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Microplate Alamar Blue Assay versus BACTEC 460 System for High-Throughput Screening of Compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*

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In response to the need for rapid, inexpensive, high-throughput assays for antimycobacterial drug screening, a microplate-based assay which uses Alamar blue reagent for determination of growth was evaluated. MICs of 30 antimicrobial agents against *Mycobacterium tuberculosis* $H_{37}Rv$, *M. tuberculosis* $H_{37}Ra$, and *Mycobacterium avium* were determined in the microplate Alamar blue assay (MABA) with both visual and fluorometric readings and compared to MICs determined in the BACTEC 460 system. For all three mycobacterial strains, there was ≤1 dilution difference between MABA and BACTEC median MICs in four replicate experiments for 25 to 27 of the 30 antimicrobics. Significant differences between MABA and BACTEC MICs were observed with 0, 2, and 5 of 30 antimicrobial agents against $H_{37}Rv$, $H_{37}Ra$, and *M. avium*, respectively. Overall, MICs determined either visually or fluorometrically in MABA were highly correlated with those determined in the BACTEC 460 system, and visual MABA and fluorometric MABA MICs were highly correlated. MICs of rifampin, rifabutin, minocycline, and clarithromycin were consistently lower for $H_{37}Ra$ compared to $H_{37}Rv$ in all assays but were similar for most other drugs. *M. tuberculosis* $H_{37}Ra$ may be a suitable surrogate for the more virulent $H_{37}Rv$ strain in primary screening of compounds for antituberculosis activity. MABA is sensitive, rapid, inexpensive, and nonradiometric and offers the potential for screening, with or without analytical instrumentation, large numbers of antimicrobial compounds against slow-growing mycobacteria.

With the increased need for drugs to combat multidrug-resistant tuberculosis (TB), there is an urgent need for rapid, low-cost, high-throughput assays for screening new drug candidates. Ideally, such assays would also be useful for assessing activity against *Mycobacterium avium*, an opportunistic pathogen which is resistant to most existing antimicrobials. Due to the slow growth of *Mycobacterium tuberculosis*, *M. avium*, and related species, incubation times for drug susceptibility assays which rely on the development of colonies or turbidity are excessively long. Susceptibility can be determined more rapidly with the radiometric BACTEC 460 system, the clinical drug susceptibility system of choice for most of the last decade (9, 10), but the high cost, high volume, lack of high-throughput format, and requirement for radioisotope disposal all limit its usefulness for mass screening. Recently, there have been a number of new mycobacterial drug susceptibility assays described (2, 4, 5, 12, 16). Most of these assays, however, lack one or more of the desired attributes of a mass screening assay: rapidity, high throughput, and low cost of supplies and equipment.

The Alamar blue oxidation-reduction dye is a general indicator of cellular growth and/or viability; the blue, nonfluorescent, oxidized form becomes pink and fluorescent upon reduction (1). Growth can therefore be measured with a fluorometer or spectrophotometer or determined by a visual color change. Alamar blue has been used as a nonradioactive assay to monitor and quantify lymphocyte proliferation (1, 6). It has also been used for detection of methicillin-resistant *Staphylococcus aureus* (13) and vancomycin-resistant enterococci (19, 23) and of susceptibility of yeasts to amphotericin B, fluconazole, flucytosine, and itraconazole (15, 20) and of gram-negative bacteria to a variety of antibacterial agents (3). Yajko et al. (22) found agreement in MICs of four first-line anti-TB drugs for 50 clinical isolates of *M. tuberculosis* when comparing the agar proportion method and a microbroth tube dilution technique which employed visual determinations of Alamar blue reduction for growth determination.

This study assesses the antimycobacterial mass drug screening potential of visual and fluorometric microplate-based Alamar blue assays (MABAs) by comparing MICs of 30 established antimicrobial agents with those obtained with the BACTEC 460 system against *M. tuberculosis* $H_{37}Ra$ ($H_{37}Ra$), *M. tuberculosis* $H_{37}Rv$ ($H_{37}Rv$), and *M. avium*. The comparative MIC data also allow for a determination of the degree of similarity between the less virulent $H_{37}Ra$ (14) and the more virulent $H_{37}Rv$ with respect to drug susceptibility and thus the suitability of using the former as a screening surrogate for the latter.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. tuberculosis* $H_{37}Ra$ ATCC 25177 ($H_{37}Ra$), *M. tuberculosis* $H_{37}Rv$ ATCC 27294 ($H_{37}Rv$), and *M. avium* ATCC 25291 were obtained from the American Type Culture Collection (Rockville, Md.). For the first three (of four) replicate experiments, $H_{37}Ra$ and $H_{37}Rv$ inocula were first passaged in radiometric 7H12 broth (BACTEC 12B; Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) until the growth index (GI) reached 800 to 999.

For the fourth replicate experiment, $H_{37}Ra$ was grown in 100 ml of Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.2% (vol/vol) glycerol (Sigma Chemical Co., Saint Louis, Mo.), 1.0 g of Casitone (Difco) per liter, 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase; Difco), and 0.05% (vol/vol) Tween 80 (Sigma). The complete medium was referred to as 7H9GC-Tween. $H_{37}Rv$ was cultured identically except that Casitone was omitted. Cultures were incubated in 500-ml nephelometer flasks on a rotary shaker (New Brunswick Scientific, Edison, N.J.) at 150 rpm and 37°C until they reached an optical density of 0.4 to 0.5 at 550 nm. Bacteria were washed and suspended

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in 20 ml of phosphate-buffered saline and passed through an 8-μm-pore-size filter to eliminate clumps. The filtrates were aliquoted, stored at −80°C, and used within 30 days. *M. avium* inoculum was prepared for all experiments by passage in BACTEC 12B medium until the GI reached 999.

Chemicals and media. Amikacin sulfate (AM), bacitracin (BAC), capreomycin (CAP), cefotaxime (CEF), cloxacillin (CLX), cycloserine (CS), doxycycline HCl (DOX), ethambutol HCl (EMB), ethionamide (ETA), fusidic acid (FUS), gentamicin sulfate (GEN), isoniazid (INH), kanamycin sulfate (KM), lincomycin HCl (LIN), minocycline HCl (MIN), *p*-aminosalicylic acid (PAS), paromomycin sulfate (PAR), rifampin (RMP), streptomycin sulfate (SM), thiacetazone (TAC), and tobramycin (TOB) were obtained from Sigma. The following compounds were kindly provided by the respective manufacturers: azithromycin (AZI; Pfizer Central Research Division, Groton, Conn.), clarithromycin (CLA; Abbott Laboratories, North Chicago, Ill.), clofazimine (CLF; Ciba-Geigy Corp., Summit, N.J.), ofloxacin (OFX; R. W. Johnson Pharmaceutical Research, Raritan, N.J.), rifabutin (RBT; Pharmacia Adria, Dublin, Ohio), rifapentine (RPT; Hoechst Marion Roussel, Cincinnati, Ohio), sparfloxacin (SPX; Parke-Davis Pharmaceutical, Ann Arbor, Mich.), ampicillin and sulbactam (AMP/S; Lederle Inc., Carolina, Puerto Rico), and piperacillin and tazobactam (PIP/T; Roerig-Pfizer, New York, N.Y.). Drugs were solubilized according to the manufacturers' recommendations, and stock solutions were filter sterilized (0.22-μm pore size) and stored at −70°C for not more than 30 days. MICs were determined four times on four different days. A common set of frozen drug stock solutions was used for all experiments, with a previously unthawed aliquot being used for each experiment. Maximum final drug concentrations in testing media were either limited by compound solubility or chosen with consideration of maximum serum concentrations following customary dosages in humans or expected MICs based on published reports.

Radiometric susceptibility test. A total of 1/10 ml of BACTEC 12B-passaged inoculum was delivered without prior dilution into 4 ml of test medium. Subsequent determination of bacterial titers yielded average titers (three experiments) of 1×10^5, 2.5×10^5, and 3.25×10^4 CFU/ml of BACTEC 12B medium for $H_{37}Rv$, $H_{37}Ra$, and *M. avium*, respectively. Frozen inocula were initially diluted 1:20 in BACTEC 12B medium, and then 0.1 ml was delivered to test medium. This yielded 5.0×10^5 and 1.25×10^5 CFU per BACTEC vial for $H_{37}Rv$ and $H_{37}Ra$, respectively. Twofold drug dilutions were prepared in either dimethyl sulfoxide (Sigma) or distilled deionized water and delivered via a 0.5-ml insulin syringe in a 50-μl volume. Drug-free control vials consisted of solvent with bacterial inoculum and solvent with a 1:100 dilution of bacterial inoculum (1:100 controls). Vials were incubated at 37°C, and the GI was determined in a BACTEC 460 instrument (Becton Dickinson) until the GI of the 1:100 controls reached at least 30. All vials were read the following day, and the GI and daily change in GI (ΔGI) were recorded for each drug dilution. The MIC was defined as the lowest concentration for which the ΔGI was less than the ΔGI of the 1:100 control (10). If the GI of the test sample was greater than 100, the sample was scored as resistant even if the ΔGI was less than the ΔGI of the 1:100 control.

Alamar blue susceptibility test (MABA). Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water, and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates. BACTEC 12B-passaged inocula were initially diluted 1:2 in 7H9GC, and 0.1 ml was added to wells. Subsequent determination of bacterial titers yielded 1×10^6, 2.5×10^6, and 3.25×10^5 CFU/ml in plate wells for $H_{37}Rv$, $H_{37}Ra$, and *M. avium*, respectively. Frozen inocula were initially diluted 1:20 in BACTEC 12B medium followed by a 1:50 dilution in 7H9GC. Addition of 1/10 ml to wells resulted in final bacterial titers of 2.0×10^5 and 5×10^4 CFU/ml for $H_{37}Rv$ and $H_{37}Ra$, respectively. Wells containing drug only were used to detect autofluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 4 of incubation, 20 μl of 10× alamarBlue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5 μl of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of ≥50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorometer (PerSeptive Biosystems, Framingham, Mass.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. If the B wells became pink by 24 h, reagent was added to the entire plate. If the well remained blue or ≤50,000 FU was measured, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition. Visual MICs were defined as the lowest concentration of drug that prevented a color change. For fluorometric MICs, a background subtraction was performed on all wells with a mean of triplicate M wells. Percent inhibition was defined as 1 − (test well FU/mean FU of triplicate B wells) × 100. The lowest drug concentration effecting an inhibition of ≥90% was considered the MIC.

Statistical analysis. All analyses were performed with the program SAS (SAS Institute Inc., Cary, N.C.). Correlation coefficients were defined according to Spearman for ranked data analysis and Pearson for raw data analysis to determine differences between the BACTEC system and the MABA either fluorometrically or visually for determination of MIC (18). A general linear model procedure using analysis of variance of ranked data was performed for each of the three variables of MIC generation to determine significant differences between the replicate comparisons of the four experiments. Tukey's Studentized range test was also used for pairwise post hoc comparison of variable analysis of ranked measure for significant differences among techniques of MIC determination and for differences between techniques for individual antimicrobial agents for each bacterial strain. Significance was determined at $P \leq 0.05$.

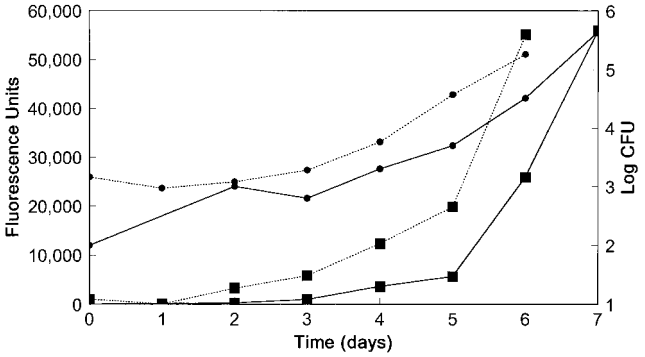

FIG. 1. CFU (●) and Alamar blue reduction FU (■) during growth of *M. tuberculosis* $H_{37}Rv$ (solid lines) and $H_{37}Ra$ (broken lines).

RESULTS

For both $H_{37}Rv$ and $H_{37}Ra$, reduction of Alamar blue dye, as determined by fluorometric measurement, paralleled the increase in CFU during incubation in 7H9GC for 5 days, after which the fluorescence appeared to increase more rapidly (Fig. 1). Preliminary experiments (data not shown) examined the effect of culture medium components on MABA MICs (relative to BACTEC 460 system MICs) for $H_{37}Ra$ and time to completion of the assay. Essentially identical results were obtained when either OADC or ADC (Difco) supplements were used together with glycerol and Casitone. Elimination of either glycerol or Casitone in either OADC- or ADC-supplemented medium had no effect on MICs or time to completion of the assay. Elimination of both glycerol and Casitone in OADC-supplemented medium resulted in slower growth without alteration of MICs while elimination of these two nutrients in ADC-supplemented medium resulted in erratic growth and difficulty in making MIC determinations.

MICs of the 30 antimicrobial agents for $H_{37}Rv$ (Table 1) were available in 9 to 10 days and 7 to 9 days with the BACTEC 460 system and MABA, respectively. MICs of six drugs were greater than the maximum drug concentrations tested in at least one assay, and with the exception of TAC, this was reflected in the other assays as well. In considering individual results for each of the four replicate experiments, MIC differences of ≤1 twofold dilution were found in (73 ± 11.1)%, (76 ± 10.6)%, and (96 ± 3.4)% of observations for the BACTEC system versus fluorometric MABA, the BACTEC system versus visual MABA, and fluorometric MABA versus visual MABA, respectively. Median MICs of four drugs differed by ≥1 dilution for the BACTEC system versus fluorometric MABA, and median MICs of three drugs differed by ≥1 dilution for the BACTEC system versus visual MABA. Pearson and Spearman correlation coefficients were, respectively, 0.95144 and 0.92519 for the BACTEC system versus fluorometric MABA, 0.97212 and 0.94145 for the BACTEC system versus visual MABA, and 0.98665 and 0.98903 for fluorometric MABA versus visual MABA. Statistically significant differences of individual drugs were not observed between any assay for any of the 30 antimicrobial agents.

TABLE 1. MICs of 30 antimicrobial agents versus *Mycobacterium tuberculosis* $H_{37}Rv$ determined by the BACTEC system, fluorometric MABA, and visual MABA

Drug	MIC (μg/ml)[a]								
	BACTEC system			Fluorometric MABA			Visual MABA		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
RMP	0.16	0.2	0.025–0.2	0.11	0.1	0.05–0.2	0.075	0.075	0.05–0.1
RBT	0.034	0.025	0.025–0.063	0.04	0.38	0.025–0.063	0.038	0.038	0.012–0.063
RPT	0.013	0.013	0.013	0.006	0.006	0.006	0.006	0.006	0.006
INH	0.031	0.025	0.025–0.05	0.05	0.05	0.05	0.044	0.05	0.025–0.05
ETA	1.17	0.94	0.31–2.5	0.94	0.94	0.63–1.25	0.94	0.94	0.63–1.25
TAC	0.20	0.13	0.063–0.5	>2.0	>2.0	0.125–>2.0	>2.0	>2.0	0.13–>2.0
PAS	2.31	0.5	0.25–8.0	1.25	1.25	0.5–>2.0	0.69	0.75	0.25–1.0
EMB	1.17	0.94	0.94–1.88	1.64	1.88	0.94–1.88	1.64	1.88	0.94–1.88
SM	0.35	0.28	0.094–0.75	0.26	0.28	0.094–0.38	0.26	0.29	0.094–0.38
AM	0.42	0.47	0.16–0.63	0.88	0.47	0.078–2.5	0.59	0.47	0.16–1.25
GEN	6.0	6.0	4.0–8.0	3.5	4.0	2.0–4.0	3.5	4.0	2.0–4.0
KM	2.0	1.25	0.63–5.0	2.19	1.25	1.25–5.0	1.56	1.25	1.25–2.5
PAR	2.75	3.0	1.0–4.0	2.5	2.0	2.0–4.0	2.25	2.0	1.0–4.0
TOB	10.0	8.0	8.0–16	7.0	8.0	4.0–8.0	7.0	8.0	4.0–8.0
OFX	0.75	0.75	0.5–1.0	0.75	0.75	0.5–1.0	0.75	0.75	0.5–1.0
SPX	0.25	0.12	0.078–0.63	0.15	0.12	0.039–0.31	0.15	0.12	0.039–0.31
CLA	26	32	8.0–32	10.0	8.0	8.0–16	8.0	8.0	8.0
AZI	>128	>128	128–>128	>128	>128	>128	>128	>128	>128
LIN	>256	>256	>256	>256	>256	128–>256	>256	>256	>256
DOX	12	12	8.0–16	8.0	8.0	8.0	8.0	8.0	8.0
MIN	5.0	4.0	4.0–8.0	3.5	4.0	2.0–4.0	3.5	4.0	2.0–4.0
CS	15.6	12.5	12.5–25	28.13	25	12.5–50	22	25	12.5–25
CEF	84	96	16–128	192	192	128–256	176	192	64–256
CLX	>256	>256	64–>256	256	256	256–>256	256	256	256–>256
AMP/S	>128	>128	128–>128	>128	>128	128–>128	>128	>128	128–>128
PIP/T	11.25	12	5.0–16	18.5	16	10–32	16.5	13	8.0–32
BAC	>256	>256	>256	>256	>256	>256	>256	>256	>256
CAP	0.78	0.63	0.63–1.25	1.72	1.88	0.63–2.5	1.41	1.25	0.63–2.5
FUS	8.5	8.0	2.0–16	12.0	12	8.0–16	8.0	8.0	8.0
CLF	0.22	0.23	0.078–0.31	2.73	0.31	0.31–10	0.86	0.31	0.31–2.5

[a] Data are expressed as means, medians, and ranges of four replicate experiments. In cases in which one or more replicate experiments yielded a MIC greater than the highest tested concentration, the mean MIC of that drug is also expressed as a ">" value.

MICs of the test agents for $H_{37}Ra$ (Table 2) were available in 7 to 8 days and 5 to 6 days with the BACTEC 460 system and MABA, respectively. Five drugs had MICs in at least one assay which were greater than the highest drug concentrations tested. MIC differences of ≤1 twofold dilution were found in (85.5 ± 10.3)%, (84 ± 11)%, and (94 ± 3.9)% of observations for the BACTEC system versus fluorometric MABA, the BACTEC system versus visual MABA, and fluorometric MABA versus visual MABA, respectively. Median MICs of three drugs differed by ≥1 dilution for the BACTEC system versus fluorometric MABA, and median MICs of five drugs differed by ≥1 dilution for the BACTEC system versus visual MABA. MICs of ≥2 twofold dilutions between the BACTEC system and fluorometric MABA were observed with CLA and AZI and between the BACTEC system and visual MABA were observed with CLA, CLF, and AZI. Pearson and Spearman correlation coefficients were, respectively, 0.94217 and 0.94433 for the BACTEC system versus fluorometric MABA, 0.95248 and 0.95368 for the BACTEC system versus visual MABA, and 0.97132 and 0.97814 for fluorometric MABA versus visual MABA. Statistically significant differences of individual antimicrobial agents were observed between the BACTEC system and visual MABA for CLF and between the BACTEC system and both MABA determinations for MIN.

MICs for *M. avium* (Table 3) were available after 8 to 10 and 10 to 11 days for the BACTEC system and MABA, respectively. MICs of 14 drugs were greater than the maximum concentration tested in at least one assay, and in most such cases, this was reflected in the other assays as well, a notable exception being TAC. MIC differences of ≤1 twofold dilution in individual assay results were found in (74 ± 9.8)%, (78 ± 9.5)%, and (93 ± 5.4)% of observations for the BACTEC system versus fluorometric MABA, the BACTEC system versus visual MABA, and fluorometric MABA versus visual MABA. BACTEC MICs were generally higher than MABA MICs for OFX. Pearson and Spearman correlation coefficients were, respectively, 0.91277 and 0.89662 for the BACTEC system versus fluorometric MABA, 0.93603 and 0.91673 for the BACTEC system versus visual MABA, and 0.97739 and 0.97251 for fluorometric MABA versus visual MABA. BACTEC MICs for CAP, CEF, PAS, SPX, and TAC were statistically different from those determined by either fluorometric MABA or visual MABA.

With respect to comparative susceptibility of $H_{37}Rv$ versus $H_{37}Ra$, there were differences in MICs of ≥1 twofold dilution with nine drugs, five of which (RMP, RBT, TAC, CLA, and AZI) had MIC differences of >2 twofold dilutions. MICs determined by each of the three assays for RMP, RBT, CLA, and MIN were significantly lower for $H_{37}Ra$ relative to $H_{37}Rv$.

Overall, there were no statistically significant differences ($P \geq 0.05$) among MICs determined in the BACTEC 460 system, visual MABA, and fluorometric MABA for either of the *M. tuberculosis* strains or for the *M. avium* strain, nor were there any significant differences in MICs for these assays among the

TABLE 2. MICs of 30 antibacterial agents versus *Mycobacterium tuberculosis* $H_{37}Ra$ determined by the BACTEC system, fluorometric MABA, and visual MABA

Drug	MIC (μg/ml)[a]								
	BACTEC system			Fluorometric MABA			Visual MABA		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
RMP	0.006	0.005	0.003–0.013	0.005	0.005	≤0.003–0.006	0.006	0.0047	≤0.003–0.013
RBT	≤0.006	≤0.006	≤0.006	≤0.003	≤0.003	≤0.003	≤0.003	≤0.003	≤0.003
RPT	≤0.006	≤0.006	≤0.006	0.004	0.003	0.003–0.006	0.004	0.0031	≤0.003–0.006
INH	0.025	0.025	0.025	0.05	0.05	0.05	0.05	0.05	0.05
ETA	1.02	1.25	0.31–1.25	2.97	3.125	0.63–5.0	1.09	0.63	0.63–2.5
TAC	0.078	0.063	0.063–0.13	0.54	0.063	≤0.03–>2.0	≤0.31	≤0.31	≤0.31–0.63
PAS	>2.0	>2.0	1.0–>2.0	1.75	2.0	1–2	1.34	1.5	0.5–2.0
EMB	1.06	0.94	0.47–1.88	3.05	1.88	0.94–7.5	1.64	1.88	0.94–1.88
SM	0.94	0.94	0.38–1.5	0.56	0.56	0.38–0.75	0.38	0.38	0.38
AM	0.74	0.70	0.31–1.25	0.6	0.6	0.31–0.78	0.59	0.63	0.31–0.78
GEN	8.0	8.0	8.0	7.0	8.0	4.0–8.0	7.0	8.0	4.0–8.0
KM	2.81	2.5	1.25–5.0	3.13	2.5	2.5–5.0	2.5	2.5	2.5
PAR	3.5	4.0	2.0–4.0	5.0	4.0	4.0–8.0	4.5	4.0	2.0–8.0
TOB	10	8.0	8.0–16	10	8.0	8.0–16	10	8.0	8.0–16
OFX	0.75	0.75	0.5–1.0	0.44	0.5	0.25–0.5	0.44	0.5	0.25–0.5
SPX	0.12	0.07	0.039–0.31	0.084	0.07	0.039–0.16	0.047	0.039	0.03–0.078
CLA	5.5	6.0	2.0–8.0	1.5	1.5	1.0–2.0	1.88	1.5	0.5–4.0
AZI	104	>128	32–>128	44	48	16–64	36	32	16–64
LIN	>256	>256	>256	>256	>256	>256	>256	>256	>256
DOX	4.5	4.0	2.0–8.0	4.25	4.0	1.0–8.0	3.75	3.0	1.0–8.0
MIN	2.0	2.0	2.0	0.88	1.0	0.5–1.0	0.88	1.0	0.5–1.0
CS	25	25	25	20	25	6.25–25	17	19	6.25–25
CEF	88	96	32–128	120	96	32–256	92	48	16–256
CLX	112	128	64–128	160	128	128–256	80	64	64–128
AMP/S	>128	>128	>128	128	128	128	128	128	128
PIP/T	21	16	16–32	16	16	16	13	16	8.0–16
BAC	>256	>256	>256	>256	256	256–>256	>256	256	256–>256
CAP	2.03	2.5	0.63–2.5	2.5	1.88	1.25–5.0	2.19	1.25	1.25–5.0
FUS	5.0	4.0	4.0–8.0	3.5	4.0	2.0–4.0	3.0	3.0	2.0–4.0
CLF	1.33	1.25	0.31–2.5	0.51	0.625	≤0.16–0.625	0.23	0.23	0.16–0.31

[a] Data are expressed as means, medians, and ranges of four replicate experiments. In cases in which one or more replicate experiments yielded a MIC greater than the highest tested concentration, the mean MIC of that drug is also expressed as a ">" value.

four experiments. Fluorometric MABA MICs correlated with visual MABA MICs for all drugs tested against all three mycobacterial strains.

DISCUSSION

During the past 3 years, we have used the BACTEC 460 system to screen over 14,000 compounds for activity against $H_{37}Rv$. As a rapid broth dilution technique, the BACTEC 460 system is superior to agar-based techniques with respect to concerns over compound stability (7) or inactivation by agar medium components (4, 8). It also provides for excellent biocontainment and allows for the easy monitoring of the kinetics of drug inhibition. Major drawbacks include expense (BACTEC 12B medium currently costs approximately $2.50 per vial) and a high medium volume of 4 ml (and thus a high test sample requirement) as well as the lack of a high-throughput format and cumbersome data acquisition.

The visually read, tube-based Alamar blue assay, as described by Yajko et al. (22) for clinical use, was modified in the current study with respect to format, medium composition, and reaction temperature. The use of microplates allowed for rapid automated reading in a microplate fluorometer as well as facilitating the preparation of multiple dilutions. Although using an incubation temperature of 37°C after adding Alamar blue reagent necessitated at least 12 h of incubation for sufficient reduction of the Alamar blue (compared to 2 h at 50°C as used by Yajko et al. [22]), the lower temperature allowed for reuse of the same plate for subsequent testing of additional wells in the event that growth was insufficient during the first test of B wells. We avoided the use of Tween 80 in the test medium in order to preclude possible enhancement of drug activity. In our laboratory, this medium gave sharper visual cutoffs than that used by Yajko et al. (22). Although inoculum densities in the BACTEC system and MABA were not identical, this did not appear to affect a high correlation between the two assays.

In order to conduct a relatively comprehensive evaluation of MABA, we included among the test compounds (i) most established and experimental compounds recognized as having clinical efficacy in infections by *M. tuberculosis*, *M. avium*, and *Mycobacterium leprae*; (ii) additional representatives of the aminoglycosides, macrolides, tetracyclines, fluoroquinolones, and polypeptides; and (iii) several cephalosporins, penicillins, and lincomycins. Based on the high degree of correlation between the MABA and the BACTEC 460 system for most of the 30 antimicrobial agents tested with all three strains of mycobacteria, use of MABA for mass screening should allow a high degree of confidence in detecting active congeners of these compounds as well as novel structures.

A major advantage of MABA is that growth can be evaluated fluorometrically, spectrophotometrically (21), or visually (3, 13, 15, 19, 20, 22, 23), the last without the use of specialized equipment. Our preliminary experiments (data not shown) indicated that fluorometric readings were more sensitive and more consistent than colorimetric (spectrophotometric) mea-

TABLE 3. MICs of 30 antibacterial agents versus *Mycobacterium avium* determined by the BACTEC system, fluorometric MABA, and visual MABA

Drug	MIC (μg/ml)[a]								
	BACTEC system			Alamar fluorometric			Alamar visual		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
RMP	>8.5	8.0	2–>16	9.25	10	1.0–16	6.25	4.0	1.0–16
RBT	>0.56	0.5	0.25–>1.0	>0.53	0.5	0.13–>1.0	>0.56	0.56	0.13–>1.0
RPT	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
INH	>32	>32	>32	>32	>32	>32	>32	>32	>32
ETA	2.65	2.65	0.31–5.0	>4.5	>5.0	0.31–>5.0	>2.8	>2.8	0.63–>5.0
TAC	0.33	0.25	0.25–0.5	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
PAS	28	16	16–64	80	64	64–128	72	64	32–128
EMB	5.63	5.63	3.75–7.5	5.16	5.63	1.88–7.5	5.16	5.63	1.88–7.5
SM	1.25	1.5	0.75–1.5	1.03	1.13	0.38–1.5	1.88	1.13	0.38–1.5
AM	5.8	5.0	2.5–10	6.25	5.0	5.0–10	6.25	5.0	5.0–10
GEN	9.3	8.0	4.0–16	6.25	4.0	1.0–16	5.0	4.0	4.0–8.0
KM	3.3	2.5	2.5–5.0	3.13	2.5	2.5–5.0	2.5	2.5	2.5
PAR	13	16	4.0–16	11.5	6.0	2.0–32	11.5	6.0	2.0–32
TOB	4.0	4.0	4.0	6.5	4.0	2.0–16	4.5	4.0	2.0–8.0
OFX	>8.0	>8.0	8.0–>8.0	6.25	8.0	1.0–8.0	6.25	8.0	1.0–8.0
SPX	8.0	8.0	8.0	3.0	3.0	2.0–4.0	3.0	3.0	2.0–4.0
CLA	2.0	2.0	2.0	3.25	2.0	1.0–8.0	5.0	5.0	2.0–8.0
AZI	16	16	16	28	20	8.0–64	16	12	8.0–32
LIN	112	128	64–128	120	96	32–256	120	96	32–256
DOX	64	48	32–128	72	64	32–128	56	64	32–64
MIN	32	32	32	>28	32	16–>32	28	32	16–32
CS	44	50	25–50	37.5	37.5	25–50	37.5	37.5	25–50
CEF	>128	128	128–>128	>160	128	128–>256	160	128	128–256
CLX	>256	>256	>256	>213	>256	128–>256	>256	>256	128–>256
AMP/S	>128	>128	>128	107	96	64–128	107	96	64–128
PIP/T	128	128	128	69	64	16–128	53	64	32–64
BAC	>256	>256	>256	>256	>256	>256	>256	>256	>256
CAP	>4.4	>5.0	2.5–>5.0	>5.0	>5.0	>5.0	5.0	5.0	5.0–>5.0
FUS	>24	24	16–>32	19	20	4.0–32	11	12	4.0–16
CLF	0.39	0.31	0.31–0.63	0.61	0.47	0.25–1.25	0.38	0.31	0.25–0.63

[a] Data are expressed as means, medians, and ranges of four replicate experiments. In cases in which one or more replicate experiments yielded a MIC greater than the highest tested concentration, the mean MIC for that drug is also expressed as a ">" value.

surements; therefore, we did not include the latter in this study. Similarly, fluorescent readings in bottom-reading mode were higher than those in top-reading mode. The high correlation of fluorometric and visual MABA MICs supports the use of the latter in settings where automation is not a priority and/or the cost of a microplate fluorometer is prohibitive or the test compounds are fluorescent at the excitation and emission settings used. Another advantage, in contrast with reporter assays such as the firefly luciferase method (2, 5), is the ability to screen compounds against any isolate, not only recombinant strains.

Current reagent costs for MABA are less than $0.03/well. Estimated costs per sample for primary screening with 30 samples per plate (two wells per sample; one with drug only) would range from approximately $0.26 for fluorometric MABA in bottom-reading mode using a black, sterile view plate with a transparent bottom to $0.10 for visual MABA using a standard translucent, sterile, microplate. Fluorometric measurements can also be performed in top-reading mode, which obviates the need for a clear-bottomed plate, thus reducing costs further for fluorometric readings.

From a biosafety perspective, MABA would appear to have a lower probability of generating aerosols than would the luciferase assays. The latter requires removal of the plate lid prior to placement in the microplate luminometer and the addition of reagent into wells by an automated injector, thus creating an aerosol and necessitating that the assay be conducted within a laminar flow cabinet. A common drawback of luciferase and MABA is that both are endpoint assays and thus drug inhibition kinetics cannot be easily monitored.

Ideally, a high-throughput assay for identifying compounds with anti-TB activity would also be useful with *M. avium*. Although *M. avium* tends to show much greater heterogeneity than *M. tuberculosis* with respect to strain variation in drug susceptibility (17), only a single isolate, the American Type Culture Collection type strain, was evaluated in the current study. The well-known resistance of *M. avium* to many antimicrobials was reflected in both BACTEC and Alamar blue assays, and this had the effect of decreasing the number of agents for which actual MICs could be determined. Nonetheless, the overall results suggest good correlation of MABA with the BACTEC 460 system, and thus MABA should also be useful for discovery of new drugs against *M. avium*.

In order to obtain more rapid results and/or because of biosafety concerns in working with virulent *M. tuberculosis*, some researchers have used surrogate mycobacteria in screening for antituberculosis compounds, often choosing saprophytic, rapidly growing species. The newer, rapid assays for *M. tuberculosis* (including MABA), however, have significantly lessened the time differential in obtaining results for slow-growing and rapidly growing mycobacteria. The critical issue would appear to be the degree of similarity in drug susceptibility between any surrogate mycobacterium and virulent *M. tuberculosis* with particular concern over species or strains

which might be less sensitive. Chung et al. (4) in describing a uracil uptake assay using the rapidly growing species *Mycobacterium aurum* compared susceptibilities to $H_{37}Rv$ with only four first-line anti-TB drugs. The MIC of one drug, RMP, was eightfold higher (three twofold dilutions) for *M. aurum* than for $H_{37}Rv$. Our own preliminary studies using an agar dilution technique (data not shown) suggested that rapidly growing mycobacteria such as *Mycobacterium smegmatis*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, and *Mycobacterium neoaurum* were less similar to (and usually less sensitive than) $H_{37}Rv$ than were the slow-growing *Mycobacterium bovis* BCG and $H_{37}Ra$ with respect to MICs of 15 compounds. While others have demonstrated the similarity in drug susceptibility between *M. bovis* BCG and $H_{37}Rv$ (2), the insensitivity of the former to pyrazinamide causes concern that other effective anti-TB agents could be missed when this species is used in a primary screen. Use of a less virulent strain of *M. tuberculosis* such as $H_{37}Ra$ might be superior in this regard. The difficulty in identifying phenotypic or genotypic differences between $H_{37}Rv$ and $H_{37}Ra$ (14) strains of *M. tuberculosis* would seem predictive of very similar drug susceptibility profiles; however, we are not aware of any published reports of comparative MICs of a variety of compounds for these strains. Although our $H_{37}Ra$ inoculum density was somewhat higher than that for $H_{37}Rv$ in three of four experiments (with a resulting shorter time to assay completion), MICs of 26 (of 30) drugs were very similar for the two strains in one or more of the different assays. Significantly, $H_{37}Ra$ appeared more sensitive than $H_{37}Rv$ to those four drugs whose MICs were discordant in all three assays. This suggests that the use of $H_{37}Ra$ for primary screening in a TB drug discovery program might result in a few false leads but would be less likely to miss active compounds. Thus, *M. tuberculosis* $H_{37}Ra$ would appear to be a suitable surrogate for the virulent $H_{37}Rv$ strain in a primary screening assay, thereby decreasing biosafety concerns and the required level of biocontainment.

MABA is a nonradiometric, inexpensive, rapid, high-throughput assay which should prove useful for large-scale screening of compounds against any strain of *M. tuberculosis*. MABA also appears promising for *M. avium*, although additional strains should be evaluated to confirm the results obtained here. Fluorometric MABA facilitates data acquisition for large-scale efforts while visual MABA is a very inexpensive alternative, providing nearly identical results. The latter, when used with *M. tuberculosis* $H_{37}Ra$, provides a primary anti-TB screen which can be conducted inexpensively in the most basic biocontainment level 2 laboratory.

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