RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Issue 5: 818-827 (May 2025)

Break Point of Enrofloxacin for *Mycoplasma hyopneumoniae*

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10.18805/IJAR.BF-1807

ABSTRACT

Background: There are currently no clinical breakpoints available to facilitate data interpretation and the correlation of MICs with *in vivo* efficacy. Defining the break point of enrofloxacin for *Mycoplasma hyopneumoniae* can determine the choice of empirical treatment and targeted therapy, predict the efficacy of drugs against specific animals and analyze treatment failure.

Methods: We determined the breakpoint of *M. hyopneumonaie* to enrofloxacin by susceptibility testing and pharmocokinetics/ pharmacodynamics model.

Result: The pharmacodynamic cutoff value of *M. hyopneumoniae* against enrofloxacin was 0.25 µg/mL and the clinical break point was 2 µg/mL. After comprehensive weighing, the final sensitivity break point was set at 2 µg/mL.To provide reference for *in vitro* bacteriostatic test of *M. hyopneumonaie* against enrofloxacin.To provide the basis for the actual dosage of enrofloxacin in the treatment of mycoplasma pneumonia in pigs.

Key words: Break point, Enrofloxacin, MIC, *Mycoplasma hyopneumoniae*, Pharmacodynamics.

INTRODUCTION

Enrofloxacin was developed in 1987 as the first fluoroquinolone used specifically in animals. It is widely used for the prevention and treatment of respiratory tract, gastrointestinal and mycoplasma infections in livestock and poultry. Enrofloxacin has a broad antibacterial spectrum, rapid and efficient bactericidal action, high bio availability, low toxicity, few side effects and no cross-resistance to other antibacterial agents. It has a good therapeutic effect against mycoplasma pneumonia in swine, *Streptococcus suis*, porcine pleuropneumonia and other bacterial respiratory diseases (Wang *et al.,* 2010).

Mycoplasma hyopneumoniae is the main pathogen causing mycoplasma pneumonia in swine. This disease is prevalent worldwide. According to a recent survey, 72.69% of Tibetan pigs tested positive for the disease (Gang *et al.,* 2018). The annual loss of China's pig industry due to mycoplasma pneumonia from 2016-2019 was as high as 8.28 billion yuan (approximately US$1.163 billion) (Zhiqiang *et al.,* 2020).

Pharmacokinetic/pharmacodynamic (PK/PD) modeling is a key tool that can help identify clinically relevant relationships between time and the effects of drugs. The PK data of drugs in target tissues and the minimum inhibitory concentration (MIC) distribution data of *M. hyopneumoniae* have been simulated using oracle crystal ball 11.1.3.0.000 software. The probability of target attainment (PTA) of each MIC was obtained when the target value was reached. The PTA data was used to calculate the cumulative fraction of responses (CFR). The maximum MIC corresponding to a CFR>90% was considered the PD breakpoint (CO_{PD}) value.

There is a pressing need for the establishment of standardized laboratory protocols and interpretive guidelines for the determination of minimum inhibitory concentrations (MICs) in veterinary mycoplasmas. Presently, the absence of defined clinical thresholds hinders the accurate interpretation of data and the correlation between MIC values and *in vivo* effectiveness (Klein *et al*., 2017; de Jong *et al*., 2021). The present study was undertaken to address this need.

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How to cite this article: Qiu, G., Rui, Y., Ma, Q., Ma, B. and Xun, L. (2025). Break Point of Enrofloxacin for *Mycoplasma hyopneumoniae.* Indian Journal of Animal Research. 59(5): 818-827. doi: 10.18805/IJAR.BF-1807.

Submitted: 25-04-2024 **Accepted:** 14-07-2024 **Online:** 26-12-2024

MATERIALS AND METHODS

The experiment was conducted in the Central Laboratory of Xinyang Agriculture and Forestry University, Laboratory of Clinical Veterinary Medicine of Huazhong Agricultural University, Laboratory of Veterinary Pharmacology of Xizang Agriculture and Animal Husbandry College and four farms from March 2023 to January 2024.

Test strain

One hundred and forty field strains of *M. hyopneumoniae* can cause *Mycoplasma pneumonia* in pigs. The strains can be detected by collecting throat swabs from infected pigs. Three vaccine strains were tested using PCR.

Drug and reagents

Enrofloxacin standard (National Institutes for Food and Drug Control, ≥99% purity, lot number: 92HZ-8NN7) and

HPLC-grade chromatographic solvents were used. Additional analytical grade chemical reagents were acquired from common commercial sources. Water was purified using double distillation or passed through a Milli-Q Advantage A10 ultrapure water preparation system. The actual concentration of the enrofloxacin (ENR) base in the product formulation was determined using a validated high performance liquid chromatography-ultraviolet (HPLC-UV) detector-based analytical method (Souza *et al.,* 2002).

Instruments

The following instruments were used: Waters Alliance E2695 HPLC (Alliance); TGL18 high speed centrifuge (YINGTAI); HZK-FA210 electronic balance (Fuzhou Huazhi Scientific Instrument Co., Ltd.); HP-030A drying/training box, refrigerator and MS105DU semi-trace electronic balance (0.0001 g) (all from METTLER TOLEDO International Trade Co., Ltd.); Direct-Q®5 pure water/ultrapure water integrated system (Merck Millipore); D3024R high speed micro-refrigerated centrifuge (Beijing Dalong Experimental Instrument Co., Ltd.); MX-F vortex oscillator (Wuhan Sevier Biotechnology Co., Ltd.); vacuum pump (Shanghai Yarong Biochemical Instrument); JJ-2BS homogenizer and QL-902 vortex instrument (Haimen Qilin Bell); and LKTC-L water bath heating pot and L400 centrifuge (Hunan Xiangyi Laboratory Instrument Development Co., Ltd.).

Determination of PK/PD threshold

The first step was to select the test bacteria. To accomplish this, *M. hyopneumoniae* near the epidemiological cutoff value (ECV) fold-point was selected to determine its pathogenicity in pigs. A field strain with strong pathogenicity was selected as the test bacterium. Next, to determine the *in vitro* PD indices, the minimum bactericidal concentration (MBC) and minimum mutation-proof concentration (MPC) of enrofloxacin were measured *in vitro* and the mutation selection window (MSW) was analyzed. Based on the MIC, the antimicrobial concentration was linearly decreased by 20% to make drug sensitive papers. Bacterial suspensions of different dilutions were inoculated on the drug sensitive paper to produce 3×10^2, 3×10^3, 3×10^4 and 3×10^5 colony forming units (CFU). Modified friis liquid medium (KM_2) was used as diluent. After culture at 30°C for 24 h, appropriate Petri dishes are selected for counting. The drug sensitive paper that limited the growth of 90% of the inoculated bacteria represented the MIC_{90} (µg/mL).

Antibacterial drugs at concentrations of 2×, 4×, 8× and 16× MIC were used to prepare drug tablets, concentrate the bacterial solution and adjust the concentration. Each drug plate was inoculated with 0.1 mL of a bacterial suspension, with four plates being used for each drug concentration. The final quantity of inoculated bacteria for each concentration was 1.2×10^{10} CFU. The minimum concentration that permitted bacterial growth after 96 h culture was the MPC. A drug tablet was prepared with a linear decrease of 20% based on the MPC and the bacteria were inoculated as described above. The lowest concentration that restricted the growth of bacteria represented the MPC (µg/mL). The MSW range is MIC_{90}<MSW (µg/mL) <MPC (Guanquan *et al.,* 2012).

Several types of *in vitro* analyses were performed. In one analysis, a concentration gradient of enrofloxacin was co-incubated with 1.5×10^8 CFU/mL of bacteria for 72 h. Bacteria were collected and enumerated at different time points. Sterilization curves were drawn to determine whether enrofloxacin had a concentration- or time-dependent effect on mycoplasma pneumoniae in pigs. Another *in vitro* analysis assessed the post-antibiotic effect (PAE). For this analysis, bacteria (10^7 CFU/mL) were pulsed with different concentrations of enrofloxacin for 1-2 h. The 10-fold increase in the number of viable bacteria in the reconstructed culture was considered the PAE value.

In brief, a single colony was incubated in KM_2 at 37°C for approximately 2 h to obtain a logarithmic growth phase bacterial suspension of 10^7 to 10^8 CFU/mL. Aliquots (0.9 mL) of the bacterial solution were individually added to four test tubes, followed by 0.1 mL of different concentrations of enrofloxacin (4×, 2×, 1× and 1/2× MIC). The fifth tube received 0.1 mL sterile physiological saline and was the control. All tubes were incubated at 37°C for 2 h. Each sample was then diluted 100 times in fresh KM_2 and immediately incubated at 37°C. Immediately and after defined times, 0.1 of each bacterial suspension was collected and enumerated using the plate method. At the same time, a maximum residual drug control tube was established to investigate the impact of the maximum residual drug on bacterial growth after drug removal by dilution. The procedure was repeated four times. PAE was calculated (h) as;

$$T - C$$

Where,

T= Time required for the addition of one logarithmic order (1 lg) of bacteria (CFU/mL) in the drug group.

C= Time required for the addition of 1 lg of bacteria (CFU/mL) in the drug-free control group (Dijie *et al.,* 2002).

Healthy piglets that have not received the *M. hyopneumoniae* vaccine were used to construct the *M. hyopneumoniae* infection model. PCR examination of nasopharyngeal swabs confirmed the absence of *M. hyopneumoniae* infection. After one week of observation, the piglets were infected with *M. hyopneumoniae* (10^8 CFU/mL) using an intranasal drip. Subsequent detection of the bacteria confirmed a successful infection. Blood samples were collected in real-time by a needle tube implanted into the jugular vein of the piglets. The nasopharynx was sampled with a sterile cotton swab.

The piglets were divided into a healthy control group and an infected group (n= 4 per group). The dose of the single intramuscular injection was 2.5 mg/kg body weight. Samples were taken from target tissues before administration and 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 72 and 96 h after administration of drugs. The enrofloxacin

concentration was detected at each time point using HPLC with a Waters e2695 instrument equipped with a model 2998 photodiode array detector and WAT054275 chromatographic column (C18, 4.6 mm × 250 mm × 5 µm) at a UV detection wavelength of 278 nm. The mobile phase was composed of 10% methanol (Phase A), 10% acetonitrile (Phase B), 0.03M ammonium acetate and 0.02 M citric acid (Phase C). The injection volume was 20 µL and the detection temperature was 30°C.

In the sample pretreatment method, plasma (0.5 mL) was accurately measured, acetonitrile (0.5 mL) was added and the mixture was oscillated for 4 min. The samples were then centrifuged at 4000 rpm (1789.19 g) for 25 min. The supernatant was collected and filtered through a 0.22 µm microporous membrane to obtain the sample for HPLC analysis.

Specificity and sensitivity assessments were conducted using five control pig plasma samples and five samples spiked with standard solutions. The samples were subjected to pretreatment and analyzed for specificity and sensitivity. A working curve was generated by adding various volumes of enrofloxacin standard stock solutions to blank pig plasma to produce mixed standard solutions having final concentrations of 100, 200, 500, 1000 and 2500 µg/mL. Next, an equivalent amount of acetonitrile was added to each mixed standard solution and agitated in a shaker for approximately 4 min before centrifugation at 4000 rpm for 25 min. Each supernatant was collected and filtered through a 0.22 µm microporous membrane to produce samples for HPLC analysis. The same process was repeated five times at each concentration daily for five consecutive days. Regression analysis of the average peak area values and corresponding concentrations was performed to obtain a regression curve.

To determine the limit of quantitation (LOQ) and limit of detection (LOD), mixed standard solutions of different final concentrations (20, 40, 60, 80 and 100 µg/mL) were prepared by spiking blank pig plasma with enrofloxacin standard stock solutions in equal volumes. The samples were treated as described above and analyzed by HPLC. This process was repeated five times at each concentration daily for five consecutive days. The lowest sample concentration was determined when the signal-to-noise ratio exceeded three, which was considered the LOD. The LOQ was determined as the lowest concentration of the sample that met the quantitative analysis requirements for recovery and relative standard deviation.

The accuracy was determined as a percentage using the recovery rate (%). Precision was expressed using the relative standard deviation (RSD). Enrofloxacin and ciprofloxacin were added to pig plasma at 60, 120 and 240 µg/mL. The within-day coefficient of variation was calculated by averaging five repeated measurements on the same day. Similarly, the between-day variation ratio was calculated using the average concentration over five consecutive days. The recovery rate (%) was determined using a single-point calibration method as,

$$X = \frac{A \times V}{As \times U} \times 100$$

Where,
X= Recovery rate.
A = Peak area of the sample.
As= Peak area of the standard working solution.
V= Sample volume.
U= Total volume of the sample extraction solution before injection.

The residual amount of drug in each sample was calculated from the regression equation of the working curve, using the calculation formula:

$$X = [(A - b)/a] \times (V/W)$$

Where,
X= Residual drug amount in the sample (µg/mL).
A= Peak area of the sample.
b= Intercept of the regression equation of the working curve.
a= Slope of the regression equation of the working curve.
V= Volume of the plasma sample.
W= Total volume of the sample extraction solution before injection.

The pharmacological switch point was determined using PK Solver 2.0 software for data analysis, according to the absorption one-compartmental model, which was consistent with relevant literature methods. The PK formula for the elimination half-life was:

$$T_{1/2}Kb = \frac{0.693}{Kb}$$

Where,
Kb= Elimination rate constant.
The absorption half-life was calculated as:

$$T_{1/2}Ka = \frac{0.693}{Ka}$$

Where,
Ka= Elimination rate constant.
The overall elimination rate was calculated as:

$$CL = Kb \times Vd$$

Where,
Kb= Elimination rate constant.
Vd= Apparent distribution volume.

The area under the concentration-time curve (AUC) was calculated as:

$$A(1/Kb-1/Ka).$$

To calculate PK/PD parameters for the clinical efficacy of fluoroquinolones, the AUC_{24}/MIC parameter needed to be considered. It was calculated by dividing the AUC_{0-24} by 0.125,0.25,0.5,1,2,4 µg/mL,respectively.

To simulate the data distribution and calculate the PTA to achieve the PK/PD breakpoint, we used a Monte Carlo simulation with the Oracle Crystal Ball 11.1.3.0.000 software on a population of 10,000 healthy pigs. This simulation generated data for the AUC_{24}/MIC distribution for each drug dilution.

Using the predicted PK/PD breakpoint value of 125 for enrofloxacin against *Salmonella*, we calculated the CFR using PTA values for each MIC concentration and the weight of each MIC value within the strain population (Pan 2012, Mi 2021). This provides the probability of achieving the desired clinical efficacy at each MIC value, which can help guide antibiotic dosing decisions.

For PK data processing of *in vivo* pharmacokinetics, the PK solver program was used to calculate PK parameters of half-life ($T_{1/2}$) and AUC using non-atrioventricular model fitting. The PK differences of enrofloxacin in healthy and diseased piglets were compared using SPSS software.

PK/PD were also determined *in vivo*. Target tissue samples collected at different time points after a single administration of the drug as described above were incubated with 10^7 CFU/mL of bacterial suspension for 72 h after filtration and sterilization. During this period, samples were collected at different time points for enumeration of bacteria, drug-resistant bacteria and drug-resistant mutant bacteria, as well as to construct indirect *in vivo* bactericidal curves and growth curves of drug-resistant bacteria.

The PK/PD model was combined to calculate the critical values of the PK/PD parameters (Xiao *et al.,* 2018). PKSolver software was used for PK/PD data *in vitro*, *in vivo* and indirectly *in vivo* after a single administration. The Sigmaid Emax equation (Hill equation) was utilized to deal with the relationship between PK/PD parameters (AUC/MIC, AUC/MPC, Cmax/MPC). The higher the values of AUC/MIC, AUC_{0-24}/MPC and Cmax/MPC, the stronger the ability of drugs to inhibit the enrichment of drug-resistant strains (Lu *et al.,* 2010; Yuhan *et al.,* 2011) and antibacterial efficacy.

The antibacterial efficacy of antibiotics against drug-resistant mutant strains was evaluated using the parameter of mutant resistance concentration (MPC). MPC is the minimum concentration of an antibacterial drug that is required to prevent the selective enrichment and amplification of single-step mutant strains. MPC was determined using 10^{10} CFU/mL of bacteria in suspension. Based on the concept of MPC, a selection index (SI) and MSW were utilized. SI is the ratio of MPC to MIC. The index is mainly used to compare the ability of antibiotics to select resistant mutant strains. MSW is the concentration range between MIC and MPC. The smaller the SI, the narrower the MSW and the less likely it is to selectively enrich drug-resistant mutant strains. In contrast, the larger the SI, the wider the MSW and the more likely it is to screen for drug-resistant mutant strains. The proposed MSW theory provides a basis for developing new treatment strategies characterized by changing drug dosage or methods. The theory also provides a more scientific and reliable method for evaluating the antibacterial efficacy of antibiotics and their ability to inhibit the growth of drug-resistant mutant strains (Yuhan *et al*., 2011).

Formulation of PD breakpoint for COPD

The enrofloxacin MIC distribution data of 143 strains of *M. hyopneumoniae* were obtained from the wild type break point value (CO_{WT}). PK data of the drug in target tissue after a single administration were calculated. The critical value of PK/PD parameters that eliminated bacteria was calculated and was the target value. PK data of drugs in target tissues and MIC distribution data of clinical bacteria were simulated using Oracle Crystal Ball 11.1.3.0.000 software. The PTA corresponding to each MIC when reaching the target value represents the CFR. The maximum MIC corresponding to a CFR >90% was the PD breakpoint (CO_{PD}) value.

Comprehensive trade-off to develop sensitivity breakpoints

According to CLSI guidelines, the final sensitivity breakpoint was determined by weighing the wild type breakpoint value (CO_{WT}), epidemiological breakpoint (ECOFF), clinical breakpoint (CO_{CL}) and CO_{PD}. The dose estimation for the treatment of swine mycoplasma pneumonia was calculated as:

$$(AUC/MIC) \times MIC \times CL/fu \times F$$

The targeted endpoint for optimal efficacy is the area under the concentration-time curve to minimum inhibitory concentration ratio (AUC/MIC). The minimum inhibitory concentration (MIC) refers to the lowest concentration of

Table 1: The linear relationship, repeatability, precision and sample recovery rate,the lowest quantitation limit (LOQ), the lowest detection limit (LOD)of Enrofloxacine.

Characteristic component	Linear relationship			Repeat ability RSD/%	Precision RSD/%	Sample recovery rate/%	The lowest quantitation limit (LOQ) µg/mL		Lowest detection limit (LOD) µg/mL
Enrofloxacine	Linear equation	R^2	Linear range/µg		Intra-day precision	Inter-day precision			
	y=1.893143e+03x +1.380940e+02	0.9984	0.00-500	2.0	2.0	2.1	85.91-88.27	0.005	0.001

a drug that inhibits the growth of bacteria. Clearance per day (CL) and the free fraction of drug in the plasma (fu) are also important factors, although fu is disregarded if binding is minimal. In this particular study, the protein binding of enrofloxacin was not taken into account due to higher concentrations of enrofloxacin in the lungs compared to the plasma. Previous studies have shown that the bioavailability (F) of enrofloxacin is 100% (Wang *et al.,* 2016; Wang *et al.,* 2022).

Results and Discussion

The MIC distribution of *M. hyopneumoniae* strains for enrofloxacin is shown in Fig 1. The linear relation ships, repeatability, precision, sample recovery rate, LOQ and LOD of enrofloxacin are provided in Table 1. The chromatograms of enrofloxacin are shown in Fig 2 and 3. Serum concentrations of enrofloxacin applied by intramuscular injection at 2.5 mg/kg body weight in Huainan pigs are provided in Table 2. Pharmacokinetic parameters of enrofloxacin in the administration procedure for swine mycoplasma pneumoniae were calculated and are presented in Table 3. The MIC_{90}, MPC_{90} and MSW values of enrofloxacin hydrochloride against *M. hyopneumoniae* are shown in Table 4. The data for the antibacterial activity of enrofloxacin against *M. hyopneumoniae* are presented in Table 5. PAE data for enrofloxacin against *M. hyopneumoniae* are shown in Table 6. MICs of the antimicrobial agents used against field strains of *M. hyopneumoniae* determined using a serial broth dilution method are shown in Table 7. MICs were determined when color changes had stopped for 1 d (*M. hyopneumoniae* grew steadily) after approximately 7 days after inoculation (final MICs). Drug time curves of enrofloxacin in administration Procedure of Swine Mycoplasma pneumonia are shown in Fig 4.

Table 2: Serum concentration of enrofloxacine (2.5 mg/kg.bw) after intramuscular injection (IM) in Huainan pigs.

Unit: μg·mL^{-1}

Blood sample collection time (h)	Huainan pig number								Blood drug concentration
	A	B	C	D	E	F	G	H	X±SD
0.25	0.68	0.73	0.74	0.59	0.71	0.84	0.71	0.77	0.72±0.07
0.5	1.26	1.23	1.24	1.27	1.12	1.15	1.06	1.14	1.18±0.08
1	1.57	1.48	1.36	1.59	1.29	1.77	1.41	1.32	1.47±0.16
2	1.63	1.65	1.57	1.65	1.66	1.83	1.62	1.56	1.65±0.08
4	1.49	1.45	1.55	1.45	1.54	1.67	1.53	1.41	1.51±0.08
8	1.25	1.21	1.27	1.24	1.16	1.22	1.08	1.12	1.19±0.07
12	0.65	0.72	0.77	0.79	0.68	0.63	0.52	0.61	0.67±0.09
24	0.23	0.17	0.28	0.27	0.33	0.15	0.21	0.19	0.23±0.06
36	0.11	0.08	0.13	0.10	0.17	0.07	0.12	0.09	0.11±0.03
48	0.06	0.04	0.06	0.05	0.08	0.03	0.05	-	0.05±0.02
72	0.005	-	-	-	0.006	-	-	-	0.0055±0.00
96	-	-	-	-	-	-	-	-	-

- : Below the minimum detection limit.

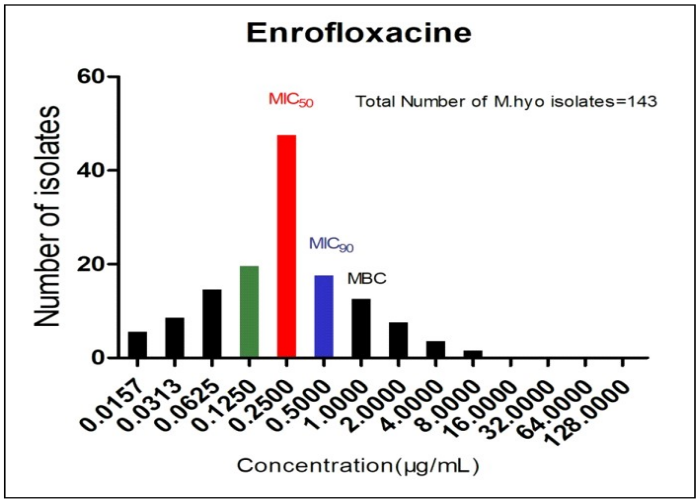


Fig 1: MIC distribution of *M. hyopneumoniae* field strains to enrofloxacin.

Table 3: Pharmacokinetic values of 8 samples (Non-compartmental analysis of plasma data after extravascular of pksolver 2.0).

Parameter	Unit	A	B	C	D	E	F	G	H
Lambda_z	1/h	0.0807	0.0602	0.064	0.0796	0.0590	0.0670	0.0632	0.0885
		78076	88291	18521	94813	29106	59913	0861	76286
t1/2	h	8.5808	11.4972	10.799	8.6975	11.74	10.336	10.966	7.825
		82475	1065	1729	19358	24645	23739	02472	42609
Tmax	h	2	2	2	2	2	2	2	2
Cmax	μg/ml	1.63	1.65	1.57	1.65	1.66	1.83	1.62	1.56
Tlag	h	0.27	0.19	0.11	0.23	0.12	0.05	0.17	0.21
Clast_obs/Cmax		0.00306	0.0242	0.0382	0.030	0.0481	0.0163	0.0308	0.0576
		7485	42424	16561	30303	92771	93443	64198	92308
AUC 0-t	μg/ml*h	24.155	22.41875	25.195	24.66125	25.235	22.46375	21.3925	20.36
AUC 0-inf_obs	μg/ml*h	24.216	23.082	26.129	25.288	26.590	22.911	22.183	21.376
		89798	22875	79479	6434	26362	11115	53147	07331
AUC 0-t/0-inf_obs		0.9974	0.9712	0.9642	0.9751	0.9490	0.9804	0.964	0.9524
		44017	55863	24947	90705	31584	74053	341499	66793
AUMC	μg/ml	301.76	269.46	352.68	312.68	406.61	236.87	286.20	239.4
0-inf_obs	*h^2	91768	83314	29217	48329	37726	0027	47516	616803
MRT 0-inf_obs	h	12.461	11.674	13.497	12.364	15.291	10.338	12.901	11.202
		09956	27696	34755	63451	82931	65296	67672	32313
Vz/F_obs	μg/ml	1.277	1.7965	1.4906	1.2404	1.5927	1.6271	1.7829	1.3203
		99157	08578	27204	64726	63007	62289	25039	66848
Cl/F_obs	(μg/ml)/h	0.1032	0.1083	0.095	0.0988	0.0940	0.1091	0.1126	0.1169
		33701	08432	67622	58605	19376	17362	96214	5319

Lambda_z: The terminal elimination rate constant, which describes the rate at which a drug is removed from the body during the terminal phase.

t1/2: The half-life of the drug, which is the time it takes for the concentration of the drug in the body to decrease by half.

Tmax: The time at which the maximum concentration of the drug is observed in the body.

Cmax: The maximum concentration of the drug observed in the body.

Tlag: The lag time, which is the time delay between the administration of the drug and the onset of its absorption.

Clast_obs/Cmax: The ratio of the last observed concentration to the maximum concentration, which can provide information about the rate of drug elimination.

AUC 0-t: The area under the concentration-time curve from time zero to the last measurable concentration, which represents the total drug exposure over time.

AUC 0-inf_obs: The area under the concentration-time curve from time zero to infinity, which represents the total drug exposure over an infinite period of time.

AUC 0-t/0-inf_obs: The ratio of the AUC from time zero to the last measurable concentration to the AUC from time zero to infinity, which can provide information about the completeness of drug absorption.

AUMC 0-inf_obs: The area under the first moment curve from time zero to infinity, which represents the total time the drug spends in the body.

MRT 0-inf_obs: The mean residence time from time zero to infinity, which represents the average time the drug stays in the body.

Vz/F_obs: The apparent volume of distribution during the terminal phase, which describes the extent of drug distribution in the body.

Cl/F_obs: The apparent clearance, which describes the rate at which a drug is removed from the body.

Table 4: MIC_{90}, MPC_{90} and MSW values of Enrofloxacin hydrochloride for M. Hyo.

Strain number	MIC_{90} (μg/mL)	MPC_{90} (μg/mL)	SI (MPC_{90} /MIC_{90})	MSW (μg/mL)
1	0.5	2	4	0.5<MSW<2
2	0.5	2	4	0.5<MSW<2
3	0.25	2	8	0.25<MSW<2
4	0.25	2	8	0.25<MSW<2
5	0.5	2	4	0.5<MSW<2

The MIC distribution data of clinical bacteria were simulated using Oracle Crystal Ball 11.1.3.0.000 software. The PTA corresponding to each MIC is shown in Fig 5.

When AUC_{0-t}/MIC >125 and there was a significant difference between adjacent MIC values, the previous MIC value was used as the CO_{PD}. The enrofloxacin CO_{PD}) of *M. hyopneumoniae* was set at 0.25 μg/mL.

Based on the bactericidal AUC_{24}/MIC ratio (40.72) and MIC of 0.5 μg/mL in the lungs, an intramuscular dosage of 2.036 mg/kg/day of enrofloxacin was used to calculate

M. hyopneumoniae bactericidal activity. Moreover, given an AUC_{24}/MIC ratio of 50.48 for bacterial eradication, a dosage of 2.52 mg/kg/day is recommended to achieve virtual elimination of *M. hyopneumoniae*.

Animal-derived enrofloxacin-resistant mycoplasmas are being detected more frequently by veterinarians worldwide (Hannan *et al*., 1997; Aarestrup *et al.,* 1998; Gautier-Bouchardon *et al.,* 2002; Fehri *et al.,* 2007; Vicca *et al.,* 2007; Thongkamkoon *et al.,* 2013; Tavio *et al.,* 2014; Antunes *et al.,* 2015; Felde *et al.,* 2018; Faucher 2019; Gonzaga *et al.,* 2020; Ammar *et al.,* 2022; Buni *et al.,* 2022; Zhang *et al.,* 2022). According to the 2016-2019 survey of most farms in China, the empirical drug used is actually the preventive drug used with reduced dosage on the basis of the original dose and the therapeutic drug used is with increased dosage. This situation has undoubtedly provided the basis for the development of drug resistance. Farm veterinarians often choose not to perform antimicrobial susceptibility testing due to the perception that it is time-consuming and the lack of standardized protocols or clinical breakpoints. (Bokma *et al.,* 2020; Bokma *et al.,* 2021).

Developing a break point value is a long-term process that requires timely correction based on the current drug resistance situation in veterinary clinical practice.

Table 5: Antibacterial activity of enrofloxacine against *M. hyo*.

Bacterial concent--ration (cfu/mL)	Drug	MIC (μg/mL)	MBC (μg/mL)
10^5	Enrofloxacine	0.25-0.5	0.5-1.0

Table 6: PAE of enrofloxacin against *M. hyo* (χ±s, n=4).

Drug	Concentration of bacterial solution at time 0	PAE(h) with different MIC				
Enrofloxacine	6.23±0.14	4 MIC	2 MIC	1 MIC	1/2 MIC	Drug residue control
		1.87±0.21	0.74±0.13	0.51±0.04	0.26±0.03	<0

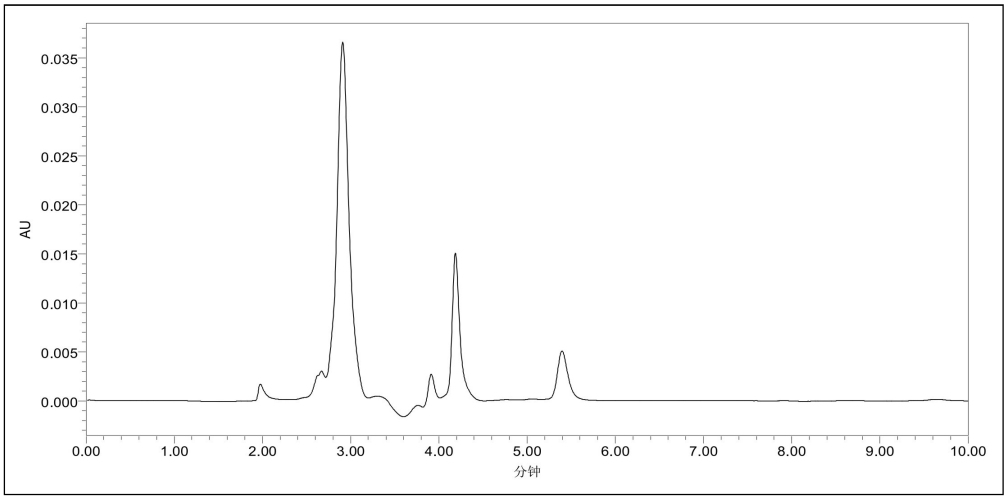


Fig 2: Chromatogram of the control sample.

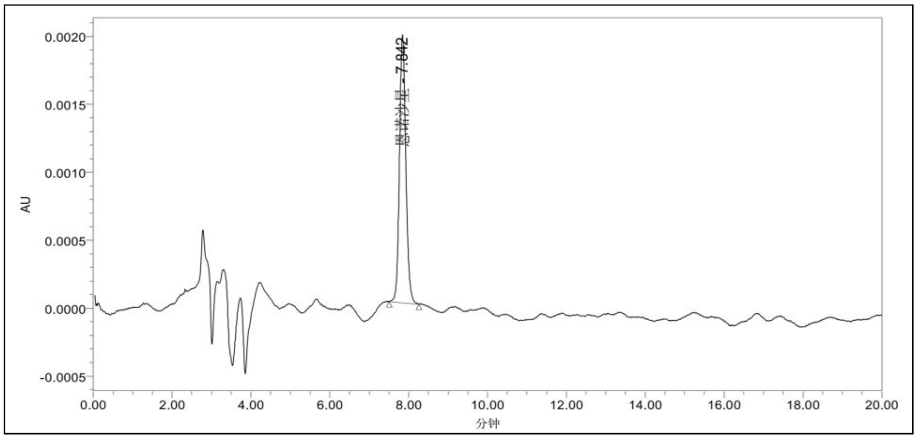


Fig 3: Chromatogram of the enrofloxacin standard sample.

Table 7: MICs of antimicrobial agents used against field strains of M. hyo, determined by a serial broth dilution method.

Drugs μg/mL)	M. hyo (n=143) TB (field strain from tibetan pigs, n=80) MI MI RAN C_{50} C_{90} GE	168L (vaccine strain)	RM48 (vaccine strain)	J(CVC C359)	HNSH (field strain, n=10)	HNPQ (field strain, n=10)	HNHC (field strain, n=10)	HNNY (field strain, n=10)	HNZZ (field strain, n=10)	AHFY (fiel strain, n=10)
enroflo--xacin	0.2 0.5 0.00 5	0.5 8-0.5	1	0.5	0.125 -16	0.008 -0.5	0.125 -0.5	0.008 -0.25	0.125 -4	0.125 -1

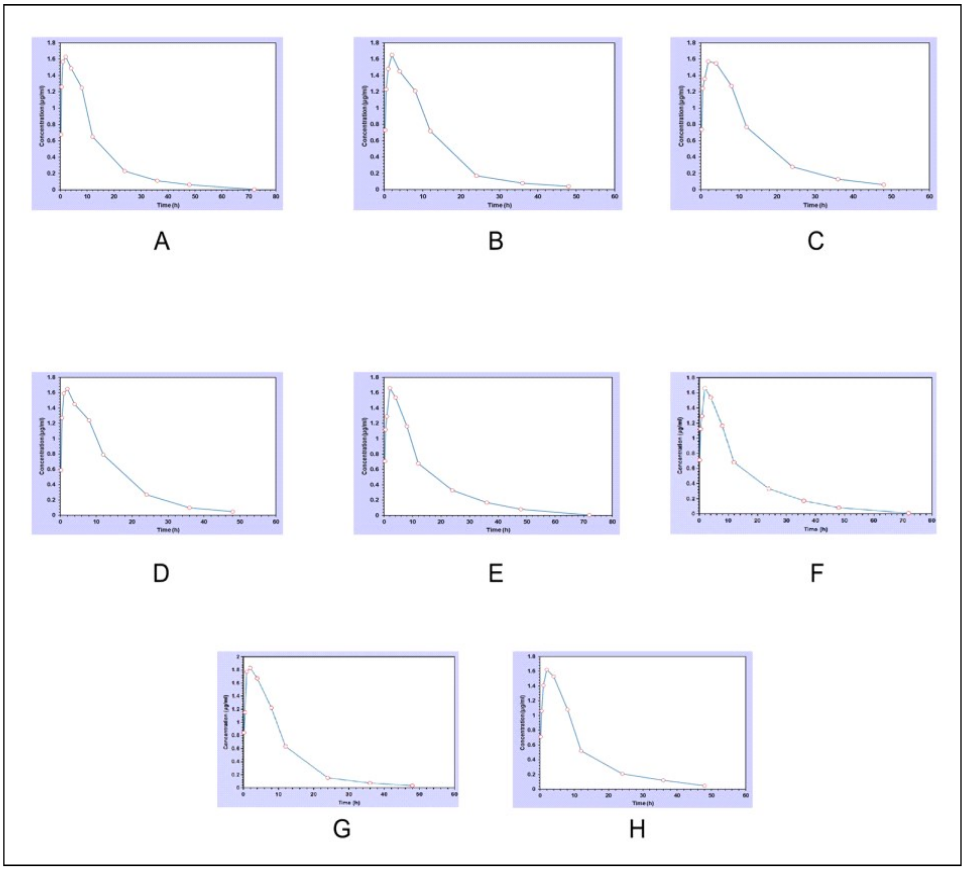


Fig 4: Drug time curve of enrofloxacin in administration procedure of swine mycoplasma pneumonia.

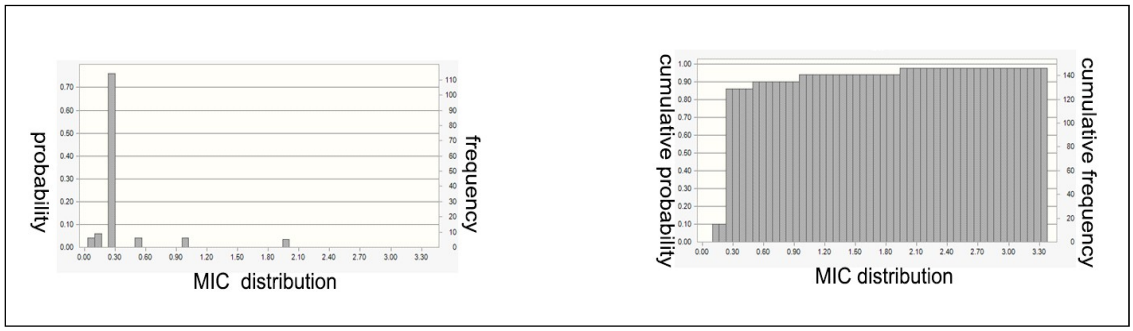


Fig 5: MIC distribution data of clinical bacteria simulated using oracle crystal ball 11.1.3.0.000 software and PTA corresponding to each MIC.

The selected drug sensitivity test method is different. The selected culture medium, inoculation volume and concentration, cultivation conditions, temperature, cultivation time and accuracy of configuring different concentrations of bacterial solution vary. Due to different geographical locations, the inflection points may also vary. The incidence as well as the selected treatment drugs vary in different regions. The distribution of bacteria also varies in different regions, as can the sensitivity of the same bacteria, leading to different inflection points. Different clinical doses and processes for obtaining clinical breakpoints vary. For some tissues, clinical breakpoints are established according to a given formula, while others use Monte Carlo simulations to determine breakpoints. The process of obtaining PD breakpoints varies, with some experimental animals being healthy and others clinically diseased (Pan, 2012).

There are corresponding detection requirements for different specimens and bacteria; different specimens and bacteria have specific detection requirements, which are outlined by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). CLSI has established official breakpoints for certain antibiotics, but only for mycoplasmas that affect humans. The procedures and media used for testing may vary depending on the species being examined (Felde *et al.,* 2018). In this study, the CLSI and EUCAST standard methods for mycoplasmas were adopted to make the test results as comparable as possible.

It is important to note that *in vitro* MIC values do not always correlate with the effectiveness of antimicrobials *in vivo*. Interpreting MIC distributions can be challenging, especially since there are no official clinical breakpoints for Mycoplasma species relevant to veterinary medicine. Additionally, herds may contain strains with varying susceptibilities to antibiotics. Therefore, pharmacokinetic/pharmacodynamic (PK/PD) analysis is a valuable tool for maximizing the *in vivo* antimicrobial activity (Felde *et al*., 2018).

The collection, isolation, purification, identification and preservation of typical clinical samples are important for drug resistance analysis and breakpoint formulation. This study used 12 batches of reagent drugs from five laboratories and 140 strains of *M. hyopneumoniae.* Certain differences between the measured values and reference data were found. The ECOFF should be set to 1 μg/mL (Rui *et al.,* 2023).

Conclusion

The pharmacodynamic cutoff value (CO_{PD}) of Mycoplasma hyopneumoniae against enrofloxacin should be set to 0.25 μg/mL, the clinical break point CO_{CL} should be set to 2 μg/mL, After comprehensive weighing, the final sensitivity break point should be set to 2 μg/mL.Defining the break point of enrofloxacin for *M. hyopneumoniae* can provide reference for determine the choice of empirical treatment and targeted therapy, predict the efficacy of drugs against specific animals and analyze treatment failure.

Funding

We thank the following for their support: the Foundation of Central Laboratory of Xinyang Agriculture and Forestry University (FCL202013, Gang Qiu). Research team on drug resistance mechanism of pathogenic bacteria in livestock and poultry and development of new alternative drugs (2024, Gang Qiu,Yapei Rui, Quanchao Ma, Bingjie Ma, Xun Li).

Conflict of interest

All authors declare that they have no conflicts of interest.

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