In vitro susceptibility of Stenotrophomonas maltophilia isolates: comparison of disc diffusion, Etest and agar dilution methods


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The disc diffusion, Etest and agar dilution techniques were compared to evaluate the antimicrobial susceptibility profile of 70 Stenotrophomonas maltophilia isolates to seven antimicrobial agents. The S. maltophilia isolates were consecutively collected from May 2000 to May 2002 from individual patients, who were hospitalized in a private Brazilian hospital. The antimicrobial susceptibility tests were carried out and interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations. The Etest was carried out according to the manufacturer’s instructions. There was good agreement among the distinct susceptibility testing results for chloramphenicol, doxycycline, gatifloxacin, trimethoprim–sulfamethoxazole and ticarcillin–clavulanate, suggesting that the disc diffusion and Etest methods are reliable for testing this group of antimicrobials against S. maltophilia. In contrast, a weak correlation was found between the disc diffusion and agar dilution techniques for testing polymyxin B and colistin with unacceptable very major error rates (18.1% and 22.7% for polymyxin B and colistin, respectively). Trimethoprim–sulfamethoxazole (MIC50, 0.06 mg/L; 98.5% susceptible) and gatifloxacin (MIC50, 0.12 mg/L; 98.5% susceptible) were the most potent antimicrobial agents tested against S. maltophilia isolates. In contrast, the worst in vitro activity was found for ticarcillin–clavulanate (MIC50, 16 mg/L; 59.1% susceptible). Although our results confirm that trimethoprim–sulfamethoxazole, gatifloxacin and doxycycline have an excellent in vitro activity against S. maltophilia, further clinical studies are necessary to evaluate the clinical efficacy of these compounds for the treatment of S. maltophilia infections, since no randomized controlled trials have been carried out and no correlation between the clinical response and susceptibility testing results has been reported.

Keywords: susceptibility testing, drug resistance, S. maltophilia

Introduction

Stenotrophomonas maltophilia has emerged as an important nosocomial pathogen capable of causing respiratory, urinary and bloodstream infections in hospitalized patients. The most susceptible patients are usually those submitted to previous antibiotic therapy, those who often require invasive procedures, patients with severe underlying comorbidities, immunocompromised patients and patients admitted to intensive care units.

The treatment of S. maltophilia infections is often problematic since this pathogen shows intrinsic resistance to many antimicrobial agents. In addition, emergence of antimicrobial resistance during therapy has also been reported. The resistance is generally the result of the reduction in permeability or the expression of efflux pumps. However, specific mechanisms of resistance, such as aminoglycoside-modifying enzymes and heterogeneous production of metallo-β-lactamases, have contributed to the multidrug-resistant phenotype displayed by this pathogen.

Since there have still been doubts concerning the accuracy and interrelation among the distinct methods for evaluating the in vitro susceptibility of S. maltophilia, this study was carried out with the objective of evaluating and comparing the methods of disc diffusion, Etest and agar dilution to determine the susceptibility profile of S. maltophilia to seven antibiotics.

Materials and methods

A total of 70 clinical isolates of S. maltophilia were consecutively collected from May 2000 to May 2002 from individual patients, hospitalized at Hospital Sírio Libanês, a tertiary private hospital located in the city of São Paulo, Brazil. The isolates were obtained from the following specimens: respiratory (47), urine (6), biopsy tissues (4), bloodstream (3)

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and others (10). Identification of S. maltophilia was carried out using the Vitek System (bioMérieux, Hazelwood, MO, USA). The isolates were stored at −20°C in the Clinical Microbiology Laboratory of the Hospital Sírio Libanês until studied further.

Antimicrobial susceptibility testing was carried out using the disc diffusion and agar dilution techniques as described by the National Committee for Clinical Laboratory Standards (NCCLS).5,6 The Etest technique (AB Biodisk, Solna, Sweden) was carried out according to the manufacturer’s instructions. The susceptibility test medium was Mueller–Hinton agar (Bio-Rad, Richmond, CA, USA). All bacterial isolates were tested by disc diffusion and Etest techniques. However, only 66 isolates were tested by agar dilution because four isolates became non-viable during the study period.

The antimicrobial discs were obtained from Oxoid (Basingstoke, UK) and possessed the following concentrations: chloramphenicol 30 µg, doxycycline 30 µg, gatifloxacin 5 µg, trimethoprim–sulfamethoxazole 2.75/1.25 µg, ticarcillin–clavulanic acid 75/10 µg, polymyxin B 300 IU and colistin 10 µg. The drug powders for the agar dilution test were obtained commercially or provided by their respective manufacturers. The minimal inhibitory concentration (MIC) was determined as the lowest antimicrobial concentration inhibiting visible growth after 18 h of incubation at 35°C.

Since there are no specific NCCLS susceptibility breakpoints for S. maltophilia, the antimicrobial susceptibility testing results were interpreted using the NCCLS criteria established for non-Enterobacteriaceae.7 Isolates having MICs ≥ 4 mg/L were considered resistant to both polymyxin B and colistin. Zone diameter breakpoints for susceptible and resistant isolates were set at ≥12 and ≤8 mm for polymyxin B, and ≥11 and ≤8 mm for colistin.8

Escherichia coli ATCC 25922 and P. aeruginosa ATCC 27853 were used as quality control (QC) strains. All the QC results were within the recommended breakpoints.7 The NCCLS documents do not currently provide the expected range for polymyxin B and colistin when testing the QC strains. Thus, polymyxin B and colistin were tested by disc diffusion for 30 consecutive days to observe if the obtained diameter zones would be coincident with those recommended by the disc manufacturer. When testing E. coli ATCC 25922, the expected zones of inhibition were from 12 to 16 mm for polymyxin B, and from 11 to 15 mm for colistin. The acceptable MIC range for the QC strains was obtained from the Etest package insert. MICS between 0.5 and 2.0 mg/L were considered acceptable for both polymyxin B and colistin, when testing P. aeruginosa ATCC 27853. For E. coli ATCC 25922, the QC ranges for polymyxin B and colistin were 0.25–1.0 and 0.125–0.5 mg/L, respectively. All the QC results were within the expected ranges.

Categorical agreement was defined if the tests results were within the same susceptibility category, and errors were determined by methods published in NCCLS M23-A2 and ranked as follows: very major error, false-susceptible result by the disc diffusion test; major error, false-resistant result produced by the disc diffusion test; and minor error, intermediate result by the disc diffusion method and a resistant or susceptible category for the dilution test.2

Statistical analysis
Simple linear regression analysis was applied to define linear functions correlating the zone of inhibition (mm) with MICs obtained by the disc diffusion and Etest or agar dilution methods (mg/L). The Etest and agar dilution variables were linearized by logarithmic conversions.

The Etest or agar dilution results were also compared to the zones of inhibition using the method of least squares as applied to computers. The strength of the linear association between pairs of variables was determined by coefficients of determination (R-square): R-square ≥ 50%, strong correlation; R-square ≥0.50%, moderate correlation; and R-square < 25%, weak correlation.

### Table 1. Comparative percentage susceptibility of the seven antimicrobial agents against S. maltophilia obtained by the three methods studied

<table>
<thead>
<tr>
<th>Agent</th>
<th>disc diffusion</th>
<th>Etest</th>
<th>agar dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>77.1</td>
<td>81.4</td>
<td>80.3*a</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0*a</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>98.6</td>
<td>97.1</td>
<td>97.0*a</td>
</tr>
<tr>
<td>SXT</td>
<td>98.6</td>
<td>98.6</td>
<td>100.0*a</td>
</tr>
<tr>
<td>Ticarcillin–clavulanate</td>
<td>60.0</td>
<td>58.6</td>
<td>59.1*a</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>90.9*a</td>
<td>78.8*</td>
<td>77.3*</td>
</tr>
<tr>
<td>Colistin</td>
<td>92.8</td>
<td>80.0</td>
<td>75.7*</td>
</tr>
</tbody>
</table>

SXT, trimethoprim–sulfamethoxazole.
aOnly 66 S. maltophilia isolates were tested.

The validation of these linear models was carried out by F-test. All P values reported were two-tailed and values lower than 0.05 were considered significant. The data were analysed with the Minitab statistical package.

### Results

#### Antimicrobial susceptibility testing

Table 1 summarizes the percentages of susceptibility of the seven antimicrobial agents against S. maltophilia obtained by the three susceptibility methods. All S. maltophilia isolates were susceptible to doxycycline, independent of the susceptibility method used. Susceptibility to trimethoprim–sulfamethoxazole was 98.6% by disc diffusion and Etest, whereas by agar dilution it was 100.0%. This occurred because the single isolate detected as resistant to trimethoprim–sulfamethoxazole became non-viable during the study, and was not evaluated by the agar dilution technique. In general, the susceptibility rates were similar among the methodologies evaluated for all antibiotics tested, except for polymyxin B and colistin. For these agents, a significant difference in the susceptibility rates was observed between disc diffusion and the agar dilution methods, with higher susceptibility rates obtained by the disc diffusion technique. For instance, 90.9% and 77.3% of the S. maltophilia isolates were considered susceptible to polymyxin B by the disc diffusion and agar dilution methods, respectively.

#### Comparison of disc diffusion and dilution methods

Table 2 shows the discordant results split by error category, antimicrobial agent and susceptibility method. Again, a good correlation among the susceptibility tests was observed for all antimicrobial agents tested, except for the polymyxins. No very major errors (false susceptible) were detected when testing chloramphenicol, doxycycline, gatifloxacin, trimethoprim–sulfamethoxazole and ticarcillin–clavulanate. A single major error (false resistant; 1.4%) was identified for chloramphenicol. When comparing chloramphenicol results by disc diffusion with those obtained by Etest and agar dilution methods, minor error rates of 4.3% and 10.0% were detected, respectively. Most of the minor errors (five out of seven) were the result of isolates showing inhibition zones at the intermediate range and agar
dilution MICs of 32 mg/L. Testing ticarcillin–clavulanate by disc diffusion gave minor error rates of 14.3% and 11.4% compared with the agar dilution and Etest techniques, respectively. These percentages could be explained in part because of the miscategorization of five isolates, exhibiting MICs of 64 mg/L.

A weak correlation was found between the disc diffusion and agar dilution techniques for polymyxin B (log agar dilution = 1.03 – 0.06 zone diameter; R-square = 11.6%; P = 0.005) and colistin (log agar dilution = 1.05–0.06 zone diameter; R-square = 18.5%; P < 0.0001). When comparing the polymyxin B results between these two methods, very major and minor error rates of 18.1% and 9.0% were detected. The comparison between the colistin results produced by disc diffusion and agar dilution methods also revealed an elevated rate of very major errors (22.7%).

Comparison of Etest and agar dilution methods

The Etest method showed excellent agreement with the reference method, the agar dilution method. Even though the comparison between the Etest and agar dilution techniques produced elevated rates of very major and major errors when testing the polymyxins, polymyxin B and colistin Etest MICs showed good correlation with agar dilution test results, exhibiting an essential agreement of 96.7% and 89.4% (± 1 log2 dilution) for colistin and polymyxin B, respectively. A trend toward higher MIC results with the agar method was noticed. Thus, the difference in the susceptibility rates and occurrence of errors could be explained by the existence of isolates demonstrating MICs at 4 mg/L, which is the polymyxin breakpoint of resistance.

<table>
<thead>
<tr>
<th>Table 2. Discordant results among the susceptibility methods (number and category of error for each antimicrobial agent tested against <em>S</em>. <em>maltophilia</em>)</th>
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<tbody>
<tr>
<td><strong>Agent</strong></td>
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<td></td>
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<tr>
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SXT, trimethoprim–sulfamethoxazole.

*Category of error: VM, very major error; M, major error; MI, minor error.

<table>
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<th>Table 3. Antimicrobial activity of the seven antimicrobial agents tested against <em>S</em>. <em>maltophilia</em> by the agar dilution method</th>
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<tr>
<td><strong>Agent</strong></td>
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SXT, trimethoprim–sulfamethoxazole.

*MIC50 and MIC90 defined as the minimal concentration of antibiotic capable of inhibiting 50% and 90% of the isolates tested, respectively.

**Comparison of the antimicrobial activity**

Table 3 shows the activity of the seven antimicrobial agents tested against *S*. *maltophilia* by the agar dilution method. Trimethoprim–sulfamethoxazole was the most potent drug tested against *S*. *maltophilia* (MIC90 0.06 mg/L) followed by gatifloxacin (MIC90 0.12 mg/L). In contrast, the worst in vitro activity was found for ticarcillin–clavulanate (MIC90 16 mg/L; 59.1% susceptible). Colistin and polymyxin B (MIC90 2 mg/L) were equally potent against *S*. *maltophilia*; 56.1% and 48.5% of the *S*. *maltophilia* isolates exhibited MICs 1 log2 dilution above or below the breakpoint of resistance for polymyxin B and colistin, respectively. Total cross-resistance between the two polymyxins was not observed because a single isolate exhibited MICs of 4 and 2 mg/L for colistin and polymyxin B, respectively.
Discussion

*S. maltophilia* is characterized by an intrinsic resistance to many classes of antibiotics. Thus, the therapeutic options for the treatment of serious infections caused by *S. maltophilia* are very limited. This scenario becomes more complicated as a result of the lack of NCCLS guidelines for testing *S. maltophilia* and through previous reports which verified an inconsistent correlation between the disc diffusion and dilution methods when testing this pathogen. 10,11

Since the dilution methods are more cumbersome or expensive than the disc diffusion test to be applied in the routine clinical microbiology laboratory, the aim of this study was to compare the performance of disc diffusion to other susceptibility methods to evaluate the antimicrobial susceptibility of *S. maltophilia* to seven antimicrobial agents.12 The Etest method showed a good agreement with the reference agar dilution method, indicating that this test is a suitable and reliable method for determining the susceptibility of *S. maltophilia* to this group of antibiotics.

The highest percentage of susceptibility to polymyxins was obtained with the disc diffusion technique, and this seems a paradox for antibiotics such as polymyxins, which diffuse poorly in agar. 13 Comparison of the discordant results between the disc diffusion and agar dilution methods for both antibiotics demonstrated an unacceptable rate of very major errors (false susceptible) for polymyxin B (18.1%) and colistin (22.7%), confirming the low accuracy of this method for testing polymyxins.

Despite the bacteriostatic action and the emergence of resistant strains, trimethoprim–sulfamethoxazole has been the drug of choice for treatment of *S. maltophilia* infections. 14–18 In this study, trimethoprim–sulfamethoxazole was the most potent drug tested. One *S. maltophilia* isolate only was resistant to this combination (MIC >32 mg/L), but it was susceptible to gatifloxacin (MIC, 0.125 mg/L), doxycycline (MIC, 2 mg/L), chloramphenicol (MIC, 2 mg/L) and colistin (MIC, 0.5 mg/L). Unfortunately, this strain became non-viable during the study and its resistant phenotype could not be confirmed by the agar dilution method.

The newer fluoroquinolones such as gatifloxacin, moxifloxacin and clinafloxacin have shown enhanced activity against *S. maltophilia* compared with ciprofloxacin. 15,16,19–23 In this study, gatifloxacin also displayed an excellent *in vitro* activity against the isolates tested (MIC90, 0.12 mg/L; 97.0% susceptible). In fact, no fully resistant isolate of *S. maltophilia* was detected. These results indicate that gatifloxacin may be used as a therapeutic option for the treatment of *S. maltophilia* infections.

*S. maltophilia* may be resistant to β-lactams by means of β-lactamases L1 and L2. However, ticarcillin–clavulanate has shown good *in vitro* activity against this pathogen, and has been the second drug of choice for treatment of *S. maltophilia* infections. Unexpectedly, a low susceptibility rate (59.1%) to ticarcillin–clavulanate was observed among the *S. maltophilia* studied. The SENTRY Antimicrobial Surveillance Program has documented a wide geographical difference in the resistance rates to ticarcillin–clavulanate (10–29%) among *S. maltophilia*. 15 Even though the mechanism of resistance has not been determined in this work, the production of L1 metallo-β-lactamases and/or the acquisition of plasmid-mediated extended-spectrum β-lactamases could explain the high percentage of ticarcillin–clavulanate resistance detected in this study.

Polymyxin B and colistin have frequently been used for the treatment of multidrug-resistant *Acinetobacter* spp. and *Pseudomonas aeruginosa* infections in Brazilian hospitals. 24 Moreover, polymyxins and trimethoprim–sulfamethoxazole have demonstrated an additive or synergic effect against *S. maltophilia*. 25,26 Resistance rates obtained in this study were similar to those reported previously. 16 The high resistance rates associated with the need for reliable polymyxin susceptibility testing demand further studies to evaluate the benefit of polymyxins as therapeutic agents against *S. maltophilia* infections.

Our results confirm that trimethoprim–sulfamethoxazole, gatifloxacin and doxycycline display excellent *in vitro* activity against *S. maltophilia*. However, further clinical studies are necessary to confirm the real effectiveness of these compounds for the treatment of *S. maltophilia* infections, and also to evaluate the correlation between the susceptibility testing results and the clinical outcome.

References


