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Inter- and Intra-Assay Reproducibility of Microplate Alamar Blue Assay Results for Isoniazid, Rifampicin, Ethambutol, Streptomycin, Ciprofloxacin, and Capreomycin Drug Susceptibility Testing of *Mycobacterium tuberculosis*▽†

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The intersample and intrasample variability of the results obtained with the microplate Alamar blue assay for the indirect drug susceptibility testing of *Mycobacterium tuberculosis* was investigated. Between 1.2 and 8.5% of paired MICs differed by more than one twofold dilution, resulting in discordant susceptible-resistant designations at frequencies between 0.6% (rifampin) and 18.9% (ethambutol).

The widespread need for rapid, accurate tuberculosis (TB) drug susceptibility testing (DST) is highlighted by the steady emergence of multidrug-resistant and extensively drug-resistant TB (1, 5). As more laboratories utilize low-cost DST methods (10), it is essential to determine if the methodologies currently in use are robust and provide accurate, reproducible results.

The inexpensive microplate Alamar blue assay (MABA) is an indirect colorimetric DST method for determining the MICs of TB drugs for strains of *Mycobacterium tuberculosis* (10). In this assay, the redox indicator Alamar blue turns from blue to pink in the presence of mycobacterial growth. When compared with "gold standards" such as BACTEC 460 (3, 4) and the proportion method (12), the MABA has been shown to give results with high levels of agreement for rifampin (RIF) and isoniazid (INH) and lower levels of concordance for ethambutol (EMB) and streptomycin (SM). In view of the lack of published data on the reproducibility of MABA results, and in recognition that variability in MIC results is clinically important only for MICs near the critical concentration of the drug, this analysis addresses the following questions: for each of six antimycobacterial drugs, what is the MIC drift (how much do MICs vary) upon repeated microplate Alamar blue testing and how important is this variability in defining strains as susceptible or resistant?

Strains were first harvested from sputum cultures positive for *M. tuberculosis* by the microscopic observation drug susceptibility assay (MODS), which uses Middlebrook 7H9 liquid medium (11), by Lowenstein-Jensen (LJ) culture, or by both methods (Fig. 1). These strains were then each subjected to indirect DST by the MABA (3, 4, 6, 12, 13). We analyzed the internal and external reproducibility of MIC results and susceptible-resistant designations by (i) reviewing the consistency of assay results for strains from the same sample cultured on different media and (ii) comparing results for strains from consecutive, but distinct, sputum samples from a single patient, cultured on the same medium. The parent study for this analysis was an evaluation of novel diagnostics for TB and multidrug-resistant TB described in detail previously (11). Patients contributed one or two samples within the same week, and the samples were cultured in parallel in MODS and LJ media. In the parent study, MABA results were used for discrepant analyses when reference test results were discordant, and thus, the MABA was performed on all strains from both MODS and LJ cultures. An analysis of these MABA results is reported here.

The MABA was performed as described previously (see appendix Fig. S1 in the supplemental material); briefly, 200-μl volumes of sterile deionized water were added to outer-perimeter wells of sterile 96-well plates. Wells in columns 3 to 11 of rows B to G received 100 μl of Middlebrook 7H9-oleic acid-albumin-dextrose-catalase broth. Aliquots of 100 μl of the highest-concentration drug solutions were added to each well in columns 2 and 3; after mixing, a 100-μl sample was transferred from each column 3 well to the corresponding well in column 4, and the contents of the column 4 wells were then

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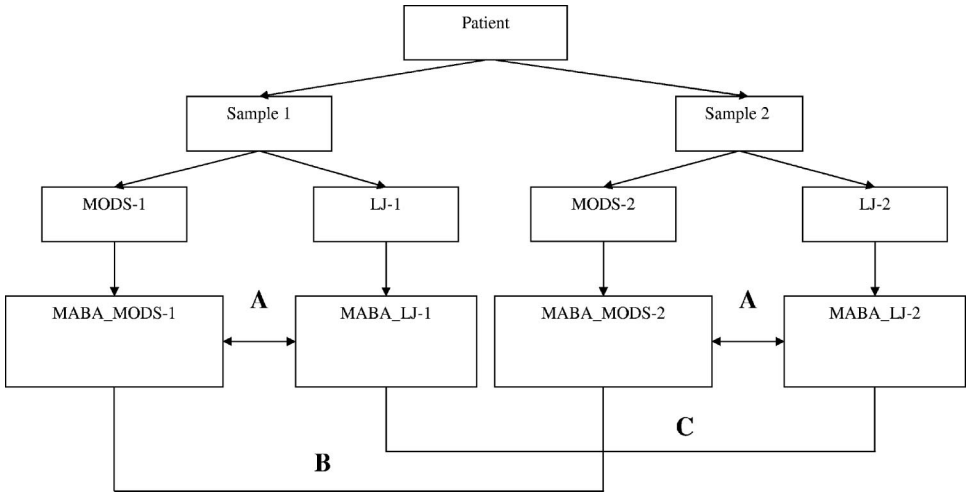

FIG. 1. Derivation of strains tested by the MABA (designated MABA_MODS-1, MABA_LJ-1, MABA_MODS-2, and MABA_LJ-2). The top row corresponds to the subject, the second row corresponds to sputum samples, the third row corresponds to cultures, and the bottom row corresponds to the strains derived from each culture. (A) Intrasample analysis. (B) Intersample analysis of paired MODS cultures. (C) Intersample analysis of paired LJ cultures.

thoroughly mixed. Identical serial 1:2 dilutions were continued until desired concentrations were reached; 100 μl of excess medium was discarded from each column 10 well. Final drug concentration ranges were as follows: INH, 0.125 to 32.0 μg/ml; RIF, 0.063 to 16 μg/ml; SM, 0.125 to 32.0 μg/ml; EMB, 0.5 to 128 μg/ml; capreomycin (CAP), 0.031 to 8 μg/ml; and ciprofloxacin (CIP), 0.063 to 16 μg/ml. *M. tuberculosis* suspensions at a McFarland standard of 1 were prepared from LJ or MODS cultures and diluted 1:25 in Middlebrook 7H9-oleic acid-albumin-dextrose-catalase to produce samples of 100 μl, which were added to wells in columns 2 to 11 of rows B to G, yielding a final well volume of 200 μl. Column 11 wells served as drug-free (inoculum-only) controls. Plates were sealed in individual Ziplock bags and incubated at 37°C for 5 days, after which the first control well (B11) was examined under an inverted light microscope for evidence of growth; if growth was observed, a freshly prepared 50-μl 1:1 mixture of Alamar blue (Trek Diagnostic Systems, OH) and 10% Tween 80 was added to this well. Plates were reincubated for 24 h, and if well B11 turned pink, the reagent mixture was added to all wells; if the well remained blue, the next control well (C11) was examined for growth and the reagent mixture was added if growth was observed. After the first day that a control well turned pink and reagent was added to all wells, the microplate was resealed and incubated for an additional 24 h at 37°C, after which all well colors were recorded. Blue was interpreted to indicate no growth, and pink was interpreted to indicate growth. The MIC was defined as the lowest drug concentration which prevented a blue-to-pink color change.

Two matched-pair analyses were performed using STATA 9.0 (Stata Corporation, College Station, TX). First, the general reproducibility of MABA results was examined by comparing the results for two samples provided by the same patient. Second, the intra-assay reproducibility of MABA results was evaluated for single samples that were aliquoted and cultured simultaneously in both LJ and MODS media. The percent agreement and the kappa statistics (to determine agreement beyond chance) were calculated for paired MABA results. CAP currently has no defined MABA breakpoint for susceptibility, so the resistant-susceptible designation analysis was not performed. DST thresholds employed for the other drugs (2, 8, 10) were as follows: INH susceptibility, MIC of ≤0.25 μg/ml, and INH resistance, MIC of ≥0.5 μg/ml; RIF susceptibility, MIC of ≤1.0 μg/ml, and RIF resistance, MIC of ≥2.0 μg/ml; EMB susceptibility, MIC of ≤2.5 μg/ml, and EMB resistance, MIC of ≥4.0 μg/ml; SM susceptibility, MIC of ≤1.0 μg/ml, and

TABLE 1. Concordance of MIC measurements and susceptible-resistant assignments in the intrasample analysis[a]

Drug	% of pairs resistant by both MABAs	% of pairs for which MICs differed by:		Kappa	% Agreement of MICs	% Susceptible-resistant assignment discordance
		>1 doubling dilution	>2 doubling dilutions			
INH	16.67	1.21	0.00	0.70	87.88	2.72
RIF	10.00	1.51	0.91	0.61	87.88	0.61
EMB	19.09	8.48	1.52	0.27	50.00	19.70
SM	4.24	5.45	0.30	0.42	58.18	6.97
CIP	0.00	3.03	0.61	0.19	67.58	2.72
CAP	NA	2.42	0.00	0.20	70.30	NA

[a] MABAs were performed on isolates recovered from parallel MODS (Middlebrook 7H9) and LJ cultures of the same sputum samples (n = 330). NA, not applicable.

TABLE 2. Concordance of MIC measurements and susceptible-resistant assignments in the intersample analysis[a]

Drug	% of LJ culture pairs resistant by both MABAs	% of LJ culture pairs for which MICs differed by:		Kappa for LJ culture pairs	% Agreement of MICs for LJ culture pairs	% Susceptible-resistant assignment discordance for LJ culture pairs	% of MODS culture pairs resistant by both MABAs	% of MODS culture pairs for which MICs differed by:		Kappa for MODS culture pairs	% Agreement of MICs for MODS culture pairs	% Susceptible-resistant assignment discordance for MODS culture pairs
		>1 doubling dilution	>2 doubling dilutions					>1 doubling dilution	>2 doubling dilutions			
INH	15.63	1.56	0.00	0.65	86.72	2.34	15.69	1.96	0.00	0.77	90.85	3.27
RIF	9.38	2.34	0.78	0.58	87.50	1.56	9.15	1.31	0.00	0.58	86.93	1.31
EMB	21.09	7.03	0.00	0.37	55.47	16.41	17.00	7.19	0.65	0.28	52.29	18.95
SM	10.94	2.34	0.00	0.47	62.50	6.25	8.50	3.92	0.00	0.41	56.86	6.54
CIP	0.00	4.69	0.78	0.20	72.66	1.56	0.65	3.27	0.65	0.24	66.01	3.27
CAP	NA	3.13	0.00	0.29	75.00	NA	NA	3.27	0.00	0.31	77.78	NA

[a] MABAs were performed on isolates recovered from pairs of LJ cultures ($n = 128$ pairs) and pairs of MODS cultures ($n = 153$ pairs) of two separate samples from the same patient. NA, not applicable.

SM resistance, MIC of ≥2 μg/ml; and CIP susceptibility, MIC of ≤1.0 μg/ml, and CIP resistance, MIC of ≥2.0 μg/ml.

Three-hundred and thirty samples yielded positive cultures in both MODS and LJ media; the levels of concordance of paired MICs of each drug and the resulting susceptible-resistant designations are shown in Table 1. For 128 patients, paired LJ culture-positive samples were available, and for 153 patients, paired MODS culture-positive samples were available for comparison; the concordance of paired MICs of each drug and the measures of discordance regarding susceptibility designations in the intersample analysis are shown in Table 2. The cross-tabulated raw data, with corresponding scatterplots, are available in the appendix in the supplemental material.

This analysis demonstrates that, regardless of the drug tested, MABA result reproducibility is not affected by whether the tested isolate is derived from LJ or MODS (Middlebrook 7H9) cultures, and intra- and intersample comparisons showed similar degrees of variability. The MIC drift varied according to the drug under consideration, with higher levels of variability noted for EMB, SM, CIP, and CAP than for INH and RIF. This variability translated into important discrepant susceptible-resistant assignments for all drugs except INH and RIF. Even paired MICs of the drugs with which the MABA performed best (INH and RIF) showed only moderate concordance. However, for these two drugs, this discrepancy had little impact on susceptible-resistant assignments because the MIC drift was generally over concentrations distant from the breakpoint.

Two limitations of this analysis were that sensitivity results for CIP were skewed due to the low number of resistant strains and that the lack of a defined MABA breakpoint for CAP precluded the assessment of the importance of CAP MIC drift.

This analysis shows that while the paired-MIC correlation was moderate at best (kappa > 0.5) (7), the majority of pairs differed by one doubling dilution or less, as a result of which the susceptible-resistant assignments were generally robust except for EMB, an agent for which DST is recognized to be challenging (9). The MABA generates more information than is generally needed by clinicians, who need to know about susceptibility or resistance for effective patient care and who rarely require specific MICs. We recommend that MABA results be reported as susceptible or resistant and that, if the measured MIC is within one dilution of the breakpoint, the assay be repeated using the same isolate.

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