Application of the Etest to the Antimicrobial Susceptibility Testing of Mycobacterium marinum Clinical Isolates

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Mycobacterium marinum, a well-recognized cutaneous pathogen, is usually treated by chemotherapy without available standardized in vitro susceptibility testing information. In this study, we have attempted to apply the stable-gradient method (Etest; AB Biodisk, Solna, Sweden) to susceptibility testing of M. marinum in order to evaluate Etest reproducibility. Results were observed to be within 1 log2 dilution of the all-test median MIC for 97.5% of the Etests. Our MIC results for the 60 strains clearly demonstrate the best in vitro potency against M. marinum isolates to be as follows (rank order): trimethoprim-sulfamethoxazole (MIC at which 90% of the isolates are inhibited [MIC90], 0.25 and 4.25 μg/ml, respectively) = ethambutol > clarithromycin (MIC90, 1 μg/ml) > minocycline = doxycycline (MIC90, 4 μg/ml) > amikacin (MIC90, 8 μg/ml). Rifampin was only marginally active against the M. marinum strains tested (MIC90 at the National Committee for Clinical Laboratory Standards) breakpoint of 1 μg/ml), and ciprofloxacin was not active (MIC90 8 μg/ml). These data should enhance the empiric drug selection for contemporary M. marinum infections and also provide evidence that the Etest can be utilized to guide chemotherapy with alternative agents.

Mycobacterium marinum, a so-called atypical mycobacterium classified in Runyon group 1, is known to inhabit swimming pools, aquariums, salt or brackish water, and some of the flora and fauna of these locations (4–6). M. marinum was first isolated in 1926 by J. D. Aronson, who observed the organism in fish that had died in the Philadelphia Aquarium (1). However, the organism was not recognized as a significant human pathogen until 1954, when M. marinum was isolated from superficial skin lesions of children who had swum in contaminated public pools (swimming pool granulomas) and was described in association with skin lesions of butchers (2, 12, 14, 19).

M. marinum is a well-recognized cutaneous pathogen which generally causes infections of the extremity most commonly associated with minor trauma. Rarely, the organism can cause deep-tissue infection or disseminated disease (6–12, 22, 25). Infection incidence depends on location, but rates of up to 0.27 per 100,000 population have been observed (4). The infection is commonly misdiagnosed, probably due to this infrequent occurrence, its indolent presentation, and the fact that M. marinum grows maximally at 31 to 33°C and is generally static at 35 to 37°C, making isolation with routine cultures unlikely (6–12, 18, 25). The organism usually causes granulomatous lesions which vary from ulcerated to nodular and sporotrichoid to involving deep-tissue structures (4, 6, 10, 25).

Treatment recommendations have been variable, but most commonly used have been physiotherapy, surgical excision, and a number of chemotherapy regimens. Recent clinical reports have suggested that antimicrobial therapy can lead to a clinical cure and that surgical debridement should be reserved for nonresponsive cases (4, 6, 10). The literature discussing antimicrobial therapy for M. marinum is limited, and the reported treatments have rarely been based on in vitro susceptibility testing information. In this report, we describe the in vitro test results (Etest; AB Biodisk, Solna, Sweden) for 60 strains of M. marinum isolated from clinical cases in 10 geographic locations (states), and we report the optimal test conditions for eight possible therapeutic antimicrobial agents.

MATERIALS AND METHODS

Antimicrobial agents. Etest strips were obtained from AB Biodisk NA, Inc. (Piscataway, N.J.), containing the following antimicrobial agents: amikacin, ciprofloxacin, clarithromycin, doxycycline, ethambutol, minocycline, rifampin, and trimethoprim-sulfamethoxazole.

Strains tested. The M. marinum isolates consisted of 64 organisms (four reference strains and 60 clinical isolates). Reference M. marinum organisms were obtained from the American Type Culture Collection (ATCC) (Rockville, Md.): ATCC 927, 11564, 11566, and 15069 were utilized as characterized control strains. The 60 clinical strains were initially isolated by laboratories located within the following states of the continental United States: Arizona (10 strains), California (3 strains), Florida (10 strains), Massachusetts (4 strains), New York (1 strain), North Carolina (6 strains), Oregon (3 strains), Texas (11 strains), Virginia (1 strain), and Washington (11 strains). Upon receipt from the above locations, all strains were subcultured on Lowenstein-Jensen nonselective slants. Although the strains were initially identified to genus and species levels by the collection laboratories, their identities were confirmed by our laboratory prior to inclusion in this study. This confirmation consisted of a negative nitrate reduction test, preferred growth at 30°C, and a positive photochromogen test performed according to published procedures (18).

Susceptibility testing. An inoculum of each M. marinum strain equivalent to a 1.0 McFarland turbidity standard was prepared from a 5-day-old subculture growing on a Lowenstein-Jensen slant. This inoculum was applied to 150-mm-diameter plates (16) upon which four Etest strips, each containing one antimicrobial agent, were placed (Fig. 1) (eight drugs on two plates per organism). The 5% sheep blood Mueller-Hinton agar (SBMHA) and/or Middlebrook 7H11 (7H11) agar plates used in these experiments were obtained from Prepared Media Laboratories (Tualatin, Ore.). Following inoculation, all strains were incubated for 5 days at 30°C. Results were interpreted by applying National Committee for Clinical Laboratory Standards (NCCLS) criteria (15, 17).

A selected subset of 10 strains were inoculated on both SBMHA and 7H11
agar and then incubated for 3 to 5 days at 30°C. The four reference strains obtained from the ATCC were tested on five occasions with all antimicrobial agents on 7H11 agar (incubated for 5 days at 30°C) as a reproducibility evaluation of the Etest susceptibility method. Endpoints were determined according to the manufacturers’ package insert instructions, with complete inhibition being the result for all antimicrobial agents except trimethoprim-sulfamethoxazole, for which 80% inhibition was considered the endpoint.

RESULTS

Ten strains were tested on both SBMHA and 7H11 media, and the growth and MICs for each medium were compared (Table 1). Four of the 10 strains did not grow sufficiently on SBMHA to be evaluable. Of the remaining six strains, the growth on 7H11 (Fig. 1) at 5 days was markedly superior to the growth observed on SBMHA. The ratios of MICs determined by Etest on SBMHA and 7H11 are listed in Table 1 for the six strains which grew on both media. MIC ratios of >1 (five drugs) suggested a possible adverse interaction between the organism and SBMHA. On the basis of these results and those of others (11, 13, 24), 7H11 agar was chosen for further investigations mainly because of more-uniform growth support of strains and the ease of interpretation of the zone of inhibition at 5 days of incubation.

Four ATCC control strains of M. marinum were tested on five occasions on 7H11 medium to evaluate Etest reproducibility. The results are shown in Table 2, which summarizes 160 tests (five replicates of eight drugs for four reference organisms). Compared to the modal or median for all replicates, the results were ±1 log₂ dilution for 97.5% of Etests. For five of eight tested drugs, all Etest results were within the acceptable limits for reproducibility.

Sixty clinical isolates of M. marinum from various areas of the United States were obtained, reidentified, and tested (Etest) against eight antimicrobial agents. These agents were selected on the basis of previous clinical reports of successful M. marinum therapy or on the basis of their effectiveness against other mycobacterial species. In addition, the majority of the tested drugs were available in oral formulations, thus offering convenient regimens for long-term clinical application. These drugs have been listed in rank order of potency in Table 3, along with their corresponding MIC distribution and the proportion of strains judged to be susceptible. Six of the drugs demonstrated greater than 95% susceptibility, as categorized by the NCCLS standards used (15–17). The observed MICs of trimethoprim-sulfamethoxazole (MIC at which 90% of the isolates are inhibited [MIC₉₀], 0.25 µg/ml) and ethambutol (MIC₉₀, 0.25 µg/ml) were well below commonly utilized NCCLS MIC breakpoints for susceptibility. However, the MICs of trimethoprim-sulfamethoxazole (read at 80% inhibition) were more difficult to interpret than complete-inhibition endpoints, but potential reader variances of endpoints of three log₂ dilution increments would have little effect on the category interpretation of Etest results. The MICs of the remaining drugs that demonstrated 100% susceptibility (clarithromycin, amikacin, and minocycline) were all at the breakpoint concentration and distributed over a narrow MIC range (three or four log₂ dilution increments). Doxycycline also showed measurable activity (MIC₉₀, 4 µg/ml), but the 60 organisms were generally resistant to ciprofloxacin (MIC₉₀, 8 µg/ml; susceptible at ≤1 µg/ml).

The antimycobacterial agent rifampin was marginally active against M. marinum strains, with a MIC₉₀ at the NCCLS break-

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**TABLE 1. Ratios of MICs determined by using the Etest applied to SBMHA and 7H11 agar**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. of occurrences of SBMHA/7H11 MIC ratio of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.12</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>0</td>
</tr>
</tbody>
</table>

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**TABLE 2. Reproducibility of the Etest when four M. marinum strains on five occasions were tested with eight drugs**

<table>
<thead>
<tr>
<th>Antimicrobial agent (no. of tests)</th>
<th>No. of results with the indicated variation (log₂ dilution) from modal value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−2</td>
</tr>
<tr>
<td>Amikacin (20)</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin (20)</td>
<td>1</td>
</tr>
<tr>
<td>Clarithromycin (20)</td>
<td>0</td>
</tr>
<tr>
<td>Doxycycline (20)</td>
<td>0</td>
</tr>
<tr>
<td>Ethambutol (20)</td>
<td>0</td>
</tr>
<tr>
<td>Minocycline (20)</td>
<td>0</td>
</tr>
<tr>
<td>Rifampin (20)</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole (20)</td>
<td>1</td>
</tr>
<tr>
<td>Total (160)</td>
<td>2</td>
</tr>
</tbody>
</table>

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*M. marinum ATCC 927, 11656, 11566, and 15069 were tested.*

A modal value was established from the five separate tests, and all results were compared to that MIC. When a mode was not clear (equal occurrences at two MIC results), the median result was applied.

The reproducibility was only 90% (±1 log₂ dilution), but a small sample size was evaluated.
point (1 µg/ml) (17). In contrast, ethambutol (MIC90, 0.25 µg/ml) was very potent against the clinical strains of *M. marinum*, and the modal MIC was 32-fold below the susceptible breakpoint (15). The use of the stable-gradient antimicrobial strip (Etest) in the evaluation of in vitro susceptibility of rapidly growing species *Mycobacterium tuberculosis* and *Mycobacterium avium-M. intracellulare* has been well described (13, 24). We utilized Etest strips on Middlebrook 7H11 plates to develop reproducible susceptibility test results for *M. marinum* in the same manner as described earlier for rapidly growing species (13). This method produces MICs within 1 week for antimicrobial agents known to contribute to favorable clinical outcomes.

We tested a large sample of clinical isolates from a wide sampling of geographic sites (10 states). These results allowed the development of potential empiric treatment choices based on quantitative susceptibility testing data. Previously reported in vitro test information has been more limited and unstandardized as to method, medium, and drugs selected. Our results clearly demonstrate the best choices (potency) for *M. marinum* infection therapy to be (rank order): trimethoprim-sulfamethoxazole (MIC90, 0.25 and 4.25 µg/ml, respectively) = ethambutol > clarithromycin (MIC90, 1 µg/ml) > minocycline (MIC90, 4 µg/ml) > doxycycline > amikacin (MIC90, 8 µg/ml). These data should allow selections of therapy for clinically suspected disease while culture or susceptibility results are awaited or simple empiric therapy when culture or susceptibility testing data may not be available.

The preferred treatment of *M. marinum* infection will likely remain a combination of chemotherapy and surgical debride-ment due to the granulomatous nature of the inflammatory response, decreased concentrations of therapeutic agents within the infection tissues, and the relative infrequency of infections available for structured clinical trials. Also, selection of agents with greater lipophilicity and intracellular concentra-tions would be expected to optimize bioavailability and the subsequent clinical response in *M. marinum* disease. Regardless of the specific therapy initially selected, the use of a simple stable-gradient susceptibility test (Etest) can now provide susceptibility results within 1 week of primary isolation, thus allowing modifications of therapy to more-potent agents or combinations.

**DISCUSSION**

Several methods have been used to determine in vitro suscep-tibility for *M. marinum*, including a radiometric respiromet-ric technique, agar disk elution methods, and agar dilution methods (3, 5, 20, 21, 23). The use of the stable-gradient antimicrobial strip (Etest) in the evaluation of in vitro susceptibility of rapidly growing species *Mycobacterium tuberculosis* and *Mycobacterium avium-M. intracellulare* has been well described (13, 24). We utilized Etest strips on Middlebrook 7H11 plates to develop reproducible susceptibility test results for *M. marinum* in the same manner as described earlier for rapidly growing species (13). This method produces MICs within 1 week for antimicrobial agents known to contribute to favorable clinical outcomes.

**ACKNOWLEDGMENTS**

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**REFERENCES**


