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 thiacetazole, 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).16,19 Although such resistance can be overcome by appropriate multi-drug regimens of second-line agents,10,19,20 inadequate antimicrobial chemotherapy can lead to the emergence of strains resistant to virtually all antituberculous agents.3,11

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.2 While testing of first-line antituberculous agents, namely isoniazid (INH), rifampicin (RMP), ethambutol (EMB), streptomycin (SM), and pyrazinamide (PZA)7-10 is routinely performed, testing of second-line antituberculous drugs is usually done only by laboratories with specific expertise. Despite extensive reports on antituberculosis drug activity of first-line agents,9 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) analysis18 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°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 method7–9 in Middlebrook 7H11 agar (Difco), which better supports the growth of multidrug resistant (MDR) strains.8 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 agar10 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 μg/ml; OFL, 2 μg/ml;12 SPA, 0.5 μg/ml.12 As for TC, a tentative critical concentration was used corresponding to the highest MIC of drug-susceptible MTB strains in 7H10 agar (2.5 μg/ml).6 Other tentative critical concentrations were: VM, 10 μg/ml7 and AK, 8 μg/ml3. Susceptibility to PZA was determined by the BACTEC method.17

MIC values were determined in Middlebrook 7H11 agar (Difco). Plates containing different drug concentrations were inoculated in triplicate with approximately 2 × 102 and 2 × 103 CFU by a semiautomated inoculator (Multipoint Inoculator A400, Denley, West Sussex, UK) and incubated at 37°C in plastic bags for 14–21 days. The following drug concentration ranges were used: from 0.125 to 64 μg/ml for INH, SM, RMP, CIP, OFL, SPA, AK, PAS; from 0.117 to 120 μg/ml for EMB and CS; from 0.094 to 96 μg/ml for KM; from 0.078 to 80 μg/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, ≤0.125 μg/ml; SM, 1 μg/ml; RMP, 0.25 μg/ml; CIP, 1 μg/ml; OFL, 1 μg/ml; KM, 1.5 μg/ml; AK, 2 μg/ml; SPA, ≤0.125 μg/ml; CM, 2.5 μg/ml; EMB, 0.47 μg/ml; CS, 7.5 μg/ml; ETH, 1.25 μg/ml; TC, 0.156 μg/ml; PAS, 0.25 μg/ml; VM, 5 μg/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 ≥64, ≥64, ≥64, and 32 μg/ml, respectively.

RFLP

Cultures of MTB strains and DNA extraction were performed as previously described.18 Briefly, mycobacterial strains were grown for 3–4 weeks at 37°C in agitation (120 rpm) in 50 ml of Middlebrook 7H9 broth (Difco). Bacteria were pelleted by centrifugation and heat-killed at 80°C for 1 hr. Chromosomal DNA (2.5–3 μg) was incubated for 4 hr with PvuII restriction enzyme (Life Technologies, Grand Island, NY) in a total volume of 20 μl. Restriction fragments were separated in a 0.8% agarose gel in 1 X 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, Halington heights, IL) by the capillary method. The probe used in the study was a 245-bp PCR product of IS611018 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.
INH, resistance to SM, RMP, and PZA occurred in 80.4%, 71.7%, 39.1% of strains, respectively, and resistance to EMB in 8.7% of strains. Among second-line antituberculous drugs, resistance to aminoglycosides (AK and KM) and quinolones (CIP, OFL, and SPA) was approximately 20%; resistant to CM and CS, was lower, at approximately 10%. Two strains were resistant to ETH (4.3%) and no strain was resistant to TC, PAS, or VM. The resistance profiles of these strains are shown in Table 1. Ten strains (21.7% of the total) were resistant to two first-line drugs only (INH and SM), and 33 (71.7%) were MDR strains (resistant to at least INH + RMP) (about 2% of the total MDR strains isolated in Italy in the period 1995–1997). These latter strains showed complex resistance patterns, and 12 of them were resistant to seven antimicrobial agents or more. Whereas strains resistant to INH plus SM or RMP were rarely resistant to other drugs, strains resistant to INH + SM + RFM were sometimes resistant to second-line agents. Resistance to PZA and EMB was often associated with resistance to second-line drugs. A strain resistant to KM (MIC of >64 μg/ml) was susceptible to AK (MIC of 4 μg/ml).

The MIC distribution of these strains to 15 antimicrobial drugs is shown in Fig. 2. Ranges of MIC values for the first-line drugs, except EMB, were usually wider (≥10 dilutions) than for second-line drugs. Although all drugs displayed a rather continuous distribution of the MIC values, RMP-resistant and RMP-susceptible strains appeared as two well-separated populations, with low or high MIC values, respectively. A bimodal distribution of MICs was also observed for the aminoglycosides AK and KM, with very high MICs for resistant strains. Relatively low MIC values were found for fluoroquinolone-resistant strains; the highest MICs were not greater than four times the critical concentrations. Few strains were resistant to second-line antituberculous agents, with MICs four times (CM and ETH) or two times (CS) higher than susceptibility cut-offs.

RFLP analysis

RFLP analysis and drug-susceptibility patterns of the 46 strains are shown in Fig. 3. A variable number of IS6110 copies was observed, but the strains contained a mean copy number of 10.2 ± 2.6 copies (range 6–18) per isolate. Comparison of DNA fingerprinting patterns indicated that 31 patients were infected with unique strains and 15 patients with strains belonging to three clusters of two strains (F26 and F27, R18 and R39, R14 and R16) and three strains (A7, A5 and F5; F9, F11 and F18; A1, A3 and A4), respectively. Although most unique strains were resistant to two to four drugs, few strains were resistant to seven drugs or more; of these one strain (R24) was resistant to 13 drugs. No apparent relationship between resistant phenotypes and RFLP types was observed, with the exception of clustered strains, in which similar or identical resistant profiles were seen. The cluster F9-F11-F18 showed the same RFLP pattern found in other strains recently isolated in Italy from an outbreak of MDR tuberculosis and was composed by strains resistant to many first- and second-line antituberculous agents. Besides resistance to first-line drugs, strains belonging to the clusters R18-R39 and A1-A3-A4 contained strains resistant to aminoglycosides or fluoroquinolones, respectively.

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**Table 1. Resistance Pattern of 46 Drug-Resistant Strains of M. tuberculosis to 16 Antituberculous Agents**
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 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 β-subunit gene (rpoB), that are associated with high-level resistance against this drug were the genetic mechanism of RMP resistance; indeed, recent studies of our group 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. 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 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 and used for treatment of known or suspect MDR TB, could lose their efficacy as a consequence of point mutations in the target genes. KM and AK also, which are presently good alternative drugs because of the low cross-resistance to SM, 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. 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 such as TC, a thiosemicarbazone, 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., 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-
SUSCEPTIBILITY OF DRUG-RESISTANT M. TUBERCULOSIS

FIG. 3. Dendogram, RFLP patterns, and drug-resistance profiles of 46 drug-resistant strains of MTB. The similarity among RFLP patterns is indicated over the dendogram 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 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.3,5,15 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.

ACKNOWLEDGMENTS

We thank Clark B. Inderlied, Children Hospital, Los Angeles, and Antonio Cassone, Istituto Superiore di Sanità, Rome, for help in reviewing the manuscript. We thank Yuming Fan, Giovanna Alfarone and Laura Parisi, Istituto Superiore di Sanità, for valuable technical assistance. This work was supported in part by the Italian AIDS Project, Istituto Superiore di Sanità, Ministero della Sanità, Contract number 10/A/Z and by the National Tuberculosis Project of the Istituto Superiore di Sanità, Grant number 96/D/T95.
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