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French Multicenter Study Involving Eight Test Sites for Radiometric Determination of Activities of 10 Antimicrobial Agents against *Mycobacterium avium* Complex

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The radiometric BACTEC 460-TB methodology has filled an increased need in the screening of a wide range of antimicrobial agents against *Mycobacterium avium* (MAC) isolates on a patient-to-patient basis. In this context, a multicenter study involving eight test sites across France was performed to determine the MICs of 10 antimicrobial agents for MAC organisms. The aim of the investigation was to compare the in vitro activities of D-cycloserine, ethambutol, ethionamide, rifampin, amikacin, streptomycin, ciprofloxacin, sparfloxacin, clofazimine, and clarithromycin against MAC isolates. All of the test sites received the same clinical isolates of MAC, and the MICs were determined by a common protocol. The overall interlaboratory reproducibility of the MICs within ±1 dilution of the modal MICs varied from 79.70 to 100% (mean, 95.2% ± 2.1%), whereas overall agreement of the MICs among the test sites varied from a mean of 91% ± 4.1% to a mean of 98 ± 1.3%. We confirmed that the proposed methodology is easy, accurate, and sufficiently reproducible to be used routinely in a clinical laboratory. Despite variations in the MICs of the same drug among strains, no link between the origin of MAC isolates (from human immunodeficiency virus-positive or -negative patients) and their drug susceptibilities was established. On the basis of the MICs that inhibited 50 and 90% of isolates tested for the drugs used, clarithromycin, clofazimine, ethambutol, and streptomycin were the most uniformly active against MAC; this was followed by amikacin, rifampin, and sparfloxacin. On the other hand, ciprofloxacin, D-cycloserine, and ethionamide showed only marginal in vitro activities.

Since the advent of the AIDS pandemic, the opportunistic human pathogens of the *Mycobacterium avium* complex (MAC) have emerged as major causes of opportunistic infections among human immunodeficiency virus (HIV)-infected patients, resulting in grave consequences as far as the morbidities, mortalities, and qualities of life of terminally ill patients are concerned (18, 27). Recently, the possibility of severe pulmonary disease in persons without predisposing conditions has also been raised (16). However, until now established methods for drug susceptibility testing of these organisms and appropriate therapeutic protocols for treating the afflicted are still lacking.

Because routine in vitro drug susceptibility testing of MAC organisms should not be performed by using the critical concentrations for *Mycobacterium tuberculosis* recommended previously (2), an alternative radiometric methodology which permits the MICs of potential anti-MAC drugs to be determined in a standardized, rapid, and reproducible way has recently been proposed (29). One of the main advantages of the radiometric method is that it is rapid (drug susceptibility results can be obtained within a week instead of 18 to 21 days with solid agar media), with a substantially lower probability of drug degradation at 37°C. However, unlike in the United States, where this method has been widely used for routine drug susceptibility testing of *M. tuberculosis* since 1981 (30), it was only upon an independent study of the radiometric method compared with the conventional 1% proportional method with solid medium by a French group (20) that the BACTEC 460-TB method was introduced in France as late as 1989.

In the context of the discussion presented above and keeping in mind the recent upsurge in MAC infections, a multicenter study involving eight test sites across France was planned to determine the MICs of various drugs for MAC organisms in routine clinical microbiology laboratories. This investigation was planned by following the protocol of a recently published U.S. multicenter study involving five test sites (29) to compare the in vitro activities of 10 potential anti-MAC drugs, i.e., D-cycloserine, ethambutol, ethionamide, rifampin, amikacin, streptomycin, ciprofloxacin, clofazimine, and two newer drugs, sparfloxacin and clarithromycin, which were not used in the recently published study (29).

MATERIALS AND METHODS

Test cultures and preparation of mycobacterial inoculum. The 10 clinical isolates of MAC (five each from HIV-positive and HIV-negative patients [see

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TABLE 1. Concentrations and commercial sources of antimicrobial agents used for radiometric MIC determinations

Antimicrobial agent (source)	Concn tested (μg/ml)
D-Cycloserine (Sigma Chemical Co., St. Louis, Mo.)	4.0, 8.0, 16.0, 32.0
Ethambutol (Lederle, Rungis, France)	2.0, 4.0, 8.0, 16.0
Ethionamide (Théraplix, Paris, France)	2.0, 4.0, 8.0, 16.0
Rifampin (Sigma Chemical Co.)	2.0, 4.0, 8.0, 16.0
Amikacin (Bristol, Paris, France)	2.0, 4.0, 8.0, 16.0
Streptomycin (Sigma Chemical Co.)	2.0, 4.0, 8.0, 16.0
Ciprofloxacin (Bayer, Sens, France)	1.0, 2.0, 4.0, 8.0
Sparfloxacin (Rhône-D.P.C. Europe, Antony, France)	0.25, 0.5, 1.0, 2.0, 4.0, 8.0
Clofazimine (Ciba, Basel, Switzerland)	0.125, 0.25, 0.5, 1.0
Clarithromycin (Abbott, Rungis, France)	1.0, 2.0, 4.0, 8.0, 16.0, 32.0

Table 2]) used in the study were supplied to each test site by the coordinating laboratory (Institut Pasteur) as fresh Löwenstein-Jensen slants. Upon receipt, the bacteria were scraped from the slants, resuspended in 3 ml of the BACTEC diluting fluid (Becton Dickinson, Towson, Md.), and homogenized with 2-mm-diameter glass beads. The suspension was allowed to stand for a few minutes to sediment the bacterial clumps. The homogeneous supernatant was taken, and the turbidity was adjusted to a McFarland no. 1 standard with diluting fluid. A total of 0.1 ml of this suspension was injected into a BACTEC 12B vial and the vial was used as the seed vial after the growth index (GI) reached 999 (29).

Radiometric MIC determination and analysis of data. The contents of the seed vial were homogenized and diluted 1:100 by adding 0.1 ml to 9.9 ml of diluting fluid as reported earlier (29). This 100-fold-diluted working suspension was then used for inoculating all of the drug-containing vials (0.1 ml per vial) as well as a vial with no drug (hereafter termed the control). The working solution was then further diluted 1:100 (total dilution of 1:10,000 compared with the vial containing the seed culture), and 0.1 ml was inoculated into a second drug-free vial. This second control vial was termed the 1:100 control. The test and control vials were incubated at 37°C, and growth was followed once daily. All of the drugs including clarithromycin were tested with BACTEC 12B broth at a routine pH of 6.8.

The MICs determined with the BACTEC 460-TB apparatus (Becton Dickinson) were essentially established as reported recently (29), except that a GI cutoff of 100 was selected for analysis. As in the reference study (29), the MIC was considered the lowest concentration of a drug that inhibited more than 99% of the bacterial population. On the basis of the GIs, the MIC was interpreted as the lowest drug concentration in the presence of which the daily GI increase (called ΔGI) was less than that in the 1:100 control, provided that the final GI reading in the drug-containing vial was not greater than 100.

According to the previously described quality control and standardization criteria in the reference study (29), the majority of the experiments were finished within 6 to 7 days; however, if the requirements were not met because of underinoculated or overinoculated samples, the tests were repeated. In the routine functional setting of a clinical microbiology laboratory in France, mycobacterial drug susceptibility testing is not performed in duplicate. For confirming the potential of using the BACTEC methodology in such a setting, MICs for each strain were determined in a single experiment with an internal control which included a mycobacterial strain for which MICs were determined previously. The MIC_{50} was the minimal drug concentration to which 50% of the test isolates were found to be susceptible, whereas the MIC_{90} was the minimal drug concentration to which at least 90% of the test isolates were found to be susceptible.

Interlaboratory variation was evaluated by the Box and Whisker method with Microsoft-Statwork software after entering the MICs of each of the drugs determined at the eight test sites. Modal MICs (MICs found in most of the tests, i.e., by at least five of the eight test sites) were preferentially considered for further analysis. However, in rare cases in which there was no clear majority that could be used to establish the modal MIC, the median values were considered for analysis (29). In accordance with the recently published multicenter study (29), the numbers and percentages of findings within ±1 dilution of the modal MICs were considered agreement, whereas any difference beyond the ±1 dilution limit was taken as a disagreement.

Drugs. The drugs used in the present investigation, their commercial sources, and the concentrations at which they were tested are summarized in Table 1. The drug concentrations screened were chosen on the basis of preliminary studies performed by the coordinating laboratory. Stock solutions of D-cycloserine, ethambutol, streptomycin, amikacin, and ciprofloxacin were dissolved in processed water, rifampin and ethionamide were dissolved in ethylene glycol, clofazimine was dissolved in methyl cellosolve (2-methoxyethanol, clarithromycin was dissolved in methanol, and sparfloxacin was initially dissolved in a minimal volume of 0.1 N NaOH and then in water. The stock solutions were sterilized by filtration through 0.2-μm-pore-size filters except that the solutions made in organic solvents were self-sterilized. Except for clofazimine, which was kept at room temperature in a dark bottle, all other drugs were kept frozen either at −70°C (maximum of 6 months) or at −20°C (maximum of 2 months) as aliquots and were thawed prior to use. A total of 0.1 ml of the stock or serially diluted working suspensions adjusted to give the desired drug concentrations was injected into each vial.

RESULTS

The results obtained in the present investigation are summarized in Tables 2 to 4 and Fig. 1 to 5. Typical radiometric data permitting MIC determinations (one curve each per drug) are illustrated in Fig. 1 to 3, whereas the composite picture of modal MICs determined by the Box and Whisker analytical method is provided in Fig. 4 (only two examples are shown for each drug). The modal MICs for all 10 antibiotics determined in multiple tests are illustrated in Table 2, and there were wide

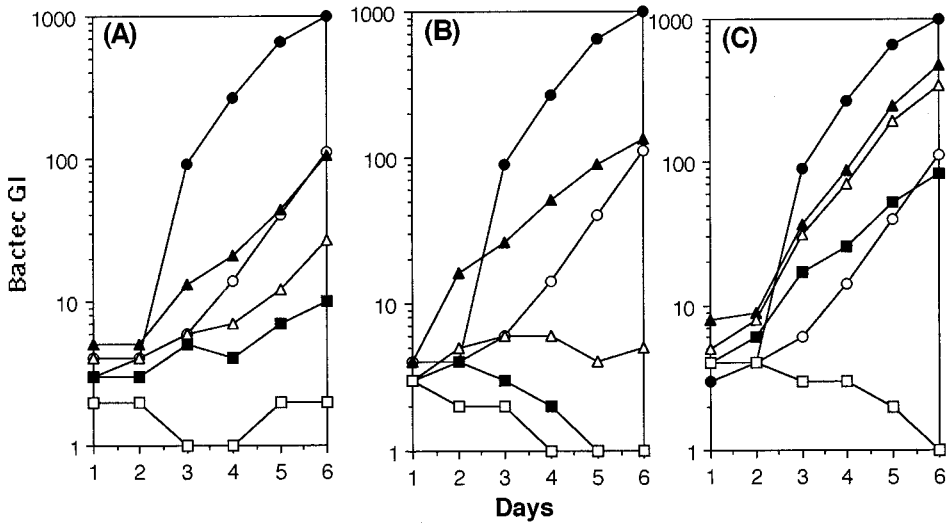

FIG. 1. Radiometric data showing results of typical MIC determinations for MAC clinical isolate 733 from an HIV-positive patient with successive onefold dilutions of rifampin (A), amikacin (B), and sparfloxacin (C). (A and B) ●, control; ○, 1:100 control; ▲, 2 μg/ml; △, 4 μg/ml; ■, 8 μg/ml; □, 16 μg/ml. (C) ●, control; ○, 1:100 control; ▲, 1 μg/ml; △, 2 μg/ml; ■, 4 μg/ml; □, 8 μg/ml.

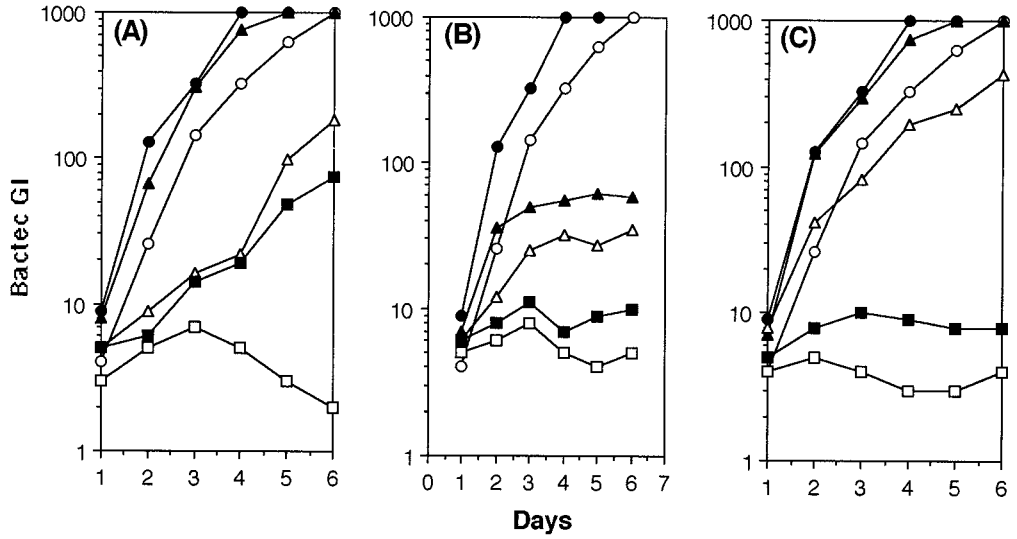

FIG. 2. Radiometric data showing results of typical MIC determinations for MAC clinical isolate 804 from an HIV-positive patient with successive onefold dilutions of D-cycloserine (A), streptomycin (B), and ciprofloxacin (C). (A) ●, control; ○, 1:100 control; ▲, 4 μg/ml; △, 8 μg/ml; ■, 16 μg/ml; □, 32 μg/ml. (B) ●, control; ○, 1:100 control; ▲, 2 μg/ml; △, 4 μg/ml; ■, 8 μg/ml; □, 16 μg/ml. (C) ●, control; ○, 1:100 control; ▲, 1 μg/ml; △, 2 μg/ml; ■, 4 μg/ml; □, 8 μg/ml.

variations in the MICs of some of the drugs tested, particularly the quinolone drugs ciprofloxacin and sparfloxacin, for the strains. However, despite these variations in MICs of the same drug for the various isolates that were screened, no link between the origins of the MAC isolates (from HIV-positive or HIV-negative patients) and their drug susceptibilities was found.

As shown in Table 3, the overall interlaboratory reproducibilities of the MICs, within ±1 dilution of the modal MICs, varied from 79.70 to 100%, with a mean of 95.2% ± 2.1%, whereas overall agreement of the MICs among the test sites varied from a mean value of 91% ± 4.1% to a mean value of 98% ± 1.3% (Table 4). Only 65.4% ± 3.1% of all the MICs determined had no difference from the modal MIC of each individual drug (Table 3); however, if the ±1 dilution difference in individual MICs compared with the modal MICs was considered agreement, the present study gave results comparable to those obtained in the reference study (29).

The MIC_{50}s and MIC_{90}s of the 10 antimicrobial agents tested are summarized in Fig. 5. Figure 5 shows that both MIC_{50}s and MIC_{90}s of D-cycloserine, ethambutol, streptomycin, and clofazimine were identical. Compared with the MIC_{50}s, onefold higher MIC_{90}s were noted for ethionamide, amikacin, and clarithromycin, whereas twofold higher MIC_{90}s were observed for rifampin, ciprofloxacin, and sparfloxacin. The difference between the MIC_{50}s and the MIC_{90}s of the drugs studied, alternatively, can be considered an index of the variability in the MICs for the MAC clinical isolates.

On the basis of the MIC_{50}s and MIC_{90}s of the drugs used in the study (Fig. 5), clarithromycin, clofazimine, ethambutol, and

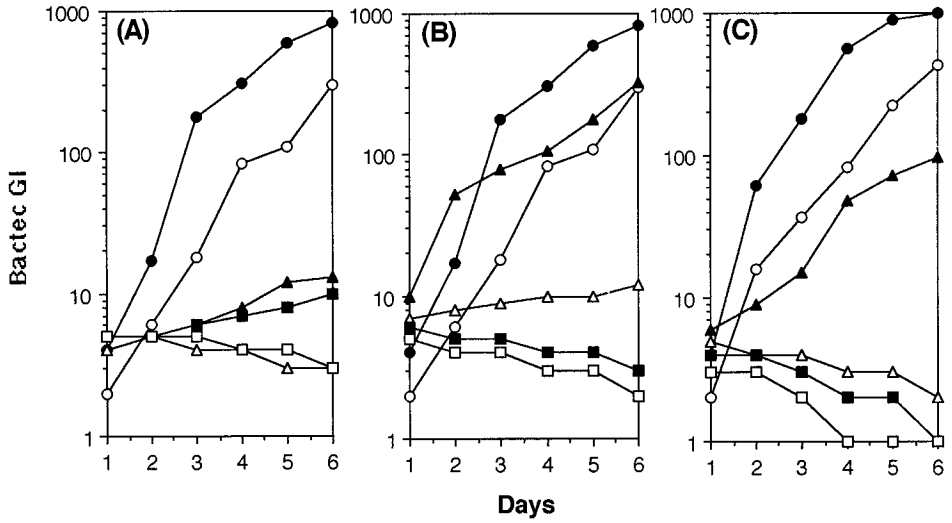

FIG. 3. Results of typical MIC determinations for MAC clinical isolates 969 (A and B) and 1110 (C) from HIV-negative patients with onefold dilutions of ethambutol and ethionamide (A), clofazimine (B), and clarithromycin (C). (A) ●, control; ○, 1:100 control; ▲, ethambutol at 2 μg/ml; △, ethambutol at 4 μg/ml; ■, ethionamide at 2 μg/ml; □, ethionamide at 4 μg/ml. (B) ●, control; ○, 1:100 control; ▲, 0.12 μg/ml; △, 0.25 μg/ml; ■, 0.5 μg/ml; □, 1 μg/ml. (C) ●, control; ○, 1:100 control; ▲, 1 μg/ml; △, 2 μg/ml; ■, 4 μg/ml; □, 8 μg/ml.

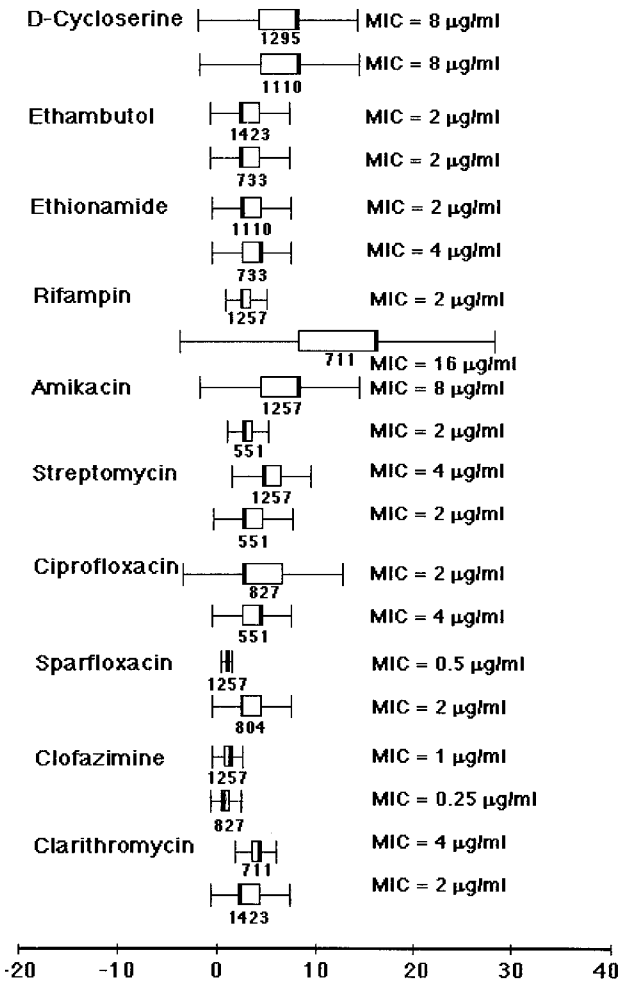

FIG. 4. Composite of interlaboratory variations in MICs among the eight test sites determined by Box and Whisker's analytical method (only two examples are shown for each drug).

streptomycin were uniformly the most active drugs against MAC; this was followed by amikacin, rifampin, and sparfloxacin. On the other hand, ciprofloxacin, D-cycloserine, and ethionamide showed only marginal in vitro activities.

DISCUSSION

Of the several opportunistic pathogens inflicting HIV-infected patients, both *M. tuberculosis* and MAC pose significant problems in the clinical management of patients infected with this immunosuppressive virus. Contrary to *M. tuberculosis* in which drug resistance usually appears as a result of inadequate therapy (23), drug resistance in MAC is apparently due to factors other than those associated with genetic events. It has been suggested that the multiple drug resistance of MAC isolates arises because of their cell envelope architecture, which acts as a barrier for the exclusion of some drugs (6, 17, 18, 19, 27).

From a medical viewpoint, tubercle bacilli are classified as resistant with respect to the critical drug concentrations beyond which treatment is no longer effective. These critical concentrations for *M. tuberculosis* were developed empirically by finding the highest MICs in Löwenstein-Jensen medium to which all wild-type strains were susceptible (4), and later, equivalent concentrations were found for 7H10 and 7H11 agar media (15). In addition the BACTEC radiometric method, which employs ^{14}C-labelled palmitic acid in an enriched 7H12 broth, was used (28, 30). However, these critical concentrations per se are not related to defined pharmacokinetic parameters and may simply reflect the susceptibility thresholds that are best able to reflect the clinical outcome of treatment of tuberculosis (7, 9). On the other hand, the wide variations in the drug susceptibility profiles of MAC organisms as opposed to the uniform susceptibility patterns of the wild-type strains of tubercle bacilli do not permit use of the 1% proportional method with fixed critical concentrations; indeed, in a recent study testing the activities of 13 drugs against 181 clinical MAC isolates, only 1 drug (clofazimine) had a MIC_{90} at the critical concentrations tested (22). Judging from the evidence that has accumulated through the years, the American Thoracic Society recently concluded that susceptibility testing of MAC to antituberculous agents by using the critical concentrations previously developed for *M. tuberculosis* does not provide useful clinical information and should be discouraged (2).

Keeping the information presented above in mind, Heifets and coworkers (7–9) proposed the determination of radiometric MICs as a quantitative measurement of the drug susceptibilities of MAC organisms in liquid medium. The present study therefore corroborates the previous conclusions of Heifets and coworkers (7–9) as well as those of the U.S. multicenter study

TABLE 2. Radiometric MICs of 10 antimicrobial agents for clinical MAC isolates determined by multiple testing

Antimicrobial agent	MIC (μg/ml) for clinical MAC isolates from[a]:									
	HIV-positive patients					HIV-negative patients				
	733[b]	551	804	827	1423	711	969	1110	1257	1295
D-Cycloserine	≤4	8	8	8	8	≤4	8	8	8	8
Ethambutol	≤2	≤2	≤2	≤2	≤2	4	≤2	≤2	≤2	≤2
Ethionamide	4	4	8	4	4	8	16	≤2	≤2	4
Rifampin	≤2	≤2	≤2	4	8	16	8	≤2	≤2	≤2
Amikacin	4	≤2	≤2	4	≤2	≤2	≤2	≤2	8	≤2
Streptomycin	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	4	≤2
Ciprofloxacin	>8	4	4	2	4	>8	>8	≤1	2	8
Sparfloxacin	8	1	2	1	1	>8	8	≤0.25	0.5	1
Clofazimine	≤0.12	0.25	0.25	0.25	0.25	0.25	≤0.12	0.25	1	≤0.12
Clarithromycin	2	4	2	2	2	4	≤1	2	4	2

[a] The modal or median MICs are indicated. The modal MIC was the MIC of the same antimicrobial agent for a single strain found in most tests across the eight test sites. In cases without a majority, the median value was considered for analysis.

[b] The numbers correspond to an individual isolate from each patient.

TABLE 3. Overall interlaboratory reproducibilities of radiometric MICs for MAC determined by multiple testing

Antimicrobial agent	% of MAC isolates with the following difference in dilution from the modal value[a]:					
	±1 (A)	−2 (B)	−1 (C)	0 (D)	+1 (E)	+2 (F)
D-Cycloserine	100	0	14.71	70.59	14.71	0
Ethambutol	100	0	5.8	68.12	26.09	0
Ethionamide	100	0	26.53	54.98	24.49	0
Rifampin	89.74	3.85	12.82	57.70	19.23	6.41
Amikacin	100	0	5.13	79.49	15.39	0
Streptomycin	98.72	0	1.28	79.49	17.95	1.28
Ciprofloxacin[b]	91.0	3.85	24.36	60.25	6.41	2.56
Sparfloxacin	96.15	3.85	14.10	71.80	10.25	0
Clofazimine[b]	79.70	2.90	8.70	55.73	15.29	15.94
Clarithromycin	97.40	1.30	20.78	55.84	19.48	2.60
Mean ± SE	95.2 ± 2.1	1.6 ± 0.6	12.8 ± 2.4	65.4 ± 3.1	16.9 ± 1.9	2.9 ± 1.6

[a] The modal value was obtained from all sites combined.
[b] The total of columns B, C, D, E, and F was <100 for ciprofloxacin and clofazimine (97.43% for ciprofloxacin and 98.56% for clofazimine) because some MICs did not fall within ±2 dilutions of the modal or median value.

(29), further extending the previous observations to two more drugs, namely, clarithromycin and sparfloxacin. Indeed, the overall interlaboratory reproducibilities of the MICs within ±1 dilution of the modal MICs in the present study varied from 79.70 to 100% (mean of 95.2% ± 2.1% compared with the mean of 99% ± 0.5% reported recently [29]), whereas the overall agreement of the MICs among all eight test sites varied from a mean value of 91% ± 4.1% to a mean value of 98% ± 1.3% (instead of 92.8% ± 2.7% to 97% ± 1.0% in the previous study [29]). The differences between the results of the present study compared with those published recently (29) are not statistically significant; our results therefore corroborate the conclusions of the recent U.S. study (29) that, considering the MICs within the ±1 dilution limit as agreement for routine purposes, the proposed methodology is easy, accurate, and sufficiently reproducible for use in a clinical laboratory.

The MIC_{50}s and MIC_{90}s were identical for D-cycloserine, ethambutol, streptomycin, and clofazimine, whereas onefold higher MIC_{90}s were noticed in the cases of ethionamide, amikacin, and clarithromycin and twofold higher MIC_{90}s were observed in the cases of rifampin, ciprofloxacin, and sparfloxacin. Although these differences between MIC_{50}s and MIC_{90}s for various drugs may alternatively serve as an index of the variabilities in the MICs for different clinical isolates, no link between the origin of MAC isolates (from HIV-positive or HIV-negative patients and their drug susceptibilities was found. On the basis of their respective MICs (Fig. 5), clarithromycin, clofazimine, ethambutol, and streptomycin were uniformly the most active drugs against MAC; this was followed by amikacin, rifampin, and sparfloxacin. On the other hand, ciprofloxacin, D-cycloserine, and ethionamide showed only marginal in vitro activities (Fig. 5). Whether the clinical efficacies of these drugs will corroborate the present in vitro results remains to be investigated among both immunocompromised and immunocompetent patient populations.

Considering the wide variations in susceptibility profiles of individual MAC isolates, both Rastogi et al. (21, 24, 26) and Hoffner et al. (10, 11) have previously suggested that, in addition to MIC determinations, in vitro assays of combined drugs by the BACTEC radiometric method should also be performed to establish better therapeutic protocols on a patient-to-patient basis. Many investigators have tested a variety of drug combinations, and the synergistic effects of ethambutol with clarithromycin and/or rifampin (13, 14, 24, 32), sparfloxacin and/or rifampin (26), and amikacin (25) have been reported. The three-drug combination of clarithromycin, ethambutol, and rifampin was shown to be the most bactericidal against both extracellularly and intracellularly growing MAC organisms (24, 31). Ethambutol has also been included with rifabutin in the three most successful reported series of regi-

TABLE 4. Overall agreement[a] of MICs of 10 antimicrobial agents among eight test sites

Antimicrobial agent	% Agreement for test site:							
	1	2	3	4	5	6	7	8
D-Cycloserine	100	100	100	100	ND[b]	100	100	100
Ethambutol	100	100	100	100	80	100	ND	80
Ethionamide	90	ND	100	100	ND	100	ND	100
Rifampin	90	100	80	80	90	80	100	90
Amikacin	100	100	100	100	100	100	100	100
Streptomycin	100	100	100	100	90	100	100	100
Ciprofloxacin	100	80	100	100	100	70	90	90
Sparfloxacin	100	100	90	100	100	80	100	100
Clofazimine	100	100	90	90	80	50	ND	60
Clarithromycin	100	100	100	100	100	90	90	90
Mean ± SE	98 ± 1.3	97.8 ± 2.2	96 ± 2.2	97 ± 2.1	92.5 ± 3.1	97 ± 5.4	97.1 ± 1.8	91 ± 4.1

[a] Agreement was defined as MICs within ±1 dilution of the modal or median value across all sites.
[b] ND, not done.

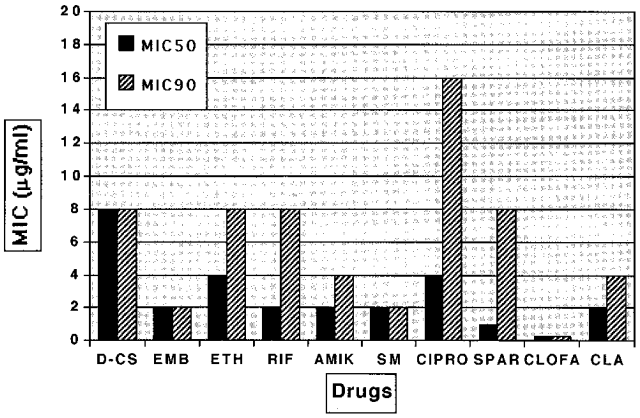

FIG. 5. Graphic representation of MIC_{50}s and MIC_{90}s of the 10 antibiotics for MAC clinical isolates. The graph is based on the modal MICs determined by multiple testing. D-CS, D-cycloserine; EMB, ethambutol; ETH, ethionamide; RIF, rifampin; AMIK, amikacin; SM, streptomycin; CIPRO, ciprofloxacin; SPAR, sparfloxacin; CLOFA, clofazimine; CLA, clarithromycin.

mens for the treatment of MAC infections in patients with AIDS (1, 3, 5). Apart from ethambutol, both clofazimine (1, 12) and amikacin (3, 5), which were included in the present study, have served as components of various MAC treatment regimens, giving favorable results. In our opinion, apart from developing individual drugs for the treatment of MAC infections, one of the major issues today is the development of appropriate drug combination regimens. Despite its elevated cost and the need to use radioactivity, the BACTEC methodology contributes significantly toward the development of an acceptable, standardized, and reproducible technique for providing rapid results of the in vitro drug susceptibilities of MAC isolates to two- and three-drug combinations (10, 11, 21, 24, 25, 26). Upon validation in multicenter trials, assays of drugs used in combination may routinely be performed in vitro on a patient-to-patient basis and should be correlated with the therapeutic outcome.

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