds form bimodal distributions (INH, KAN, AMI, RIF, RFB) and therefore conform to the classical binary paradigm whereby an isolate is either 'resistant' or 'susceptible'. CRYPTIC aimed for half the isolates collected to be multi-drug resistant (MDR) and the MIC histograms for isoniazid and rifampicin are consistent with this. Given this bias towards MDR in the dataset, one would expect appreciable resistance to ethambutol, ethionamide, both fluoroquinolones and both aminoglycosides. Both drugs belonging to the latter class indeed have a subset of isolates with very high MICs. The MIC histograms for the remaining compounds (EMB, ETH, MXF, LEV) are not bimodal hence it is unclear whether they can be adequately described by two log-normal distributions. Since the remaining drugs on the plates (BDQ, DLM, CFZ, LZD) have not yet been widely used, and for some countries were not even available to treat tuberculosis, one expects little resistance in the dataset and hence it is likely these MIC histograms are 'phenotypically wild-type' (pWT). All the MIC histograms are truncated/censored at either one or both ends, and some are severely truncated with the mode MIC occurring in the lowest dilution (AMI, RFB, DLM). Our large dataset allows us to use reproducible, algorithmic approaches for estimating the 99th percentile of the wild-type population.

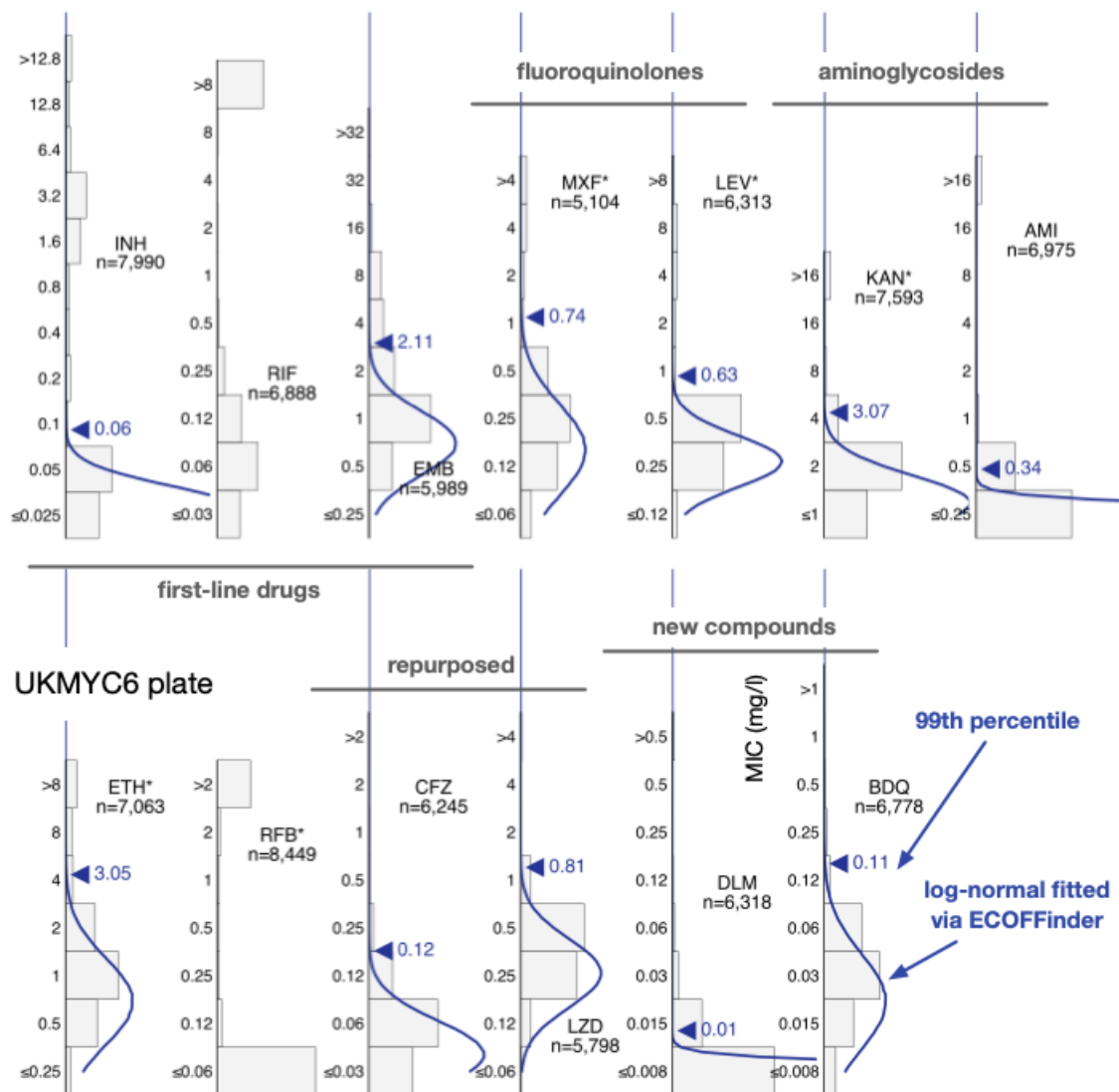


Figure 2. The MIC histograms for the 13 antibiotics on the UKMYC6 plate. Only MICs which have passed the quality assurance process described in the Methods are shown. ECOFFinder was used to fit a log-normal distribution to each histogram; this is drawn in blue and the resulting 99th percentile is labelled. ECOFFinder was unable to fit a log-normal to both rifampicin (RIF) and rifabutin (RFB). See Fig. S6 for the UKYMYC5 histograms and the Supplemental Information for the numerical data. The histograms can be reproduced online (21).

Iteratively fitting a log-normal distribution

ECOFFinder is a heuristic approach that attempts to iteratively fit a log-normal distribution to the MIC histogram and is recommended by both EUCAST (20, 25) and the CLSI. EUCAST advise that ECOFFinder should not be applied to truncated data, but here we apply it to demonstrate how it performs for different levels of censored data. Distributions derived using ECOFFinder (Fig. 2, S6 & Table 2) describe our data well where the MIC histogram is minimally truncated (ETH, LZD, BDQ), however where the MIC histogram is heavily truncated (AMI, RFB, DLM) the resulting log-normal distribution does not fit the MIC histogram, and where the mode MIC is

resistant (RIF) it fails to perform a fit at all. In addition, since ECOFFinder requires a single consistent MIC distribution and our dataset is composed of two plate designs, two ECOFF/ECVs are returned for each drug. For many drugs these are very similar but for ethambutol and delamanid the estimates are almost a doubling dilution different.

To overcome the problem that our MIC histograms are truncated we applied *interval regression* – an established statistical method for fitting normal distributions to truncated data (26, 27) which, unlike conventional maximum-likelihood algorithms, takes into account that observations are properly represented by *intervals*. The entire dataset containing measurements from both plate designs can then be considered simultaneously, resulting in a single pair of log-normal distributions that describe the MIC histograms on both plate designs (Fig. S7). The model fails to converge for kanamycin and ethambutol and for several drugs the second distribution has a variance much larger than the MIC range which is nonsensical (AMI, ETH, RFB, CFZ, LZD, DLM, BDQ), although for the new- and repurposed compounds this is understandable since we do not expect many resistant isolates. Where the two distributions describe the data reasonably well (INH, RIF, MXF, LEV), they are well-separated, as defined by the 99th percentile of the lower distribution (ECOFF/ECV) being smaller than the 1st percentile of the upper distribution (the non-wild-type cut-off value, NCOFF), with the exception of isoniazid where NCOFF < ECOFF.

Defining a *genotypically wild-type* population

Using these approaches we were not able to produce acceptable results when the MIC histogram is truncated and/or is not clearly bimodal. In the latter case it is probable that the overall MIC histogram is a convolution of several smaller, narrower distributions. Genetics offers a way to disentangle these sub-populations: one can predict genetically the susceptibility of strains to most, but not all, of the 13 anti-TB compounds of interest (6–8). Note that we were unable to use the newer and more comprehensive genetic catalogue released by the WHO since its derivation set included these samples (9, 10). We predicted the antibiogram for the first-line (INH, RIF, EMB and also PZA – see below) and second-line (AMI, KAN, LEV, MXF, ETH) compounds (Methods). No predictions were made for the other anti-TB compounds on the plate since the association between genetics and their resistance is poorly understood at present.

We defined an isolate as being *genotypically wild-type* (gWT) if it is predicted to be susceptible to the four first-line compounds and not resistant to the second-line compounds (see ref (6) for the distinction). The laxer criterion for the second-line compounds allowed for the fact that our understanding for these drugs is less complete. Epidemiologically, it is the case that if an isolate is susceptible to all four first-line antibiotics, it is also likely to be susceptible to second-line antibiotics (except perhaps for prior fluoroquinolone exposure or deeply rooted second-line resistance mutations). To contribute a laboratory had to have collected susceptible samples

which had undergone whole genome sequencing and also had a high quality photograph taken of the UKMYC plate after 14 days incubation so we could run the QA process. As a result isolates from only nine CRyPTIC laboratories made up this dataset (Table S8) and the number of confirmed MICs varied between 2,594 and 4,078 by drug, with a mean of 3,263 (Table S9). Visually, the resulting MIC histograms are simpler and more likely to be adequately described by a single log-normal distribution (Fig. S8).

Directly measuring the ECOFF/ECV from the gWT population

Directly determining the MIC of the 99th percentile from the gWT wild-type population is an attractive option since it requires no further assumptions. This is not usually possible since typically either one cannot discern the wild-type population and/or there are an insufficient number of isolates. The large size of our dataset and the inclusion of genetic information enables us to directly measure the ECOFF/ECV (Fig. 3, S9). Our dataset is enriched for resistance, hence the proportion of resistant samples misclassified as susceptible due to sample mislabelling is likely to be of the order of a few percentage points, even after we have removed some putative mislabelled samples (Methods). This makes directly identifying the 99th percentile challenging, hence we shall also consider the 97.5th and 95th percentiles.

All three percentiles for the MIC histograms of the gWT population are at most two doubling dilutions apart, except for levofloxacin (UKMYC5) and isoniazid (UKMYC6). The latter has an appreciable number of isolates that, despite being classified as gWT, have elevated MICs. These are likely due to some remaining samples that were mislabelled and illustrates the difficulty in using the 99th percentile to define an ECOFF/ECV due to its sensitivity to errors in the dataset, especially when the prevalence of resistance is high, as in the case for isoniazid in our dataset. We shall take forward the values for the 99th percentiles (Table 2) but will bear in mind that a high amount of variation may indicate strain mis-labelling.

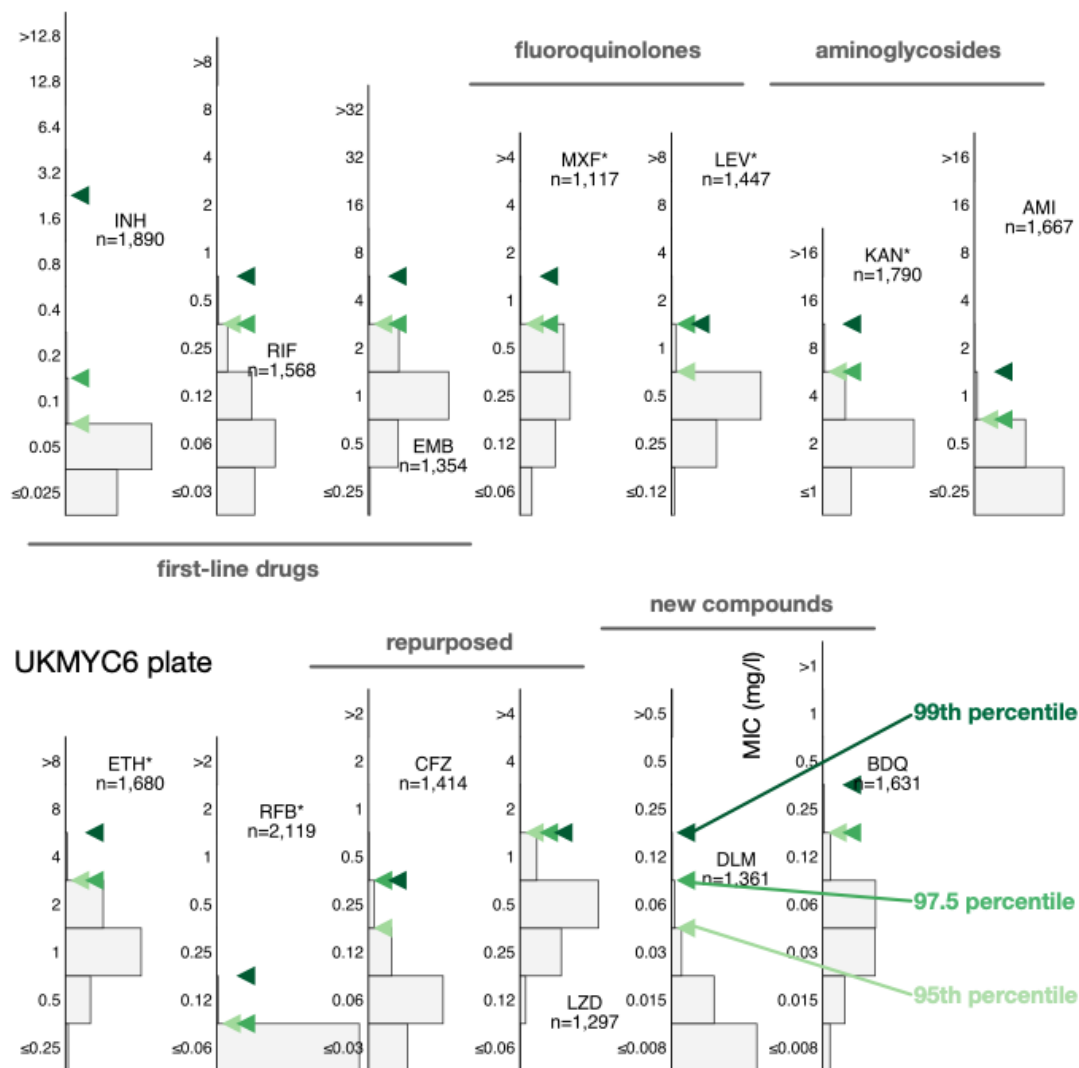


Figure 3. Directly measuring the ECOFF/ECVs from the gWT population on the UKMYC6 plate. To illustrate the sensitivity to the precise percentile used in the definition, the 95th, 97.5th and 99th percentiles are all shown. The analysis and figure can be reproduced (21).

Interval regression takes account of the truncated distributions

To avoid the identification of the 99th percentile being disproportionately affected by a small number of mislabelled resistant samples one usually fits a log-normal distribution to the pWT (here gWT) population and then calculate from the resulting function the MIC of the 99th percentile. We cannot apply ECOFFinder here since its heuristic requires the presence of non-susceptible isolates in the distribution so we instead simultaneously fit a single log-normal distribution using interval regression to the MIC histograms from both plate designs (Fig. 4, S10, Table 2). With the exceptions of isoniazid, rifabutin and delamanid, the resulting log-normal distributions describe the MIC histograms well, even when there is moderate truncation due to the plate design. The rifabutin MIC distribution is, however, extremely truncated, and hence there

are insufficient data to perform a fit - the concentration range for this drug should be lowered in future designs.

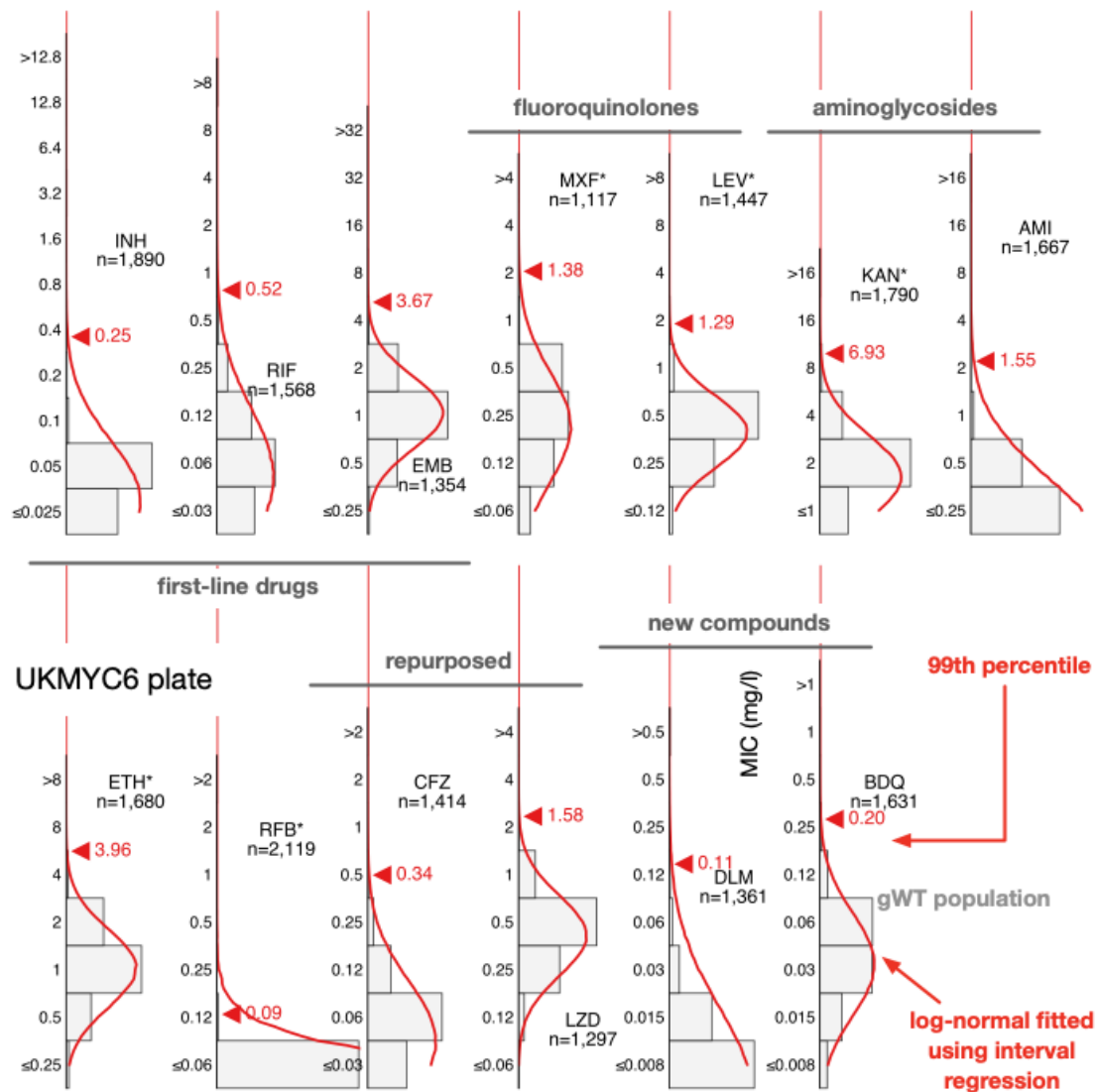


Figure 4. Interval regression is able to fit a log-normal distribution to the MIC histograms of the genotypically wild-type isolates for all 13 drugs on the UKMYC6 plate. Data from both plate designs were considered simultaneously, hence the resulting distributions are those the algorithm considers to best describe both the UKMYC5 (Fig. S10) and UKMYC6 data sets. See the Supplemental Information for the numerical data. The data can be reproduced (21).

Table 2. The 99th percentiles of the wild-type population as determined by three different algorithmic approaches and the resulting proposed ECOFF/ECVs for the thirteen drugs on the UKMYC6/5 plates.

Drug		ECOFFfinder (mg/L)		Direct measurement on the gWT (mg/L)		Interval regression on gWT (mg/L)	Proposed ECOFF/ECVs (mg/L)
		UKMYC6	UKMYC5	UKMYC6	UKMYC5	Both	
Isoniazid	INH	0.06	-	1.6	0.1	0.25	0.1
Rifampicin	RIF	-	0.08	0.5	0.25	0.52	0.5
Ethambutol	EMB	2.1	4.0	4	4	3.7	4
Moxifloxacin	MXF	0.74	0.75	1	2	1.4	1
Levofloxacin	LEV	0.63	0.72	1	4	1.3	1
Kanamycin	KAN	3.1	2.9	8	4	7.0	4
Amikacin	AMI	0.34	0.31	1	1	1.6	1
Ethionamide	ETH	3.1	3.0	4	4	4.0	4
Rifabutin	RFB		-	0.12	0.12	0.09	0.12
Clofazimine	CFZ	0.12	0.078	0.25	0.5	0.34	0.25
Linezolid	LZD	0.81	0.95	1	1	1.6	1
Delamanid	DLM	0.010	0.019	0.12	0.12	0.11	0.12
Bedaquiline	BDQ	0.11	0.11	0.25	0.25	0.20	0.25

Proposed ECOFF/ECV values

We infer that direct measurement is the most reliable method since it makes the fewest assumptions. However for drugs where there is variation of more than a doubling dilution between the 95th, 97.5th and 99th percentiles, which may indicate the gWT population includes a small but unknown number of isolates with elevated MICs, we shall place greater weight on the result obtained by interval regression. When the MIC histogram is not heavily truncated, we shall also include the 99th percentile reported by ECOFFinder. Note that to convert an MIC that is reported as a real number into an ECOFF/ECV it should be rounded up to the next value in the doubling dilution series. All these data and the resulting ECOFF/ECV values are shown in Fig. 5 & Table 2.

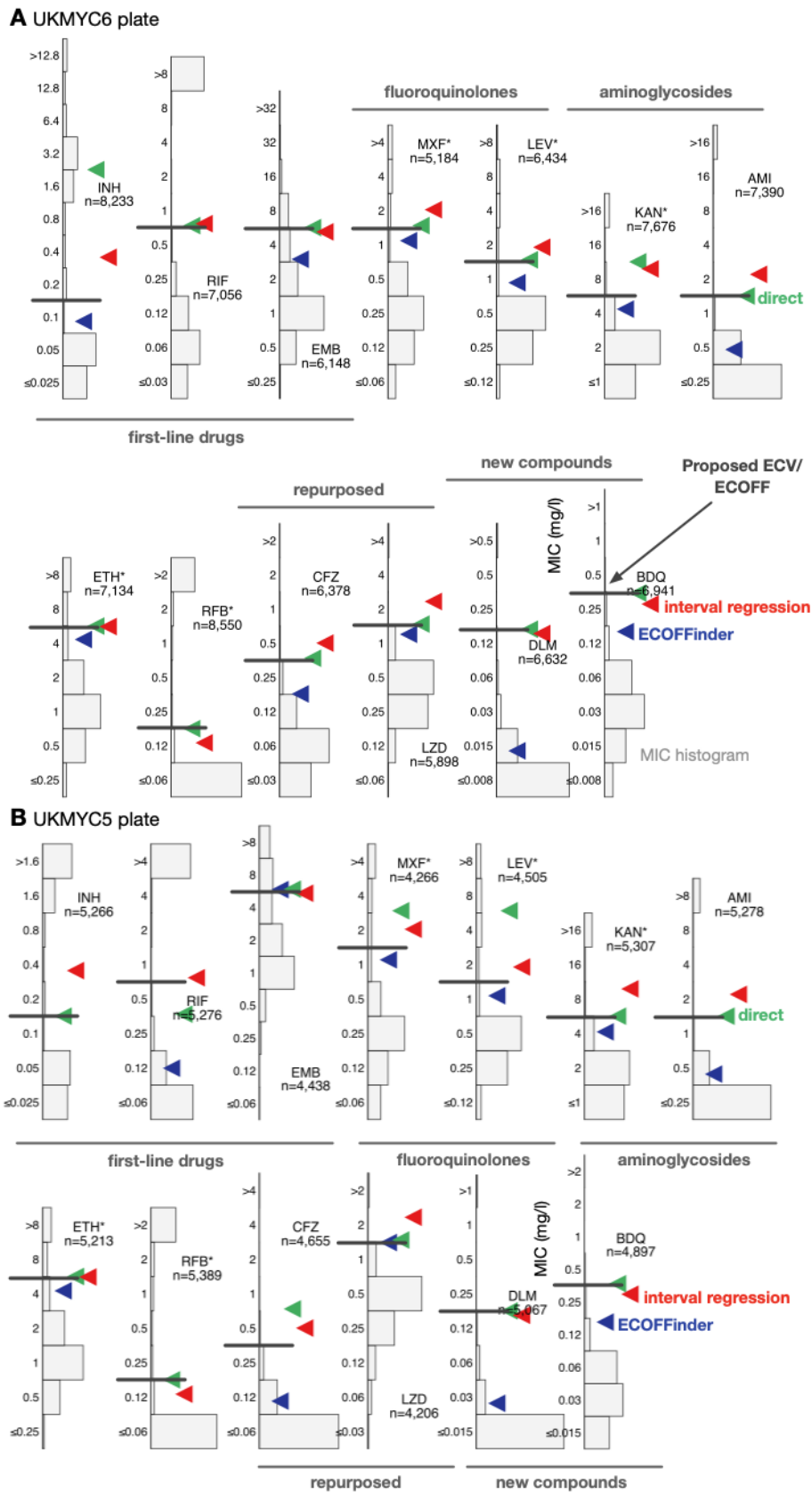


Figure 5. The 99th percentiles of the wild-type populations for the 13 drugs on the (A) UKMYC6 and (B) UKMYC5 plate designs as calculated by ECOFFinder, direct measurement and interval regression. The ECOFF/ECV values are drawn on each graph as a horizontal line.

The 99th percentile determined by direct measurement for isoniazid for the UKMYC6 dataset was discounted due to the large range of MICs spanned by the percentiles – this is most likely due to resistant samples mis-identified as susceptible due to laboratory mislabelling. INH has the highest prevalence of resistance in the dataset, hence would be affected most. Our starting point is therefore the corresponding value for the UKMYC5 dataset (0.1 mg/L). The ECOFFfinder results were ignored for INH since the gWT MIC histogram is truncated on both plate designs and hence the fits were poor. Visually the gWT MIC histograms (Fig. S8) do not appear to follow a log-normal distribution and consequently interval regression over-estimates the 99th percentile (Fig. 4, S10). The ECOFF/ECV of 0.1 mg/L for Isoniazid is therefore less well supported than the ECOFFs for the remaining drugs.

Direct measurement of the 99th percentiles for rifampicin were 0.5 and 0.25 mg/L for the UKMYC6 and UKMYC5 datasets, respectively. Again, ECOFFfinder was not used due to concerns about truncation. Visually the gWT MIC histogram appears more normal in character and the 99th percentile derived from the interval regression fit is 0.52 mg/L. Our proposed consensus ECOFF/ECV for rifampicin is hence 0.5 mg/L. Direct measurement produced a consistent value of 4 mg/L for ethambutol which is supported by interval regression and ECOFFfinder for the UKMYC5 dataset (the concentration range on the UKMYC6 plate was more truncated). Both fluoroquinolones behaved similarly: direct measurement gave a value of 1 mg/L for the 99th percentile for both compounds for the UKMYC6 dataset, but 2 mg/L and 4 mg/L for the UKMYC5 dataset (MXF and LEV, respectively). These values for the latter dataset were very sensitive to the exact percentile used in the definition (Fig. S9), again suggesting that these gWT populations may contain a small number of mislabelled resistant samples. Both drugs have the same concentration range on both plate designs, are only moderately truncated and hence ECOFFfinder would be expected to give reasonable results. These, along with the result of the interval regression (Fig. 5), result in an ECOFF/ECV of 1 mg/L for both fluoroquinolones.

Direct measurement indicates the 99th percentile for kanamycin is 8 mg/L and 4 mg/L for the UKMYC6 and UKMYC5 datasets, respectively, whilst it produces the consistent value of 1 mg/L for amikacin. The MIC histogram of the latter is too truncated for ECOFFfinder to function correctly and visually the log-normal fitted by interval regression appears to have over-estimated the 99th percentile as 1.6 mg/L, hence we propose an ECOFF/ECV for amikacin of 1 mg/L. The kanamycin MIC histograms are less truncated and interval regression better describes the gWT population; these data support an ECOFF/ECV of 4 mg/L for kanamycin. For ethionamide direct measurement produces a consistent value of 4 mg/L which is supported by interval regression and ECOFFfinder. The MIC histogram of rifabutin is extremely truncated and hence only direct measurement is likely to be effective; it estimates that 0.12 mg/L to be the 99th percentile for both datasets, which is therefore our ECOFF/ECV.

Direct measurement yields consistent values of 0.25 mg/L and 1 mg/L for bedaquiline and linezolid respectively, with each supported by both interval regression and ECOFFinder. Our ECOFF/ECV for delamanid is 0.12 mg/L since this is the direct measurement which is the same for both datasets and it is supported by interval regression. Lastly, direct measurement for clofazimine suggests the 99th percentiles are 0.25 mg/L and 0.5 mg/L for the UKMYC6 and UKMYC5 datasets, respectively. The latter has more variation and hence we propose its ECOFF/ECV is 0.25 mg/L.

Comparison against genetic variants known to confer resistance

Using the subset of the isolates with genetic information, we can examine our proposed ECOFF/ECVs by plotting the MIC histograms of several genetic variants that are widely accepted to confer resistance to key anti-TB drugs (Fig. 6 & S11). The *rpoB* S450L and *katG* S315T single nucleotide polymorphisms substantially increase the MICs of rifampicin and isoniazid, respectively, and the majority (96.9% & 99.5%) of isolates with these mutations had MICs greater than the ECOFF/ECV. The c-15t mutation in the promoter of the *fabG1/inhA* operon was associated with borderline (0.2 mg/L) isoniazid MICs unless present in combination with a *katG* S315T mutation (MIC >1.6 mg/L), as observed elsewhere (22, 28). It is likely that this promoter mutation, and others like it, are responsible for the small peak in the MIC histogram observed for isoniazid at 0.2 mg/L.

Substituting isoleucine or valine at position 306 in the *embB* gene was associated with elevated ethambutol MICs, however, the increase in MIC is much less than observed for either of the rifampicin or isoniazid resistance-conferring mutations mentioned above, leading to only 58.3% and 39.6% of isolates containing these mutations, respectively, having an MIC above the ECOFF/ECV. This is expected since it is known that isolates containing these variants can have variable or discordant MGIT results (29). For both fluoroquinolones, the *gyrA* D94G mutation increases the MIC more than the *gyrA* A90V mutation (30), however for levofloxacin the wild-type and non wild-type populations appear slightly better separated with the result that for these mutations 90.0% & 90.7% of isolates lie above the ECOFF/ECV whilst for moxifloxacin the equivalent values are 87.0% & 58.2%. The majority of isolates (90.7% & 89.3%) with the a1401g mutation in the *rrs* gene have an MIC above the ECOFF/ECV for kanamycin and amikacin, respectively. Finally, whilst the c-15t mutation in the promoter of the *fabG1/inhA* operon increases the MIC of ethionamide more than it does isoniazid, only 80.4% of isolates with this variant lie above the ECOFF/ECV.

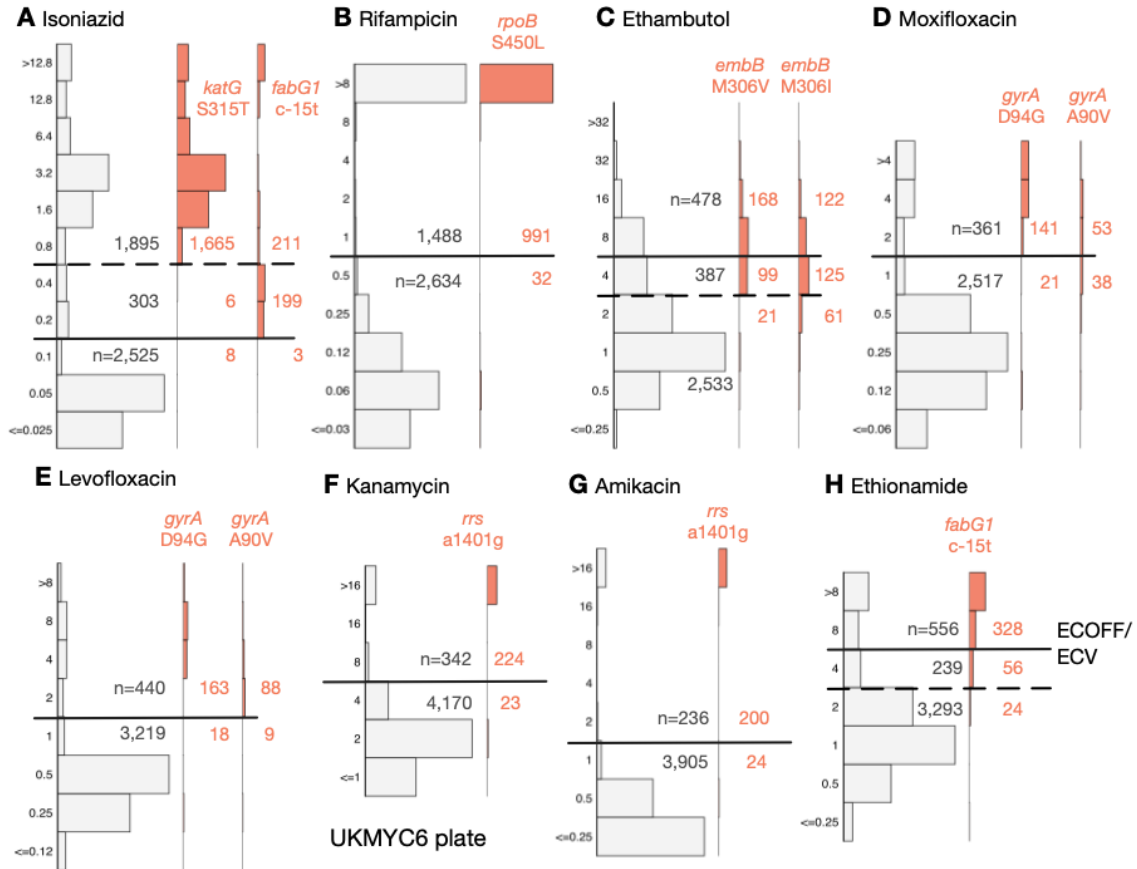


Figure 6. The MICs of isolates containing genetic variants known to confer resistance to different drugs tend to lie above the ECOFF/ECV on the UKMYC6 plate. The number of isolates lying above and below the ECOFF/ECV is annotated. The dashed line indicates the margin of a proposed ‘borderline’ category for isoniazid, ethambutol and ethionamide. The same analysis has been repeated on the UKMYC5 dataset (Fig. S11) and can be reproduced (21).

A role for a Borderline category?

The ECOFF/ECV merely defines an MIC below which the majority of the ‘wild-type’ isolates should lie. It does not necessarily follow that the majority of non wild-type isolates have an MIC above the ECOFF/ECV and therefore care needs to be taken when using an ECOFF/ECV to define susceptibility and resistance. Although for most drugs an MIC below or equal to the ECOFF/ECV can be categorised as ‘susceptible’ and those with MICs above the ECOFF/ECV are ‘resistant’, the MIC histograms of isoniazid, ethambutol and ethionamide are more complex. There is genetic evidence (Fig. 6) that this is due to a multitude of genetic variants, each with a different effect on the MIC. We therefore propose a third category, ‘borderline’, for isoniazid, ethambutol and ethionamide (Fig. 6, Table 3).

Table 3. The proposed ECOFF/ECV values and suggested borderline MICs for three compounds.

Drug	ECOFF/ECV	Borderline
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		(mg/L)	(mg/L)
Isoniazid	INH	0.1	0.2, 0.4
Rifampicin	RIF	0.5	-
Ethambutol	EMB	4	4
Moxifloxacin	MXF	1	-
Levofloxacin	LEV	1	-
Kanamycin	KAN	4	-
Amikacin	AMI	1	-
Ethionamide	ETH	4	4
Rifabutin	RFB	0.12	-
Clofazimine	CFZ	0.25	-
Linezolid	LZD	1	-
Delamanid	DLM	0.12	-
Bedaquiline	BDQ	0.25	-

Validation by comparison to MGIT and MODS results

The resistance of a subset of isolates was independently tested to a range of compounds using either the Mycobacteria Growth Indicator Tube (MGIT) system or the microscopic-observation drug-susceptibility (MODS) assay (31). We can therefore validate our MIC-based categorisation by directly comparing between the binary (or ternary) phenotype derived from an MIC and the result from one of these well-established clinical microbiology methods (Fig. 7 & S12, Table S10).

The agreement between MGIT and UKMYC is good, with a sensitivity of 93.4% and a specificity of 97.0% (Table S10). Since the borderline category lies above the ECOFF/ECV, it is interpreted as providing a way of discriminating between isolates with a moderately elevated MIC and those with a high MIC. For rifampicin, the agreement between MGIT and UKMYC is excellent with a sensitivity of 96.5% and a specificity of 96.6%. The borderline category for ethambutol provides a “buffer zone” since isolates with an MIC of 4 mg/L are only 70.4% resistant according to MGIT. Ignoring these isolates, the sensitivities and specificities are 91.4% and 91.9%, respectively, for ethambutol. Since the ‘borderline’ category in this case lies below the ECOFF/ECV, these isolates would otherwise be classified as ‘susceptible’ but in reality have a mixed character. Hence not assigning a borderline category would result in the sensitivities and specificities becoming 72.8% and 92.5%, respectively.

The aminoglycosides behave similarly to one another with sensitivities and specificities of 76.2% and 99.1% for kanamycin and 84.3% and 99.3% for amikacin, respectively. The 'borderline' category for ethionamide (MIC of 4 mg/L) are 79.4% resistant according to MGIT. Excluding these isolates, the sensitivity is 63.0% and 97.0%, respectively. Limited number of isolates were tested for moxifloxacin or levofloxacin resistance using MGIT (Fig. S12). Although large number of isolates were tested for clofazimine and linezolid resistance by MGIT (Fig. S12), the low prevalence of resistance ensures no useful conclusions can be drawn.

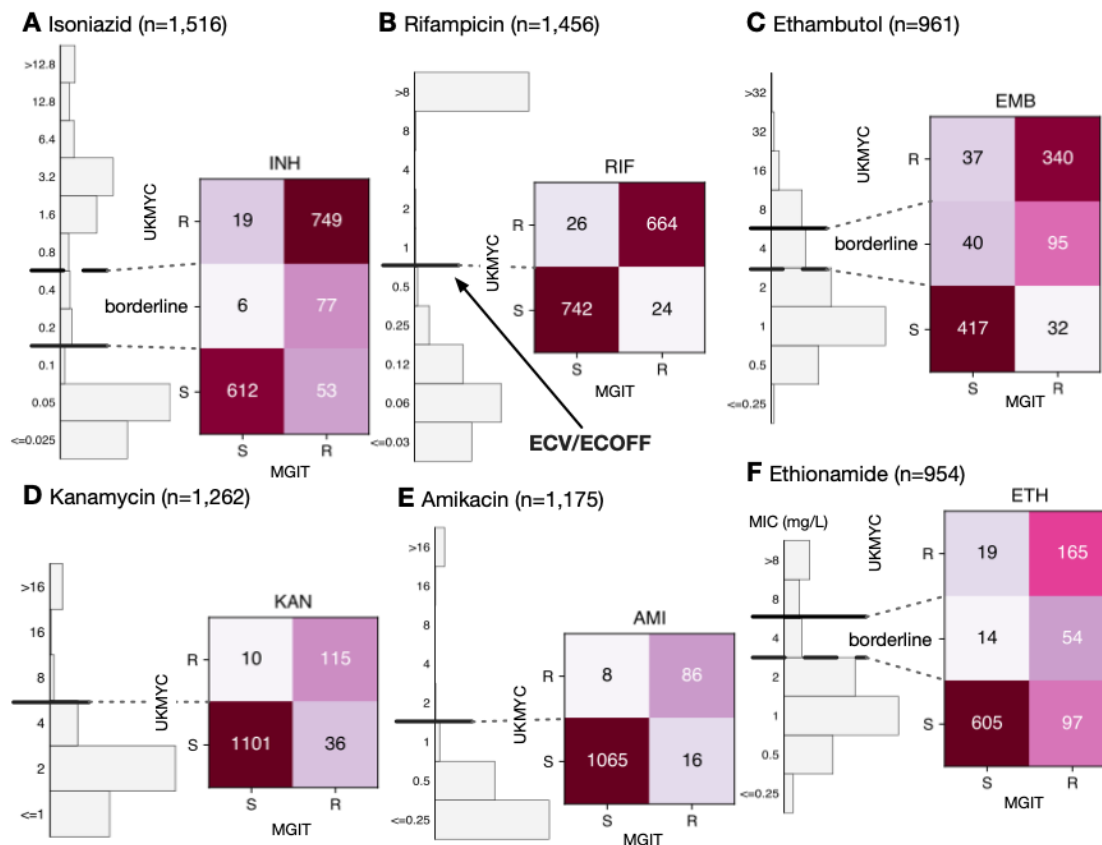


Figure 7. The binary (or ternary) classification derived from the MIC using the ECOFF/ECVs and MIC-based categorisation in Table 3 agrees well with MGIT results for the samples for (A) isoniazid, (B) rifampicin, (C) ethambutol, (D) kanamycin, (E) amkacin and (F) ethionamide. These data and figures can be reproduced (21).

A different set of samples were tested in parallel using the MODS assay (Fig. S13, Table S11). The sensitivities and specificities for isoniazid (n=1,888) and rifampicin (n=1,857) were 95.3% & 98.9% and 95.1% & 99.2%, respectively.

DISCUSSION

We have proposed epidemiological cut-offs (ECOFF/ECVs) for research-use for 13 different anti-TB compounds for the UKMYC series of broth microdilution plates using an aggregated dataset of 20,637 tuberculosis samples collected worldwide by 14 CRyPTIC laboratories based in 11 countries on five continents. The UKMYC6 plate design (Fig. 1B) not only contain the first-line drugs rifampicin, isoniazid and ethambutol, but also all of the Group A drugs, one of the two Group B compounds (clofazimine) and five of the seven Group C medicines recommended by the World Health Organisation for treating cases of multi-drug resistant tuberculosis (19). As such these plates offer near comprehensive phenotypic AST as well as a standardised, scalable phenotype that, once analysed with linked genomic data, will inform clinical decisions where routine diagnostics switch to genome sequencing.

We caution that whilst the ECOFF/ECVs proposed herein have been derived using the largest collection of *M. tuberculosis* samples to date, the methods do not conform with those laid out by EUCAST. That said, our analyses illustrate that the EUCAST definition of an ECOFF/ECV as the 99th percentile of the wild-type population (20) is difficult to apply in practice since firstly it is not always possible to define which isolates are *phenotypically* wild-type without engaging in a circular argument. We were able to avoid this here by defining a *genotypically* wild-type (gWT) population. The second problem is that using the 99th percentile to define the ECOFF/ECV places a very stringent upper limit on the total error rate which becomes harder to meet as the prevalence of resistance in any dataset increases. Despite our efforts, we see evidence that our gWT populations for some compounds contain >1% resistant isolates for some drugs which e.g. hampers the use of direct measurement. In contrast the CLSI have a less-stringent definition for the ECOFF/ECV which avoids this issue (32) but in turn can create inconsistencies between studies. As suggested elsewhere, using a lower percentile (e.g. 97.5th) could help (22).

The CLSI recently proposed breakpoints for the MYCOTB plate (33). There are a few minor differences: our proposed ECOFF/ECV for rifampicin is one doubling dilution lower at 0.5 mg/L. The impact of this is difficult to assess due to the paucity of isolates with MICs of 0.5 and 1.0 mg/L. For isoniazid, although it is not possible to make an exact comparison between the ECOFF/ECVs since the doubling dilution series used on the UKMYC plates and by the CLSI are different, the value proposed by CLSI (0.12 mg/L) is close the value proposed here (0.1 mg/L). The CLSI breakpoints for ethambutol exactly agree with the MIC-based classification adopted by CRyPTIC. Our ECOFF/ECVs are different to those of a recent MYCOTB study (22), however we note that the number of samples was modest (385) and originated from a single country. In addition, ECOFF/ECVs were determined using ECOFFinder, which given the truncated nature of the MIC histograms for many of the drugs, is not now advised and may have biased some of the

results. Our ECOFF/ECVs for rifampicin and isoniazid are, however, consistent with a recent recommendation made by the WHO, albeit for MGIT

Critical concentrations for several of the drugs on the UKMYC plates (which are inoculated with 7H9 growth media) exist for *M. tuberculosis* grown in other growth media, such as Löwenstein-Jensen, 7H10 and 7H11, and also other AST methods, such as the BACTEC Mycobacterial Growth Indicator Tube 960 (34–36). Caution must, of course, be applied when comparing cut-offs derived using fundamentally different growth media and AST methods.

The ECOFF/ECVs for nine drugs proposed by a series of 7H10 agar studies all either agree or are one doubling dilution different to our proposed ECOFF/ECVs for the UKMYC plates (37–39). More recently, there has been a push to set breakpoints for the new compounds delamanid and bedaquiline so that AST can be performed for these important drugs (40). An early MGIT study using 194 isolates proposed an ECOFF/ECV for delamanid of 0.125 mg/L (41), which is identical to our value. An ECOFF/ECV of 0.125 mg/L for bedaquiline on broth microdilution plates was proposed (42), however the 95th percentile of the wild-type population was used to define the ECOFF/ECV since CLSI guidelines were followed (32) and ECOFFinder was used despite the truncated nature of the MIC histograms. This value was supported by two subsequent studies, the first of which showed that the sensitivity and specificity is maximised with a ECOFF/ECV of 0.12 mg/L compared to 0.25 mg/L (43). The second confirmed this value, however also stated that the 99th percentile of the wild-type population was 0.25 mg/L (44). This illustrates that the exact value can be difficult to pin down when different ECOFF/ECV definitions are used; hopefully the number and diversity of samples in our study will help resolve this important question. Lastly, the ECOFF/ECVs proposed here lie within the range of breakpoints recommended by the WHO for different growth media, with the exception of clofazimine for which the WHO recommends a cut-off of 1 mg/L in MGIT (35, 36).

Deriving ECOFF/ECVs from MICs relies on several assumptions, foremost that applying a binary resistant/susceptible classification to a clinical infection is a reasonable and helpful way to proceed. That simplifying the description of the results of clinical microbiology investigations helps interpretation is not in doubt (45), however problems with reproducibility can arise depending on the character of the underlying MIC histogram. If the MIC histogram is 'bimodal' (i.e. has two narrow peaks separated by an interval greater than their individual variance) then placing the ECOFF/ECV between the peaks leads to a helpful and reproducible classification system (46). On the UKMYC series of plates, the only drugs that conform to this ideal are the rifamycins and the aminoglycosides; the other compounds either have more complex distributions (INH, EMB, MXF, LEV, ETH) or resistance is not sufficiently prevalent for us to fully characterise their MIC distributions (CFZ, LZD, DLM, BDQ).

There is a further implicit (weak) assumption that the 'susceptible' and 'resistant' subpopulations can each be described by a *single* MIC distribution, which is not necessarily true, as exemplified by the effect of the *fabG1* promoter mutations on isoniazid (Fig. 6A) (28). In addition this assumption implies that different lineages behave similarly when exposed to an antibiotic, which is unlikely to be true (47). Finally, the wild-type distribution is usually assumed to be log-normal, however our data do not support this for all drugs (e.g. LEV, Fig 4) resulting in the log-normal distributions fitted by interval regression apparently over-estimating the 99th percentile. Should this turn out to be generally true, this would invalidate methods based on fitting such distributions, making direct measurement more appealing (25).

One can deconstruct the error in determining an ECOFF/ECV using a microtitre plate into sample selection biases, data entry and labelling errors, inoculation and incubation error, measurement error, error in defining the wild-type population, uncertainties arising from censored data and error in fitting a curve to the resulting MIC histogram. In addition to the obvious benefits in collecting such a large and diverse dataset (Table 1, Fig 1, Table S2), we have been careful to minimize measurement error (Fig. S3) and have also used a principled method to attempt to remove some putative mislabelled samples (Methods). By defining a genotypically wild-type population and either applying interval regression to fit normal distributions (Fig. 4) or directly measuring the 99th percentile (Fig. 5), we have also minimized the final two sources of error. Despite these steps, further sources of error no doubt remain. Another key weakness of this study is the lack of pyrazinamide, which due to its preference for acidic conditions, is currently unable to be successfully incorporated onto broth microdilution plates, although there is hope that this could be rectified in future (48).

The debate about how to define and calculate ECOFF/ECVs will continue and new approaches will be suggested (49–52). However it evolves, larger and more geographically diverse tuberculosis datasets, such as presented here, will bring more confidence and rigour to the antibiotic susceptibility testing of clinical tuberculosis samples. We hope also, that as clinical microbiology transitions into a data-driven science, our proposed method of directly measuring the required percentile of the gWT population will gain traction due to its simplicity and reproducibility as the genetics of *M. tuberculosis* resistance becomes better understood and accepted.

Although the main objective of the CRyPTIC project is to map the genetic variations in the *M. tuberculosis* genome that confer resistance to many antibiotics, the sheer number of samples collected provides a body of evidence to support the use of 7H9 broth microdilution plates in clinical mycobacteriology. Applications potentially include antibiotic susceptibility testing for

samples that are predicted to be MDR or XDR by the GeneXpert RIF/MDR assay system or surveying the prevalence of different patterns of resistance by region or country, allowing regional regimens to be designed and their impact monitored. Finally, even in settings which adopt genetics-based clinical microbiology (11), it would be prudent to maintain culture-based testing not only to identify new genetic variants as they arise but also to continuously monitor the performance of the genetic resistance catalogue which are likely to change over time as such catalogues are only likely partly causal.

In future work the CRyPTIC project will apply the ECOFF/ECVs proposed here not only to further optimise a genetic catalogue for the first-line anti-TB compounds (6) but also to extend coverage to second-line, repurposed and new compounds, with the aim of covering as many of the drugs recommended by the WHO for treating MDR and XDR tuberculosis (19). Clearly the numerical data being collected by the consortium also lends itself to the development of a genetic catalogue for anti-TB compounds that can make *quantitative* predictions; such a catalogue would naturally take account of additivity, epistasis and non-linear effects. Finally, the tools and data-driven approaches developed here could be applied to other pathogens, especially other mycobacteria.

METHODS

Ethics review

Approval for the CRyPTIC study was obtained by Taiwan Centers for Disease Control IRB No. 106209, University of KwaZulu Natal Biomedical Research Ethics Committee (UKZN BREC) (reference BE022/13), University of Liverpool Central University Research Ethics Committees (reference 2286), Institutional Research Ethics Committee (IREC) of The Foundation for Medical Research, Mumbai (Ref nos. FMR/IEC/TB/01a/2015 and FMR/IEC/TB/01b/2015), Institutional Review Board of P.D. Hinduja Hospital and Medical Research Centre, Mumbai (Ref no. 915-15-CR [MRC]), scientific committee of the Adolfo Lutz Institute (CTC-IAL 47-J / 2017) and in the Ethics Committee (CAAE: 81452517.1.0000.0059) and Ethics Committee review by Universidad Peruana Cayetano Heredia (Lima, Peru) and LSHTM (London, UK). No ethics approval was required for the remaining laboratories since at no time was any patient identifiable information shared with the consortium.

Sample selection

The CRyPTIC project aimed for around half the samples collected to be susceptible to the first-line compounds with the remainder MDR/XDR. There was, however, large variation between the different participating laboratories.

Incubation and inoculation protocol

Each laboratory followed a standard operating protocol laid out by the CRyPTIC consortium, which was similar to that described previously (17). Clinical samples were sub-cultured either using Lowenstein-Jensen tubes, 7H10 agar plates or MGIT tubes. The protocol specified that first a suspension at 0.5 McFarland standard in saline Tween with glass beads (Thermo Fisher, Scientific Inc., USA) from 20- to 25-day-old colonies. These were then diluted 100-fold by adding 100 μ l of suspension to 10 ml of enriched 7H9 broth (17). A semi-automated Sensititre Autoinoculator (Thermo Fisher, Scientific Inc., USA) was used to dispense 100 μ l of inoculum (1.5×10^5 CFU/ml, with approximate range from 5×10^4 CFU/ml to 5×10^5 CFU/ml) into a well of a UKMYC5/6 microdilution plate. The plate was then sealed using transparent plastic provided by the manufacturer. The UKMYC5 and UKMYC6 microdilution plates were designed by the CRyPTIC consortium and manufactured by Thermo Fisher Inc., U.K. The drugs included and their concentrations are described in Fig. 1. Delamanid and bedaquiline pure substances were provided by Otsuka Pharmaceutical Co., Ltd. and Janssen Pharmaceutica, respectively. The H37Rv ATCC 27294 was used to perform periodic quality control runs since it is susceptible to all the drugs on both plate designs.

Measurement of MICs after 14 days incubation

In each laboratory a scientist read each plate after 14 days incubation using a Thermo Fisher Sensititre Vizion digital MIC viewing system, with results entered via a bespoke web portal (<https://clires2.oucru.org>). In those cases where this was not possible, spreadsheets were sent. A photograph was also taken using the Vizion system and also stored in CliRes2. Two laboratories used a mirrored-box to read the plates and one of these also took a photograph using a DSLR. A plate was marked as invalid if it did not have adequate bacterial growth in both positive control wells. A small subset of plates with poor growth at day 14 were incubated for a further week and then read again.

Other AST measurements

Where available, the results of standard AST tests conducted by the participating laboratory were also entered via the CliRes2 online portal, or in some cases shared via spreadsheet. The methods used were mainly either the Mycobacteria Growth Indicator Tube (MGIT) system or the microscopic-observation drug-susceptibility (MODS) assay (31). All MGIT tests used standard critical concentrations (CC) – for the moxifloxacin results only those with a CC of 0.5 mg/L were included.

Genetic sequencing and interpretation

Sequencing arrangements differed slightly between each CRyPTIC participating laboratory. All sequencing was performed using Illumina machines and hence the input to our genetic sequencing pipelines was a matched pair of FASTQ files containing the short reads. Data integrity was ensured throughout by tracking the MD5SUM hashes of the FASTQ files.

Human and HIV reads were removed from the raw sequence data as follows. Reads were mapped to the reference genome H37Rv, the human genome version GRC38, the HIV reference NC_001802.1, various other viral genomes (so that, if any reads mapped to HIV, no-one would only know that they mapped to some virus), and nasopharyngeal flora genomes from the human microbiome project, using BWA MEM. First, a read pair was kept if either read matched H37Rv, then removed if either read matched one of the other genomes, and finally kept if both reads were unmapped.

Variants were initially called using SAMtools and Cortex, two variant callers with orthogonal strengths (samtools a high sensitivity SNP caller, and cortex a high specificity SNP and indel caller). These calls were then passed to the adjudication software minos, which produces a graph representation of the reference genome plus conflicting calls from the two callsets, and then remaps reads to the graph to adjudicate statistically. This adjudication process, and the

performance of the combined samtools/cortex callset, are documented (53). All of this process, including versions of samtools and cortex and the reference genomes for filtering) is encapsulated in Clockwork version 0.8.3 (54).

Samples were excluded from the dataset if they had either more than 100,000 unfiltered samtools variant calls (a weak filter applied to detect samples contaminated with the wrong species) or an average read coverage of 15 or less when mapped to reads covering the H37Rv reference. The samples that pass these criteria and have paired phenotype data are named the GPI (geno-pheno intersection). Variant calls were removed if they overlapped a set of masked positions as previously defined (55). This mask consists of 324,971 positions from the H37Rv reference with self-blast matches, and can be found here: https://github.com/iqbal-lab-org/cryptic_tb_callable_mask/commit/43ec21319209b23f648f32e4868bdf07cf09f2a0.

Version 3 of the H37Rv strain (NC_000962.3) was used as the TB reference genome throughout. The resulting VCF files were then transferred to the CRyPTIC data warehouse where they were interpreted.

Genetic resistance catalogue

A hybrid TB genetic resistance catalogue was constructed by merging two published catalogues, the first more recent catalogue contained rows for the four first-line drugs (INH, RIF, EMB, PZA) (6). The second also contained rows for MXF, LEV, STM, OFX, AMI, KAN, CAP, ETH, LZD, CFZ, DLM, BDQ, RFB, PTO, PAS (8). Since each catalogue was constructed with respect to version 2 of the H37Rv *M. tuberculosis* reference genome, they were first translated to version 3 of the reference. These catalogues are freely available to download (56) and use a standard grammar, GARC, that is both machine- and human-readable. To avoid putting as few assumptions into downstream code as possible, default rules are included that e.g. specify that non-synonymous amino acid mutations that match no other row have an unknown effect. The hybrid catalogue was constructed by taking the rows for the first-line compounds from the first catalogue and rows for all other drugs from the second. This catalogue, called CRyPTICv1.31, is freely available for download (56) and is also provided in the attendant repository (21).

Genetic analysis

Each sample VCF was compared to a reference genome object using the Python gumpy module (57), thereby creating a table of genetic variants (both single nucleotide polymorphisms, SNPs, and insertions/deletions). Both the individual SNPs were stored and also their aggregated effect on any coding region of gene encoding a protein sequence. An intergenic region of up to 100 bases upstream of the start codon was assumed to be the promoter sequence and hence was associated with the gene. This list of variants was then parsed by a second bespoke Python

module, *piezo*, that reads the hybrid catalogue and understands the GARC grammar and so returns a resistant, susceptible or unknown prediction for each drug in the catalogue (58). The species and, if *M. tuberculosis*, lineage of all samples was determined by SNP-IT (23).

Data warehousing

With the exception of the compressed FASTQ files, all data (VCFs, images, MIC metadata, genetic variants and catalogue predictions) were aggregated and stored in a hierarchical file system using the Python *datreant* 1.0.2 module (59) which allowed for data discovery, tagging and filtering. Updates were performed by inhouse Python scripts. Plate metadata was downloaded from *CliRes2* using the *zeep* Python SOAP client.

Quality assurance of minimum inhibitory concentration readings

Central to our quality assurance (QA) process is the photograph taken of the plate after 14 days of incubation using the *Vizion* instrument by the laboratory scientist. Images were deduplicated by checking the MD5SUM was unique. The remaining images were first read by bespoke software, *AMyGDA* (60, 61), which detects the locations of the wells and, by measuring the growth in each well, estimates an MIC for all drugs. For 54.7% of all measurements the MICs measured by the laboratory scientist and *AMyGDA* were identical (Fig. S4, S5A) and therefore passed the quality control process.

Images of the 45.3% of cases where these two methods disagreed were uploaded to a Citizen Science project, hosted by the *Zooniverse* platform, called *BashTheBug* (62). Each image was classified by at least 11 different volunteers and the median reading was taken to be the consensus. In 38.1% of the images sent (17.3% of the total) the consensus MIC agreed with the MIC measured by the laboratory scientist using the *Vizion* instrument. Visual inspection of a random subset (Fig. S5B) suggested that these were mostly cases where *AMyGDA* incorrectly estimated the MIC, usually because the growth was too small to be programmatically detected. For a smaller proportion (12.0% of the images completed by *BashTheBug*, 5.4% of the total – Fig. S3), the *BashTheBug* consensus agreed with the MIC measured by *AMyGDA*. Visual inspection of a random subset (Fig. S5C) indicated that, for the most part, these were errors made by the laboratory scientist. An error rate of 5.4% for a subjective laboratory-based measurement is reasonable and catching and correcting these errors is the main goal of this quality control process. Overall, therefore, we have a high degree of confidence in 77.4% of the MIC measurements since two or more independent methods concur on the value. Finally, in 22.6% of cases all three methods gave a different answer (Fig. S5D); these are excluded from further analysis. All these proportions are averaged over all drugs; there is significant variation between drugs (Table S7 & S8).

Putative mislabelled samples

Some 44 samples were assumed to be mislabelled samples as defined by being genotypically wild-type but having both an INH MIC ≥ 1.6 mg/L and an RIF MIC ≥ 4 mg/L. This corresponds to 0.8% of the dataset which is likely an underestimate. All 44 samples were removed.

ECOFFinder

A version of ECOFFinder (ECOFFinderXL2011forMac.xlsx) that worked on Microsoft Excel running on Apple Mac computers was provided by Dr Claudio Köser (25).

Interval regression

The intreg function in STATA version v15.1 (Stata Corp.) was used.

Data analysis and graphs

All data analysis, with the exception of the interval regressions and ECOFFinder, were performed using Python 3.8 in conjunction with Pandas 1.2.1 (63), numpy 1.19.5 (64). Graphs were plotted using matplotlib 3.3.4 (65) and GeoPandas 0.8.2.

Reproducibility

The raw data (photographs of 96-well plates, genetic variant call files) along with a series of data tables related by a schema can be downloaded from the European Bioinformatics Institute at <http://ftp.ebi.ac.uk/pub/databases/cryptic/>. In addition, one can reproduce nearly all the tables and figures, along with the Supplemental Data, in a browser window (i.e. no installation required) using Python code we have made publicly available (21).

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AUTHOR CONTRIBUTIONS

- All contributed laboratories collected samples and provided data.
- DWC, TEAP, SH, ALGC, AWS, TMW, PWF, DMC designed the study.
- PWF, SH, ALGC and retrieved and analysed the MIC data.
- ZI, MH, JK and PWF analysed all the genetic information.
- PWF, ASW, TMW performed all analysis.
- PWF wrote the manuscript with all partners offering feedback.

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Epidemiological cutoffs for a 96-well broth microdilution plate for high-throughput research antibiotic susceptibility testing of *M. tuberculosis*: Supplemental Information

The CRyPTIC Consortium*

February 2, 2022

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*see list at end of this document

Laboratory	Plate Design Location	Total	UKMYC5	UKMYC6
African Health Research Institute	South Africa	124	0	124
Brazil	Brazil	368	68	300
Centers for Disease Control and Prevention	United States	136	1	135
Centre for Tuberculosis, NICD	South Africa	1912	895	1017
Chinese Center for Disease Control and Prevention	China	2768	1409	1359
Hinduja Hospital and Foundation for Medical Research Mumbai	India	4992	2327	2665
Institute of Microbiology and Laboratory Medicine	Germany	1773	104	1669
Oxford University Clinical Research Unit	Vietnam	1509	679	830
Public Health Sweden	Sweden	457	100	357
San Raffaele Scientific Institute	Italy	2367	1134	1233
TORCH	South Africa	147	0	147
Taiwan Centers for Disease Control	Taiwan	171	171	0
Universidad Peruana Cayetano Heredia	Peru	3452	1077	2375
University of Capetown	South Africa	461	0	461
Total		20637	7965	12672

Table S1: Total number of samples collected by laboratory, split by microtitre plate design. This table can be reproduced online¹.

Country	Samples
India	4992
Peru	3451
China	2767
South Africa	2638
Italy	1769
Vietnam	1509
Germany	1133
Sweden	457
Pakistan	408
Brazil	368
Nepal	302
Turkmenistan	256
Taiwan	171
Belarus	165
United States	136
Ukraine	30
Kyrgyzstan	28
Algeria	25
Tajikistan	19
Kazakhstan	4
Somalia	3
Unknown	1
South Georgia and the South Sandwich Islands	1
New Caledonia	1
Chile	1
Slovenia	1
Japan	1
Total	20,637

Table S2: Total number of samples grouped by country where collected¹. Some laboratories only collected samples from within the country where they are based whilst others collected samples from several countries, hence this list is longer than Table S1.

Species	Number of isolates
<i>M. tuberculosis</i>	12348
<i>M. bovis</i> BCG	7
<i>M. orygis</i>	4
<i>M. bovis bovis</i>	2
<i>M. bovis caprae</i>	1

Table S3: All isolates contained a species belonging to the Mycobacterium complex¹, as determined by SNP-IT². Since this was done by inspecting the genetics, this was only possible for isolates which had their whole genomes sequenced.

Lineage	Number of isolates	Proportion (%)
Lineage 1	692	5.6
Lineage 2	4358	35.3
Lineage 3	1065	8.6
Lineage 4	6227	50.4
Lineage 6	6	0.0

Table S4: The majority of *M. tuberculosis* isolates belonged to either lineage 2 or 4¹, as determined by SNP-IT².

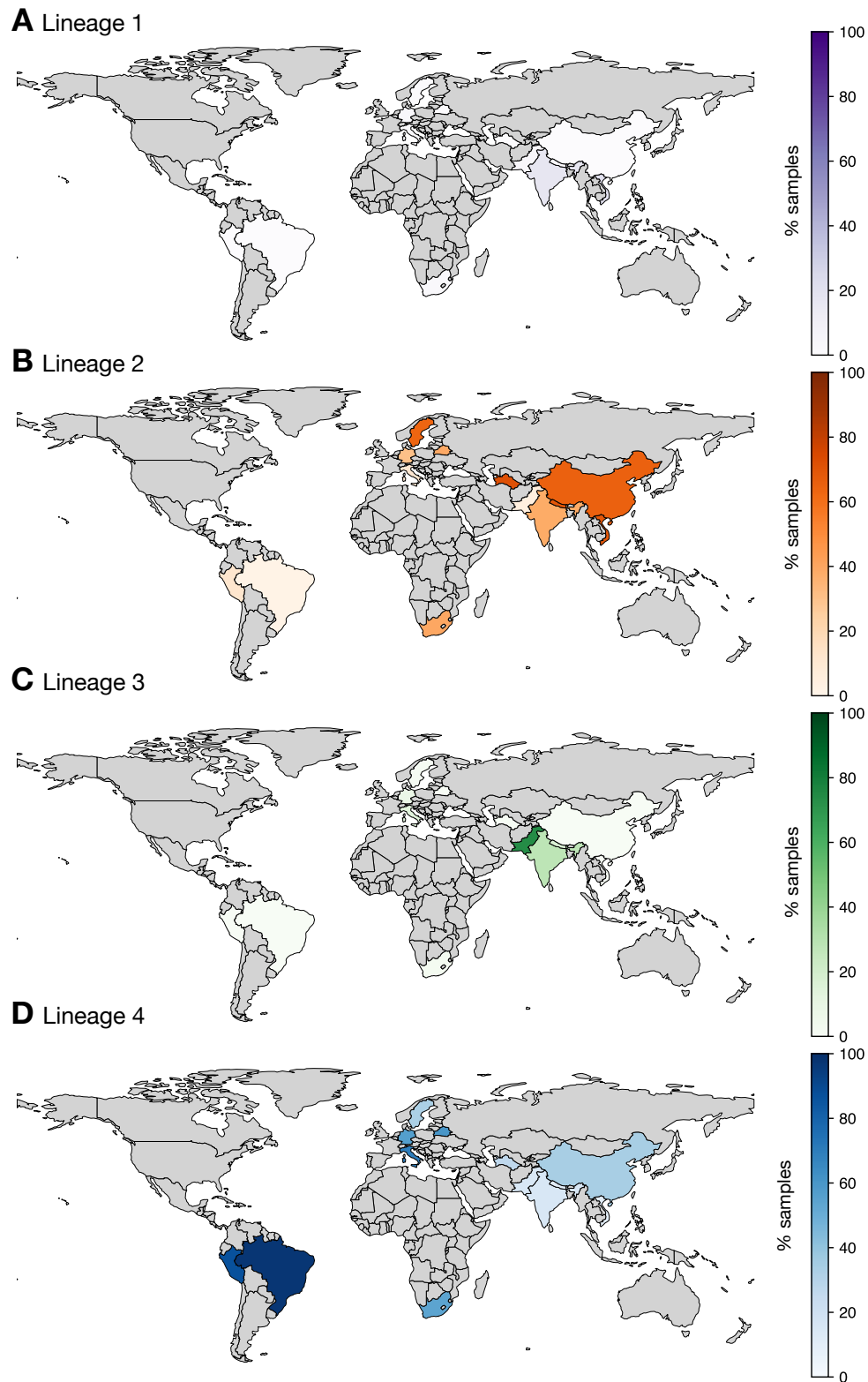


Figure S1: Choropleths showing the proportion of Lineages 1-4 in the isolates containing *M. tuberculosis* collected from each country¹. Only countries where more than 100 samples were collected are shown and Lineage 6 is excluded due to the small number of samples.

Lineage Country	Lineage 1	Lineage 2	Lineage 3	Lineage 4	Total
Algeria	0	0	0	25	25
Belarus	0	40	0	62	102
Brazil	1	8	0	334	343
China	0	702	1	371	1074
Germany	25	218	60	400	703
India	261	581	425	239	1506
Italy	52	148	179	909	1288
Japan	0	0	0	1	1
Kyrgyzstan	0	25	0	3	28
Nepal	5	137	34	21	197
Pakistan	18	21	300	64	403
Peru	1	339	0	2303	2643
Slovenia	0	0	0	1	1
Somalia	0	1	0	1	2
South Africa	61	858	55	1171	2145
South Georgia and the South Sandwich Islands	0	0	0	1	1
Sweden	6	271	6	141	424
Tajikistan	0	15	0	4	19
Turkmenistan	0	85	1	32	118
Ukraine	0	21	0	9	30
Vietnam	262	888	4	134	1288
Total	692	4358	1065	6227	12342

Table S5: Distribution of *M. tuberculosis* lineages by country where collected¹. For clarity only isolates with Lineages 1-4 are shown.

Drug	Plate design	H37Rv mode MIC	Essential agreement	Number of isolates
INH	UKMYC6	0.05 mg/L	94.9-96.6 %	(165-168)/174
RIF	UKMYC6	0.12 mg/L	80.7 %	146/181
EMB	UKMYC6	1 mg/L	97.9 %	183/187
MXF	UKMYC6	0.25 mg/L	99.5 %	184/185
LEV	UKMYC6	0.5 mg/L	100 %	188/188
KAN	UKMYC6	2 mg/L	90.9-98.3 %	(159-172)/175
AMI	UKMYC6	0.5 mg/L	89.7-99.4 %	(148-164)/165
ETH	UKMYC6	1 mg/L	97.9 %	185/189
RFB	UKMYC6	≤ 0.06 mg/L	–	–
CFZ	UKMYC6	0.06 mg/L	84.4-90.3 %	(157-168)/186
LZD	UKMYC6	1 mg/L	91.5 %	173/189
DLM	UKMYC6	0.015 mg/L	67.7-92.1%	(111-151)/164
BDQ	UKMYC6	0.03 mg/L	97.3 %	179/184
INH	UKMYC5	0.05 mg/L	96.3-97.5 %	(78-79)/81
RIF	UKMYC5	≤ 0.06 mg/L	–	–
EMB	UKMYC5	1 mg/L	97.6 %	80/82
MXF	UKMYC5	0.25 mg/L	100 %	82/82
LEV	UKMYC5	0.5 mg/L	98.8 %	81/82
KAN	UKMYC5	2 mg/L	83.6-100 %	(66-79)/79
AMI	UKMYC5	0.5 mg/L	55.6-100 %	(45-81)/81
ETH	UKMYC5	1 mg/L	100%	80/80
RFB	UKMYC5	≤ 0.06 mg/L	–	–
CFZ	UKMYC5	≤ 0.06 mg/L	–	–
LZD	UKMYC5	0.5 mg/L	98.8 %	81/82
DLM	UKMYC5	≤ 0.015 mg/L	–	–
BDQ	UKMYC5	0.06 mg/L	98.7 %	77/78

Table S6: The proportion of strains that lie within 1 dilution of the mode MIC of the standard H37Rv reference strain varies between drugs. Data for both plate designs are shown separately. For some of the drugs the mode is different on the two plates (LZD, BDQ) – the MIC distributions for these drugs are narrow with the majority of isolates contained within two doubling dilutions and hence it is difficult to define the mode. For other drugs the mode lies one well above the lowest dilution (INH, KAN, AMI, CFZ, DLM) on one or both of the plate designs, thereby introducing ambiguity into the essential agreement (EA). In these cases, the EA is calculated as a range that either includes or excludes the isolates in the bottom well. For yet other drugs, the mode lies in the lowest dilution (CFZ, RIF, RFB, DLM); the dilution range for CFZ, RIF and DLM was extended to lower values in the UKMYC6 plate design which partially resolved this issue. Five and eight laboratories contributed variable numbers of H37Rv readings to the UKMYC5 and UKMYC6 datasets, respectively. This study was not designed to determine the quality control ranges for H37Rv on the UKMYC series of plates, however this data could be used to guide future plate designs and experiments.

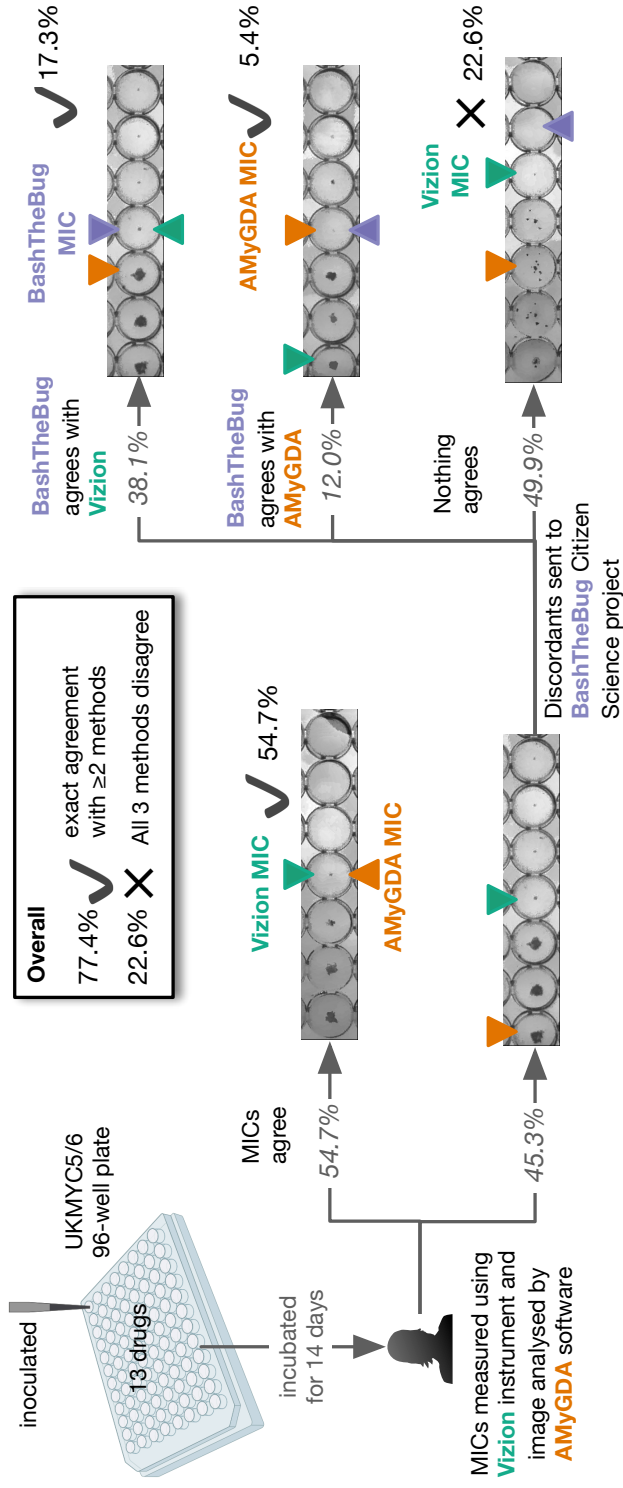


Figure S3: In our quality assurance workflow each minimum inhibitory concentration (MIC) was measured by two or three independent methods. (A) Following culture in a MGIT tube (Methods), a sample was inoculated onto a UKMYC plate. (B) After 14 days incubation, each plate was placed in a Thermo Fisher Sensitire Vizion MIC Viewing System and the MICs were read by a trained laboratory scientist and stored in a central database³. A photograph of the plate was then taken using the Vizion instrument and the image uploaded to the same central database. (C) Each image was subsequently analysed centrally using the AMyGDA plate-reading software specifically developed for this purpose⁴. If the two MICs for a drug were identical (55% of all measurements), the process halts and that measurement is annotated as passing the control quality process. (D) If the MICs were different, an image of that drug doubling dilution series was uploaded to BashTheBug, a citizen science project. At least 11 different volunteers then measured the MIC and a consensus was taken. (E) Of the images sent to BashTheBug, the consensus MIC agreed with either the Vizion or AMyGDA reading in 39% and 12% of cases (Fig. S2). This resulted in a further 23% of the measurements passing the quality control process. (F) For the remaining 22% of measurements, all three MICs differed. These data were excluded from analyses.

Antibiotic	Passed QA (%)	Failed QA (%)
AMI	81.1	18.9
BDQ	78.6	21.4
CFZ	73.3	26.7
DLM	76.0	24.0
EMB	67.1	32.9
ETH	81.0	19.0
INH	88.7	11.3
KAN	84.4	15.6
LEV	71.5	28.5
LZD	68.3	31.7
MXF	63.5	36.5
RFB	91.7	8.3
RIF	80.6	19.4
Average	77.4	22.6

Table S7: The proportion of readings for each antibiotic where at least two methods agreed on the result and therefore have passed the quality control process. Cases where all three methods disagree fail the process. Only measurements where an image is available, a result was determined by the laboratory scientist and, if required, a consensus had been returned by the BashTheBug volunteers are included.

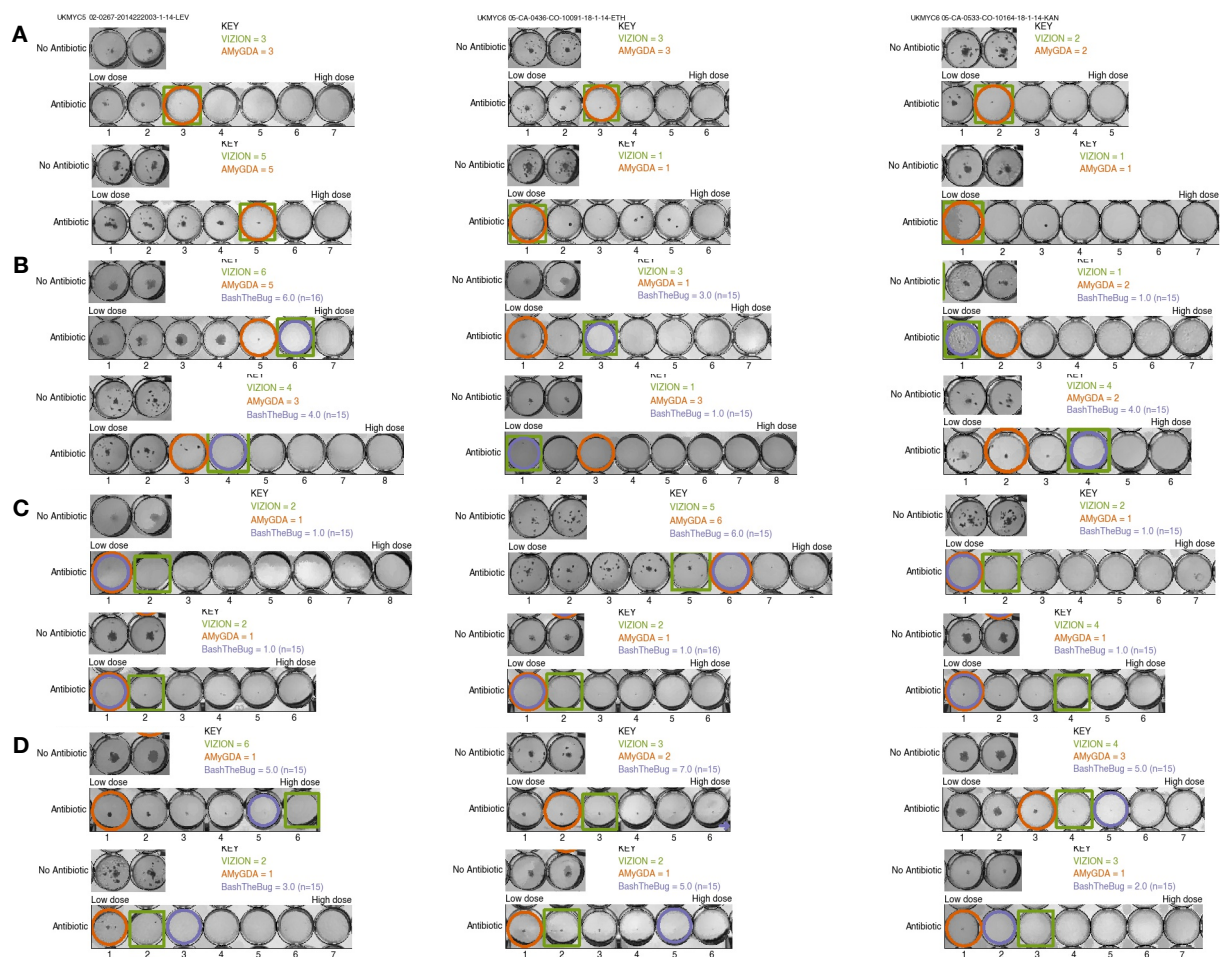


Figure S4: (Related to Figure S3) Randomly selected example images from different parts of the quality control workflow. **(A)** Six examples where the MIC measured by the laboratory scientist using the Vizion instrument and the AMyGDA software agree. **(B)** Six examples where the MIC measured by the laboratory scientist using the Vizion instrument and the consensus measured by the BashTheBug citizen scientists agree. In four cases AMyGDA assessed a lower MIC than the other methods as it incorrectly did not detect small growth in wells. In the remaining two cases, AMyGDA called a higher MIC since it incorrectly assessed growth due to artefacts. **(C)** Six examples where the MIC measured by AMyGDA and the BashTheBug consensus agree. The Vizion measurement appears to be either simply incorrect or the operator has assessed very small dots as growth, which is contrary to the CRyPTIC standard operating procedure. **(D)** Six examples where all three methods yield different MICs. These are a mixture of cases where e.g. all three methods call consecutive MICs and the tendency of the citizen scientists to be conservative in their assessment of growth can be seen.

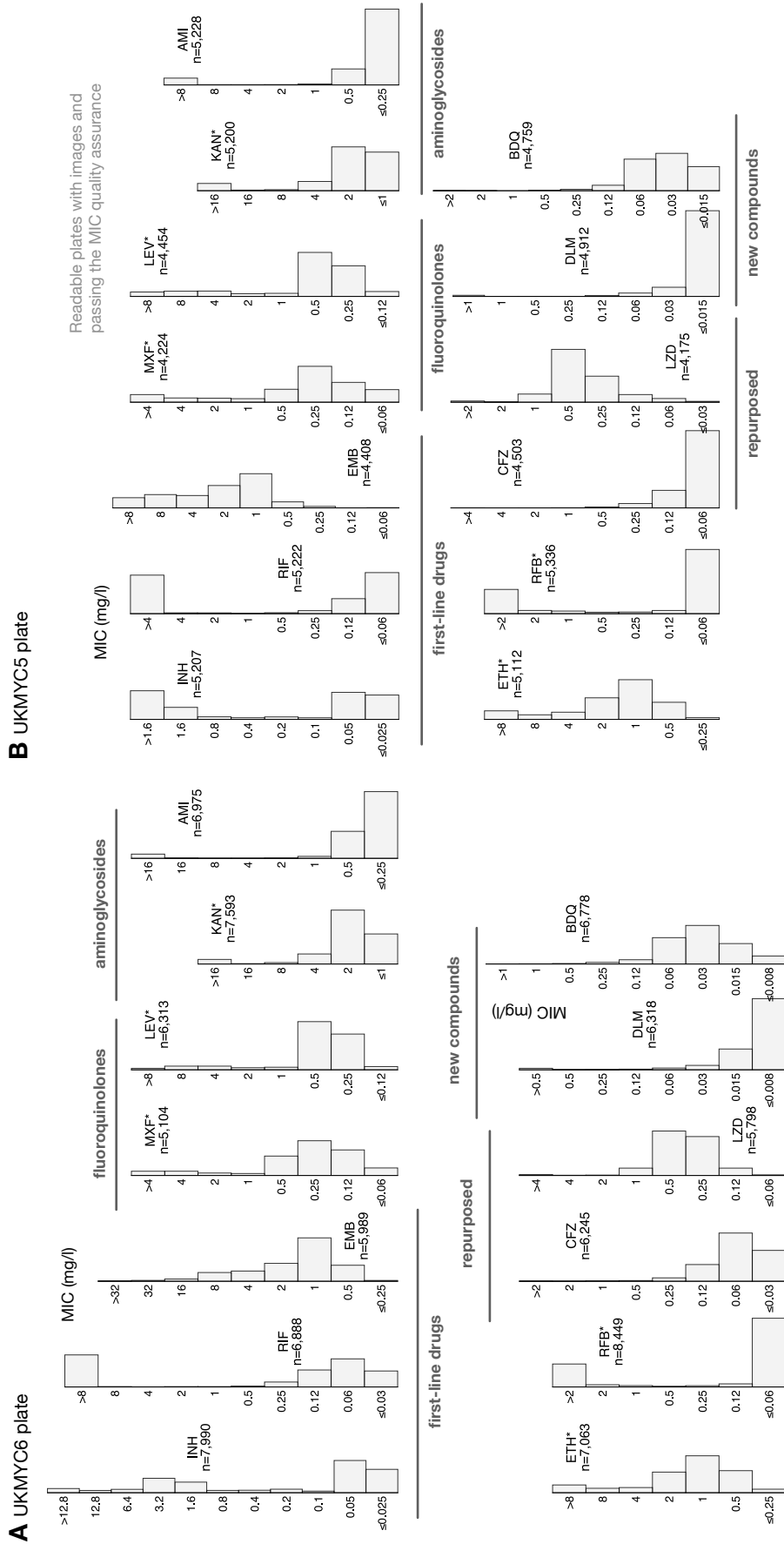


Figure S5: The histograms of the MICs, by drug, that passed the quality assurance process for the (A) UKMYC6 and (B) UKMYC5 plates¹. Each MIC reading has therefore been confirmed by at least two independent methods. See the separate Supplemental CSV file for the numerical data.

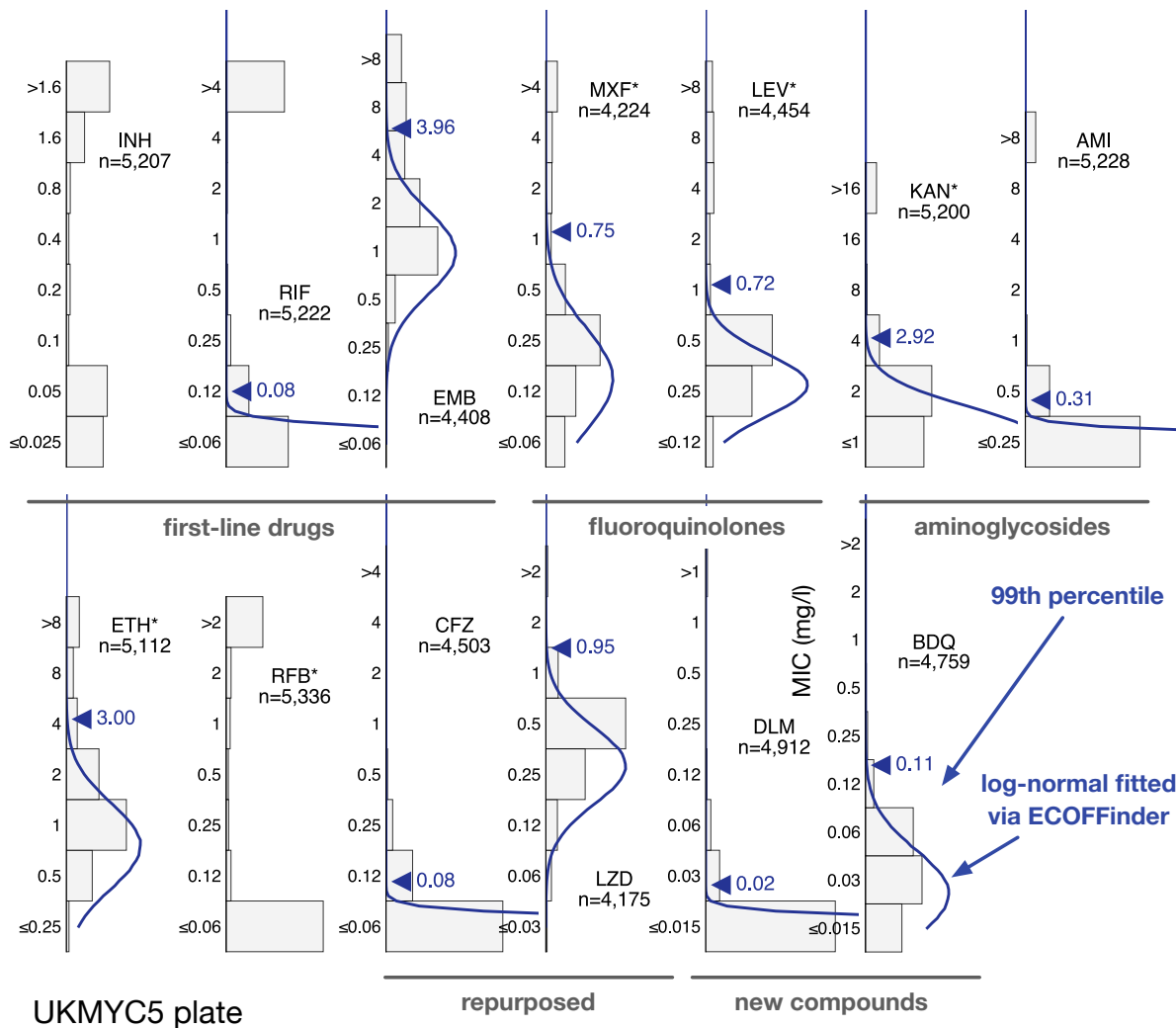


Figure S6: (Related to Figure 2) The MIC histograms for the 13 antibiotics on the UKMYC5 plate. Only MICs which have been confirmed by two independent measurement methods are shown. ECOFFinder was used to fit a log-normal distribution to each histogram; this is drawn in blue and the resulting 99th percentile is labelled. ECOFFinder was unable to fit a log-normal to isoniazid (INH) and rifabutin (RFB). See the separate Supplemental CSV file for the numerical data.

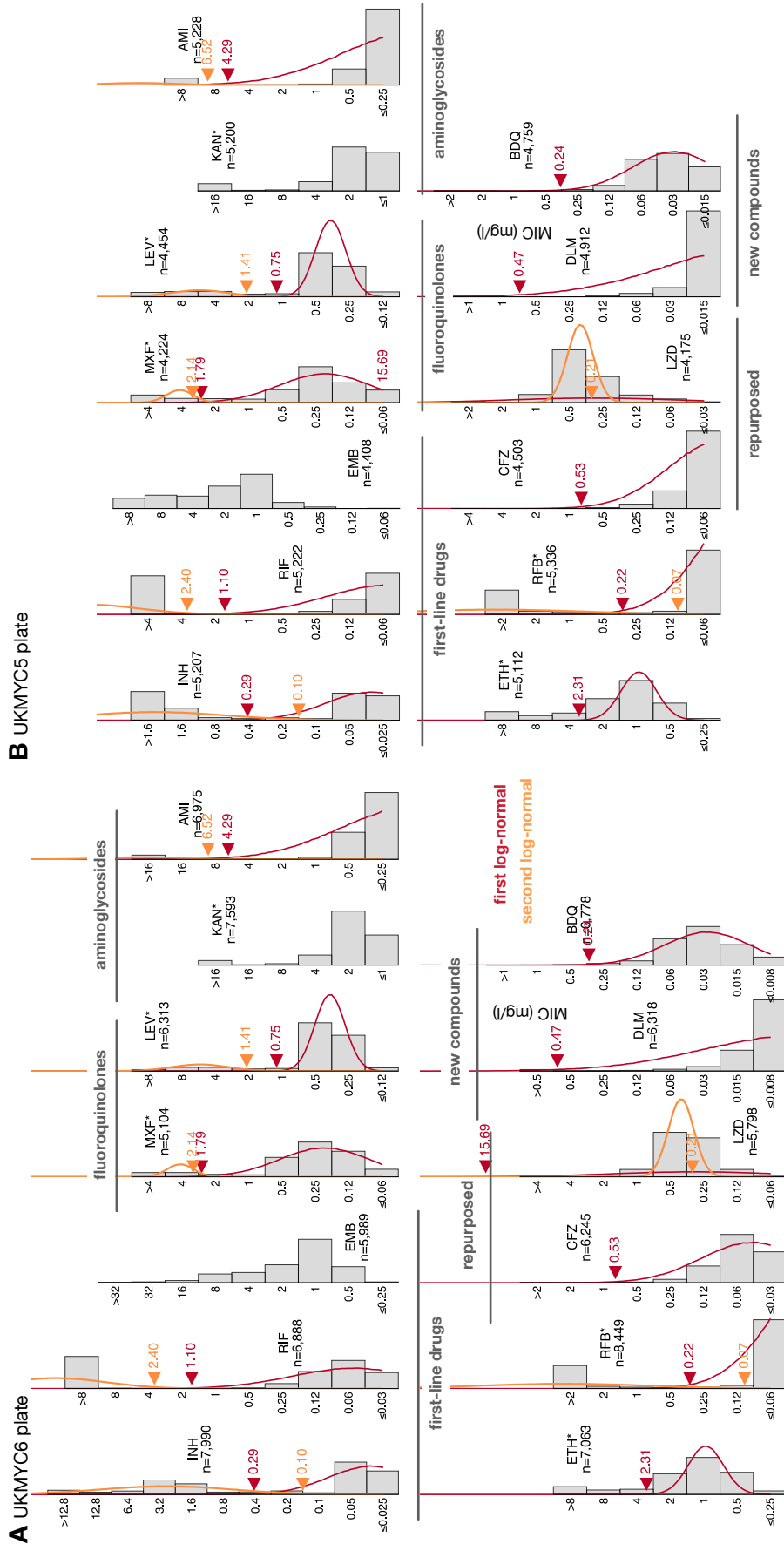


Figure S7: Two log-normal distributions were simultaneously fitted to the MIC histograms from each plate design for each drug using interval regression. The log-normal distribution with the smaller mean is coloured red and the other is coloured orange. The method fails to converge for kanamycin (KAN) and the variance of the second log-normal distribution is occasionally much larger than the range of the data (DLM, ETH)

Laboratory	Plate Design Location	Total	UKMYC5	UKMYC6
Brazil	Brazil	126	48	78
Centre for Tuberculosis, NICD	South Africa	592	333	259
Chinese Center for Disease Control and Prevention	China	463	463	0
Hinduja Hospital and Foundation for Medical Research Mumbai	India	365	365	0
Institute of Microbiology and Laboratory Medicine	Germany	439	8	431
Oxford University Clinical Research Unit	Vietnam	526	370	156
San Raffaele Scientific Institute	Italy	411	109	302
Universidad Peruana Cayetano Heredia	Peru	1039	354	685
University of Capetown	South Africa	342	0	342
Total		4303	2050	2253

Table S8: Total number of genetically wild-type (gWT) isolates collected by laboratory, split by microtitre plate design.

PLATEDESIGN DRUG	UKMYC5	UKMYC6	Total
AMI	1840	1667	3507
BDQ	1666	1631	3297
CFZ	1586	1414	3000
DLM	1687	1361	3048
EMB	1505	1354	2859
ETH	1832	1680	3512
INH	1813	1890	3703
KAN	1834	1790	3624
LEV	1587	1447	3034
LZD	1483	1297	2780
MXF	1477	1117	2594
RFB	1959	2119	4078
RIF	1810	1568	3378

Table S9: Number of genetically wild-type MICs by drug that have passed the quality assurance process. Note that only definite numerical MICs have been included.

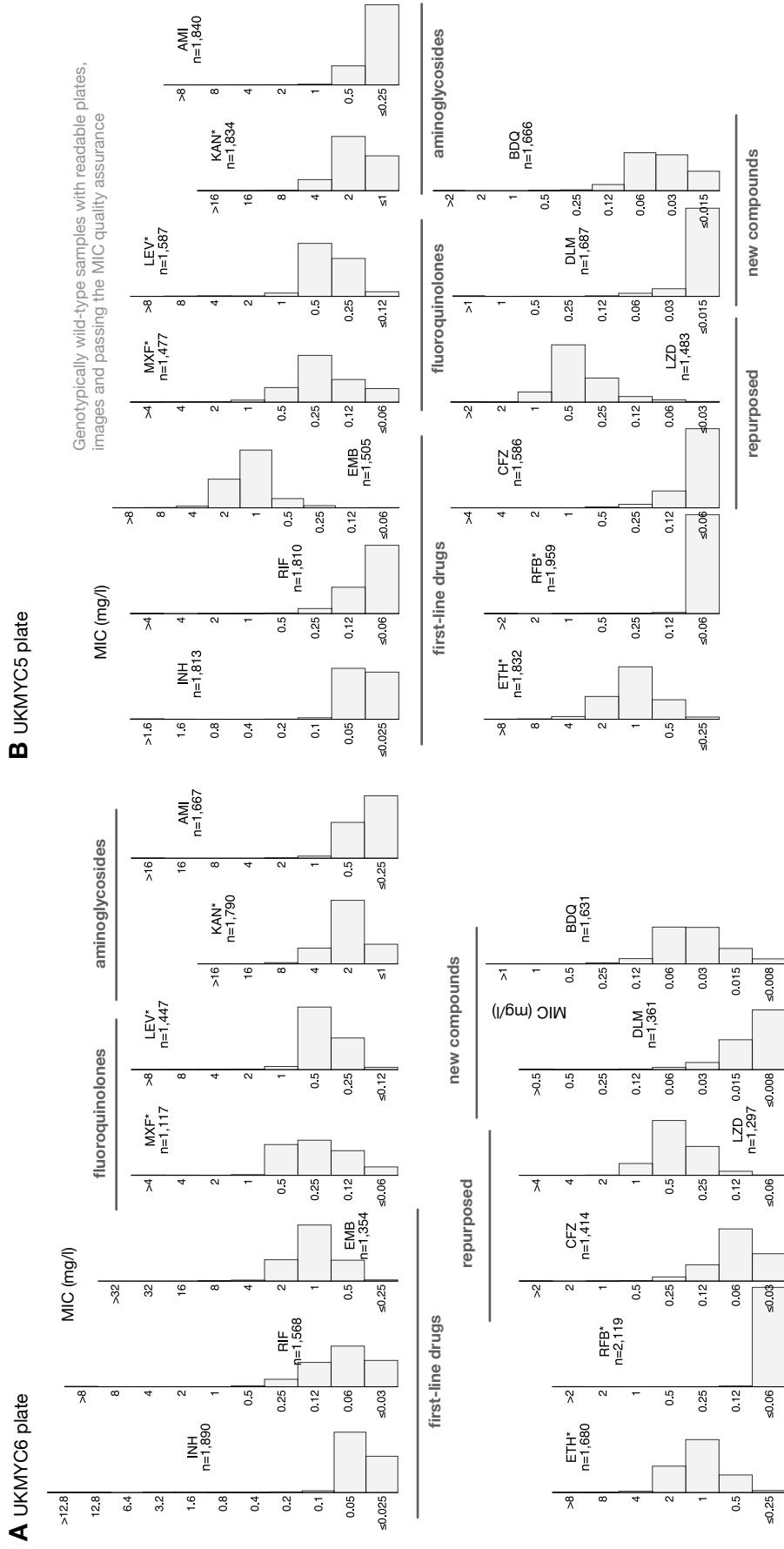


Figure S8: The histograms of all MICs that passed the quality assurance process and are *genotypically wild-type* for the (A) UKMYC6 and (B) UKMYC5 plates. Each MIC reading has therefore been confirmed by at least two independent methods.

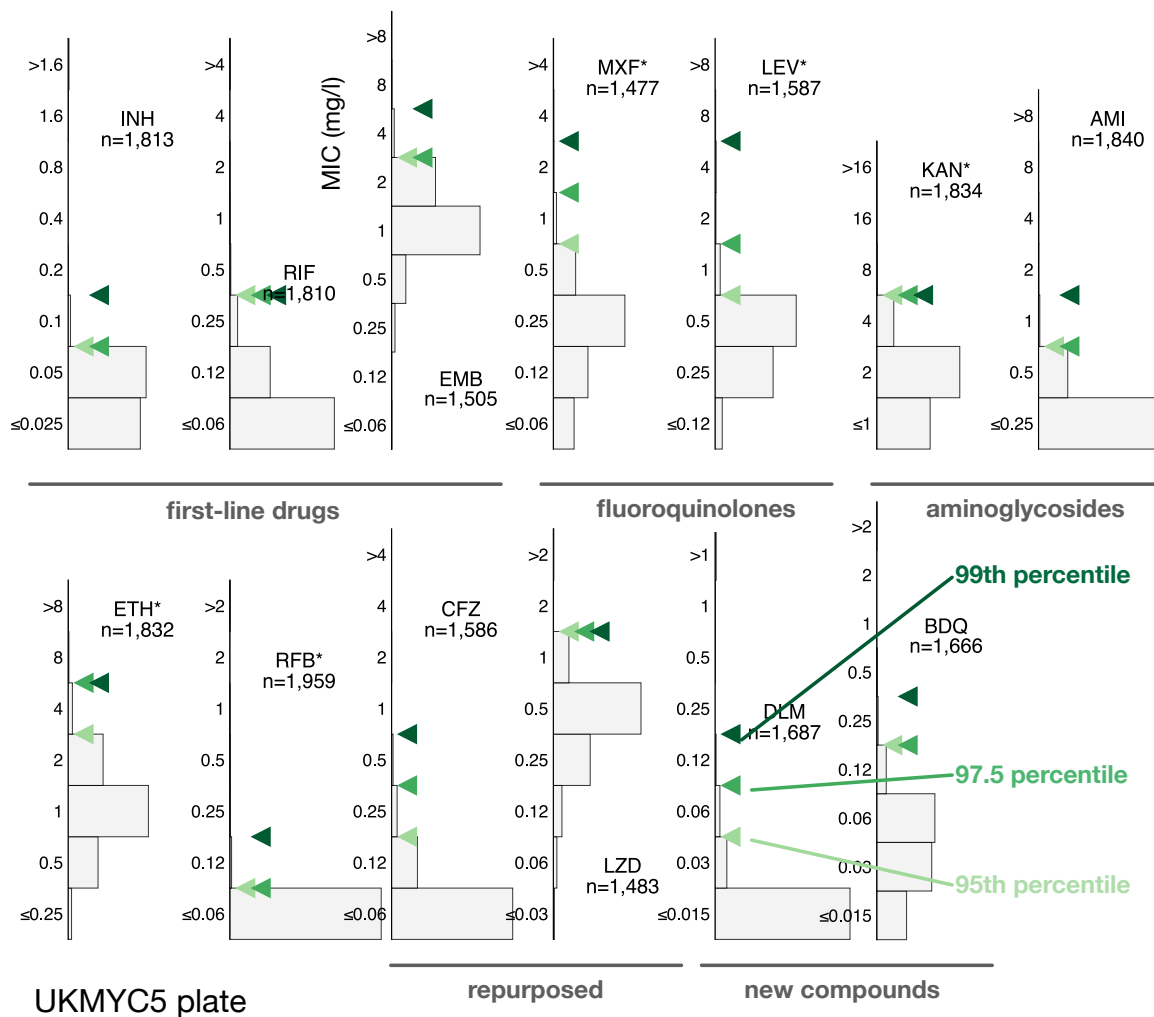


Figure S9: (Related to Figure 3) Direct measurement of ECOFF/ECVs from the gWT population on the UKMYC5 plate. To illustrate the sensitivity to the precise percentile used in the definition, the 95th, 97.5th and 99th percentiles are all labelled.

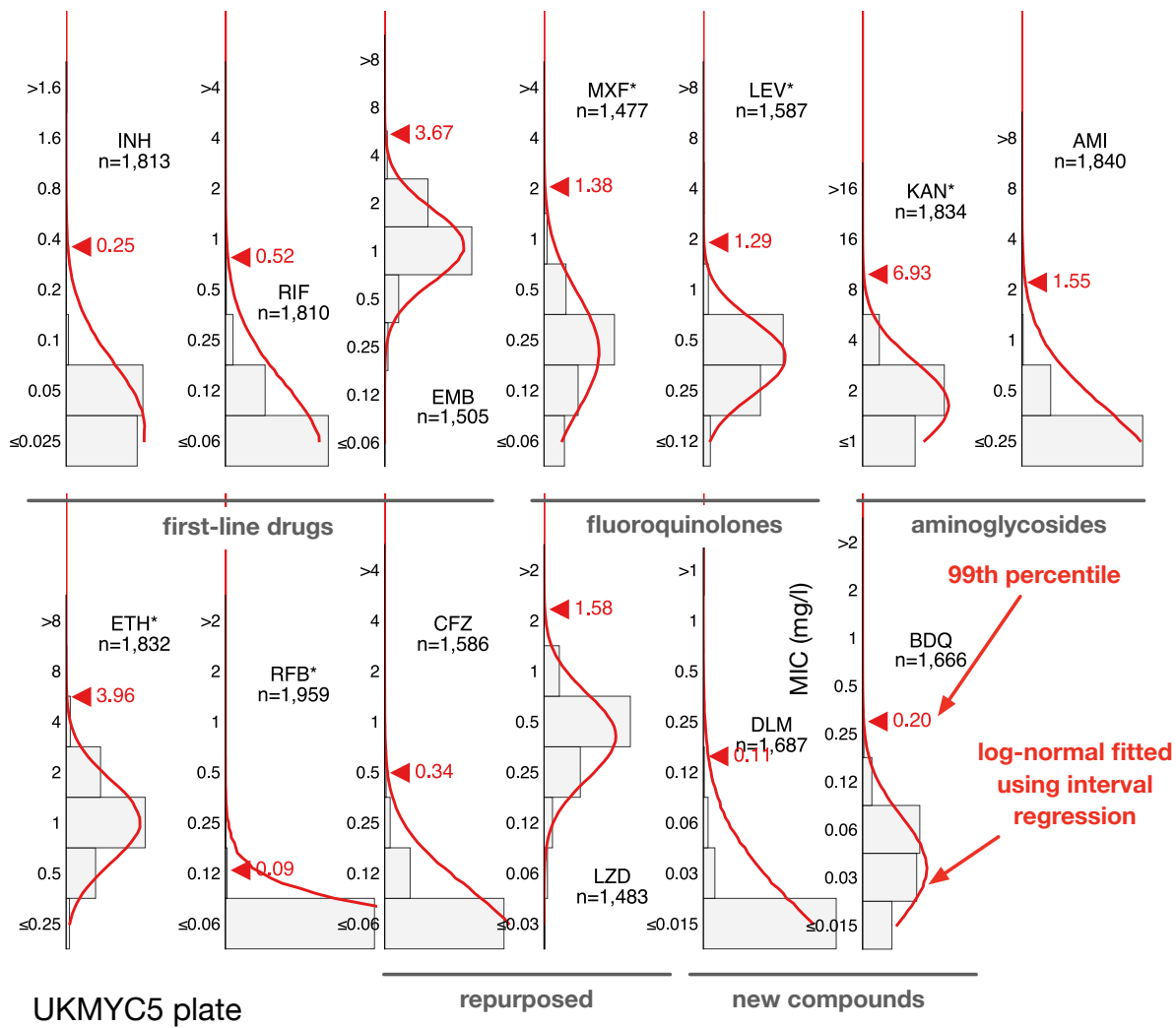


Figure S10: (Related to Figure 4) Interval regression is able to fit a log-normal distribution to the MIC histograms of the genetically wild-type isolates for the 13 drugs on the UKMYC5 plate. Data from both plate designs were considered simultaneously, hence the resulting distributions are those the algorithm considers to best describe both the UKMYC6 (Fig. 4) and UKMYC5 data sets.

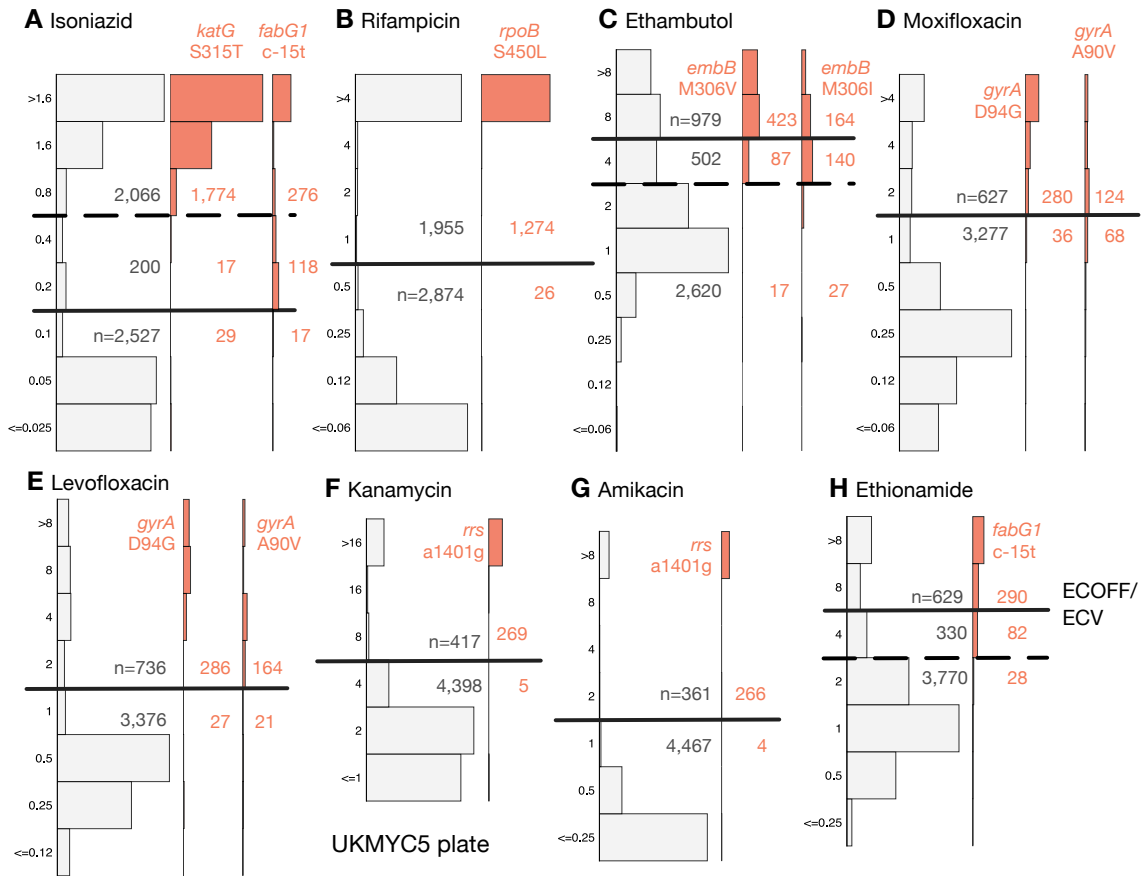
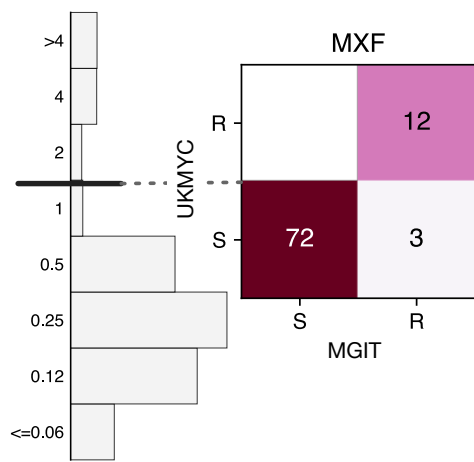
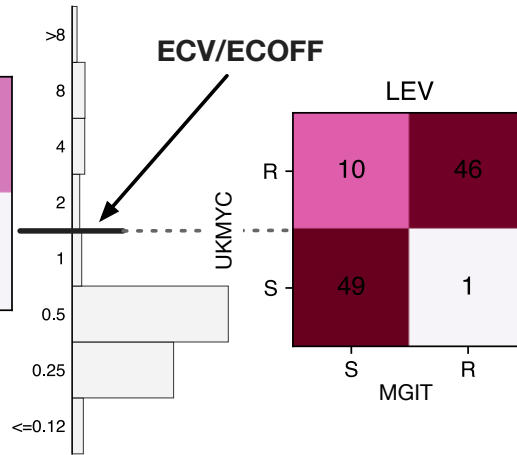


Figure S11: (Related to Figure 6) The MICs of isolates containing genetic variants known to confer resistance to different drugs tend to lie above the proposed ECOFF/ECV on the UKMYC6 plate. The number of isolates lying above and below the ECOFF/ECV is annotated. The dashed line indicates the margin of a proposed borderline category for isoniazid, ethambutol and ethionamide. The same analysis repeated on the UKMYC5 dataset is Figure 6 in the main body of the manuscript.

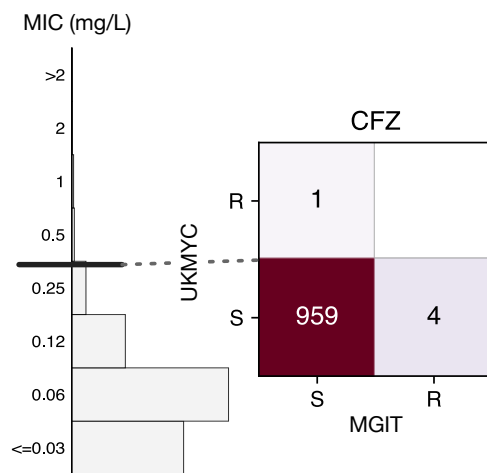
A Moxifloxacin (n=87)



B Levofloxacin (n=106)



C Clofazimine (n=964)



D Linezolid (n=889)

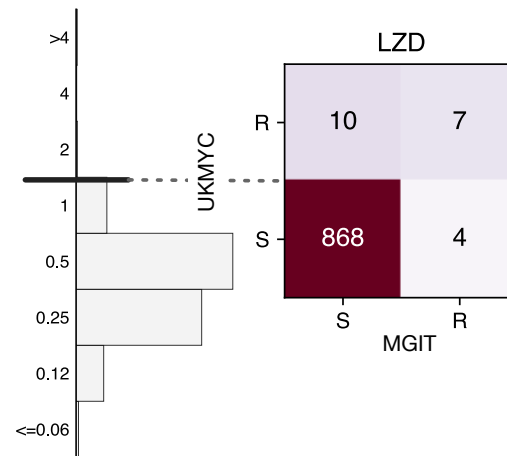


Figure S12: (Related to Figure 7) There is reasonable agreement between the phenotypes measured by the UK-MYC plates (assuming the ECOFF/ECVs) and the MGIT960. There are comparatively few isolates for the fluoroquinolones and very few resistant isolates for clofazimine and linezolid.

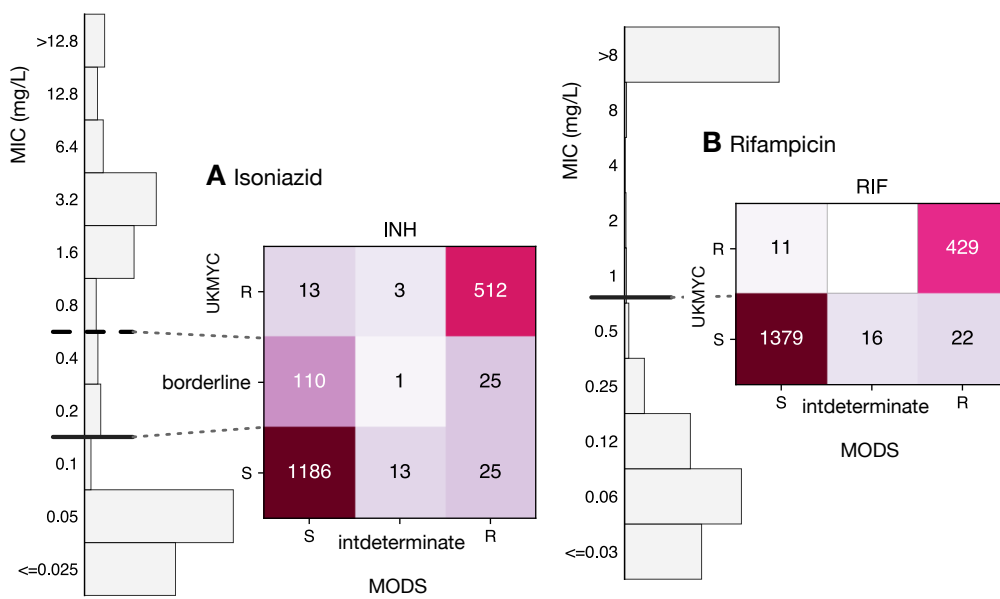


Figure S13: There is reasonable agreement between the phenotypes measured by the UKMYC plates (assuming the ECOFF/ECVs) and the MODS assay⁵. Only isoniazid and rifampicin were tested.

Drug	Number of isolates	Sensitivity (%)	Specificity (%)	Categorical agreement (%)	MD (%)	VMD (%)
INH	1516	93.4	97.0	95.0	6.6	3.0
RIF	1456	96.5	96.6	96.6	3.5	3.4
EMB	961	91.4	91.9	91.6	8.6	8.1
MXF	87	80.0	NaN	NaN	20.0	NaN
LEV	106	97.9	83.1	89.6	2.1	16.9
KAN	1262	76.2	99.1	96.4	23.8	0.9
AMI	1175	84.3	99.3	98.0	15.7	0.7
ETH	954	63.0	97.0	86.9	37.0	3.0
LZD	889	63.6	98.9	98.4	36.4	1.1
CFZ	964	NaN	99.9	NaN	NaN	0.1

Table S10: Comparing the binary phenotypes derived from a UKMYC plate results to MGIT960. The sensitivity and specificity were calculated ignoring any borderline categorisation.

Drug	Number of isolates	Sensitivity (%)	Specificity (%)	Categorical agreement (%)	MD (%)	VMD (%)
INH	1888	95.3	98.9	97.8	4.7	1.1
RIF	1857	95.1	99.2	98.2	4.9	0.8

Table S11: Comparing the binary phenotypes derived from a UKMYC plate results to MODS⁵. The sensitivity and specificity were calculated ignoring any borderline categorisation.

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