Effect of Dosing and Dosing Frequency on the Efficacy of Cefoxizime and the Emergence of Ceftizoxime Resistance During the Early Development of Bacteroides fragilis /Enterobacter cloacae Mixed Infection Murine Abscesses.

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Running title: Pharmacodynamic indices and cephalosporin resistance

Keywords Pharmacodynamic index, animal model, mutant prevention concentration, cephalosporins, resistance

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Summary

The efficacy of β-lactams is thought to be dependent on the time that the unbound concentrations exceed the MIC (fT>MIC). However, the pharmacodynamic index (PDI) that correlates best to selection of resistance is not yet clear. The selection of ceftizoxime (CZX)-resistant Enterobacter cloacae during the development of murine mixed-infection abscesses was studied to determine the PDI important to the emergence of resistance and the PDI value needed for prevention of resistance. Studies were carried out 24 h after inoculation with Bacteroides fragilis ATCC 23745 and E. cloacae 22491. CZX 6 - 1536 mg/kg/day given q2h, q4h, q6h or q8h, was started 30 min before inoculation and continued for 24h. Resistant mutants were isolated to determine mutant frequencies (MF). The fT>MIC varied from 9 - 98% for E. cloacae, fCmax was 0.6 – 578 mg/l and fAUC 1.9 – 553 mg.h/l. The fAUC/MIC best explained the in vivo efficacy. CZX-resistant B. fragilis and E. cloacae mutants were isolated from untreated controls at a MF of 10^{-5}-10^{-7}. The MF of resistant B. fragilis did not increase during therapy. Selection of resistant E. cloacae at a MF of 10^{-1}-10^{-2} was related to the fT>MIC and the fAUC/MIC following an inverse U-shape. However, the fAUC/MIC was the stronger driver of resistance. The highest MF were 0.7 – 0.9 at a fAUC/MICs of approximately 250. We conclude that the fAUC/MIC is the PDI that correlated best to the in vivo efficacy of CZX and probably also to the emergence of E. cloacae resistant mutants. A fAUC/MIC of 1000 was needed to prevent emergence of this resistance.
Introduction

The emergence of resistant bacterial strains during beta-lactam therapy is associated with the intensity of beta-lactam use (13, 21, 33) and with prolonged antibiotic exposure (15). Until now, antibiotic dosing regimens used to treat infections have been based primarily on pharmacodynamic indices (PDI) (19) that describe optimal efficacy and/or prevention of toxicity. However, the increasing problem of emergence of resistance under the influence of antibiotic selection necessitates the need to determine the PDI that correlates best to the development of this resistance.

To date, there have been very few studies that have investigated the PDI important to the emergence of beta-lactam resistance. While a 24 h AUC/MIC ratio of $\geq 100$ (10, 34, 37) or a peak/MIC ratio of 8 to 10 (3, 8) may significantly reduce the emergence of resistant subpopulations during treatment with fluoroquinolones and aminoglycosides, it has been reported that these indices do not appear to play an important role in the suppression of resistance during beta-lactam therapy (34). However, recent findings have indicated the importance of a high dose, short-elimination half-life regimen to minimise the emergence of cephalosporin-resistant

*Escherichia coli* (25).

In a recent study, we found that the preferential selection of beta-lactam-resistant mutants during the treatment of mixed-infection abscesses was dependent not only on the type of beta-lactam used for therapy but also on the antibiotic doses employed. Ceftizoxime (CZX)-resistant mutants of *Enterobacter cloacae* were selected within 24 h of treatment with lower doses of this cephalosporin but was not found with higher doses of the antibiotic. Since a single dosing frequency (q2h) was used for these studies, it was not possible to distinguish between the $fT>MIC$ and other PDIs describing efficacy and emergence of resistance (31). In the present investigation, we have extended our previous study to include different dosing regimens to determine the PDI important to the emergence of resistant mutants and to establish the PDI value needed for prevention of this resistance.
Materials and Methods

**Antibiotics and media.** Ceftizoxime (Cefizox ®) (CZX) was supplied by Fujisawa Holland B.V. (Houten, The Netherlands). Wilkens Chalgren (WC) broth, WC agar, Eosine Methylene Blue (EM) agar, Brain Heart Infusion broth (BHI) and DST agar were all supplied by Unipath Ltd (Haarlem, The Netherlands).

**Bacterial strains.** *Bacteroides fragilis* ATCC 23745 and a clinical isolate *Enterobacter cloacae* 22491 were used. The MICs of CZX for these strains were 1 and 0.25 µg/ml respectively (31). Overnight cultures were obtained by inoculating 30 ml volumes of WC broth with 0.1 ml of standardized frozen bacterial suspensions (29) and incubating aerobically (*E. cloacae*) or anaerobically (*B. fragilis*) at 37 °C for 18 h.

**Determination of mutant prevention concentration (MPC).** The MPC of *E. cloacae* 22491 was determined by the method as described by Lu et al. (17). An overnight culture of *E. cloacae* 22491 was concentrated to $10^{10}$ CFU/ml by centrifugation during 10 min. at 3000 g. Subsequently, 1 ml of this suspension was applied to 5 plates (200 µl per plate) containing various concentrations of CZX. 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 concentrations 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 sub-cultured on antibiotic free agar plates to test the stability of the mutants.

**Animals.** Female specified pathogen free (SPF) BALB/c mice (IFFA Credo, l’Arbresle, France) 12-18 weeks and weighing 20-25 g were used throughout the study. The caecal contents of male SPF Swiss mice (Broekman Institute B.V., The Netherlands) were used for the production of autoclaved caecal contents (ACC) (30). All animals received water and food *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee of the Erasmus University, Rotterdam, The Netherlands.

**Mouse model.** The subcutaneous abscess model described previously (29) was employed. Briefly, inocula were prepared by diluting overnight cultures of *B. fragilis* and *E. cloacae* 22491 in WC broth which were then mixed together with ACC in a volume ratio of 1:1:2. Final inocula contained $10^7$ cfu *B. fragilis*, $10^7$ cfu *E. cloacae* and 4 mg ACC (dry weight) in a total volume of 0.25 ml. Mice were injected
subcutaneously on both flanks. Abscesses were allowed to develop for 24 h. Mice were then killed by CO$_2$ asphyxiation and the abscesses were dissected, weighed and homogenized in 1 ml PBS for 10 seconds (Pro 200, B.V. Centraal Magazijn, Abcoude, The Netherlands). Total bacterial counts were determined on the resulting suspensions by making duplicate serial 10-fold dilutions in PBS and plating 20 µl of each dilution onto EM agar (E. cloacae) or WC agar containing 100 mg/l gentamicin (B. fragilis). Plates were incubated at 37 °C aerobically for 24 h (EM agar) or anaerobically for 48 h (WC agar). Bacterial counts were expressed as the mean ± SEM log$_{10}$ cfu/abscess of 4 abscesses per treatment group. The lower threshold limit was 1.7 log$_{10}$ cfu/abscess.

**Pharmacokinetic studies.** Single dose pharmacokinetic studies with 100 mg/kg CZX were performed on groups of 3 mice 4 days after inoculation. Blood was removed by orbital puncture 10, 20, 30, 45, 60, 120, 240 and 360 min after drug administration and serum samples stored at – 80°C until they were assayed. Multiple dose studies were carried out on mice treated with 36 doses of 100 mg/kg CZX q2h. Antibiotic concentrations were determined in duplicate by the agar diffusion bioassay outlined previously (29) using E. coli 62 as test strain. Pharmacokinetic parameters were determined using the MW/Pharm computer program package (Mediware, Groningen, The Netherlands) with a one-compartment open model. The obtained parameters were used to simulate various dosing regimens and determine pharmacokinetic properties of each regimen, such as fT>MIC, fCmax and fAUC, allowing for a protein binding of 13% of the antibiotic in mouse serum (22). As each dosing regimen in the mouse model was started 30 min before inoculation (see below), the exposure time of the bacteria to the antibiotic was 23.5h. Therefore, to accurately determine the pharmacokinetic properties of each regimen in this model, the fT>MIC and fAUC values for all dosing regimens for the first 30 min were calculated separately and subsequently subtracted from the 24h values. The fCmax value was not corrected assuming only differences in absolute values but not in values relative to each other of the various dosing regimens.

**Antibiotic treatment and emergence of resistance in early abscess development.** Groups of 2 mice were treated with subcutaneous daily doses of 6 to 1536 mg/kg/day CZX. Two-fold increasing doses were given q2h and 4-fold increasing doses were given q4h and q6h. Daily doses of 384 and 1536 mg/kg/day were given q8h. Treatment was started 30 min before inoculation with B. fragilis/ E. cloacae and continued for 24 h. Resistant E. cloacae mutants were isolated from
treated and untreated abscesses on WC agar plates containing 16 x MIC of CZX that had been incubated aerobically for 48 h at 37 °C. To isolate *B. fragilis* mutants, the WC agar plates, in addition to the CZX concentration mentioned above, also contained 100 mg/l gentamicin that inhibited the growth of the *E. cloacae* mutants. These plates were incubated anaerobically at 37 °C for 72 h. (Control experiments showed that gentamicin had no synergistic or antagonistic effect on the number of CZX-resistant *B. fragilis* mutants isolated on plates containing 16 x MIC CZX). The mutant frequency (MF) was expressed as the ratio of the number of resistant colonies isolated per total bacterial counts found on antibiotic-free agar.

**Pharmacodynamic analysis.** The PDIs that correlated best to efficacy and emergence of resistance were determined by visual inspection and non-linear regression using GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, California, USA). The Emax model with variable slope was used to fit to the $f_T>MIC$, $f_{AUC/MIC}$, $f_{C_{max}}/MIC$ and the total bacterial counts while a Gaussian type function was used to fit to resistance data.

**Results**

**Pharmacokinetics.** After single and multiple doses of 100 mg/kg, the half-life of CZX was 0.23 h and 0.22 h respectively indicating that there was no change in the pharmacokinetics of this cephalosporin during therapy. The pk data of CZX described in the literature by Murakawa et al. (22)(20 mg/kg) are similar to our findings (100 mg/kg); the half-life was 0.26 h. The model parameters used to calculate pharmacokinetic and pharmacodynamic parameters for each dosing regimen resulted in $f_{AUC_{0.5-24h}}$ of 1.9-553 mg.h/l, and peak concentrations ($f_{C_{max}}$) from 0.6 to 578 mg/L. The $f_T>MIC$s ranged from 9 to 98% of the dosing interval for *E. cloacae*.

**Effect of CZX dosing regimens on the total bacterial populations of abscesses.** The total bacterial counts of *B. fragilis* and *E. cloacae* in untreated abscesses 23.5 h after inoculation were $8.0 \pm 0.1$ and $8.8 \pm 0.1 \log_{10} \text{cfu/abscess}$ respectively. When treated with CZX during the development of these abscesses, there was no bacterial killing with all dosing regimens < 96 mg/kg/d (Fig. 1A). With dosing regimens ≥ 96 mg/kg/d, the killing of both strains was very similar reaching a maximum log reduction of $\geq 5 \log_{10} \text{cfu/abscess}$ compared to untreated abscesses. The efficacy of CZX against the *E. cloacae* and *B. fragilis* strains was reduced as the frequency of the dosing regimens decreased (Fig. 1A).

**Effect of CZX dosing regimens on mutant frequency.**
Figure 2 shows the effect of increasing daily doses on both the total population as well as the resistant (MIC >16 µg/ml) population of *E. cloacae* for various dosing intervals. With all regimens up to 96 mg/kg/d, the numbers of *E. cloacae* resistant mutants increased. The most striking effect was observed for the q2h regimens, where a maximum increase of 3.8 log_{10} cfu/abscess was reached when the total population comprised almost exclusively resistant cells. If the same daily doses were given less frequently, i.e. q4h, significantly fewer mutant strains were selected (maximum increase 2 log_{10} cfu/abscess) and higher CZX doses were required before this selection occurred. The q6h dosing regimen did not preferentially select CZX-resistant *E. cloacae* mutants. Emergence of resistant *B. fragilis* strains was not observed. Figure 1B shows the mutant frequency with respect to total daily dose. In the absence of ceftizoxime, CZX-resistant mutants of *B. fragilis* and *E. cloacae* were isolated from the total bacterial population at respective frequencies of 0 to 10^{-7} and 10^{-5} to 10^{-7}. CZX treatment had no effect on the frequency that CZX-resistant mutants of *B. fragilis* were isolated from the abscesses. However, with all dosing regimens > 24 mg/kg/d, the average mutant frequency of CZX-resistant *E. cloacae* strains increased during therapy to between 0.01 and 0.9. At the highest total daily doses, the mutant frequencies decreased for the q2h and the q4h regimens.

**Pharmacodynamic analysis.** The regression analyses of the PDIs, *f*T > MIC, *f*AUC/MIC and *f*C_{max}/MIC, in relation to CZX efficacy and emergence of resistance of *E. cloacae* are presented in Figures 3 and 4 respectively. The *f*AUC/MIC was the PDI that correlated best to the in vivo efficacy of CZX against the total bacterial populations of the abscesses (Figure 3). Surprisingly, the relationship between *f*T > MIC and in vivo efficacy was not as good as for *f*AUC/MIC; indeed no fit could be obtained. In contrast, the frequency that CZX–resistant *E. cloacae* mutants were isolated from the abscesses was related to both the *f*T > MIC and *f*AUC/MIC. (It should be noted that, because the values of the first 30 min were taken out of the calculations, the peak concentration in the *f*C_{max}/MIC graph corresponds to a first compartment model at t = 0, that is after the first dose and before inoculation, therefore the *f*C_{max}/MIC peak extends beyond the graph). (Figure 4). Bell-shaped curves fitted the relationship between the MF and the *f*T > MIC and the *f*AUC/MIC with similar R^2 values of 0.624 and 0.597 respectively. However, the points that result in the fit with the *f*T > MIC curve are almost completely driven by one (q2h) dosing regimen with no clear relationship of the points from the other dosing regimens while the points that result in the fit of the *f*AUC/MIC curve are derived from all regimens.
with only one outlier from the highest dosing of 1536 mg/kg. This indicates that the $f_{\text{AUC/MIC}}$ is probably the PDI that drives the selection of resistant *E. cloacae* in this model and that a $f_{\text{AUC/MIC}}$ value of more than approximately 1000 would be required to prevent emergence of this resistance.

**Relation of MPC to the number of ceftizoxime resistant *E. cloacae***.

*E. cloacae* 22491 had a MPC of 384 µg/ml and a MIC$_{99}$ of 0.125 µg/ml. Thus, the MPC was relatively high. Because of this, the time within the mutant selection window (tMSW) was more or less equal to the $T_{> \text{MIC}}$ and no distinction could be made between the effects of tMSW and $T_{> \text{MIC}}$. The conclusions with respect to the $f_{T_{> \text{MIC}}}$ therefore also apply to tMSW.

**Discussion**

It is reported that the $f_{T_{> \text{MIC}}}$ is the most important PDI to explain the efficacy of beta-lactam antibiotics against *Enterobacteriaceae* and, for maximum efficacy, cephalosporin serum concentrations should be above the MIC for 60% to 70% of the dosing interval (4). However, the present study has demonstrated that, in this mixed infection abscess model, the $f_{\text{AUC/MIC}}$ was the PDI that correlated best to the *in vivo* efficacy of CZX and probably also to the emergence of *E. cloacae* resistant mutants. Presumably, this was due to the high antibiotic concentrations required to kill the large numbers of resistant mutants present in the abscesses and also the reason efficacy was not related to $f_{T_{> \text{MIC}}}$.

In our abscess model, the selective pressure of CZX was correlated to both the $f_{\text{AUC/MIC}}$ and $f_{T_{> \text{MIC}}}$, although the $f_{\text{AUC/MIC}}$ seems to explain the emergence of resistance better over all dosing regimens. The Gaussian distribution was used to fit the MF data. This function was chosen because the MIC distributions are log normally distributed (16, 35). Thus, if the probability of emergence of resistance is to be related to the MIC, and independent of the MIC, it follows that the distribution of the MF is distributed in a similar fashion. An $f_{\text{AUC/MIC}}$ of approximately 1000 would be required to suppress the selection of CZX-resistant *E. cloacae* mutants. This value is much higher than the value that is needed for optimal efficacy. If $f_{T_{> \text{MIC}}}$ is regarded as a predictor too, the data seem to suggest values to nearly 100% are required to prevent the emergence of resistance and this value is also relatively high (4). In a study looking at the exposure required to prevent resistance to ceftriaxone in *Enterobacter* in the neutropenic thigh model, Berkhout al. found that $T_{> 32}$ times the MIC was important for efficacy, probably because that prevented emergence of the
resistant clone (2). Thus, the pk/pd relationship found here indicate that higher values of the pk/pd index are needed for prevention of emergence of resistance compared to those needed for efficacy in this experimental setting.

In earlier studies, a mutant selection window (MSW), when the risk of mutant selection is greatest, has been defined by some authors as the drug concentration range that extends from the MIC to the mutant prevention concentration (MPC). In these investigations, the preferential selection of antimicrobial resistance was prevented when drug concentrations fell outside this MSW and it is postulated that a window of opportunity is created in which antibiotic levels are sufficient to kill the susceptible population yet allowed the increase of the resistant population. This MSW hypothesis has been used to explain the results of several in vitro studies using a 3rd generation cephalosporin (23) and quinolones (7, 10, 36) against both gram positive and gram negative bacterial strains. For quinolones, this was achieved with dosing regimens producing AUC_{24h}/MICs of > 100 (10, 34, 37). In the present study, no distinction could be made between the effects of fT>MIC and tMSW because of the relative high MPC values of the E. cloacae strain. The relationships between these two PDI’s and the MF are therefore similar, and the conclusions with respect to the fT>MIC also apply to the tMSW. That is, a reasonable correlation is found with the MF using a Gaussian distribution but with the same limitations in the interpretation.

Previous studies using in vitro models have investigated the importance of dosing regimens (23), T > MIC (24) and fAUC (25) to the selection of beta-lactam-resistant mutants. The results of our study concur with those reported in other in vivo models (1, 28). Bakker-Woudenberg et al (1) have demonstrated that the PDI that correlated best to the therapeutic effect of ceftazidime in an immuunocompetent rat model of Klebsiella pneumoniae lung infection was dependent on the duration of treatment and/or the parameter of outcome. Concomitantly, the reduction of susceptible gut commensal E. cloacae during this treatment was significantly correlated to the fAUC/MIC (12). Importantly, this abscess model, as well as the rat model described both immunocompetent animals and this may be part of the explanation that the effect is better correlated to AUC than to T>MIC.

The elucidation of the relationship between PDIs and emergence of resistance during therapy facilitates the design of more effective dosing regimens. The results presented here could be relevant to the clinical situation in which a 3rd generation cephalosporin would be used to treat an infection resulting from complications following abdominal surgery, such as leakage of an intestinal suture (5, 6).
case, antibiotic treatment has to be started prior to re-operation. *Enterobacter* strains can be involved in such infections (9, 32). We acknowledge that the PDI values reported here were obtained from experiments using a single *E. cloacae* – cephalosporin combination and that this preferential selection may not be common to all *Enterobacter/Enterobacteriaceae* strains (26, 31). Indeed, none of the dosing regimens increased the frequency that CZX-resistant strains of *B. fragilis* were isolated from the abscesses (31). Nevertheless, 3rd generation cephalosporins are still widely used for empirical treatment (5) and surgical prophylaxis (11) and they are more likely to select resistant strains of *Enterobacteriaceae* than any other beta-lactam (27, 31).

The findings of this study also challenge the practice of administering beta-lactams in small frequent doses or by continuous infusion (20) as an appropriate procedure for treatment with certain cephalosporins. In our quest to find dosing regimens that will prevent the emergence of resistance, perhaps higher doses given less frequently may be more beneficial (12, 25). Alternatively, the use of antibiotics with enhanced activity against resistant mutants, for example cefepime (18, 26, 31), may be more advantageous in the treatment of infections involving ‘high risk’ strains.

Although not an objective of this investigation, the duration of therapy may also be a contributing factor to the emergence of antimicrobial resistance. Indeed, this appears to be an important aspect in the selection of quinolone resistance (10) and in the pharyngeal colonization of beta-lactam resistant strains (14). However, at infection sites where bacterial numbers are high, the selection of resistance may occur more readily. We found that CZX-resistant *E. cloacae* strains could be selected within 24h of treatment. In previous studies involving in vitro kinetic models, the preferential selection of beta-lactam resistant strains occurred within 6h (24) and 14 h (23) of antibiotic exposure while prolonged exposure increased the risk of selecting mutants with an additional mutation (23).

In conclusion, this is a useful animal model to investigate the PDIs important to the emergence of antimicrobial resistance during therapy. The fAUC/MIC is probably the best PDI that explains the emergence of CZX-resistant *E. cloacae* during the early development of mixed infection abscesses.

**Acknowledgements.**
This study was financially supported by an unrestricted grant of Wyeth Lederle, Hoofddorp, The Netherlands and was presented, in part, at the 2003 43rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Illinois, USA.
References


Legends

Fig. 1. In vivo effect of increasing dosings of CZX on the total bacterial counts of *B. fragilis* ATCC 23745 and *E. cloacae* 22491 isolated from mixed-infection abscesses on antibiotic-free media (A), and on the frequency of CZX-resistant *B. fragilis* ATCC 23745 and *E. cloacae* 22491 isolated from mixed-infection abscesses on media containing 16 x MICs CZX (B). The mutant frequency is expressed as the ratio of the number of resistant colonies isolated per total bacterial numbers isolated on antibiotic-free agar as a function of total daily doses. Dosing regimens of 6 to 1536 mg/kg/day were started 30 min before inoculation and continued for 24h. Daily doses were divided into different dosing regimens given q2h, q4h, q6h or q8h.

Fig. 2 The effect of CZX dosing frequency on the total and CZX-resistant populations of *E. cloacae* 22491 isolated from mixed-infection abscesses. Dosing regimens of 6 to 1536 mg/kg/day were started 30 min before inoculation and continued for 24h. Daily doses were divided into different dosing regimens given q2h, q4h or q6h. The total bacterial population was isolated on antibiotic-free media and the resistant population on media containing 16 x MICs CZX.

Fig. 3 Relationship between the $f_T > MIC$, $f_{AUC}/MIC$ and $f_{C_{max}}/MIC$ of CZX and the total bacterial counts of *E. cloacae* 22491 isolated from mixed-infection abscesses 24h after treatment. Lines indicate the best model fit for the Emax model.

Fig. 4 Relationship between the $f_T > MIC$, $f_{AUC}/MIC$ and $f_{C_{max}}/MIC$ of CZX and the mutant frequency of CZX-resistant *E. cloacae* 22491 isolated from mixed-infection abscesses 24h after treatment.
Fig. 1

A.  

B. fragilis

E. cloacae

Total daily dose (mg/kg)

Total bacterial population (Log_{10} cfu/abscess)

B. fragilis

E. cloacae

Total daily dose (mg/kg)

Mutant frequency

Dosing frequency  ○ q2h  ● q4h  □ q6h  ▲ q8h
Fig. 2

![Graph showing log cfu/abscess vs. total daily dose (mg/kg) for different dosing regimens (q2h, q4h, q6h). The graph compares total population vs. resistant population.](http://aac.asm.org/)
Fig. 3.

Dosing frequency

- q2h
- q4h
- q6h
- q8h
Fig. 4.