Role of ceftazidime dose regimen on the selection of resistant *Enterobacter cloacae* in the intestinal flora of rats treated for an experimental pulmonary infection

W. H. F. Goessens¹*, J. W. Mouton², M. T. ten Kate¹, A. J. Bijl¹, A. Ott¹ and I. A. J. M. Bakker-Woudenberg¹

¹Department of Medical Microbiology & Infectious Diseases, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands; ²Department of Medical Microbiology & Infectious Diseases, Canisius Wilhelmina Ziekenhuis, Nijmegen, The Netherlands

Received 8 September 2006; returned 28 October 2006; revised 6 December 2006; accepted 6 December 2006

**Objectives:** The effect of ceftazidime dosing increments and frequency of dosing on the selection of ceftazidime-resistant *Enterobacter cloacae* in the intestine was studied in rats, during treatment of a pulmonary infection caused by *Klebsiella pneumoniae.*

**Methods:** Rats with pulmonary infection (*n* = 10 per group) received therapy with doses of ceftazidime at 3.1 to 400 mg/kg per day at a frequency of every 6, 12 or 24 h for 18 days, starting 24 h after bacterial inoculation of the lung. Emergence of resistance in intestinal *E. cloacae* was monitored by culturing fresh stool specimens at days 0, 8, 15, 22, 29, 36 and 43 on agar plates with (6.4 mg/L) and without ceftazidime. Pharmacodynamic indices and time within the mutant selection window (MSW) were assessed in infected rats for each regimen. Ceftazidime-resistant *E. cloacae* mutants were characterized by determination of the β-lactamase activity under cefoxitin-induced and non-induced conditions.

**Results:** A reduction of intestinal ceftazidime-susceptible *E. cloacae* was observed and showed a significant correlation with the *fAUC/MIC* at days 8, 15 and 22 and with the *fC* max on days 8, 15, 22, 29 and 36. More rats treated with 12–25 and 50–100 mg/kg per day every 6 h were found colonized with ceftazidime-resistant *E. cloacae* mutants than animals treated every 12 h or every 24 h. The proportion of rats colonized with ceftazidime-resistant *E. cloacae* mutants at days 15, 36 and 43 correlated with the time during which ceftazidime plasma concentrations were within the boundaries of the MSW. Only at day 15 was a correlation demonstrated between the *fC* max and significantly fewer rats colonized with ceftazidime-resistant *E. cloacae.* Ceftazidime-resistant *E. cloacae* mutants (MIC ≥ 128 mg/L) were characterized as stable derepressed mutants.

**Conclusions:** Colonization with stable derepressed ceftazidime-resistant *E. cloacae* mutants particularly occurred when rats were exposed to moderate doses of ceftazidime (12–25 or 50–100 mg/kg per day) administered every 6 h. Emergence of resistance was correlated with time within the MSW.

Keywords: PK/PD indices, mutation prevention concentration, mutant selection window, collateral damage

**Introduction**

In antimicrobial treatment of infections the proper antibiotic, dosing regimen and treatment duration are primarily based upon characteristics of the infecting organism(s), including antibiotic susceptibility profile, and the clinical conditions of the patient.³ Often, due to laboratory delay, clinicians start empirical therapy with broad-spectrum antibiotics. In such cases the likelihood of enhancing selection of antibiotic-resistant subpopulations in the bacterial population causing the infection, or bacteria residing in the endogenous flora, is not always taken into account.

Resistant subpopulations selected from the endogenous flora may affect the patient’s condition. Particularly, in critically ill patients, such resistant organisms selected from the endogenous flora during treatment may lead to invasive infection.²³
Thomas et al., Fish et al. and Muller et al. demonstrated that treatment with β-lactams may result in emergence of resistance, especially in genera with a natural AmpC β-lactamase. The incidence was highest in Pseudomonas aeruginosa followed by Enterobacter and Serratia species. These genera are often isolated from Intensive Care Units as colonizers of wounds and they are known to consist of populations with a mutation frequency towards β-lactam antibiotics of $10^{-6}$ to $10^{-7}$. Exposure of bacterial populations to a β-lactam antibiotic will lead to eradication of the susceptible organisms, and at the same time to the selection of resistant organisms that increase in number during further antibiotic treatment.

Experimental studies demonstrating the side effects of dose increments or frequency of dosing of antibiotics on the selection of resistant microorganisms in the residing endogenous flora have not been performed for β-lactams. Pharmacodynamic and pharmacokinetic indices are studied in relation to the efficacy of an antibiotic treatment, rather than being investigated as a parameter in the selection of resistant subpopulations.

Drlica proposed, and demonstrated for quinolones, the relevance of an antibiotic concentration range, known as the mutant selection window (MSW), within which mutants are preferably selected as opposed to concentrations beyond these boundaries. The upper boundary is defined as the mutant prevention concentration (MPC), while the lower boundary is approximated by the MIC.

Consequently, dosing regimens that result in concentrations within the MSW for an extended period would result in enhancement of mutant selection. However, for classes of agents other than the quinolones, there is less evidence in favour of this hypothesis. In addition, little is known about other pharmacodynamic indices that might play an important role as well.

The primary objective of the present study was to investigate the role of dose and dosing frequency of the β-lactam ceftazidime in the selection of resistant Enterobacter cloacae isolates in the intestinal flora of rats treated for a Klebsiella pneumoniae lung infection to determine the relationship between drug exposure in the serum and emergence of resistance in bacteria residing in the endogenous intestinal flora.

## Materials and methods

### Bacterial strains

*Klebsiella pneumoniae* (ATCC 43816, capsular serotype 2) was used for establishment of the lung infection throughout all experiments. The MIC of ceftazidime for the *K. pneumoniae* and the *E. cloacae* residing in the endogenous intestinal flora was 0.5 mg/L and 0.125 mg/L, respectively, as determined by the macro-broth tube dilution test.

### Animals

Male strain albino RP/AEur/RijHsd albino rats were used in all experiments. Animals 11–15 weeks of age, body weight 250–350 g with specified pathogen-free status were used. The experimental protocols adhered to the rules laid down in the Dutch Council of the European Communities (1986). The study protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus University Medical Center Rotterdam.

### Animal model of unilateral pneumonia

A left-sided unilateral pneumonia was induced as has been described in detail elsewhere. In brief, rats were anaesthetized with fluanisone and fentanyl citrate (Hynnorm) (Janssen, Animal Health, Saunderton, UK), followed by pentobarbital (Nembutal) (Sanofi Santé b.v., Maassluis, The Netherlands). After intubation of the left primary bronchus, a cannula was passed through the tube and the left lung was inoculated with 10⁶ viable *K. pneumoniae* bacteria in the logarithmic phase of growth suspended in 20 μL of PBS. After bacterial inoculation, the narcotic antagonist naloxone hydrochloride (Narcan) (Bristol-Myers Squibb, Woerden, The Netherlands) was injected. Inoculation of the lung resulted in an acute unilateral pneumonia. Untreated rats developed septicaemia and pleuritis, and eventually died from day 3 after bacterial inoculation. Death of all untreated rats had occurred by day 8. Rats were housed individually with free access to water and SRMA chow (Hope Farms b.v., Woerden, The Netherlands).

### Antimicrobial treatment

Treatment was always started 24 h after inoculation of the left lung with *K. pneumoniae*. At that time the bacterial numbers in the left lung had increased 10³-fold up to log₁₀ cfu 9.8 (range, 9.1–9.9). Ceftazidime (GlaxoSmithKline, Zeist, The Netherlands) was administered intramuscularly in a volume of 0.1 mL once a day (every 24 h), two times daily (every 12 h) or four times daily (every 6 h) for 18 days. From 10 to 12 rats were included per treatment group. Ceftazidime doses varied, ranging from 3.1 to 400 mg/kg per day, by 2-fold increases.

### Determination of therapeutic efficacy

Therapeutic efficacy was assessed by the animal survival rate. Rats were monitored for survival daily until day 43 after bacterial inoculation. Post-mortem cultures of the left lung and blood from succumbing rats were performed to check for the presence of *K. pneumoniae* only, as well as for determination of their susceptibility to ceftazidime.

### Serum ceftazidime concentrations

Serum ceftazidime levels were determined after intramuscular injections of the agent at various doses. Blood specimens for antibiotic assay were obtained by orbital puncture under isoflurane anaesthesia. Determination of ceftazidime levels and the details with regard to the correlation coefficient and the coefficient of variation has been previously described.

### Isolation of susceptible and resistant *E. cloacae* in faeces of ceftazidime-treated infected rats

The numbers of susceptible and resistant *E. cloacae* mutants in the intestine were determined by culturing fresh stool specimens.
of animals which survived the infection with *K. pneumoniae* at day 0, 8, 15, 22, 29, 36 and 43. At the indicated time intervals fresh faeces (250–500 mg) were sampled and suspended in 1 mL of saline. The suspension was vortexed and diluted 10-fold in saline. Volumes of 200 μL were plated on MacConkey agar containing 8 mg/L amoxicillin/clavulanic acid and on MacConkey agar containing 35 mg/L vancomycin and 6.4 mg/L ceftazidime. After incubation for 48 h colony counts were performed and converted into colony-forming units per gram of faeces.

**Characterization of ceftazidime-resistant *E. cloacae* mutants**

The stability of ceftazidime-resistant *E. cloacae* mutants from each treatment group was characterized in various ways and compared to the initial ceftazidime-susceptible *E. cloacae* isolates. Colonies of *E. cloacae* mutants (one colony per rat per treatment group) were randomly selected from the plates containing ceftazidime and vancomycin (the latter suppresses overgrowth of enterococci).

**Stability**

After multiple (at least five) subcultures in antibiotic-free broth, MICs were tested by using an inoculum of $10^5$ cfu/mL.

**Antibiotic susceptibility profiles**

These were determined by VITEK 2 (BioMérieux, Marcy l’Etoile, France) following the manufacturer’s instructions.

**β-Lactamase activity**

This was determined under cefoxitin-induced and non-induced conditions. Overnight cultures of resistant *E. cloacae* mutants in brain heart infusion broth (Becton Dickinson, Sparks, MD, USA) were diluted 1 : 20 in 5 mL of fresh broth and incubated while shaking at 37°C. A duplicate set of cultures was similarly inoculated, and after 2 h of incubation, 10 mg of cefoxitin per litre (0.2 times the MIC) was added to induce β-lactamase production. After 4 h, bacteria were harvested from both induced and non-induced cultures by centrifugation at 1500 g for 10 min at 4°C. The pellets were resuspended in 500 μL of 50 mM Tris–HCl (pH 7.4), and β-lactamase was extracted by the lysozyme-based method outlined by Paterson *et al.* The protein concentrations of the enzyme extractions were measured using a dye-binding assay (Bio-Rad, Marnes la Coquette, France). Enzyme activity was measured by the technique of O’Callaghan, as described previously, with slight modifications. Briefly, 100 μL of 1 mM nitrocefin solution was added to 100 μL of the enzyme extract and after 10 min nitrocefin destruction was measured at a wavelength of 490 nm (Bio-Rad model 550 microplate reader). Enzyme activity was defined as micromoles of nitrocefin destroyed per minute per millilitre of added enzyme extract and calculated as follows: Δ extinction/minute × 0.1 μmol of nitrocefin × 10 × dilution factor of enzyme extract. Enzyme specific activity was expressed as μmol/min per mg of protein.

**Macrophrestriction analysis of DNA by PFGE**

Colonies were suspended in 100 μL of EET buffer (100 mM EDTA/10 mM EGTA/10 mM Tris–HCl). Bacterial suspensions were embedded in agarose plugs by mixing with equal volumes of 1% agarose solution; 200 μL plugs were prepared. Plugs were incubated overnight with protease K (1 mg/mL) and SDS (1%) and were subsequently washed with buffer (10 mM Tris/1 mM EDTA) six times for 30 min each time. Subsequently, the plugs were stabilized twice for 30 min each time in 120 μL of a buffer (SuRe cut H buffer; Boehringer, Mannheim, Germany) and were digested with 40 U of XbaI during overnight incubation at 37°C. Plugs were washed four times, for 30 min each time with 0.5× Tris/borate/EDTA. The DNA present in the agarose plugs was analysed on a 1% agarose gel by PFGE (CHEF DR III) at 14°C and 6 V/cm in 0.5× Tris/borate/EDTA by using pulse times of 5–35 s at an angle of 120° (~60° + 60°) for 20 h. The agarose gel was stained afterwards in ethidium bromide (5 mg/L) and photographed under ultraviolet illumination.

**Determination of mutant prevention concentration (MPC)**

The MPC for *E. cloacae* was determined by the method described by Lu *et al.* An overnight culture of *E. cloacae* was concentrated to $10^{10}$ cfu/mL by centrifugation for 10 min at 3000 g. Subsequently, 1 mL of this suspension was applied to each of five plates (200 μL per plate) containing various concentrations of ceftazidime. Preliminary determinations using 2-fold dilutions of drug provided an approximate value of the MPC. This was followed by a second more precise determination of the MPC by using plates containing linear drug concentration increments. Agar plates were incubated for 18 h at 37°C. The MPC was defined as the lowest drug concentration that prevented bacterial colony formation from a culture containing $10^{10}$ bacteria. Colonies growing at the highest antibiotic concentration were subcultured on antibiotic-free agar plates to test the stability of the mutants.

**Pharmacokinetic/pharmacodynamic (PK/PD) and statistical analysis**

The PK/PD indices were determined for the unbound fraction of ceftazidime using Miclab 2.32 (Medimatics, Maastricht, The Netherlands). Values were assumed to follow steady-state conditions. Data analysis was performed using Graphpad Prism version 3.0 (Graph Pad Software, Inc., San Diego, CA, USA).

The number of ceftazidime-susceptible *E. cloacae* isolates was determined by subtraction of the overall number of *E. cloacae* colony-forming units (cfu) minus the number of ceftazidime-resistant *E. cloacae* per gram of faeces. Subsequently, log transformation of the susceptible and resistant *E. cloacae* cfu numbers was performed to enable comparison within and between groups. Thus, geometric means could be calculated as well as differences between groups tested with the two-sample Student’s *t*-test. Changes in colonizing numbers of *E. cloacae* between sampling days were tested for significance with the paired-samples *t*-test on log-transformed cfu number. The influence of dosing (dosage, administration frequency) and pharmacodynamic indices ($\Delta$AUC/MIC, $\Delta T > MIC$, MPC, ΔMSW) on log cfu (susceptible or resistant *E. cloacae*) was assessed with linear regression analysis, with log cfu as outcome, and the
logarithm of dosing, or the pharmacodynamic indices, as independent variables in the regression model. The association between proportions of rats colonized with ceftazidime-resistant *E. cloacae* and treatment regimens was analysed with the χ² test.

**Results**

**Therapeutic efficacy of different ceftazidime treatment regimens on *K. pneumoniae* lung infection**

Untreated infected animals died between day 3 and day 7 after bacterial inoculation of the lung. As previously studied, the effective dosages of ceftazidime approaching 50% and 90% survival of the animals determined at day 22 and day 43 were 12.5 and 50 mg/kg per day for all dosing frequencies, every 24 h, 12 h and 6 h, respectively. It was concluded that rat survival depended on the total daily dosage, and was independent of the dosing frequency. The PK/PD indices determining the animal survival rate are described and discussed in a previous paper.14

**Composition of the intestinal aerobic flora of rats before and after ceftazidime treatment**

Before assessment of the effect of ceftazidime treatment, the aerobic intestinal flora of uninfected animals were cultured, quantified and identified. The intestinal flora consisted mainly of *E. cloacae*, coagulase-negative staphylococci, *Streptococcus* spp., *Enterococcus* spp. and *Lactobacillus* spp. All animals harboured the same *E. cloacae* strain (see below). The numbers of *E. cloacae* cfu before treatment ranged from $2 \times 10^5$ to $3 \times 10^7$ per gram of faeces. In one pilot experiment, ceftazidime treatment of rats (*n* = 5) at 200 mg/kg per day, administered every 12 h for 18 days, revealed no significant change in the number of Gram-positive organisms.

**Effect of ceftazidime treatment regimens on the number of intestinal ceftazidime-susceptible *E. cloacae* during and after treatment**

The influence of ceftazidime treatment regimens on intestinal ceftazidime-susceptible *E. cloacae* is shown in Figure 1. In all three dosing frequencies, depending on the dose administered, a decrease was observed in the number of ceftazidime-susceptible *E. cloacae* isolates during treatment followed by a gradual increase to pre-treatment levels after termination of therapy. Recolonization of the intestine with *Enterobacter cloacae* after cessation of therapy in rats treated every 24 h with a relatively high dose of ceftazidime did not occur in the observation period of 43 days. However, variability between animals was large, reflected by considerable standard deviations. A statistically significant reduction in the number of ceftazidime-susceptible *E. cloacae* isolates was observed in all dosing regimens and frequencies from day 0 to day 8 except for the 3–6 mg/kg per day administered 4 times a day. After termination of treatment the number of susceptible *E. cloacae* isolates increased. The increase from day 22 to 29 was statistically significant in all groups except those treated once a day with 3–6 and 12–25 mg/kg per day.

**Relationship between pharmacodynamic indices and reduction of intestinal ceftazidime-susceptible *E. cloacae***

The relationship between pharmacodynamic indices and alteration in the number of ceftazidime-susceptible *E. cloacae* during treatment was analysed by linear regression. The weighted association between $f\text{AUC/MIC}$ ratios, $fT_{\text{MIC}}$, $C_{\text{max}}$ and log cfu of ceftazidime-susceptible *E. cloacae* was calculated for each sampling moment. The calculated associations for day 8 are depicted in Figure 2(a–c). In Figure 2(a), a scatterplot and corresponding regression line are shown, demonstrating the association calculated for day 8. According to the regression
In vivo selection of ceftazidime-resistant Enterobacter cloacae

Effect of ceftazidime treatment regimens on selection of intestinal ceftazidime-resistant E. cloacae mutants during and after treatment

The effect of ceftazidime treatment on the selection of ceftazidime-resistant E. cloacae mutants in the intestine was investigated by determination of the proportion of rats with ceftazidime-resistant E. cloacae at the indicated time intervals during and after treatment. Figure 3 illustrates that ceftazidime-resistant E. cloacae were selected during the ceftazidime treatment period, in a number of rats. Significantly more rats treated every 6 h compared with 12-hourly or once daily treated rats were found to be colonized with ceftazidime-resistant E. cloacae mutants during one or more sampling days, particularly in the 12–25 mg/kg per day groups (P < 0.001, χ²), but also in the 50–100 mg/kg per day groups (P = 0.02).

Absolute numbers of intestinal ceftazidime-resistant E. cloacae mutants

As the proportion of rats colonized with ceftazidime-resistant E. cloacae mutants appeared associated with dosing frequency, we also determined the absolute number of ceftazidime-resistant mutants per gram of faeces. Irrespective of treatment group, in all rats with resistant E. cloacae mutants, a steady increase in number of ceftazidime-resistant mutants was observed starting on day 8, with mean numbers of 10³ cfu/g, increasing to 10⁵ cfu/g at day 22. This level of ceftazidime-resistant numbers of E. cloacae was maintained until day 43 (data not shown).

Relation between pharmacodynamic indices and emergence of intestinal ceftazidime-resistant E. cloacae mutants

No correlation could be found between the proportion of rats colonized with ceftazidime-resistant E. cloacae mutants and the fAUC/MIC ratio as well as fT>MIC for E. cloacae (data not shown). However, a significant negative correlation was demonstrated for the fCmax on day 15. A high fCmax resulted in significantly fewer rats colonized with ceftazidime-resistant E. cloacae, as shown in Figure 3(a).

Figure 2. Degree of E. cloacae suppression by various ceftazidime (CAZ) pharmacodynamic indices. The relationship on day 8 between fAUC/MIC ratio (a), T \text{MIC} (b) or fCmax (c) and \log_{10} cfu counts of CAZ-susceptible E. cloacae. The line resembles the outcome of linear weighted regression. A regression coefficient of 0 results in a horizontal line and implies absence of correlation. Linear regression coefficients (with 95% confidence intervals) of the association between fAUC/MIC ratio (d) or fCmax (e) and \log_{10} cfu counts of CAZ-susceptible E. cloacae are shown for all sampling days. Bars with confidence intervals excluding 0 represent a significant correlation, e.g. significant negative correlations on days 8, 15 and 22, when higher fAUC/MIC ratio or fCmax were associated with lower \log_{10} cfu counts.

In conclusion, the reduction in the susceptible E. cloacae population is correlated with fAUC/MIC and C_{\text{max}}, which is in contradiction with other papers demonstrating a correlation with fT>\text{MIC}.¹⁸
Relation of MPC to emergence of intestinal ceftazidime-resistant E. cloacae mutants

The E. cloacae isolates from rats before treatment had an MPC of 16 mg/L and an MIC of 0.125 mg/L. With these parameters the time that ceftazidime-serum levels fell into the mutation selection window (tMSW) was determined. In Figure 4 the MSWs are depicted for the three dosing frequencies at a daily dose of 50 mg/kg per day administered every 24 h, 12 h or 6 h a day. When administered every 6 h, the time in the MSW of the regimen 400 mg/kg per day is paradoxically less than the 200 and 100 mg/kg per day regimens. This effect is due to accumulation of the drug, thereby increasing trough levels above the MIC and hence decreasing time in the MSW.

The proportion of rats colonized with ceftazidime-resistant E. cloacae mutants correlated with the time that serum concentrations of ceftazidime were within the boundaries of the MSW (Figure 6). This correlation was significant at days 15, 36 and 43 (P values 0.028, 0.008 and 0.008, respectively).

Phenotypic characterization of intestinal ceftazidime-resistant E. cloacae mutants

To determine the antibiotic susceptibility profile of the E. cloacae mutants growing from ceftazidime-containing agar plates, one colony per treatment group was randomly picked. MICs of ceftazidime were determined before and after mutants had been subcultured successively for five times in ceftazidime-free broth. The results of the initially ceftazidime-susceptible E. cloacae isolates and the ceftazidime-resistant E. cloacae mutants are shown in Table 1.

Besides determining MICs of ceftazidime, susceptibilities to other antibiotics were also determined by the VITEK 2 system,
as shown in Table 2. The mutants resistant to ceftazidime appeared also to be resistant to amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam and cefoxitin. This change in antibiotic susceptibility pattern is presumed to be the result of a derepression of the class I chromosomal $\beta$-lactamase. Direct proof for this presumption was obtained by experiments in which we quantified the $\beta$-lactamase of the initially ceftazidime-susceptible *E. cloacae* and the ceftazidime-resistant *E. cloacae* mutants. The $\beta$-lactamase enzyme was isolated under non-induced and induced conditions using sub-MIC concentrations of cefoxitin. From the data in Table 3 it is concluded for the ceftazidime-resistant *E. cloacae* mutants that the reduced susceptibility for ceftazidime is the result of increased activity of the $\beta$-lactamase enzyme following a mutation leading to derepression of the class I $\beta$-lactamase gene.

**Genotypic characterization of intestinal ceftazidime-resistant *E. cloacae* mutants**

From all treatment groups, *E. cloacae* colonies growing on ceftazidime-containing plates were randomly picked and analysed by PFGE, together with the initial ceftazidime-susceptible *E. cloacae* isolate. From the PFGE patterns it was concluded that all ceftazidime-resistant mutants had the same restriction patterns as the initial isolate. Clearly, the mutants originated from the initial population and were not the result of selection of a different minor *E. cloacae* subpopulation.

**Discussion**

Emergence of antimicrobial resistance during antibiotic treatment is an increasing problem. Resistance can occur in the infecting target organism(s) and/or in the colonizing normal flora. During treatment of infection the host’s normal flora is unintentionally exposed to antibiotics, which may lead to secondary colonization by potentially pathogenic, often multiple antibiotic-resistant, organisms.

In our animal model of severe infection we demonstrated that treatment with third generation cephalosporins in the therapeutic range had a profound effect on intestinal colonization with *E. cloacae*. We noticed a strong reduction of the ceftazidime-susceptible bacteria and, in some animals, selection of

---

**Table 1.** MICs for *Enterobacter cloacae* isolates obtained before and after therapy

<table>
<thead>
<tr>
<th>Ceftazidime dosage (mg/kg per day)</th>
<th>Initial isolate MIC (mg/L)$^b$</th>
<th>Mutant$^a$</th>
<th>MIC (mg/L)$^c$</th>
<th>MIC (mg/L)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.06</td>
<td>128</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>50</td>
<td>0.125</td>
<td>128</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>25</td>
<td>0.125</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>12.5</td>
<td>0.125</td>
<td>64</td>
<td>64</td>
<td>128</td>
</tr>
</tbody>
</table>

$^a$Four representative ceftazidime (CAZ)-resistant *E. cloacae* mutants were obtained at day 43 from rats treated every 6 h with CAZ at various dose schedules for 18 days.

$^b$MICs for *E. cloacae* isolates obtained before CAZ treatment.

$^c$MICs for *E. cloacae* mutants obtained directly after CAZ treatment.

$^d$MICs for *E. cloacae* mutants obtained after CAZ treatment and being successively subcultured (5×) in antibiotic-free broth.
pre-existing ceftazidime-resistant mutants. These mutants originated from the ceftazidime-susceptible E. cloacae population initially present in the intestine.

We also showed that particularly frequent administration of relatively small doses resulted in emergence of resistance. The PK/PD index that predicted resistance selection was the period of time that ceftazidime plasma levels fell within the boundaries of the MSW. This is in accordance with the theoretical concept of the MSW, but in contradiction with the in vivo studies of Campion et al.

We observed that during treatment, the intestinal ceftazidime-susceptible E. cloacae population reduced by four to six log10 steps, and after termination of therapy this population gradually returned to normal levels. However, in rats treated once a day with high doses, the return to normal levels took longer. This reduction in numbers of ceftazidime-susceptible E. cloacae is probably not harmful, in terms of causing a short period of imbalance in the microflora which normally acts as a barrier against colonization by potentially pathogenic microorganisms and against overgrowth of microorganisms that are already present.

More important, however, is the enrichment of ceftazidime-resistant E. cloacae mutants. This occurred more often in rats treated every 6 h with relatively low doses than in rats treated with any dose once daily. So, a once daily dose frequency minimizes ‘collateral damage’ of the therapeutic agent by preventing the selection of ceftazidime-resistant E. cloacae mutants in the intestine. This observation correlates with the concept of the MSW as advocated by Drlica et al. and others. In our animal model the number of rats that became colonized with ceftazidime-resistant E. cloacae mutants correlated strongly with the time that ceftazidime plasma concentrations were inside the MSW for E. cloacae, as shown in Figure 6. Etienne et al. found similar results with quinolones, using a model of experimental pneumococcal infection in rabbits. They found that serum concentrations above the MPC prevented the selection of fluoroquinolone-resistant Streptococcus pneumoniae in the lungs. These data, in combination with the results of Cui et al. and the results of the present study, support the concept that resistant mutants are selectively enriched when antibiotic concentrations fall within the boundaries of the MSW. For determination of the MSW, the MIC was applied instead of the MIC99 (Lu et al.) as both parameters gave the same results. For determination of the time that ceftazidime levels fell within the boundaries of the MSW, ceftazidime plasma levels were applied. Ceftazidime levels determined in the intestine would be more appropriate, however, due to binding of ceftazidime to faecal material, no trace amounts of ceftazidime could be detected.

Other investigators demonstrated that the duration of time that antibiotic plasma levels exceeded 32 times the MIC was also important in preventing the emergence of resistance. Whereas the results of the present study fit in the model of the MSW, part of the present data are also in agreement with the concept that selection of resistant organisms can be prevented by achieving high peak plasma levels exceeding the MIC. In the concept of equal daily dosing, higher peak plasma levels are obtained in the once-daily dosing regimen, which again is correlated with a short time in the MSW, but also with a considerable time above the MPC value. Etienne et al. and Cui et al. demonstrated in their in vivo studies that the time above the MPC is an important parameter in preventing selection of resistant mutants. Olofsson et al. defined an AUC/MIC ratio of ≥22 to prevent in vivo selection of ciprofloxacin-resistant Escherichia coli. The objective of the present study was to define the parameters involved in selection of resistance instead of investigation of the parameters involved in prevention of resistance. Furthermore, it is demonstrated in the present animal model, as well as in patients that being colonized with Enterobacteriaceae possessing a class I β-lactamase, that antimicrobial therapy does not necessarily lead to selection of resistant subpopulations. Factors such as the absolute numbers of intestinal microorganisms and variation in individual plasma levels of cephalosporins probably have substantial impact on the selection enrichment of mutants during therapy. Especially, the absolute number of resistance-prone microorganisms is critical. For the susceptible E. cloacae population present in the intestine the mutant frequency at 16× the MIC is 5×10−6. In cases where the susceptible E. cloacae population exceeds numbers of 109–1010 it is relatively easy to select resistant mutants, as 200 bacteria out of 109 are resistant. However, in our model the number of E. cloacae in the intestine is 108−109 per gram of faeces, indicating that the chance of encountering and selecting a resistant E. cloacae is less obvious. So, in infections with relatively large numbers of microorganisms, selection of resistant mutants is ‘easy’ to achieve. Furthermore, it is demonstrated that once selection of resistant organisms has occurred during antibiotic exposure, the mutants remain present in considerable numbers up to 25 days after termination of antibiotic therapy. These findings demonstrate that the ‘collateral effect’

<table>
<thead>
<tr>
<th>Ceftazidime dosage (mg/kg per day)</th>
<th>non-induced</th>
<th>induced</th>
<th>non-induced</th>
<th>induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.08</td>
<td>0.6</td>
<td>13.4</td>
<td>11.0</td>
</tr>
<tr>
<td>50</td>
<td>0.03</td>
<td>0.6</td>
<td>12.0</td>
<td>12.4</td>
</tr>
<tr>
<td>25</td>
<td>0.1</td>
<td>1.0</td>
<td>12.0</td>
<td>10.8</td>
</tr>
<tr>
<td>12.5</td>
<td>0.4</td>
<td>0.5</td>
<td>11.4</td>
<td>12.7</td>
</tr>
</tbody>
</table>

*Four representative ceftazidime (CAZ)-resistant E. cloacae mutants were obtained at day 43 from rats treated every 24 h with CAZ at various dose schedules for 18 days.
In vivo selection of ceftazidime-resistant Enterobacter cloacae

i.e. selection and enrichment of resistant mutants, is not reversible. The ceftazidime-resistant population is not replaced by the initial susceptible population.

Despite the strong mutant selecting capacity of broad-spectrum cephalosporins on coliforms, clinicians sometimes have no other choice of antibiotics when treating critically ill patients with mixed infections. Our animal model supports the concept that the use of a high dose administered once daily should be recommended to reduce ‘collateral damage’ of antibiotic to the endogenous flora, i.e. reduce the risk of selecting resistant subpopulations, thereby circumventing long-term problems in these particular patients. Our previous study, has shown that the fAUC/MIC ratio was the PK/PD index that best correlated with therapeutic efficacy of ceftazidime administered for 18 days. Combining these findings on treatment efficacy and the outcome of the present study, the optimal regimen of a β-lactam antibiotic for both infection treatment and preventing selection of resistant endogenous subpopulations is administration once-daily at high dose. Future studies focused on other Enterobacteriaceae are needed to generalize the present observations.

Acknowledgements

We wish to thank S. Voermans and J. Laurijssens for their technical assistance. This work was supported by the AstraZeneca/ESCMID Unrestricted Research Grant 2005 awarded to I.A.J.M. Bakker-Woudenberg, W.H.F Goessens and J.W. Mouton and was presented, in part, at the 16th European Congress of Clinical Microbiology and Infectious Diseases, Nice, France, 2006.

Transparency declarations

None to declare.

References


Goessens et al.


