In vitro efficiency of the piperacillin/tazobactam combination against inhibitor-resistant TEM- and complex mutant TEM-producing clinical strains of Escherichia coli

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Objectives: We investigated the bacteriostatic and bactericidal activities of piperacillin/tazobactam against 16 clinical Escherichia coli producing inhibitor-resistant TEM β-lactamases (IRT; 13/16) and complex mutant TEM enzymes (CMT; 3/16).

Methods: Bacteriostatic activity was evaluated by three methods (disc diffusion, Vitek2 automated system, MIC determination by a microdilution method) and a time–killing study was used to investigate the bactericidal effect against standard (5 × 10^5 cfu/mL) and high inocula (5 × 10^6 cfu/mL).

Results: Piperacillin/tazobactam was bacteriostatic against most of the tested strains (15/16). Using a high inoculum, the piperacillin/tazobactam combination was not bactericidal against the 13 IRT-producing strains and one of the CMT-producing strains (1/3). A loss of bactericidal activity was still observed for seven IRT-producing strains (7/13) with a standard bacterial inoculum (<99.9% killing over 24 h).

Conclusions: Despite usual in vitro bacteriostatic activity, the piperacillin/tazobactam combination was not bactericidal against most IRT-producing clinical strains of E. coli, especially for the treatment of a high bacterial inoculum. This possible loss of bactericidal effect should be brought to the attention of physicians and may require high dosing regimens for the treatment of severe infections.

Keywords: resistance, β-lactamases, β-lactamase inhibitors, bactericidal activity, antibiotics

Introduction

The production of β-lactamases is the main mechanism of resistance to β-lactam antibiotics in Enterobacteriaceae. The most prevalent enzymes (TEM-1 and SHV-1) are able to inactivate penicillins and narrow-spectrum cephalosporins, but they are susceptible to β-lactamase inhibitors. Two strategies have been developed to thwart the activity of these enzymes: the use of stable β-lactam antibiotics such as oxymino cephalosporins, and the combination of penicillins and β-lactamase inhibitor. However, oxymino cephalosporin-hydrolysing TEM and SHV mutants, designated extended spectrum β-lactamases (ESBLs), have emerged since the 1980s.1 Likewise, the intensive use of penicillin/β-lactamase inhibitor combinations has been followed by the emergence of inhibitor-resistant TEMs (IRTs) harbouring point mutations conferring resistance to β-lactam-based inhibitors.2 In addition, TEM and SHV enzymes combining IRT- and ESBL-type substitutions have been discovered since the mid-1990s. Most enzymes of this new subgroup (10/11) belong to the TEM family and are called complex mutant TEMs (CMTs).3 These inhibitor-resistant enzymes have been mainly observed in Escherichia coli, which is one of the major bacteria isolated in clinical microbiology.

Despite lower activity of the inhibitors, most IRT- and CMT-producing strains are susceptible to the piperacillin/tazobactam combination in vitro. Chaibi et al.2 have suggested a possible loss of bactericidal activity. However, to our knowledge, the bactericidal activity of this combination has never been investigated. The aim of the present study was therefore to evaluate the bacteriostatic and bactericidal effects of the piperacillin/tazobactam combination against 16 E. coli clinical strains that produce representative IRT and CMT enzymes.
Materials and methods

Bacterial isolates

A collection of 16 non-duplicate clinical strains of *E. coli* was used throughout the study. This collection comprised 13 IRT-producing isolates and 3 CMT-producing isolates (Table 1). The β-lactamases produced by these strains were identified by isoelectric focusing and specific PCR and sequencing experiments. All these strains produced only the TEM enzymes that are indicated in Table 1. *E. coli* ATCC 25922 and *E. coli* ATCC 35218 and the strains CF001, CF002 and 2300 were used as standard reference strains.

Vitek2 automated system

The AST-N052 card (bio-Mérieux, Marcy l’Etoile, France) was used for each isolate using an inoculum comprising between 0.52 and 0.60 McFarland units according to the manufacturer’s recommendations for susceptibility testing of the piperacillin/tazobactam combination. The interpretation of the evaluated MIC was based on the breakpoints of the European Committee on Antimicrobial Susceptibility testing (EUCAST) (piperacillin/tazobactam): susceptible (S) ≤ 8/4 mg/L, resistant (R) > 16/4 mg/L.3

Disc diffusion method

The susceptibility to the piperacillin/tazobactam combination was tested by the disc diffusion method with discs containing 75 mg of piperacillin and 10 mg of tazobactam in accordance with the Comité de l’Antibiogramme de la Société Française de Microbiologie (CA-SFM) recommendations.2

MIC determination

MIC was determined in duplicate by a microdilution method and interpreted according to the EUCAST guidelines (S ≤ 8/4 mg/L, R > 16/4 mg/L).3 The following antimicrobial concentration range was used: piperacillin (0.5 - 1024 mg/L) for a fixed concentration of tazobactam at 4 mg/L for all piperacillin concentrations. All tests were performed in duplicate.

Specific activity

The amounts of produced β-lactamase were evaluated for each strain by determining by three independent experiments the specific activity of a crude extract against piperacillin by a microacidimetric method as previously described.5

Time–kill study

Standard antibiotic powders of piperacillin and tazobactam (Wyeth Laboratories, Pearl River, NY) were used throughout the study. Time–kill studies were performed using a final volume of 100 mL of

Table 1. MICs, inhibition diameters and clinical categorization obtained by the Vitek2 system (AST-N052 card), by the disc diffusion method and by the microdilution method for the 16 clinical strains. *E. coli* ATCC 25922 and ATCC 35218 were used as quality controls.

<table>
<thead>
<tr>
<th>Strain</th>
<th>TEM enzyme</th>
<th>Specific activity (µmol/min/mg)</th>
<th>IC₅₀ (µM)</th>
<th>Vitek2 system</th>
<th>Disc-diffusion method</th>
<th>Microdilution method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MIC (mg/L)</td>
<td>Zone diameter (mm)</td>
<td>MIC (mg/L)</td>
</tr>
<tr>
<td>ATCC 25922</td>
<td>—</td>
<td>—</td>
<td></td>
<td>≤4/4 S</td>
<td>30 S</td>
<td>1/4 S</td>
</tr>
<tr>
<td>CF001</td>
<td>TEM-1</td>
<td>2.2</td>
<td>0.13³</td>
<td>≤4/4 S</td>
<td>29 S</td>
<td>4/4 S</td>
</tr>
<tr>
<td>CF002</td>
<td>TEM-1</td>
<td>13.5</td>
<td>0.13³</td>
<td>≤4/4 S</td>
<td>25 S</td>
<td>2/4 S</td>
</tr>
<tr>
<td>ATCC 35218</td>
<td>TEM-1</td>
<td>1.7</td>
<td>0.13³</td>
<td>≤4/4 S</td>
<td>29 S</td>
<td>1/4 S</td>
</tr>
<tr>
<td>2300</td>
<td>TEM-28</td>
<td>0.5</td>
<td>0.02³</td>
<td>≤4/4 S</td>
<td>30 S</td>
<td>4/4 S</td>
</tr>
<tr>
<td>CF0012</td>
<td>TEM-31</td>
<td>4.8</td>
<td>12³</td>
<td>≤4/4 S</td>
<td>25 S</td>
<td>2/4 S</td>
</tr>
<tr>
<td>CF0022</td>
<td>TEM-30</td>
<td>10.6</td>
<td>2.9³</td>
<td>≤4/4 S</td>
<td>24 S</td>
<td>4/4 S</td>
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<tr>
<td>CF0032</td>
<td>TEM-32</td>
<td>1.2</td>
<td>1.5³</td>
<td>≤4/4 S</td>
<td>24 S</td>
<td>8/4 S</td>
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<tr>
<td>CF0042</td>
<td>TEM-35</td>
<td>3.5</td>
<td>1.8³</td>
<td>8/4 S</td>
<td>21 S</td>
<td>16/4 I</td>
</tr>
<tr>
<td>CF0052</td>
<td>TEM-33</td>
<td>20.8</td>
<td>1.9³</td>
<td>64/4 R</td>
<td>19 S</td>
<td>128/4 R</td>
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<tr>
<td>CF0062</td>
<td>TEM-34</td>
<td>2</td>
<td>1³</td>
<td>≤4/4 S</td>
<td>25 S</td>
<td>8/4 S</td>
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<tr>
<td>CF0072</td>
<td>TEM-36</td>
<td>3.9</td>
<td>0.9³</td>
<td>8/4 S</td>
<td>22 S</td>
<td>16/4 I</td>
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<tr>
<td>CF0082</td>
<td>TEM-37</td>
<td>1.5</td>
<td>1³</td>
<td>8/4 S</td>
<td>23 S</td>
<td>16/4 I</td>
</tr>
<tr>
<td>CF0092</td>
<td>TEM-38</td>
<td>22.5</td>
<td>2.5³</td>
<td>8/4 S</td>
<td>21 S</td>
<td>16/4 I</td>
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<tr>
<td>CF0102</td>
<td>TEM-39</td>
<td>27.9</td>
<td>1³</td>
<td>≤4/4 S</td>
<td>24 S</td>
<td>8/4 S</td>
</tr>
<tr>
<td>P37</td>
<td>TEM-45</td>
<td>8.6</td>
<td>1.4⁸</td>
<td>≤4/4 S</td>
<td>28 S</td>
<td>4/4 S</td>
</tr>
<tr>
<td>CF0152</td>
<td>TEM-51</td>
<td>5.6</td>
<td>3.5³</td>
<td>≤4/4 S</td>
<td>29 S</td>
<td>4/4 S</td>
</tr>
<tr>
<td>BM4511</td>
<td>TEM-103</td>
<td>6.2</td>
<td>ND</td>
<td>≤4/4 S</td>
<td>26 S</td>
<td>2/4 S</td>
</tr>
<tr>
<td>CF349</td>
<td>TEM-109+TEM-1</td>
<td>1.1</td>
<td>0.27</td>
<td>≤4/4 S</td>
<td>28 S</td>
<td>4/4 S</td>
</tr>
<tr>
<td>T0799</td>
<td>TEM-125+TEM-1</td>
<td>2.5</td>
<td>0.27</td>
<td>8/4 S</td>
<td>26 S</td>
<td>4/4 S</td>
</tr>
<tr>
<td>CF1295</td>
<td>TEM-151+TEM-1</td>
<td>1.3</td>
<td>0.27</td>
<td>≤4/4 S</td>
<td>26 S</td>
<td>4/4 S</td>
</tr>
</tbody>
</table>

S, susceptible; I, intermediate; R, resistant.

³S ≤ 16/4 mg/L, I = 16/4 mg/L, R > 16/4 mg/L.3

⁴S ≥ 21 mm, I between 20 and 17 mm, R < 17 mm.⁴

Data from Chaibi et al.2
cation-adjusted Mueller-Hinton broth to which was added 40 mg/L of piperacillin and 5 mg/L of tazobactam based on the mean steady-state serum concentration obtained using a dosing regimen of 3.375 g of piperacillin/tazobactam every 6 h. Standard inoculum time–kill studies contained an initial inoculum of $5 \times 10^5$ cfu/mL, while high inoculum time–kill studies contained an initial inoculum of $5 \times 10^6$ cfu/mL. An antibiotic-free control containing the tested strain and a negative control containing E. coli ATCC 25922 were included for each test. Organisms and antibiotic(s) were added and a sample was removed from each flask after 0, 2, 4, 6, 8, 10, and 24 h of incubation at 37°C. Colony counts were performed by diluting the sample in sterile water and logarithmically plating 50 μL samples on trypticase soy agar plates. The plates were incubated at 35°C for 24 h and time–kill profiles were constructed (log cfu/mL versus time). The limit of quantification was 100 cfu/mL, thus any colony count lower than this limit was rounded to 100 cfu/mL. All tests were performed in duplicate.

**Results and discussion**

The IRT panel comprised 13 clinical strains that all produced different enzymes harbouring most IRT-type substitutions. The CMT panel corresponded to three of the four piperacillin/tazobactam-susceptible strains that produced a CMT with an

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**Figure 1.** Time–killing curves of piperacillin/tazobactam (40/5 mg/L) against (a) E. coli strains that produce TEM-30, TEM-31 or TEM-51 (high inoculum); (b) E. coli that produce TEM-32, TEM-33 or TEM-34 (high inoculum and standard), TEM-38 or TEM-103 (high inoculum); (c) E. coli that produce TEM-35, TEM-36 or TEM-37 (high inoculum and standard); (d) E. coli that produce TEM-39 or TEM-125 (high inoculum).
IC$_{50}$ higher than that of TEM-1. These strains were chosen to be representative of the diversity of clinical IRT- and CMT-producing *E. coli* strains.

Bacteriostatic tests showed that 15 of the 16 strains were partially or fully susceptible to the combination (Table 1). Only minor discrepancies were observed between the three methods. Vitek2 MICs were slightly lower than those obtained by the microdilution method. However, a loss of bactericidal activity was observed for seven IRT-producing strains with standard bacterial inoculum (<99.9% killed over 24 h) (Figure 1a–d). In a recent study, Tam et al. observed that, with the same inoculum, a piperacillin/tazobactam concentration two times higher than the MIC was sufficient to obtain a bactericidal effect over 24 h, even when the strain produced an ESBL. In our study, despite a concentration two to four times higher than the MIC, we observed no such bactericidal activity for 6 of 12 IRT-producing strains.

Using a high inoculum (5 x 10$^8$ cfu/mL), the combination was not bactericidal against any IRT-producing strains and one CMT-producing *E. coli*, which exhibited regrowth (Figure 1a and Figure S1 as Supplementary data at JAC Online) after 4–10 h of incubation. This loss of bactericidal effect was not observed with the control *E. coli* strains producing TEM-1 or the ESBL TEM-28, highlighting the importance of the enzymatic resistance to tazobactam for this behaviour. The clinical relevance of such an inoculum effect is not widely accepted. However, Rice et al. observed a loss of activity of $\beta$-lactam antibiotics, such as cefotaxime and cefpirome, during the treatment of experimental high inoculum infections with ESBL-producing strains even when the MICs were in the susceptible range. In addition, our high inoculum was only 10 times higher than the standard inoculum in order to limit the possibility of artefacts caused by $\beta$-lactamase release.

Overall, these results suggest an increased risk of clinical failure for the treatment of infections due to IRT-producing strains, and to a lesser extent due to CMT-producing strains, with piperacillin/tazobactam at the standard dosing regimen (3.375 g every 6 h), especially in immunocompromised patients or for the treatment of high bacterial inoculum associated infections in which a bactericidal treatment is essential. Although some publications have reported that the production of IRT enzymes is associated with clinical failure for the amoxicillin/clavulanate combination, to our knowledge there are no clinical data concerning the use of piperacillin/tazobactam against clinical strains that produce IRT $\beta$-lactamases to confirm our results.

Unexpectedly, the strains that produced IRT with low sensitivity to tazobactam, such as TEM-31, TEM-30 and TEM-51 (IC$_{50}$: 2.9–12 $\mu$M), were killed, unlike strains producing IRT more sensitive to tazobactam, such as TEM-32, TEM-44, TEM-35, TEM-36 and TEM-37 (0.9–1.8 $\mu$M). A low specific activity against piperacillin (<4 $\mu$mol/min/mg) was observed for five of the seven IRT-producing strains that were not killed by the piperacillin/tazobactam combination. Higher specific activities (ranging from 5.6 to 27.9 $\mu$mol/min/mg) were observed for all the killed IRT-producing strains. According to these results, it seems that neither the IC$_{50}$ nor the specific activity were predictable of the bactericidal activity of piperacillin/tazobactam against an IRT-producing *E. coli*. The loss of the bactericidal effect probably involves other parameters, such as the level of production of the penicillin binding proteins, PBP2 and PBP3. However, it was not predictable by the MIC values. The combination was not bactericidal against susceptible strains with MICs ranging from 4/4 to 8/4 mg/L (TEM-32-, TEM-34-, TEM-45-producing strains), whereas it was bactericidal against a strain with a 16/4 mg/L MIC (TEM-38-producing *E. coli*).

In conclusion, despite usual in vitro bacteriostatic activity, the piperacillin/tazobactam combination was not always fully bactericidal against CMT- and IRT-producing *E. coli*. The possible loss of bactericidal effect should be noted by physicians when the production of an inhibitor-resistant $\beta$-lactamase such as IRT is suspected, as the treatment of severe infections due to such strains may require higher dosing regimens of piperacillin/tazobactam. This observation needs to be confirmed by further pharmacokinetic–pharmacodynamic and clinical investigations.

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### Transparency declarations

None to declare.

### Supplementary data

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

### References


