In Vitro Activities of β-Lactam–β-Lactamase Inhibitor Combinations against Stenotrophomonas maltophilia: Correlation between Methods for Testing Inhibitory Activity, Time-Kill Curves, and Bactericidal Activity

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The activities of ampicillin, ampicillin-sulbactam, amoxicillin, amoxicillin-clavulanic acid, ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, aztreonam, and aztreonam-clavulanic against Stenotrophomonas maltophilia strains for which the MICs of penicillins and commercially available β-lactam-β-lactamase inhibitor combinations were higher than the breakpoints usually recommended for Pseudomonas aeruginosa in commercially available broth microdilution methods were tested by the agar diffusion, agar dilution, and broth microdilution methods. Time-kill curve studies were performed when discrepancies between these methods were observed. The MICs obtained by the commercially available broth microdilution method, the agar dilution method, and the broth microdilution method were almost identical. Twenty-five percent of the strains tested showed inhibition diameters of ≥15 mm for ticarcillin-clavulanic acid, and 43.7% of the strains tested showed inhibition diameters of ≥18 mm for piperacillin-tazobactam by the agar diffusion method. The time-kill curves for these strains confirmed the results obtained by dilution methods. Aztreonam-clavulanic acid (2:1) at concentrations of ≤16 μg/ml inhibited all of these strains (MIC range, 1 to 16 μg/ml). The time-kill curves confirmed this activity. The addition of piperacillin to this combination did not modify the MICs. The combination aztreonam-clavulanic acid-ticarcillin was two- to fourfold more active than aztreonam-clavulanic acid alone. We studied the inhibitory and bactericidal activities of the two most active combinations (aztreonam-clavulanic acid and aztreonam-clavulanic acid-ticarcillin) against the standard inoculum and 10 and 50 times the standard inoculum. Inoculum modifications did not modify the MICs. Both combinations showed good bactericidal activity against the standard inoculum. With 10 times the standard inoculum, minimum bactericidal concentration (MBC) results were heterogeneous (for 55% of the strains, MBCs were between the MIC and 4-fold the MIC, and for 45% of the strains MBCs were between 8- and >32-fold the MIC). With 50 times the standard inoculum, MBCs were at least 32-fold the MICs for all the strains tested.

Stenotrophomonas maltophilia is an aerobic, nonfermenting, gram-negative bacillus that causes significant morbidity and mortality in hospitalized patients, mainly debilitated, neutropenic, and immunosuppressed patients (4). The treatment of infections caused by this microorganism is difficult, because S. maltophilia is frequently resistant to most of the widely used antibiotics (5, 7, 15). This resistance is usually attributed to low cell wall permeability (3, 16) and, in the case of β-lactams, mainly to two β-lactamases (L1, a metallo-β-lactamase included in group 3 of the Bush classification, and L2, a β-lactamase included in group 2e among the cephalosporinases inhibited by clavulanic acid [2]).

The activities of β-lactam–β-lactamase inhibitor combinations against S. maltophilia vary notably in different studies (1, 5, 11, 13, 14). The combination aztreonam-clavulanic acid (AZT-C), although not available commercially, almost systematically has MICs for S. maltophilia lower than the breakpoint usually considered for aztreonam alone against other gram-negative bacteria, according to previous studies (1, 5, 6).

Otherwise, some studies have indicated that in vitro susceptibility tests for S. maltophilia are problematic and that the dilution methods currently recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (9) can lead to erroneous results (1, 11, 13, 17). We have tested the in vitro inhibitory and bactericidal activities of several β-lactam–β-lactamase inhibitor combinations against S. maltophilia strains for which the MICs of several β-lactams and β-lactam-β-lactamase inhibitor combinations have been shown by various methods to be high to determine the correlation between standard sensitivity methods when testing these combinations against this microorganism. Moreover, we have tested the combinations that showed the best behaviors against different inocula to determine the influence of the inoculum on the efficacies of these combinations.

MATERIALS AND METHODS

Microorganisms. Thirty-two epidemiologically unrelated clinical strains of S. maltophilia from different sources were used. The MICs of penicillins and commercially available β-lactam–β-lactamase inhibitor combinations for the S. maltophilia strains were higher than the breakpoints usually recommended for Pseudomonas aeruginosa (10) by the broth microdilution method (PASCO); Escherichia coli ATCC 25922 and P. aeruginosa ATCC 27853 were used as control strains.

Antibiotics. Ampicillin, amoxicillin, ticarcillin, piperacillin, aztreonam, clavulanic acid, sulbactam, and tazobactam were kindly provided as standard powders by their respective manufacturers. Amoxicillin plus clavulanic acid (A-C) and amoxicillin plus sulbactam (A-S) were combined at a penicillin:inhibitor ratio of 2:1, ticarcillin plus clavulanic acid (T-C) were combined by using a fixed clavulanic concentration of 2 μg/ml and tazobactam, and tazobactam plus tazobactam (P-T) were combined by using a fixed tazobactam concentration of 4 μg/ml AZT and
clavulanic acid were combined at a β-lactam:inhibitor ratio of 2:1: this has previously been shown to be the most active ratio against this species (5). Combinations of penicillins (amoxicillin, ticarcillin, and piperacillin) plus aztreonam and clavulanic acid were tested at penicillin:monobactam:inhibitor concentration ratios of 2:2:1. Fixed concentrations of clavulanate were not used in these triple combinations, because ratios of AZT-C of about 2:1 are necessary to maintain the synergism of AZT-C according to previous results (5).

Susceptibility testing. Agar diffusion, agar dilution, and broth microdilution studies were performed according to NCCLS standards (9, 10). The agar diffusion studies with all antibiotics except AZT-C were performed with commercially available disks (Oxoid, Basingstoke, United Kingdom). Since disks with AZT-C are not commercially available, we added disks containing 10 μg of AZT with 2 μg of a solution of clavulanic acid at 2.5 g/liter (final amount, 5 μg per disk). Agar dilution and broth microdilution studies were performed for all antibiotics individually and the combinations A-C, A-S, T-C, P-T, AZT-C, amoxicillin-clavulanic acid-AZT (A-C-AZT), ticarcillin-clavulanic acid-AZT (T-C-AZT), and piperacillin-clavulanic acid-AZT (P-C-AZT). Time-kill curve studies were performed as described by NCCLS (8) with a high inoculum (10⁶ CFU/ml), peak concentrations of antibiotics in serum (32 μg of ticarcillin per ml for T-C and 64 μg of piperacillin per ml for P-T), and fixed concentrations of inhibitors (2 mg of clavulanate per liter and 4 mg of tazobactam per liter) for all strains for which discrepant results were obtained between these methods. We considered the results to be discrepant when the MICs obtained by the broth microdilution and agar dilution methods were different by more than two twofold concentrations, and when the MICs obtained by any of the dilution methods were higher than the breakpoints usually recommended for P. aeruginosa, but when the diameters obtained by the agar dilution method were wider than those recommended as being the minimum for consideration of the microorganisms as sensitive, or vice versa. For AZT-C, the breakpoints recommended for aztreonam alone against P. aeruginosa were used. Once our results indicated the methods that were the most reliable for testing β-lactam–β-lactamase inhibitor combinations against S. maltophilia and the most active combinations, we tested the inhibitory and bactericidal activities of these combinations against S. maltophilia by the broth microdilution method with different inocula (the standard inoculum recommended by NCCLS and inocula 10 and 50 times the standard inoculum) to determine the influence of the inoculum size on the activities of these combinations.

RESULTS

There was a correlation between the results obtained by the three methods used to test ampicillin, amoxicillin, ticarcillin, piperacillin, AZT, A-C, and A-S for their inhibitory activities against all strains. The MICs for the 32 strains tested were higher than the breakpoints previously established for all these antibiotics and combinations by the three methods. By the agar diffusion method, 8 strains (25%) had diameters of ≥15 mm with T-C and 14 strains (43.7%) had diameters of ≥18 mm with P-T (6 strains were included in both groups). The MICs for all of these strains were higher than the breakpoints established by the agar dilution and broth microdilution methods (Table 1).

The combination AZT-C showed a correlation for all strains, and for all of the strains the MICs were lower than the breakpoint defined for AZT against P. aeruginosa (MIC at which 50% of isolates are inhibited [MIC₅₀], 4 mg/liter; MIC₉₀, 8 mg/liter; MIC range, 1 to 16 mg/liter), and the diameters were wider than the minimum recommended diameters required to consider P. aeruginosa sensitive to AZT.

To assess the differences observed between the diffusion and the dilution methods, we performed eight time-kill studies with six strains (two strains with diameters of ≥15 mm with T-C by the agar diffusion method, two strains with diameters of ≥18 mm with P-T, and two strains with both characteristics) (Fig. 1). Time-kill studies confirmed the resistance of these strains to both combinations and then confirmed the results of the dilution methods (Fig. 1).

The triple combination P-C-AZT had the same activity as AZT-C alone (MIC₅₀, 4 μg/ml; MIC₉₀, 8 μg/ml; MIC range, 1 to 16 μg/ml) by both the broth microdilution and the agar dilution methods. The activity of the triple combination T-C-AZT was two- to fourfold higher than that of AZT-C (MIC₉₀, 1 μg/ml by the agar dilution method and 2 μg/ml by the broth microdilution method; MIC₉₀, 4 μg/ml by both methods; MIC range, 0.5 to 4 μg/ml by the agar dilution method and 1 to 4 μg/ml by the broth microdilution method).

Since the MICs of AZT-C for S. maltophilia strains were low but the MICs of all other β-lactam–β-lactamase inhibitor combi-

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### Table 1. Proportion of S. maltophilia isolates tested resistant to β-lactams and β-lactam–β-lactamase inhibitor combinations by three methods

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>% Resistant by the following method:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agar diffusion</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>100</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>100</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>100</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>100</td>
</tr>
<tr>
<td>AZT</td>
<td>100</td>
</tr>
<tr>
<td>AZT</td>
<td>100</td>
</tr>
<tr>
<td>A-C</td>
<td>100</td>
</tr>
<tr>
<td>A-S</td>
<td>100</td>
</tr>
<tr>
<td>T-C</td>
<td>75</td>
</tr>
<tr>
<td>P-T</td>
<td>56.3</td>
</tr>
<tr>
<td>AZT-C</td>
<td>0</td>
</tr>
</tbody>
</table>

* Resistance was defined according to the NCCLS breakpoints for P. aeruginosa. For AZT-C, NCCLS breakpoints for AZT and P. aeruginosa were used.

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![Time-kill curves for T-C against four strains with diameters of ≥15 mm by the agar diffusion method and resistant by the broth microdilution method.](http://aac.asm.org/)

![Time-kill curves for P-T against four strains with diameters of ≥18 mm by the agar diffusion method and resistant by the broth microdilution method.](http://aac.asm.org/)
of non-metallo-

bications were high and since ticarcillin is the only penicillin that increased the activity of this combination, we tested the MICs and minimum bactericidal concentrations (MBCs) by the agar microdilution method with the standard inoculum and with inocula 10 and 50 times the standard inoculum. MICs were similar for all three inocula for all strains (Table 2). The with inocula 10 and 50 times the standard inoculum. MICs and minimum bactericidal concentrations (MBCs) by that increased the activity of this combination, we tested the usefulness may be impaired by the fact that a proportion of AZT-C of about 2:1 is important for the activity of the combina- tion, and the pharmacokinetics of AZT and clavulanic acid are quite different. Both AZT-C and T-C-AZT had good bactericidal activities against all strains tested except when very large inocula were used. Then, if pharmacokinetic profiles that allow for the proportions necessary for synergy are reached, monobactam–β- lactam–inhibitor combinations and, eventually, T-C-mono- bactam combinations might be good alternatives for the treatment of infections caused by multidrug-resistant strains of S. maltophilia.

**REFERENCES**


**DISCUSSION**

The results obtained in the present study confirm previously published data with respect to the usefulness of some methods of testing the susceptibility of this microorganism (1, 11, 13, 17). Agar diffusion seems to lead to false results of sensitivity to β-lactam–β-lactamase inhibitor combinations with some frequency. The results of the agar dilution and broth dilution methods correlate perfectly with those of time-kill studies. Other interesting data are those related to the activities of AZT-C and T-C-AZT against S. maltophilia. This microorganism usually harbors two types of β-lactamases: L1, a metallo-β-lactamase that hydrolyzes all β-lactams except aztreonam and that is not inhibited by clavulanic acid, and L2, a Bush group 2e β-lactamase that hydrolyzes aztreonam but that is inhibited by clavulanic acid (2). More recently, another group of non-metallo-β-lactamases has been described in S. maltophilia; the pl of these β-lactamases range from 5.2 to 6.6, and in general they hydrolyze all β-lactams except imipenem and aztreonam and are not inhibited by clavulanic acid (12). This profile of β-lactamase production can explain the behaviors of the S. maltophilia strains tested. S. maltophilia strains harboring these β-lactamases can hydrolyze almost all β-lactams and β-lactam–β-lactamase inhibitor combinations with the exception of AZT-C, since the only β-lactamase from S. maltophilia that hydrolyzes AZT (L2) is inhibited by clavulanic acid. The fact that the high level of activity of the combination AZT-C is, in vitro, much pronounced at ratios of about 2:1, as has been reported previously (3), remains unexplained. The activity of T-C-AZT is higher than that of AZT-C in strains for which ticarcillin and T-C MICs are high, and this is also difficult to explain. Nevertheless, synergy between ticarcillin and T-C and other antibiotics (co-trimoxazole and ciprofloxacin) against S. maltophilia has already been described (13). According to our results, the in vitro sensitivity of S. maltophilia to β-lactam–β-lactamase inhibitor combinations by the agar diffusion method should be considered with caution and the results should be confirmed by other methods. As we reported previously (5), the combination AZT-C (2:1) is, in our experience, active against all S. maltophilia strains tested, including strains for which the MICs of all other β-lactam–β-lactamase inhibitor combinations tested are high. Other investigators (1) have reported that 85% of S. maltophilia strains are sensitive to the combination AZT-C (1:1). This activity is even increased by the addition of ticarcillin, although not by the addition of amoxicillin, ampicillin, or pipercillin. The clinical usefulness may be impaired by the fact that a proportion of AZT-C of about 2:1 is important for the activity of the combina- tion, and the pharmacokinetics of AZT and clavulanic acid are quite different.

**REFERENCES**


