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PHARMACOLOGY

Pharmacodynamics of Temocillin in Neutropenic Murine Infection Models

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ABSTRACT Temocillin is used for the treatment of various infections caused by *Enterobacterales*. The pharmacokinetic (PK)/pharmacodynamic (PD) index that is best correlated with the activity of beta-lactams is the percentage of time that the unbound concentration exceeds the MIC (%*f*T>MIC). However, the %*f*T>MIC needed for a bacteriostatic or killing effect of temocillin is unknown in thigh and lung infection models. In the present study, we studied the temocillin PK in plasma and epithelial lining fluid (ELF) of infected neutropenic mice and determined the plasma exposure-response relationships for *Escherichia coli* and *Klebsiella pneumoniae*. Neutropenic murine thigh and lung infection models were used. The bacterial loads in the thighs or lungs were determined. A sigmoid maximum-effect model was used to fit the plasma exposure-response relationship. A one-compartment model with first-order absorption best described temocillin PK (clearance [CL], 1.03 L/h/kg; volume of distribution [*V*], 0.457 L/kg). Protein binding was 78.2% ± 1.3% across different plasma concentrations. A static effect was achieved for all strains in both the thigh and lung infection models. However, the median %*f*T>MIC needed for a static effect was much lower in the lung infection model (27.8% for *E. coli* and 38.2% for *K. pneumoniae*) than in the thigh infection model (65.2% for *E. coli* and 64.9% for *K. pneumoniae*). A 1-log kill was reached for all strains in the lung infection model (median %*f*T>MIC values of 42.1% for *E. coli* and 44.1% for *K. pneumoniae*) and 7 out of 8 strains in the thigh infection model (median %*f*T>MIC values of 85.4% for *E. coli* and 74.5% for *K. pneumoniae*). These data support the use of temocillin in patients with pneumonia.

KEYWORDS PK/PD index, efficacy, pharmacodynamics

Temocillin is a parenteral beta-lactam antibiotic developed in the 1980s and is indicated for the treatment of bloodstream infections, complicated urinary tract infections, lower respiratory tract infections, and wound infections. It is gaining popularity since it is active against many *Enterobacterales* while being stable against most types of beta-lactamases, including AmpC and extended-spectrum beta-lactamases (1). However, it is not stable against most carbapenemases and is not active against *Pseudomonas aeruginosa* and *Acinetobacter* spp.

In the process of drug development for antibiotics, an important part thereof is to determine the potency of the drug against the infecting pathogen. To this end, it is important to determine the exposure-response relationship. To determine this relationship, pathogens are exposed to various dosing regimens of the drug, usually in neutropenic murine infection models. By using these relationships, the minimal exposure in humans for which there is a high probability of a positive treatment outcome can be determined.

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With regard to the beta-lactam antibiotics, it is known that the pharmacokinetic (PK)/pharmacodynamic (PD) index (PDI) that is best predictive of efficacy is the percentage of time that the unbound concentration exceeds the MIC (%*f*T>MIC). Optimized dosing of antimicrobials requires the determination of the magnitude of the PDI for various antibacterial effects. Nowadays, only some data are available for urinary tract infection models (2, 3), but data for thigh and lung infection models are lacking. We therefore performed studies in neutropenic murine thigh and lung infection models to determine the plasma pharmacodynamic target of the %*f*T>MIC resulting in bacteriostatic, 1-log kill, and 2-log kill effects against both *Escherichia coli* and *Klebsiella pneumoniae* strains.

RESULTS

Plasma protein binding. The relationship between the total concentration in murine plasma and the free fraction resulted in a linear relationship (R^2 = 0.9769). In total, 80 observations of total-versus-free-concentration pairs were evaluated, resulting in protein binding of 29.8% ± 12.7% (mean ± standard deviation [SD]). Linear regression resulted in an unbound fraction of 78.2% ± 1.3% (95% confidence interval [CI], 75.6 to 80.9%). For the calculations of exposures to unbound temocillin, a single value of 78.2% was used over a total concentration range from 1 to 376 mg/L.

Pharmacokinetics. The total murine plasma concentration-time curves for the different doses are presented in Fig. 1. The PK of the total concentrations of temocillin in mice was best described by a one-compartment model with first-order absorption. To increase the stability of the model, the absorption rate constant (K_a) value was fixed to the estimated value after analyses of the structural model. The inclusion of between-subject variability (BSV) on clearance (CL) improved the model fit significantly. A significant correlation between the dose and the place of infection was found on clearance. The parameter estimates for the final model are presented in Table 1. The goodness-of-fit plots showed good agreement between the observed and the model-predicted values. Validation of the model using bootstrap methods (Table 1) and visual predictive checks (VPCs) (Fig. 2) showed that the model described the data well. Figure 3 shows the area under the concentration-time curve (AUC) and CL values (for both the thigh and lung infection models) plotted versus the different dose levels, showing the influences of place of infection and dose on CL as well as the limited influence on the AUC.

Bronchoalveolar lavage (BAL) fluid samples were also obtained to determine the temocillin concentration in the epithelial lining fluid (ELF) at various time intervals after the subcutaneous dose. In all samples taken from mice dosed at up to 32 mg/kg of body weight, the temocillin concentrations were below the limit of quantification (LOQ) at all time points. In the ELF from mice dosed with 64 mg/kg temocillin or higher, temocillin was detected in 34 of the 96 samples, all within 1 h after drug administration. Due to the dilution of the ELF in BAL fluid samples, only 8 of these samples had concentrations that were equal to or higher than the LOQ within 30 min after drug administration. After the dose of 256 mg/kg, the mean unbound temocillin concentration in ELF was 86.7 mg/L over the first 30 min, with a range from 56.6 to 145 mg/L. Based on the ratio of the urea concentrations in plasma/BAL fluid, the dilution factor of ELF was ~30, i.e., an LOD for ELF of 30 mg/L, as the LOD was 1 mg/L. Due to the low number of quantifiable samples in the ELF, these data were not added to the population PK model for plasma.

Pharmacodynamics. Limited dose fractionation studies have indicated that the maximum-effect (E_{max}) model describes well the plasma exposure-response relationship using the %*f*T>MIC (Fig. 4). The %*f*T>MIC fitted the data slightly better than the *f*AUC/MIC ratio. The %*f*T>MIC-versus-ΔCFU/organ curves in both the thigh and lung infection models in neutropenic mice are shown in Fig. 5 for 3 *E. coli* and 3 *K. pneumoniae* strains using every-2-h (q2h) dosing regimens. The %*f*T>MIC values corresponding to static, 1-log kill, and 2-log kill effects in both infection models for all isolates are shown in Table 2. For all strains, a static effect was achieved in both the thigh and lung infection models. There was also no statistical difference found for static effects

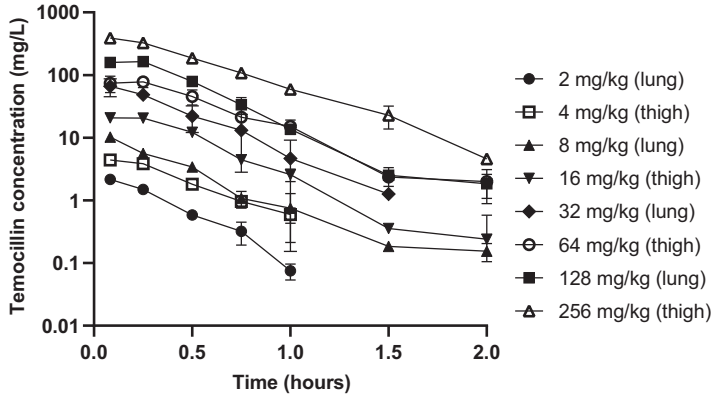

FIG 1 Total murine plasma concentration-time curves (means and SD) for the different doses in the thigh and lung infection models (190 mice in total). The concentrations in the PK controls were undetectable and are not presented.

between *E. coli* and *K. pneumoniae* strains in the thigh ($P = 0.9837$ by an unpaired two-tailed *t* test) and lung ($P = 0.5328$ by an unpaired two-tailed *t* test) infection models.

However, the plasma %*f*T>MIC correlating with a static effect in the lung infection model was significantly lower than that in the thigh infection model, with mean values ± SD of 33.0% ± 21.3% and 65.1% ± 16.9% for the lung and thigh infection models, respectively ($P = 0.0049$ by an unpaired two-tailed *t* test). A 1-log kill effect was achieved for all strains in the lung infection model and 7 of 8 strains in the thigh infection model. A 2-log kill was achieved for 7 of 8 strains in the lung infection model and 1 of 8 strains (for *K. pneumoniae* ATCC 43816) in the thigh infection model. In one *K. pneumoniae* isolate, a 2-log kill effect could not be demonstrated since the maximum exposure reached in this experiment was 60% *f*T>MIC.

Analysis of the strains simultaneously using comodeling resulted in slightly higher values of 71.2% *f*T>MIC and 69.9% *f*T>MIC in the thigh infection model for stasis for *E. coli* and *K. pneumoniae*, respectively. In the lung infection model, these values were 29.5% and 35.5% for *E. coli* and *K. pneumoniae*, respectively. All values are presented in Fig. 6.

DISCUSSION

In the current study, we describe plasma protein binding in mice and found a fixed percentage of 78.2% ± 1.3% for the unbound concentration across a range of concentrations. The PK in mice was described using a population PK model, and clearance was found to be influenced by the place of infection (thigh versus lung) and the temocillin dose. ELF penetration was poor and was observed at detectable levels only during the first hour after the 256-mg/kg dose. A static effect was achieved for all *E. coli*

TABLE 1 Parameter estimates of the final model and results of bootstrapping

Parameter	Value	
	Estimate of the final model	Bootstrap [median (90th percentile range)]
K_a (h^{-1})	20 (fixed)	20 (fixed)
CL/*F* (L/h/kg)[a]	1.03	1.02 (0.97 to 1.08)
V_c/F (L/kg)	0.457	0.457 (0.427 to 0.488)
Infection place on CL	1.25	1.24 (1.17 to 1.33)
Dose on CL	−0.102	−0.102 (−0.131 to −0.073)
BSV on CL (%)	11.3	10.9 (7.5 to 13.9)
Residual error (additional) (mg/L on ln scale)	0.219	0.213 (0.175 to 0.252)

[a]The covariates infection place (INFP) (thigh = 0 and lung = 1) and dose (in milligrams per kilogram) are implemented in the model as follows: $CL = 1.03 \times 1.25^{INFP} \times (dose/100)^{-0.102}$.

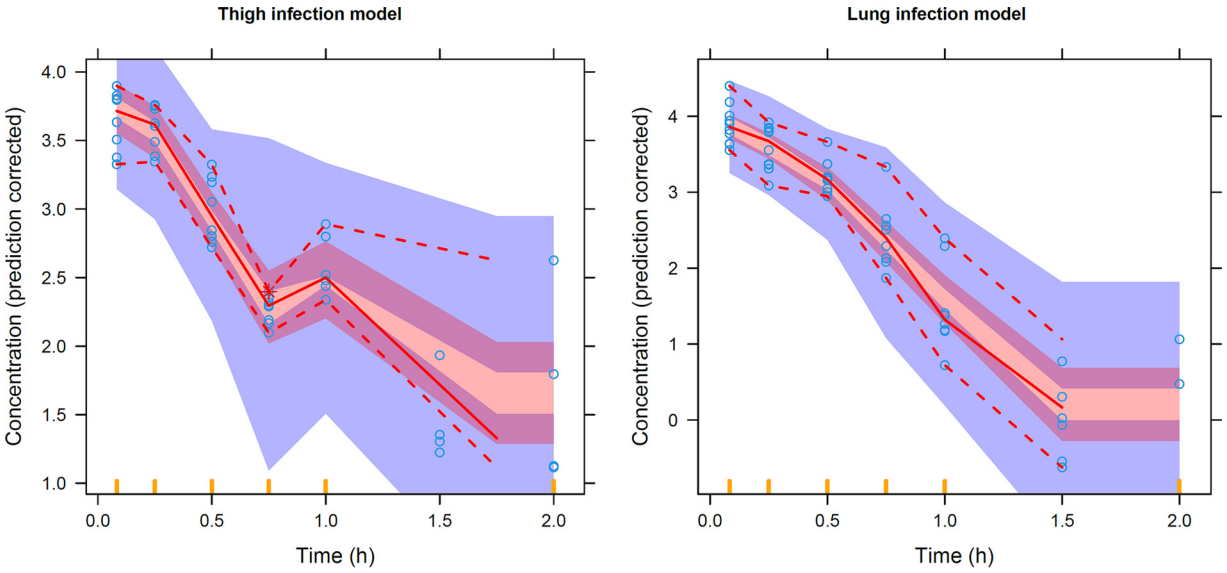

FIG 2 Prediction-corrected VPCs stratified by infection type of the final pharmacokinetic model. The circles represent the observed data. The middle continuous line is the 50th percentile of the observed data, and the upper and lower dashed lines are the 95th and 5th percentiles of the observed data, respectively. The shaded regions represent the 95% prediction intervals of the 5th, 50th, and 95th percentiles. Times are in hours, and concentrations are in milligrams per liter (natural log scale).

and *K. pneumoniae* strains in both the thigh and lung infection models at mean plasma %*f*T>MIC values of 33.0% in the lung model and 65.1% in the thigh model. A 1-log kill was reached for all strains in the lung infection model at a median value of 42.1% *f*T>MIC and for 7 out of 8 strains in the thigh infection model at a median value of 78.8% *f*T>MIC. Overall, the %*f*T>MIC values were slightly higher when strains were comodeled.

Although the antibacterial effect in the lung infection model requires a lower plasma %*f*T>MIC than the thigh infection model, the temocillin concentrations in ELF in both infection models were unexpectedly low. Since single-dose PK experiments were performed, temocillin may accumulate in ELF after repeated dosing, which could explain the killing effect that was observed in exposure-response experiments. Biopsy studies of 8 patients indicated serum/lung tissue penetration of 0.26 (approximately) 30 min after the intravenous administration of a single dose of 2 g temocillin (4). However, a recent study employing microdialysis in the soft tissue of healthy volunteers indicated that temocillin might have good tissue penetration in muscles and the subcutis (5). Furthermore, temocillin accumulation was noted in peritoneal secretions 12 h after infusion, with a tissue/plasma penetration value of 1.7 (6). Therefore, the effect of temocillin on lung infections might be explained by the penetration of temocillin into the pulmonary tissue instead of the ELF.

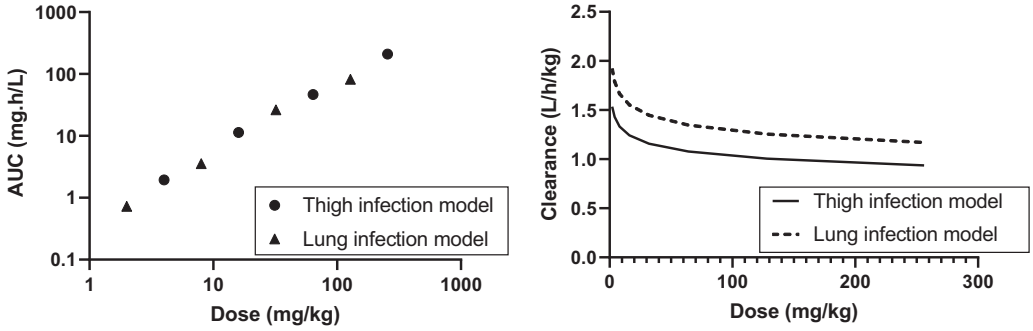

FIG 3 Plasma AUC and clearance for both the thigh infection and lung infection models versus the different dose levels.

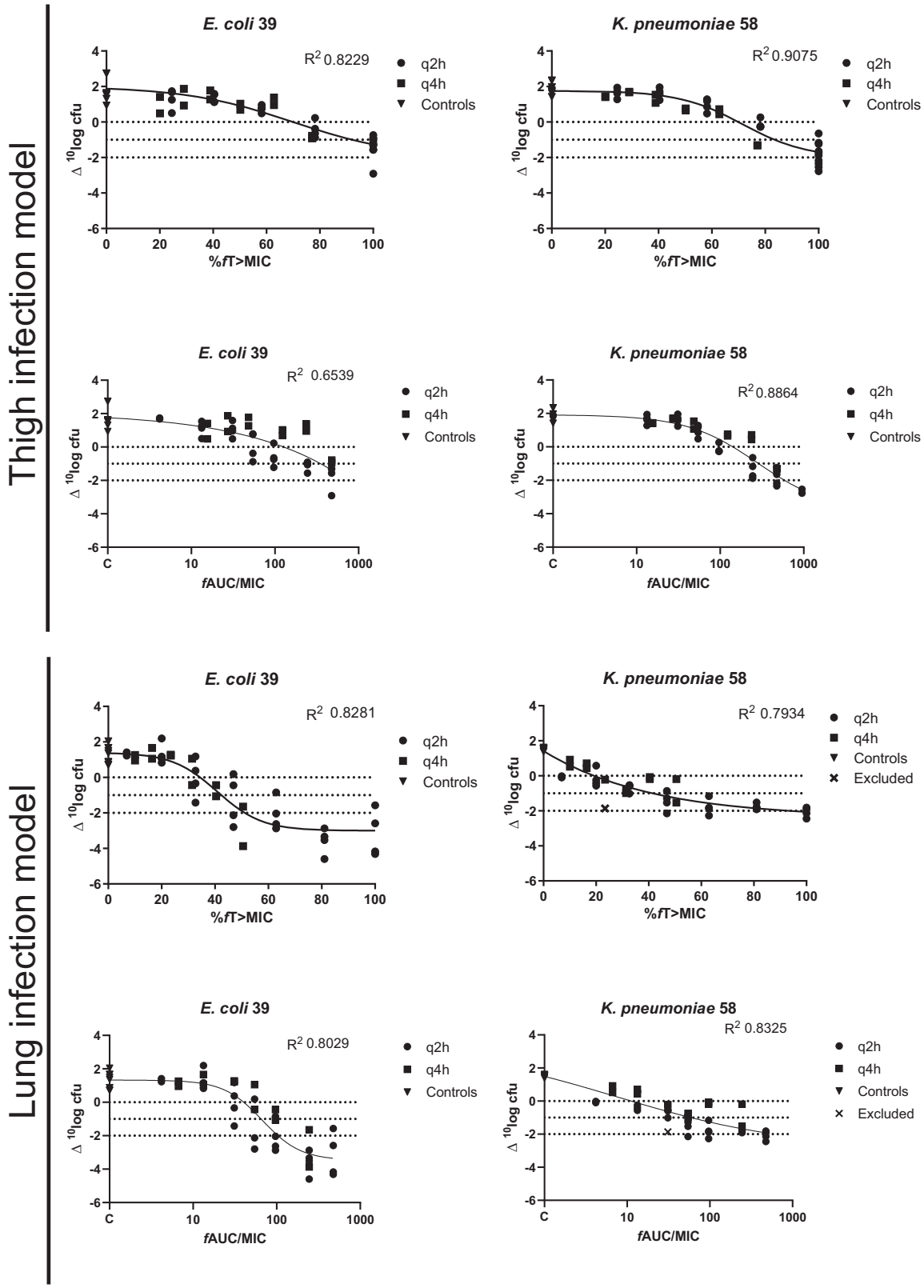

FIG 4 Plasma %*f*T>MIC and *f*AUC/MIC ratio versus the change in CFU for *E. coli* 39 and *K. pneumoniae* 58 in both the thigh infection (32 mice) and lung infection (64 mice) models. Each data point represents the bacterial load in an individual thigh or lung. Controls represent the placebo-treated controls.

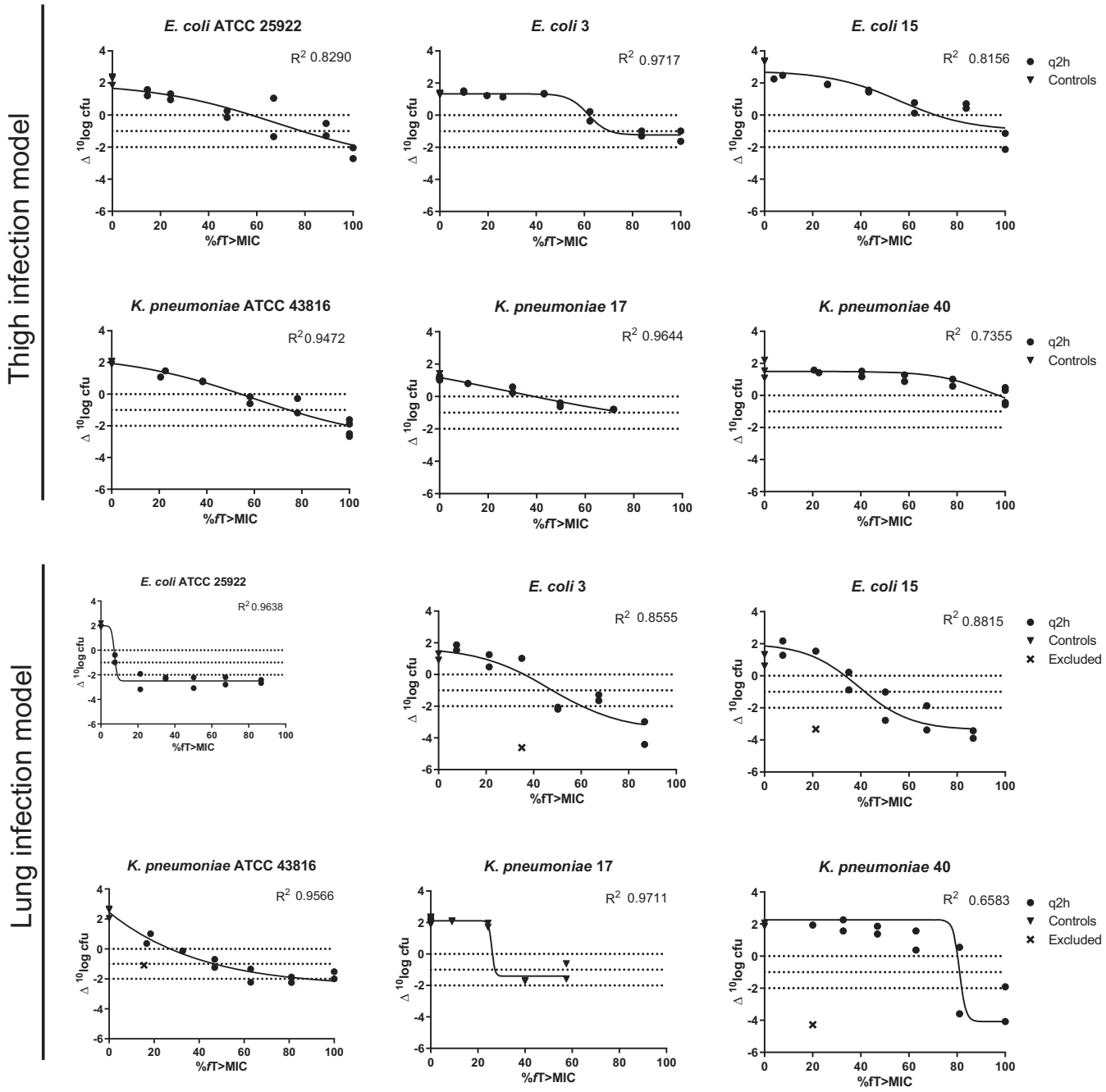

FIG 5 Plasma exposure-response curves for 3 *E. coli* and 3 *K. pneumoniae* isolates in both the thigh infection (54 mice) and lung infection (108 mice) models for %*f*T>MIC. Each data point represents the bacterial load in an individual thigh or lung. Controls represent the placebo-treated controls.

Protein binding in mice was found to be linear in the range of 1 to 376 mg/L (total concentrations). This is in contrast to data on protein binding in humans, which is complex (7). There is clear nonlinearity in the human data, but there is also a clear difference between protein binding in healthy volunteers and those in various patient groups. Four different nonlinear relationships were found for healthy volunteers (mean unbound fraction of 4 to 16% at total concentrations of 20 to 200 mg/L), hospitalized patients with urinary tract infections (mean unbound fraction of 12 to 29% at total concentrations of 20 to 200 mg/L), patients admitted to the intensive care unit (ICU) with (suspected) ventriculitis (mean unbound fraction of 23 to 42% at total concentrations of 20 to 200 mg/L), and ICU patients with sepsis/septic shock (mean unbound fraction of 32 to 52% at total concentrations of

TABLE 2 Pharmacodynamic targets in plasma associated with stasis, 1-log kill, and 2-log kill in both the neutropenic murine thigh infection (86 mice in total) and lung infection (172 mice in total) models for *E. coli* and *K. pneumoniae*

Organism	Median MIC (range) (mg/L)	Resistance mechanism(s)	%*f*T>MIC					
			Thigh infection model			Lung infection model		
			Stasis	1-log kill	2-log kill[a]	Stasis	1-log kill	2-log kill
E. coli								
ATCC 25922	16 (8–16)	Wild type	57.8	78.8	NA	7.1	7.8	8.9
3	16 (16)	TEM-48, CTX-M-15	61.9	70.9	NA	36.0	47.9	60.3
15	16 (16)	CTX-M-15	71.2	96.9	NA	33.5	41.7	50.7
39	8 (8)	TEM-1	69.9	92.1	NA	34.5	42.4	51.2
Mean			65.2	84.6	NA	27.8	35.0	42.8
Median			65.9	85.4	NA	34.0	42.1	51.0
SD			6.4	12.0	NA	13.8	18.3	23.0
K. pneumoniae								
ATCC 43816	8 (4–8)	Wild type	54.4	74.5	100	27.1	46.6	87.5
17	64 (16–64)	SHV-1, OXA-1, CTX-M-15	38.3	74.4	NA	26.2	27.2	NA
40	8 (8–16)	LEN, GES-1	97.1	NA	NA	80.2	81.1	81.9
58	8 (8)	TEM-84, SHV-11	69.9	83.5	NA	19.2	41.5	93.3
Mean			64.9	77.5[b]	NA	38.2	49.1	87.6[b]
Median			62.2	74.5[b]	NA	26.7	44.1	87.5[b]
SD			25.0	5.2[b]	NA	28.2	22.9	5.7[b]

[a]NA, not achieved.
[b]Based on 3 observations.

20 to 200 mg/L) (7). This is important for the interpretation of human data. The PK/PD index target values found in this analysis are presented as unbound concentrations. To calculate target attainment in patients with various dosing regimens, unbound human concentrations for the specific population should be compared to these target values.

Among the exposure-response curves for the lung infection model, *E. coli* strain ATCC 25922 resulted in extremely low %*f*T>MIC values required for stasis, 1-log kill, or 2-log kill. It could be questioned whether this is caused by the absence of resistance

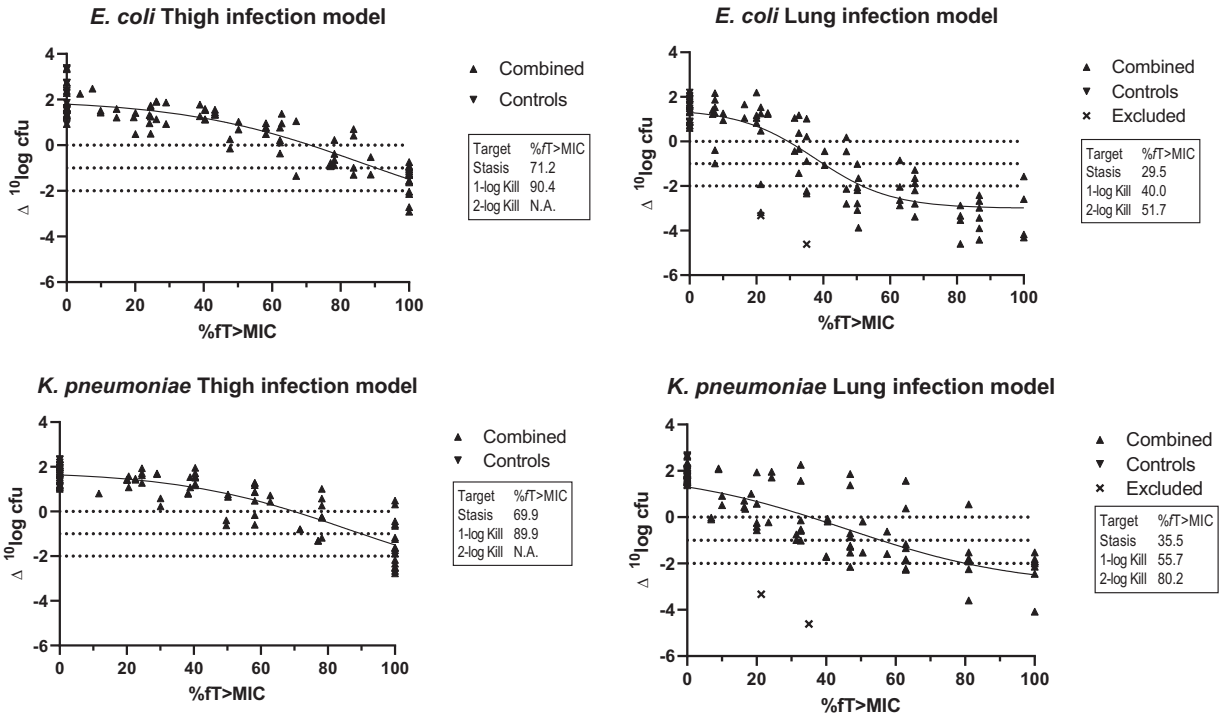

FIG 6 Plasma exposure-response curves for all *E. coli* and *K. pneumoniae* isolates comodeled per species and infection model.

mechanisms. However, this strain in the thigh infection model did not result in lower target values than those for the other strains. Also, when the targets for clinical strains were compared to the results for both the *E. coli* and *K. pneumoniae* ATCC strains, there was no indication that the activity was influenced by the presence or absence of resistance mechanisms. It is therefore not likely that the lower %*f*T>MIC is caused by the absence of resistance mechanisms. Inaccuracies in MIC measurements might contribute to the variations in the exposure-response analysis found. The MIC values of the isolates used were tested in triplicate because it is known that there is a considerable amount of variability between measurements, which cannot be avoided (8).

Current EUCAST breakpoints for temocillin apply only to infections originating from the urinary tract since the available data on the PK/PD target values were limited to data from a murine urinary tract model (2) as well as a murine pyelonephritis model (3). These breakpoints are based on a PK/PD target of 40 to 50% *f*T>MIC for stasis. This target value used in breakpoint settings is lower than the value found in the thigh infection model in the current study (i.e., 66% for stasis) but higher than the target values found in the lung infection model (i.e., 30% for stasis). Despite the fact that the breakpoints apply only to urinary tract infections, temocillin is being used for the treatment of respiratory tract infections as well. A recent pharmacokinetic study of 32 mechanically ventilated patients with pneumonia used a PK/PD target of 50% *f*T>MIC as the bacteriostatic target and estimated that with intermittent dosing with 2 g every 8 h, a probability of target attainment (PTA) of at least 90% would be reached for strains with MICs of up to 4 mg/L (9). Continuous infusions (6 g per 24 h after a loading dose of 2 g) resulted in higher PTAs, with a >90% PTA for a target of 100% *f*T>MIC, and strains with MICs of up to 8 mg/L (9). With the lower PK/PD target values found in the lung model in our study, it is likely that adequate target attainment will be reached for strains with even higher MIC values. This is promising for the use of temocillin for pneumonia since the epidemiological cutoff values (ECOFFs) for *E. coli* and *K. pneumoniae* are 16 mg/L and 8 mg/L, respectively.

In conclusion, the PK/PD relationships of temocillin in neutropenic murine thigh and lung infection models are described. A static effect was reached for all strains in both infection models. The %*f*T>MIC values needed for stasis were similar for *E. coli* and *K. pneumoniae*. The median %*f*T>MIC values required to achieve stasis and 1-log kill in the lung infection model are relatively low, at 30.3% and 42.1%, respectively. These data support clinical studies on the use of temocillin in patients with pneumonia.

MATERIALS AND METHODS

Bacteria, media, and antibiotics. Experiments were performed with 4 *E. coli* and 4 *K. pneumoniae* strains. For both species, ATCC strains (*E. coli* ATCC 25922 and *K. pneumoniae* ATCC 43816) as well as three clinical strains with several resistance mechanisms were used (Table 1). MICs were determined in triplicate by standard broth microdilution procedures with geometric 2-fold serial dilutions in cation-adjusted Mueller-Hinton II (CAMHII) broth according to EUCAST guidelines (10). The median values and the ranges for replicates are reported, and the median values were subsequently utilized for PK/PD analyses.

A freezer stock of approximately 1×10^9 CFU/mL in CAMHII broth was made for all strains. Prior to infection, a culture of this stock grown overnight was prepared at 37°C. On the day of infection, log-phase cultures were made from this culture in fresh CAMHII broth by incubation for 1 or 2 h (depending on the growth rate of the isolate) at 37°C under shaking conditions. The log-phase cultures were diluted with CAMHII broth (thigh infection) or normal saline (lung infection) to a final inoculum of approximately 10^8 CFU/mL for both thigh and lung infections. The number of CFU in the inoculum was counted on Mueller-Hinton II agar (MHA) plates.

All media were obtained commercially from Becton, Dickinson (Olen, Belgium). Temocillin was kindly provided by Eumedica Pharmaceuticals (Manage, Belgium). Solutions of temocillin were prepared in saline 1 h prior to treatment and stored at 4°C until use.

Thigh and lung infection models in neutropenic mice. *In vivo* experiments were performed in the Erasmus Laboratory Animal Science Center (EDC) in Rotterdam, The Netherlands, according to European Union Animal Directive 2010/63/EU 2010 (11) under license number AVD101002016702, and study plans were approved by the Institutional Animal Welfare Body. Seven- to eight-week-old outbred female CD-1 mice (weight of 25 ± 5 g on the day of infection) were obtained from Charles River (Germany). Mice were housed under standard conditions, with food and water *ad libitum*. After 1 week of acclimatization, mice were rendered neutropenic by the intraperitoneal injection of cyclophosphamide 4 days (150 mg/kg of body weight) and 1 day (100 mg/kg of body weight) before the onset of the experiment.

Buprenorphine analgesia was applied (Indivior Europe Limited, Dublin, Ireland). Infection in the thigh infection model was induced by the intramuscular injection of 0.05 mL of approximately 10^8 CFU/mL of different strains in CAMHBII broth into each thigh. Lung infection was induced in isoflurane-anesthetized mice by applying 0.05 mL of approximately 10^8 CFU/mL in saline intranasally.

Pharmacokinetics. Temocillin treatment was started 2 h after the mice (190 mice in total) were infected in the thighs or lungs (time zero). Single doses of 2 to 256 mg/kg were administered subcutaneously in 0.1 mL saline to determine the PK, and all doses were studied in two mice. Blood and bronchoalveolar lavage (BAL) fluid samples were concomitantly taken directly postmortem at 12 time points before (time zero) (PK controls) and after (5, 15, 30, and 45 min and 1, 1.5, 2, 4, 6, 8, and 12 h) the administration of temocillin. PK controls were obtained to check whether the temocillin concentrations in blood were indeed undetectable before the onset of treatment. Blood samples were centrifuged immediately in a precooled centrifuge to separate plasma. For BAL fluid, the trachea was approached by a cervical incision in the neck to insert a cannula. Using this cannula, the lungs were instilled two times each with 1 mL of saline. The samples were pooled and placed directly on ice. Plasma and BAL fluid were stored at −80°C until analysis.

Pharmacodynamics. Limited dose fractionation experiments were performed for 1 *E. coli* isolate and 1 *K. pneumoniae* isolate. Doses of 8 to 512 mg/kg in the lung infection model (64 mice in total) and 16 to 1,024 mg/kg in the thigh infection model (32 mice in total) were administered every 2 or 4 h. In addition, 3 *E. coli* and 3 *K. pneumoniae* isolates were tested with doses of 16 to 512 mg/kg in the lung infection model (108 mice in total) and 16 to 512 mg/kg in the thigh infection model (54 mice in total) administered every 2 h. At time zero, two mice were humanely killed to determine the bacterial loads in the thighs or lungs at the start of the treatment (start-of-treatment controls). The experiments also included placebo-treated controls to check the growth of the microorganisms. After 24 h, the remaining mice were humanely killed for the determination of CFU. The thighs or lungs were excised and homogenized in 2 mL of phosphate-buffered saline (PBS) by using a GentleMACS Octo dissociator (Miltenyi Biotec, Leiden, The Netherlands). The bacterial burdens in both the thigh infection and lung infection models were quantified by culturing 3 × 10 μL of 10-fold serial dilutions of the ~2 mL homogenized tissue on MHA, with each thigh or lung resulting in a separate data point for bacterial burdens. In addition, 200 μL of the undiluted homogenate was plated onto MHA, resulting in a lower limit of detection (LOD) of 10 CFU/tissue.

Temocillin concentrations in plasma and BAL fluid. The temocillin concentrations in plasma and BAL fluid (of 190 mice infected in the thigh or lung) were determined using a validated ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method. A Dionex Ultimate UPLC system consisting of an Ultimate UPLC pump, an Ultimate RS autosampler, and an Ultimate RS column compartment connected to a Thermo TSQ Vantage triple-quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used. This method requires only 50 μL of plasma or BAL fluid for analysis of the total temocillin concentration. Samples were prepared using protein precipitation with methanol. Cefazoline was used as an internal standard to correct for any sample loss, ion enhancement, or ion suppression. Temocillin standard curves were linear for the concentration range of 1.0 to 100 mg/L (R^2 of 0.9950). The lower LOQ was 1 mg/L. Several parameters, such as accuracy and precision, were validated at three quality control (QC) levels: low (QC L), medium (QC M), and high (QC H). The validation results for accuracy were −2.0% for QC L, −0.1% for QC M, and 7.8% for QC H. The within-day precision values were 4.3%, 0.6%, and 5.0% for QC L, M, and H, respectively. The between-day precision values were 4.1%, 6.9%, and 7.6% for QC L, M, and H, respectively.

Temocillin concentrations in ELF were calculated from the BAL fluid. The BAL fluid was not centrifuged before analysis. Since urea concentrations are usually the same in plasma and ELF, the ratio of the urea concentration in BAL fluid to that in plasma can be used to determine the apparent ELF volume (12). The urea measurements were carried out using the QuantiChrom urea assay kit (BioAssay Systems, Hayward, CA, USA). To convert BAL fluid concentrations into ELF concentrations, the following formula was used: $[\text{temocillin}]_{ELF} = [\text{temocillin}]_{BAL} \times ([\text{urea}]_{plasma}/[\text{urea}]_{BAL})$. Since the protein binding of antibiotics in the ELF is expected to be negligible, the concentrations in the ELF were assumed to be equal to the unbound concentrations (13, 14).

Plasma protein binding of temocillin in mice. Free concentrations were obtained in 80 selected individual samples from mice infected in the thigh or lung (as described above) over the entire total concentration range after temperature-controlled ultrafiltration of 200 μL of plasma using Nanosep 30K Omega centrifugal devices (VWR International BV, Amsterdam, The Netherlands). Next, 50 μL of the filtrate was used for sample preparation.

To determine protein binding and whether there is a concentration dependence on plasma protein binding, the percentages of protein binding (means ± SD) for the individual observations were plotted over the total concentrations and analyzed by linear regression analysis in order to assess whether the slope deviates significantly from zero (GraphPad Prism, version 9.4.0; GraphPad Software, Inc., San Diego, CA, USA).

Temocillin pharmacokinetic analysis in mice (population modeling). To describe the PK, a population model was developed using nonlinear mixed-effects modeling (NONMEM, version 7.4.2; Icon Development Solutions, Ellicott City, MD, USA). For the analysis, the total concentration data were used, natural logarithm transformed, and analyzed using the first-order conditional estimation (FOCE) method with interaction. Two mice were used per time point. All time points were included until both concentrations at a specific time point were below the LOQ. The PK parameters were scaled per kilogram of mouse weight. To initiate the analysis, several 1- and 2-compartment models were tested to develop a structural model. An absorption rate constant (K_a) was implemented in the model to describe the absorption

following subcutaneous injection. Typical values for clearance (CL), the central volume of distribution (V_c), the peripheral volume of distribution (V_p), and intercompartmental clearance (Q) were estimated. As bioavailability (F) could not be estimated, CL, V_c, V_p, and Q values corresponded to the ratios of CL/F, V_c/F, V_p/F, and Q/F, respectively. For each of the parameters of the structural model, the addition of between-subject variability (BSV), as described by an exponential-error model, was evaluated. For the comparisons between various structural and statistical models, a level of significance of a P value of <0.001 was used. The residual error between the observed and the predicted plasma concentrations was described using an additional error model for logarithmically transformed data. For data below the LOQ, an extra residual error of 0.5 mg/L (0.5× LOQ) was taken into account. Afterward, we evaluated whether part of the variability found could be explained by a potential covariate. The covariates assessed were the place of infection (using a proportional equation) and the temocillin dose (using an exponential equation). Covariates were included using forward inclusion ($P < 0.05$) and backward elimination ($P < 0.001$). For model selection, the minimum objective function values (OFVs), parameter precision, error estimates, and visual inspections of the goodness-of-fit plots were considered. The final model was validated internally using a bootstrap resampling method ($n = 1{,}000$) and a visual predictive check (VPC) ($n = 1{,}000$).

Pharmacodynamic and statistical analyses. Values derived from the population PK model were used to calculate the plasma %fT>MIC over a period of 24 h in MicLab (Medimatics, Maastricht, The Netherlands). The changes in the counts of CFU per tissue after 24 h of treatment compared to the counts of CFU per tissue at 0 h (ΔCFU) were calculated for all dosing regimens in both infection models. All data were inspected visually, and data points were excluded from the analysis when the individual data point deviated by >1.5-log kill from the fitted curve. PK/PD response curves were fit to the ΔCFU data by using a sigmoid maximum-effect (E_{max}) model (GraphPad Prism, version 9.4.0; GraphPad Software, Inc., San Diego, CA, USA) to determine the effect of temocillin on the change in colony counts. PK/PD magnitudes for stasis, 1-$\log_{10}$ kill, and 2-$\log_{10}$ kill were determined. Differences in values for the PK/PD indices between the groups were compared using the unpaired t test. Values of the static effect in the thigh infection model for *E. coli* and *K. pneumoniae* were compared, as were these values in the lung infection model. Also, the values of the static effect in the lung infection model and thigh infection model were compared.

Additionally, all isolates were comodeled simultaneously per species and per infection model to correct for isolates that did not reach certain targets. The analyses were performed using a sigmoid E_{max} model (GraphPad Prism, version 9.4.0; GraphPad Software, Inc., San Diego, CA, USA).

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