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Effect of Dosing and Dosing Frequency on the Efficacy of Ceftizoxime and the Emergence of Ceftizoxime Resistance during the Early Development of Murine Abscesses Caused by *Bacteroides fragilis* and *Enterobacter cloacae* Mixed Infection▽

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The efficacy of β-lactams is thought to be dependent on the time that the unbound concentrations exceed the MIC (*fT*>MIC). However, the pharmacokinetic/pharmacodynamic index (PDI) that correlates best to the selection of resistance is not yet clear. The selection of ceftizoxime (CZX)-resistant *Enterobacter cloacae* mutant strains during the development of murine mixed-infection abscesses was studied to determine the PDI that is important for the emergence of resistance and the PDI value needed for the prevention of resistance. Studies were carried out 24 h after inoculation with *Bacteroides fragilis* ATCC 23745 and *E. cloacae* 22491. Six to 1,536 mg of CZX/kg of body weight/day given every 2 h (q2h), q4h, q6h, or q8h was started 30 min before inoculation and continued for 24 h. Resistant mutants were isolated to determine mutant frequencies (MF). The *fT*>MIC varied from 9 to 98% for *E. cloacae*, the peak concentration (unbound fraction) was 0.6 to 578 mg/liter, and the area under the concentration-time curve (unbound fraction) (*f*AUC) was 1.9 to 553 mg · h/liter. The *f*AUC-to-MIC ratio best explained the in vivo efficacy. CZX-resistant *B. fragilis* and *E. cloacae* mutants were isolated from untreated controls at an MF of 10^{-5} to 10^{-7}. The MF of resistant *B. fragilis* did not increase during therapy. The selection of resistant *E. cloacae* strains at an MF of 10^{-1} to 10^{-2} was related to the *fT*>MIC and the ratio of *f*AUC to MIC following an inverse U shape. However, the ratio of *f*AUC to MIC was the stronger driver of resistance. The highest MFs were 0.7 to 0.9 at an *f*AUC-to-MIC ratio of approximately 250. We conclude that the ratio of *f*AUC to MIC is the PDI that correlated best to the in vivo efficacy of CZX and probably also to the emergence of resistant *E. cloacae* mutants. An *f*AUC-to-MIC ratio of 1,000 was needed to prevent the emergence of this resistance.

The emergence of resistant bacterial strains during β-lactam therapy is associated with the intensity of β-lactam use (12, 20, 32) and with prolonged antibiotic exposure (14). Until now, antibiotic dosing regimens used to treat infections have been based primarily on the pharmacokinetic/pharmacodynamic index (PDI) (18) that describes the 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 that is important for the emergence of β-lactam resistance. While the ratio of the area under the concentration-time curve (AUC) at 24 h to MIC of ≥100 (10, 33, 36) or a peak-to-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 β-lactam therapy (33). However, recent findings have indicated the importance of a high-dose, short-elimination half-life regimen to minimize the emergence of cephalosporin-resistant *Escherichia coli* strains (25).

In a recent study, we found that the preferential selection of β-lactam-resistant mutants during the treatment of mixed-infection abscesses was dependent not only on the type of β-lactam used for therapy but also on the antibiotic doses employed. Ceftizoxime (CZX) (Cefizox)-resistant mutants of *Enterobacter cloacae* were selected within 24 h of treatment with lower doses of this cephalosporin but were not found with higher doses of the antibiotic. Since a single dosing frequency (every 2 h [q2h]) was used for these studies, it was not possible to distinguish between the time that the unbound concentrations exceed the MIC (*fT*>MIC) and other PDIs describing efficacy and emergence of resistance (30). In the present investigation, we have extended our previous study to include different dosing regimens to determine the PDI important for the emergence of resistant mutants and to establish the PDI value needed for the prevention of this type of resistance.

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MATERIALS AND METHODS

Antibiotics and media. CZX was supplied by Fujisawa Holland B.V. (Houten, The Netherlands). Wilkens Chalgren (WC) broth, WC agar, eosin methylene blue (EM) agar, brain heart infusion broth, 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 (30). Cultures grown overnight were obtained by inoculating 30-ml volumes of WC broth with 0.1 ml of standardized frozen bacterial suspensions (28) and incubating them aerobically (*E. cloacae*) or anaerobically (*B. fragilis*) at 37°C for 18 h.

Determination of MPC. The mutant prevention concentration (MPC) of *E. cloacae* 22491 was determined by use of a method described previously by Lu et al. (16). A culture of *E. cloacae* 22491 grown overnight was concentrated to 10^{10} CFU/ml by centrifugation during 10 min at 3,000 × *g*. Subsequently, 1 ml of this suspension was applied to five plates (200 μl per plate) containing various concentrations of CZX. Preliminary determinations using twofold 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.

Animals. Female specific-pathogen-free BALB/c mice (IFFA Credo, l'Arbresle, France) that were 12 to 18 weeks of age and weighing 20 to 25 g were used throughout the study. The cecal contents of male specific-pathogen-free Swiss mice (Broekman Institute B.V., The Netherlands) were used for the production of autoclaved cecal contents (ACC) (29). All animals received water and food ad libitum. The study was approved by the Institutional Animal Care and Use Committee of Erasmus University, Rotterdam, The Netherlands.

Mouse model. A subcutaneous abscess model described previously (28) was employed. Briefly, inocula were prepared by diluting cultures of *B. fragilis* and *E. cloacae* 22491 grown overnight 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* cells, 10^7 CFU *E. cloacae* cells, and 4 mg (dry weight) ACC 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 phosphate-buffered saline for 10 s (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 phosphate-buffered saline and plating 20 μl of each dilution onto EM agar (*E. cloacae*) or WC agar containing 100 mg/liter 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 $\log_{10}$ CFU/abscess of four abscesses per treatment group ± standard error of the mean. The lower threshold limit was 1.7 $\log_{10}$ CFU/abscess.

Pharmacokinetic studies. Single-dose pharmacokinetic studies with 100 mg of CZX/kg of body weight were performed on groups of three mice 4 days after inoculation. Blood was removed by orbital puncture at 10, 20, 30, 45, 60, 120, 240, and 360 min after drug administration, and serum samples were 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 an agar diffusion bioassay outlined previously (28) using *E. coli* strain 62 as the 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, fC_{max}, and *f*AUC, allowing for a protein binding of 13% of the antibiotic in mouse serum (21). 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 23.5 h. Therefore, to accurately determine the pharmacokinetic properties of each regimen in this model, the $fT>$MIC and *f*AUC values for all dosing regimens for the first 30 min were calculated separately and subsequently subtracted from the 24-h values. The fC_{max} 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 two mice were treated with subcutaneous daily doses of 6 to 1,536 mg/kg/day CZX. Twofold-increasing doses were given q2h, and fourfold increasing doses were given q4h and q6h. Daily doses of 384 and 1,536 mg/kg/day were given q8h. Treatment was started 30 min before inoculation with *B. fragilis* or *E. cloacae* and continued for 24 h. Resistant *E. cloacae* mutants were isolated from treated and untreated abscesses on WC agar plates containing 16× the 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/liter gentamicin, which 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× the MIC of 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 nonlinear regression using GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, CA). The E_{max} model with variable slope was used to fit to the $fT>$MIC, the ratio of *f*AUC to MIC, the ratio of fC_{max} to 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-lives of CZX were 0.23 h and 0.22 h, respectively, indicating that there was no change in the pharmacokinetics of this cephalosporin during therapy. The pharmacokinetic data for CZX described previously by Murakawa et al. (21) (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 from 0.5 to 24 h of 1.9 to 553 mg · h/liter and peak concentrations (fC_{max}) of 0.6 to 578 mg/liter. The $fT>$MICs 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 ± 0.1 and 8.8 ± 0.1 $\log_{10}$ CFU/abscess, respectively. When treated with CZX during the development of these abscesses, there was no bacterial killing with all dosing regimens of <96 mg/kg/day (Fig. 1A). With dosing regimens of ≥96 mg/kg/day, the killings of both strains were very similar, reaching a maximum log reduction of ≥5 $\log_{10}$ 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 and the resistant (MIC > 16 μg/ml) population of *E. cloacae* for various dosing intervals. With all regimens up to 96 mg/kg/day, the numbers of resistant *E. cloacae* 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 of 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

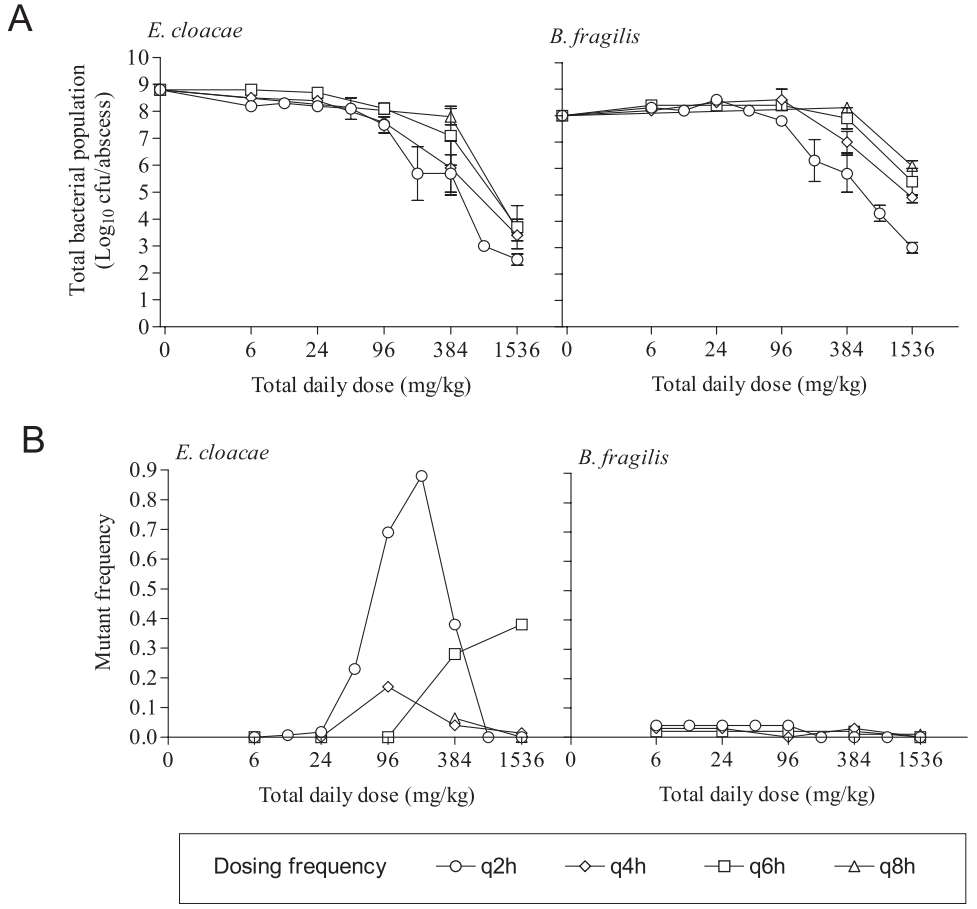

FIG. 1. In vivo effect of increasing dosings of CZX on the total bacterial counts of *B. fragilis* ATCC 23745 and *E. cloacae* 22491 isolated from mixed-infection abscesses on antibiotic-free medium (A) and on the frequency of CZX-resistant *B. fragilis* ATCC 23745 and *E. cloacae* 22491 cells isolated from mixed-infection abscesses on medium containing 16× the MIC of CZX (B). The MF is expressed as the ratio of the number of resistant colonies isolated to total bacterial numbers isolated on antibiotic-free agar as a function of total daily doses. Dosing regimens of 6 to 1,536 mg/kg/day were started 30 min before inoculation and continued for 24 h. Daily doses were divided into different dosing regimens given q2h, q4h, q6h, or q8h.

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 with which CZX-resistant mutants of *B. fragilis* were isolated from the abscesses. However, with all dosing regimens of >24 mg/kg/day, 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, *fT*>MIC, *f*AUC-to-MIC ratio, and fC_{max}-to-MIC ratio in relation to CZX efficacy and emergence of resistance of *E. cloacae* are presented in Fig. 3 and 4, respectively. The ratio of *f*AUC to MIC was the PDI that correlated best to the in vivo efficacy of CZX against the total bacterial populations of the abscesses (Fig. 3). Surprisingly, the relationship between *fT*>MIC and in vivo efficacy was not as good as that for the ratio of *f*AUC to 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 *fT*>MIC and *f*AUC-to-MIC ratio. (It should be noted that because the values of the first 30 min were taken out of the calculations, the peak concentration in the graph of the ratio of fC_{max} to MIC corresponds to a first compartment model at time zero, that is, after the first dose and before inoculation; therefore, the fC_{max}-to-MIC peak extends beyond the graph [Fig. 4].) Bell-shaped curves fitted the relationship between the MF and the *fT*>MIC and the *f*AUC-to-MIC ratio, with similar R^2 values of 0.624 and 0.597, respectively. However, the points that result in the fit with the *fT*>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-to-MIC ratio curve are derived from all regimens, with only one outlier from the highest dosing of 1,536 mg/kg. This indicates that the ratio of *f*AUC to MIC is probably the PDI that drives the selection of resistant *E. cloacae* strains in this model and that a ratio of *f*AUC to MIC of more than approximately 1,000 would be required to prevent the emergence of this resistance.

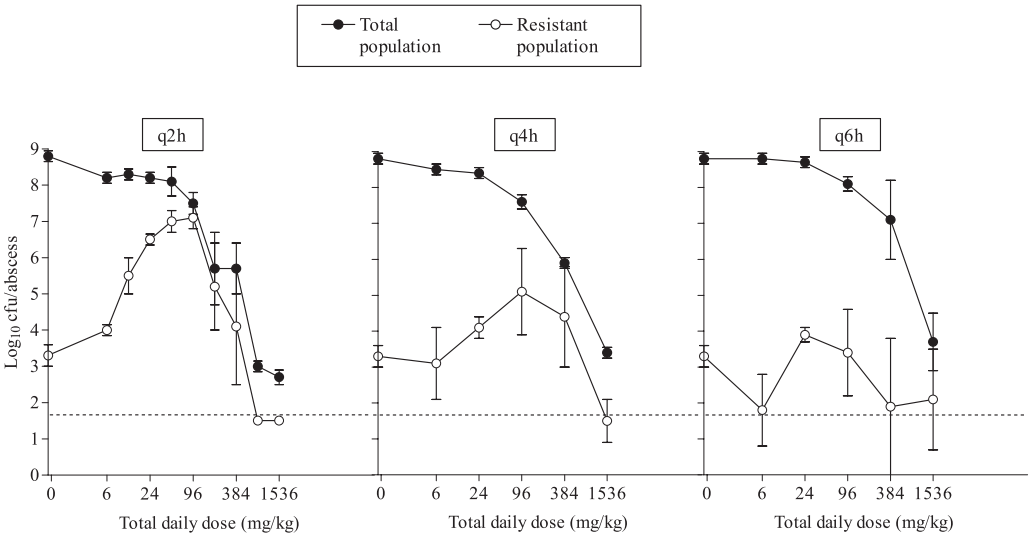

FIG. 2. Effect of CZX dosing frequency on the total and CZX-resistant populations of *E. cloacae* 22491 isolated from mixed-infection abscesses. Dosing regimens of 6 to 1,536 mg/kg/day were started 30 min before inoculation and continued for 24 h. Daily doses were divided into different dosing regimens given q2h, q4h, or q6h. The total bacterial population was isolated on antibiotic-free medium, and the resistant population was isolated on medium containing 16× the MIC of CZX.

Relation of MPC to the number of ceftizoxime-resistant *E. cloacae* strains. *E. cloacae* 22491 had an MPC of 384 μg/ml and an MIC_{99} of 0.125 μg/ml. Thus, the MPC was relatively high. Because of this, the time within the mutant selection window (MSW) (tMSW) was more or less equal to the *T*>MIC, and no distinction could be made between the effects of tMSW and *T*>MIC. The conclusions with respect to the *fT*>MIC therefore also apply to tMSW.

DISCUSSION

It is reported that the *fT*>MIC is the most important PDI to explain the efficacy of β-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*AUC-to-MIC ratio was the PDI that correlated best to the in vivo efficacy of CZX and probably also to the emergence of resistant *E. cloacae* 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 *fT*>MIC.

In our abscess model, the selective pressure of CZX was correlated to both the *f*AUC-to-MIC ratio and *fT*>MIC, although the *f*AUC-to-MIC ratio 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 (15, 34). 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*AUC-to-MIC ratio of approximately 1,000 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 *fT*>MIC is also regarded as a predictor, the data suggest that values of 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 et al. previously found that *T*>32 times the MIC was important for efficacy, probably because that prevented the emergence of the resistant clone (2). Thus, the pharmacokinetic-pharmacodynamic relationship found here indicates that higher values of the pharmacokinetic-pharmacodynamic index are needed for the prevention of emergence of resistance compared to those needed for efficacy in this experimental setting.

In previous studies, an MSW, when the risk of mutant selection is greatest, was defined by some authors as the drug concentration range that extends from the MIC to the MPC. In those 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 allow the increase of the resistant population. This MSW hypothesis has been used to explain the results of several in vitro studies using an expanded-spectrum cephalosporin (22) and quinolones (7, 10, 35) against both gram-positive and gram-negative bacterial strains. For quinolones, this was achieved with dosing regimens producing ratios of the AUC at 24 h to MIC of >100 (10, 33, 36). 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 PDIs 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 investigated the importance of dosing regimens (22), *T*>MIC (23), and *f*AUC

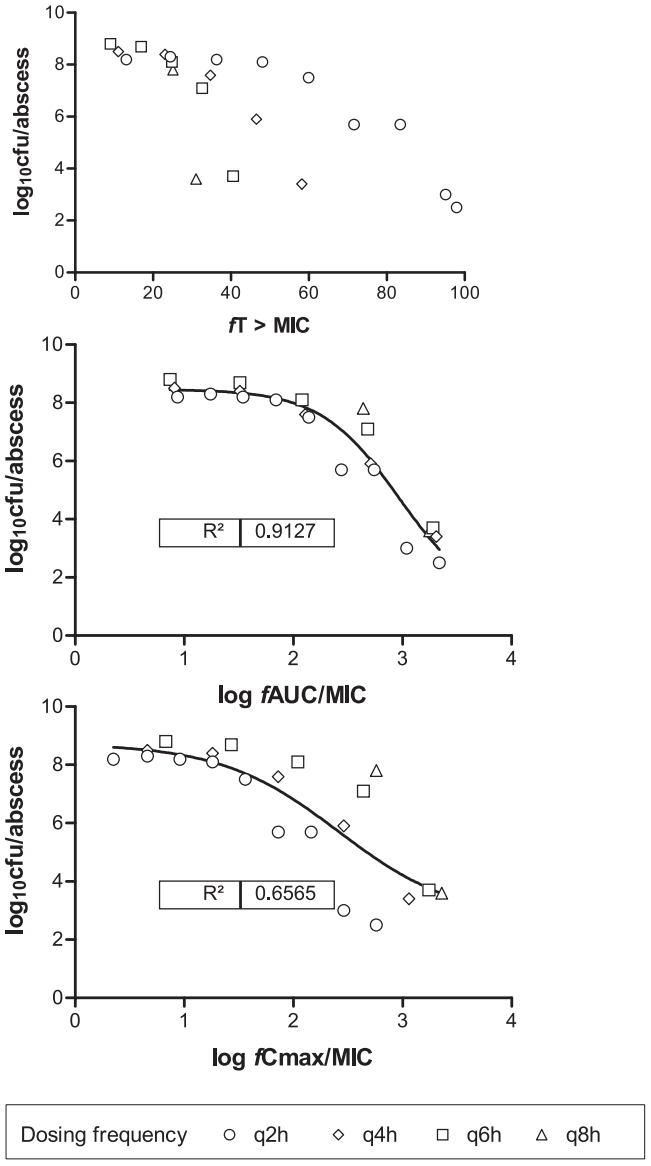

FIG. 3. Relationship between the *fT*>MIC, *f*AUC-to-MIC ratio, and fC_{max}-to-MIC ratio of CZX and the total bacterial counts of *E. cloacae* 22491 isolated from mixed-infection abscesses 24 h after treatment. Lines indicate the best model fit for the E_{max} model.

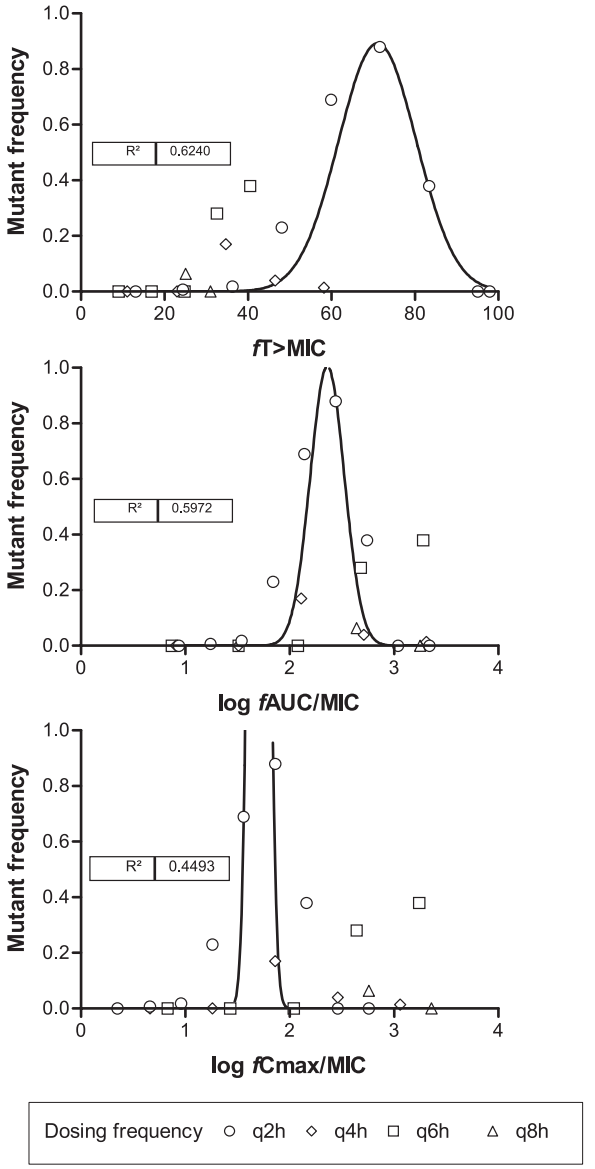

FIG. 4. Relationship between the *fT*>MIC, *f*AUC-to-MIC ratio, and fC_{max}-to-MIC ratio of CZX and the mutant frequency of CZX-resistant *E. cloacae* 22491 cells isolated from mixed-infection abscesses 24 h after treatment.

(24) to the selection of β-lactam-resistant mutants. The results of our study concur with those reported previously for other in vivo models (1, 27). Bakker-Woudenberg et al. (1) previously demonstrated that the PDI that correlated best to the therapeutic effect of ceftazidime in an immunocompetent 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* isolates during this treatment was significantly correlated to the *f*AUC-to-MIC ratio (11a). Importantly, this abscess model as well as the rat model described immunocompetent animals, and this may be part of the explanation of why the effect is better correlated to AUC than to *T*>MIC.

The elucidation of the relationship between PDIs and the 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 an expanded-spectrum cephalosporin would be used to treat an infection resulting from complications following abdominal surgery, such as leakage of an intestinal suture (5, 6). In this case, antibiotic treatment has to be started prior to reoperation. *Enterobacter* strains can be involved in such infections (9, 31). 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 (25, 30). Indeed, none of the dosing regimens increased the frequency with which CZX-resistant strains of *B. fragilis* were

isolated from the abscesses (30). Nevertheless, expanded-spectrum 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 β-lactam (26, 30).

The findings of this study also challenge the practice of administering β-lactams in small frequent doses or by continuous infusion (19) 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 (11a, 24). Alternatively, the use of antibiotics with enhanced activity against resistant mutants, for example, cefepime (17, 25, 30), 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 factor contributing 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 β-lactam-resistant strains (13). 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 24 h of treatment. In previous studies involving in vitro kinetic models, the preferential selection of β-lactam-resistant strains occurred within 6 h (22) and 14 h (24) of antibiotic exposure, while prolonged exposure increased the risk of selecting mutants with an additional mutation (22).

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

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