

# Automated detection of bacterial growth on 96-well plates for high-throughput drug susceptibility testing of *Mycobacterium tuberculosis*

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## Abstract

*M. tuberculosis* grows slowly and is challenging to work with experimentally compared with many other bacteria. Although microtitre plates have the potential to enable high-throughput phenotypic testing of *M. tuberculosis*, they can be difficult to read and interpret. Here we present a software package, the Automated Mycobacterial Growth Detection Algorithm (AMyGDA), that measures how much *M. tuberculosis* is growing in each well of a 96-well microtitre plate. The plate used here has serial dilutions of 14 anti-tuberculosis drugs, thereby permitting the MICs to be elucidated. The three participating laboratories each inoculated 38 96-well plates with 15 known *M. tuberculosis* strains (including the standard H37Rv reference strain) and, after 2 weeks' incubation, measured the MICs for all 14 drugs on each plate and took a photograph. By analysing the images, we demonstrate that AMyGDA is reproducible, and that the MICs measured are comparable to those measured by a laboratory scientist. The AMyGDA software will be used by the Comprehensive Resistance Prediction for Tuberculosis: an International Consortium (CRyPTIC) to measure the drug susceptibility profile of a large number (>30000) of samples of *M. tuberculosis* from patients over the next few years.

Tuberculosis (TB) kills more people globally than any other infectious disease [1, 2]. In 2016 the World Health Organization estimated that only 22 % of the 600000 people who required treatment for multi-drug resistant tuberculosis (MDR-TB) were diagnosed and received appropriate therapy [1]. In order to control the epidemic, priority should be given to the fast detection of MDR-TB cases and the identification of a proper and effective therapeutic regimen [3], which is currently done by culture-based drug-susceptibility testing (DST).

Existing liquid and solid media culture-based DST methods require significant infrastructure and highly trained laboratory scientists and, due to the inherent slow-growth rate of

*M. tuberculosis*, take at least 4–5 weeks to return a result to the clinician [4]. Although genotypic assays such as the Cepheid Xpert MTB/RIF [5] have been developed for *M. tuberculosis* (MTB) that overcome some of these challenges, no single molecular test exists that determines the effectiveness of a large number of anti-TB compounds simultaneously. One solution is to sequence the whole genome of each patient sample of MTB and infer the effectiveness, or otherwise, of a large number of drugs from the presence of genetic variants known to confer resistance [6, 7]. Using whole-genome sequencing (WGS) in this way has been shown to not only be much faster, but is already cheaper than the existing culture-based methods [4]. It critically depends, however, on a comprehensive and accurate

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**Abbreviations:** AMyGDA, Automated Mycobacterial Growth Detection Algorithm; AST, antimicrobial-susceptibility testing; CRyPTIC, Comprehensive Resistance Prediction for Tuberculosis: an International Consortium; DST, drug-susceptibility testing; MDR, multi-drug resistant; MGIT, mycobacterial growth indicator tube; MTB, *Mycobacterium tuberculosis*; REMA, resazurin microtitre assay; RIF, rifampicin; TB, tuberculosis; WGS, whole-genome sequencing.

Supplementary material is available with the online version of this article.

catalogue that relates genetic variants to their effect on different anti-TB compounds. Whilst existing catalogues can reasonably accurately predict the effect of specific genetic mutations on the first-line anti-TB drugs (isoniazid, rifampicin, pyrazinamide, ethambutol) [8], more work is required to construct a catalogue allowing the effect of mutations on second-line, repurposed and novel compounds to be inferred, as well as improving the existing knowledge of first-line drugs. A comprehensive and accurate catalogue of genetic variants is vital for a WGS-based clinical microbiology to be able to accurately recommend treatments for MDR-TB [7].

The Comprehensive Resistance Prediction for Tuberculosis: an International Consortium [9] (CRyPTIC) is collecting over 30 000 samples of MTB world-wide at approximately 20 participating laboratories over the next 3 years with the objective of identifying the majority of genetic variation in MTB responsible for antibiotic resistance in a large number of second-line, repurposed and novel anti-TB compounds, as well as the first-line compounds used in the standard regimen. The large number of samples involved make it too challenging and expensive to use traditional cultured-based methods. Microtitre plates are an attractive option for high-throughput culture-based DST, as they enable testing of a large number of strains in the presence of different anti-MTB drugs at a range of concentrations. A range of microtitre 96-well plates have been developed and tested in recent years, including frozen plates [10], a dry-form plate [11] (both of which are manufactured by Thermo Fisher) and plates that indirectly detect growth through a colorimetric assay, for example the resazurin microtitre assay (REMA) plate [12]. The CRyPTIC project chose to use a dry-form plate since transportation and storage is straightforward and, unlike the REMA plate, the plate remains sealed following inoculation for the entire incubation period, improving biosafety. The best-known example of a dry-form 96-well plate is the Thermo Fisher Sensititre *M. tuberculosis* MIC Plate (MYCOTB), which assays the MIC for 12 anti-MTB drugs. Following inoculation of a cultured isolate into each hemi-spherical bottomed well, the plate is sealed and then incubated for 2 weeks, and the presence or absence of growth in each well, and hence the MIC, is then visually assessed by a trained laboratory scientist. The accompanying Sensititre Vizion Digital MIC viewing system is designed to help with this process. CRyPTIC has developed a variant of the standard MYCOTB plate, called the UKMYC5 plate, containing 14 different anti-MTB drugs, that includes two repurposed compounds (linezolid and clofazimine) and two new compounds (bedaquiline and delamanid, Fig. 1a). The drugs are present at a minimum of five and a maximum of eight doubling dilutions each. An initial study by the CRyPTIC project has demonstrated that the UKMYC5 plate is as accurate as either the frozen-form or REMA plate [13]. Each *M. tuberculosis* patient sample collected by the CRyPTIC project will have its drug-susceptibility profile determined using this plate and its genome sequenced. Since the main goal of the project is to combine

the genetic and phenotypic data and statistically infer the effect of specific genetic variants, it is essential that all errors and biases in the data are minimized. Any system based on visual assessment by a trained laboratory staff member is, however, subject to some degree of variability between operators. Automated reading, using specifically designed computer software, offers the promise of greater consistency but of course may not be as accurate, given a human's superior ability to recognize and discriminate visual patterns.

In this paper, we describe the design and parametrization of software, the Automated Mycobacterial Growth Detection Algorithm (AMyGDA), that measures the growth of MTB on 96-well plates. To test its reproducibility and accuracy, we apply it to 114 UKMYC5 plates inoculated with 15 different strains of *M. tuberculosis*, including the H37Rv ATCC 27294 MTB reference strain [14]. We are deploying AMyGDA within the international CRyPTIC tuberculosis project to help check and validate the MICs measured by the laboratory scientists for the 14 anti-TB compounds on the UKMYC5 plate for all >30 000 samples collected [13].

## METHODS

### Culturing

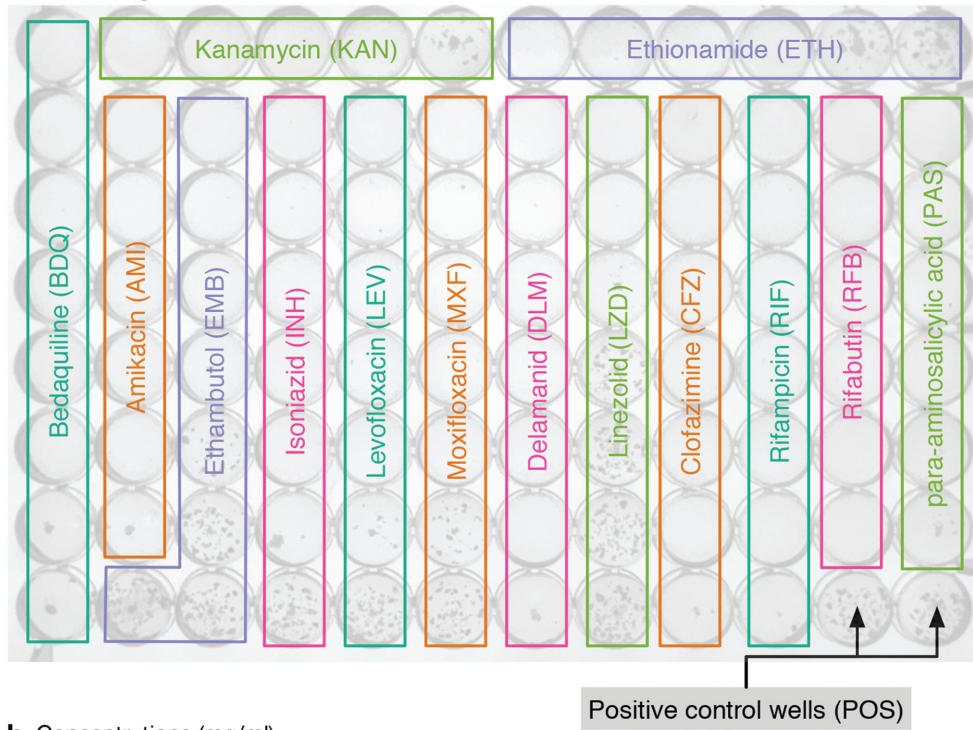
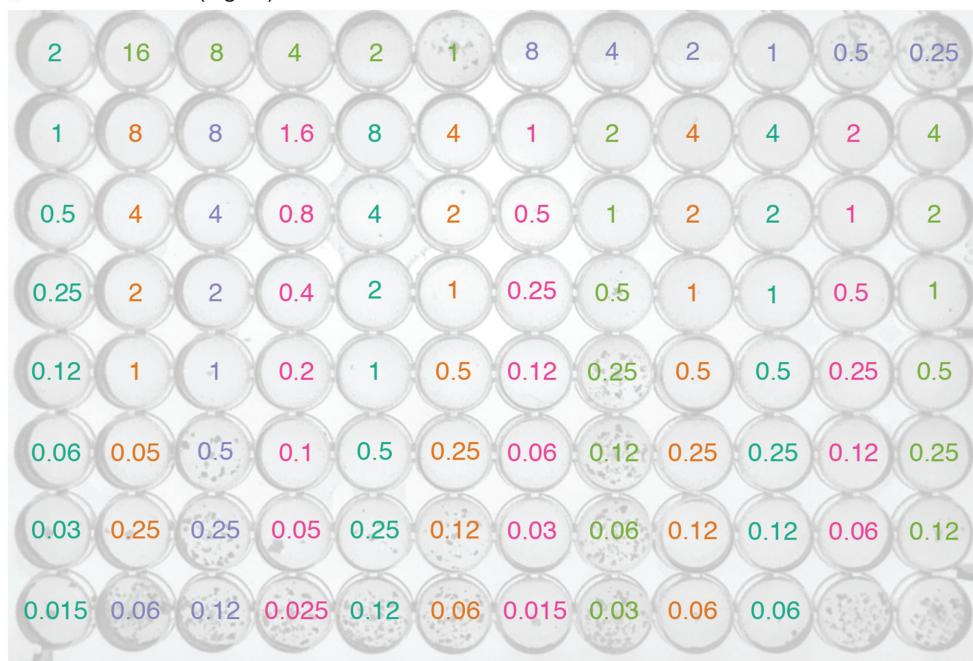
Each of the three laboratories received one screw-capped plastic cryovial with approximately 1000 µl of mycobacterial suspension, and sub-cultured 200 µl in a mycobacterial growth indicator tube (MGIT; Becton Dickinson). A second subculture step was then performed: 200 µl of a well-mixed MGIT broth was inoculated on a solid Löwenstein–Jensen (LJ) medium, and incubated at 37 °C for 3–5 weeks. The MTB reference strain H37Rv, American Type Culture Collection 27294, obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources), USA was used and the other strains were as described herein [13].

### Plate inoculation

Overall, 2 to 5 mg of growth was harvested from LJ media after 3 to 5 weeks' incubation, and re-suspended in 5 ml of saline solution, homogenized, and the supernatant density adjusted to a McFarland standard 0.5 suspension ( $\sim 1.5 \times 10^7$  c.f.u. ml<sup>-1</sup>). Then, 100 µl of this suspension was inoculated into a MGIT and 100 µl dispensed from the MGIT into each of the plate's 96 wells. Each plate was covered with an optically clear adhesive seal supplied by the manufacturer of the plate and transferred to aerobic incubation at 35–37 °C. The seal remains in place during the entire incubation period. More details can be found here [13].

### Plate reading by the Vizion Digital MIC viewing system

Fourteen days after inoculation, laboratory staff read the plate using a Thermo Fisher Vizion Digital MIC reading system. MICs were recorded in a database. Vizion images were then generated according to the manufacturer's guidance. Various combinations of Vizion lighting parameters – colour of background and level of illumination – were tested

**a** Anti-TB drugs and abbreviations**b** Concentrations (mg/ml)

**Fig. 1.** The UKMYC5 96-well dry microtitre plate contains 14 different anti-TB compounds and two positive control wells. (a) In addition to first-line (rifampicin, isoniazid and ethambutol) and second-line compounds, the plate contains repurposed (clofazimine and linezolid) and new (delamanid and bedaquiline) compounds. (b) Each drug has between five and eight wells forming a doubling dilution series and there are also two positive control wells, which contain no drug.

and, for the purposes of this study, the combination of 'white background' and 'level 7', respectively, was found to produce images with the highest contrast. Each image was

manually cropped so it would fit on the Vizion Read window screen, with the result that final images had as similar dimensions as possible. Images were stored locally as

lossless bitmap files (Fig. S1, available with the online version of this article) by all laboratories before uploading to the database. In one laboratory, the plate was then removed from the Vizion and a second laboratory scientist re-inserted the plate and took a second photograph (Fig. S13), which was also uploaded to the database.

## Software

AMyGDA is written in object-oriented Python3. Each image is stored as a numpy array [15] and all image processing is done using the OpenCV2 Python API [16]. To allow for straightforward metadata storage we use the datreant module [17]. The algorithm assumes that each well is inoculated in the centre and mycobacteria will grow out radially from the centre, and that the images are 8-bit greyscale, allowing quantification of intensity of each pixel (range 0 to 255). Dark pixels (with low numerical values) were assumed to represent bacterial growth and light pixels to represent no growth. Growth was quantified according to the number of dark pixels per well. The AMyGDA software can be downloaded from <http://fowlerlab.org/software/amygda> and the package includes all 15 images in Fig. S1 so the reader can reproduce Figs S2–S4 and S12 for themselves.

## Filtering, image processing and growth detection

The raw images tend to be noisy, lacking in contrast and unevenly illuminated (Figs 2a and S1). To correct for these problems a mean shift filter [18] is applied (Figs 2b and S2), followed by a contrast-limited adaptive histogram equalization filter (Figs 2c and S3) and finally the pixel histogram was normalized so that the mode has a pixel value of 180 (out of 255) and the fifth and ninety-fifth percentiles of the distribution occur at pixel values of 70 and 220 (again out of 255), respectively (Figs 2d and S4). The positions of the wells are then detected using a Hough transform optimized for circles as implemented in OpenCV; this is applied iteratively until only 96 circles are detected in the image. Next, the histogram of pixel intensities in a central circular region of each well is calculated and the proportion of bacterial growth inferred. If above a specified threshold, the well is labelled with a coloured circle that also defines the region analysed for growth. This process, including optimizing the choice of parameters, is described in detail in the online Supplementary Material. Finally, in addition to saving the MICs to disc, each image is annotated with the name and concentration of the drug in each well, providing a final composite image (Figs 2e, S11 and S12) that forms a complete, auditable record of the process.

## RESULTS

### Study design

Three TB reference laboratories (Birmingham, UK and Milan, Italy and Gauting, Germany) received 15 vials of *M. tuberculosis*. Fourteen of these were blinded and one contained the *M. tuberculosis* ATCC27294 H37Rv reference strain. The 14 blinded strains were sub-cultured by each lab isolate and inoculated onto two UKMYC5 plates;

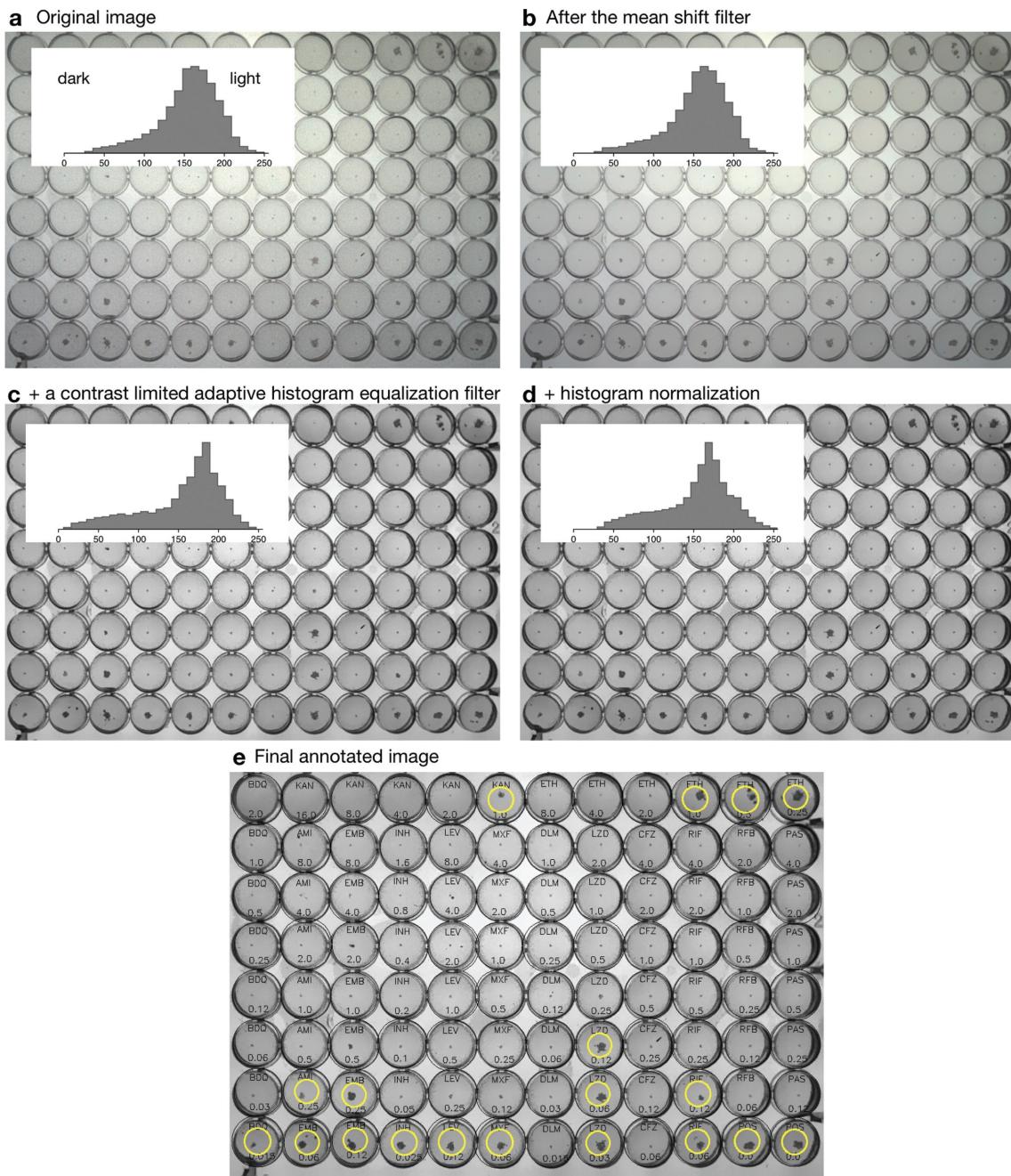
the H37Rv strain was sub-cultured and then inoculated onto 10 UKMYC5 96-well microtitre plates, making a total of 38 UKMYC5 plates per laboratory. After 2 weeks of incubation, the MICs of the 14 anti-TB drugs on each plate were read by a trained laboratory scientist using a Vizion instrument, then a photograph of the plate was taken, which was analysed at a later date by the AMyGDA software. To further test reproducibility, in one of the three laboratories a second scientist removed and re-inserted the plate into the Vizion and took a second photograph, which was also analysed by the software. In total, 114 UKMYC5 plates were inoculated and incubated; 38 of these were photographed twice and the remaining 76 were only photographed once, making a total of 152 images analysed producing 2128 MICs in all. The laboratory scientist and AMyGDA determined that *M. tuberculosis* appeared not to be growing in one or more of the two positive control wells of 13 and four plates, respectively. Three plates were determined by both the scientist and AMyGDA to be lacking growth in one or more of the control wells, making a total of 14 plates (12 %) flagged by one or both of the methods as having insufficient growth after 14 days of incubation. All 14 plates were therefore excluded from subsequent analysis. In total, 100 plates were considered, resulting in 1400 pairs of MICs – one measured by laboratory scientist and the other by AMyGDA. Out of these 100 valid plates, 37 plates were photographed twice and both images analysed by AMyGDA, resulting in 518 pairs of MICs that should, of course, be identical.

### Sources of error

AMyGDA can wrongly report growth in a well – usually due to the presence of one of a range of image artefacts – and it can miss existing growth where only small and/or faint patches of growth are apparent. A trade-off between sensitivity and specificity is hence inevitable as these sources of error are inversely coupled. Artefacts that systematically affect a region of a plate or even the entire plate are more challenging to avoid or detect; these include shadows (Fig. 3a) and remnants of the original inoculation ('sediment', Fig. 3b). Artefacts that occur more randomly usually result in nonsensical growth patterns – e.g. the bacteria appear to grow at high, but not low, concentrations of antibiotic – which can be detected by the software and flagged for further investigation. These include air bubbles (Fig. 3c), condensation (Fig. 3d), contamination (Fig. 3e) and possible failure of the integrity of a plate, e.g. evaporation of inoculum during incubation due to inadequate plate sealing (Fig. 3f). For more information, including a detailed description of how the parameters were set within AMyGDA to maximize its sensitivity and specificity, please see the online Supplementary Material.

### Reproducibility of the software

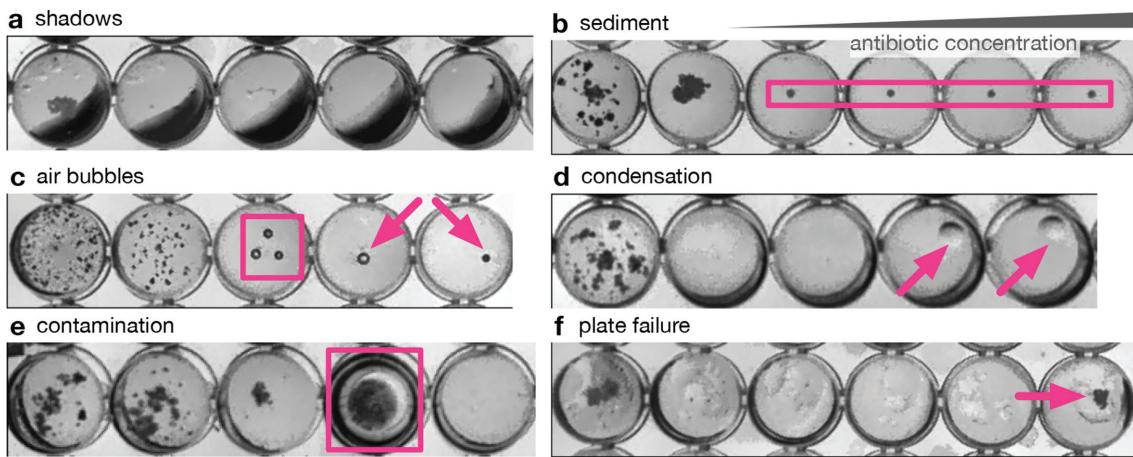
As expected, AMyGDA produced identical results when applied ten times to the same image and is therefore trivially reproducible (data not shown). Giving the software different



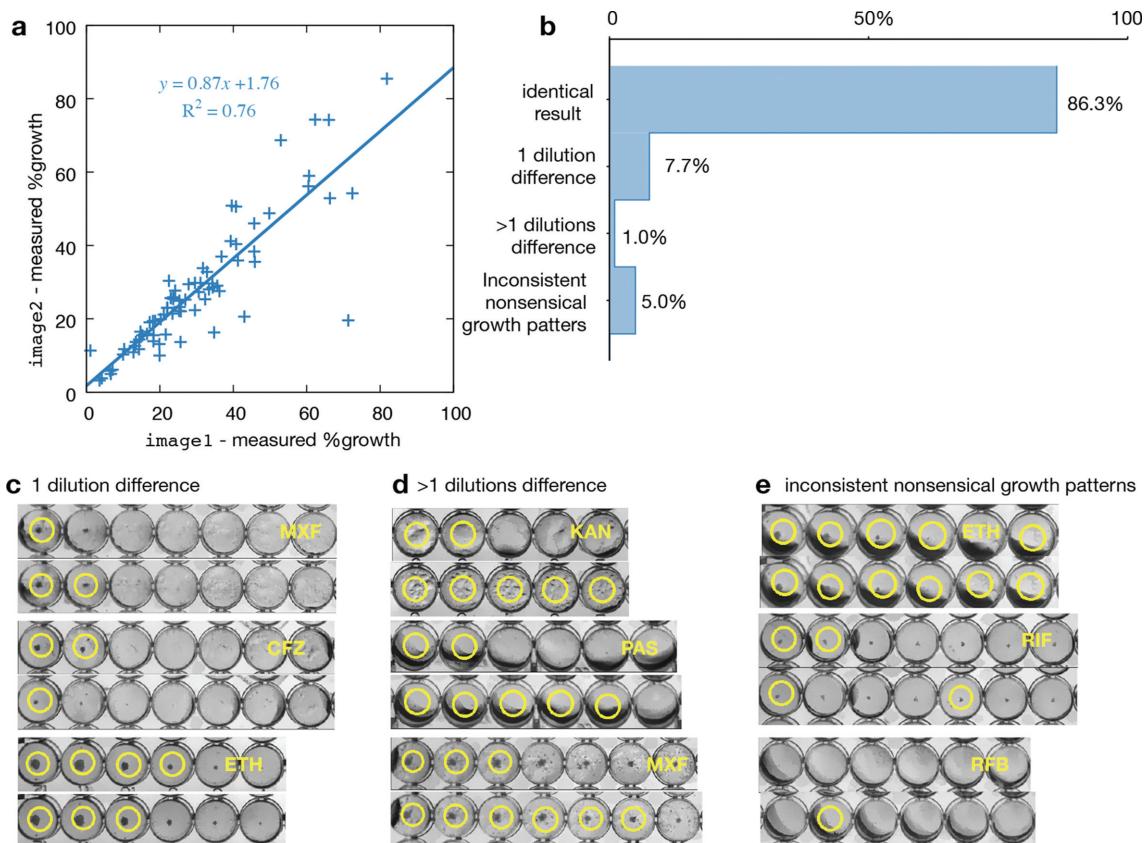
**Fig. 2.** The AMyGDA software produces a filtered, annotated composite image with all detected growth labelled. (a) The original grey-scale image is noisy and has poor contrast. To correct for this, (b) a mean shift filter, then (c) a contrast-limited adaptive histogram equalization filter are applied and finally (d) the pixel histogram is normalized as described in Methods. In all cases, the pixel histogram is shown. (e) The wells with detected bacterial growth are marked with a yellow circle and each well is labelled with its drug and concentration and the estimated circumference of the well is marked.

images of the same plate is a more stringent test of its reproducibility. Of the 38 plates for which two images were taken by two different laboratory scientists, one plate did not have growth in both control wells and another had two images that were obviously not of the same plate, leaving 36 UKMYC5 plates with two images available for this analysis,

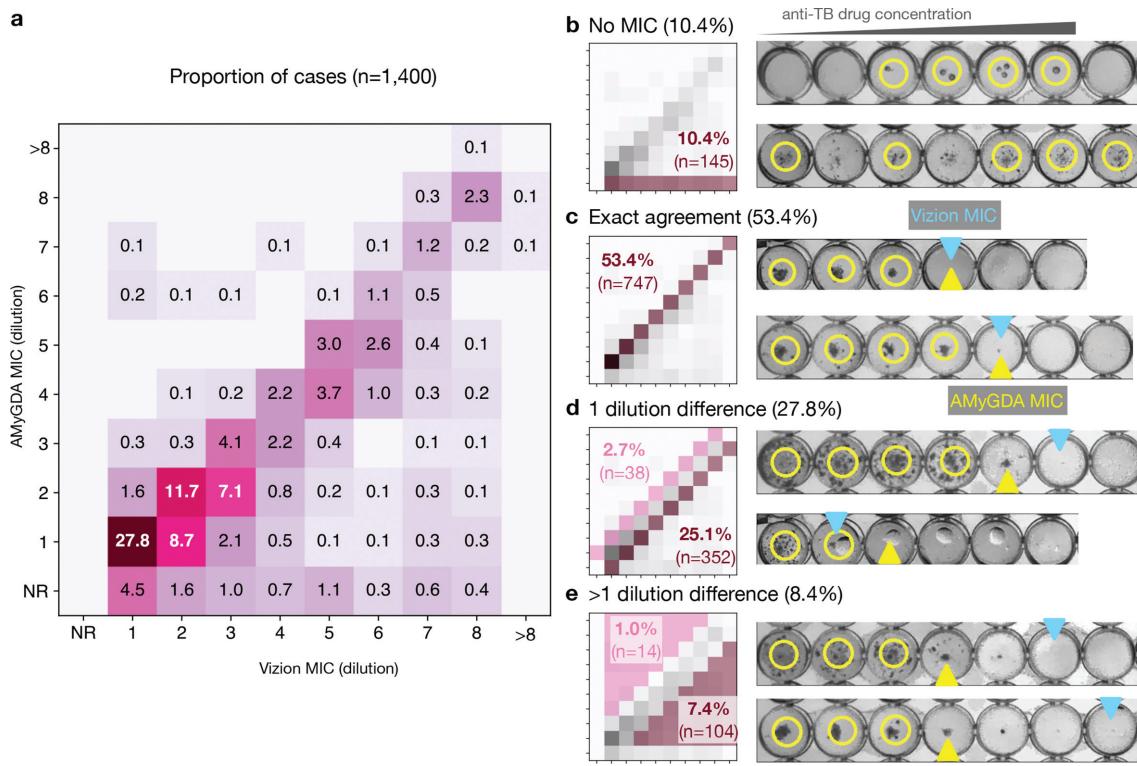
making a total of 504 pairs of MIC measurements that can be compared. The amount of detected growth in each of the control wells varied slightly from one image to the other (Fig. 4a). Although a linear fit explains the data well, there are outliers and there is clearly some scatter as evidenced by a coefficient of determination of 0.76.



**Fig. 3.** There are variety of artefacts that AMyGDA can mistake for growth, including (a) shadows, (b) sediment, (c) air bubbles, (d) condensation, (e) contamination and (f) possible failure of the plate integrity.



**Fig. 4.** Applying AMyGDA to the 36 valid plates (504 pairs of MICs) that were photographed twice (Fig. S13) measures the same MIC for 86 % of wells. (a) The growth measured in the control wells between both images is correlated. (c) There are 39 cases (7.7 %) where there is a single doubling dilution difference. (e) There are five cases where there are >one doubling dilution difference. (f) Finally, in 25 cases (5.0 %) a nonsensical growth pattern is returned for one image, but not the other.



**Fig. 5.** Results of the AMyGDA were validated by comparing to independent measurements made by laboratory scientists using the Vizion Digital MIC viewing instrument. (a) A heat map for all MIC doubling dilutions from all 100 valid UKMYC5 plates showing the concordance between the human-based measurement and the AMyGDA software. Note that since this is an aggregate of all 14 drugs, some of which are only present in 5, 6 or 7 dilutions, the distribution will be biased. Fourteen plates were excluded from this analysis as either or both measurements indicated that there was insufficient growth in one or both control wells. (b) The AMyGDA software identified nonsensical growth in 10.4% ( $n=145/1400$ ) of cases. In all those cases, the laboratory scientist was able to read an MIC using the Vizion instrument. (c) Both methods infer the same MIC in 53.4% of cases ( $n=747/1400$ ) with (d) a further 27.8% ( $n=390/1440$ ) within  $\pm$  one doubling dilution. (e) In only 8.4% of cases ( $n=118/1440$ ) do the methods disagree by more than a doubling dilution.

In 86.3 % of cases (435/504), AMyGDA estimated an identical MIC (Figs 4b and S13) when analysing a second image of the same plate, including 23 cases where a nonsensical growth pattern was detected in both images, namely where growth was detected in higher but not in lower concentration wells. The MIC measured by AMyGDA from the second image was only one doubling dilution different in 7.7 % (39/504) of cases (Fig. 4c). Examining the images in more detail shows that these cases were a mixture of wells, which either had little growth or artefacts. Five MICs (1.0 %) measured from the second image were  $>1$  doubling dilutions different to the MIC measured from the first image (Fig. 4d), due again to artefacts or low growth. The differences in the remaining 5.0 % (25/504) of MICs pairs were when the algorithm detected a nonsensical growth pattern in one image, but not in the other.

There is no international standard for Mycobacterial anti-microbial-susceptibility testing (AST), therefore there is no agreed threshold that any new AST method must clear before it can be considered for clinical use. We shall therefore apply with caution the standard defined for aerobic

bacteria [19]. This states that the proportion of any readings within a doubling dilution of the mode must be  $\geq 95\%$ . On the basis of this dataset comprising 36 UKMYC5 plates, AMyGDA achieves a reproducibility of 94.0 % (474/504), which is promising but not yet conclusive.

#### Validation against measurements made by a laboratory scientist

As mentioned above, prior to analysis by AMyGDA, all UKMYC5 plates were read by a trained laboratory scientist using the Vizion Digital MIC instrument, allowing us to compare the MICs obtained by both methods (Fig. 5a). There were no systematic differences in the MICs measured by all three laboratories.

The AMyGDA software was unable to return an MIC in 145/1400 of cases (10.4 %) due to nonsensical growth patterns (Table 1). These were either due to artefacts being incorrectly classified as bacterial growth or limited bacterial growth ‘confusing’ the algorithm (Fig. 5b). In all cases, the laboratory scientists were able to discern an MIC, demonstrating the superior ability of a human to identify and

**Table 1.** Validation of the AMyGDA software by comparing the measured MIC to that determined by a laboratory scientist using the Vizion Digital MIC viewing instrument. If we exclude the cases where AMyGDA was unable to determine an MIC, then the MICs measured by AMyGDA and the laboratory scientist were within one doubling dilution of each other in 90.6 % (1137/1255) of cases

Description	No. of cases	Proportion of cases
AMyGDA unable to determine an MIC	145	10.4 %
AMyGDA and laboratory scientist measure the same MIC	747	53.4 %
AMyGDA measures MIC one doubling dilution lower than the laboratory scientist	352	25.1 %
AMyGDA measures MIC one doubling dilution higher than the laboratory scientist	38	2.7 %
The MICs measured by AMyGDA and the laboratory scientist are two or more doubling dilutions different	118	8.4 %

ignore artefacts. In 747/1400 (53.4 %) of cases, both methods gave exactly the same MIC (Table 1, Fig. 5c). In 352/1400 (25.1 %) of cases AMyGDA reported an MIC one doubling dilution lower than the laboratory scientist. In contrast, in only 38/1440 (2.7 %) of cases did the software predict an MIC one doubling dilution higher than the scientist (Table 1, Fig. 5d). This disparity is due to the relatively conservative setting of the growth classification parameters to minimize the detection of artefacts as described in the online Supplementary Material. Finally, in 118/1440 MICs (8.4 %), the laboratory scientist and the AMyGDA software assigned MICs that were two or more dilutions different (Table 1, Fig. 5e), usually because there was little growth in the wells containing higher concentration of drug.

There is no accepted threshold for accuracy due to the lack of a Mycobacterial AST standard, so again we shall tentatively apply the criteria any new AST method for aerobic bacteria must satisfy when its results are compared to those of an accepted AST method [19]: the new method must be in essential agreement in  $\geq 90$  % of cases. Essential agreement is defined as the two MICs lying within a single doubling dilution of one another. If we exclude the cases where AMyGDA is unable to return a reading, the software has an essential agreement of 90.6 % (1137/1255) with the Vizion readings (Table 1). This should not be taken as evidence that the software is therefore sufficiently accurate to be deployed clinically, however, since there are several problems with this analysis that we will discuss shortly.

## DISCUSSION

We have shown how AMyGDA, a Python package, can detect and measure the growth of *M. tuberculosis* in images of a 96-well microtitre plate, each having been inoculated and incubated with one of 15 known TB strains, one of which was the H37Rv *M. tuberculosis* strain. Here we used a variant of the standard Thermo Fisher Sensititre MYCOTB 96-well plates, UKMYC5, that has been designed by the international CRyPTIC tuberculosis consortium and uniquely includes two repurposed (clofazimine and linezolid) and two new (bedaquiline and delamanid) compounds. Since UKMYC5 contains 14 different anti-TB compounds, each forming a doubling dilution series, the software therefore can determine the MIC for each of the 14

drugs on the UKMYC5 plate. AMyGDA can be downloaded from <http://fowlerlab.org/software/amygda>.

Ideally one applies an external set of success criteria when assessing the effectiveness of new software. As there are no AST standards for Mycobacteria, we have tentatively applied an international AST standard for aerobic bacteria [19]. This is a stringent test since any new AST test must pass the appropriate standard before it can be considered for use clinically. The results indicate that AMyGDA is suitable for use in a high-throughput research setting. It would be inappropriate, however, to conclude that AMyGDA is suitable for deploying in clinical microbiology laboratories. For that to be determined, a much larger and more comprehensive validation study using clinical isolates will be required to more systematically evaluate the accuracy and reproducibility of the AMyGDA software.

The CRyPTIC project has therefore adopted AMyGDA primarily to assist in the quality control and identification of discrepancies in readings of MICs taken by laboratory scientists. Since photographs will be taken of all plates after 2 weeks' incubation, the intention is to retrospectively analyse all these images and, by merging with the measurements taken by the expert, identify and investigate plates where there is insufficient agreement between the MICs measured by human and computer. In this way we hope to identify measurement errors and so minimize the biases and errors in the large phenotype dataset that the CRyPTIC project is collecting. This dataset will also, we hope, be sufficiently large enough to conclusively determine, given a Mycobacterial AST standard, whether AMyGDA is sufficiently reproducible and accurate for clinical use.

Since AMyGDA measures the amount of growth in each well, it also potentially opens up new research questions. For example, as the concentration of drug is increased, how does the apparent growth of *M. tuberculosis* change from well to well? Or, more ambitiously, by linking to the genomic data that is being collected, can we identify strains or mutations that grow quickly or better (at least on the UKMYC5 plate)? These and other questions will be the focus of future studies.

An additional use for this and similar automated plate-reading software could be for high-throughput phenotypic screening for *M. tuberculosis* drug discovery. Unlike a

human, the software is quantitative and so could, given enough samples, detect small changes in the rate of growth due to mild inhibition, enabling the use of microtitre plates to identify potential leads for novel antibiotic compounds, which otherwise would be missed by high-throughput phenotypic screening of a compound- or fragment-library.

Conventionally culture-based methods are read after a fixed time period: here 2 weeks following inoculation [13]. We observed, however, significant variation in the amount of growth between plates inoculated with the same strain, as well as variation between different strains. Software, like AMyGDA, could instead prospectively monitor the growth in the control wells during the incubation period, allowing a plate to only read once it is deemed to have sufficient growth ('read-when-ripe'), further reducing the mean time between sample collection and result.

There are a number of limitations to our study in addition to the ones mentioned above. The Vizion was not designed to capture photographs that could be processed by software and hence it has uneven illumination and the digital camera is, by today's standards, of low resolution. An alternative, cheaper way of capturing images with more diffuse illumination and a higher resolution camera would help considerably. Secondly, whilst we have minimized the false detection of artefacts, they remain a problem, especially those that tend to systematically afflict whole regions of a plate. Improved experimental protocols may help avoid some of these (e.g. air bubbles) and a re-design of the plate layout would help with others (e.g. shadows).

In conclusion, combining computer software, such as AMyGDA, with microtitre plate-based MIC measurement can facilitate high-throughput culture-based drug susceptibility testing for tuberculosis.

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

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