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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 n-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, n-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 morbidity, 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., n-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

* Corresponding author. Phone: (590)89.38.81. Fax: (590)89.38.80.
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 pro-
cessed water, rifampin and ethionamide were dissolved in ethylene glycol, clo-
fazimine was dissolved in methyl cellulosolve (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 in-
jected into each vial.

RESULTS

The results obtained in the present investigation are sum-
marized 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

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).

The MICs determined with the BACTEC 460-TB apparatus (Becton Dickin-
son) 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
calculated as the lowest drug concentration to which at least 90% of the test isolates
were found to be susceptible, whereas the MIC<sub>50</sub> was the minimal drug concentration
in which 50% 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 pro-
cessed water, rifampin and ethionamide were dissolved in ethylene glycol, clo-
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The MIs determined with the BACTEC 460-TB apparatus (Becton Dickin-
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analysis (29). In accordance with the recently published multicenter study (29),
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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 in-
jected into each vial.
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} and MIC_{90} of the 10 antimicrobial agents tested are summarized in Fig. 5. Figure 5 shows that both MIC_{50} and MIC_{90} of d-cycloserine, ethambutol, streptomycin, and clofazimine were identical. Compared with the MIC_{50}, onefold higher MIC_{90} were noted for ethionamide, amikacin, and clarithromycin, whereas twofold higher MIC_{90} were observed for rifampin, ciprofloxacin, and sparfloxacin. The difference between the MIC_{50} and the MIC_{90} 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} and MIC_{90} of the drugs used in the study (Fig. 5), clarithromycin, clofazimine, ethambutol, and

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.

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.
D-Cycloserine

MIC = 8 µg/ml

MIC = 8 µg/ml

Ethambutol

MIC = 2 µg/ml

MIC = 2 µg/ml

Ethionamide

MIC = 2 µg/ml

MIC = 2 µg/ml

Rifampin

MIC = 2 µg/ml

MIC = 2 µg/ml

Amikacin

MIC = 16 µg/ml

MIC = 8 µg/ml

Streptomycin

MIC = 4 µg/ml

MIC = 2 µg/ml

Ciprofloxacin

MIC = 2 µg/ml

MIC = 2 µg/ml

Sparfloxacin

MIC = 0.5 µg/ml

MIC = 2 µg/ml

Clarithromycin

MIC = 4 µg/ml

MIC = 2 µg/ml

Clofazimine

MIC = 1 µg/ml

MIC = 0.25 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.

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

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>HIV-positive patients</th>
<th>HIV-negative patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>733&lt;sup&gt;a&lt;/sup&gt;</td>
<td>551</td>
</tr>
<tr>
<td>D-Cycloserine</td>
<td>≤4</td>
<td>8</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Rifampin</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>Amikacin</td>
<td>4</td>
<td>≤2</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;8</td>
<td>4</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Clofazimine</td>
<td>≤0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> The numbers correspond to an individual isolate from each patient.

DISCUSSION

Of the several opportunistic pathogens infecting HIV-infected patients, both <i>M. tuberculosis</i> and MAC pose significant problems in the clinical management of patients infected with this immunosuppressive virus. Contrary to <i>M. tuberculosis</i> 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 <i>M. tuberculosis</i> 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 14C-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<sub>90</sub> 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 <i>M. tuberculosis</i> 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 3. Overall interlaboratory reproducibilities of radiometric MICs for MAC determined by multiple testing

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>% of MAC isolates with the following difference in dilution from the modal value:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>±1 (A)</td>
</tr>
<tr>
<td>d-Cycloserine</td>
<td>100</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>100</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>100</td>
</tr>
<tr>
<td>Rifampin</td>
<td>89.74</td>
</tr>
<tr>
<td>Amikacin</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>98.72</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>91.0</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>96.15</td>
</tr>
<tr>
<td>Clofazimine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.70</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>97.40</td>
</tr>
</tbody>
</table>

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

<sup>a</sup> The modal value was obtained from all sites combined.
<sup>b</sup> 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% ± 1.6% 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<sub>50</sub> and MIC<sub>90</sub> were identical for d-cycloserine, ethambutol, streptomycin, and clofazimine, whereas onefold higher MIC<sub>50</sub> were noticed in the cases of ethionamide, amikacin, and clarithromycin and twofold higher MIC<sub>90</sub> were observed in the cases of rifampin, ciprofloxacin, and sparfloxacin. Although these differences between MIC<sub>50</sub> and MIC<sub>90</sub> 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<sup>a</sup> of MICs of 10 antimicrobial agents among eight test sites

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>% Agreement for test site:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>d-Cycloserine</td>
<td>100</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>100</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>90</td>
</tr>
<tr>
<td>Rifampin</td>
<td>90</td>
</tr>
<tr>
<td>Amikacin</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>100</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>100</td>
</tr>
<tr>
<td>Clofazimine</td>
<td>100</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>100</td>
</tr>
</tbody>
</table>

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

<sup>a</sup> Agreement was defined as MICs within ±1 dilution of the modal or median value across all sites.
<sup>b</sup> ND, not done.
men 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 method, despite its elevated 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 90. Enhancement of drug susceptibility testing in Mycobacterium avium-intracellulare and other slowly growing nontuberculous mycobacteria, p. 123–141. In L. B. Heifets (ed.), Drug susceptibility in the chemotherapy of mycobacterial infections. CRC Press, Inc., Boca Raton, Fl.


FIG. 5. Graphic representation of MIC_{S0} and MIC_{C90} of the 10 antibiotics for MAC clinical isolates. The graph is based on the modal MICs determined by multiple testing. D-CS, t-cycloserine; EMB, ethambutol; ETH, ethionamide; RIF, rifampin; AMIK, amikacin; SM, streptomycin; CIPRO, ciprofloxacin; SPAR, sparfloxacin; CLOFA, clofazimine; CLA, clarithromycin.


