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Resazurin reduction assays for screening of anti-tubercular compounds against dormant and actively growing *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*

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***Objectives*: To develop simple, rapid, low-cost and robust assays for screening drugs against dormant and actively growing mycobacteria.**

***Methods*: Actively growing aerobic and hypoxia-adapted dormant cultures of *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG and *Mycobacterium smegmatis* were tested for susceptibility to standard antimicrobial drugs by resazurin reduction assay. The visual and fluorimetric MICs were compared with those obtained by the standard cfu assay.**

***Results*: Drug MICs for *M. tuberculosis* and *M. bovis* BCG were determined by the aerobic resazurin microplate assay (REMA) and correlated well with those obtained by the cfu assay. Metronidazole and nitrofurans showed comparable bactericidal activity in the hypoxic resazurin reduction assay (HyRRA). The HyRRA assay was noted to be superior to the cfu assay in that it distinguished between metabolically active dormant bacteria and non-viable organisms, unlike the cfu assay that could not differentiate between these two populations. The HyRRA assay performed with good concordance in both fluorimetric and visual formats to distinguish between bactericidal and bacteriostatic effects of a drug.**

***Conclusions*: The REMA and HyRRA assays will be useful for anti-tubercular anti-dormancy compound screening and drug susceptibility testing in a safe, reliable, easy and cost-effective manner particularly in low resource countries. The application of the assays in *M. smegmatis* or *M. bovis* BCG offers the distinct advantage of rapidly and safely screening anti-tubercular compounds in a high-throughput format.**

Keywords: *M. tuberculosis*, *M. bovis* BCG, *M. smegmatis*, dormancy, REMA, HyRRA

Introduction

Effective tuberculosis (TB) treatment is dogged by multiple challenges. First, anti-tubercular treatment over a prolonged period (6–8 months) is associated with a significant degree of non-compliance among patients, which has contributed to the emergence of multidrug-resistant strains that are difficult to eradicate. Another challenge is to eliminate tubercle bacilli from the huge reservoir of latently infected individuals who stand the risk of progressing to active TB. Therefore, there is an urgent need to develop novel drugs to treat latent TB infections. New drug discovery is dependent on whole cell assays to reliably screen for compounds with anti-dormancy, anti-tubercular activities. Until recently, the physiology of dormant tubercle bacilli was poorly understood and they were believed to persist in an ill-defined non-replicative state for indefinite periods of time only to reactivate later, sometimes even decades after the primary infection, upon weakening of the host immune response. In a hypoxia-induced *in vitro* model of dormancy, bacteria were resistant to the standard antimycobacterials but susceptible to metronidazole.[1,2] Subsequently, nitrofurans were shown to inhibit actively growing and dormant *Mycobacterium bovis* BCG.[3]

The search for drugs targeting dormant bacteria is hampered by the limitations of the currently used cfu assay. A resazurin colorimetric assay enables rapid drug susceptibility testing of mycobacterial isolates under aerobic conditions, whereby the blue non-fluorescent oxidized form of the dye is reduced within

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the environment of viable cells to form resorufin, which is pink and fluorescent.[4,5] We combined the resazurin reduction assay with an *in vitro* model of mycobacterial dormancy to develop assays for screening anti-dormancy anti-tubercular compounds under hypoxic conditions. Because of the simplicity of testing, rapid growth rate, non-pathogenic nature of *Mycobacterium smegmatis* and its capacity to adapt to hypoxia, [6,7] we standardized the aerobic and hypoxia-based assays in *M. smegmatis* and also extended them to *M. bovis* BCG. Unlike many other laboratories which test only one or two critical concentrations of drug, we tested anti-tubercular drugs over a wide concentration range to correctly determine the drug MICs and establish the robustness of the assays developed.

Materials and methods

Bacterial strains and growth conditions

To prepare stocks, *Mycobacterium tuberculosis* H37Rv (henceforth referred to as H37Rv), *M. bovis* BCG (henceforth referred to as BCG) and *M. smegmatis* mc^{2}155 were grown to logarithmic phase (OD_{595} ~0.5) in Middlebrook 7H9 broth supplemented with 10% albumin dextrose complex (ADC) (7H9-S for H37Rv) or Dubos broth supplemented with 10% ADC (Dubos-S for BCG and *M. smegmatis*); 0.05% glycerol and 0.05% Tween 80 were also added to the media although no glycerol was added for BCG culture. Stocks were prepared by harvesting the bacteria, resuspending them in one-fifth volume of the original culture and storing in aliquots at −80°C. For resazurin microplate assay (REMA) and hypoxic resazurin reduction assay (HyRRA), the bacterial stock was subcultured in 7H9-S or Dubos-S medium supplemented with 0.05% glycerol and 0.05% Tween 80 under aerobic conditions with shaking at 190 rpm to logarithmic phase (OD_{595} ~0.5). The culture was resuspended by passaging it 10–15 times through a $26\frac{1}{2}$ gauge needle and diluting in growth medium (without Tween 80) to OD_{595} ~0.001 for aerobic assays; or OD_{595} ~0.003 for H37Rv/BCG and OD_{595} ~0.001 for *M. smegmatis*, respectively, for hypoxic assays. All cultures were grown at 37°C in a shaker incubator.

Drug and reagent preparation

Rifampicin, streptomycin, ethambutol, isoniazid, ethionamide, ofloxacin, *p*-aminosalicylic acid, metronidazole, nitrofurantoin, nitrofurazone and furaltadone were procured from Sigma. Drugs were solubilized according to manufacturers' recommendations, and stock solutions were filter sterilized and stored in aliquots at −20°C. Resazurin sodium salt powder (Acros Organics, Belgium) was prepared as a 0.02% w/v solution in sterile distilled water and filter sterilized; it was stored at 4°C for not more than 10 days.

REMA

Drug susceptibility testing (DST) using resazurin was performed under aerobic conditions in a similar manner to that described for *M. tuberculosis* by Palomino *et al.*[5] with minor modifications. The assay was performed in black, clear-bottomed, 96-well microplates (catalogue no. 353948; Becton Dickinson, USA). Initial drug dilutions were prepared in either DMSO or sterile deionized water, and subsequent 2-fold serial dilutions were performed in 0.1 mL of 7H9-S/Dubos-S medium supplemented with 0.05% glycerol (without Tween 80) in the microplates. Approximately 5×10^4 cfu was added per well in a volume of 0.1 mL. Control wells contained bacteria only (B), medium only (M) and drug only (to detect autofluorescence of drug) and were used to calculate percentage inhibition of viability (see below). The plates were incubated at 37°C for varying periods (see the Results and discussion section). Thereafter, 30 μL of 0.02% resazurin and 12.5 μL of 20% Tween 80 were added. The wells were observed after 24 and 48 h for a colour change from blue to pink and fluorescence of control wells ≥50 000 relative fluorescence units (RFU). The assay was also standardized for BCG and *M. smegmatis*. Fluorescence was measured by excitation at 530 nm and emission at 590 nm using Gemini XS spectrofluorimeter (SpectraMAX) in bottom reading mode. Visual MIC was defined as the lowest concentration of drug that prevented a colour change. For fluorimetric MIC, background fluorescence from medium (M) and drug wells was subtracted. Percentage inhibition of viability was defined as: 1−(test well fluorescence/mean fluorescence of triplicate B wells)×100 as described previously.[8] The lowest drug concentration effecting an inhibition of 90% was considered as the MIC.

HyRRA

For the HyRRA assay, 3 mL culture aliquots (A_{595} ~0.003 containing ~1.5×10^6 cfu/mL for H37Rv/BCG or A_{595} ~0.001 containing ~5×10^5 cfu/mL for *M. smegmatis*), were injected into 5 mL uncoated vacutainer tubes (Becton Dickinson, USA, catalogue no. 367614) and kept static at 37°C to allow for self-generation of hypoxia in the cultures. Fading/decolorization of Methylene Blue (at final concentration of 1.5 mg/L) was used to determine hypoxia/anoxia. Various drugs were subsequently injected (100 μL/tube) at different concentrations and the tubes were further incubated for 96 h at 37°C under static conditions. Metronidazole (active only on anaerobically grown organisms) and isoniazid (acting only under aerobic conditions),[2,9] were used to confirm the existence of anoxic culture conditions. Three hundred and fifty microlitres of 0.02% resazurin and 100 μL of 20% Tween 80 were injected into each tube and the tubes were incubated overnight. Two hundred microlitres culture aliquots were transferred to a 96-well black microplate and fluorescence determined as described above. Thereafter visual and fluorimetric MICs were determined for the test drugs in the same manner as for aerobic REMA.

Determination of MIC by cfu assay

On day 7 (for H37Rv and BCG) or day 2 (for *M. smegmatis*) of incubation in the aerobic REMA assay and on day 4 of incubation post-drug addition for all three species in the HyRRA assay, cfu assay was performed to determine drug MICs. Briefly, 10-fold serial dilutions were plated on 7H10-S agar. The plates were incubated at 37°C and colony counts were finalized after 6 weeks of incubation. MIC was considered to be the lowest concentration of drug that permitted growth of ≤1% of the bacteria in comparison with that observed in the B wells/tubes on day zero.

Results and discussion

The development of a hypoxia-induced *in vitro* model of dormancy in *M. tuberculosis* by Wayne,[10] has provided a valuable experimental system to understand the dormancy response and also offers an opportunity to develop screening assays for anti-dormancy anti-tubercular drugs. BCG and *M. smegmatis* have also been shown to transition into a non-replicative dormant state under conditions of oxygen limitation.[6,7,11] In recent years,

redox dye-based aerobic assays (Alamar Blue assay, tetrazolium-based assay, resazurin reduction assay) have been used to assess drug susceptibility of *M. tuberculosis* isolates.[4,5,8,12–15] Here we combined the *in vitro* model of dormancy with the resazurin reduction assay to develop robust whole cell assays to screen for anti-dormancy anti-tubercular compounds using H37Rv, BCG and *M. smegmatis*.

Experimental set-up for aerobic and anaerobic testing of antimycobacterial compounds

Resazurin reduction assays were developed with aerobic (REMA) and hypoxia-adapted mycobacteria (HyRRA) to screen for drugs and compounds active against growing and dormant mycobacteria. The parameters that were standardized included initial culture density, drug exposure time and duration of incubation post-resazurin addition. The use of black microtitre plates permitted measurement of fluorescence with a minimum background and subsequent MIC determination post-resazurin reduction. When we used an initial cell density ranging between OD_{595} ~0.003–0.005, the drug MICs in the H37Rv REMA assay were noted to be higher than the previously reported MICs [8,15,16] (data not shown). MICs were similar to the reported values when the assay was optimized with $\sim 5 \times 10^4$ cells per well (OD_{595} ~0.001) and a post-drug incubation period of 7 days and 1 day for H37Rv/BCG and *M. smegmatis*, respectively. With *M. smegmatis*, exposure of the culture to drugs for 1 or 2 days followed by an overnight or 2 h incubation with resazurin, respectively, yielded identical drug MICs.

For the HyRRA assay, an initial cell density of 0.003 OD ($\sim 1.5 \times 10^6$ cfu/mL) and 0.001 OD ($\sim 5 \times 10^5$ cfu/mL) was optimal for H37Rv/BCG and *M. smegmatis*, respectively. Vacutainer tubes were ideal for HyRRA as they are leak proof and have self-sealing rubber septa that can withstand multiple needle punctures necessary for introduction of culture/drug/Tween 80/resazurin. Anaerobic conditions within the tubes were established within 30–32 days or 5 days for H37Rv/BCG and *M. smegmatis*, respectively, and a drug exposure time of 96 h was allowed for the dormant cultures. Post-resazurin addition, the microplate/vacutainer tubes were incubated overnight (16–20 h) until the dye colour changed from blue to pink and the fluorescence in control cultures reached 45 000–50 000 RFU [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. This permitted a clear and distinguishable readout to determine loss of viability due to drug activity.

Comparative MIC determination under aerobic and anaerobic conditions

In general, there was a fairly good correlation between the MICs obtained by aerobic REMA (visual and fluorimetric methods) and the cfu assay against H37Rv/BCG and MICs were well within the range of previously reported MICs (Table 1). A slight variation in the MICs was noted with streptomycin, ethambutol and isoniazid; for these drugs the MICs obtained were one/two 2-fold dilutions lower by the cfu assay than the REMA assay. This may be attributable to more favourable growth conditions provided by liquid versus solid culture medium to drug-treated bacilli which may be compromised in their physiology. Furthermore, all the nitrofurans showed substantial bactericidal activity in the aerobic REMA assay; these results were in agreement with previous observations,[3] in BCG by the cfu assay. MICs of streptomycin, ethambutol and ofloxacin against the fast-growing mycobacterium *M. smegmatis* matched with those obtained for H37Rv and BCG and also correlated well among the REMA and cfu assays. The MIC obtained by both REMA and cfu assays for rifampicin was higher than that obtained with H37Rv and BCG but similar to a previous finding in *M. smegmatis*.[17] For isoniazid, ethionamide and *p*-aminosalicylic acid, higher MICs were obtained by the REMA assay. Since the MICs for these drugs by the cfu assay were comparable to those for slow-growing mycobacteria, the apparently discrepant REMA MICs may be ascribed to the rapid growth of *M. smegmatis* in the liquid medium with an associated overexpression of the target genes,[18,19] and not due to inherent differences in the mechanism(s) of drug action between the species. Broadly speaking, the efficiency and the advantages of using *M. smegmatis* for the rapid and safe screening of anti-tubercular compounds outweigh the quantitative discrepancies in MICs that may be noted between H37Rv and the surrogate strain.

Four drugs including metronidazole, nitrofurazone, furaltadone and nitrofurantoin, were used to develop the hypoxic HyRRA assay, as they were previously reported to exhibit anti-mycobacterial activity under anoxic conditions.[2,11] In the presence of metronidazole at 1711.6 mg/L (10 mM) the cultures remained blue post-resazurin addition and thereby confirmed bactericidal activity of metronidazole against dormant mycobacteria (Figure S1). Furthermore, isoniazid-treated H37Rv cultures remained pink even at test concentrations that were >10-fold higher than the MIC for aerobically cultured H37Rv (Figure S1); this confirmed the dormant nature of hypoxia-adapted cultures in the HyRRA assay. At higher concentration of nitrofurans, a concordance in drug MICs was noted for HyRRA and cfu assays (Figure 1a). However, at 10-fold lower drug concentrations, a disparity was noted for some of the drugs. For instance, by the cfu assay >99% loss of H37Rv viability was noted in the presence of 9.9 mg/L (50 μM) of nitrofurazone; however, by the HyRRA assay only 49% loss of viability was observed. Similarly, only 43% and 15% loss in viability was observed in the presence of 171.16 mg/L (1 mM) and 85.58 mg/L (500 μM) of metronidazole in contrast to 95% and 89% inhibition obtained with the cfu assay. On the other hand, a consensus in MIC was noted with nitrofurantoin by both the assays. The differences in MICs by the two assays might be explained by the fact that dormant cultures of H37Rv were unable to grow on solid medium plates but were maintained in viable form in broth cultures.[20] Therefore, broth culture-based HyRRA assay truly reports the loss of viability in drug-treated dormant cultures and determines the MICs more accurately. Analogous drug assays were also established for BCG and *M. smegmatis* and MICs of test drugs were comparable to those obtained for H37Rv (Figure 1b and c). In *M. smegmatis* MICs of nitrofurans were more than 10-fold higher (≥500 μM) by the HyRRA assay in comparison with those determined by the cfu assay.

A useful feature of the HyRRA assay is its ability to distinguish between the bactericidal or bacteriostatic effect of a drug. An overnight incubation of drug-treated dormant culture with resazurin reported bacterial viability by a change in colour of the redox dye (Figure S1). Prolonged incubation of these

Table 1. Comparative MICs of 11 anti-tubercular drugs for *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis* determined by resazurin microtitre plate assay (REMA) and conventional plate counts (cfu) under aerobic conditions

	MIC (mg/L)[a]									Previously determined MIC for *M. tuberculosis* by Alamar Blue assay (mg/L)[b]	Previously determined MIC for *M. tuberculosis* by proportion method (mg/L)[b]
	M. tuberculosis H37Rv			*M. bovis* BCG			*M. smegmatis* mc^{2}155				
Drug	visual REMA	fluorimetric REMA	cfu technique	visual REMA	fluorimetric REMA	cfu technique	visual REMA	fluorimetric REMA	cfu technique		
STR	0.125	0.125	<0.03	0.25	0.25	0.25–0.5	0.25	0.25	≤0.5–2	0.5–2	2–3
ETM	1	1	0.5–0.25	0.5	0.5	0.5	0.5	0.5–2	≤4–5	4–5	5–7.5
RIF	0.002	0.002	0.002	0.002	0.002	0.004–0.008	0.5–1	0.5–1	0.25–1	0.05–0.2	0.01–1
INH	0.125	0.125	<0.031	0.063	0.063	0.015	4	4	≤0.06–0.125	0.06–0.2	0.01–0.2
OFX	0.25	0.25	0.25	0.5	0.5	0.5	0.5–1	0.5–1	0.5–1	0.5–1	0.5–1
ETH	0.63	0.63	0.63	0.63	0.63	0.63	10	10–20	0.63–2.5	0.63–1.25	0.31–2.5
PAS	1	1	4	1	1	4	512	256–512	0.5–>2	0.5–>2	0.25–8
NFS	49.5	49.5	ND	ND	ND	ND	49.5	49.5	ND	ND	ND
FLD	81.1	81.1	ND	ND	ND	ND	81.1	81.1	ND	ND	ND
NIT	29.8	29.8	ND	ND	ND	ND	29.8	29.8	ND	ND	ND
MTZ	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	ND

RIF, rifampicin; ETM, ethambutol; STR, streptomycin; INH, isoniazid; OFX, ofloxacin; ETH, ethionamide; PAS, *p*-aminosalicylic acid; NFS, nitrofurazone; FLD, furaltadone; NIT, nitrofurantoin; MTZ, metronidazole; ND, not done; NI, no inhibition.

MIC is defined as lowest concentration of drug/test compound that results in ≥90% inhibition of viability by REMA assay and ≥99% loss of viability by the cfu assay.

[a]Data are ranges of six independent experiments. In cases where one or more experiments yielded an MIC greater/less than the highest/lowest tested concentration, the MIC is expressed as a '>/<'value, respectively.

[b]According to Yajko *et al.*,[15] Collins and Franzblau[8] and Collins *et al.*[16]

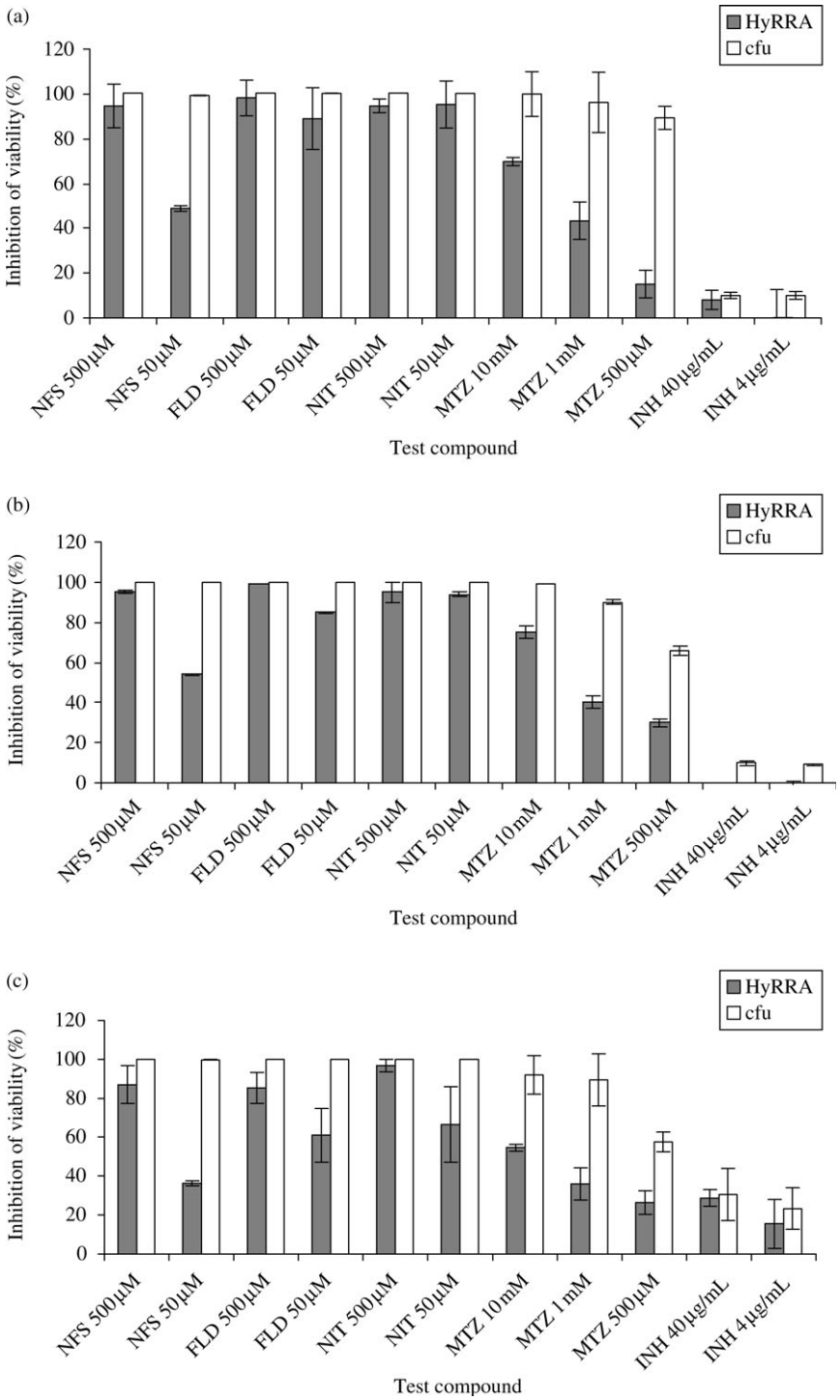

Figure 1. Comparison of the HyRRA assay and the cfu assay. The percentage inhibition of viability observed in the presence of nitrofurans, metronidazole and isoniazid in hypoxia-adapted dormant bacilli is shown for: (a) *M. tuberculosis*; (b) *M. bovis* BCG and (c) *M. smegmatis*. Results are shown as averages ± SD of three independent experiments. NFS, nitrofurazone; FLD, furaltadone; NIT, nitrofurantoin; INH, isoniazid; MTZ, metronidazole.

tubes often resulted in further reduction of the dye by the slowly metabolizing dormant cultures. For example, dormant H37Rv culture exposed to 119.08 mg/L (500 μM) nitrofurantoin remained blue after 1 and 5 days of resazurin addition (Figure S1); this established nitrofurantoin at 119.08 mg/L (500 μM) to be bactericidal. In contrast, cultures incubated with 99.07 mg/L (500 μM) nitrofurazone were blue after 1 day of incubation with resazurin but changed to pink after 5 days, indicating that nitrofurazone at this concentration only slowed down cellular metabolism and was not bactericidal. These subtle differences were not evident from the cfu assay. For example, Murugasu-Oei and Dick,[3] determined 500 μM nitrofurazone to be bactericidal against BCG by cfu assay. The same interpretation would be made in the present study if viability loss in cfu

assays were matched. Therefore, the HyRRA assay is advantageous and simply informs about the bactericidal or bacteriostatic nature of compound by incubation of tubes for a longer time period (5 days).

Comparison between visual and fluorimetric resazurin reduction assay

The visual assay offers several advantages over the fluorimetric assay. A complete correlation in the MICs was obtained by visual and fluorimetric REMA and HyRRA assays for all the test drugs in all three mycobacterial species (Table 1 and Figure S1). The fluorimetric assay requires additional plasticware, expensive instrumentation and maintenance, thereby increasing the cost of assay. Furthermore, transfer of the culture to microplates for measurement carries the associated risk of aerosol generation. Therefore, visual REMA/HyRRA can be safely used for performing DST in mycobacteria.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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