Variable susceptibility to piperacillin/tazobactam amongst *Klebsiella* spp. with extended-spectrum $\beta$-lactamases

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MICs of piperacillin/tazobactam are conventionally determined by varying the concentration of piperacillin in the presence of a fixed 4 mg/L tazobactam. When tested in this way, the MIC distribution for *Klebsiella* isolates with extended-spectrum $\beta$-lactamases (ESBLs) is strongly bimodal, such that many producers are inhibited at 16 + 4 mg/L whilst others require MICs of $\geq 512 + 4$ mg/L. When, however, piperacillin/tazobactam was tested as a fixed 8:1 ratio, the MIC distribution became unimodal. If clavulanate 4 mg/L was combined with piperacillin, a unimodal MIC distribution was seen for ESBL-producing *Klebsiella* spp. but a bimodal distribution arose if the clavulanate concentration was reduced to 0.25 mg/L. These data for alternative combinations suggested that the bimodal MIC distribution seen for piperacillin + tazobactam 4 mg/L was a titration effect, not a reflection of some ESBLs being resistant to tazobactam. Even within single strains, as defined by serotype and DNA fingerprints, there was considerable variation in susceptibility to piperacillin + tazobactam 4 mg/L, with some representatives highly susceptible and others highly resistant. Some of the more resistant representatives produced more of their ESBL, or had a greater number of $\beta$-lactamase types, but these associations were not universal. Elevated resistance to piperacillin + tazobactam was not associated with porin change in any ESBL producer examined, but has been found by others.

Keywords: piperacillin/tazobactam, *Klebsiella*, extended-spectrum $\beta$-lactamases

Introduction

Piperacillin/tazobactam is the most active penicillin/$\beta$-lactamase inhibitor combination against *Klebsiella* isolates with extended-spectrum $\beta$-lactamases (ESBLs); nevertheless, some ESBL producers are resistant. Such variation is not surprising, since it is a general observation that the MICs of $\beta$-lactamase inhibitor combinations vary among producers of the same $\beta$-lactamase type, contingent on the permeability of individual strains and the amount of $\beta$-lactamase that they synthesize.$^1$

What is surprising is that the MIC distributions of piperacillin/tazobactam for ESBL-producing *Klebsiella* spp. are strongly bimodal (see Results for examples), with values clustered either around 4 + 4 to 8 + 4 mg/L or $\geq 512 + 4$ mg/L.$^{2,3}$ Even when ESBL-producing isolates belong to a single outbreak strain, it is common to find that some representatives are susceptible to piperacillin + tazobactam, whereas others are highly resistant. Such stratification has not been reported with piperacillin/tazobactam for producers of other $\beta$-lactamases, nor for other inhibitor combinations against ESBL producers. Skewed unimodal MIC distributions were found when tazobactam, clavulanate and sulbactam combinations were tested against *Escherichia coli* isolates with various amounts of TEM-1 enzyme; moreover, ESBL producers are consistently susceptible to the cephalosporin/clavulanate combinations used in ESBL detection tests.$^5$

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To investigate why bimodal MIC distributions arise for ESBL producers with piperacillin + tazobactam 4 mg/L, we tested piperacillin in combination with different concentrations of tazobactam or, as a control, with clavulanate. We also compared the β-lactamase expression, kinetics and outer membrane profiles of piperacillin/tazobactam-susceptible and -resistant representatives of ESBL-producing outbreak strains of *Klebsiella pneumoniae*.

### Materials and methods

#### Bacterial strains

ESBL-producing isolates were collected in two surveys of *Klebsiella* from European ICUs, undertaken in 1994 and 1997/8.²,³ The 1994 survey included 220 ESBL producers from 27 hospitals, the 1997/8 survey included 161 producers from 16 hospitals. Species identification was with the API20E system (bioMérieux, La Balme les Grottes, France), and strains were typed by capsular serotyping and by pulsed-field gel electrophoresis (PFGE) of genomic DNA digested with *Xba*I. The methods of DNA digestion and PFGE were as described previously⁶ for the isolates collected in 1994; the method of Gautom² was used for the isolates collected in 1997/8 as it was more convenient than the earlier method and gave comparable results. MICs of antibiotics were determined on Iso-Sensitest agar (Oxoid, Basingstoke, UK), as previously described.²,³ Piperacillin and tazobactam were from Wyeth (Taplow, UK), lithium clavulanate from Smith-Kline Beecham (Welwyn Garden City, UK) and cefotaxime from Sigma (Poole, UK).

Isolates were identified as ESBL producers on the basis of ceftazidime/ceftriaxone + clavulanate MIC ratios of ≥16. β-Lactamase profiles were examined by isoelectric focusing (IEF) of extracts prepared by the sonication (1994) or repeated freezing and thawing (1997/8) of cells harvested from nutrient agar plates;⁸ *bla*TEM and *bla*SHV genes were sought with the primers and conditions described previously.⁹ In a few cases, the PCR products of amplification of *bla*SHV were sequenced by chain termination, using the method and conditions described elsewhere;⁹ otherwise the PCR products of *bla*SHV genes were profiled by single-stranded conformational polymorphism (SSCP), as described previously.⁵

#### Quantification of β-lactamase activity

Cultures were grown overnight, with shaking, in 10 mL amounts of nutrient broth at 37°C, then diluted 10-fold into fresh identical broth at 37°C. After incubation for a further 4 h, these cultures were harvested at 5000g and 37°C. The cells were washed in 10 mL of 0.1 M phosphate buffer pH 7.0, resuspended in 1.5 mL of the same buffer, chilled on ice and disrupted by sonication. The resulting extracts were used in spectrophotometric assays at 37°C and pH 7.0, with 0.1 mM cefotaxime or 0.2 mM piperacillin as substrates; assay wavelengths were 255 and 235 nm, respectively.

To assay inhibition of β-lactamase activity, sonicates (250–100 µL) were mixed with various concentrations of tazobactam, from 0.01 µM to 0.1 mM, in 1 mL volumes of 0.1 M phosphate buffer. After incubation for 10 min at 37°C, 100 µL amounts of 2 nM piperacillin were added, giving a final piperacillin concentration of 0.182 mM. Piperacillin hydrolysis was then measured by spectrophotometry at 235 nm and the *I*₅₀ was defined as the tazobactam concentration reducing the piperacillin hydrolysis rate by 50%. Specific activity was defined as nmol β-lactam hydrolysed/min per mg cell protein, with protein measured by the Lowry method or with the Bradford Reagent (Sigma). In either case, bovine serum albumin was used for the standards.

#### Extraction and electrophoresis of outer membrane proteins

Outer membrane proteins were extracted as the Sarkosyl-insoluble fraction from exponential phase cells that had been grown in Nutrient Broth No. 2 (Oxoid).⁵ These preparations were suspended in distilled water and adjusted to a protein concentration of 1 mg/mL, as assayed by the Lowry method.¹⁰ Protein profiles were then obtained by SDS–PAGE, using the methods described by Livermore & Williams.⁸

#### Results

### Effect of inhibitor concentrations on MIC distributions

Susceptibility tests with piperacillin in the presence of various concentrations of tazobactam or clavulanate were carried out for the 220 ESBL-producing *Klebsiella* spp. from the 1994 survey and for the 161 from the 1997/8 collection. All except six of the isolates were resistant to piperacillin 16 mg/L and the great majority (95.4%) required piperacillin MICs ≥ 128 mg/L. MIC distributions of piperacillin + tazobactam 4 mg/L were bimodal for both collections (Figure 1a), with peaks at 4 + 4 to 8 + 4 mg/L and at ≥1024 + 4 mg/L, and with the ‘trough’ at MICs of 32 + 4 to 256 + 4 mg/L. These distributions have been published previously²,³ but are reproduced here to illustrate the phenomenon under investigation.

Further studies were then carried out by varying the inhibitor or its concentration, using only the 161 ESBL producers from the 1997/8 collection. When piperacillin was combined with tazobactam at a fixed 8:1 ratio, the MIC distribution for the ESBL producers became unimodal (Figure 1b). A unimodal distribution was also seen when the isolates were tested with piperacillin + clavulanate 4 mg/L. In this latter case, the single mode was at 4 + 4 mg/L, but there was a small trail of resistant isolates with MICs up to 512 + 4 mg/L (Figure 1c).
With the clavulanate concentration reduced to 1 mg/L, the MIC distribution for piperacillin + clavulanate retained a single mode, now at 8 mg/L, but the proportion of resistant isolates increased (Figure 1d); when, however, the clavulanate concentration was lowered to 0.25 mg/L, the distribution became bimodal, with peaks at 16 + 0.25 and 256 + 0.25 mg/L (Figure 1e).

**Figure 1.** MIC distributions of piperacillin + β-lactam inhibitor combinations versus ESBL-producing *Klebsiella* spp. (a) Piperacillin + tazobactam 4 mg/L versus the 220 ESBL producers from the 1994 survey (grey) and 161 ESBL producers from the 1997/8 survey (black); (b) piperacillin/tazobactam 8:1 versus the 161 ESBL producers from the 1997/8 survey; (c–e) piperacillin + clavulanate 4, 1 and 0.25 mg/L, respectively, versus the 161 ESBL producers from the 1997/8 survey. Piperacillin MICs ≥ 1024 mg/L in (b) (not other panels) need to be treated with caution since they correspond to tazobactam concentrations ≥128 mg/L, which may inhibit the growth of *Klebsiella* spp.

Piperacillin/tazobactam resistance in relation to strain structure

The 220 ESBL producers collected in 1994 comprised 85 strains as defined by serotyping and PFGE, together with seven isolates for which satisfactory PFGE profiles could not be obtained. Fully 61% of these 220 isolates belonged to 16 ‘outbreak’ strains, each with three or more representatives, whereas 39% were ‘sporadics’, with only one or two representatives. Of the 16 outbreak strains, eight exclusively comprised isolates susceptible to piperacillin + tazobactam at the breakpoint of 16 + 4 mg/L, whereas three comprised only resistant organisms and five included representatives of both phenotypes. Similar patterns were seen among the isolates collected in 1997/8. Among the 161 ESBL producers, 115 (71%) belonged to 17 outbreak strains, each with three or more representatives, whereas the remaining 46 (29%) belonged to 35 sporadic strains. Among the outbreak strains from 1997/8, four solely comprised organisms susceptible to piperacillin + tazobactam 4 mg/L, six had only resistant organisms and seven included both resistant and susceptible organisms. In those instances where outbreak strains included both susceptible and resistant isolates (Table 1), the MIC distributions of...
Piperacillin + tazobactam 4 mg/L were extremely wide and, so far as could be judged (allowing for the small numbers of isolates per strain), recapitulated the bimodal pattern seen for the collections as a whole.

**β-Lactamase profiles**

Among the 220 ESBL producers collected in 1994, multiple β-lactamases were found by IEF in 27/154 (17.5%) of those susceptible to piperacillin + tazobactam (16 + 4 mg/L) compared with 29 of the 76 (38.2%) that were resistant ($\chi^2 = 16.29$, $P < 0.001$). If the division was drawn at 64 + 4 mg/L, corresponding to the 'natural' trough in the bimodal distribution rather than the breakpoint (Figure 1a) then the proportions of 'susceptible' and 'resistant' isolates with multiple β-lactamases were 31/167 (18.6%) and 25/63 (39.7%), respectively ($\chi^2 = 15.6$, $P < 0.001$). These analyses indicated an association between production of multiple β-lactamases and resistance to piperacillin + tazobactam 4 mg/L, but this conclusion was not supported by results from the 1997/8 collection, when multiple β-lactamases were found in 43 of the 66 isolates susceptible at breakpoint and in 72 of the 95 found resistant ($\chi^2 = 2.01$, $P > 0.05$). Similarly, multiple β-lactamases were observed in 51 of the 77 isolates from the 1997/8 collection that were susceptible to piperacillin + tazobactam at the natural trough of ≤64 + 4 mg/L, compared with 65 of 84 that were resistant ($\chi^2 = 2.47$, $P > 0.05$). A complicating factor was that a greater proportion of isolates had multiple enzymes in the 1997/8 study, and that more had TEM enzymes (G. S. Babini, L. M. C. Hall, M. Yuan & D. M. Livermore, unpublished data).

### Table 1. MIC distributions of piperacillin + tazobactam 4 mg/L for those outbreak strains that included susceptible and resistant representatives

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Origin</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>≥1024</th>
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</thead>
<tbody>
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<td>K25/PN1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>22</td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>2</td>
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<td>2</td>
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<td>1</td>
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<td>2</td>
<td>2</td>
<td>2</td>
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<td>1</td>
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<tr>
<td>K16-PN29&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1</td>
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<td>1</td>
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<tr>
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<td>1994, Istanbul</td>
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<td>1</td>
<td>2</td>
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<tr>
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<td>1994, Istanbul</td>
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<td>2</td>
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<td>1</td>
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<tr>
<td>K16-PN29&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>14</td>
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<tr>
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<td>2</td>
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<tr>
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<td>172</td>
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<tr>
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<td>2</td>
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<td>1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tr>
</tbody>
</table>

<sup>a</sup>The strains are defined by their capsular serotype (e.g. K25) and by an arbitrary code given to their PFGE profile (e.g. PN1). Some strains agglutinated multiple antisera and are designated, e.g. K81/33/35.

<sup>b</sup>Strains encountered in both survey years.

### Basis of variation in susceptibility to piperacillin/tazobactam within strains

Additional studies sought explanations for variation in susceptibility to piperacillin/tazobactam within strains. Investigation centred on the 52 representatives of the K25/PN1 strain from the 1994 survey (Table 1) and on six pairs of isolates from the 1997/8 study. One of these latter pairs (pair 1) also comprised organisms belonging to the K25/PN1 strain, which continued to be isolated at two centres in the more recent survey.

Members of the K25/PN1 strain were variously collected in Bordeaux, Clermont-Ferrand and Ghent during the 1994 survey, and were consistently found to carry a β-lactamase gene with the PCR-SSCP profile of $\text{bla}_{\text{SHV}}$, also to have the $\pi 7.8$ band characteristic of this enzyme. This β-lactamase identification was confirmed by gene sequencing for one representative, and is consistent with many other reports for this epidemic strain, which is long-established and widely distributed in France.6,11,12 Among the 20 representatives from Clermont-Ferrand, 15 were susceptible to piperacillin + tazobactam at 16 + 4 mg/L, whereas five had a low-level resistance, with MICs of 32–64 + 4 mg/L. PCR indicated that these latter five organisms had $\text{bla}_{\text{TEM}}$ genes as well as $\text{bla}_{\text{SHV}}$, whereas $\text{bla}_{\text{TEM}}$ was not detected in the 15 susceptible representatives. Of the 20 K25/PN1 isolates from Bordeaux, 18 were susceptible to piperacillin + tazobactam 16 + 4 mg/L, whereas two were resistant. Among the 12 representatives from Ghent, eight were susceptible and four were highly resistant, with MICs ≥512 + 4 mg/L. None of the isolates from Ghent or Bordeaux gave a PCR product with primers to
Piperacillin/tazobactam versus ESBL producers

blaTEM and none had other β-lactamases besides SHV-4 (pI 7.8), as revealed by IEF. β-Lactamase quantification was undertaken on 10 representatives of the K25/PN1 strain that lacked blaTEM; three each were from Clermont-Ferrand and Bordeaux, and four from Ghent. These organisms were selected as ranging widely in their susceptibility to piperacillin + tazobactam 4 mg/L (Table 2); one was exceptional in retaining borderline susceptibility to unprotected piperacillin (MIC 16 mg/L), whereas the others were highly resistant. SHV-4 β-lactamase activities ranged between 21 and 97 nmol cefotaxime hydrolysed/min per mg protein, and a relationship was evident between specific activity and the MIC of piperacillin/tazobactam (Table 2). Nevertheless, this relationship was imperfect and the two most resistant isolates (nos 619 and 613, MICs ≥ 1024 + 4 mg/L) had lower β-lactamase-specific activities than no. 266, which was much less resistant (MIC 64 + 4 mg/L).

Outer membrane protein profiles were also examined for these 10 representatives of strain K25/PN1 (Figure 2). All yielded two major bands, with molecular weights of 39 and 40.5 kDa. There was no obvious relationship between the intensity of these bands and the MIC of piperacillin or piperacillin + tazobactam 4 mg/L. Minor protein components were also visible and varied among the isolates, but also without apparent relationship to piperacillin + tazobactam MICs. The representatives with the highest level of resistance to piperacillin + tazobactam 4 mg/L were also the least susceptible to ceftazidime + clavulanate, requiring MICs of 1 to 2 + 2 mg/L, compared with 0.25 + 2 to 0.5 + 2 mg/L for the other representatives, but were not unusually resistant to other antibiotics (Table 2). Most notably, they did not have increased resistance to cefoxitin, which is often a good indicator of impermeability or efflux-type resistance in Klebsiella spp.13,14

Studies on the further six pairs of isolates, which each comprised one susceptible and one resistant isolate of the same PFGE and serotype, revealed no general relationship between the MICs of piperacillin + tazobactam 4 mg/L and any other parameter measured (Table 3). Thus, three pairs (nos 4, 5 and 6 in Table 3) varied in their β-lactamase profiles, but there was no tendency for the more resistant organism within the pair to have more nitrocefin-reactive bands. The resistant isolates in pairs 1, 2, 3 and 4 did have higher β-lactamase-specific activities than their susceptible counterparts, but these differences were modest and the relationships were reversed for pairs 5 and 6, with lower specific activities for the resistant isolates.

To determine whether enzymic susceptibility to inhibition varied within the pairs, I50 values were calculated for tazobactam. These assays were carried out with crude extracts containing multiple enzymes, so the values obtained were comparable only within pairs, not between them. Extracts from the resistant and susceptible isolates of the two pairs (nos 2 and 3) with conserved IEF profiles required virtually identical tazobactam I50 values (Table 3). In the case of pair 4, where the IEF profile was not conserved, the I50 values also differed, but the susceptible isolate required the higher I50. The isolates of pair 5 also had different IEF profiles, but required similar I50 values of tazobactam.

The organisms of pair 1 showed the characteristic outer membrane profile of the K. pneumoniae K25/PN1 strain to which they belonged, with the two major bands separated only very narrowly (Figure 3, cf. Figure 2). Isolates belonging to the remaining five pairs showed the more typical profiles for the species, with major bands at 43 and 39 kDa. Only minor variations in profile were seen within the pairs and none of these was consistently associated with the differences in resistance phenotype.

Table 2. MICs and β-lactamase-specific activities of members of the K25/PN1 strain

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>PIP (mg/L)</th>
<th>PIP/TZB</th>
<th>ATM</th>
<th>CAZ</th>
<th>CAZ/CLA</th>
<th>CRO</th>
<th>FOX</th>
<th>nmol cefotaxime hydrolysed/min per mg protein</th>
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<td>626</td>
<td>Ghent</td>
<td>32</td>
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<td>Ghent</td>
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<td>2</td>
<td>128</td>
<td>8</td>
<td>65</td>
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</table>

ATM, aztreonam; CAZ, ceftazidime; CLA, clavulanate; CRO, ceftriaxone; FOX, cefoxitin; PIP, piperacillin; TZB, tazobactam.
These studies investigated why piperacillin + tazobactam 4 mg/L gives strongly bimodal MIC distributions for ESBL producers. The bimodal distribution was found to be abolished if piperacillin/tazobactam was tested as an 8:1 ratio rather than with a fixed 4 mg/L tazobactam (Figure 1). Moreover, although piperacillin + clavulanate 4 mg/L had a unimodal MIC distribution, a bimodal distribution arose when the clavulanate concentration was reduced to 0.25 mg/L. Both approaches (testing with a fixed ratio rather than fixed inhibitor concentration, and varying a fixed inhibitor concentration) thus led to the same conclusion: that a bimodal distribution depended on the conditions and was not contingent upon...
some ESBLs being susceptible to tazobactam (or other inhibitors) whilst others were resistant. This is in keeping with the observations that all of the many ESBLs that have been tested directly for inhibition by tazobactam (or clavulanate) have proved to be susceptible.15

The most plausible explanation of a bimodal distribution is that it is a titration effect, reflecting the steady-state proportion of enzyme that can be kept in an inactive form by the amount of inhibitor available. Aside from the inhibitor concentration itself, factors likely to co-determine the amount of enzyme that can be titrated into an inactive form include the permeability of the strain, the amount of β-lactamase produced, the rate of enzyme re-synthesis, and the rate of enzyme regeneration by breakdown of inhibitor complexes.

Further investigations studied why some ESBL producers were more resistant to piperacillin/tazobactam than others. The results indicated mechanisms for individual groups of strains, but not a global explanation. Thus, electrofocusing results for isolates from the 1994 survey suggested that the more resistant organisms more often had multiple β-lactamases. Likewise, some (but not all) of the more resistant members of the K25/PN1 strain had TEM as well as SHV-4 β-lactamases. These data were in keeping with those of Rice et al.,16 who associated resistance to piperacillin/tazobactam with the simultaneous expression of TEM and SHV enzymes, but were not supported by the 1997/8 survey, where the prevalence of multiple enzyme types appeared to be as high among the susceptible as the resistant isolates. It should perhaps be added that production of multiple TEM and SHV enzymes does not guarantee resistance to piperacillin/tazobactam; up to five TEM and SHV variants were found in most of a series of K. pneumoniae isolates from Durban, South Africa, yet these were consistently susceptible to piperacillin + tazobactam.9

Variation in quantity of a single β-lactamase is a further and obvious explanation for variation in susceptibility to inhibitor combinations, since it is evidently easier to inhibit a small amount of enzyme activity than a large amount.1 Studies on representatives of the K25/PN1 strain (Table 2) suggested such a pattern, with the MIC of piperacillin + tazobactam 4 mg/L broadly relating to specific activity against cefotaxime. Nevertheless, the variation in piperacillin + tazobactam MIC was ~500-fold, whereas that in β-lactamase specific activity was only five-fold, suggesting that some other factor(s) must also be implicated. Moreover, no clear relationship between β-lactamase-specific activity and piperacillin/tazobactam MIC was seen for a further six pairs of ESBL-producing isolates from the 1997/8 collection. Thus, although some relationships existed between the amount and numbers of β-lactamase(s) and the level of piperacillin + tazobactam resistance for individual strains, these relationships were neither consistent nor universal. Relationships between porin loss and piperacillin/tazobactam resistance were not found, although they have been demonstrated in individual K. pneumoniae isolates by others.17

There remains, critically, the question of whether piperacillin/tazobactam should be used in infections caused by ESBL producers. On the one hand, many producers are susceptible in vitro and the combination has been successfully used in experimental infections caused by susceptible producers.18–20 On the other hand, some ESBL producers are resistant, and failure of empirical piperacillin + tazobactam has been reported in some infections caused by ESBL producers, which were subsequently shown to express in vitro
resistance. In these circumstances the best advice is to be guided by susceptibility results for the individual isolates and to consider piperacillin/tazobactam as a therapeutic option when ESBL producers are demonstrably susceptible, but to be cautious with empirical use in settings where ESBL producers are prevalent, even when these belong to outbreak strains that are generally susceptible.

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References


