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Multicenter Laboratory Validation of Susceptibility Testing of *Mycobacterium tuberculosis* against Classical Second-Line and Newer Antimicrobial Drugs by Using the Radiometric BACTEC 460 Technique and the Proportion Method with Solid Media

GABY E. PFYFFER,[1]* DONALD A. BONATO,[2] ADELEH EBRAHIMZADEH,[3] WENDY GROSS,[2] JACQUELINE HOTALING,[4] JOHN KORNBLUM,[3] ADALBERT LASZLO,[5] GLENN ROBERTS,[6] MAX SALFINGER,[4] FRANZISKA WITTWER,[1] AND SALMAN SIDDIQI[7]

Swiss National Center for Mycobacteria, Department of Medical Microbiology, University of Zurich, Zurich, Switzerland[1]*; Veterans Affairs Medical Center, West Haven, Connecticut*[2]*; New York City Department of Health, New York,*[3] *and Wadsworth Center, New York State Department of Health, Albany,*[4] *New York; Laboratory Center for Disease Control, Ottawa, Canada*[5]*; Mayo Clinic, Rochester, Minnesota*[6]*; and Becton Dickinson Microbiology Systems, Sparks, Maryland*[7]

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In a large multicenter study involving six major study sites in the United States, Canada, and Europe, the susceptibilities of 272 *Mycobacterium tuberculosis* strains to classical second-line antituberculosis (anti-TB) drugs (capreomycin, cycloserine, ethionamide, and kanamycin) and newer compounds (amikacin, clofazimine, ofloxacin, and rifabutin) were determined by the radiometric BACTEC 460 procedure and the conventional proportion method on Middlebrook 7H10 agar. Previously established critical concentrations for classical second-line anti-TB drugs were compared with several concentrations in liquid medium to establish equivalence. MICs of newer compounds determined in liquid medium were either the same or up to four times lower than those determined in agar medium. After establishing critical concentrations (breakpoints) in the extended testing of clinical isolates, we obtained an excellent overall correlation between the two systems, with no errors with amikacin, kanamycin, and ofloxacin and very few major or very major errors with the other drugs; however, for cycloserine, no breakpoint concentration could be recommended due to repeatedly inconsistent results by both methods. Based on these data we conclude that the BACTEC 460 procedure is a simple and rapid method requiring 4 to 8 days on average to generate accurate antimicrobial susceptibility testing (AST) results for eight anti-TB drugs other than those considered primary ones. These data not only fill a major gap of knowledge regarding the critical test concentrations of secondary anti-TB drugs but also provide a baseline for future evaluations of *M. tuberculosis* AST with the more recently developed, nonradiometric broth-based culture systems.

With the recent global resurgence of tuberculosis (TB) and concomitant rise in multidrug-resistant strains of *Mycobacterium tuberculosis* (37, 38), there is an increasing demand for determining the in vitro susceptibilities of clinical isolates to antimicrobial agents other than those considered primary drugs (isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin). Conventional antimicrobial susceptibility testing (AST) with solid media such as Löwenstein-Jensen (LJ) or Middlebrook 7H10 agar requires 3 or more weeks to be completed, and for some classical second-line drugs as well as newer antimicrobial agents, appropriate critical drug concentrations have not fully been established.

AST by the radiometric BACTEC method (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), introduced in 1980, is an efficient way to test frontline drugs (27, 30–33) and results in a significantly shorter turnaround time than that of the traditional proportion method. Susceptibility testing by this procedure has become the current method of choice (34), even when the hazards arising from the use of radioisotopes are taken into account. However, data on critical concentrations for classical second-line and other newer drugs are largely fragmentary or lacking altogether. Some data from AST of second-line drugs with BACTEC 12B medium were presented quite early (11) but have never been published. Allen et al. (1) defined the MICs on Middlebrook agar of aminoglycosides while Heifets' group undertook several investigations into AST with the BACTEC 12B medium, e.g., with aminoglycosides (13), ethionamide (16, 22), and quinolones (15), the last group of compounds having also been studied by others (4, 5, 8, 9). The MICs of rifabutin, generated either with the radiometric BACTEC 12B medium (17) or with Middlebrook 7H9 broth (7), are also available. However, as a whole, most of these studies have either included only a narrow spectrum of drugs and/or tested only a rather limited number of *M. tuberculosis* strains.

In light of this situation, the primary aim of our study was to develop a basic protocol which defines appropriate critical concentrations for secondary drugs to allow reliable testing with both BACTEC 12B and agar medium. With more than 270 strains of *M. tuberculosis*, the reproducibility of results generated by different laboratories was also assessed. Once vali-

* Corresponding author. Mailing address: Swiss National Center for Mycobacteria, Department of Medical Microbiology, University of Zurich, Gloriastrasse 30, 8028 Zurich, Switzerland. Phone: 41 1 634 27 86. Fax: 41 1 634 49 18. E-mail: pfyffer@immv.unizh.ch.

dated, the radiometric procedure should allow clinical laboratories to provide physicians with accurate and timely drug susceptibility information. In addition, since quite a few nonradiometric mycobacterial culture systems based on liquid medium, such as the Mycobacteria Growth Indicator Tube (manual version [23]; BACTEC 960 automated version [12]), the MB/BacT (2), and the ESP Culture System II (35), have recently been developed, this study may also provide a guideline for future development of AST procedures with these novel devices.

MATERIALS AND METHODS

Study sites. This study was conducted at six mycobacteriology laboratories, including the Mycobacteriology Laboratory, Veterans Affairs Medical Center, West Haven, Conn.; the Bureau of Laboratories, New York City Department of Health, New York, N.Y.; the Laboratory Center for Disease Control, Ottawa, Ontario, Canada; the Mycobacteriology Laboratory, Mayo Clinic, Rochester, Minn.; the Mycobacteriology Laboratory, Wadsworth Center, New York State Department of Health, Albany, N.Y.; and the Swiss National Center for Mycobacteria, Department of Medical Microbiology, University of Zurich, Zurich, Switzerland. A seventh site, the Research and Development Division, Becton Dickinson Microbiology Systems, Sparks, Md., was the coordination site, from which cultures and antimicrobial agents were supplied and at which data were collected.

Antimicrobial agents. All drugs were obtained from the manufacturers in a chemically pure form. The drugs used were capreomycin sulfate, D-cycloserine, ethionamide, and kanamycin monosulfate from Sigma, St. Louis, Mo.; amikacin from Bristol-Myers Squibb, Syracuse, N.Y.; clofazimine from Ciba-Geigy, Suffern, N.Y.; ciprofloxacin from Miles, West Haven, Conn.; ofloxacin from Ortho/R. W. Johnson Pharmaceutical, Raritan, N.J.; and rifabutin from Adria Laboratories, Dublin, Ohio. The compounds were supplied by the coordinator of the study. Amikacin, capreomycin, ciprofloxacin, and kanamycin were dissolved in sterile distilled water (DW). Ofloxacin was dissolved in a 0.1 N NaOH solution. Ethionamide was dissolved in ethylene glycol and incubated overnight for sterilization. Subsequent dilutions of these two drugs were made in sterile DW. Clofazimine was dissolved in dimethyl sulfoxide and stored at room temperature in the dark. Subsequent dilutions were made in the same solvent. For D-cycloserine a 0.1% solution of Na_2CO_3 was prepared and added to 100 ml of DW until a pH of 10 was achieved. Dilutions were made in sterile DW. Rifabutin was dissolved in methanol and diluted in DW. Stock solutions of each drug were made at least 40 times higher than the highest test concentration used. Except for clofazimine and ethionamide, which are considered self-sterilizing, all stock solutions were filtered in a sterile manner with a 0.22-μm-pore-size polycarbonate filter; approximately 20% of the initial filtrate was discarded. All stock solutions except clofazimine were stored at −70°C in small aliquots. Once thawed, the remaining solutions were discarded.

Culture media and quality control. Middlebrook 7H12 broth (BACTEC 12B; Becton Dickinson Microbiology Systems) was used for radiometric testing, and Middlebrook 7H10 agar plates (BBL, Cockeysville, Md.) were used for AST by the proportion method. All media were supplied by the coordinator of the study. *M. tuberculosis* H37Rv (ATCC 27294; susceptible to all drugs tested) was used as a strain for internal quality control, which was done on a weekly basis, along with the other strains included in the study.

AST. (i) BACTEC 460. For radiometric AST, the standard protocol was followed (31). Each drug was tested at several concentrations. Antimicrobials were always added in 0.1-ml quantities to a BACTEC 12B vial to achieve the desired concentrations. Susceptibility and resistance were judged by comparison of the change in the growth index of the control with that of the test drug as recommended. This interpretation gave susceptibility results at the 1% proportion basis.

(ii) Traditional proportion method. AST was carried out with Middlebrook 7H10 agar according to the standard procedure (18, 20). For some classical second-line antituberculotics (capreomycin, cycloserine, ethionamide, and kanamycin), the recommended critical concentrations (20) were used. For all other drugs, several concentrations were tested to establish the MICs and critical concentrations. The MIC was defined as the lowest concentration of drug that inhibited more than 99% of the bacterial population. As a consequence, the critical concentration was defined as the concentration of drug required to prevent growth above the 1% threshold (critical proportion) of the test population of TB bacilli (18). Results were read 21 days after inoculation of the medium.

Study design and strains. The study was carried out concomitantly in three phases at all six centers.

Phase I. Phase I was designed to establish a basic test procedure and to determine a working range for the antimicrobial drug concentrations to be used in liquid medium (BACTEC 12B) and on conventional solid media. In addition, the reproducibility of the results of the test systems was evaluated. For this purpose, a total of 10 clinical isolates of *M. tuberculosis* which were susceptible to all primary drugs in earlier tests and stored in a strain collection were subcultured onto LJ slants. As soon as growth appeared on the slants (10 to 15 days after inoculation) these isolates were shipped by overnight delivery to the six clinical testing sites. In an effort to minimize variations of results, the cultures were used directly for AST without subculturing. For those antibiotics whose critical concentrations had been established previously on solid medium, i.e., capreomycin, cycloserine, ethionamide, and kanamycin, only one drug concentration was tested on Middlebrook 7H10 agar but at least three concentrations were tested in BACTEC 460. For all other drugs at least three different concentrations were tested by the proportion method as well as with BACTEC 460.

Phase II. The aims of phase II were to establish equivalent breakpoint drug concentrations for the BACTEC 460 and conventional solid medium methodologies and to evaluate interlaboratory reproducibility of test results for susceptible and resistant isolates. Drug concentrations used in phase II were adjusted and finalized based on the information obtained from phase I. Strains of *M. tuberculosis* with known drug resistance, especially to the test drugs, were selected from several clinical sources. Twenty isolates were selected and subcultured onto LJ medium. Once growth appeared, sets of 20 isolates each were shipped to the six test sites and were used for AST as in phase I.

Phase III. Phase III was extended testing with both BACTEC 460 and Middlebrook 7H10 agar with the drug concentrations established in phase II. Staff at each site independently tested at least 24 strains of *M. tuberculosis* which had recently been isolated from clinical specimens at their own laboratory.

RESULTS

In phases I and II of this multicenter AST study sets of TB strains containing 10 and 20 isolates, all from stock cultures, were analyzed. Strains tested in phase I were confirmed to be susceptible to all drugs. Among the 20 strains tested in phase II, 6 were resistant to capreomycin, 0 were resistant to cycloserine, 10 were resistant to ethionamide, 9 were resistant to kanamycin, 8 were resistant to amikacin, 1 was resistant to clofazimine, 1 was resistant to ofloxacin, and 13 were resistant to rifabutin. In phase III, AST was performed with a total of 242 *M. tuberculosis* strains which had recently been isolated from clinical specimens by staff at the six participating laboratories. Nineteen strains were resistant to capreomycin, 19 were resistant to cycloserine, 56 were resistant to ethionamide, 34 were resistant to kanamycin, 32 were resistant to amikacin, 0 were resistant to clofazimine, 20 were resistant to ofloxacin, and 83 were resistant to rifabutin.

Phase I. The 10 fully drug-susceptible isolates supplied to all sites were tested against various concentrations of eight drugs. Most BACTEC 460 MICs determined at the test sites agreed within ±1 serial dilution (Table 1). For example, the MIC of amikacin was ≤1.0 μg/ml for 100% of the strains and that of capreomycin was ≤1.25 μg/ml for 98.3% of the strains at all test sites combined. With a few drugs, MICs extended over a wider range than that noted above. This held true for the quinolones, whose MICs varied by more than 1 dilution from site to site, although in the majority of cases the values were between ≤0.5 and 1.0 μg/ml. Cycloserine gave the least-reproducible results for both BACTEC 460 and conventional testing (proportion method). Based on phase I results, drug concentrations were adjusted for subsequent testing in phase II. For amikacin, clofazimine, and ethionamide, drug concentrations to be used with BACTEC 460 were lowered by 1 dilution, whereas for cycloserine and ofloxacin, concentrations were increased for both solid and liquid medium systems. Ciprofloxacin testing was discontinued; on the other hand, rifabutin testing was included in phases II and III.

Phase II. Results of phase II testing of the 20 susceptible and resistant strains of *M. tuberculosis* sent to all six study sites are reported in Table 2. Interlaboratory reproducibility of results by the BACTEC 460 and conventional AST methods was good, with MICs being within ±1 dilution of each other in most cases. It appeared that agar AST results varied more from site to site than those obtained by the BACTEC 460 method. In general, interlaboratory variation in results was observed more frequently with cycloserine and to some extent

TABLE 1. Results of phase I generated by the six study sites in AST of 10 susceptible strains of *M. tuberculosis*

Antimicrobial agent	BACTEC 460 medium		Middlebrook 7H10 agar	
	Inhibitory concn (μg/ml)	Count (%)[a]	Inhibitory concn (μg/ml)	Count (%)
Capreomycin	≤1.25	59 (98.3)	10.0	59 (98.3)
	2.5	1 (1.7)		
Cycloserine	≤50	27 (45)	25.0	36 (60)
	60.0	23 (38.3)		
	70.0	9 (15)		
	>70.0	1 (1)		
Ethionamide	≤1.25	59 (98.3)	5.0	59 (98.3)
	2.5	1 (1.7)		
Kanamycin	≤1.25	48 (80)	5.0	60 (100)
	2.5	12 (20)		
Amikacin	≤1.0	60 (100)	≤2.0	54 (90)
			>4.0	6 (10)
Clofazimine	≤0.25	59 (98.3)	≤0.25	47 (78.3)
	0.5	1 (1.7)	0.5	13 (21.7)
Ciprofloxacin	≤0.5	46 (76.7)	≤0.5	41 (68.3)
	1.0	13 (21.7)	1.0	18 (30)
	2.0	1 (1.7)	2.0	1 (1.7)
Ofloxacin	≤0.5	21 (35)	≤0.5	19 (31.7)
	1.0	34 (56.7)	1.0	38 (63.3)
	2.0	4 (6.7)	2.0	3 (5)
	>4.0	1 (1.7)		

[a] The count is the total number of susceptible strains.

with ethionamide and capreomycin than with the other drugs, regardless of the AST method applied.

For some drugs BACTEC 460 medium-based MICs were lower than agar-based MICs (up to four times), while for others they were slightly lower than or equal to agar-based MICs. MICs for susceptible strains in 12B medium were mainly between ≤1.25 and 2.5 μg/ml for capreomycin, ≤0.625 and 1.25 μg/ml for ethionamide, and ≤1.25 and 2.5 μg/ml for kanamycin. Amikacin susceptibility test results with MICs of ≤0.5 μg/ml in 12B medium and ≤2.0 and 4.0 μg/ml in Middlebrook 7H10 medium were equivalent. Clofazimine was more active in liquid medium, with MICs between ≤0.125 and 0.25 μg/ml in the BACTEC 460 and ≤0.5 and 1.0 μg/ml in the Middlebrook 7H10 medium. Ofloxacin and rifabutin were found to be equally active in BACTEC 12B medium and on Middlebrook 7H10 medium, with MICs between ≤1.0 and 2.0 μg/ml and ≤0.5 and 1.0 μg/ml, respectively. Cycloserine susceptibility testing continuously yielded inconsistent results. Therefore, no breakpoint could be established. Results for cycloserine on Middlebrook 7H10 agar were even less reliable.

Overall, the results allowed distinct MICs to be determined for susceptible and resistant strains by both techniques. This information was used to establish tentative breakpoint concentrations for further data analysis (Table 3). When results were analyzed with these critical concentrations, there was good agreement of results with both susceptible and resistant TB strains (Table 4). In some instances (clofazimine and ofloxacin), the total number of resistant strains was, however, too small to draw any definite conclusions.

Phase III. Phase III extended testing with 242 clinical isolates of *M. tuberculosis* among the six study sites, with 27 isolates at site 1, 42 isolates at site 2, 48 isolates at site 3, 24 isolates at site 4, 68 isolates at site 5, and 33 isolates at site 6. Tentative breakpoint concentrations elaborated in phase II (Tables 3 and 4) were acceptable with some minor changes (Table 5). Generally, we looked at the concentrations which gave the fewest total errors (ethionamide at 1.25 μg/ml in the BACTEC 460 and 5.0 μg/ml in conventional AST, kanamycin at 5.0 μg/ml in the BACTEC 460 and 5.0 μg/ml in conventional AST, amikacin at 1.0 μg/ml in the BACTEC 460 and 4.0 μg/ml in conventional AST, clofazimine at 0.5 μg/ml in the BACTEC 460 and 1.0 μg/ml in conventional AST, ofloxacin at 2.0 μg/ml in the BACTEC 460 and 2.0 μg/ml in conventional AST, and rifabutin at 0.5 μg/ml in the BACTEC 460 and 1.0 μg/ml in conventional AST). For kanamycin, amikacin, clofazimine, ciprofloxacin, and rifabutin these concentrations also gave the fewest very major errors. The only exception was capreomycin. Although overall agreement for this drug at 2.5 μg/ml (8 very major errors, 2 major errors) was better than at 1.25 μg/ml (4 very major errors, 13 major errors), we have chosen 1.25 μg/ml as the breakpoint (only 4 very major errors instead of 8). Cycloserine testing was, again, very inconsistent by both the BACTEC 460 and agar methodologies. At one site, cycloserine testing by the method of proportion did not yield reportable results at all, with false resistance being shown even with the *M. tuberculosis* H37Rv control. These data were excluded from our analysis (Table 5).

Results obtained from the BACTEC 460 and the conventional agar methods were compared by constructing 2-by-2 tables for each drug concentration. Critical concentrations were eventually established in such a way that they yielded the least number of very major errors (false susceptibility) and major errors (false resistance). With the critical test concentrations established in this phase, neither false resistance between BACTEC 460 and conventional AST (resistance by BACTEC 460, susceptibility by conventional AST) nor false susceptibility (susceptibility by BACTEC 460, resistance by conventional AST) was observed for amikacin, kanamycin, or ofloxacin. Results with other antimicrobial agents like clofazimine and rifabutin showed very minor discordance. Ethionamide results produced 7 (12.5%) very major disagreements, while capreomycin results produced 13 major but only 4 (21.1%) very major disagreements. Cycloserine testing resulted in 17 (89.5%) very major disagreements (Table 5).

Eventually, this information led to the definition of the critical concentrations (breakpoints) for testing in BACTEC 12B medium and on Middlebrook 7H10 agar (Table 6).

DISCUSSION

The data presented here constitute the first comprehensive, multicenter AST study of second-line anti-TB drugs and newer compounds currently being used for the treatment of TB, validating both broth-based and solid-medium-based systems. Given the fact that MICs, as a whole, are highly dependent on a large array of different factors such as medium composition, pH, inoculum size, and incubation time, the primary aim of this study was to establish critical concentrations with which to perform AST on a wide spectrum of anti-TB drugs under defined conditions.

Our data, encompassing more than 17,000 individual susceptibility test results generated with 272 strains of *M. tuberculosis* in six centers, corroborate the fact that the radiometric BACTEC 460 method is feasible for AST of most of these drugs as it has previously been shown to be for frontline an-

TABLE 2. Reproducibility data generated at the six study sites (phase II) during AST of 20 strains of *M. tuberculosis*

Drug	Strain category[a] (no. of strains)	Test system	Inhibitory concn (μg/ml)	No. of cultures at site:						Total no. of cultures
				1	2	3	4	5	6	
Capreomycin	S (14)	BACTEC 460	≤1.25	14	13	14	11	7	13	72
			2.5	0	1	0	2	7	0	10
			5	0	0	0	1	0	1	2
		Middlebrook 7H10	≤10.0	13	13	12[c]	14	14	12	78
	R (6)	BACTEC 460	>5.0	6	6	6	6	6	6	36
		Middlebrook 7H10	>10.0	6	6	1	6	0[c]	6	25
Cycloserine	S (20)	BACTEC 460	≤75	20	20	18	17	14	—[b]	89
			100	0	0	1	3	5	—	9
			200	0	0	1	0	1	—	2
		Middlebrook 7H10	≤30	19	20	18	18	16	—	91
Ethionamide	S (10)	BACTEC 460	≤0.625	6	6	6	3	3	5	29
			1.25	3	4	4	6	6	5	28
			2.5	1	0	0	1	1	0	3
		Middlebrook 7H10	≤5.0	7	9	10	10	10	9	55
	R (10)	BACTEC 460	≤0.625	0	0	0	0	1	0	1
			1.25	1	2	1	1	1	0	6
			2.5	2	2	1	2	2	1	10
			>2.5	7	6	8	7	6	9	43
		Middlebrook 7H10	≥5.0	10	9	2[c]	10	4[c]	2	37
Kanamycin	S (11)	BACTEC 460	≤1.25	8	7	7	6	7	8	43
			2.5	3	4	4	5	4	3	23
		Middlebrook 7H10	≤5.0	11	11	11	11	11	10	65
	R (9)	BACTEC 460	≤1.25	0	0	1	0	1	3	1
			2.5	1	1	0	1	0	0	3
			5	8	8	8	8	8	8	48
		Middlebrook 7H10	≥5.0	9	9	9	9	7	9	52
Amikacin	S (12)	BACTEC 460	≤0.5	12	12	11[c]	12	12	12	71
		Middlebrook 7H10	≤2.0	11	11	11	10	12	12	67
			4.0	0	0	0	1	0	0	1
			>4.0	1	1	1	1	0	0	4
	R (8)	BACTEC 460	>2.0	8	8	8	8	8	8	48
		Middlebrook 7H10	>4.0	8	8	8	8	7[c]	8	47
Clofazimine	S (19)	BACTEC 460	≤0.125	13	19	18	19	18	19	106
			0.25	6	0	1	0	1	0	8
		Middlebrook 7H10	≤0.5	19	19	18[c]	19	18[c]	17	110
			1	0	0	0	0	0	2	2
	R (1)	BACTEC 460	≤0.125	0	0	0	1	0	0	1
			0.5	0	1	1	0	1	0	3
			>0.5	1	0	0	0	0	1	2
		Middlebrook 7H10	≤0.5	0	0	1	1	1	0	3
			1.0	1	1	0	0	0	0	2
			>1.0	0	0	0	0	0	1	1
Ofloxacin	S (19)	BACTEC 460	≤1.0	16	15	14	16	18	15	94
			2.0	3	4	5	3	1	4	20
		Middlebrook 7H10	≤1.0	18	19	17[c]	14	18[c]	12	98
			2.0	1	0	1	5	0	7	14
	R (1)	BACTEC 460	4.0	1	0	0	0	0	0	1
			>4.0	0	1	1	1	1	1	5
		Middlebrook 7H10	>4.0	1	1	1	1	1	1	6
Rifabutin	S (7)	BACTEC 460	≤0.5	6	6	6	7	7	7	39
			1.0	1	1	1	0	0	0	3
		Middlebrook 7H10	≤0.5	7	7	7	7	7	7	42
	R (13)	BACTEC 460	1.0	1	1	0	0	1	1	4
			2.0	0	0	1	1	0	0	2
			>2.0	12	12	12	12	12	12	72
		Middlebrook 7H10	≤0.5	1	1	0	0	1	1	4
			1.0	0	0	0	1	2	0	3
			2.0	0	0	3	0	2	0	5
			>2.0	12	12	9[c]	12	5[c]	12	62

[a] S, susceptible; R, resistant.
[b] Site 6 was excluded (see Results).
[c] Results of some susceptibility tests are missing.

TABLE 3. Tentative breakpoints and ranges of presumptive critical concentrations for AST in liquid and on solid media (phase II)

Drug	Range of critical concn or critical concn[a]	
	In BACTEC 460	On Middlebrook 7H10 agar
Capreomycin	1.25–2.5	10
Cycloserine	75–100	30
Ethionamide	1.25–2.5	5
Kanamycin	2.5–5.0	5
Amikacin	0.5–1.0	2.0–4.0
Clofazimine	0.125–0.25	0.5–1.0
Ofloxacin	1.0–2.0	1.0–2.0
Rifabutin	0.5–1.0	0.5–1.0

[a] Single values indicate previously established critical concentrations (20).

ti-TB drugs (30–33). Complying with the recommendation of the Centers for Disease Control and Prevention of using broth-based methods for AST (34), more and more laboratories in the United States have now adopted the BACTEC 460 technique so that test results can be reported to the clinician with a minimal turnaround time, i.e., within 4 to 8 days, as was the average in our study. Besides the longer turnaround time, there are several other drawbacks of AST on solid media, such as drug inactivation during agar preparation and degradation of the antimicrobial agent during the extended period of incubation (10), which can, at least to some extent, be circumvented easily by using broth-based media. Another important goal of the study was to achieve a high degree of interlaboratory reproducibility of AST results. As indicated by our data, the agreement in results among the six laboratories was quite high throughout the three phases.

The drug panel remained the same throughout the study, except that (i) ciprofloxacin was dropped after phase I because of its known poor in vivo response against TB, and (ii) rifabutin, a promising anti-TB drug, was added for phases II and III. For the first two phases, each of the six sites was provided with identical sets of *M. tuberculosis* strains. In testing of these strains by all participants, susceptibility data generated in these two initial phases were found to be highly reliable and reproducible. Further testing eventually led to the establishment of the final critical test concentrations for seven of the eight second-line drugs. Basically, establishment of the final concentrations was achieved in three major steps: (i) by establishing MICs by testing fully susceptible TB strains (phase I), (ii) by adjusting drug concentrations and testing susceptible and resistant strains (phase II), and (iii) by testing a large number of clinical *M. tuberculosis* isolates ($n = 242$) with particular emphasis on isolates that are resistant to primary drugs. This process helped in establishing the final breakpoints by selecting those drug concentrations which gave minimum false susceptible and false resistant results with both test systems.

Allen et al. (1) have defined critical concentrations in Middlebrook 7H11 agar as 9.5 μg/ml for capreomycin, 2.5 μg/ml for kanamycin, and 1.0 μg/ml for amikacin. These concentrations are different from ours on Middlebrook 7H10, as we tested 10.0 μg of capreomycin per ml and 5.0 μg of kanamycin per ml, which have been established previously as critical concentrations, and 4.0 μg of amikacin per ml.

Important work in radiometric AST, though with a very limited number of strains of *M. tuberculosis*, has been carried out by Heifets et al. (13–17). This group has suggested the following critical drug concentrations for BACTEC 12B medium: 5.0 μg/ml (MIC range, 1.5 to 3.0) for capreomycin, 4.0 μg/ml (indicating an MIC range of 0.5 to 1.0) for amikacin, 2.0 μg/ml (MIC range, 0.25 to 2.0) for ofloxacin, and 0.12 μg/ml (MIC range, 0.015 to 0.06) for rifabutin. In general, these MICs differ by 1 to 2 serial dilutions from ours. This divergence is not surprising when one considers those authors' notion that, at these suggested critical concentrations, the test strain, if susceptible, should be considered only moderately susceptible. In their hands, fully susceptible strains were those which were susceptible to a concentration 1 dilution (twofold) lower than the critical concentration while resistant strains were those which were resistant to a concentration 1 serial dilution higher than the critical concentration. Even a fourth category, designated very resistant, was suggested. Though the concept of four categories of susceptibility, i.e., susceptible, moderately susceptible, resistant, and very resistant, might be helpful to some physicians, in particular when dealing with multidrug-resistant TB, this concept has, however, not been widely accepted.

Additional information is available for the quinolones (4, 5, 19, 21). Chen et al. (5) established MIC ranges in BACTEC 12B medium for ciprofloxacin between 0.25 and 2.0 μg/ml and for ofloxacin between 0.5 and 2.0 μg/ml and set the breakpoint concentration at 2.0 μg/ml. These values agree perfectly with

TABLE 4. Agreement of test results based on the tentative breakpoint concentrations (phase II)

Drug	Strain category[a] (no. of strains)	BACTEC 460		Middlebrook 7H10	
		Inhibitory concn (μg/ml)	Agreement (%)	Inhibitory concn (μg/ml)	Agreement (%)
Capreomycin	S (14)	1.25	86	10	94
		2.5	98		
	R (6)	1.25	100	10	71
		2.5	100		
Cycloserine	S (20)	75	89	30	91
		100	98		
Ethionamide	S (10)	1.25	95	5	92
		2.5	100		
	R (10)	1.25	88	5	65
		2.5	72		
Kanamycin	S (11)	2.5	100	5	98
		5.0	100		
	R (9)	2.5	89	5	96
		5.0	89		
Amikacin	S (12)	0.5	100	2.0	93
		1.0	100	4.0	94
	R (8)	0.5	100	2.0	100
		1.0	100	4.0	100
Clofazimine	S (19)	0.125	93	0.5	98
		0.25	100	1.0	100
	R (1)	0.125	83	0.5	50
		0.25	83	1.0	17
Ofloxacin	S (19)	1.0	100	1.0	88
		2.0	100	2.0	100
	R (1)	1.0	100	1.0	100
		2.0	100	2.0	100
Rifabutin	S (7)	0.5	93	0.5	100
		1.0	100	1.0	100
	R (13)	0.5	100	0.5	95
		1.0	95	1.0	91

[a] S, susceptible; R, resistant.

TABLE 5. Analysis of false susceptibility (very major error) and false resistance (major error) results at various drug concentrations (phase III)

Drug	Inhibitory concn (μg/ml)		No. of isolates tested by BACTEC 460 and Middlebrook 7H10 methods[a] that were:				Errors (%)	
	In BACTEC 460 medium	On Middlebrook 7H10 agar	S by both tests	R by both tests[b]	S by BACTEC 460 and R by Middlebrook 7H10	R by BACTEC 460 and S by Middlebrook 7H10	VM[c] (false S)	M[d] (false R)
Capreomycin	1.25	10.0	210	15	4	13	21.1	6.2[h]
	2.5		221	11	8	2	42.1	0.9
	5.0		223	7	12	0	63.2	None
Cycloserine[e]	75.0	30.0	175	5	14	15	73.7	8.6
	100		187	2	17[f]	3	89.5	1.6
	200		190	0	19	0	100	None
Ethionamide	0.625	5.0	149	56	0	37	None	24.8
	1.25		177	49	7	9	12.5	5.1[h]
	2.5		182	41	15	4	26.8	2.2
Kanamycin	2.5	5.0	206	34	0	2	None	1.0
	5.0		208	34	0	0	None	None[h]
Amikacin	0.5	4.0	209	32	0	1	None	0.5
	1.0		210	32	0	0	None	None[h]
Clofazimine	0.125	0.5	202	3	2	35	40.0	17.3
	0.25		227	2	3	10	60.0	4.4
	0.5		234	0	5	3	98.7	1.3
	0.125	1.0	204	0	0	38	NA[g]	18.6
	0.25		230	0	0	12	NA	5.2
	0.5		239	0	0	3	NA	1.3[h]
Ofloxacin	1.0	1.0	211	21	4	6	16.0	2.8
	2.0		217	20	5	0	20.0	None
	4.0		217	18	7	0	28.0	None
	1.0	2.0	215	20	0	7	None	3.3
	2.0		222	20	0	0	None	None[h]
	4.0		222	18	2	0	10.0	None
	1.0	4.0	215	18	0	9	None	4.2
	2.0		222	18	0	2	None	0.9
	4.0		223	17	1	1	5.6	0.5
Rifabutin	0.5	1.0	158	83	0	1	None	0.6[h]
	1.0		158	82	1	1	1.2	0.6
	0.5	2.0	158	81	0	3	None	1.9
	1.0		159	81	0	2	None	1.3

[a] S, susceptible; R, resistant.
[b] Reliability of the analyses was questionable due to the very low number of resistant isolates.
[c] VM, very major disagreement (susceptible by BACTEC 460 and resistant by the proportion method).
[d] M, major disagreement (resistant by BACTEC 460 and susceptible by the proportion method).
[e] Site 6 was excluded. For cycloserine no recommendation concerning the critical concentration is being made.
[f] Fourteen of 17 isolates that were resistant by the Middlebrook 7H10 method but susceptible by the BACTEC 460 method were from a single laboratory (site 3).
[g] NA, not applicable since none of the tested organisms were resistant at the conventional concentration.
[h] Critical concentration.

our ofloxacin data (MICs, 1.0 to 2.0 μg/ml; breakpoint, 2.0 μg/ml). Others, however, have reported MICs of ofloxacin between 0.5 and 1.0 μg/ml and a critical concentration of 1.0 μg/ml (4). The range of MICs for our test strains on solid medium appeared, however, to be narrow (1.0 to 4.0 μg/ml, with a critical concentration of 2.0 μg/ml), compared to MICs of 0.3 to 4.0 μg/ml and 0.125 to 4.0 μg/ml for ciprofloxacin and ofloxacin, respectively, in Middlebrook 7H10 medium (5).

The reported MICs of rifabutin in BACTEC 12B medium extend over a broad range. Our values (0.5 to 1.0 μg/ml) are perfectly concordant with those of Della Bruna and Olliaro (6). However, other groups have found both lower ranges (0.015 to 0.125 μg/ml [5] and 0.015 to 0.6 μg/ml [17]) and a higher range (3.6 μg/ml [7]), the last having been generated in Middlebrook 7H9 broth. One of the most recent studies of AST of secondary anti-TB drugs (25) confirms largely our values for capreomycin (1.0 to 2.0 versus 1.25 to 5.0 μg/ml [this study]), kanamycin (5.0 versus 2.0 to 4.0 μg/ml), amikacin (0.5 to 1.0 versus 0.5 μg/ml), and clofazimine (0.5 versus 0.1 to 0.4 μg/ml). Such low MICs of the latter drug were also found by others (26). Rastogi et al. (25) used a human macrophage system and demonstrated that, of a large variety of drugs tested, clofazimine was the only one which yielded discrepant results between its extracellular and intracellular activities. This may be due to the fact that this drug concentrates within phagosomes and phagolysosomes of infected macrophages (24), which might, at least to some extent, explain its ineffectiveness as an anti-TB agent.

Even though for some of the compounds tested it was not

TABLE 6. Final recommendations for critical test concentrations for second-line drugs

Drug	Recommended test concn	
	In BACTEC 460	On Middlebrook 7H10 agar
Capreomycin	1.25	10.0
Cycloserine	—[a]	—
Ethionamide	1.25	5.0
Kanamycin	5.0	5.0
Amikacin	1.0	4.0
Clofazimine	0.5	1.0
Ofloxacin	2.0	2.0
Rifabutin	0.5	1.0

[a] No recommendation due to inconsistent results.

possible to include a fair amount of resistant strains in the test panels, which would call for additional studies with genetically well-characterized, resistant strains, several important conclusions can be drawn from our study. (i) Except with cycloserine, the radiometric BACTEC 460 procedure is a simple and rapid method requiring 4 to 8 days on average to generate reliable AST results for second-line anti-TB drugs. (ii) Cycloserine testing should be discontinued, due to difficulties with the drug at all six study sites, despite repeated testing. (iii) With these comprehensive AST data it is anticipated that our evaluation may also provide a guideline for future studies, especially when AST procedures are established for the newer, nonradiometric, broth-based systems such as the MB/BacT (28), the ESP Culture System II (3), the manual Mycobacteria Growth Indicator Tube (29, 36) or its automated version (BACTEC 960). The drug concentrations defined here for BACTEC 12B medium and Middlebrook 7H10 agar will undoubtedly serve as a reliable baseline for establishing critical test concentrations for AST by these recently developed growth-based technologies.

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