

## Activity of 16 Antimicrobial Agents Against Drug-Resistant Strains of *Mycobacterium tuberculosis*

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### ABSTRACT

The *in vitro* activity of 16 antimicrobial agents against 46 drug-resistant strains of *Mycobacterium tuberculosis* recently isolated from Italian patients was determined. As for first-line antituberculosis drugs, while isoniazid was ineffective against all the strains tested, resistance to streptomycin, rifampicin, pyrazinamide, and ethambutol was 80.4%, 71.7%, 39.1%, and 8.7%, respectively. Among second-line antituberculous drugs, resistance to ciprofloxacin, ofloxacin, and sparfloxacin and to amikacin and kanamycin was around 20%. About 10% of the strains were resistant to capreomycin and cycloserine and 4.3% were resistant to ethionamide; no strain was found to be resistant to thiacetazone, *para*-aminosalicylic acid, and viomycin. Although all strains displayed a rather continuous distribution of minimal inhibitory concentrations (MICs), a bimodal distribution was observed for rifampicin, amikacin, and kanamycin, with very high MIC values for resistant strains; relatively low MICs were found for fluoroquinolone-resistant strains. Among the small number of strains resistant to second-line agents, low resistant levels were observed. Restriction fragment length polymorphism analysis showed few strain clusters with resistance to first-line antituberculous drugs and aminoglycosides, fluoroquinolones, or both. Altogether, these results showed that second-line agents were still active against the isoniazid-resistant and multiply first-line resistant strains tested, with none or low resistance levels; these observations can be of importance for the treatment of multidrug-resistant tuberculosis in Italy.

### INTRODUCTION

THE RECENT REPORTS of drug-resistant tuberculosis (TB) arising in many countries have caused great concern about the potential spread of resistant strains of *Mycobacterium tuberculosis* (MTB).<sup>16,19</sup> Although such resistance can be overcome by appropriate multi-drug regimens of second-line agents,<sup>10,19,20</sup> inadequate antimicrobial chemotherapy can lead to the emergence of strains resistant to virtually all antituberculous agents.<sup>5,11</sup>

The Advisory Council of the Centers for Disease Control recommended that *in vitro* susceptibility tests be done on initial MTB isolates from all tuberculous patients.<sup>2</sup> While testing of first-line antituberculous agents, namely isoniazid (INH), ri-

fampicin (RMP), ethambutol (EMB), streptomycin (SM), and pyrazinamide (PZA)<sup>7-10</sup> is routinely performed, testing of secondary antituberculous drugs is usually done only by laboratories with specific expertise. Despite extensive reports on antituberculous drug activity of first-line agents,<sup>9</sup> not many investigations have been performed to evaluate the *in vitro* activity of second-line antituberculous agents against multiply drug-resistant MTB strains.

The aim of the present study was to assess the activity of a large panel of drugs against MTB strains resistant to at least two first-line antituberculous agents collected from different Italian regions. In addition, to determine some relationships between the strains, restriction fragment length polymorphism (RFLP) analysis<sup>18</sup> of these isolates was performed.

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## MATERIALS AND METHODS

### Microorganisms

Forty-six strains of MTB isolated from human immunodeficiency virus (HIV)-negative (43 strains) and HIV-positive (3 strains) patients selected on the basis of their resistance to at least two first-line antituberculous drugs were used in the study. Isolates were collected in the period 1995–1997 in three different Italian mycobacteriology laboratories (Rome, Florence, Ancona) receiving specimens from various general hospitals of central and northern Italy. All strains were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) and stored at  $-80^{\circ}\text{C}$ .

### Antimicrobial agents

INH, EMB, SM, PZA, ofloxacin (OFL), amikacin (AK), kanamycin (KM), capreomycin (CM), cycloserine (CS), *para*-aminosalicylic acid (PAS), viomycin (VM) (Sigma Chemical, St. Louis, MO), and ciprofloxacin (CIP) (Bayer, Milan, Italy) were dissolved in distilled water; RMP (Sigma) was dissolved in methanol; sparfloxacin (SPA) (Rhône Poulenc Rorer, Vitry-Alfortville, France) was dissolved in ethanol; thiacetazone (TC) and ethionamide (ETH) (Sigma) were dissolved in propylene glycol.

### Antimicrobial susceptibility

Susceptibility to INH, RMP, SM, EMB, and to second-line antituberculous agents (CM, CS, ETH, TC, PAS, VM, AK, KM, CIP, OFL, SPA) was determined by the proportion method<sup>7–9</sup> in Middlebrook 7H11 agar (Difco), which better supports the growth of multidrug resistant (MDR) strains.<sup>8</sup> A strain was considered resistant if the proportion of bacilli resistant to the critical concentration of a drug exceeded 1%. Recommended critical concentrations in 7H11 agar<sup>10</sup> were used. Tentative critical concentrations, usually corresponding to the highest minimal inhibitory concentrations (MICs) of drug-susceptible MTB strains in 7H11 agar, were as follows: CIP,  $2\ \mu\text{g/ml}$ <sup>7</sup>; OFL,  $2\ \mu\text{g/ml}$ <sup>7,12</sup>; SPA,  $0.5\ \mu\text{g/ml}$ <sup>12</sup>. As for TC, a tentative critical concentration was used corresponding to the highest MIC of drug-susceptible MTB strains in 7H10 agar ( $2.5\ \mu\text{g/ml}$ ).<sup>6</sup> Other tentative critical concentrations were: VM,  $10\ \mu\text{g/ml}$ <sup>3</sup> and AK,  $8\ \mu\text{g/ml}$ <sup>3</sup>. Susceptibility to PZA was determined by the BACTEC method.<sup>17</sup>

MIC values were determined in Middlebrook 7H11 agar (Difco). Plates containing different drug concentrations were inoculated in triplicate with approximately  $2 \times 10^2$  and  $2 \times 10^3$  CFU by a semiautomated inoculator (Multipoint Inoculator A400, Denley, West Sussex, UK) and incubated at  $37^{\circ}\text{C}$  in plastic bags for 14–21 days. The following drug concentration ranges were used: from 0.125 to  $64\ \mu\text{g/ml}$  for INH, SM, RMP, CIP, OFL, SPA, AK, PAS; from 0.117 to  $120\ \mu\text{g/ml}$  for EMB and CS; from 0.094 to  $96\ \mu\text{g/ml}$  for KM; from 0.078 to  $80\ \mu\text{g/ml}$  for CM, ETH, TC, VM. The MIC was defined as the lowest drug concentration inhibiting more than 99% of the inoculum. MICs of the drug-susceptible MTB reference strain ATCC 27294 (H37Rv), as determined for control, were: INH,  $\leq 0.125\ \mu\text{g/ml}$ ; SM,  $1\ \mu\text{g/ml}$ ; RMP,  $0.25\ \mu\text{g/ml}$ ; CIP,  $1\ \mu\text{g/ml}$ ; OFL,  $1\ \mu\text{g/ml}$ ; KM,  $1.5\ \mu\text{g/ml}$ ; AK,  $2\ \mu\text{g/ml}$ ; SPA,  $\leq 0.125$

$\mu\text{g/ml}$ ; CM,  $2.5\ \mu\text{g/ml}$ ; EMB,  $0.47\ \mu\text{g/ml}$ ; CS,  $7.5\ \mu\text{g/ml}$ ; ETH,  $1.25\ \mu\text{g/ml}$ ; TC,  $0.156\ \mu\text{g/ml}$ ; PAS,  $0.25\ \mu\text{g/ml}$ ; VM,  $5\ \mu\text{g/ml}$ . MICs of MTB reference strains resistant to SM (ATCC 35820), INH (ATCC 35822), RMP (ATCC 35838), EMB (ATCC 35837), as determined for control, were  $\geq 64$ ,  $\geq 64$ ,  $\geq 64$ , and  $32\ \mu\text{g/ml}$ , respectively.

### RFLP

Cultures of MTB strains and DNA extraction were performed as previously described.<sup>18</sup> Briefly, mycobacterial strains were grown for 3–4 weeks at  $37^{\circ}\text{C}$  in agitation (120 rpm) in 50 ml of Middlebrook 7H9 broth (Difco). Bacteria were pelleted by centrifugation and heat-killed at  $80^{\circ}\text{C}$  for 1 hr. Chromosomal DNA ( $2.5\text{--}3\ \mu\text{g}$ ) was incubated for 4 hr with *Pvu*II restriction enzyme (Life Technologies, Grand Island, NY) in a total volume of  $20\ \mu\text{l}$ . Restriction fragments were separated in a 0.8% agarose gel in  $1 \times \text{TBE}$  (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA pH 8.2) at 29 V for 17 hr and then transferred onto a nylon membrane (Hybond N-Plus, Amersham, Harlington heights, IL) by the capillary method. The probe used in the study was a 245-bp PCR product of IS6110<sup>18</sup> purified by a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA) and nonradioactively labeled with a chemiluminescent kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Boehringer Mannheim, Indianapolis, IN). The reference DNA included in all experiments as a standard was the chromosomal DNA extracted from MTB 14323. RFLP patterns were compared with the assistance of a computerized system (Gel Doc 1000, Molecular Analyst Fingerprinting Program, Biorad Laboratories, Hercules, CA).

## RESULTS

### Antimicrobial susceptibility

The susceptibility pattern of 46 MTB strains to all drugs tested is shown in Fig. 1. While all strains were resistant to

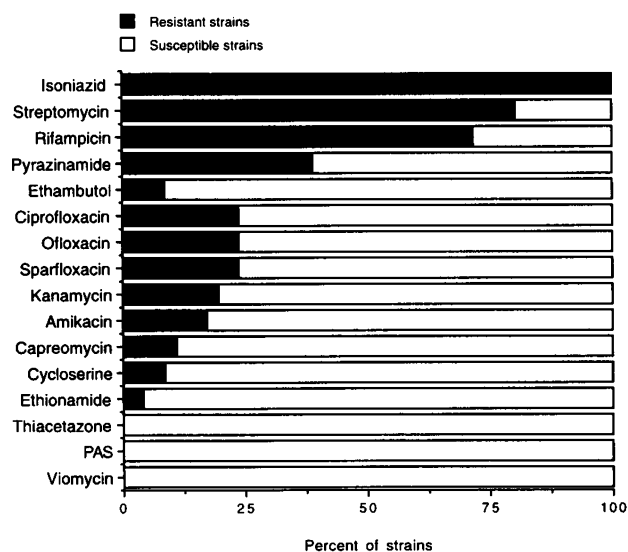


FIG. 1. Susceptibility pattern of 46 drug-resistant strains of MTB against 16 antimycobacterial drugs.



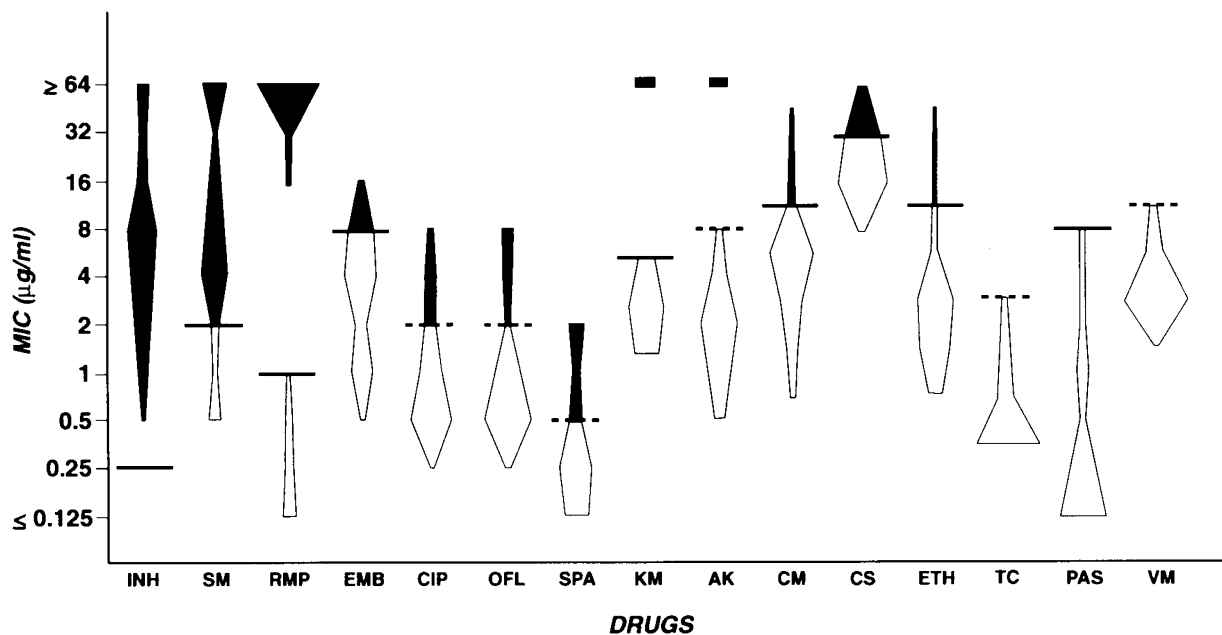


FIG. 2. MIC distribution for 46 drug-resistant strains of MTB for 15 drugs reported as polygonal diagrams. For each drug, the number of strains with the same MIC corresponds to the width of the diagram at the indicated concentration. Recommended critical concentrations in Middlebrook 7H11 agar<sup>10</sup> are indicated as continuous bars; tentative critical concentrations (Materials and Methods) as hatched bars. Resistant strains are over the bars, and susceptible strains are under the bars.

## DISCUSSION

The MTB strains tested in this study were selected on the basis of their resistance to at least two first-line antituberculous agents. Among primary agents, INH was ineffective against all the strains and most isolates were resistant to SM and RMP, but showed limited resistance to PZA and EMB. The low number of EMB-resistant strains is in accordance with a recent WHO report<sup>19</sup> in which the worldwide prevalence of primary and acquired resistance to this drug was lower than for the other first-line agents. Low-level RMP-resistant strains were not observed among our isolates and suggested that specific alterations of RNA polymerase  $\beta$ -subunit gene (*rpoB*), that are associated with high-level resistance against this drug<sup>1</sup> were the genetic mechanism of RMP resistance; indeed, recent studies of our group<sup>14</sup> have shown that *rpoB* mutations occurred in these high-level resistant strains.

Resistance to second-line drugs, such as the aminoglycosides KM and AK and fluoroquinolones CIP, OFL, and SPA, was observed in about 20% of the strains tested. RFLP study showed that among four strains (R24, F9, F11, F18) which showed resistance both to aminoglycosides and fluoroquinolones, three strains (F9, F11, F18) belonged to a cluster associated to a recent outbreak of MDR resistant tuberculosis in Italy.<sup>13</sup> In the other strains, the resistance to aminoglycosides and to fluoroquinolones, occurred separately. This is an important point and suggests that when the resistance develops to both these classes of drugs, the risk of an outbreak of MDR strains as a consequence of poor compliance or inadequate therapy<sup>5</sup> is more

likely. On the basis of these observations, particular caution in the use of these drugs should be used because it is known that fluoroquinolones, which are presently suggested<sup>5</sup> and used<sup>11</sup> for treatment of known or suspect MDR TB, could lose their efficacy as a consequence of point mutations in the target genes.<sup>4</sup> KM and AK also, which are presently good alternative drugs because of the low cross-resistance to SM,<sup>11</sup> could suffer the same problem in the short term. The 20% resistance observed in both classes of drugs in this study is alarming.

Second-line antituberculous drugs have, in general, the disadvantage of being less effective and more toxic than the first-line drugs.<sup>5</sup> Nevertheless, when the efficacy of one or two major antituberculous drugs is lost, these second-line drugs represent the only way to treat the patient. For this reason, we tested whether susceptibility to these antituberculous agents could guide the design of regimens using the remaining drugs. Our data indicated that, even in strains with very high antituberculous resistance to first-line drugs, some of the drugs traditionally considered as second-line agents<sup>10</sup> such as TC, a thiosemicarbazole, PAS, an antifolate, and VM, a basic polypeptide with a mechanism of action similar to that of aminoglycosides, were still active *in vitro* against all strains tested. Others drugs like ETH, a derivative of isonicotinic acid with little cross-resistance to INH, CM, a polypeptide antibiotic, and CS, an inhibitor of cell wall synthesis, were active against most of the strains tested. These results are in keeping with those of a previous study by Goble *et al.*,<sup>3</sup> but differences in the resistance to some drugs were found, likely due to differences in drug regimens used in our country to treat the pa-

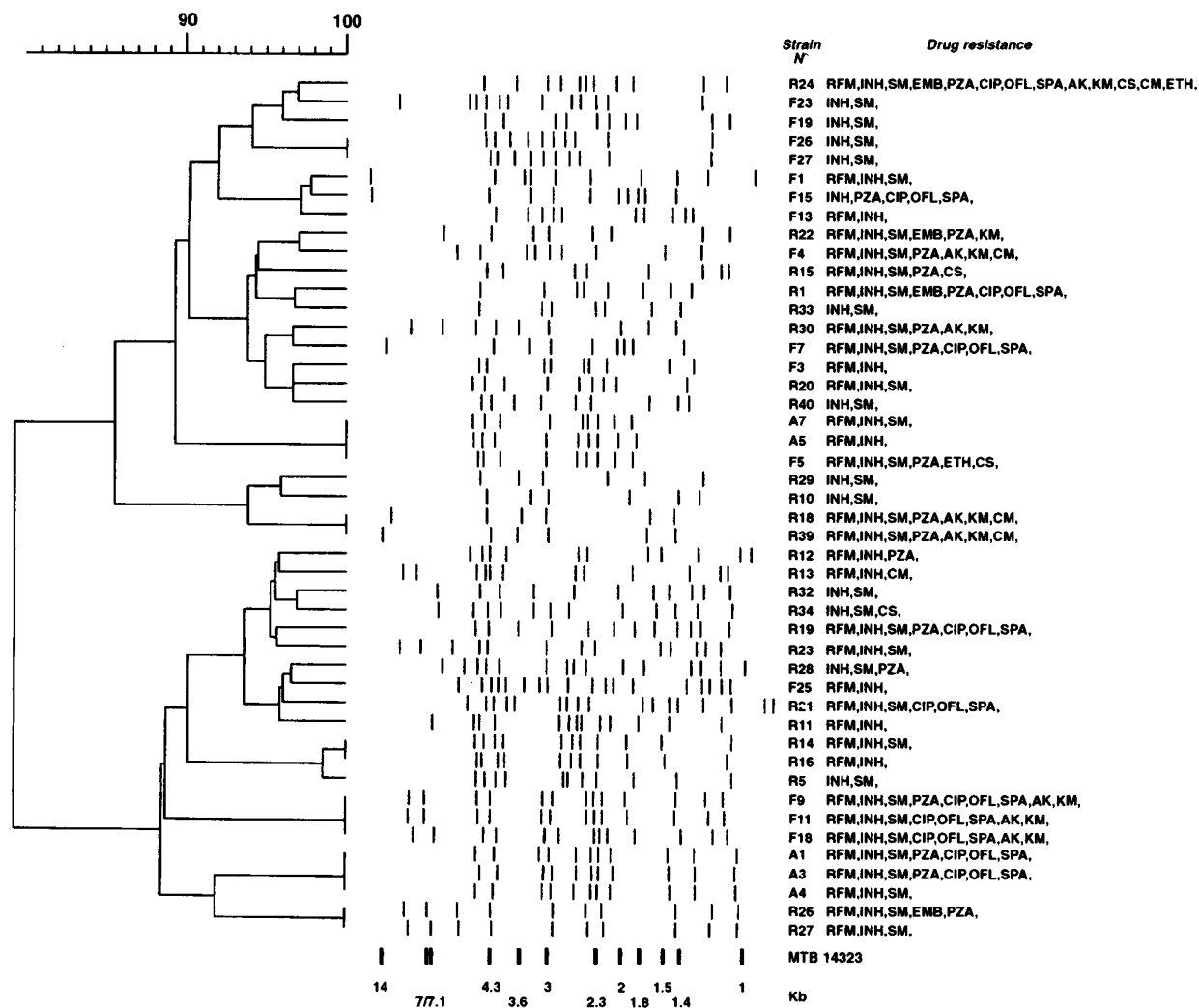


FIG. 3. Dendrogram, RFLP patterns, and drug-resistance profiles of 46 drug-resistant strains of MTB. The similarity among RFLP patterns is indicated over the dendrogram as a percentage. Last lane shows the fingerprinting of the reference strains MTB 14323.

tients. On the other hand, recent WHO guidelines for protocol development of a standard third-line treatment regimen for tuberculosis patients failing treatment on a standard WHO/IUATLD retreatment regimen<sup>20</sup> indicated that therapy with ETH + CM + EMB + OFL + PZA for 3 months followed by ETH + EMB + OFL for 18 months was the regimen of choice to treat "chronic excretors" of microorganisms.

Unfortunately, not many studies exist on the susceptibility of MDR strains to second-line antituberculous drugs.<sup>3,9,15</sup> Our work provides this information on a relatively large number of multiply first-line resistant strains and gives some insight into the potential use of second-line agents against difficult-to-treat MTB strains. Moreover, on the basis of the results obtained, we think that the activity of these agents, alone and in combination, should be re-examined in animal models of drug-resistant

infections. This could help to find new regimens for the treatment of patients infected with MDR strains.

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