Antibiotic Susceptibility Pattern of *Mycobacterium marinum*

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In vitro activities of 17 antibiotics against 53 clinical strains of *Mycobacterium marinum*, an atypical mycobacterium responsible for cutaneous infections, were determined using the reference agar dilution method. Rifampin and rifabutin were the most active drugs (MICs at which 90% of the isolates tested were inhibited [MIC90], 0.5 and 0.6 μg/ml, respectively). MICs of minocycline (MIC90, 4 μg/ml), doxycycline (MIC90, 16 μg/ml), clarithromycin (MIC90, 4 μg/ml), sparfolacin (MIC90, 2 μg/ml), moxifloxacin (MIC90, 1 μg/ml), imipenem (MIC90, 8 μg/ml), sulfamethoxazole (MIC90, 8 μg/ml) and amikacin (MIC90, 4 μg/ml) were close to the susceptibility breakpoints. MICs of isoniazid, ethambutol, trimethoprim, azithromycin, ciprofloxacin, ofloxacin, and levofloxacin were above the concentrations usually obtained in vivo. For each drug, the MIC90 geometric mean MIC, and modal MIC were very close, showing that all the strains had a similar susceptibility pattern. Percent agreement (within ±1 log2 dilution) between MICs yielded by the Etest method and by the agar dilution method used as reference were 83, 59, 43, and 24% for minocycline, rifampin, clarithromycin, and ciprofloxacin, respectively. Reproducibility with the Etest was low, in contrast to that with the agar dilution method. In conclusion, *M. marinum* is a naturally multidrug-resistant species for which the agar dilution method is more accurate than the Etest for antibiotic susceptibility testing.

*Mycobacterium marinum* is an atypical photochromogenic mycobacterium belonging to group I of Runyon’s classification (18). This mycobacterium was successively named *M. piscium*, *M. marinum* (1), *M. platypoecilus*, *M. anabanti*, and *M. balnei*. Comparative sugar fermentative reaction data together with published morphological, cultural, and pathogenic data suggested that they were all synonymous with *M. marinum* (17). *M. marinum* inhabits fresh and salt water and causes disease in many fish species and occasionally in humans (24, 28). Human infections are generally limited to cutaneous diseases and are referred to as “swimming pool granuloma” and “fish tank granuloma” in reference to the epidemiology and the inoculation mode (24, 28). The frequency of *M. marinum* in bacteriology laboratories is low, since less than 1% of the mycobacterial clinical isolates belong to this species (11). Susceptibility data on *M. marinum* are scarce and rely upon the small numbers of strains and antibiotics tested (20, 23, 25). As a consequence, intrinsic antibiotic susceptibilities of *M. marinum* are not well defined, and methods for their routine determination have not been evaluated.

In this study we looked for the antibiotic susceptibilities of 53 clinical isolates of *M. marinum* by determining the MICs of 17 antibiotics using the agar dilution method. Antibiotics tested were tetracyclines, rifampin, and cotrimoxazole, which were reported to be effective for treating *M. marinum* infections (8), and antimycobacterial antibiotics active against *Mycobacterium tuberculosis* (isoniazid, rifabutin, ethambutol, and aminoglycosides) or atypical mycobacteria (clarithromycin, azithromycin, and imipenem). In addition, we tested fluoroquinolones, since new derivatives (levofloxacin, sparfolacin, and moxifloxacin) appear particularly active against mycobacteria (15). We compared the reference (but cumbersome) method used to a more practical and routine method of antibiotic susceptibility testing, the new stable gradient method known as Etest.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The study involved 53 clinical strains of *M. marinum* that were isolated over a period of 3 years (1995 to 1997) in bacteriology laboratories located in all parts of France. These strains were referred to the National Reference Centre for the Surveillance of Mycobacterial Infections and their Resistance to Antituberculous Agents (Laboratory of Bacteriology, Groupe Hospitalier Pitié-Salpêtrière, Paris, France), working for the present study in collaboration with the AZAY-Mycobacterium Group of the University hospitals of France. The strains of *M. marinum* were identified on the basis of phenetic characters as described previously (4). Strains were stored at −80°C in Youmans broth supplemented with 20% fetal bovine serum until the MICs were determined. *M. marinum* ATCC 927, *Mycobacterium smegmatis* ATCC 19420 and mc2 155, and *Escherichia coli* ATCC 25922 were used as controls for MIC determination. Mycobacteria were grown in Middlebrook 7H9 broth for 3 to 5 days (2 days for *M. smegmatis*), and the culture suspension was adjusted with additional sterile distilled water to equal a McFarland 1.0 turbidity standard (approximately 106 CFU per ml).

**Antimicrobial agents.** Rifampin, ofloxacin, and levofloxacin (Hoechst Marion Roussel, La Défense, France), rifabutin (Pharmacia & Upjohn, Rueil Malmaison, France), ethambutol (Lederle, Paris La Défense, France), amikacin (Bristol-Myers Squibb, Paris La Défense, France), imipenem (Merek Sharp & Dohme Chimret, Paris, France), minocycline (Wyeth Lederle, La Défense, France), doxycycline (Elerte, Aubervilliers, France), azithromycin (Pfizer, Orsay, France), clarithromycin (Abbott, Saint Rémy sur Avre, France), ciprofloxacin and moxi- floxacin (Bayer Pharma, Puteaux, France), sparfolacin (Rhône Poulenc Rorer, Vitry sur Seine, France), and isoniazid, sulfamethoxazole, and trimethoprim (Roche, Fontenay sous Bois, France) were kindly provided by the manufacturers. Etest strips containing either rifampin, clarithromycin, ciprofloxacin, or minocycline were obtained from AB Biodisk, BMD, Marne-la-Vallée, France.

**Determination of the MICs by the agar dilution method.** The agar dilution method was performed on Mueller-Hinton agar (Difco, Serlabo, Bonneuil sur Marne, France) supplemented with 5% Middlebrook OADC (olive acid, albumin, dextrose and catalase [OADC, Elancourt, France]). The 5% (vol/vol) ratio of OADC was found optimal for *M. marinum* growth by inoculating in preliminary tests 16 strains in duplicate on Mueller-Hinton agar prepared with 0% OADC (8 of 16 grew normally), 2.5% (14 of 16 grew normally), 5% (16 of 16 grew normally), and 10% (16 of 16 grew normally).

Twofold dilutions of the antibiotics were added in order to obtain the following final concentrations: amikacin, 0.25 to 64 μg/ml; imipenem, 0.06 to 32 μg/ml;
riparin, 0.015 to 32 μg/ml; rifabutin, 0.001 to 4 μg/ml; ethambutol, 0.03 to 8 μg/ml; azithromycin, 0.015 to 64 μg/ml; ofloxacin and ciprofloxacin, 0.06 to 128 μg/ml; sparfloxacin, levofloxacin, and moxifloxacin, 0.03 to 128 μg/ml; minocycline, doxycycline, and clarithromycin, 0.06 to 32 μg/ml; sulfamethoxazole and trimethoprim, 1 to 512 μg/ml; and isoniazid, 0.5 to 32 μg/ml. A 1/100 dilution of a McFarland 1.0 turbidity standard suspension was inoculated with a Steers replicator delivering approximately 10^6 CFU per spot. Plates were incubated at 30°C in a 5% CO2 incubator. MICs for E. coli ATCC 25922, M. smegmatis mc^2 155, and M. smegmatis ATCC 19420 were determined after 24 and 48 h of incubation, respectively. MICs for M. marinum strains were determined after 7 days (i.e., when half of the strains had grown) or 11 days (i.e., when all of the strains had grown) of incubation. The MIC was defined as the lowest concentration of antibiotic resulting in complete inhibition of growth or in growth of fewer than 10 colonies (<1% of the inoculum).

To evaluate the reproducibility with the method, independent tests were performed for M. marinum ATCC 927 (six tests) and 37 clinical strains (two tests each).

**Etest method.** A suspension, equal to a McFarland 1.0 turbidity standard suspension, was applied onto the surface of a 5% sheep blood Mueller-Hinton agar plate (Sanofi Diagnostic Pasteur; 15 by 15 mm) using a sterile cotton swab. The plate was incubated at 30°C in 5% CO2 as described above. The MIC was the lowest concentration of antibiotic yielding a zone of inhibition that was greater than the MIC standard strip, taking into account even faint growth as recommended for other mycobacteria (3, 27).

To evaluate the reproducibility with the method, 10 independent tests were performed for M. marinum ATCC 927. In addition, Etests were performed by two distinct operators, one operating under research conditions and one under routine conditions, for 39 clinical strains.

**Results.** The reproducibility was evaluated on the basis of the MIC differences in log2 dilution between the tests with the same strain. The reproducibility value was defined as the percentage of strains which yielded the same MIC within ±1 log2 dilution. The agreement between agar dilution and Etest methods was the percentage of strains which yielded the same MIC value within ±1 log2 dilution, by the two methods. Category discrepancies were evaluated using the breakpoints for determining susceptibility and resistance categories recommended by the NCCLS for aerobic bacteria (14). A major interpretive discrepancy was defined as resistance by the reference agar dilution method and susceptibility by the Etest method, a major interpretive discrepancy was defined as resistance by the Etest method and susceptibility by the agar dilution method, and a minor discrepancy was defined as intermediate susceptibility by one method and susceptible or resistance by the other method.

**Results.**

**Agar dilution method.** The reproducibility of results with the agar dilution method was good (>80% agreement) for all antibiotics except for sulfamethoxazole (<50% reproducibility). The MIC results are presented in detail in Table 1. MICs of rifampin and rifabutin (MICs at which 90% of the isolates were inhibited [MIC_{90}], 0.5 and 0.06 μg/ml, respectively) were far lower than those of other antibiotics. The MIC_{90}S of minocycline (4 μg/ml), doxycycline (16 μg/ml), clarithromycin (4 μg/ml), imipenem (8 μg/ml), and amikacin (4 μg/ml) were close to the breakpoints. MICs of isoniazid (MIC_{90}, 8 μg/ml), ethambutol (MIC_{90}, 4 μg/ml), trimethoprim (MIC_{90}, 128 μg/ml), and azithromycin (MIC_{90}, 128 μg/ml) were above the breakpoints. Among the fluoroquinolones tested, MICs of sparfloxacin (MIC_{90}, 2 μg/ml) and moxifloxacin (MIC_{90}, 1 μg/ml) were four- to eightfold lower than those of ciprofloxacin (MIC_{90}, 8 μg/ml), ofloxacin (MIC_{90}, 16 μg/ml), and levofloxacin (MIC_{90}, 8 μg/ml). For each antibiotic, the MICs were distributed in a narrow range (see the examples of doxycycline and moxifloxacin in Fig. 1) with an overall standard deviation comprised of between 1.5 and 2.6 log2 dilution. As a consequence, modal MICs were equal to or within 1 log2 dilution of MIC_{90}S and were close to geometric mean MICs (Table 1). The MICs for the reference strain, ATCC 927, were within 0 to 1 dilutions of the modal MIC for 53 clinical strains.

**Etest method.** We determined by Etest the MICs of rifampin, clarithromycin, minocycline, and sparfloxacin, which were the most active drugs against *M. marinum* as determined by the agar dilution method and for which Etest strips were available. For these four antibiotics, the ellipse inhibition zone was clear, without trailing growth, and thus reading the MIC was not ambiguous. The reproducibility with the Etest method for the reference strain was 100% in the case of minocycline (10 out of the 10 tests yielded the same MICs within ±1 log2) and 70% for rifampin and clarithromycin but only 40% for sparfloxacin. The results of duplicate tests performed by independent laboratories agreed with those of the Etest method.
those designed for slowly growing mycobacteria, such as the fluoroquinolones (19). To date no method has been recommended as the standard for determining the in vitro susceptibility of mycobacterial agents, such as new macrolides, imipenem, and clarithromycin and rifampin (20, 25). 2. The present study allowed us to delineate the susceptibility pattern of M. marinum towards antituberculous drugs and new drugs which have been shown to be active against other antimycobacterial species. Clearly, M. marinum is susceptible to rifampin, rifabutin, and amikacin but resistant to isoniazid and ethambutol. With regard to susceptibilities to fluoroquinolones, on one hand M. marinum is resistant to ofloxacin, ciprofloxacin, and levofloxacin, and on the other hand the majority of the strains were susceptible to moxifloxacin and sparfloxacin (MIC50 and geometric mean MIC, 0.5 and 1 μg/ml, respectively). As described previously for M. tuberculosis and atypical mycobacteria (12), fluoroquinolones were arranged from that with the lowest MIC to that with the highest MIC as follows: moxifloxacin, sparfloxacin, levofloxacin, ciprofloxacin, and ofloxacin. The results also confirmed the moderate susceptibility of M. marinum to tetracyclines (20, 23, 25) and the higher activity of minocycline than of doxycycline (MICs being constantly twofold lower). A majority of strains were susceptible to clarithromycin, to sulfamethoxazole, and to imipenem, but modal MICs of these drugs were close to the breakpoints. Finally, M. marinum was found to be resistant to azithromycin and to trimethoprim.

The geometric mean MIC, the modal MIC, and the MIC50 for each antibiotic taken separately were close, and the geometric standard deviations were very low (Table 1), strongly suggesting a homogeneous susceptibility pattern for the M. marinum species. This fact was confirmed by the narrow MIC distribution for each antibiotic, as shown for doxycycline and moxifloxacin in Fig. 1. The susceptibility pattern of M. marinum described herein likely corresponds to the wild-type susceptibility pattern. Until now, no relapse due to the selection of a resistant mutant has been reported, and acquired resistance is not known for M. marinum.

Therefore, on the basis of in vitro susceptibilities, candidates for treatment of M. marinum can be chosen. Cases of success have seldom been reported after treatment of M. marinum infections by rifampin (7, 8), and MICs of the rifamycins and rifampin and rifabutin are indeed the lowest and are close to those found for M. tuberculosis. Minocycline and to a lesser extent doxycycline, clarithromycin, imipenem, and amikacin dilution using a Steers replicator (20, 25), M. marinum grows rapidly enough, indeed, to be tested by the method used for rapidly growing mycobacteria, despite the fact that it belongs to the slowly growing mycobacteria on the basis on genetic and mycolic acid analysis (5, 21). We confirmed the rapid growth of M. marinum and showed that its growth required a lower level of OADC supplementation than is required for other slowly growing mycobacteria. Consequently, we used as a reference the agar dilution method using a Steers replicator (25) for its convenience and because of the large worldwide experience of this method of testing antibiotic activity that has been acquired with nonfastidious organisms.

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are serious candidates for the treatment of *M. marinum* infections, since their MICs are in the range of blood levels. Moreover, these MICs are close to those found for other atypical mycobacteria, such as *M. avium*, and rapidly growing mycobacteria, for which in vitro activity has been correlated with in vivo efficacy (6, 26). The activities of streptomycin and amikacin, even if higher than those of other fluoroquinolones, remained lower than those against *M. tuberculosis*, and thus in vivo efficacy should be carefully assessed. Pharmacokinetic considerations might also influence the therapeutic value of the antibiotics. However, all the antibiotics with good in vitro activity cited above also have a very high intracellular penetration and extravascular distribution.

Our second objective was the evaluation of a routine method for susceptibility testing of *M. marinum*. Etest has been demonstrated to be an accurate and precise method of MIC determination for bacteria other than mycobacteria (2). The results yielded by the Etest method were shown to agree with those yielded by the agar dilution method for rapidly growing and some slowly growing mycobacterial species (3, 9, 13, 16, 27). In the present study on *M. marinum*, the level of agreement between results for Etest and those for agar dilution was high only for minocycline (83% agreement) but in contrast was low for rifampin, streptomycin, and clarithromycin. The low agreement rates found for *M. marinum* were not expected, since more than 70% agreement was reported between results for Etest and those for agar dilution for *M. fortuitum* and *M. chelonae* (3) and for *M. avium* (16), and Etest MICs determined in the present study were close to those reported by Flynn et al. (9). If reproducibility of results with the Etest was excellent for minocycline, it was poor for clarithromycin, rifampin, and streptomycin. Consequently, we cannot recommend the Etest method for antibiotic susceptibility testing of *M. marinum*. Nevertheless, no acquired resistance has been described so far, and routine susceptibility testing seems unnecessary except for relapse cases, as for other atypical mycobacteria (24).

In conclusion, we described the wild-type susceptibility pattern of *M. marinum* for 17 antibiotics. Among these antibiotics, rifampin, rifabutin, tetracyclines (particularly minocycline), amikacin, imipenem, and clarithromycin are good candidates for testing in vivo efficacy.

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