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

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Received 14 October 1994/Returned for modification 18 November 1994/Accepted 20 December 1994

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 morbidity, mortalities, and quality 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, unlike in the United States, 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

<table>
<thead>
<tr>
<th>Antimicrobial agent (source)</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Cycloserine (Sigma Chemical Co., St. Louis, Mo.)</td>
<td>4.0, 8.0, 16.0, 32.0</td>
</tr>
<tr>
<td>Ethambutol (Lederle, Rungis, France)</td>
<td>2.0, 4.0, 8.0, 16.0</td>
</tr>
<tr>
<td>Ethionamide (Théraplix, Paris, France)</td>
<td>2.0, 4.0, 8.0, 16.0</td>
</tr>
<tr>
<td>Rifampin (Sigma Chemical Co.)</td>
<td>2.0, 4.0, 8.0, 16.0</td>
</tr>
<tr>
<td>Amikacin (Bristol, Paris, France)</td>
<td>2.0, 4.0, 8.0, 16.0</td>
</tr>
<tr>
<td>Streptomycin (Sigma Chemical Co.)</td>
<td>1.0, 2.0, 4.0, 8.0</td>
</tr>
<tr>
<td>Ciprofloxacin (Bayer, Sens, France)</td>
<td>1.0, 2.0, 4.0, 8.0</td>
</tr>
<tr>
<td>Sparfloxacin (Rhône-D.P.C., Antony, France)</td>
<td>1.0, 2.0, 4.0, 8.0, 16.0</td>
</tr>
<tr>
<td>Clofazimine (Ciba, Basel, Switzerland)</td>
<td>0.25, 0.5, 1.0, 2.0, 4.0, 8.0</td>
</tr>
<tr>
<td>Clarithromycin (Abbott, Rungis, France)</td>
<td>1.0, 2.0, 4.0, 8.0, 16.0, 32.0</td>
</tr>
</tbody>
</table>

Table 2) were used in the study. 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 propan-2-ol and ethambutol, streptomycin, amikacin, and ciprofloxacin were dissolved in propylene glycol. 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 5, 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 ciprofloxacin (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.
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 \(\pm 1\) dilution of the modal MICs, varied from 79.70 to 100%, with a mean of 95.2% \(\pm 2.1\%\), whereas overall agreement of the MICs among the test sites varied from a mean value of 91% \(\pm 4.1\%\) to a mean value of 98% \(\pm 1.3\%\) (Table 4). Only 65.4% \(\pm 3.1\%\) of all the MICs determined had no difference from the modal MIC of each individual drug (Table 3); however, if the \(\pm 1\) 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 \(\beta\)-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. 2. Radiometric data showing results of typical MIC determinations for MAC clinical isolate 804 from an HIV-positive patient with successive onefold dilutions of \(\beta\)-cycloserine (A), streptomycin (B), and ciprofloxacin (C). (A) ● control; ○, 1:100 control; ▲, 4 \(\mu\)g/ml; △, 8 \(\mu\)g/ml; ■, 16 \(\mu\)g/ml; □, 32 \(\mu\)g/ml. (B) ● control; ○, 1:100 control; ▲, 2 \(\mu\)g/ml; △, 4 \(\mu\)g/ml; ■, 8 \(\mu\)g/ml; □, 16 \(\mu\)g/ml. (C) ● control; ○, 1:100 control; ▲, 1 \(\mu\)g/ml; △, 2 \(\mu\)g/ml; ■, 4 \(\mu\)g/ml; □, 8 \(\mu\)g/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 \(\mu\)g/ml; △, ethambutol at 4 \(\mu\)g/ml; ■, ethionamide at 2 \(\mu\)g/ml; □, ethionamide at 4 \(\mu\)g/ml. (B) ● control; ○, 1:100 control; ▲, 0.12 \(\mu\)g/ml; △, 0.25 \(\mu\)g/ml; ■, 0.5 \(\mu\)g/ml; □, 1 \(\mu\)g/ml. (C) ● control; ○, 1:100 control; ▲, 1 \(\mu\)g/ml; △, 2 \(\mu\)g/ml; ■, 4 \(\mu\)g/ml; □, 8 \(\mu\)g/ml.](http://aac.asm.org/DownloadedFrom)
DISCUSSION

Of the several opportunistic pathogens infecting 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 Lowenstein-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 corresponding to 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 susceptibility 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 on January 7, 2014 by guest http://aac.asm.org/ Downloaded from

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**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>MIC (μg/ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>733a</td>
<td>551</td>
</tr>
<tr>
<td></td>
<td>711</td>
<td>969</td>
</tr>
<tr>
<td>D-Cycloserine</td>
<td>≤8</td>
<td>≤8</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>Rifampin</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td>Amikacin</td>
<td>≤2</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>&gt;8</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>≤2</td>
</tr>
</tbody>
</table>

* 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.
* The numbers correspond to an individual isolate from each patient.
further extending the previous observations to two more drugs, namely, clarithromycin and sparflxacin. 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% ± 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% ± 1.8% 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 MICs were identical for D-cycloserine, ethambutol, streptomycin, and clofazimine, whereas onefold higher MICs were noticed in the cases of ethionamide, amikacin, and clarithromycin and twofold higher MICs were observed in the cases of rifampin, ciprofloxacin, and sparflxacin. Although these differences between MICs and MICs 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 sparflxacin. 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-
mements 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.

ACKNOWLEDGMENTS

We thank S. H. Siddiqi (Becton Dickinson, Sparks, Md.) for helpful discussions and B. Quiviger and B. Gasparello (Becton-Dickinson, Meylan, France) for lending the BACTEC 460 apparatus to N. Rastogi. The mycobacteria and tuberculosis project at Guadeloupe, French West Indies, in the Caribbean was partially financed through the Projet CORDET (Ministry of Overseas Departments and Territories, French Republic) and Fondation Francaise Raoul Follereau, Paris, France.

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FIG. 5. Graphic representation of MIC50 and MIC90 of the 10 antibiotics for MAC clinical isolates. The graph is based on the modal MICs determined by

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