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In Vivo Pharmacokinetic/Pharmacodynamic Profiles of Valnemulin in an Experimental Intratracheal *Mycoplasma gallisepticum* Infection Model

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Valnemulin, a semisynthetic pleuromutilin antibiotic derivative, is greatly active against *Mycoplasma*. The objective of our study was to evaluate the effectiveness of valnemulin against *Mycoplasma gallisepticum* in a neutropenic intratracheal model in chickens using a pharmacokinetic/pharmacodynamic (PK-PD) method. The PK of valnemulin after intramuscular (i.m.) administration at doses of 1, 10, and 20 mg/kg of body weight in *M. gallisepticum*-infected neutropenic chickens was evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Real-time PCR (RT-PCR) was used for quantitative detection of *M. gallisepticum*. The ratio of the 24-h area under the concentration-time curve divided by the MIC (AUC_{24}/MIC) correlated well with the *in vivo* antibacterial effectiveness of valnemulin ($R^2 = 0.9669$). The AUC_{24}/MIC ratios for mycoplasmastasis (a reduction of 0 $\log_{10}$ color-changing unit [CCU] equivalents/ml), a reduction of 1 $\log_{10}$ CCU equivalents/ml, and a reduction of 2.5 $\log_{10}$ CCU equivalents/ml are 28,820, 38,030, and 56,256, respectively. In addition, we demonstrated that valnemulin at a dose of 6.5 mg/kg resulted in a reduction of 2.5 $\log_{10}$ CCU equivalents/ml. These investigations provide a solid foundation for the usage of valnemulin in poultry with *M. gallisepticum* infections.

Mycoplasma gallisepticum is a primary pathogen in chickens, turkeys, pheasants, red-legged partridges, chukar, and members of the *Fringillidae* family (1). It is the etiologic agent of chronic respiratory disease (CRD), with clinical symptoms of rales, nasal discharge, air sacculitis, and depression (2). It has been deemed the most economically important of the four pathogenic *Mycoplasma* species, as it causes vast losses in the commercial poultry industry (3). *M. gallisepticum* infection causes not only direct losses, such as reduced hatchability, increased feed conversion ratio, lower quality of processed chicken carcasses, and increased mortality, but it also causes indirect losses, like costs of eradication procedures, monitoring programs, and control procedures (4). Moreover, it leads to a negative effect on foreign trade (2). *M. gallisepticum* can be transmitted vertically through eggs and horizontally by susceptible individuals through direct/indirect contact with contaminated surfaces, infected equipment, and airborne particles (2). Although both vaccination and antimicrobial drugs are utilized against *M. gallisepticum*, vaccination for *M. gallisepticum* control has been greatly discouraged, because of considerable organism strain variability and the uselessness of stopping horizontal spread (5). Given this, antimicrobial chemotherapy plays a significant role in treating infections of *M. gallisepticum*. Valnemulin, as a semisynthetic pleuromutilin antibiotic derivative, was developed solely for veterinary use due to its potent activity against *Mycoplasma* (6). This peculiarity indicates valnemulin to be another prospective therapeutic choice in *M. gallisepticum* infections.

A rational antimicrobial drug dosage to be used in veterinary clinical practice should be based on the relationship of pharmacokinetics (PK) and pharmacodynamics (PD) (7). Therefore, in the current study, we investigated the *in vivo* PK-PD profiles of valnemulin against *M. gallisepticum*. This *in vivo* PK-PD model avoids the limitations of *ex vivo* PK-PD. For instance, for *ex vivo* PK-PD, there is continuous exposure to a fixed concentration of agent for a defined time (24 h), which does not mimic the concentration of antibacterial decline in animals because of body clearance and drug metabolism. In addition, the concentration in the target site usually varies from that in serum. Therefore, an *in vivo* PK-PD model provides more actual approximate clinical data. Unfortunately, due to the difficulty of isolating and culturing *M. gallisepticum* from chickens, little is known about the *in vivo* PK-PD profiles of valnemulin against *M. gallisepticum*. Molecular methods, such as hybridization techniques, PCR, or combinations of both techniques to detect the presence of bacterium-specific DNA sequences, were used in both qualitative and quantitative detection of the organism (8, 9). Recently, real-time PCR (RT-PCR) methods that monitor the production of specific PCR products by fluorimetric detection of amplified products and use melting curve analysis or specific hybridization probes for identification have been used for qualitative and quantitative detection of various pathogens (10, 11). This method for the quantitative detection of *M. gallisepticum* was also reported in several studies (12, 13).

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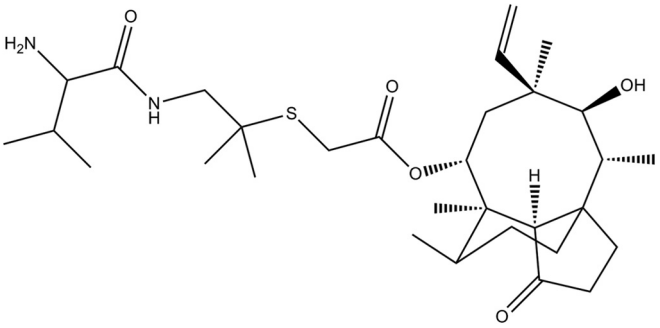

FIG 1 Chemical structure of valnemulin.

In the current study, an experimental *M. gallisepticum*-infected neutropenic chicken model was developed, and the PK of valnemulin at 1, 10, and 20 mg/kg of body weight was evaluated. The dose proportionality of valnemulin was evaluated in a range of 1 to 20 mg/kg. RT-PCR was used for quantitative detection of *M. gallisepticum in vivo*. An *in vivo* PK-PD study of valnemulin against *M. gallisepticum* was established to identify the PK-PD index associated with variable valnemulin effectiveness and to elucidate the magnitude of the PK-PD parameters most predictive of effectiveness. These studies were used with MICs to provide a rational approach to the design of a dosage regimen that optimizes effectiveness with respect to bacteriological and clinical outcomes.

MATERIALS AND METHODS

Organisms, chemicals, and susceptibility assay. The *M. gallisepticum* standard strain S6 was purchased from the Chinese Veterinary Microorganism Culture Collection Center (Beijing, China). Valnemulin hydrochloride (>99%) was kindly supplied by Guangdong Dahuanong Animal Health Products Co., Ltd. (Guangdong, China). The structure of valnemulin is shown in Fig. 1. The MIC of valnemulin against the study strain *M. gallisepticum* S6 was determined by a broth dilution method, according to recommended protocols (14). Briefly, the *M. gallisepticum* strain was cultured to reach exponential phase and diluted to $\sim 2 \times 10^5$ color-changing units (CCU)/ml. A series of concentrations of valnemulin were prepared by doubling dilution (final concentration, 3.5×10^{-4} to 1.44 μg/ml) with *M. gallisepticum* medium. A 0.1-ml aliquot of the medium with different concentrations of valnemulin was added to a 96-well plate, and another 0.1-ml aliquot of prepared *M. gallisepticum* S6 was supplemented to each well, achieving final titers of $\sim 1 \times 10^5$ CCU/ml. The tests were conducted in triplicate and included growth controls (*M. gallisepticum* in the medium only), endpoint controls (blank medium adjusted to pH 6.8), and germfree controls (blank medium only). The plates were cultured at 37°C. Changes in color were monitored every 4 h until the color of the growth control was the same as that of the endpoint control. The MIC was determined as the minimal concentration of valnemulin that resulted in no change in color.

***In vitro* standard DNA preparation.** Overnight cultures of the *M. gallisepticum* strain were used to inoculate 20 ml of medium specifically sold as "*M. gallisepticum* medium," purchased from Qingdao Hope Bio-Technology Co., Ltd. (Shandong, China), to a starting concentration of 10^5 CCU/ml, and these were allowed to grow for 36 h at 37°C. At 36 h of incubation, 20 ml of the sample was centrifuged for 10 min at 1,500 rpm, and the pellet was resuspended in 0.6 ml of *M. gallisepticum* medium. The sample was serially diluted for organism counting by culture. Meanwhile, DNA was isolated from serial 10-fold dilutions (10^0 to 10^{-6}) prepared from the 0.6-ml sample with a bacterial DNA kit (Omega Bio-Tek, Inc., Norcross, GA). Isolated DNA was aliquoted in 30-μl volumes and stored at −80°C. The DNA copies of *M. gallisepticum* were determined by real-time PCR (RT-PCR) (12). The *in vitro* standard DNA curve was plotted by the number of *M. gallisepticum* organisms derived from the culture method and the cycle threshold (C_T) values obtained using RT-PCR results, as previously described (12, 13).

Neutropenia model. In order to study the effectiveness of valnemulin solely and to eliminate immunity caused by different variables among different chickens, a neutropenic model was used. The South China Agriculture University Animal ethics committee approved all *in vivo* experiments (March 2014). In addition, all husbandry practices and experimental operations were performed with full consideration of animal welfare. One-day-old chickens (weighing 35 to 45 g) were provided from the Guangdong Academy of Agricultural Sciences (Guangzhou, Guangdong, China). The chickens were *M. gallisepticum* free and fed with antibacterial-free food and water *ad libitum*. Two days postarrival, the chickens were rendered neutropenic with cyclophosphamide (Puboxin Biotechnology Co., Ltd., Beijing, China) administered intramuscularly (i.m.) at 60 mg/kg for 3 days (15). Blood was drawn from the neck vein, and leukocytes were counted with smears. The animals were severely granulocytopenic (absolute leukocyte count, $<1{,}000/mm^3$) and remained so for 8 days after the last injection of cyclophosphamide.

***M. gallisepticum* intratracheal infection model.** *M. gallisepticum* is a major cause of CRD in chickens. Thus, a *M. gallisepticum* intratracheal infection model was utilized in this study. Briefly, neutropenic chickens were inoculated with a 0.2-ml aliquot solution containing approximately 10^8 CCU of the *M. gallisepticum* strain, a 95% infective dose (ID_{95}) for the study strain, as established in pilot studies, via intratracheal injection for 3 days. Clinical symptoms and bacteriological examinations were performed to ensure infection. To quantify the pathogen load, tracheae, air sacs, and lungs were removed at the time of sacrifice, homogenized in 2 ml of phosphate-buffered saline (PBS), and centrifuged at 500 rpm for 5 min. A 0.5-ml aliquot of supernatant was used for DNA extraction with a bacterial DNA kit (Omega Bio-Tek, Inc., Norcross, GA). A DNA template was used for C_T value determination through RT-PCR. To confirm the recovery rate of DNA extraction from chicken lung, trachea, and air sac tissue samples, the same amount of 10-fold dilutions (10^0 to 10^{-6}) of an *M. gallisepticum* sample from an *in vitro* DNA standard curve was added to blank chicken lung, trachea, and air sac tissue samples, and the same procedure as described above for the samples was used.

Determination of DNA copies of *M. gallisepticum* using RT-PCR. RT-PCR was used to identify DNA copies of *M. gallisepticum* using *M. gallisepticum*-specific primers Mg14F (5′-GAG CTA ATC TGT AAA GTT GGT C-3′; melting temperature [T_m], 57.80°C) and Mg13R (5′-GCT TCC TTG CGG TTA GCA AC-3′; T_m, 63.6°C), as described in a previous study (12). All RT-PCRs were performed on a Bio-Rad iQ5 (Bio-Rad Laboratories, Inc., USA) using the SYBR *Ex Taq* premix (TaKaRa, Shiga, Japan). The DNA template extracted from samples or elution buffer (negative control), the forward and reverse primers per reaction, SYBR premix, and water were mixed according to recommended procedures, at a final volume of 25 μl. The PCR mixture was incubated for 40 cycles, and fluorescence was monitored during each cycle at 72°C. After amplification, a melting curve analysis was performed starting at 65°C, using a temperature transition rate of 0.2°C/s to determine the T_m values of the PCR products. The C_T value was defined as the cycle number yielding a maximum value of the second derivative of the amplification curve of the sample. Samples were regarded as positive when both a measurable C_T and the expected T_m (± 0.5°C) were seen.

Valnemulin PK in the neutropenic intratracheal infection model. Infected neutropenic chickens were treated with i.m. valnemulin at a single doses of 1, 10, or 20 mg/kg. Blood samples in 1.5-ml aliquots were collected from the neck vein at 10 min, 30 min, and 1, 2, 4, 6, 8, 12, and 24 h after drug administration. Ten chickens were sampled at each time point. Serum was obtained by centrifuging blood samples at 3,000 rpm for 10 min and freezing at −20°C immediately until analysis (within 2 weeks). The valnemulin concentrations in serum were determined via a high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, as described previously (16–18). The limit of quantita-

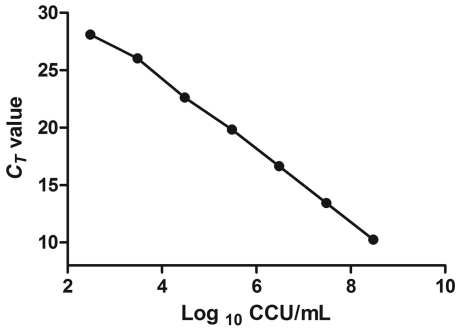

FIG 2 *In vitro* DNA standard curve. Relationship of DNA standards between C_T value and culture results (in $\log_{10}$ CCU per milliliter). The mean values from three different RT-PCR runs are shown.

tion was confirmed to be 2.5 ng/ml (19). The coefficient of correlation (R^2) was 0.9992 for the linear range of 2.5 to 500 ng/ml. To overcome the carryover effect, water was inserted in the detection queue for every 10 samples. The intra- and interday coefficients of variation were determined to be 5.2% and 8.7%, respectively.

Efficacy of valnemulin in neutropenic chicken intratracheal infection model. To evaluate the effectiveness of valnemulin, infected neutropenic chickens were treated with either 0.85% NaCl (control) or i.m. valnemulin at 1, 2, 4, 6, 8, 10, 15, or 20 mg/kg once daily at 24 h postinfection for 3 days. At 24 h after the last drug administration, the animals were euthanized, and the tracheae, air sacs, and lungs were collected, homogenized in 2 ml of PBS, and centrifuged at 500 rpm for 5 min. A 0.5-ml aliquot of supernatant was used for DNA extraction with a bacterial DNA kit (Omega Bio-Tek, Inc., Norcross, GA), as described above. The number of DNA copies of *M. gallisepticum* in these samples was measured by RT-PCR, as described above. The amount of *M. gallisepticum* was expressed as color-changing unit equivalents per milliliter.

PK and PD analysis. The PK profiles of valnemulin were analyzed by the noncompartmental model with uniform weighting using the WinNonlin software (version 6.1; Pharsight, CA, USA). The surrogate marker of antibacterial effectiveness, AUC_{24}/MIC, was calculated using *in vitro* MIC values and PK parameters derived from three i.m. administrations of valnemulin. The bacterial load for each sample was calculated according to the *in vitro* standard DNA curve of the C_T value and bacteria counting by the culture method, measured as the color-changing unit equivalents per milliliter. The effectiveness of valnemulin was expressed as the reduction in *M. gallisepticum* load after treatment compared to that before treatment. The *in vivo* PK-PD relationship of valnemulin was described using a sigmoid maximum effect (E_{max}) model in the WinNonlin software (version 6.1; Pharsight), with the following equation:

$$E = E_0 + \frac{E_{max} \times C_e^N}{EC_{50}^N + C_e^N}$$

where E is the change in $\log_{10}$ color-changing unit (CCU) equivalents per milliliter for different dosage regimens, E_0 is the change in $\log_{10}$ CCU equivalents per milliliter in the control sample (absence of valnemulin), E_{max} is the difference in effect between the greatest amount of growth (as seen for the growth control, E_0) and the greatest amount of kill, C_e is the AUC_{24}/MIC in the effect compartment, EC_{50} is the AUC_{24}/MIC value producing a 50% reduction in bacterial counts, and N is the Hill coefficient that describes the steepness of the AUC_{24}/MIC effect curve (20).

Dosage calculation. In order to deduce a more rational regimen, the general formula adapted by Toutain, Bousquet-Mélou, and Martinez (21) was employed to estimate the dosages for different magnitudes of efficiency:

$$\text{Dose} = \frac{\text{CL} \times (\text{AUC}_{24}/\text{MIC}) \times \text{MIC}_{90}}{F \times fu}$$

where dose is the optimal dose (in milligrams per kilogram of body weight per day), CL is body clearance (in liters per kilogram per hour), AUC_{24}/MIC is the breakpoint marker for the desired effect, MIC_{90} is the MIC inhibiting 90% of strains (in milligrams per liter), F is the bioavailability, and *fu* is the free drug fraction.

RESULTS

Susceptibility testing. The MIC of valnemulin against the study strain was 0.0014 μg/ml.

***In vitro* standard DNA curve and recovery rate.** There was a significant correlation between the C_T values and $\log_{10}$ color-changing units per milliliter ($y = -3.028x + 36.155$; $R^2 = 0.9976$; Fig. 2), indicating that the correlation between the molecular and culturing methods is significant. The limit of detection was 3×10^2 CCU equivalents/ml. A small range (0.087 to 0.45) of standard deviations of the C_T values was observed. The recovery rates at different dilutions were 56.5% ± 5.08%.

Neutropenia model. The animals were severely granulocytopenic (absolute leukocyte count, <1,000/mm^3) and remained so for 8 days after the last injection of cyclophosphamide.

***M. gallisepticum* intratracheal infection model.** An evaluation of the *M. gallisepticum* infection model mainly depended on clinical symptoms and bacteriological assays. Depression, mouth breathing, and eye closures were observed in infected animals. In addition, air sacculitis, the cardinal sign of *M. gallisepticum*, infection was noticed in 95% of the infected chickens. The mean *M. gallisepticum* load was 0.3×10^6 CCU for all inoculated chickens. The morbidity and mortality rates were 95% and 17%, respectively, at 5 days after infection. Neither clinical signs nor air sacculitis were observed in noninfected control animals. The bacteriological assay was negative in the control chickens.

PK profiles of valnemulin in neutropenic intratracheal infection model. The main PK parameters are presented in Table 1. The maximum concentration of drug in serum (C_{max}) values were 6.34, 71.32, and 111.13 μg/ml for 1-, 10-, and 20-mg/kg doses, respectively, which were observed at 30 min after administration (Fig. 3). The half-life ($t_{1/2\beta}$) was approximately 2.8 h for all three different doses. In addition, a concentration-dependent AUC_{24} was seen (13.88, 125.96, and 213.61 μg · h/ml for 1-, 10-, and 20-mg/kg doses, respectively). Importantly, a significant correlation between dose and AUC_{24} or C_{max} was observed ($R^2 = 0.9899$ and 0.972 for dose-to-AUC_{24} and dose-to-C_{max} ratios, respectively; Fig. 4). As the AUC_{24} increased in a dose-dependent manner from 1 to 20 mg/kg, the AUC_{24} of 2, 4, 6, 8, and 15 mg/kg was

TABLE 1 Pharmacokinetic parameters of valnemulin in serum following single-dose intramuscular administration various doses in *M. gallisepticum* infected chickens ($n = 10$/group)

Parameter[a]	Results for valnemulin dose (mg/kg) of:		
	1	10	20
C_{max} (μg/ml)	6.34	71.32	111.13
T_{max} (h)	0.5	0.5	0.5
$t_{1/2\beta}$ (h)	2.78	2.81	2.71
AUC_{24} (μg · h/ml)	13.88	125.96	213.61
CL_β/F (liters/h/kg)	0.072	0.079	0.093
V_z/F (liters/kg)	0.29	0.32	0.36

[a] C_{max}, maximum serum concentration; T_{max}, time of maximum serum concentration; $t_{1/2\beta}$, elimination half-life; AUC_{24}, 24-h area under serum concentration-time curve; F, bioavailability; CL_β/F, clearance divided by bioavailability; V_z/F, volume of distribution scaled by bioavailability.

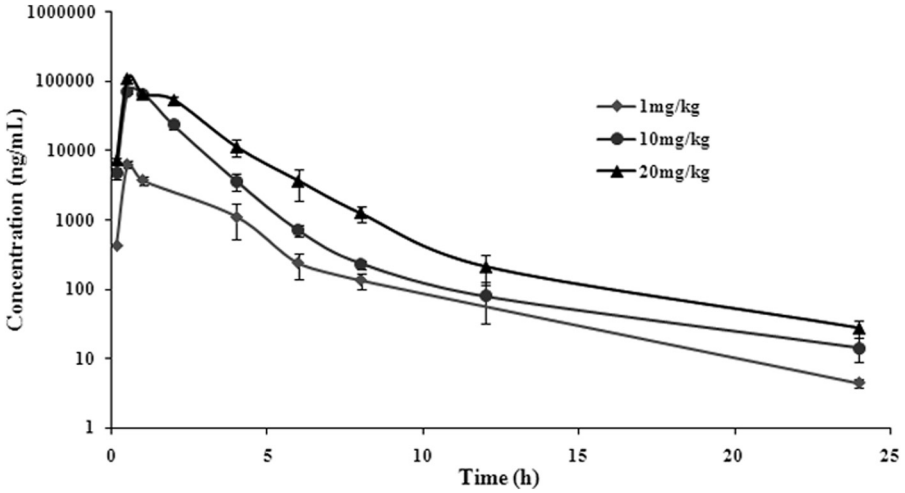

FIG 3 Serum concentrations of valnemulin following single-dose intramuscular administration at 1, 10, or 20 mg/kg in *M. gallisepticum* intratracheal infection model ($n = 10$/time point).

calculated according to the linear relationship. These results indicate that the analytic chemistry method is a reasonable tool to derive meaningful PK profiles and may predict valnemulin efficacy.

***In vivo* effectiveness of valnemulin in neutropenic chicken intratracheal infection model.** The C_T values of respiratory tissues from chickens administered valnemulin doses of 0, 1, 2, 4, 6, 8, 10, 15, or 20 mg/kg increased with increasing dose, implying that the bacteria load decreased. The C_T value increased sharply between the doses of 1 and 8 mg/kg and gently from 8 to 20 mg/kg. The calculated numbers *in vivo* of *M. gallisepticum* after drug treatment are shown in Fig. 5.

Valnemulin PK-PD profiles. The AUC_{24}/MIC values for doses of 0, 1, 2, 4, 6, 8, 10, 15, and 20 mg/kg were 0, 9,917, 21,778, 36,750, 51,714, 66,692, 89,971, 119,092, and 152,579, respectively. The parameter AUC_{24}/MIC correlated significantly with effectiveness ($R^2 = 0.9669$). The profile of sigmoid E_{max} model describing the relationship of antibiotic effectiveness and AUC_{24}/MIC is presented in Fig. 6. The AUC_{24}/MIC ratios for mycoplasmastasis (reduction of 0 $\log_{10}$ CCU/ml), a reduction of 1 $\log_{10}$ CCU/ml, and a reduction of 2.5 $\log_{10}$ CCU/ml were 28,820, 38,030, and 56,256, respectively (Table 2). The EC_{50} was 39,299, and the slope of the graph of the AUC_{24}/MIC ratio versus effectiveness was 9.98.

Dosage calculation. For dosing calculations, bioavailability had been taken into account, owing to the extravascular route of administration, and the free-drug fraction was not required for using PD data generated *in vivo*. The MIC of S6 was substituted for the MIC_{90}, because not enough valnemulin MIC data were available to provide an estimate of the MIC_{90}. A dosage of 6.5 mg/kg was calculated for a reduction of 2.5 $\log_{10}$ CCU/ml.

DISCUSSION

M. gallisepticum is a highly transmissible and persistent pathogen in chickens, turkeys, and some wild birds. Significant losses in all sectors of the poultry industry have occurred due to infection by this organism (22). Outbreaks have been seen even in vaccinated birds, demonstrating that further means of control are needed (23).

Valnemulin shows excellent effectiveness for treating mycoplasmal infection and rarely induces significant resistance (16). Its

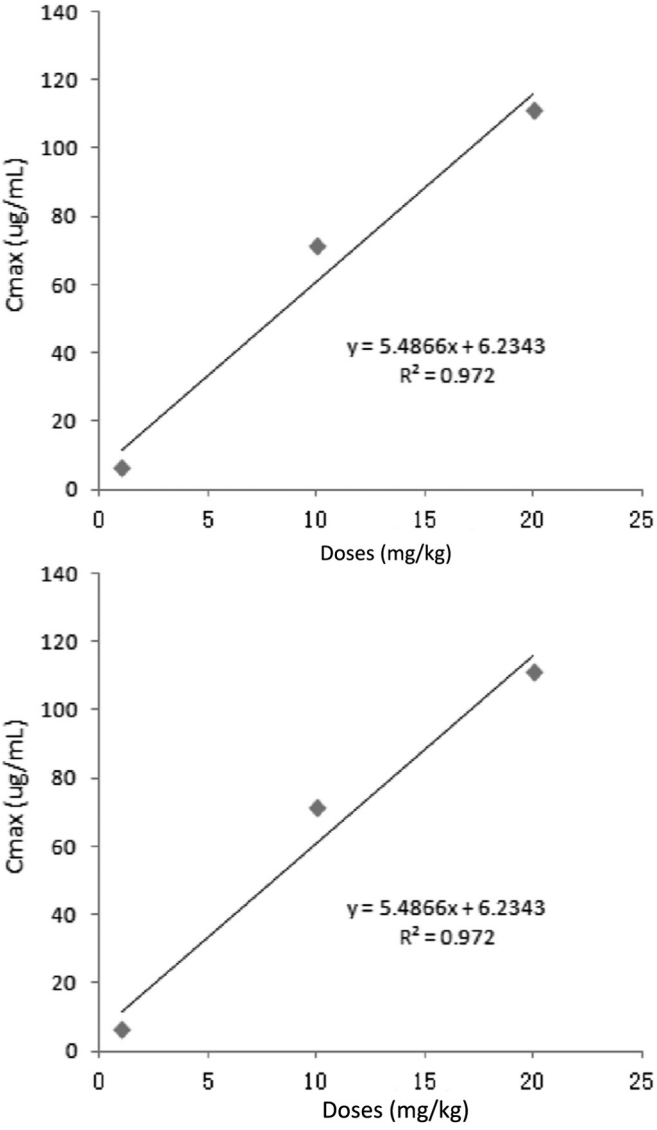

FIG 4 Linear regression plots between administered dose and C_{max} values, and between administered dose and AUC_{24} values.

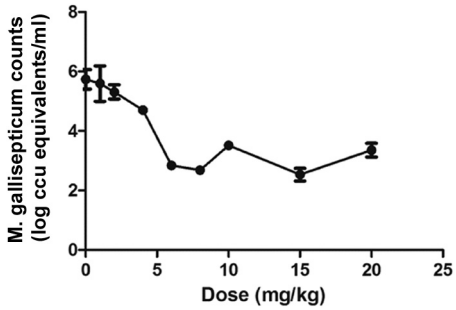

FIG 5 Calculated *in vivo M. gallisepticum* counts after valnemulin treatment ($n = 4$/dose).

TABLE 2 Pharmacodynamic analysis of valnemulin in *M. gallisepticum* intratracheal infection model

Parameter[a]	Value
E_{max} ($\log_{10}$ CCU equivalents/ml)	2.69
E_0 ($\log_{10}$ CCU equivalents/ml)	0.11
EC_{50}	39,299
AUC/MIC for reduction of ($\log_{10}$ CCU equivalents/ml):	
0	28,820
1	38,030
2.5	56,256
Slope (N)	9.98

[a] E_0, change in $\log_{10}$ CCU equivalents/ml in the control sample (absence of valnemulin); E_{max}, difference in effect between the greatest amount of growth (as seen for the growth control, E_0) and the greatest amount of kill; EC_{50}, AUC_{24}/MIC ratio producing a 50% reduction in bacterial counts; N, Hill coefficient that describes the steepness of the AUC_{24}/MIC effect curve.

PK character is also favorable; however, its chemotherapeutic properties are sparse. The use of a PK-PD model to identify the PD activity by integrating the PK character, MIC, and pathogen load outcome has been proven helpful in the design of rational dosage regimens in humans and animals and in the determination of susceptibility breakpoints (24, 25).

In the present investigation, a number of interesting findings emerged. First, to the best of our knowledge, this is the first time the dose proportionality of valnemulin PK in the range of 1 mg/kg to 20 mg/kg has been described in chickens. The rate and extent of absorption were constant among tested doses (1, 10, and 20 mg/kg), with clearance and distribution volume values of 0.072, 0.079, and 0.093 h and 0.29, 0.32, 0.36 liters/kg, respectively. The significant correlation between dose and AUC_{24} ($R^2 = 0.9899$) allows us to calculate the AUC_{24} for other dosages. This phenomenon was confirmed in other research, i.e., for marbofloxacin PK in pigs within doses ranging from 4 to 16 mg/kg (26); however, our estimated PK parameters were somewhat different from those published previously for chickens infected by *M. gallisepticum* plus *Escherichia coli* (17). After i.m. administration at 10 mg/kg, the drug was absorbed quickly, with a C_{max} of 71.32 μg/ml achieved at 0.5 h in 9-day-old infected chickens, while the mean C_{max} values of 27.94 μg/ml and time to C_{max} (T_{max}) of 1.57 h occurred in adult chickens infected with *M. gallisepticum* plus *E. coli*. In addition, the $t_{1/2\beta}$ was much shorter in our model, with a value of 2.81 h compared to the value of 6.50 h in the chickens infected with *M. gallisepticum* plus *E. coli*. This difference may be explained by age and pathological situation. In our study, *M. gallisepticum* caused only respiratory system injury in 9-day-old chickens, while the injury from *M. gallisepticum* plus *E. coli* affected the whole body (lungs, liver, kidneys, heart, and others) in the adult chicken (17). These results emphasize the influence of age and physiological status on pharmacokinetics (27, 28).

Our data show a significant correlation between the PK-PD index, AUC_{24}/MIC, and the *in vivo* antibacterial effects of valnemulin against *M. gallisepticum* ($R^2 = 0.9669$), which was in accordance with previous studies of valnemulin against *Staphylococcus aureus* (18, 29). The AUC_{24}/MIC ratios for mycoplasmastasis (a

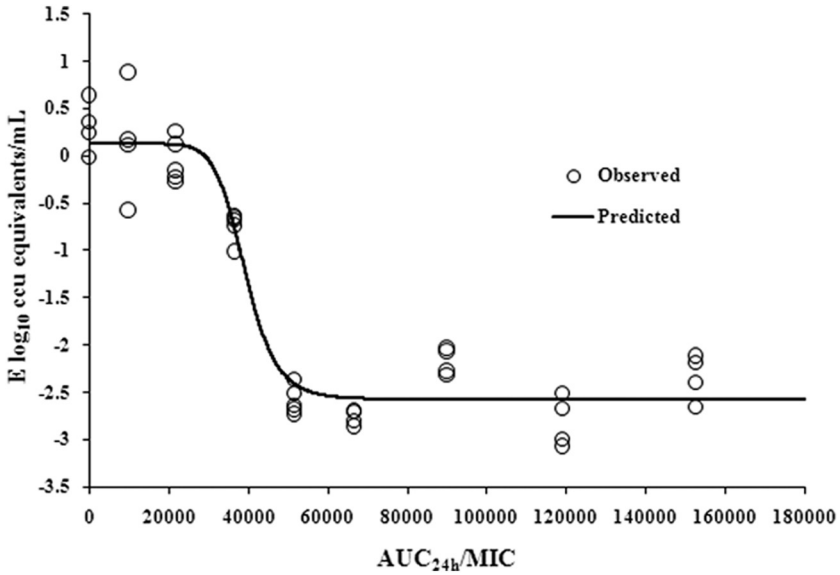

FIG 6 Sigmoid E_{max} relationships between antimycoplasmal effect (E, $\log_{10}$ CCU equivalents/ml) and *in vivo* AUC_{24}/MIC ratio against *M. gallisepticum* in serum of chickens.

reduction of 0 $\log_{10}$ CCU equivalents/ml), a reduction of 1 $\log_{10}$ CCU equivalents/ml, and a reduction of 2.5 $\log_{10}$ CCU equivalents/ml (28,820, 38,030, and 56,256, respectively) were much higher than those resulting *ex vivo* (634, 1,297, and 1,987 for reductions of 0, 3, and 4 $\log_{10}$ CCU/ml, respectively) (30). This may due to the following reasons. First, the antibacterial concentration is constant in *ex vivo* PK-PD studies, while the concentration of antibacterial drug declined in animals because of body clearance. Next, in *ex vivo* studies, the drug acts on *M. gallisepticum* directly, but *in vivo*, the concentration in serum was not the real one acting against *M. gallisepticum*, as the infection site is the air sac or respiratory system, and valnemulin concentrations in air sacs or lungs are usually different from that in serum. Finally, *in vivo* PK-PD takes all the infection factors into consideration, which may influence the ratio. The *in vivo* PK-PD model showed great advantage over *in vitro* or *ex vivo* PK-PD models, as it considered the complex environment of the infected animals. In addition, we recognize that the dose fractionation studies were not included in the current manuscript. Therefore, the PD index of valnemulin was not entirely demonstrated. Future studies will be designed to adjudicate the effects of these limitations.

We also demonstrated that in order to achieve a reduction of $\geq$2.5 $\log_{10}$ CCU equivalents/ml in bacterial counts, the recommended daily dose would be $\geq$6.5 mg/kg daily for 3 days when valnemulin was applied to treat *M. gallisepticum*-infected chickens, with an MIC of 0.0014 μg/ml, which is almost half of the recommended dose range for swine enzootic pneumonia (10 to 12 mg/kg) (31). This recommended daily dose has been proven to be effective but less expensive for farmers. Additionally, a much lower drug dosage can be more ecofriendly, cost-effective, and minimize the drug residue burden on public health via human food consumption.

The gold standard for evaluating the effectiveness of an antibacterial drug is culturing and counting bacteria; however, the results for *M. gallisepticum* were usually influenced by overgrowth of faster growing *Mycoplasma* species or were impeded by other organisms or no growth in subculture. In addition, the cultivation technique is expensive, laborious, and requires a lot of time (32). Real-time PCR methodology, which is reported to be capable of quantitative detection of *M. gallisepticum* in an *in vivo* model and from clinical samples has significantly overcome the problems with time, specificity, and sample size (12).

In this study, neutropenic chickens were used to remove host immunity factors that may play an important role in *M. gallisepticum* infections and in antimicrobial effectiveness. Previous studies suggest that the PK-PD index magnitude necessary for successful therapy is reduced in animal models with neutrophils present (33). Therefore, further studies on the elucidation of host immunity-pathogen interactions are essential. In addition, a population PK approach should be conducted to derive a population clearance of valnemulin for use in dose calculations. Moreover, MIC distributions should be evaluated to take variations in susceptibility of *M. gallisepticum* to valnemulin into account. This dose regimen should be validated in clinical practice by evaluating therapeutic outcomes and monitoring resistance development.

In conclusion, the present study characterized the *in vivo* effectiveness of valnemulin against *M. gallisepticum* in a neutropenic chicken model. The *in vivo* data suggest that animal dosage regimens should supply an AUC_{24}/MIC of valnemulin of 56,256 for a reduction of 2.5 $\log_{10}$ CCU equivalents/ml of *M. gallisepticum*. These studies suggest that valnemulin, if used for the treatment of *M. gallisepticum*, with an MIC_{90} at 0.0014 μg/ml, would benefit from a valnemulin dose of 6.5 mg/kg once daily for 3 days, which is convenient for use in the clinical setting.

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