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Development of a microdilution method to evaluate *Mycobacterium tuberculosis* drug susceptibility

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A new rapid microdilution method, employing the dye resazurin as an indicator of mycobacterial growth, was developed to evaluate drug susceptibility of *Mycobacterium tuberculosis* reference strain H37Rv and of 13 *M. tuberculosis* susceptible or multidrug-resistant clinical strains. Different growth conditions were evaluated. The MICs of isoniazid, rifampicin, streptomycin and ethambutol were determined by the Microdilution Resazurin Assay (MRA) and the results compared with those obtained by the agar proportion method; complete agreement was always obtained. MRA resulted in a rapid, reliable, simple and inexpensive coloured method suitable for testing the susceptibility of *M. tuberculosis* clinical strains to first-line drugs; its employment in evaluating new antibacterial molecules is also suggested.

Keywords: *M. tuberculosis*, resazurin, MIC evaluation, microdilution assay, first-line antitubercular drugs

Introduction

Tuberculosis is the leading cause of death due to a single infectious agent; furthermore, the most worrisome trend in recent years is the increase in multidrug-resistant tuberculosis strains.[1] There is a need for rapid determination of the drug susceptibility of clinical isolates of *Mycobacterium tuberculosis*. Culture-based techniques on solid media for susceptibility testing take almost 3 weeks to give results due to the slow growth rate of *M. tuberculosis* clinical isolates. Liquid culture systems, including BACTEC MB/BacT and MGIT, enable laboratories to determine *Mycobacterium* susceptibility to first-line drugs within 1–2 weeks incubation time.[2–4] Molecular methods for the detection of antibiotic resistance of *M. tuberculosis* complex strains are not simple, are expensive and have insufficient predictive value.[5] Some other methods have been developed with variable results and costs.[6–8] Alamar Blue, an oxidation–reduction indicator, has been used to evaluate the antifungal, antimicrobial or antitubercular activity of drugs[9–12] and to measure cell proliferation and cell toxicity.[13,14] For the assessment of cell toxicity and cell viability, identical results were obtained using resazurin and Alamar Blue.[15]

The aim of this study was to develop a new, simple and rapid microdilution plate method, employing resazurin to evaluate the MICs of the first-line antitubercular drugs isoniazid, rifampicin, ethambutol and streptomycin for *M. tuberculosis* reference and clinical strains. Resazurin is an oxidation–reduction indicator used for the evaluation of cell proliferation and microbial growth.[16,17] It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. A resazurin reduction test has also been used for decades to demonstrate bacterial and yeast contamination of milk.[18,19]

We investigated the growth conditions of different *M. tuberculosis* strains, the growth incubation time of each culture, the concentration of mycobacterium inoculum, and the incubation time of the microplate before and after resazurin addition. The results obtained were compared with those obtained by means of the reference agar dilution method; the accuracy of the method was verified by viable counting from the test wells.

Materials and methods

Bacterial strains and culture conditions

Thirteen *M. tuberculosis* strains were clinical isolates cultured from specimens submitted to the 'Modulo di Microbiologia Polmonare', Ospedale di Cattinara, Trieste, Italy, for investigation of mycobacterial disease, and consisted of respiratory isolates from different patients. Strains were identified by classical culture and biochemical characteristics and by DNA probe (GeneProbe, San Diego, CA, USA); *M. tuberculosis* reference strain H37Rv was also employed. All strains were maintained on Lowenstein–Jensen medium and grown for 7–28 days in Middlebrook 7H9 broth, supplemented with 10% OADC, 0.2% glycerol and 0.1% Bacto Casitone (Difco). Stock cultures containing 1×10^7 cfu/mL of each mycobacterium strain were saved frozen at –80°C, thawed when required to perform the test and grown for 2 days in complete 7H9 medium. The cultures obtained were vortexed, large agglomerates allowed to sediment completely and the supernatant further diluted 1:5, 1:50 and 1:500 in complete 7H9 broth. Titres were determined by viable

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Table 1. MICs (mg/L) determined by MRA on *M. tuberculosis* H37Rv resuspended from Lowenstein–Jensen (LJ) and grown in 7H9 broth[a]

	LJ	7H9	
	$0.5–1 \times 10^4$ cfu/mL	$0.5–1 \times 10^4$ cfu /mL	$0.5–1 \times 10^3$ cfu /mL
Isoniazid	0.1–0.2	0.1	0.1
Rifampicin	0.25–0.5	0.25	0.125
Streptomycin	0.125–1	1	0.25–0.5
Ethambutol	1–2	1–2	1

[a]Each number indicates the same result in duplicate experiments.

counting on 7H11 agar plates, giving $0.5–1 \times 10^4$, $0.5–1 \times 10^3$ and $0.5–1 \times 10^2$ cfu/mL, respectively. These strain dilutions were used as inoculum in the microdilution plate assay. Alternatively to frozen stock cultures, suspensions from Lowenstein–Jensen slants in complete 7H9 broth were vortexed, adjusted to a turbidity equivalent to that of a 0.5 McFarland standard, diluted as described previously and used as the inoculum in the microdilution plate assay.

Chemicals

Isoniazid, rifampicin, streptomycin sulphate and ethambutol hydrochloride were obtained from Sigma–Aldrich. Solutions were prepared in sterile water, except for rifampicin, which was diluted in dimethyl sulphoxide, to obtain 10 mg/L stock solutions. Resazurin was obtained from Sigma–Aldrich and prepared as 10 g/L sterile water stock solution, saved frozen at –20°C, thawed and diluted 1:10 in sterile water when required.

Agar proportion test

The agar proportion susceptibility test was performed according to established procedures[20] in Middlebrook 7H11 medium. Briefly, the test was performed in quadrant Petri plates, drugs were diluted in order to obtain 0.2 and 1 g/L isoniazid, 1 mg/L rifampicin, 7.5 mg/L ethambutol, and 2 and 10 mg/L streptomycin; drug-free controls were included. Each strain was classified as susceptible to a drug if the number of colonies that grew on the drug-containing medium was <1% of the number of colonies grown on the control plate and resistant if the number of colonies was >10%. In cases where two drug concentrations were tested in the agar proportion method, a strain was classified as intermediate if it showed resistance to the lower drug concentration but was susceptible to the higher drug concentration.

Microdilution Resazurin Assay (MRA)

The microdilution test was performed in 96-well plates. Two-fold dilutions of each drug were prepared in the test wells in complete 7H9 broth, the final drug concentrations being: isoniazid 128–0.00625 mg/L, rifampicin 128–0.0625 mg/L, streptomycin 128–0.125 mg/L and ethambutol 128–0.25 mg/L. Twenty microlitres of each bacterial suspension was added to 180 µL of drug-containing culture medium. Control wells were prepared with culture medium only and bacterial suspension only. The plates were sealed and incubated for 7–14 days at 37°C. After each incubation time, 5 µL of resazurin solution were added per well, colouring them blue. Plates were incubated at 37°C for additional 24, 48 or 72 h. After each incubation time intervals plates were read for colour change from blue to pink in live mycobacterium-containing wells. MIC was defined as the lowest drug concentration that prevented resazurin colour change from blue to pink. Each MIC was determined three times in duplicate experiments. Viable counting from control wells and from test wells was performed onto 7H11 agar plates.

Results

Evaluation of MRA

Different growth conditions of *M. tuberculosis* H37Rv were evaluated in the MRA. In preliminary experiments, no difference in mycobacterial growth and killing was observed after 7–14 days of plate incubation; a 7 day incubation time was therefore selected for all further experiments. Further reading of resazurin colour change in mycobacterium-containing wells gave the same results after an additional 72 h incubation as after 48 h incubation; 48 h was therefore always selected as evaluation end time. Visual observation of colour change from blue to pink to assess MICs was adequate, as confirmed by viable counting; results were always assessed without the need for instrumentation for photometric or fluorimetric reading.[19]

The MICs of the drugs obtained by MRA are given in Table 1, employing bacterial suspensions prepared at different dilutions obtained either from Lowenstein–Jensen slants or from complete 7H9 broth cultures. The results obtained using suspensions from Lowenstein–Jensen medium as the inoculum at concentrations of $0.5–1 \times 10^4$ cfu/mL indicated that the range of MICs was no more than one two-fold dilution for isoniazid, rifampicin and ethambutol. For streptomycin, a wider MIC range was determined, ranging from 0.125 to 1 mg/L. Results obtained using suspensions from 7H9 broth as the inoculum at concentrations of $0.5–1 \times 10^4$ and $0.5–1 \times 10^3$ cfu/mL indicate a limited or absent variability in MICs of all drugs, except for streptomycin. A 10-fold dilution of bacterial suspension, corresponding to $0.5–1 \times 10^2$ cfu/mL, always determined resazurin colour change from blue to pink only after additional 5 days incubation of the micro-plate.

Streptomycin showed wide MIC variability in all previously described test conditions; we therefore investigated the effect of different growth incubation time in complete 7H9 broth, together with different inoculum size, on the susceptibility of *M. tuberculosis* H37Rv to streptomycin (Table 2). A wide range of low MICs of streptomycin were obtained on cultures grown for 7–14 days at final concentrations of $0.5–1 \times 10^3$ and $0.5–1 \times 10^2$ cfu/mL; good, reproducible results were obtained on cultures grown for 21–28 days at a final concentration of $0.5–1 \times 10^4$ cfu/mL.

Table 2. MICs (mg/L) of streptomycin determined by MRA on *M. tuberculosis* H37Rv grown in 7H9 broth[a]

	Inoculum size (cfu/mL)	
Growth incubation time (days)	$0.5–1 \times 10^4$	$0.5–1 \times 10^3$
7	1	0.25–0.5
14	1	0.25–0.5
21	2	1
28	2	1

[a]Each number indicates the same result in duplicate experiments.

Table 3. MICs (mg/L) determined by standard agar proportion test (APT) and MRA on *M. tuberculosis* H37Rv[a]

	APT	MRA
Isoniazid	0.2	0.1–0.2
Rifampicin	1	0.25–0.5
Streptomycin	2	0.5–2
Ethambutol	2	1–2

[a]Each number indicates the same result in duplicate experiments.

Comparison of MRA with the agar proportion test

Table 3 reports the MICs obtained for isoniazid, rifampicin, streptomycin and ethambutol for *M. tuberculosis* strain H37Rv by the agar proportion susceptibility test performed in 7H11 agar, and the MICs obtained by MRA. A 14-day-old (21-day-old to evaluate streptomycin) *M. tuberculosis* H37Rv culture, diluted 1:50 and containing $0.5–1 \times 10^4$ cfu/mL live mycobacterium, was employed in MRA. The microplate was incubated for 7 days and read for colour change after addition of resazurin and an additional 24 h incubation time. MICs obtained by MRA were one two-fold dilution lower than MICs obtained with the agar proportion test. Viable counting performed on blue-coloured wells showed 99–100% inhibition.

MRA on clinical strains

The MICs of isoniazid, rifampicin, streptomycin and ethambutol for 13 *M. tuberculosis* clinical strains as measured by MRA are reported in Table 4, together with the results obtained by agar proportion test. The assay was performed as indicated for *M. tuberculosis* H37Rv reference strain; each determination was obtained in duplicate experiments. Five clinical strains out of the 13 tested were multidrug-resistant according to clinical laboratory indications and the agar proportion test. Three of these strains, H160, H190 and H320, were resistant to all the drugs tested; one strain, H200, was resistant to isoniazid and rifampicin; and one strain, H231, was resistant to isoniazid, streptomycin and ethambutol according to the agar proportion test. The MICs of clinical strains determined by MRA were lower than the critical concentration employed in the agar proportion method, as indicated, ranging from 0.0125 to 0.05 mg/L for isoniazid, from 0.0625 to 0.125 mg/L for rifampicin, from 0.25 to 1 mg/L for streptomycin, and from 1 to 4 mg/L for ethambutol. The MICs obtained on the multidrug-resistant strains confirmed resistance to all drugs; strain H320 showed an intermediate susceptibility to streptomycin, as did strains H260 and H310.

Discussion

The cultivation of strains in complete 7H9 broth improved both the standardization of the inoculum size and the reproducibility of MIC determination, particularly for streptomycin susceptibility testing. In fact, the employment of broth suspensions from Lowenstein–Jensen medium slants involved a variable metabolic adaptation of strains, with a variable growth response. The use of complete 7H9 broth with the addition of 0.1% casitone as culture medium and the use of a concentration of 10^4 mycobacteria/mL as inoculum were required conditions to ensure a positive growth response after a 7 day incubation. The assay performed with medium in the absence of casitone always showed an erratic growth that, in most cases, was not evaluable by colour change due to the reduction of resazurin.

The streptomycin MICs depended strictly on the growth phases of the mycobacteria, as shown in Table 2, owing to the fact that both the transport of streptomycin across the bacterial walls and its binding to the 30S ribosomal subunit are energy-dependent processes.[21] The low metabolic activity of mycobacteria during their very early growth and the slowing metabolic activity of mycobacteria during the stationary phase of growth may limit the entrance of streptomycin into bacteria, resulting in higher MICs. Both the growth incubation time and the inoculum size of the culture have been standardized to allow correct determination of streptomycin MICs.

The resazurin microdilution plate method, MRA, proved to be a reliable and reproducible assay. As shown in Table 3, MRA gave good, reproducible results compared with those obtained by the reference agar proportion method; results were obtained after 8–9 days incubation, with convenient shortening of the evaluation time. MRA performed on clinical strains (Table 4) again gave good, reproducible results, including when employing inoculum suspensions prepared directly from Lowenstein–Jensen slants. It is noteworthy that MIC determinations for isoniazid, rifampicin and ethambutol were always scarcely affected by culture conditions and inoculum size.

Different methods have been described to detect antibiotic resistance of *M. tuberculosis* complex clinical strains, some of which employed colour development like the Alamar Blue assay,[11,12,22] which is a proprietary reagent that requires stabilizing agents, or tetrazolium salt assay,[23] which requires lysing buffer for colour development. Both tests employed solutions containing either Tween 80, ethanol, formamide or SDS, which are foaming agents that do not ensure complete safety when testing mycobacterium, and could furthermore interfere with mycobacterium viability. The MRA method proved to be a safe, rapid and reliable assay; the resazurin solution we employed in the microplate test MRA did not need stabilizing agents, its stability being always ensured by diluting the frozen stock solution when needed for the test.

New candidate drugs are needed to treat multidrug-resistant tubercular bacilli, and it is therefore necessary to have a screening assay to evaluate the mycobacterium-inhibiting activity of new chemicals. In a preliminary series of experiments, we have evaluated using MRA the MICs of a number of new molecules[24] that have already been described for their interesting *in vitro* antitubercular activity.[25] Resazurin was employed as an oxidation–reduction indicator at a final concentration of 25 mg/L[19] and no interaction with drugs or any other chemical was ever detected. Intermediate-coloured wells have

Table 4. MICs (mg/L) determined by MRA on *M. tuberculosis* clinical isolates[a]

Strain	Isoniazid	Rifampicin	Streptomycin	Ethambutol
H150	0.0125 (S)	0.0625–0.125 (S)	0.25–0.5 (S)	1–2 (S)
H160	**4** (R)	**16–32** (R)	**8–16** (R)	**8–16** (R)
H180	0.025–0.05 (S)	0.0625–0.125 (S)	0.5–1 (S)	2 (S)
H190	**4 (R)**	**>128 (R)**	**>128 (R)**	**8–16 (R)**
H200	**2 (R)**	**16–32 (R)**	0.25 (S)	1 (S)
H210	0.0125 (S)	0.0625 (S)	1 (S)	2 (S)
H230	0.0125 (S)	0.0625 (S)	1 (S)	2 (S)
H250	0.0125 (S)	0.0625 (S)	0.5–1 (S)	2 (S)
H260	0.0125 (S)	0.125 (S)	**4** (I)	4 (S)
H280	0.0125 (S)	0.125 (S)	1–2 (S)	4 (S)
H310	0.0125 (S)	0.0625–0.125 (S)	**8** (I)	0.5–1 (S)
H320	**>128 (R)**	**16–32 (R)**	**4 (I)**	**8 (R)**
H231	**4 (R)**	0.0625 (S)	**128 (R)**	**8 (R)**

[a]Each number indicates the same result in duplicate experiments; bold numbers indicate resistant strains according to MRA.
S, I and R indicate susceptible, intermediate and resistant strains, respectively, according to APT.

sometimes been observed after 48 h incubation in the presence of resazurin when evaluating multidrug-resistant strains; prolonged incubation up to 72 h resulted in intermediate-coloured wells changing to pink; therefore, intermediate colours obtained after 48 h can be consequently interpreted as positive growth, as was also determined by viable counting. Visual observation of colour change from blue to pink was adequate: results were always assessed without instrumentation for photometric or fluorimetric reading.

In conclusion, MRA is a rapid, inexpensive, low technology procedure, suitable for susceptibility testing of first- and second-line antitubercular drugs and for screening new antitubercular compounds against *M. tuberculosis* clinical strains. The interesting results obtained prompt us to evaluate by means of MRA the drug susceptibility of different *Mycobacterium* species, including *Mycobacterium avium*.

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