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The dose regimen formulation of doxycycline hydrochloride and florfenicol injection based on ex vivo pharmacokinetic-pharmacodynamic modeling against the *Actinobacillus pleuropneumoniae* in pigs

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Abstract

Doxycycline hydrochloride and florfenicol combination (DoxHcl&FF) is an effective treatment for respiratory diseases. In the study, our objective was to evaluate the activity of DoxHcl&FF against *Actinobacillus pleuropneumoniae* (APP) in porcine pulmonary epithelial lining fluid (PELF) and the optimal dosage scheme to avoid the development of resistance. The DoxHcl&FF was administered intramuscularly (IM) at 20 mg/kg, and the PELF was collected at different time points. The minimum inhibitory concentration (MIC) and time-mortality curves were also included in the study. Based on the sigmoid E_{max} equation and dose equations, the study integrated the in vivo pharmacokinetic data of infected pigs and ex vivo pharmacodynamic data to obtain the area under concentration time curve ($AUC_{0\text{-}24h}$)/MIC values in PELF and achieve bacteriostatic activity, bactericidal activity and the virtual eradication of bacteria. The study showed that the combination of DoxHcl and FF caused no significant changes in PK parameters. The peak concentration (C_{max}) of FF in healthy and diseased pigs was 8.87 ± 0.08 μg/mL and 8.67 ± 0.07 μg/mL, the $AUC_{0\text{-}24h}$ were 172.75 ± 2.52 h·μg/mL and 180.22 ± 3.13 h·μg/mL, the C_{max} of DoxHcl was 7.91 ± 0.09 μg/mL and 7.99 ± 0.05 μg/mL, and the $AUC_{0\text{-}24h}$ was 129.96 ± 3.70 h·μg/mL and 169.82 ± 4.38 h·μg/mL. DoxHcl&FF showed strong concentration-dependent tendencies. The bacteriostatic, bactericidal, and elimination activity were calculated as 5.61, 18.83 and 32.68 h, and the doses were 1.37 (bacteriostatic), 4.59 (bactericidal) and 7.99 (elimination) mg/kg. These findings indicated that the calculated recommended dose could assist in achieving more precise administration, increasing the effectiveness of DoxHcl&FF treatment for APP infections.

Keywords Florfenicol, Doxycycline hydrochloride, PK-PD, Pig, Dose regimen, *Actinobacillus pleuropneumoniae*

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Introduction

Actinobacillus pleuropneumoniae (APP) refers to a bacterial pathogen that has a significant economic impact in the pig industry worldwide by inducing porcine pleuropneumonia, and the economic burden of this disease is mainly the result of the acute outbreaks, which will bring high mortality, loss of production and high medical costs (Sassu et al. 2018; Bao et al. 2019).

Florfenicol (FF) is a promising antibacterial agent for the treatment of APP in the pig industry (Shin et al. 2005), and researchers at home and abroad have conducted extensive research on its pharmacodynamic (PD) and pharmacokinetic (PK), proving it is sensitive to APP (Catry et al. 2008). FF binds to the 50S subunit and inhibits phthaloyl transferase, thereby inhibiting the extension of the phthaloyl chain and interfering with protein synthesis. In recent years, due to its unreasonable use and the serious problem of drug resistance, the use of FF is not optimistic in the field of pig pneumonia treatment (da Silva et al. 2017). Clinically, Doxycycline hydrochloride (DoxHcl) was commonly used to treat bacterial diseases in livestock such as pigs and chickens. It is sensitive to respiratory pathogens, especially APP and *Pasteurella multocida*. Some researchers have reported that the combination of two or more antibacterial drugs can appropriately improve the efficacy and reduce drug resistance (Ayukekbong et al. 2017; Lima et al. 2017). Moreover, some studies were proved that FF and DoxHcl have a certain synergy in pharmacokinetics. After intramuscular injection in pigs, it can reach the peak plasma concentration rapidly within 1–3 h, and for the concentration in plasma, the peak concentration (C_{max}) is not much different and the half-life of the two is relatively long. It shows that the effective concentration can be maintained for the same time after the combination of these two drugs, which exerts a good synergistic effect on the infection site and improves the therapeutic effect (Liu et al. 2003; Kim et al. 2008). Therefore, it is necessary to optimize the dosing regimen of the combination drug to improve the curative effect, reduce the generation of drug resistance and provide an alternative drug for clinical medication. Nevertheless, there are important issues that urgently need to be assessed, such as the formula, dosage and administration scheme of the combined drug and its safety (Rivero-Juarez et al. 2019).

Inappropriate choice and suboptimal dosing of antimicrobials have been identified as major factors driving the emergence of antimicrobial resistance (Toutain and Lees 2004; Bhardwaj et al. 2019). Inadequate dosing can cause resistance in zoonotic pathogens and/or commensal bacteria, which is a major public health concern (Toutain and Lees 2004). The PK-PD model, which reflects the relationship among the host, drug and pathogens, can predict and formulate a rational drug regimen. For time-dependent antimicrobials and concentration-dependent antimicrobials, the primary PK-PD efficacy indices are the C_{max}/minimal inhibitory concentration (MIC), AUC/MIC and peak time (T_{max}) > MIC (Nielsen and Friberg 2013; Bhardwaj et al. 2019). Currently, for PK-PD studies, when dealing with PK dates of multi-component Chinese drugs, some methods can be used, mainly including the biological effect method and the multi-effect component integrated PK method (Yan et al. 2018). In terms of the biological effect method, it was difficult to choose appropriate quantitative activity indicators, which had relatively large limitations. For the multi-effect component integrated PK method, the method is still in the theoretical stage. Some researches, have analyzed the PK and PD parameters of each component separately after the interaction of the compound drug, establishing a PK-PD model method for combination drugs and determining the best drug regimen (Louie et al. 2013; Fu et al. 2016). Those methods analyze the PK and PD of each component after drug interaction, considering not only the PD of each component against pathogens, but also calculating the concentrations and PK parameters of each component.

However, there is still no report on the comprehensive PK-PD data required for dosing optimization of DoxHcl&FF. Therefore, the study aimed to reveal the antibacterial activity of combined FF and DoxHcl against APP isolates from piglets and to formulate the optimal dose regimen of FF/DoxHcl based on PK-PD for the treatment of piglets' respiratory diseases. It was conducted with the objectives of establishing (a) the MIC, minmum bactericidal concentration (MBC), minmum prevention concentration (MPC) and sterilization curve of FF, DoxHcl and DoxHcl&FF on APP; (b) the PK of FF injection, DoxHcl injection and DoxHcl&FF injection following intramuscular injection; (c) integration and modeling of PK-PD data to determine the optimum dosage regimen of DoxHcl&FF injection against APP in piglets.

Results

The virulence of APP

The virulence test was performed on six strains of MIC_{90} APP. After resuscitation of the selected six strains of APP, the prepared concentrations of APP were 10^7 CFU/mL, 10^8 CFU/mL and 10^9 CFU/mL. The results showed that no mice died in the control group. APP BW1 was the most pathogenic strain to mice, with a mortality rate of 100% within 12 h. All results were shown in Supplementary Table 1 and were used to study the antimicrobial activity of DoxHcl&FF (1:1) both in vitro and ex vivo.

Distribution of MIC for APP

The distributions of MIC for the 131 APP strains in response to FF, DoxHcl and DoxHcl&FF (1:1) are shown in Fig. 1. The MIC_{90} values were 8, 8 and 2 μg/mL, respectively. According to the MIC_{90} values of the strains, six strains (BW1, BW48, BW4, BW45, BW42, BW14) for which MIC was similar to 131 APP strains in response to DoxHcl&FF (1:1), FF and DoxHcl were selected.

Antibacterial activity of FF, DoxHcl and DoxHcl&FF against APP

The results in Supplementary Table 2 showed that MIC of both FF and DoxHcl was 8 μg/mL. MIC of DoxHcl & FF was (2/2) μg/mL and the value of FIC was 0.5. Thus, DoxHcl and FF had a synergistic effect on APP BW1 at a ratio of 1:1, and this met the requirements of a combination medication for pharmacodynamics.

MIC, MBC, and MPC of FF, DoxHcl, and DoxHcl&FF (1:1) against APP BW1

MIC of FF, DoxHcl and DoxHcl&FF (1:1) against APP BW1 was 8, 8 and 2 μg/mL in TSB, and 8, 8 and 2 μg/mL in PELF. According to the micro-dilution technique, MBC was 8, 8, 4 μg/mL in TSB and PELF, and MPC was 12.8, 12.8, 6.4 μg/mL in TSB, respectively. The MICs and MBCs obtained from the TSB broth and PELF were not significantly different, indicating that the composition of the growth matrix does not affect the antibacterial activity of DoxHcl&FF.

Pharmacokinetic analysis of FF, DoxHcl and DoxHcl&FF (1:1) in plasma and PELF

The mean ± SD of the DoxHcl&FF (1:1) concentration-time curves in PELF after intramuscular injection are shown in Fig. 2. The drug concentration of FF and DoxHcl were best fitted by the two-compartment model, and the drug concentration could reach MIC at 0.5–24 h and MBC at 1–9 h. In addition, the main PK parameters of FF, DoxHcl and DoxHcl&FF are shown in Tables 1 and 2. The area under the drug-time curves of the healthy and compounded DoxHcl&FF in the healthy and diseased plasma FF groups were 91.86 h.μg/mL, 105.52 h.μg/mL and 115.05 h.μg/mL, respectively, with peak times of 3.41 h, 3.45 h and 3.67 h, peak concentrations of 4.13 μg/mL. The peak concentrations were 4.13 μg/mL, 4.08 μg/mL and 4.09 μg/mL, the distribution half-lives were 3.62 h, 3.04 h and 4.12 h, the elimination half-lives were 22.61 h, 20.57 h and 22.58 h, and the ratios of body clearance to bioavailability were 0.22 L/h/kg, 0.19 L/h/kg and 0.17 L/h/kg, respectively. There were no differences in pharmacokinetic parameters between healthy FF and diseased FF groups, and the absorption distribution and metabolic processes of FF in pigs after FF and DoxHcl formed a compound drug compared with the pharmacokinetic processes of single-formula FF. The area under the pharmacokinetic curves of the healthy group with mono-prescription DoxHcl and the healthy plasma DoxHcl group with compound DoxHcl&FF and the diseased plasma DoxHcl group were 68.20 h.μg/mL, 72.77 h.μg/mL and 73.29 h.μg/mL, respectively, with peak times of 1.86 h, 1.97 h and 2.04 h, and the peak concentrations were 3.63 μg/mL, 3.58 μg/mL and 3.60 μg/mL, the distribution half-lives were 4.65 h, 4.20 h and 3.76 h, the

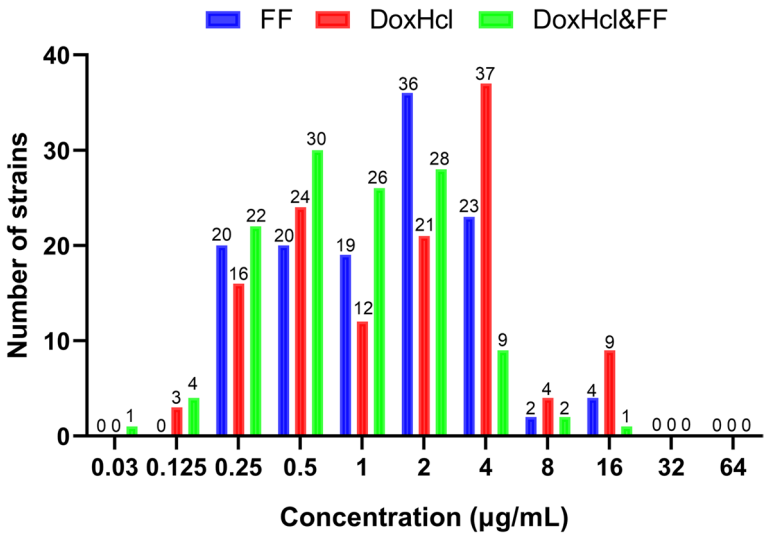

Fig. 1 MICs of APP in response to FF (blue), DoxHcl (red) and DoxHcl&FF (green)

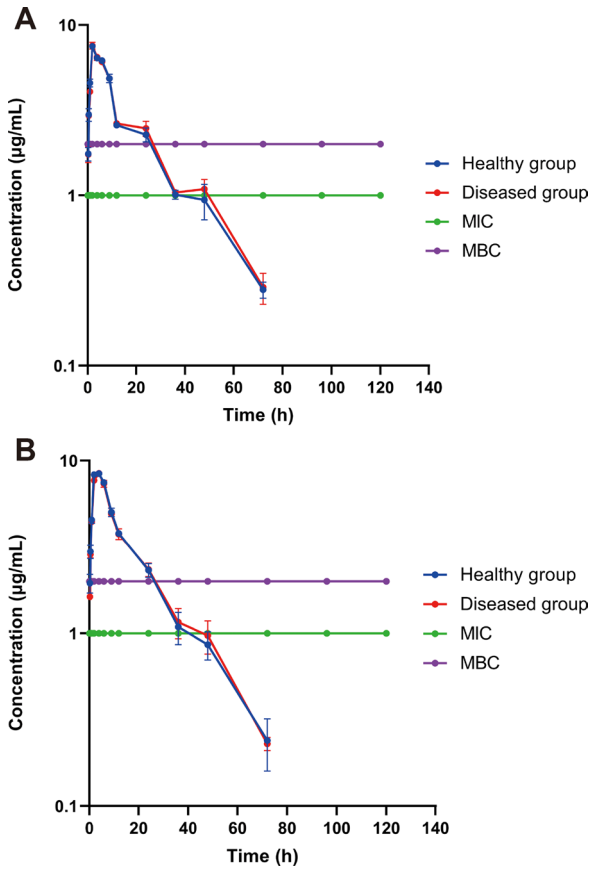

Fig. 2 Mean concentration versus time curves for DoxHcl&FF in PELF: FF (**A**), DoxHcl (**B**)

elimination half-lives were 16.32 h, 14.90 h and 15.37 h, and the ratios of body clearance to bioavailability were 0.29 L/h/kg, 0.27 L/h/kg and 0.27 L/h/kg, respectively. The pharmacokinetic parameters of the healthy DoxHcl group and the diseased DoxHcl group were not different, and the absorption distribution and metabolic process of DoxHcl in pigs after the combination of DoxHcl and FF were not different compared with the pharmacokinetic process of DoxHcl alone. In addition, the area under the drug-time curves for the healthy group with single FF and the healthy alveolar fluid FF group with compound DoxHcl&FF and the diseased alveolar fluid FF group were 144.22 h.μg/mL, 172.75 h.μg/mL and 180.22 h.μg/mL, respectively. The peak times were 3.14 h, 2.90 h and 3.07 h. The peak concentrations were 8.03 μg/mL, 8.87 μg/mL and 8.67 μg/mL, the distribution half-lives were 2.51 h, 1.72 h and 1.83 h, the elimination half-lives were 18.49 h, 17.74 h and 19.46 h, and the ratios of body clearance to bioavailability were 0.17 L/h/kg, 0.12 L/h/kg and 0.11 L/h/kg, respectively. The results showed that the area under the drug-time curve of FF was larger than that of FF alone, and the compounded diseased group was larger than the healthy group, and there were no significant differences in other pharmacokinetic parameters, indicating that the compounded DoxHcl&FF could stay in the diseased pigs for a long time and play a better therapeutic role. The areas under the drug-time curves of the healthy and the diseased plasma DoxHcl groups of the monopartite DoxHcl and the compound DoxHcl&FF were 133.26 h.μg/mL, 126.96 h.μg/mL and 169.82 h.μg/mL, respectively. The peak times were 2.19 h, 2.72 h and 2.68 h, respectively. The peak concentrations were 7.68 μg/mL, 7.91 μg/mL and 7.99 μg/mL, the distribution half-lives were 1.34 h, 1.72 h and 1.66 h, the elimination half-lives were 19.56 h, 15.44 h and 24.44 h, and the ratios of body clearance to bioavailability were 0.15 L/h/kg, 0.16 L/h/kg and 0.12 L/h/kg, respectively. The results showed that the area under the drug-time curve of DoxHcl was larger than that of DoxHcl alone after the combination of FF and DoxHcl, and there were no significant differences in other pharmacokinetic parameters, indicating that the combination of DoxHcl&FF could stay in the diseased pigs for a long time and play a better therapeutic role. The drug concentration of PELF and plasma displayed no difference between the healthy and diseased groups. Moreover, there were significant differences in drug concentrations between plasma and PELF. After intramuscular administration, FF and DoxHcl in PELF were significantly higher than those in plasma. The values for C_{max} and $AUC_{0\text{-}24h}$ in PELF were obviously higher than those in plasma. There were no significant change in metabolic process after the interaction of FF and DoxHcl.

In vitro and ex vivo antimicrobial activity of DoxHcl&FF (1:1)

As shown in Fig. 3, time-growth curves of APP BW1 in TSB and PELF indicated that the logarithmic growth stages of APP in TSB and PELF were 2–9 h and 3–9 h, respectively. The speed to reach the logarithmic growth stage of APP BW1 in TSB was faster in contrast to that of PELF, whereas the overall quantity of APP BW1 microbes in PELF was greater in contrast to that in TSB.

The time-mortality curves of DoxHcl&FF (1:1) against APP BW1 in TSB and PELF were showed in Fig. 4. There was a positive correlation between bactericidal effect of DoxHcl&FF (1:1) and APP in both TSB and PELF, according to the curves shown in Fig. 4A and B. After treatment with the 1 MIC of DoxHcl&FF (1:1) for 12 h, the microbe CFU was remarkably reduced, but it could restore growth. In addition, after treatment with DoxHcl&FF (1:1) at levels > 1 MIC for 8 h, microbe CFU remarkably reduced to non-detectable status. The time-mortality features in vitro and ex vivo were similar to each other. These outcomes revealed that DoxHcl&FF (1:1) had a typical content-dependent characteristic both in vitro

Table 1 The PK parameters after administration (20 mg/kg, IM) in plasma (mean ± SD, $n = 6$)

Parameters	Unit	Single FF	FF of compound		Single DoxHcl	DoxHcl of compound	
		Healthy	Healthy	Diseased	Healthy	Healthy	Diseased
K_{12}	h	0.08 ± 0.04	0.08 ± 0.03	0.06 ± 0.03	0.08 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
K_{21}	h	0.09 ± 0.02	0.14 ± 0.04	0.11 ± 0.01	0.16 ± 0.02	0.14 ± 0.03	0.14 ± 0.01
$AUC_{0\text{-}24h}$	h·μg/mL	91.86 ± 4.52	105.52 ± 1.46	115.05 ± 1.88	68.20 ± 3.98	72.77 ± 1.41	73.29 ± 1.05
T_{max}	h	3.41 ± 0.27	3.45 ± 0.21	3.67 ± 0.08	1.86 ± 0.14	1.97 ± 0.06	2.04 ± 0.07
C_{max}	μg/mL	4.13 ± 0.11	4.08 ± 0.09	4.09 ± 0.05	3.63 ± 0.14	3.58 ± 0.07	3.60 ± 0.09
$T_{1/2\alpha}$	h	3.62 ± 1.21	3.04 ± 0.96	4.12 ± 1.49	4.65 ± 2.73	4.20 ± 0.75	3.76 ± 0.59
$T_{1/2\beta}$	h	22.61 ± 2.15	20.57 ± 1.66	22.85 ± 1.76	16.32 ± 1.38	14.90 ± 0.54	15.37 ± 0.82
CL/F	L/kg/h	0.22 ± 0.01	0.19 ± 0.003	0.17 ± 0.003	0.29 ± 0.02	0.27 ± 0.005	0.27 ± 0.004
V_1/F	L/kg	3.03 ± 0.60	3.29 ± 0.42	3.45 ± 0.55	4.50 ± 0.69	4.81 ± 0.18	4.69 ± 0.28
V_2/F	L/kg	2.58 ± 0.74	1.75 ± 0.49	1.65 ± 0.79	1.44 ± 0.65	0.72 ± 0.20	0.94 ± 0.40

The data were substituted into the first-order absorption two-compartment model in WinNonlin software to fit. K_{12} first-order rate constant of drug transport from the central compartment to the peripheral compartment, K_{21} first-order rate constant of drug transport from the peripheral chamber to the central chamber, $AUC_{0\text{-}24h}$ the area under the concentration-time curve, C_{max} maximal drug concentration, T_{max} time to reach, C_{max}, $T_{1/2\alpha}$ the distribution half-life, $T_{1/2\beta}$ the elimination half-life, *CL/F* total body clearance as a function of bioavailability, V_1/F distribution volume of the central chamber as a function of bioavailability, V_2/F distribution volume of the peripheral chamber as a function of bioavailability

Table 2 The PK parameters after administration (20 mg/kg, IM) in PELF (Mean ± SD, $n = 6$)

Parameters	Units	Single FF	FF of compound		Single DoxHcl	DoxHcl of compound	
		Healthy	Healthy	Diseased	Healthy	Healthy	Diseased
K_{12}	h	0.11 ± 0.02	0.19 ± 0.008	0.18 ± 0.004	0.28 ± 0.01	0.18 ± 0.006	0.23 ± 0.008
K_{21}	h	0.10 ± 0.002	0.15 ± 0.008	0.13 ± 0.004	0.15 ± 0.02	0.14 ± 0.01	0.12 ± 0.007
$AUC_{0\text{-}24h}$	h·μg/mL	144.22 ± 1.98	172.75 ± 2.52	180.22 ± 3.13	133.26 ± 4.43	126.96 ± 3.70	169.82 ± 4.38
T_{max}	h	3.14 ± 0.10	2.90 ± 0.05	3.07 ± 0.01	2.19 ± 0.05	2.72 ± 0.03	2.68 ± 0.03
C_{max}	μg/mL	8.03 ± 0.20	8.87 ± 0.08	8.67 ± 0.07	7.68 ± 0.07	7.91 ± 0.09	7.99 ± 0.05
$T_{1/2\alpha}$	h	2.51 ± 0.31	1.72 ± 0.06	1.83 ± 0.03	1.34 ± 0.06	1.72 ± 0.06	1.66 ± 0.03
$T_{1/2\beta}$	h	18.49 ± 0.47	17.74 ± 0.43	19.46 ± 0.42	19.56 ± 1.71	15.44 ± 1.01	24.44 ± 1.50
CL/F	L/kg/h	0.17 ± 0.09	0.12 ± 0.002	0.11 ± 0.002	0.15 ± 0.005	0.16 ± 0.005	0.12 ± 0.003
V_1/F	L/kg	1.35 ± 0.15	1.11 ± 0.02	1.11 ± 0.02	1.21 ± 0.03	1.21 ± 0.02	1.16 ± 0.02
V_2/F	L/kg	1.48 ± 0.13	1.36 ± 0.04	1.48 ± 0.03	2.29 ± 0.22	1.55 ± 0.11	2.26 ± 0.13

The data were substituted into the first-order absorption two-compartment model in WinNonlin software to fit. K_{12} first-order rate constant of drug transport from the central compartment to the peripheral compartment, K_{21} first-order rate constant of drug transport from the peripheral chamber to the central chamber, $AUC_{0\text{-}24h}$ the area under the concentration-time curve, C_{max} maximal drug concentration, T_{max} time to reach C_{max}, $T_{1/2\alpha}$ the distribution half-life, $T_{1/2\beta}$ the elimination half-life, *CL/F* total body clearance as a function of bioavailability, V_1/F distribution volume of the central chamber as a function of bioavailability, V_2/F distribution volume of the peripheral chamber as a function of bioavailability

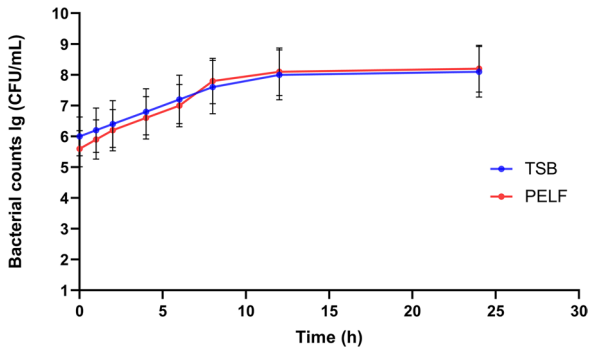

Fig. 3 The time-growth curves of APP BW1 in vitro (TSB, blue) and ex vivo (PELF, red)

and ex vivo, and that a 2 MIC content of DoxHcl&FF (1:1) could fully eradicate APP after 24 h.

PK-PD integration model

As a concentration-dependent action, the PK-PD parameter results, which were acquired from the PK data in vivo shown in Tables 1 and 2, and MIC, MBC and MPC ex vivo, were chosen. For APP, on the basis of the PK-PD data in PELF, the rates of C_{max}/MIC, $AUC_{0\text{-}24h}$/MIC, etc. are presented in Table 3. The ex vivo antibacterial activity of DoxHcl&FF (1:1) against APP (BW1) was determined in PELF samples collected before administration and at 0, 0.25, 0.5, 1, 2, 4, 6, 9, 12, 24, 36, 48, 72, 96 and 120 h after intramuscular injection.

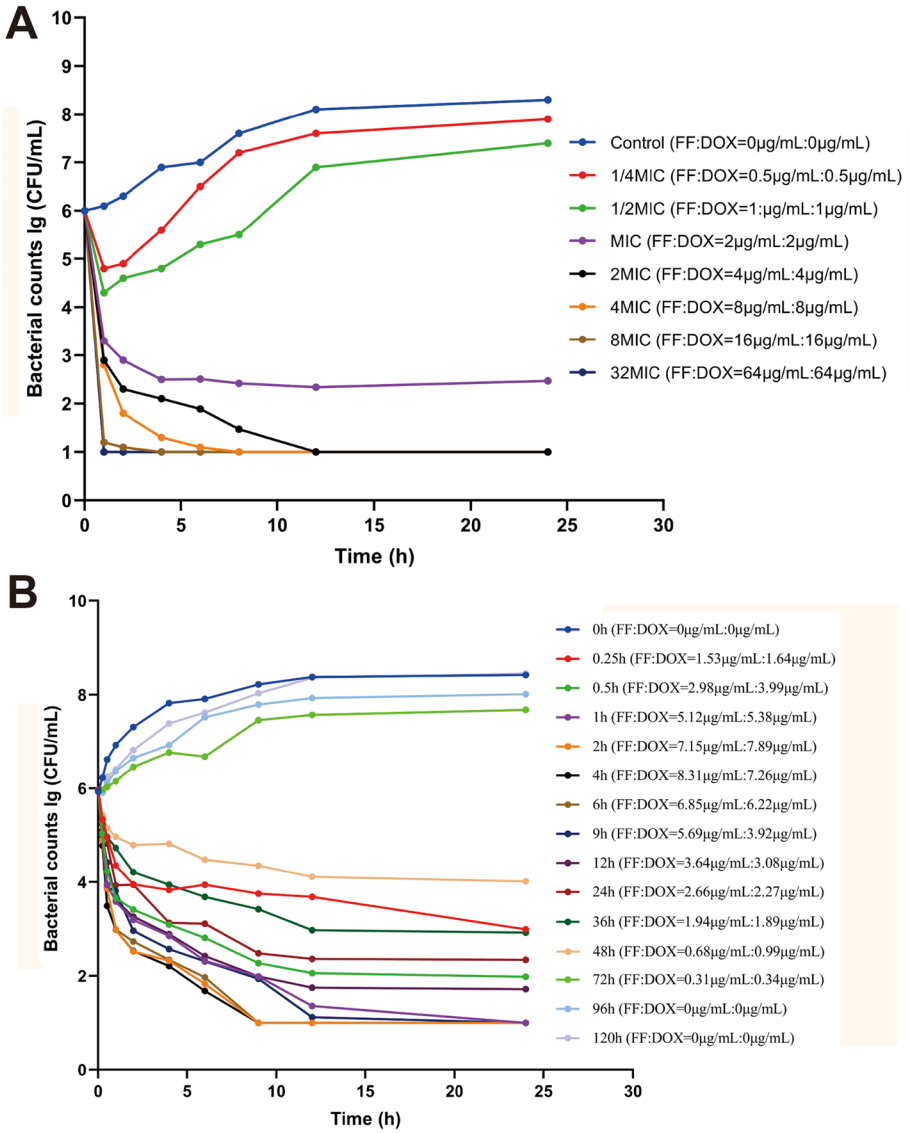

Fig. 4 The time-mortality curves of DoxHcl&FF against APP BW1 in vitro (**A**) and ex vivo (**B**)

The relationship between antimicrobial efficacy and the ex vivo PK-PD parameter of the $AUC_{0\text{-}24h}$/MIC ratio was simulated by using the inhibitory sigmoid E_{max} model. The free fraction of FF in PELF was 90.89%, and that of DoxHcl was 91.11% in this study. The model parameters of the values of the Hill coefficient E_{max}, EC_{50}, E_0, N, and $AUC_{0\text{-}24h}$/MIC are shown for three levels of growth inhibition in Table 4. The values of the $AUC_{0\text{-}24h}$/MIC ratio needed for bacteriostatic activity (E = 0), bactericidal activity (E = −3) and bacterial elimination (E = −4) are shown in Table 4.

Estimation of doses

According to the $AUC_{0\text{-}24h}$/MIC ratios for different levels of antibacterial activity, the predicted doses are shown

Table 3 The PK-PD parameters for DoxHcl&FF against APP BW1 in PELF

PK-PD Parameters	Units	FF		DoxHcl	
		Healthy	Diseased	Healthy	Diseased
C_{max}/MIC	–	4.44 ± 0.04	4.34 ± 0.04	3.96 ± 0.05	4.00 ± 0.03
$AUC_{0\text{-}24h}$/MIC	h	86.38 ± 1.26	90.11 ± 1.57	63.48 ± 1.85	84.91 ± 2.19
C_{max}/MBC	–	2.22 ± 0.02	2.17 ± 0.02	1.98 ± 0.03	2.00 ± 0.02
$AUC_{0\text{-}24h}$/MBC	h	43.19 ± 0.63	45.06 ± 0.79	31.74 ± 0.93	42.46 ± 1.10
C_{max}/MPC	–	1.10 ± 0.01	1.08 ± 0.01	0.99 ± 0.01	1.00 ± 0.01
$AUC_{0\text{-}24h}$/MPC	h	21.59 ± 0.32	22.53 ± 0.39	15.87 ± 0.46	21.23 ± 0.55

$AUC_{0\text{-}24h}$ the area under the concentration-time curve, *C_{max}* maximal drug concentration, *MIC* Minimum inhibitory concentration, *MBC* Minmum bactericidal concentration, *MPC* Minimum prevention concentration

Table 4 The PK-PD model of FF, DoxHcl and DoxHcl&FF ex vivo

Parameters	Unit	Healthy (FF)	Diseased (FF)	Healthy (DoxHcl)	Diseased (DoxHcl)
E_{max}	logCFU/mL	2.23 ± 0.006	2.41 ± 0.002	2.00 ± 0.001	2.40 ± 0.004
EC_{50}	h	8.55 ± 0.22	9.94 ± 0.36	6.58 ± 0.35	11.55 ± 0.61
E_0	logCFU/mL	−5.52 ± 0.03	− 5.27 ± 0.03	−6.85 ± 0.36	−5.16 ± 0.04
N	–	1.17 ± 0.04	1.36 ± 0.038	0.58 ± 0.07	1.73 ± 0.13
$(AUC_{0\text{-}24h}/MIC)_{ex}(E=0)$	h	3.94 ± 0.22	5.61 ± 0.26	0.78 ± 0.24	7.42 ± 0.64
$(AUC_{0\text{-}24h}/MIC)_{ex}(E=-3)$	h	15.99 ± 0.28	18.83 ± 0.49	10.32 ± 0.73	19.64 ± 0.53
$(AUC_{0\text{-}24h}/MIC)_{ex}(E=-4)$	h	28.63 ± 0.21	32.68 ± 0.64	24.06 ± 0.48	31.08 ± 0.38

$(AUC_{24h}/MIC)_{ex}$ E represents the difference of bacteria logarithmic between the initial inoculated bacteria and the alveolar fluid sample at different time points cultivated for 24 h; E_0 represents the difference of bacteria logarithmic between the initial inoculated bacteria and the alveolar fluid sample cultivated for 24 h; E_{max} represents the difference of bacteria logarithmic between the initial inoculated bacteria and the blank alveolar fluid cultivated for 24 h; EC_{50} represents the PK-PD parameter in ex vivo when the half of the maximum effect of alveolar fluid sample was produced; N represents the Hill coefficient, describing the PK-PD parameter in the half body and the slope after linearization with the effect of E

in Table 5. In this study, in order to better cure the disease, the dose against APP in diseased pigs was selected, and then the smaller dose of the combined ingredients was selected as the final dose. Finally, based on the dose equations, the suggested doses for the bacteriostatic, bactericidal and elimination activity of DoxHcl&FF (1:1) against APP after 24 h were 1.37, 4.59 and 7.99 mg/kg b.w., respectively.

Assessment of dose

Different dosage regimens of 1.37 mg/kg every 12 h, 4.59 mg/kg every 12 h, 7.99 mg/kg every 12 h, 1.37 mg/kg every 24 h, 4.59 mg/kg every 24 h and 7.99 mg/kg every 24 h (Fig. 5) were simulated. A dosage regimen of 4.59 mg/kg every 24 h should be sufficient to reach bactericidal activity. The software was Mlxplore and the initial parameter was the dosage. The dosage of Panel B was twice that in Panel A, so Panel B was administered twice, Panel A was administered four times, and the total dosage was the same.

Discussion

APP, as an important pig pathogen, has become a serious problem in animals raised for food purposes (Podolska et al. 2012). Moreover, the resistance rate of pathogenic bacteria to commonly used antimicrobial drugs has gradually increased, and almost all bacteria have acquired resistance genes, resulting from overuse and misuse (Aslam et al. 2018; Luo et al. 2019). In clinical settings, the etiology of the disease is complicated: it is often manifested as a secondary infection and as a mixed infection with multiple pathogenic bacteria. It is difficult to effectively control the disease with a single medication, which seriously damages the development of the breeding industry; therefore, effective antimicrobial agents are required to effectively manage APP.

A combination of antibacterial drugs can improve the therapeutic effect, alleviate adverse reactions and reduce drug resistance. A combination of drugs can also expand the scope of antibacterials in cases of mixed infections or bacteriological diagnosis (Ayukekbong et al. 2017). FF represents a potentially effective drug for APP. Studies in China and abroad on PD and PK showed that it is sensitive to APP (Catry et al. 2008; Jourquin et al. 2022). Moreover, early studies found that the antibacterial activity of the combination of FF and DoxHcl was significantly higher than that of just FF. Thus, in this study, the PD and PK of a compound FF and DoxHcl injection and its rational dose regimens against APP in pigs were studied via the PK-PD model approach so as to provide maximal efficacy.

The highly pathogenic clinical isolate strain APP BW1 was chosen for the study of the in vitro and ex vivo PD. In the APP populations, for FF, DoxHcl and DoxHcl&FF (1:1), the MIC_{90} was 8, 8 and 2 μg/mL, respectively (Fig. 1). According to the fractional inhibitory concentration index (FIC) (Yu et al. 2010), these results indicate that FF and DoxHcl have a synergistic or additive effect when applied in a ratio of 1:1, and it achieved higher antibacterial activity in vitro than FF or DoxHcl alone. The growth curves of APP in TSB and PELF showed no significant difference (Fig. 3). As we all know, FF and

Table 5 Dosage of FF and DoxHcl in the compound injection for different purposes

Antibacterial activity	Weight dose (mg/kg b.w.)			
	Healthy		Diseased	
	FF	DoxHcl	FF	DoxHcl
E = 0	1.00	0.27	1.37	1.92
E = −3	4.07	3.57	4.59	5.08
E = −4	7.30	8.32	7.99	8.04

E = 0, −3, −4, respectively represent the target values of AUC_{24h}/MIC under the targets of antibacterial, therapeutic and eradication

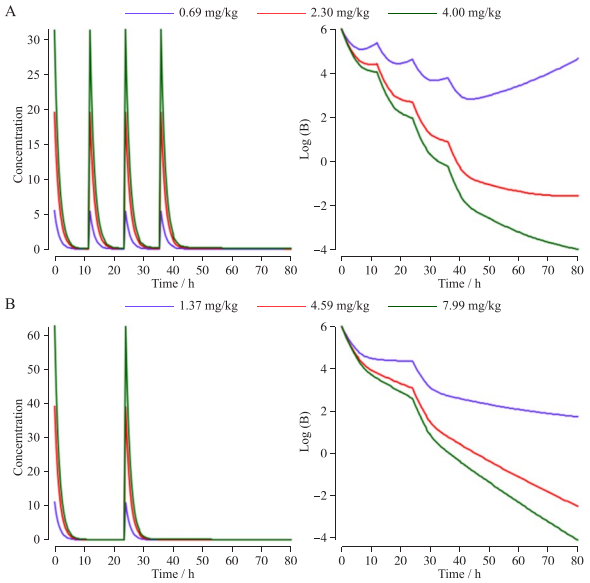

Fig. 5 Predicted growth of APP under different regimens: per 12 h (**A**), per 24 h (**B**)

DoxHcl showed a significant concentration dependence, according to the in vitro and ex vivo time-mortality curves. DoxHcl&FF showed that there was a concentration-dependent bactericidal effect induced by increasing drug concentrations. According to the results of the study, the ex vivo AUC/MIC values should be selected for PK-PD modeling.

It was found that measurement at the infection site for PK and PD was a better method for analyzing and correlating the PK-PD model (Mouton et al. 2008; Nielsen and Friberg 2013). In most published studies on the PK-PD model, the PK was studied in serum as the ex vivo data. However, the PK of DoxHcl&FF in the lung content, which acts as the target infection site of APP in pigs, was first used to investigate the effects in PELF. The collection of PELF in vivo could keep target animals in a normal physiological state, and the drug concentration in the alveolar lavage fluid obtained by the alveolar lavage technique was diluted with physiological saline. The study used urea nitrogen detection to determine the actual lung tissue (Mzyk et al. 2017). Furthermore, experts have emphasized the importance of unbound biological drug concentrations, and this played an important role in the evaluation of the antimicrobial activity. Therefore, the active unbound drug concentrations were determined to provide a better correlation with the microbiological outcome from the PK-PD model. The protein binding rate of PELF was obtained by calculating the percentage of the drug binding to protein in the total amount of the drug in PELF. The free fraction of FF in PELF was 90.89%, and that of DoxHcl was 91.11% in this study.

The PK data of single FF, single DoxHcl, and compound DoxHcl&FF in plasma after intramuscular injection of the recommended dose of 20 mg/kg for T_{max}, C_{max}, $AUC_{0\text{-}24h}$, $T_{1/2\alpha}$, $T_{1/2\beta}$ and $C_{L/F}$ are shown in Table 1. By comparing single FF, single DoxHcl and compound DoxHcl&FF (1:1), we can see that if the two drugs are combined together, their absorption, distribution and elimination processes are almost the same, which has little impact. For single FF and single DoxHcl, the pharmacokinetic variables in the plasma were similar to the variables of a previous study (Li et al. 2016; Pérez-Fernández et al. 2017). For DoxHcl&FF, in contrast to plasma, the drug contents in PELF were remarkably greater, with C_{max} and $AUC_{0\text{-}24h}$ values of 8.67 μg/mL and 180.22 μg·h/mL for FF, and C_{max} and $AUC_{0\text{-}24h}$ values of 7.99 μg/mL and 169.82 μg·h/mL for DoxHcl, respectively, which were 2.12-, 1.57-, 2.22- and 2.32-folds greater in contrast to those in plasma, respectively (Tables 1 and 2). As an example of concentration-dependent action for DoxHcl&FF, the parameters $AUC_{0\text{-}24h}$/MIC >125 were used as a threshold for the successful therapeutic outcome of fluoroquinolone against Gram negative bacteria (Toutain et al. 2002). Nevertheless, the liminal values might be diverse for diverse medicines, due to the diversity of the immunity conditions of targeted pigs and causative agents. Hence, it is imperative to explore the PK-PD parametric results of DoxHcl&FF one by one. Herein, the PD data were acquired from PELF to forecast dose usage, given that it exhibited more clinical relevance in contrast to TSB broth. For the ex vivo time-mortality curves, in order to better achieve a synergistic additive effect between the drugs and to enhance the antibacterial activity, the culture in PELF mixed with FF and DoxHcl was selected. As per the PK of infected animals and the PD parametric results, for the FF in the compound, the ex vivo $AUC_{0\text{-}24h}$/MIC rates of DoxHcl&FF required clinically for bactericidal action and elimination of the APP strain with a MIC_{90} of 2 μg/mL were 18.83 and 32.68 h, while those of the DoxHcl of the compound were 19.64 and 31.08 h. In order to better cure the disease, the dose against APP in diseased pigs was selected, and then, as the final dose, the smaller dose of the combined ingredients was selected. Moreover, based on the dose equations, the suggested doses for the bacteriostatic, bactericidal and elimination activity of DoxHcl&FF against APP after 24 h were 1.37, 4.59, and 7.99 mg/kg b.w., respectively (Table 5). Finally, it was found that a dosage regimen of 4.59 mg/kg every 24 h was enough to achieve the bactericidal effect and avoid the emergence of drug resistance.

Conclusions

In conclusion, the present study identified the PK-PD parameter results in vitro, the dosages needed to realize the goal of 90% bacteriostatic, bactericide, and

elimination activity were 1.37, 4.59, and 7.99 mg/kg, respectively. A dosage regimen of 4.59 mg/kg of FF: DoxHcl (1:1) by intramuscular injection every 24 h could be sufficient to achieve bactericidal activity and avoid the emergence of drug resistance. However, the suggested dosage regimens still need further validation in clinical practice.

Methods

Medicines and reagents

FF (1280 μg/mL) and DoxHcl (1280 μg/mL) reference standard were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and were prepared with sterile water or sterile water with 5% methanol added, respectively. DoxHcl&FF injection was prepared in our laboratory (Li et al. 2016). Acetonitrile and methyl alcohol (liquid chromatography grade) were purchased from TEDIA (Ohio, USA). Tryptone soya agar (TSA) and tryptone soya broth (TSB) were purchased from Qingdao Hai Bo Biological (Shandong, China), and 1% nicotinamide adenine dinucleotide (NAD) and 5% newborn calf serum were from Guangzhou Ruite Biological Technology Co., Ltd. (Guangdong, China). Other chemicals and reagents were of analytical grade or higher.

Animals

All animal experiments in this study were performed in compliance with the Huazhong Agricultural University animal experiment center guidelines and were approved by the Animal Ethics Committee. Twenty-four healthy three-way cross castrated piglets (HZAUSW-2019-004), which weighed 24 ± 1.0 kg and were aged 6.5 ± 0.5 weeks, were used for this study. The temperature of the animal housing was maintained at 16–28°C and the relative humidity was in the range of 50–80%. The pigs were fed with antibiotic-free feed.

Healthy Kunming mice, which weighed 16–20 g and were aged 7–10 weeks, were used for this study, and the weight difference did not exceed 20% of the average weight. The mice, purchased from the Experimental Animal Center of Huazhong Agricultural University, were evenly split between males and females.

Bacterial strains

One hundred and thirty-one APP strains were identified by polymerase chain reaction (PCR) obtained from the National Reference Laboratory of Veterinary Drug Residues of (HZAU). The *E.coli* ATCC 25922 strain was selected as the quality control strain (QC). The antibiotic susceptibility was detected according to the standards formulated by the Clinical and Laboratory Standard Institute (CLSI-M07A8–2010). The isolates were subcultured at least 3 times in TSB and TSA, and were incubated at 37°C for 16–24 h.

The APP virulence experiment

The MIC_{90} strains (100 μL) at 10^8 CFU/mL were inoculated into TSB including 5% newborn calf serum and 1% NAD at 37°C for 24 h. In total, 57 mice were randomly divided into 19 groups. Each group had 3 mice, and a negative control group (injection of TSB broth) was included. Three concentration gradients of 10^7, 10^8 and 10^9 CFU/mL were set for each strain. Each mouse was intraperitoneally injected with 200 μL. The mice were observed for 3 days after injection, and the deaths of mice in each group were counted.

Determination of combination antibacterial activity

The checkerboard method was used to determine the combined susceptibility of two antibiotics (A refers to DoxHcl and B refers to FF). The concentration gradient of A and B was set as shown in Supplementary Table 3. The model diagram of the checkerboard method for determining the combined drug sensitivity test is shown in Supplementary Fig. 1, and the specific steps are briefly described below. Take 96-well bacterial culture plate, row Y1 and column X1 are the separate drug rows and columns for drug A and drug B. Holes 2–7 of row Y1 were added with 100 μL each of standard drug solution for drug A at concentrations of 1/4, 1/2, 1, 2, 4, and 8 MIC, respectively; and holes 2–7 of column X1 were added with l00 μL each of standard drug solution for drug B at concentrations of 1/4, 1/2, 1, 2, 4, and 8 MIC, respectively. Holes 2–7 of row Y2-Y7 were added with 50 μL each of standard solution of drug A concentration of 1/4, 1/2, 1, 2, 4, 8 MIC, respectively; Holes 2–7 of column X2-X7 were added with 50 μL each of standard solution of drug B concentration of 1/4, 1/2, 1, 2, 4, 8 MIC, respectively. The total volume of liquid in the 96-well plates was 200 μL. The plates were incubated in a constant temperature incubator (37°C, 5% CO_2) for 24–48 h. The lowest concentration in the clarified wells was observed to be the MIC of the combined drug. The results were read according to the method of equivalent midpoint, each tube along the angle parallelepiped of point 0 was equivalent to the midtube, and the lowest concentration of sterile growth was read as point 1 for the combined results, which corresponded to the point on the X-axis as MIC(A) and the corresponding point on the Y-axis as MIC(B). The Fractional Inhibitory Concentration Index (FIC) was used as the basis for judging the combined drug susceptibility test, and the calculation formula was as follows:

$$FIC = \frac{MIC(combinationA)}{MIC\left(singleA\right)} + \frac{MIC(combinationB)}{MIC\left(singleB\right)}$$

An FIC index of ≤0.5 is a synergistic effect, an FIC index of 0.5–1 is additive, an FIC index of 1–2 is irrelevant and an FIC index of >2 is antagonistic.

Determination of the MIC

The minimal inhibitory concentration (MIC) of FF, DoxHcl and DoxHcl&FF against 131 isolates of APP was investigated by using a micro-dilution method, respectively, according to the CLSI-M07A8–2010. Then, the highest pathogenicity strain in populations of MIC_{90} chosen to explore the antimicrobial features of DoxHcl&FF in vitro and ex vivo. The strains (100 μL) at 10^8 CFU/mL were injected into TSA with 5% NBCS and 1% NAD, with two-fold continuous dilution of FF (0.03–64 μg/mL), DoxHcl (0.03–64 μg/mL) and DoxHcl&FF (1:1) (0.03–64 μg/mL). Afterwards, the dishes were cultivated at 37°C for 24 h. The MIC was taken into consideration when growth was no longer visible to the naked eye. *E.coli* (ATCC 25922) was included as a QC strain.

Detection of MIC, MBC and MPC in TSB and PELF

For the MIC and the minimum bactericidal concentration (MBC), the identification of APP BW1 in vitro and ex vivo was completed via the micro-dilution method as per the specifications of the CLSI-M07A8–2010, and the MBC was desaturated ≥10 times via TSB; afterwards, 100 μL of every suspension solution was spread and calculated on the TSA dishes for 48 h at 37°C. The MBC was the lowest content of FF, DoxHcl and DoxHcl&FF (1:1) suppressing 99.9% of the density of APP bacteria.

For the mutant prevention concentration (MPC), 1 × MIC, 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC, 32 × MIC, 64 × MIC of the TSA plates were prepared, and 100 μL of concentrated bacteria at 10^{10} CFU/mL was loaded on the TSA plates and incubated at 37°C in a 5% CO_2 incubator for 72 h, according to the measured MIC values of FF, DoxHcl and DoxHcl&FF (1:1). We took the lowest drug concentration without bacteria at 72 h as the initial measurement of MPC (MPC_{pr}). After the MPC_{pr} had been determined, the MPC_{pr} decreased linearly by 20% or more. The minimum drug concentration without bacterial growth was the exact MPC.

Bacterial growth and time-mortality curves after different concentrations of DoxHcl&FF (1:1) in vitro and ex vivo

The growth curves of DoxHcl&FF (1:1) against APP BW1 were identified via plate calculation. The growth curves of APP BW1 in PELF and TSB were drawn at 0, 1, 2, 4, 6, 8, 12 and 24 h.

As per the MIC results (2/2 μg/mL) of APP BW1 in response to DoxHcl&FF (1:1), TSA dishes were prepared, involving diverse DoxHcl&FF (1:1) levels varying from 1/4 to 32 MIC. For the in vitro time-mortality curve, a 10^6 CFU/mL microbe suspension was desaturated to obtain the TSB. Afterwards, 100 μL of the desaturated sample was spread on the TSA dishes at 0, 1, 2, 4, 6, 8, 12 and 24 h. Eventually, those specimens were cultured at 37°C with 5% CO_2 in an incubating device for 24 h. For the ex vivo time-mortality curves of DoxHcl&FF (1:1) acquired at diverse temporal points in PK research, the microbes (10^6 CFU/mL) were cultured with PELF specimens involving diverse DoxHcl&FF (1:1) contents. The ex vivo time-mortality curve was used to accommodate a suitable PD model, resting on the assumption that there is a logarithmic decrease in the quantity of microbes at a content of DoxHcl&FF (1:1) with the cultivation duration, in line with the suppressive sigmoid E_{max} model.

Dose administration and experimental design

The study established the prevalence model of APP, then inoculated a colony of APP BW1 with strong virulence into the TSB broth and conducted the incubation at 37°C under 5% CO_2 until 10^8 CFU/mL, and then the bacterial solution was enriched to reach 1×10^9 CFU/mL, which was used as the inoculation. After continuous inoculation for 3 days, at 3–5 mL each time, we observed whether the pigs developed symptoms such as increased body temperature, cough, loss of appetite or claudication, and scored the symptoms according to the symptom scoring shown in Supplementary Table 4. It indicated that the infection was successful when any symptom reached a score of 2. Twenty-four pigs were divided into four groups. Two groups of pigs were equally divided into healthy and diseased groups, and the diseased group was infected with APP. Twelve pigs were injected with the DoxHcl&FF compound injection at 20 mg/kg. As for the other 12 pigs, six pigs were injected with FF alone and 6 pigs were injected with DoxHcl as a pharmacokinetic comparison. These blood samples and PELF were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 9, 12, 24, 48, 72, 96 at 120 h.

Alveolar lavage fluid sampling procedure

The experimental pigs were fasted and drank freely before anesthesia. Atropine was injected intramuscularly at a dose of 0.05 mg/kg b.w., and 10 mg/kg b.w. of propofol was intravenously injected into the marginal vein of the ear. After anesthesia, bronchial intubation was performed. After finding the epiglottis, the laryngoscope pressed open the epiglottal cartilage and an electronic bronchoscope was inserted into the trachea. This process achieved simultaneous monitoring until the bronchoscope was inserted into the fourth stage bronchus, and

then 50 mL saline at 37°C was injected; after 20 s, an air pump was used to draw back the alveolar lavage fluid.

Blood and PELF sample extraction

The blood and PELF samples were collected with coagulant and then those samples were centrifuged at 3500 rpm for 10 min to obtain the serum. Next, 3 mL of acetonitrile and 0.5 mL of ethylenediaminetetraacetic acid (EDTA) were added into 0.5 mL of the serum/PELF. The tubes were vortexed for 2 min and then put into the ultrasonic cleaner for 20 min. After that, the samples were centrifuged at 10,000 rpm for 10 min. After transferring the supernatant to another clean tube, these steps were repeated. The supernatant was then dried in a water bath at 40°C under nitrogen. With the use of 1 mL sodium dihydrogen phosphate and acetonitrile (v/v = 81.5:18.5) for dissolving the pellet, the complex solution was filtered through a 0.22 μm filter membrane and loaded into a sample bottle for HPLC analysis. The samples were determined by using a Waters 2695 series reverse-phase HPLC. The ZORBAX SB C18 column (250 × 4.6 mm, i.d. 5 μm; Agilent Technology, USA) was used for separation.

The specificity of the detection method was good for both FF and DoxHcl. There was no endogenous interference on the chromatograms. The linear range of the standard curves of FF ranged from 0.05 to 10 μg/mL ($r^2 = 0.9991$) in plasma and from 0.05 to 10 μg/mL ($r^2 = 0.9991$) for PELF. For DoxHcl, it ranged from 0.1 to 10 μg/mL ($r^2 = 0.9988$) in plasma and from 0.1 to 10 μg/mL ($r^2 = 1$) in PELF, while for FF, the limit of detection (LOD) was 0.03 μg/mL and the limit of quantification (LOQ) was 0.05 μg/mL in plasma and PELF. For DoxHcl, the LOD was 0.06 μg/mL and the LOQ was 0.1 μg/mL in plasma and PELF. The mean recovery of both FF and DoxHcl was > 85% in plasma and PELF. The relative standard deviations (RSD) of FF and DoxHcl were below 9.0% in the plasma samples and PELF for intraday and inter-day variation. The external standard was used to quantify the drugs.

Binding of FF and DoxHcl to PELF protein

The PELF protein binding of FF and DoxHcl was determined in triplicate in each of six PELF samples collected from six pigs in the PK study. Through measuring the drug concentration inside and outside the dialysis bag, the blank PELF protein binding rate was calculated on the basis of the concentration. The formula for calculating the protein binding rate is as follows:

$$\text{Protein binding rate} = (\text{Dt} - \text{Df})/\text{Dt} \times 100\%$$

where Dt is the concentration of alveolar drugs in the dialysis bag (total concentration) and Df is the concentration of drugs outside the dialysis bag (free drug concentration).

PK analysis

WinNonlin software (V. 5.2.1, Pharsight Corporation, Mountain View, CA, United States) was used to calculate the PK parameters of DoxHcl&FF for plasma and PELF, including C_{max}, T_{max}, $AUC_{0\text{-}24h}$, etc. in pigs.

PK-PD integration modeling analysis

For selection of the PK-PD parameters, AUC/MIC, C_{max}/MIC and T > MIC were standardized (Lei et al. 2017). A previously published study reported that AUC/MIC might be the optimal PK-PD parameter for FF and DoxHcl (Maaland et al. 2013). The sigmoid E_{max} model was used to integrate the ex vivo $AUC_{0\text{-}24h}$/MIC ratio and the bacterial count changes (logCFU/mL) in PELF after 24 h of incubation. The model was as follows:

$$E = E_{\max} - \frac{(E_{\max} - E_0)C^N}{C^N + EC_{50}^N}$$

where E indicates the difference between the amount of bacteria in alveolar fluid at different time points after 24 h of incubation and the logarithm of the amount of bacteria initially inoculated, E_0 is the difference in antibacterial effect after 24 h of incubation in control samples (as a logarithm), E_{max} is the maximum difference of the antibacterial effect (as a logarithm) of lavage fluid samples incubated with the drug, C indicates the value of the hemi-internal PK-PD parameter, EC_{50} is the PK-PD parameter of the drug that produces 50% of the maximal antibacterial effect and N is the Hill coefficient, which describes the steepness of the parameter effect curve.

Statistical analysis

Data documented via Sequence Identification Program 1.4 were exported as Microsoft Office Excel Comma Separated Value files and were imported to Microsoft Office Excel 2003 SP2. The identified spreadsheets were subjected to analysis by Statistical Analysis System for Windows. Descriptive analysis results and computed results were studied separately (PROC MEANS) before two-sided t-tests for diversity in the data (PROC TTEST) and linear regressive analyses for assessment of the normal curve (PROC REG). A *P*-value below 0.05 was considered significant.

Abbreviations

DoxHcl	Doxycycline hydrochloride
FF	Florfenicol
APP	*Actinobacillus pleuropneumoniae*
PELF	Porcine pulmonary epithelial lining fluid
MIC	Minimum inhibitory concentration
PD	Pharmacodynamic
PK	Pharmacokinetic
MBC	Minimum bactericidal concentration
MPC	Minimum prevention concentration

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-023-00066-y.

Additional file 1: Supplementary Table 1. Virulence of APP results in mice. **Supplementary Table 2.** The results of combination MIC of DoxHcl and FF against APP. **Supplementary Table 3.** Checkerboard method for combinations of Drugs A and B. **Supplementary Table 4.** Clinical symptom rating scale of APP. **Supplementary Fig. 1.** Delineation of the checkerboard method.

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Authors' contributions
Y.Y. and D.P. contributed to the conception and design of this work; Y.Y., B.A., S.X. and W.Q. participated in the sample collection, laboratory experiments and data analysis; Y.Y., J.L. and D.P. drafted the manuscript; S.X., W.Q., H.H., L.H., W.L. and J.L revised the manuscript. All authors reviewed and approved the final version of this manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
The use of pigs in this study was according to the guide-lines of the Animal Experiment Ethical Inspection of Laboratory Animal Center, Huazhong Agricultural University, Wuhan, China (HZAUSW-2019-004). All efforts were made to minimize the suffering of the animals.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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