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A pharmacokinetic–pharmacodynamic model characterizing the emergence of resistant *Escherichia coli* subpopulations during ertapenem exposure

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Objectives: Resistant subpopulations with reduced expression of outer membrane porins have been observed in ESBL-producing *Escherichia coli* during exposure to ertapenem. The aim of this work was to develop a pharmacokinetic–pharmacodynamic (PKPD) model to characterize the emergence of resistant *E. coli* during exposure to ertapenem and to predict bacterial killing following different dosing regimens of ertapenem.

Methods: Data from *in vitro* time–kill experiments were used to develop a mechanism-based PKPD model for three *E. coli* strains: a native strain, an ESBL-producing strain, and an ESBL-producing strain with reduced expression of porins OmpF and OmpC. Each strain was exposed to static ertapenem concentrations (1–512×MIC) for 24 h using starting inocula of ~10^6 and 10^8 cfu/mL.

Results: The developed PKPD model consisted of three bacterial states: susceptible growing, less susceptible non-growing, and non-susceptible non-growing bacteria. A pre-existing bacterial subpopulation was used to describe the emergence of resistance. The PKPD model adequately characterized the data of the three *E. coli* strains investigated. Results from predictions suggest that the conventional dosage (1 g intravenously once daily) might result in regrowth of resistant subpopulations when used to treat infection caused by ESBL-producing strains.

Conclusions: Resistant subpopulations frequently emerged in *E. coli* when exposed to ertapenem, supporting that the time course of emergence of resistance should be taken into consideration when selecting dosing regimens.

Introduction

In the past decade, emerging resistance in *Escherichia coli* related to the production of ESBLs has been reported more frequently worldwide.[1] ESBLs have the capacity to hydrolyse almost all β-lactam antibiotics, with the exception of carbapenems. Therefore, it is crucial to maintain the clinical efficacy of carbapenems as the last resort for the treatment of infections caused by ESBL-producing *E. coli*. Carbapenem resistance in *E. coli*, which is still rare in most settings, is typically mediated by the production of carbapenemases[1] but can result also from combined mechanisms of reduced outer membrane permeability and ESBL or AmpC production.[2–4]

Ertapenem is a broad-spectrum carbapenem that is active against a range of Gram-positive and Gram-negative bacteria, including *E. coli*, as well as some anaerobic bacteria.[5,6] It is indicated for treatment of complicated intra-abdominal, skin and skin-structure and urinary tract infections, as well as community-acquired pneumonia.[6] Ertapenem is unique among carbapenems in that it has a relatively long half-life (~4 h), which is the result of considerable protein binding (>90%).[5,7] In healthy volunteers, the standard dose of 1 g administered as a 30 min infusion results in a maximum total plasma concentration of 155 mg/L at the end of infusion, followed by an exponential decline to <1.2 mg/L at 24 h.[7]

Since ertapenem belong to the β-lactam antibiotics, the pharmacodynamic (PD) driver correlated to antimicrobial efficacy is generally expected to be the time that the unbound drug concentrations remain above the MIC ($fT_{>MIC}$).[8] Data obtained from neutropenic murine thigh infection models have suggested that the PD target for ertapenem to produce 80% of its maximum effect is an $fT_{>MIC}$ of ~33%.[9] The corresponding target required for the bacteriostatic effect is an $fT_{>MIC}$ of ~20%.[9,10] It has also been reported that differences in bacterial species or ESBL production do not influence these target requirements.[9]

Recent results from a study with dynamic *in vitro* time–kill curve experiments demonstrated that subpopulations with reduced susceptibility frequently emerge in ESBL-producing *E. coli*

when exposed to ertapenem.[4] The resistant subpopulations were found to have elevated ertapenem MICs of 0.75–1.5 mg/L, corresponding to intermediate susceptibility or low-level resistance in accordance with the clinical breakpoints defined by EUCAST. Mutations resulting in reduced expression of outer membrane porins OmpF and OmpC, and thus impaired permeability and lower antibiotic concentrations in the periplasmic space, were found in these subpopulations.[4,11] Other studies have also reported the lack of membrane porins in ertapenem-resistant ESBL-producing *E. coli*.[12,13] This indicates the presence of pre-existing porin-deficient subpopulations that can be selected during ertapenem exposure.

Pharmacokinetic–pharmacodynamic (PKPD) models developed based on experiments with frequent bacterial count observations over time are a powerful tool to predict antimicrobial activity and determine effective dosing regimens. To date, several PKPD models have been proposed for describing the emergence of resistant bacterial subpopulations during antibiotic treatment;[14–22] however, few studies have investigated the impact of exposure to ertapenem on the emergence of resistant *E. coli* and no mechanism-based PKPD model has previously been described.

The aim of the present study was to develop a PKPD model to characterize the emergence of resistant *E. coli* during exposure to different static concentrations of ertapenem. This model was subsequently used to predict the antibacterial activity of different dosing regimens by linking the PKPD model to the pharmacokinetic (PK) profile of unbound ertapenem in plasma.

Materials and methods

Bacteria and media

Three previously characterized strains of *E. coli*, including a native non-ESBL-producing strain (DA14781), an ESBL-producing strain (DA14833) and an ESBL-producing strain with reduced expression of outer membrane porins OmpF and OmpC (DA16808), were used.[11,23] All strains originated from *E. coli* MG1655. DA14833 was constructed by conjugation of a plasmid (pUUH239.2), which encodes CTX-M-15, TEM-1 and OXA-1 β-lactamases, into native DA14781.[23] DA16808 is a mutant derivative of DA14833 with an *ompR* R209C mutation that reduces expression of both OmpC and OmpF resulting in increased MIC of ertapenem. This mutant has previously been isolated during both static and dynamic selection with ertapenem.[4,11] The bacteria were stored at −80°C and grown on Mueller–Hinton (MH) agar plates (Difco Laboratories, Detroit, MI, USA). For DA14833 and DA16808, MH agar plates and broth were supplemented with tetracycline 20 mg/L, during pre-culture, to select for the pUUH239.2 plasmid.

Antibiotics and MIC determination

Ertapenem was obtained from Merck and Co. (NJ, USA) and tetracycline hydrochloride was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Stock solutions were prepared by dissolving ertapenem in sterile distilled water and dissolving tetracycline in ethanol to a concentration of 10000 mg/L and then subsequently diluted in MH broth to obtain the desired concentrations (described below). Stock solutions were stored at −20°C and used within 1 week. MIC values were determined by the Etest™ method, according to the manufacturer's description (bioMérieux, Marcy-l'Étoile, France). To investigate changes in the MIC values for *E. coli* during exposure to different concentrations of ertapenem, MICs were measured twice: before adding antibiotic (0 h) and after the end of the experiment (24 h). The MICs for bacteria at 24 h were determined using colonies from the viable count plates (i.e. no additional period of growth in liquid medium). When the number of colonies was insufficient, bacteria were re-streaked on to fresh plates and incubated overnight at 37°C prior to MIC determination. All MIC determinations were performed at least in duplicate.

Time–kill curve experiments

A single bacterial colony was inoculated in 1 mL of MH broth and grown overnight at 37°C. The overnight culture was diluted 100-fold in 15 mL of pre-warmed MH broth, incubated further for 1.5 h to achieve bacteria in the exponential growth phase and then diluted 10-fold in 10 mL of pre-warmed MH to obtain a starting inoculum of $\sim 10^6$ cfu/mL (low-density culture; LD). To investigate the effect of the inoculum size on the PD of ertapenem, the LD culture was allowed to continue growing for ∼3.5 h, in a second set of experiments, resulting in a starting inoculum of $\sim 10^8$ cfu/mL (high-density culture; HD).

For the three strains, ertapenem was added to achieve concentrations corresponding to 1, 1.5, 2, 4, 16, 64, 256 and 512×MIC. A growth control culture without the addition of ertapenem was included in each batch of experiments. Cultures were incubated at 37°C with shaking at 190 rpm. Samples were obtained for viable counts at 0 h (before addition of ertapenem) and at 0.5, 1, 2, 4, 6, 18 and 24 h after ertapenem addition. Aliquots of 100 μL were drawn from each culture, serially diluted with PBS, plated on MH agar plates and further incubated at 37°C. Colonies were counted after 24 h. For samples from cultures containing high antibiotic concentrations (64, 256 and 512×MIC diluted $\leq 1\times10^3$), all samples were centrifuged (4000 **g** for 5 min) and resuspended in PBS prior to plating to minimize antibiotic carry-over effects. The lower limit of detection (LOD) was 10 cfu/mL. All experiments were performed at least in duplicate.

PKPD model building

A previous mechanism-based model developed by Nielsen *et al.*[24] was selected as a basic model structure. This model has one compartment for susceptible growing bacteria (S) and one compartment for non-susceptible resting (persisting) bacteria (R). Bacteria in the growing state (S) transfer to the resting state (R) with a transfer rate constant (k_{SR}), proportional to the total number of bacteria in the system (S+R) in agreement with previous studies of persister formation.[25,26] This model structure does not explain the regrowth of bacteria due to the selection of a resistant subpopulation observed during exposure to low concentrations of ertapenem. Therefore, the basic model was expanded to include two subpopulations: subpopulation 'A', which represents drug-susceptible bacteria, and subpopulation 'B', which represents pre-existing resistant bacteria.

During model development, it was found that the basic model structure including subpopulations fitted the LD data well. However, when LD and HD were pooled and modelled simultaneously, the drug efficacy was underestimated for the HD data for the high concentrations (64, 256 and 512×MIC), as the model predicted that a large proportion of bacteria were rapidly transferred to the resting state. To characterize better the inoculum effect of ertapenem, the resting compartment used in the original model was divided into two compartments, i.e. resting 1 (R1) and resting 2 (R2). Both of these represent non-growing bacteria; bacteria in R1 are drug susceptible, while R2 represents non-susceptible bacteria. The final model structure is illustrated in Figure 1.

The amount of susceptible bacteria (S) was described by first-order rate constants for growth (k_{growth}), natural death (k_{death}), drug effect (k_{drug}) and transfer between compartments (k_{SR1} and k_{R1S}) as follows:

$$\frac{dS}{dt} = k_{growth} \cdot S - k_{death} \cdot S - k_{drug} \cdot S - k_{SR1} \cdot S + k_{R1S} \cdot R1 \qquad (1)$$

Susceptible growing bacteria (S) were adapted and changed into a less susceptible non-growing state (R1) with the transfer rate constant k_{SR1}. k_{SR1} was estimated as being equal to a proportionality

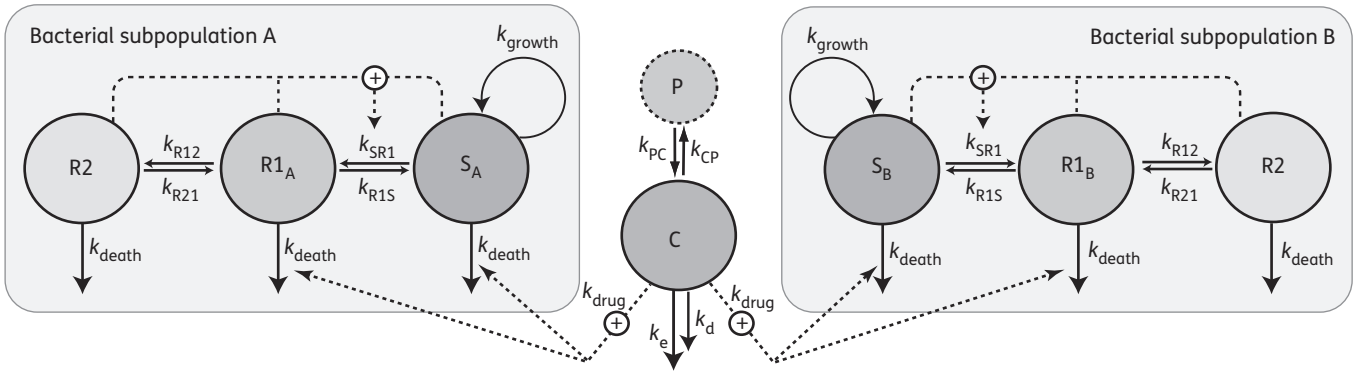

Figure 1. Schematic illustration of the final PKPD model for ertapenem and *E. coli*. S, drug-susceptible growing bacteria; R1, less susceptible non-growing bacteria; R2, non-susceptible non-growing bacteria. The total bacterial population is divided into two subpopulations: susceptible bacteria (A) and pre-existing resistant bacteria (B). k_{growth}, growth rate constant; k_{death}, natural death rate constant; k_{SR1} and k_{R1S}, transfer rate constants between the S and R1 compartments; k_{R12} and k_{R21}, transfer rate constants between the R1 and R2 compartments; k_{drug}, drug effect on bacterial killing. For model development a one-compartment PK model was used to account for drug degradation during the *in vitro* experiments according to a degradation rate constant, k_d. Transfer from S to R1 was assumed to be bacterial density-dependent according to a linear function with a proportionality constant times the total bacterial content in the system (S+R1+R2). For dose predictions, a two-compartment PK model was used with one central compartment (C) and one peripheral compartment (P), with k_{CP} and k_{PC} being transfer rate constants between the C and P compartments and k_e the elimination rate constant.

constant (PC) times the total number of bacteria in the system ($S_A+S_B+R1_A+R1_B+R2_A+R2_B$; TOT) as described in Equation (2).

$$k_{SR1} = PC \times TOT \quad (2)$$

Bacteria in R1 were then transferred to the non-susceptible non-growing state (R2) with rate constant k_{R12}, which was assumed independent of bacterial amount. The amount of bacteria in the R1 and R2 compartments was described by first-order rate constants for natural death (k_{death}), drug effect (k_{drug}) and transfer between compartments (k_{SR1}, k_{R1S}, k_{R12} and k_{R21}) as follows:

$$\frac{dR1}{dt} = k_{SR1} \cdot S - k_{R1S} \cdot R1 - k_{death} \cdot R1 - k_{drug} \cdot R1 - k_{R12} \cdot R1 + k_{R21} \cdot R2 \quad (3)$$

$$\frac{dR2}{dt} = -k_{death} \cdot R2 + k_{R12} \cdot R1 - k_{R21} \cdot R2 \quad (4)$$

Equations (1–4) were applied to describe the change of bacterial concentration over time for both subpopulation A and subpopulation B. For the experimental design used, the bacteria are not expected to resume growth once they have entered the resting state, thus k_{R1S} and k_{R21} were set to 0. The drug effect on bacterial killing (k_{drug}) was described by a sigmoidal E_{max} model (Equation 5).

$$k_{drug} = \frac{E_{max} \cdot C^{\gamma}}{EC_{50}{}^{\gamma} + C^{\gamma}} \quad (5)$$

where E_{max} represents the maximal bacterial killing effect, EC_{50} is the concentration of antibiotic required to produce 50% of E_{max} and γ is the sigmoidal factor. The EC_{50} values for a less susceptible subpopulation were constrained to be equal to or higher than the EC_{50} for the susceptible subpopulations of the native strain. To constrain the parameters the EC_{50} values were estimated using the original EC_{50} (EC_{50,SA_native}) and the fractional increase in EC_{50} (fEC_{50}) as follows:

$$EC_{50,SA\_ompR} = EC_{50,SA\_native} \times (1 + fEC_{50,SA\_ompR}) \quad (6)$$

$$EC_{50,SB} = EC_{50,SA\_native} \times (1 + fEC_{50,SB}) \quad (7)$$

$$EC_{50,R1} = EC_{50,SA\_native} \times (1 + fEC_{50,R1}) \quad (8)$$

Resistance may be associated with a fitness cost resulting in slower growth rates. k_{growth} for resistant bacteria ($k_{growth,ompR}$) was estimated using the k_{growth} of the native strain ($k_{growth,native}$) and a fractional decrease in k_{growth} (fk_{growth}) as follows:

$$k_{growth,ompR} = k_{growth,native} \times (1 - fk_{growth}) \quad (9)$$

E_{max} and γ were estimated separately for each strain, but constrained to be the same for all compartments for one strain. All bacteria were assumed to have the same natural death rate (k_{death}). Initially, all parameters were estimated individually for each strain; thereafter the model structure was simplified by estimating shared parameters across bacterial strains. A shared parameter was selected unless the objective function value (OFV) increased by >10.83 ($P<0.001$ for 1 degree of freedom). The fraction of less susceptible bacteria (f_{R1}) and resting bacteria (f_{R2}) in the starting inocula were fixed to 17000 and 700 bacteria per 10^6 cfu/mL, respectively, based on predictions for a growth control experiment grown until achieving 10^8 cfu/mL (i.e. resampling the preparation of the starting inocula).

Degradation of ertapenem during time–kill experiments was accounted for by including a first-order rate constant for degradation (k_d) of 0.0173 h^{-1} in the model.[4] During model development, other structural models were also investigated, including those describing adaptive resistance,[21,27] as well as effect delay.

Data analysis

The data were analysed using the Laplacian approximation method with ADVAN13 in NONMEM software version 7.3.[28] All bacterial count data were natural log-transformed and simultaneously fitted. The L2 data item was used to handle the correlation between replicate samples.[24] Although all experiments were performed using a standardized protocol, variation in the occurrence of regrowth was observed between replicate experiments. This reflects the stochastic emergence of resistant mutants in the starting cultures of the experiments. To account for this variability, the mixture module in NONMEM was used.[28] The starting inoculum of each experiment was allocated to either a mixture having resistant bacteria (mix 1) or a mixture containing only susceptible bacteria (mix 2). The parameters

estimated in the model were the fraction of resistant bacteria (f_{mutant}) existing in experiments belonging to mix 1 (f_{mutant} was fixed to 0 for experiments belonging to mix 2) and the proportion of experiments belonging to mix 1 (P_{mutant}). To handle observations below the LOD, the M3 method[29] was used.

Model performance was evaluated by visual predictive checks (VPCs)[30] and the OFV. The Xpose program version 4[31] and R 2.15.3 (www.R-project.org) were used for graphical evaluation. In addition to the standard errors reported by NONMEM, the uncertainty of parameter estimates was assessed using the sampling importance resampling method[32] with 10000 initial samples obtained from the covariance matrix.

Predictions of dosing regimens

The final model was linked to a two-compartment PK model proposed by Wiskirchen *et al.*[33] and used to predict unbound ertapenem concentration–time profiles and *E. coli* counts for an adult patient population receiving different ertapenem dosing regimens. This PK model was developed based on data from 12 healthy subjects, receiving 1 g of ertapenem intravenously every 24 h for 3 days and, after a 4 day washout period, having a second treatment period of 3 additional days. The parameters used for predictions were the elimination rate constant (k_e), transfer rate constant from central to peripheral compartments (k_{CP}), transfer rate constant from peripheral to central compartment (k_{PC}) and central volume of distribution (V_C) of 0.38 h^{-1}, 0.43 h^{-1}, 0.44 h^{-1} and 5.04 L, respectively. To account for non-linearity in protein binding, unbound concentrations were predicted by the polynomial equation as follows:

$$y = y_0 + a \cdot x + b \cdot x^2 \quad (10)$$

where y=percentage unbound concentration, y_0=2.4592, a=0.003528, b=0.0001241 and x=total ertapenem concentration.[33] For the predictions, the starting inocula were set to 1×10^6 and 3×10^8 cfu/mL, i.e. approximately the values obtained for the LD and HD starting inocula in the time–kill experiments, with f_{R1}, f_{R2} and f_{mutant} according to the estimated values. Several dosing regimens were explored, including: (i) single dose of 0.5, 0.75, 1, 1.5 or 2 g administered as a 30 min infusion; (ii) total daily dose of 1 g administered with infusion durations of 0.5, 1, 2, 3, 6, 12 or 24 h; and (iii) total daily dose of 1 g administered as a 30 min infusion at dosing intervals of 8, 12 or 24 h (i.e. 0.33 g every 8 h, 0.5 g every 12 h or 1 g every 24 h). The dose predictions were limited to 24 h.

Results

Time–kill curve experiments

A total of 2202 data points derived from 124 time–kill curve experiments were available for analysis. Of these, 67 (3%) were below the LOD. In Figure 2, representative time–kill profiles for the three *E. coli* strains exposed to ertapenem are illustrated. Following exposure to no (control experiments) or low ertapenem concentrations bacteria grew until they approached a maximum bacterial count of ~10^9 cfu/mL. When using high concentrations (64, 256 and 512×MIC) all experiments, both LD and HD, showed >5 $\log_{10}$ bacterial killing at 24 h. For intermediate concentrations (1–4×MIC depending on the strain and inoculum size) a killing effect was initially observed and then regrowth occurred. Discrepancies between repeated experiments were found with regard to the native and ESBL-producing strains when using concentrations of 2 and 4×MIC and 4 and 16×MIC, respectively, in that regrowth was observed in some experiments, but not in others (6 of 12 experiments in total). When compared with the LD experiments, the HD experiments showed a considerable reduction in the bacterial killing effect in all strains when exposed to intermediate concentrations of ertapenem, indicating an inoculum effect of ertapenem against *E. coli*.

Resistant subpopulations are selected during ertapenem exposure

At the start of the experiment (0 h), the MICs for the native strain, the ESBL-producing strain and the ESBL-producing *ompR* mutant strain were 0.004, 0.023 and 0.75 mg/L, respectively (Table 1). As expected, in the experiments where no ertapenem was added, the MICs at 24 h were not significantly altered. The MICs were generally increased following exposure to moderate ertapenem concentration. For example, when LD starting inocula were exposed to a concentration of 2×MIC, the MIC of the endpoint culture was increased 31-fold (0.125 mg/L) for the native strain, 33-fold (0.75 mg/L) for the ESBL-producing strain and 8-fold (6 mg/L) for the ESBL-producing *ompR* mutant strain (Table 1).

For the ESBL-producing strain, there was a tendency that HD starting inocula showed a less pronounced increase in MIC. When discrepancies were observed between replicated experiments, the MICs for experiments showing regrowth were generally higher than for experiments without regrowth. For example, one of three experiments with the ESBL-producing strain exposed to a concentration of 4×MIC resulted in regrowth with an MIC of 0.75 mg/L in the endpoint population, whereas the MIC did not significantly alter (0.047 mg/L) in experiments where no regrowth was observed.

PKPD model

The parameter estimates of the final PKPD model are given in Table 2. As indicated by the VPCs in Figure 3, the final PKPD model well described the bacterial growth and killing and the emergence of resistant *E. coli* subpopulations following exposure to a wide range of ertapenem concentrations for both LD and HD starting inocula. For all the three *E. coli* strains tested, the model allowed the starting inoculum to contain a very small amount of pre-existing resistant bacteria at the start of experiments (f_{mutant}; Table 2). Inclusion of a mixture model to explain variability in regrowth of bacteria observed in some experiments (i.e. 2 and 4×MIC for the native strain and 4 and 16×MIC for the ESBL-producing strain) significantly improved the model fit (ΔOFV −722). For the ESBL-producing *ompR* mutant strain, all experiments were estimated to have a pre-existing bacterial subpopulation at the start (i.e. all experiments allocated to mix 1). For the native and ESBL-producing strains, the probability of having pre-existing resistant bacteria was estimated to be 79% and 84%, respectively.

The *E. coli* growth rate was fast for the native strain and the ESBL-producing strain (k_{growth} of 2.48 h^{-1}) and estimated to be 17% lower for the *ompR* mutant strain (2.06 h^{-1}). No significant differences in OFV and goodness of fit were seen when k_{growth} were estimated separately for the two subpopulations of bacteria (S_A and S_B). The ordinary E_{max} model (γ equal to 1) adequately described the bacterial killing effect of ertapenem and estimation of the sigmoidal factor did not result in a statistically significant improvement. As it was expected that ertapenem affects each strain of *E. coli* differently, the E_{max} and EC_{50} were estimated to be strain-specific. The E_{max} of ertapenem were estimated to be

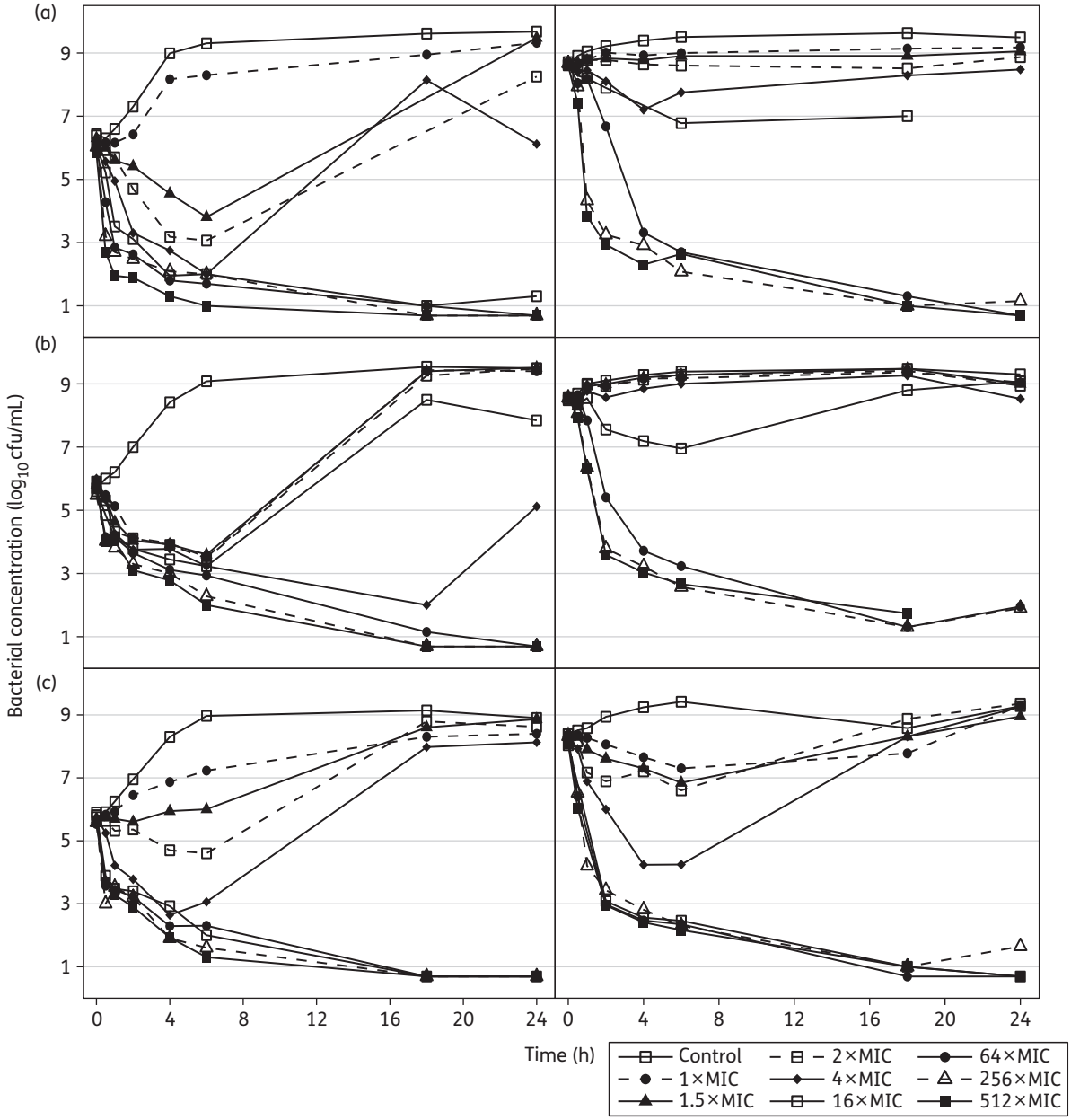

Figure 2. Typical time–kill profiles for *E. coli* following exposure to static ertapenem concentrations from 0 to 512×MIC. Plots show the results for the native strain DA14781 (a), ESBL-producing strain DA14833 (b) and *ompR* R209C ESBL-producing strain DA16808 (c) for LD starting inocula (left) and HD starting inocula (right). Observations <LOD are plotted as 5 cfu/mL.

17.4, 8.6 and 12.8 h^{-1} for the native strain, the ESBL-producing strain and the ESBL-producing *ompR* mutant strain, respectively, indicating a less drastic maximal initial reduction in bacterial count for the ESBL-producing strain (Figure 2).

A shared EC_{50} of the susceptible bacteria ($EC_{50,SA}$) of 0.029 mg/L was estimated for the native and ESBL-producing strains, but a significantly higher $EC_{50,SA}$ was estimated for the ESBL-producing *ompR* mutant strain (6.06 mg/L). The estimated EC_{50} values for pre-existing resistant bacteria ($EC_{50,SB}$) were considerably higher than those of the susceptible bacteria; 0.81, 3.55 and 37.09 mg/L for the native strain, the ESBL-producing strain and the ESBL-producing *ompR* mutant strain, respectively. For the native and ESBL-producing strains, the EC_{50} for bacteria in the R1 compartment ($EC_{50,R1}$), was estimated to be further increased by 41- and 167-fold, respectively (common parameter estimated for subpopulation A and B). For the ESBL-producing *ompR* mutant strain, there was no statistical improvement from estimating separated EC_{50} values for S and R1, indicating the same effect for both compartments (i.e. $fEC_{50,R1}$ fixed to 0).

For experiments with discrepancies with regards to regrowth (2–4×MIC for the native strain and 4–16×MIC for the ESBL-producing strain), the MIC increased 32-fold for the experiments

Table 1. MIC values for the *E. coli* native strain, ESBL-producing strain and ESBL-producing *ompR* mutant strain before and after 24 h of exposure to ertapenem for LD and HD starting inocula

Strain	Starting inoculum	MIC (mg/L)								
		before experiment (0 h)	after experiment (24 h)							
			control	1×MIC	1.5×MIC	2×MIC	4×MIC	16×MIC	64×MIC	256×MIC	512×MIC
Native strain DA14781	LD	0.004	0.008	0.012	0.064	0.047/0.125[a]	0.012/0.094[a]	0.016	0.012	ND	ND
	HD		0.008	0.008	0.008	0.012	0.064	0.064	ND	0.012	ND
ESBL-producing strain DA14833	LD	0.023	0.023	0.19	0.5	0.75	0.047/0.75[a]	0.047/1[a]	ND	ND	ND
	HD		0.023	0.016	0.023	0.047	0.032	0.032	0.032	0.047	ND
ESBL-producing *ompR* R209C mutant strain DA16808	LD	0.75	0.75	6	4	6	4	ND	ND	ND	ND
	HD		0.75	4	6	4	3	ND	ND	2	ND

ND indicates that the MIC was not determined (no detectable bacteria at 24 h).
MIC results are presented as median values.
[a]MICs for experiments without and with regrowth.

allocated to mix 1 and 4-fold for experiments allocated to mix 2, indicating agreement between the PKPD model and changes in ertapenem susceptibility observed in experiments.

Even though it was found that the adaptive resistance model[21] also well described the data, the OFV was slightly higher than for the final model (ΔOFV +21). Addition of an effect delay did not significantly improve the model fit (ΔOFV <4).

Predictions of dosing regimens

Figures 4–6 illustrate the model-predicted concentration–time profiles of ertapenem and bacterial counts for LD and HD cultures of the native strain, the ESBL-producing strain and ESBL-producing *ompR* mutant strain, following different dosage regimens. The predictions in Figure 4 indicate that all investigated ertapenem doses produce a rapid initial killing of both the native and ESBL-producing strains, with >3 $\log_{10}$ cfu/mL reduction within 1 h. As expected, the antimicrobial activity of ertapenem against the ESBL-producing *ompR* mutant strain was much lower compared with the native and ESBL-producing strains. Bacterial concentrations decreased >3 $\log_{10}$ cfu/mL during the first 4 h with doses ≥1 g and was followed by substantial regrowth of resistant bacteria. While the bacterial killing effect is predicted to be sustained for the 24 h period for the native strain, regrowth of pre-existing resistant subpopulations was predicted to occur in a dose-dependent fashion for both ESBL-producing strains.

The bacterial killing effect was not sensitive to the duration of the infusion (given the same total dose) for the native strain (Figure 5b). However, for the ESBL-producing strain and the ESBL-producing *ompR* mutant strain, the bacterial killing effect was predicted to decrease when the same total ertapenem dose (1 g) was administered over a longer period, particularly with the HD starting inoculum.

In Figure 6, the PK profiles of ertapenem and bacterial counts following fractionated drug administrations were predicted for a total dose of 1 g. Results suggests that there is no advantage of giving the total daily dose of 1 g in divided doses at 8 or 12 h intervals. Instead, more frequent administration would decrease bacterial killing activity of ertapenem for both the ESBL-producing strain and the ESBL-producing *ompR* mutant strain because of lower initial drug concentrations. It is predicted that ertapenem would have a lower bacterial killing effect against the ESBL-producing strain following the second and the third dose (<0.5 $\log_{10}$ cfu/mL) compared with >3 $\log_{10}$ cfu/mL reduction after the first dose, due to selection of resistant subpopulations.

When the density of the starting inoculum was increased to 3×10^8 cfu/mL (HD starting culture), more non-growing non-susceptible bacteria are predicted and the overall bacterial counts of all strains were ∼2 $\log_{10}$ cfu/mL higher than the LD starting culture (Figures 4–6). Nevertheless, the magnitudes of bacterial killing of ertapenem remained the same between LD and HD starting cultures for all dosage regimens tested. This indicates that the inoculum size of bacteria would have minimal effect on the killing activity of ertapenem when using doses 0.5–2 g.

Discussion

In the present study, a mechanism-based PKPD model was developed to characterize the emergence of resistant *E. coli* subpopulations following exposure to a wide range of ertapenem

Table 2. Parameter estimates for the final PKPD model

Parameter	Explanation	Parameter estimate (standard error, 95% CI)[a]		
		native strain (DA14781)	ESBL-producing strain (DA14833)	ESBL-producing *ompR* mutant strain (DA16808)
k_{growth} (h^{-1})	growth rate constant	2.48 (0.19, 2.35–2.57)		
fk_{growth}	fractional decrease in k_{growth}			0.17 (0.08, 0.11–0.24)
k_{death} (h^{-1})	natural death rate constant		0.229 (0.023, 0.208–0.245)	
E_{max} (h^{-1})	maximal achievable bacterial killing effect of ertapenem	17.35 (2.01, 14.88–20.67)	8.57 (0.54, 7.75–9.54)	12.83 (0.95, 11.36–14.27)
$EC_{50,SA}$ (mg/L)	ertapenem concentration that produces 50% of E_{max} for susceptible bacteria (subpopulation A)	0.029 (0.005, 0.024–0.036)		
fEC_{50,SA_ompR}	fractional increase in EC_{50} for the ESBL-producing *ompR* mutant strain			208 (44, 160–258)
$fEC_{50,SB}$	fractional increase in EC_{50} for pre-existing resistant bacteria (subpopulation B)	27.04 (4.35, 21.06–29.71)	121.28 (17.60, 95.48–144.67)	5.12 (1.06, 3.82–6.17)
$fEC_{50,R1}$	fractional increase in EC_{50} for less susceptible non-growing bacteria (R1)	40.25 (7.50, 30.51–52.62)	165.80 (49.50, 118.22–247.30)	0 (fix)
PC (h^{-1})	proportionality constant for transfer rate constant from growing to resting state (k_{SR1})	2.72×10^{-9} (6.18×10^{-10}, 2.06×10^{-9}–3.32×10^{-9})		
k_{R12} (h^{-1})	transfer rate from less susceptible non-growing (R1) to non-susceptible non-growing state (R2)	1.00×10^{-5} (2.05×10^{-6}, 4.00×10^{-6}–1.66×10^{-5})		
P_{mutant} (%)	proportion of experiments having pre-existing resistant bacteria (i.e. belonging to mix 1)	79 (10, 60–93)	84 (7, 69–94)	
f_{mutant}	fraction of pre-existing resistant bacteria (subpopulation B) at the start of experiment in experiments belonging to mix 1	4.43×10^{-11} (1.33×10^{-10}, 5.83×10^{-12}–2.23×10^{-10})	3.14×10^{-8} (5.38×10^{-8}, 6.76×10^{-9}–1.11×10^{-7})	3.14×10^{-6} (2.32×10^{-5}, 6.07×10^{-7}–1.08×10^{-5})
$\varepsilon_{residual}$	residual error (on ln scale)		1.28 (0.05, 1.21–1.37)	
$\varepsilon_{replicate}$	replicate error (on ln scale)		0.56 (0.03, 0.53–0.59)	

'fix' refers to the value that was fixed during the estimation.
[a]95% CI values were obtained using the sampling importance resampling method.

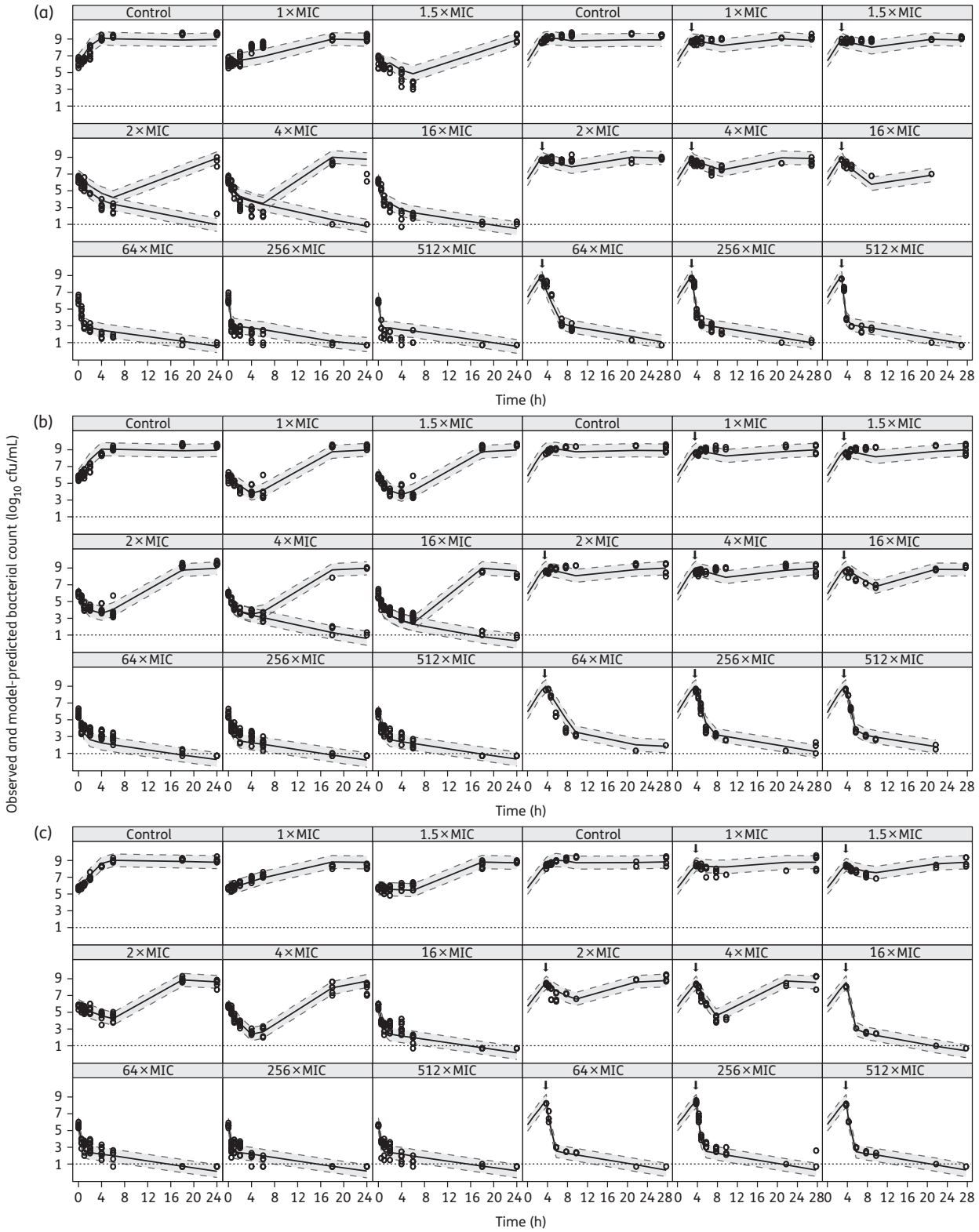

Figure 3. VPCs for the final model with observed bacterial counts (circles), presented with the median (continuous line) and the 80% prediction interval (shaded area between dashed lines) obtained from simulated data ($n=1000$). Results are shown for the native strain DA14781 (a), ESBL-producing strain DA14833 (b) and *ompR* R209C ESBL-producing strain DA16808 (c) for LD starting inocula (left) and HD starting inocula (right). Horizontal dotted lines represent the LOD of 10 cfu/mL. Times of ertapenem administration for HD (~3.5 h after the start of the LD experiments) are indicated with arrows.

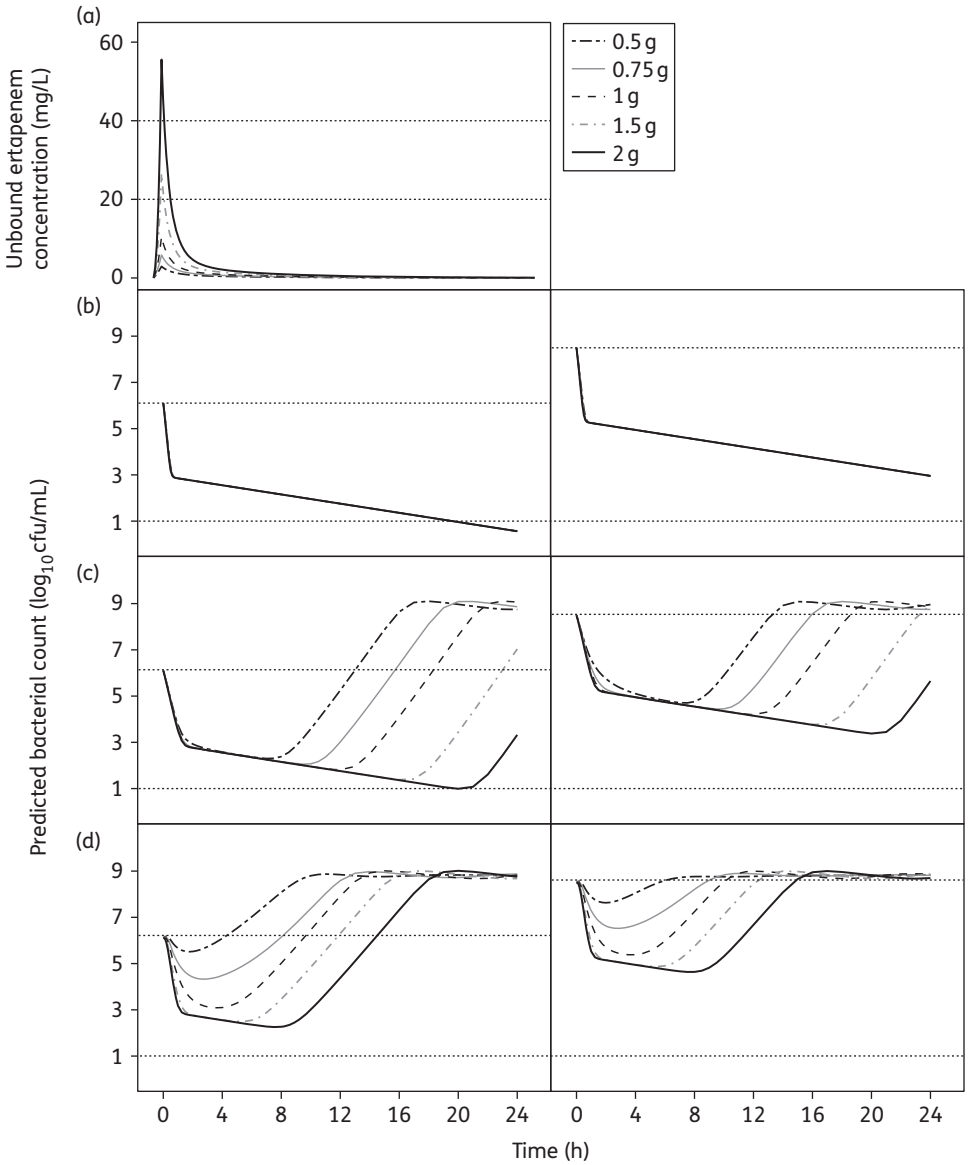

Figure 4. Model-predicted unbound ertapenem concentrations (a) and *E. coli* counts (b–d) following 30 min of intravenous administration of ertapenem doses of 0.5, 0.75, 1, 1.5 and 2 g. Results are shown for the native strain DA14781 (b), ESBL-producing strain DA14833 (c) and *ompR* R209C ESBL-producing strain DA16808 (d) for LD starting inocula (left) and HD starting inocula (right). Horizontal dotted lines represent ertapenem concentrations of 20 and 40 mg/L (a) or a bacterial count of 1×10^6 cfu/mL or 3×10^8 cfu/mL and LOD of 10 cfu/mL (b–d).

concentrations. The model includes a series of compartments, i.e. susceptible growing bacteria (S), less susceptible non-growing bacteria (R1) and non-susceptible non-growing bacteria (R2), for two co-existing bacterial subpopulations [susceptible subpopulation (S_A) and pre-existing resistant subpopulation (S_B)]. The model parameters were simultaneously estimated for three strains of *E. coli*, including the native strain, an ESBL-producing strain and an ESBL-producing *ompR* mutant strain.

For all *E. coli* strains investigated, the initial rapid killing effect was followed by a significant regrowth. This phenomenon has been shown to occur due to less susceptible bacteria that are present in the culture at the start of the experiment and then selected during exposure to ertapenem.[4,11] The emergence of resistance during the experiments was here confirmed by MIC determinations before and after exposure to ertapenem (Table 1). In the developed model, the regrowth of bacteria was taken into account by the introduction of a pre-existing less susceptible bacterial subpopulation, a commonly used strategy to describe emergence of resistance.[16]

In this study, the k_{growth} for the native strain and the ESBL-producing strain was estimated at 2.5 h^{-1}. The ESBL-producing *ompR* mutant strain was estimated to have a 17% slower growth rate (2.1 h^{-1}) compared with the other strains. This value is in agreement with a previous study that demonstrated a 20% reduction in the growth rate for the ESBL-producing *ompR* mutant *E. coli*.[11] The natural death rate (k_{death}) was estimated at 0.23 h^{-1}

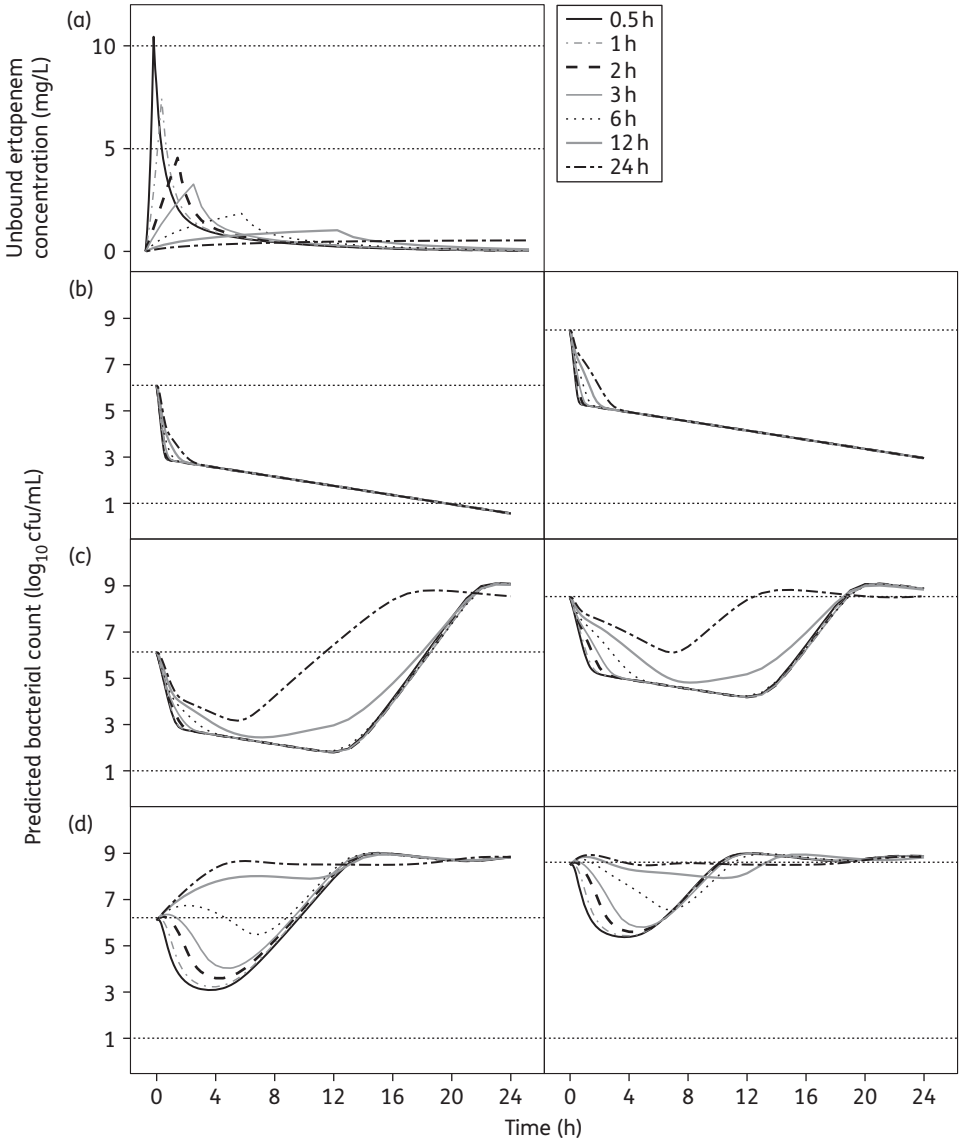

Figure 5. Model-predicted unbound ertapenem concentrations (a) and *E. coli* counts (b–d) following administration of a total dose of 1 g of ertapenem with different infusion durations (0.5, 1, 2, 3, 6, 12 or 24 h). Results are shown for the native strain DA14781 (b), ESBL-producing strain DA14833 (c) and *ompR* R209C ESBL-producing strain DA16808 (d) for LD starting inocula (left) and HD starting inocula (right). Horizontal dotted lines represent ertapenem concentrations of 5 and 10 mg/L (a) or a bacterial count of 1×10^6 cfu/mL or 3×10^8 cfu/mL and LOD of 10 cfu/mL (b–d).

for all three *E. coli* strains representing the terminal slope observed in the time–kill experiments with high ertapenem exposure after selection for the resting subpopulation.

The amount of resistant bacteria at the start of the experiments was here estimated as a fraction of the total bacterial content in the starting inoculum (f_{mutant}). This parameter was estimated with rather high uncertainty, reflecting the biological variation between experiments in the emergence of mutations and the limited information on this parameter in the data. Further, the f_{mutant} was also estimated to be very low. This parameter is likely to be affected by the assumption that k_{growth} was assumed to be constant during the experiment and that the growth rate constant was assumed to be the same for both pre-existing resistant and susceptible subpopulation. The f_{mutant} parameter should therefore be interpreted with caution, as it may not reflect a true number of bacteria in the biological system.

Previously, Mohamed *et al.*[21] have developed a mechanism-based PKPD model to describe the emergence of adaptive resistance for *E. coli* when exposed to gentamicin. This model structure was tested in this study, but was statistically inferior to the pre-existing resistance model. With the adaptive resistance model, the rate constant of resistance development (k_{on}) was found to be independent of ertapenem concentration and the rate of return to the susceptible state (k_{off}) was estimated to the lower boundary and thus fixed to 0 (i.e. assuming that resistance continue to develop over time regardless of drug exposure).

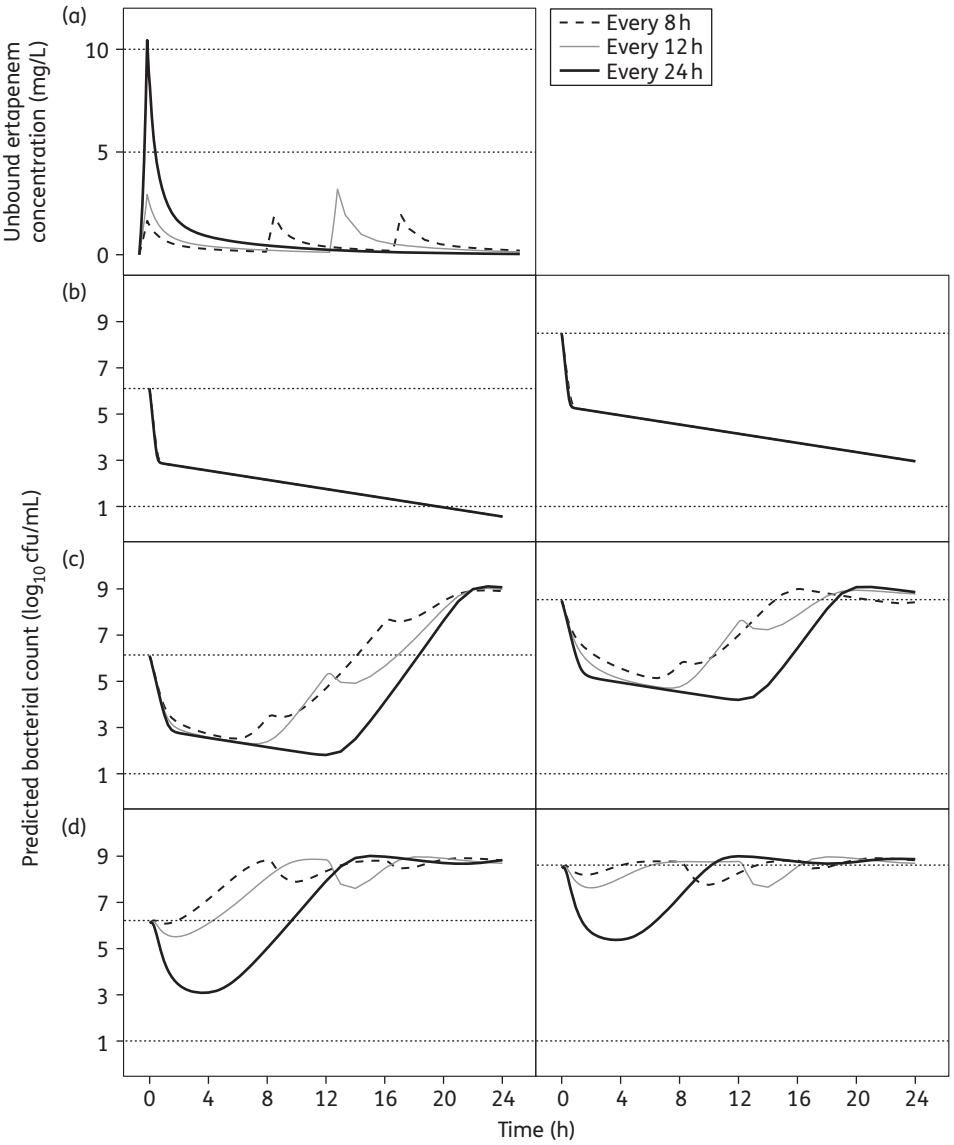

Figure 6. Model-predicted unbound ertapenem concentrations (a) and *E. coli* counts (b–d) following administration of a total dose of 1 g of ertapenem with different dosing intervals (8, 12 or 24 h). Results are shown for the native strain DA14781 (b), ESBL-producing strain DA14833 (c) and *ompR* R209C ESBL-producing strain DA16808 (d) for LD starting inocula (left) and HD starting inocula (right). Horizontal dotted lines represent ertapenem concentrations of 5 and 10 mg/L (a) or a bacterial count of 1×10^6 cfu/mL or 3×10^8 cfu/mL and LOD of 10 cfu/mL (b–d).

Prior to ertapenem exposure, the MICs for the native strain and the ESBL-producing strain were 0.004 and 0.023 mg/L, respectively, and thus classified as susceptible according to the EUCAST definition (susceptible $\leq$0.5 mg/L, intermediate 0.5–1 mg/L, resistant >1 mg/L). For the ESBL-producing *ompR* mutant strain, the MIC was 0.75 mg/L and thus classified as intermediate susceptible to ertapenem. For LD starting inocula, MICs after 24 h of ertapenem exposure were generally elevated, particularly for the ESBL-producing strain, where MIC increased up to 43-fold, resulting in MIC values of 1 mg/L. For the native strain, the MIC increased up to 31-fold (0.125 mg/L). These findings are in agreement with previously determined resistance levels for a single additional ertapenem resistance mutation in each respective genetic background.[11] Predicted mutations would probably be related to *ompR* inactivation in the absence (0.125 mg/L) and presence (1 mg/L) of ESBL production and an additional loss of function mutation in *ompF* and/or increased copy number of the β-lactamase genes on the plasmid in the ESBL-producing *ompR* R209C mutant. Such mutations have previously been determined to occur at a rate of 10^{-6}–10^{-8} per cell per generation and would thus be predicted to show a stochastic pre-existence in the starting cultures used here.[4,11]

The HD starting inoculum for the ESBL-producing strain showed less or no increase in the MIC at 24 h after ertapenem exposure. These findings may be due to the reduction of ertapenem efficacy with higher bacterial density, resulting in a higher fraction of susceptible bacteria remaining at the end of the experiment. An alternative explanation could be transient amplification

of the β-lactamase genes on the plasmid that would give increased MIC and better survival. Such amplifications have been shown to be very unstable which could lead to an underestimation of the MIC in the final population.[34] In this study, a decrease in the rate and extent of bacterial killing of intermediate ertapenem concentrations was observed when inoculum size was increased 100-fold, indicating an inoculum effect of ertapenem (Figure 2). Previous investigations concerning the inoculum effect for ertapenem show conflicting results,[35,36] probably due to variation between methods, inoculum size tested and bacterial strains. A model-based approach can here facilitate in translating observed *in vitro* effects to the clinical context.

The results from the dose predictions suggest that ertapenem doses of 0.5–2 g produce a maximal and similar bacterial killing effect against the native strain of *E. coli* and that no additional benefit is to be expected by fractioning the doses or by prolonging the infusion. However, for the ESBL-producing strain, the model predicts an early bacterial killing effect followed by bacterial regrowth occurring in a dose-dependent fashion. The dosing regimens explored in this study are therefore not expected to result in an efficient treatment for the ESBL-producing *ompR* mutant strain.

Predicted bacterial counts for the ESBL-producing *E. coli* were similar for infusion durations ranging from 0.5 to 6 h, indicating no significant advantage of extended infusion regimens. For the dose of 1 g, the model predicted that extension of the infusion duration from 0.5 to 6 h would increase $fT_{>MIC}$ from 29% to 38% for MIC 0.5 mg/L, from 21% to 27% for MIC 0.75 mg/L and from 15% to 20% for MIC 1 mg/L. An extended 12 h infusion of ertapenem or continuous infusion for the entire 24 h dosing interval is predicted to result in decreased bacterial killing and regrowth of pre-existing resistant subpopulations. The same trend, i.e. a decreased antibacterial efficacy when using extended or continuous infusion, was observed for the ESBL-producing *ompR* mutant strain.

The $fT_{>MIC}$ is widely used as the PK/PD determinant for activity of carbapenems and thus an extended infusion time is considered a strategy to optimize antimicrobial efficiency of carbapenems.[37,38] However, clinical data on the effectiveness of extended infusion regimens remain contradictory.[39,40] Further, the PK/PD indices are generally considered consistent within a drug class and in this case most *in vivo* studies have been performed with other carbapenems than ertapenem. The predictions shown in this study exemplify the problems associated with suggesting clinical dosing regimens based on the PK/PD indices and raise questions regarding the appropriateness of using the indices to extrapolate results between substances within a drug class and when there is a need also to consider the dynamics of the emergence of resistance.

The dose predictions were here limited to 24 h after the first dose, in accordance with the experimental data used for model development. Further, the dose predictions made here only reflect the killing effect imposed by the drug exposure and assumes similar growth and killing behaviour *in vivo* as observed *in vitro*. *In vivo*, the immune system will have an additional effect on bacterial killing not taken into account in these predictions and therefore these results should be investigated further *in vivo*. The predictions showed that the drop in bacterial count is expected to be similar regardless of the bacterial density in the starting inoculum (LD and HD starting inocula). However, in the case of high bacterial densities, it is predicted that a considerable number of bacteria are in the resting state at the start of treatment. This highlights the need for an extended duration of treatment and the importance of the additional bacterial killing effect imposed by the immune cells for infections associated with high bacterial burdens.

In conclusion, a mechanism-based PKPD model for ertapenem was developed to characterize the emergence of ertapenem-resistant *E. coli* subpopulations. Results from predictions suggest that the recommended dosage regimen (1 g once daily) might result in regrowth of resistant subpopulations when used to treat infection caused by ESBL-producing strains. However, higher dosing (e.g. 1.5 or 2 g once daily) would result in better bacterial killing. No clear advantage is to be expected for extended infusions or fractionated doses. This study highlights the importance of a detailed PKPD characterization taking the time course of emergence of resistance into consideration when selecting dosing regimens.

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Transparency declarations

None to declare.

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