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Comparison of the pharmacokinetics of tilmicosin in plasma and lung tissue in healthy chickens and chickens experimentally infected with *Mycoplasma gallisepticum*

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Abstract

The objectives of this study were to compare the plasma and lung tissue pharmacokinetics of tilmicosin in healthy and *Mycoplasma gallisepticum*-infected chickens. Tilmicosin was orally administered at 4, 7.5 and 10 mg/kg body weight (b.w) for the infected and 7.5 mg/kg b.w for the uninfected control group. We found no significant differences in plasma tilmicosin pharmacokinetics between diseased and healthy control chickens. In contrast, the lung tissues in *M. gallisepticum*-infected chickens displayed a $t_{1/2}$ (elimination half-life) 1.76 times longer than for healthy chickens. The C_{max} (the maximum concentration of drug in samples) of tilmicosin in *M. gallisepticum*-infected chickens was lower than for controls at 7.5 mg/kg b.w ($p < .05$), and the AUC_{inf} (the area under the concentration–time curve from time 0 extrapolated to infinity) in infected chickens was higher than for the healthy chickens ($p < .05$). The mean residence time of tilmicosin in infected chickens was also higher than the healthy chickens. These results indicated that the lungs of healthy chickens had greater absorption of tilmicosin than the infected chickens, and the rate of elimination of tilmicosin from infected lungs was slower.

KEYWORDS

lung tissue, *Mycoplasma gallisepticum*, pharmacokinetics, plasma, tilmicosin

1 | INTRODUCTION

Mycoplasma gallisepticum is the etiologic agent of chronic respiratory disease (CRD) in chickens and colonizes mucosal surfaces of the respiratory tract. Coughing, sneezing, nasal discharge, mild conjunctivitis, and tracheal rales are the visible clinical signs of *M. gallisepticum* infection (Levisohn & Kleven, 2000). *M. gallisepticum* can be transmitted horizontally from the infected chickens to healthy chickens by the aerosol route but also vertically *via* eggs. Reduced weight gain, hatchability, feed conversion efficiency, and increased mortality are observed in *M. gallisepticum*-infected chickens and cause associated economic losses to poultry farmers (Kleven, 1990). The use of vaccination for *M. gallisepticum* control has been hampered by an inability to stop horizontal spread, and the considerable strain variability found in this organism. Consequently, antimicrobials play a significant role in control and treatment of these infections (Zhang et al., 2016, 2018).

Macrolides have been used extensively to treat bacterial respiratory diseases of livestock and are characterized by low serum concentrations but large volumes of distribution with accumulation and persistence in the lung (Yang et al., 2019). Tilmicosin is a semi-synthetic 16-membered lactone ring macrolide that has

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strong antimicrobial activity in vitro against a wide variety of gram-positive and gram-negative bacteria as well as Mycoplasmas (Xiong, Zhu, Zhao, et al., 2019). It is approved for the therapy or control of CRD caused by *M. gallisepticum* in chickens. Previous studies have indicated that tilmicosin administered in drinking water at ≥50 mg/L for either 3 or 5 days was effective against *M. gallisepticum* infections. Furthermore, there were significant reductions in air sac lesions and peritonitis in *M. gallisepticum*-infected chickens by treatment with 100, 200 or 300 mg/L for 5 days (Charleston, Gate, Aitken, & Reeve-Johnson, 1998; Kempf, Reeve-Johnson, Gesbert, & Guittet, 1997). This drug is also approved for the control of pneumonia associated with *Mannheimia haemolytica* in sheep and cattle, and swine respiratory disease caused by *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* (Womble, Giguère, Murthy, Cox, & Obare, 2006). For the reason, that antimicrobials are usually applied to treat infected animals rather than healthy animals clinically (Ding et al., 2013). In addition, pharmacokinetics and tissue distribution of antimicrobials can be affected by histopathological changes during an infection (Huang, Chen, & Zhang, 2003; Van Miert, 1990; Zeng & Feng, 1997). The pharmacokinetics and tissue distribution of antimicrobials in naturally or experimentally infected animals are a better indicator of effectiveness.

The pharmacokinetics of tilmicosin in healthy and infected animals has been determined in healthy pigs and those infected with *Haemophilus parasuis* and *A. pleuropneumoniae* (Xiong, Zhu, Yang, et al., 2019; Zhang, Zhao, Liu, Liu, & Li, 2017). This type of information regarding tilmicosin pharmacokinetics in *M. gallisepticum*-infected chickens is lacking, especially in lung tissues that are the primary site of *M. gallisepticum* proliferation during infection. The purpose of the current study was to compare the pharmacokinetics of tilmicosin in plasma and lung tissue of healthy chickens and chickens infected with *M. gallisepticum*.

2 | MATERIALS AND METHODS

2.1 | Organisms and chemicals

Mycoplasma gallisepticum standard strain S6 was purchased from the China Institute of Veterinary Drug Control (Beijing). *M. gallisepticum* artificial medium base was purchased from Qingdao Hope Biological Technology. Nicotinamide adenine dinucleotide and cysteine were purchased from Guangzhou prob information Technology. Sterile swine serum was purchased from Guangzhou Ruite Biological Technology. The initial pH of the medium was adjusted to pH 7.8 with 1 M NaOH.

Tilmicosin phosphate was provided by Guangdong Dahuanong Animal Health Products. Acetonitrile, methanol, and formic acid (high-performance liquid chromatography grade) and remaining analytical grade reagents were purchased from the Guangzhou Chemical Reagent.

2.2 | Animals and inoculation

One-day-old Sanhuang broiler chickens were supplied by the Guangdong Academy of Agricultural Sciences. All in vivo experiments were approved by the animal research committee of the South China Agriculture University Animal Ethics Committee (Approval number: 2018 A009). The chickens were *M. gallisepticum*-free and given antimicrobial-free feed and water ad libitum.

A total of 608 chickens were randomly divided into five groups: (a) A group of 90 chickens as uninfected group were used to study the pharmacokinetics of tilmicosin in healthy chickens; (b) a group of 270 chickens as infected group were experimentally infected with *M. gallisepticum*; then, they were used to study the pharmacokinetics of tilmicosin in *M. gallisepticum*-infected chickens; (c) 120 chickens were used as positive group to observe the infection of *M. gallisepticum* in infected chickens; (d) 120 healthy chickens were used as the negative group to confirm the healthy chickens were not infected with *M. gallisepticum*; and (e) the remaining eight healthy chickens were used as blank control to obtain blank plasma and lung tissues.

The 3-day-old chickens were infected with *M. gallisepticum* as previously described (Zhang, Wu, et al., 2017). Briefly, the chickens were inoculated with 0.2 ml aliquots of 10^9 CFU/ml of *M. gallisepticum* (twice a day) *via* intratracheal injection over three consecutive days. The animals were continually monitored for clinical signs of respiratory tract infection such as nasal discharge, sneezing, coughing and rales. Before the inoculation of *M. gallisepticum*, before the administration of tilmicosin, 48, 96, and 144 hr after the administration, the chickens in positive and negative group were euthanized ($n = 6$ per time point). Serum samples were collected to detect *M. gallisepticum* antibodies from the positive and negative group at the time of euthanasia. *M. gallisepticum* antibodies were detected using a commercial ELISA kit and was used according to the supplied instructions using an ELx800 Microplate Reader (BioTek Instruments). Pathological changes in the trachea, air sacs, and lungs of infected animals were determined. Besides, isolation of *M. gallisepticum* from air sac and lung tissues in positive and negative group was also carried out to confirm the success of the experimental infection. All animals were housed in a large room, and healthy chickens and *M. gallisepticum*-infected chickens were housed in different compartments separated by at least 20 m. The passage was disinfected every day during the course of the experiments. Moreover, the feeding environment was controlled to prevent other bacterial infections.

2.3 | Pharmacokinetics of tilmicosin in healthy and *M. gallisepticum*-infected chickens

The groups of 90 healthy chickens were orally administered tilmicosin by oral gavage at 7.5 mg/kg b.w, and they were euthanized at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, and 144 hr

after the administration of tilmicosin. The group of 270 infected chickens was orally administered with tilmicosin at 4, 7.5, and 10 mg/kg b.w and were euthanized at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, and 144 hr after the administration of tilmicosin. Blood and lung tissues were collected from six chickens at each sampling time point per treatment group. The chickens in blank group were untreated, and the blank samples (plasma and lung tissues) were collected before the experiment. The samples of plasma and lung tissues were stored at −20°C until analyzed within 2 weeks.

2.4 | Analysis of tilmicosin

Tilmicosin concentrations in plasma and lung tissues were determined by HPLC–MS/MS (Agilent 1200 series high-performance liquid chromatography unit and an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization source), and the chromatographic separation was achieved on a Phenomenex C_{18} column (150 mm × 2 mm; 5 μm) using a mobile phase consisted of solution A (0.1% formic acid in water) and solution B (acetonitrile) at 0.25 ml/min. The gradient elution was 0–1.5 min, 10% B; 1.5–6 min, 95% B; 6–6.5 min, 5% B; and 6.5–12.5 min, 5% B. The injection volume was 5 μl. Data acquisition and processing were carried out using the Analyst 1.5 software supplied with the instrument.

Mass spectrometric analysis was performed in the positive ion multiple reaction monitoring mode at 5,000 V ion spray voltage. The ion source temperature (TEM) was maintained at 600°C. Precursor/product ion pair transitions were m/z 869.60/696.60 and m/z 869.60/174.50, respectively. The collision energies were 56 and 64 V. The fragmentation voltage was 120 V, and the dwell time was 0.15 s.

Plasma samples were pretreated as previously reported (Lombardi, Portillo, Hassfurther, & Hunter, 2011) with modifications. In brief, plasma sample (0.25 ml) was added to 1 ml of acetic acid/acetonitrile (1:99) and vortexed for 1 min and incubated at 45°C for 10 min. The solution was centrifuged at 10,000 *g* at 4°C for 10 min; then, the supernatant was removed, and the extraction was repeated. The supernatants were combined and evaporated to near dryness at 45°C under a gentle stream of nitrogen. The residue was suspended in 1 ml 0.1% formic acid. Finally, the mixture was vortexed for 30 s and filtered through a 0.22 mm syringe filter (JinTeng) prior to HPLC-MS/MS analysis.

Lung tissues were treated as previously described with modifications (Modric, Webb, & Davidson, 1999). In brief, lung tissue samples (0.2 g) were homogenized and 1.2 ml of acetonitrile was added; then, the mixture was vortexed and placed on a shaking table for 10 min. The suspension was then centrifuged at 2,000 *g* at 4°C for 10 min, the supernatant was removed, and the extraction was repeated. The supernatants were combined, and 9 ml of water was added. The sample was then applied to an SPE C_{18} cartridge that had been preconditioned with 3 ml methanol and 3 ml of water. The sample was loaded and washed with 3 ml water and 2.5 ml of acetonitrile and then dried using a low positive pressure. The column was eluted using 5 ml of 0.1 M ammonium acetate in methanol: acetonitrile (8:2, v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen at 45°C, and the residue was dissolved in 1 ml 0.1% formic acid and filtered as per above.

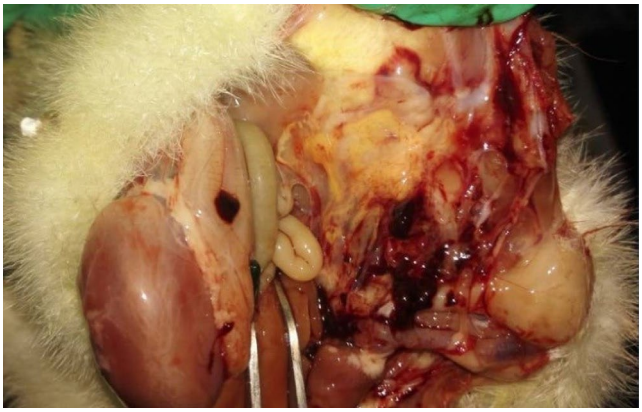

FIGURE 1 The lesion of air sac of the chickens infected with *Mycoplasma gallisepticum*

2.5 | Pharmacokinetics analysis

Pharmacokinetic profiles of tilmicosin in plasma and lung tissues were analyzed by noncompartmental model using WinNonlin software (version 5.2; Pharsight). Pharmacokinetic parameters including elimination half-life ($t_{1/2}$), the area under the concentration–time curve from time 0 extrapolated to infinity (AUC_{inf}), and maximum concentration of drug in samples (C_{max}), the time of peak concentration (t_{max}), and mean residence time (MRT) were calculated.

2.6 | Statistical analysis

The data were expressed as (mean ± *SEM*) and analyzed using SPSS software (IBM), and differences between group averages were examined using Duncan's multiple range test.

3 | RESULT

3.1 | *M. gallisepticum* infection model

Evaluation of the *M. gallisepticum* infection model was based on observable clinical signs and colonization of *M. gallisepticum* in air sac and lung tissues. Coughs, sneezing, ocular and nasal discharge, breathing difficulty/mouth breathing, and moist rales were observed in *M. gallisepticum*-infected chickens. The air sacs were thickened and contained opaque and caseous deposits. Infected lungs were

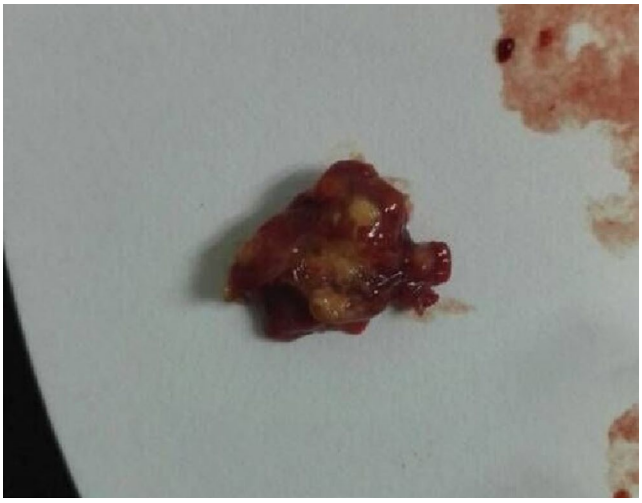

FIGURE 2 The lesion of lung tissue of the chickens infected with *Mycoplasma gallisepticum*

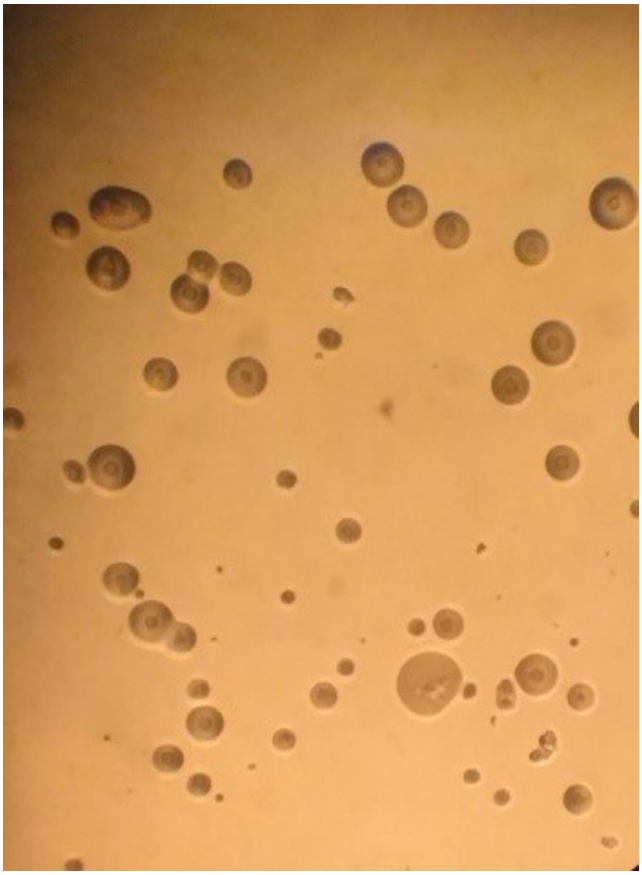

FIGURE 3 The isolated *Mycoplasma gallisepticum* under the microscope

swollen and consolidated with gray granulomas (Figures 1 and 2). *M. gallisepticum* isolated from lung tissues produced characteristic nipple shaped or fried egg appearing colonies on solid media (Figure 3). The loads of *M. gallisepticum* in air sacs and lungs 144 hr postinfection were $10^{6.15}$ and $10^{5.86}$ CFU/ml.

3.2 | Tilmicosin serum and tissue levels

In our protocol for HPLC-MS/MS analysis of tilmicosin, the compound eluted rapidly as a single symmetric peak at 5.8 min with no interfering peaks. The calibration curve in plasma and lung tissue was linear from 0.005–0.1 μg/ml and 0.005–0.5 μg/g, respectively. The limit of detection and quantification (LOQ) in plasma was 0.001 and 0.002 μg/ml and in lung tissues were 0.004 and 0.008 μg/g, respectively. The recoveries of tilmicosin were 83.84%–98.55% in plasma and 82.88%–92.37% in lung tissues. The within-run relative standard deviations and between-run were all <10.53% in both plasma and lung tissue (n = 18).

3.3 | Tilmicosin pharmacokinetics

The time–concentration curves of tilmicosin in plasma and lung tissues after oral administration at doses of 4, 7.5 and 10 mg/kg b.w in infected and at 7.5 mg/kg b.w in healthy control chickens are shown in Figures 4 and 5. Tilmicosin levels in plasma for both infected and control animals were below the LOQ at 96 hr after oral administration, and there were no differences in plasma levels of the drug ($p > .5$) (Table 1). In contrast, the lung tissue distribution of the drug differed between *M. gallisepticum*-infected and healthy chickens. The $t_{1/2}$ of tilmicosin *M. gallisepticum*-infected chickens was 1.76 times longer than for healthy chickens although no significant differences were found for t_{max} ($p > .05$). The C_{max} of tilmicosin in infected chickens was lower than for their healthy counterparts at 7.5 mg/kg b.w ($p < .05$) while the AUC_{inf} was higher ($p < .05$). The MRT of tilmicosin in *M. gallisepticum*-infected chickens was also higher than for the healthy chickens (Table 2).

A comparison of the pharmacokinetics of tilmicosin in plasma and lung tissue indicated that the C_{max} of tilmicosin in lung tissues was 12.8 and 14.28 times higher than for the corresponding plasma values for infected and healthy chickens, respectively. In addition, the t_{max} of tilmicosin in lung tissues was 6.83 and 6.67 times higher than plasma in infected chickens and healthy chickens; besides, we also found no significant differences in the $t_{1/2}$ of tilmicosin between plasma and lungs for infected and healthy chickens ($p > .05$).

4 | DISCUSSION

Mycoplasma gallisepticum is the causative agent of CRD in chickens and exerts a large financial burden on the poultry industry. Tilmicosin accumulates in lung tissue and is approved for the control and treatment of respiratory diseases caused by *Mycoplasma* spp (Womble et al., 2006). However, the current study is the first that examines the pharmacokinetics of tilmicosin in chickens with *M. gallisepticum* infections. Previous studies have reported that the pharmacokinetics at infected sites are a better approximation of the actual clinical conditions (Benet, Kroetz, Sheiner, Hardman, & Limbird, 1996; Sang,

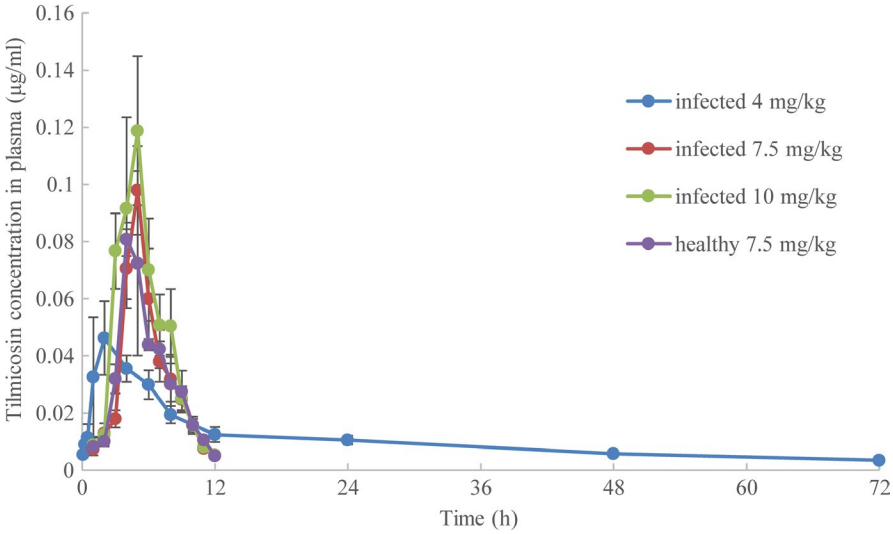

FIGURE 4 The time–concentration curves of tilmicosin in plasma after oral administration at a single dose of 4, 7.5, or 10 mg/kg b.w in *Mycoplasma gallisepticum* infection model and at s single dose of 7.5 mg/kg b.w in healthy chicken (*n* = 6/time point, per group)

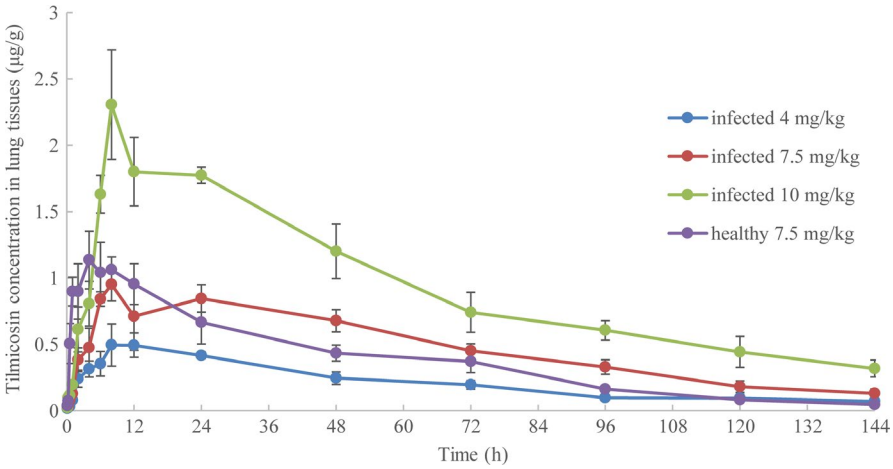

FIGURE 5 The time–concentration curves of tilmicosin in lung tissues after oral administration at a single dose of 4, 7.5, or 10 mg/kg b.w in *Mycoplasma gallisepticum* infection model and at s single dose of 7.5 mg/kg b.w in healthy chicken (*n* = 6/time point, per group)

TABLE 1 Pharmacokinetic parameters of tilmicosin in plasma following oral administration at single doses as indicated

Parameters	Units	4 mg/kg infected	7.5 mg/kg infected	10 mg/kg infected	7.5 mg/kg Not infected
$t_{1/2}$	hr	23.56	21.88	21.68	25.98
t_{max}	hr	2.00	2.17	1.42	1.50
C_{max}	μg/ml	0.07	0.11	0.16	0.11
MRT	hr	17.86	18.34	17.52	20.47
AUC_{inf}	μg·hr/ml	0.88	1.35	1.55	1.46

Abbreviations: AUC_{inf}, the area under the concentration–time curve from time 0 extrapolated to infinity; C_{max}, the maximum concentration of drug in samples; MRT, mean residence time; $t_{1/2}$, elimination half-life; t_{max}, the time of peak concentration.

Hao, Huang, Wang, & Yuan, 2016). We generated detailed values of tilmicosin concentrations in lung tissues of *M. gallisepticum*-infected chickens that will be helpful for designing and optimizing reasonable dosage regimens.

Previous studies on *M. gallisepticum* infections in chickens have used intratracheal (0.2 ml) and sinus (0.05 ml) routes with inoculum sizes of 10^6–10^7 CFU/ml using strain R-P10. After inoculation, the chickens were examined postmortem for gross lesions and sections of trachea were cultured for *M. gallisepticum* recovery. Lesions in the air sacs and peritoneum were scored from zero to four (Charleston et al., 1998; Kempf et al., 1997; Kleven, King, & Anderson, 1972; Reeve-Johnson & Otte, 1997). Our current study differed from these in a number of ways. We used a larger inoculum size (10^9 CFU/ml) that was calculated based on preliminary experiments using doses of 10^7, 10^8, and 10^9 CFU/ml twice a day for three consecutive days. We found that based on the morbidity, mortality, and *M. gallisepticum* load in air sacs and lungs, the best inoculum size for the infection model was 10^9 CFU/ml and this has been previously reported. In addition, we used a quantitative assessment of lesions in air sacs and lungs. At an inoculum size of

TABLE 2 Pharmacokinetic parameters of tilmicosin in lung following oral administration at single doses as indicated

Parameters	Units	4 mg/kg infected	7.5 mg/kg infected	10 mg/kg infected	7.5 mg/kg Not infected
$t_{1/2kel}$	hr	45.50	40.77	45.45	24.98
t_{max}	hr	11.33	9.33	8.33	9.67
C_{max}	μg/g	0.63	1.10	2.63	1.49
MRT	hr	48.34	50.65	51.07	40.79
AUC_{inf}	μg·hr/g	35.40	78.33	157.71	56.73

Abbreviations: AUC_{inf}, the area under the concentration–time curve from time 0 extrapolated to infinity; C_{max}, the maximum concentration of drug in samples; MRT, mean residence time; $t_{1/2}$, elimination half-life; t_{max}, the time of peak concentration.

10^9 CFU/ml, the mean *M. gallisepticum* load in air sacs and lungs was ~ 10^6.CFU/ml and indicated that the CFU and quantitative detection method adopted by us in this study is accurate because the *M. gallisepticum* colony is recorded from direct microscopic examination.

Our results indicated no significant differences in tilmicosin concentration in plasma between *M. gallisepticum*-infected and healthy chickens. A previous study found a similar pattern for tilmicosin between healthy and *H. parasuis*-infected pigs (Zhang, Zhao, et al., 2017). However, the $t_{1/2}$ of tilmicosin in our infected chickens was 1.76 times longer than for the healthy chickens. In addition, C_{max} was lower and the AUC_{inf} was higher for infected chickens. This revealed that healthy lungs absorbed tilmicosin better than when infected with *M. gallisepticum*. Moreover, the rate of elimination of tilmicosin from infected lungs was slower than for the healthy counterparts. A previous study produced similar results in the pharmacokinetic depletion phase of doxycycline in healthy and *M. gallisepticum*-infected chickens (Gbylik-Sikorska, Gajda, & Posyniak, 2018). This study and ours indicated that *M. gallisepticum* infection has a profound influence of drug distribution. For example, the t_{max} of doxycycline in lung tissues was 4 hr in healthy and 8 hr in infected chickens and $t_{1/2}$ was 20 hr in healthy and 28.5 hr in infected chickens (Gbylik-Sikorska et al., 2018). These differences between the plasma and lung drug levels are most likely the result of the inflammation and pneumonia caused by *M. gallisepticum* infections and lesions, hyperemia, edema, and necrosis in the lungs. Our findings were in agreement with typical pathologic changes found in other reports that also concluded a decreased circulation in infected lungs led to a decrease in drug metabolism (Ding et al., 2013; Gbylik-Sikorska et al., 2018; Xiao et al., 2015)..

In the present study, we found that the C_{max} of tilmicosin in lung tissues was 12.8 and 14.28 times higher than for plasma between *M. gallisepticum*-infected chickens and their healthy counterparts and t_{max} was 6.83 and 6.67 times higher than plasma values, respectively. We did not find any significant differences for $t_{1/2}$ of tilmicosin in plasma and lung between infected and control chickens. A previous study reported the pharmacokinetics and tissue concentrations of tilmicosin after a single oral dose (50 mg/kg b.w) in healthy Leghorn chickens indicated that the C_{max} of tilmicosin in lung tissue was 6.2 times greater than in plasma (Keles et al., 2001). Similar to other macrolide antibiotics, tilmicosin has good tissue penetration and the ability to penetrate into phagocytic cells is essential for activity against intracellular organisms (Meyer, Bril-Bazuin, Mattie, & Broek, 1993). Studies using radiolabeled tilmicosin have indicated that cellular/ extracellular drug concentration ratios at 4 hr in chicken heterophils, macrophages, and monocytes were 138, 32, and 66, respectively (Scorneaux & Shryock, 1998). This indicated that the ability of tilmicosin to penetrate into phagocytic cells was strong.

Tilmicosin has been widely used in the treatment of CRD caused by *M. gallisepticum* that colonized lung and air sacs, and its primary advantage is the high concentrations that accumulate in lung tissues. Therefore, analyses of lung tissues should be the primary focus in attempting to develop rational treatments of pulmonary infections caused by facultative intracellular pathogens such as *M. gallisepticum*. The MIC of tilmicosin for *M. gallisepticum* strain S6 is 0.038 μg/ml (Zhang, Ye, et al., 2017). Those authors found that C_{max} values in plasma were 0.07, 0.11, and 0.16 μg/ml after single oral doses of 4, 7.5, and 10 mg/kg b.w. We found C_{max} values in lung tissues were 0.63, 1.10, and 2.63 μg/ml after single oral dosings of 4, 7.5, and 10 mg/kg b.w. For both studies, the C_{max} of tilmicosin in plasma and lung exceeded the MIC. These findings indicate that tilmicosin is therapeutically effective for *M. gallisepticum* infections.

5 | CONCLUSIONS

We established an in vivo *M. gallisepticum* infection model by intratracheal inoculation of 0.2 ml of 10^9 CFU/ml twice a day for three consecutive days. The $t_{1/2}$ of tilmicosin in *M. gallisepticum*-infected chickens was 1.76 times longer than for healthy chickens, and the C_{max} for infected chickens was lower at 7.5 mg/kg b.w ($p < .05$). The AUC_{inf} of tilmicosin in infected chickens was higher than for healthy chickens at the same dose. Healthy chickens have a greater absorption of tilmicosin than *M. gallisepticum*-infected chickens, and the rate of elimination of tilmicosin in *M. gallisepticum*-infected lung tissue was lower than that for healthy chickens.

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CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

AUTHOR CONTRIBUTIONS

NZ, ZL, CZ, and YW contributed to methodology, software, validation, formal analysis, data curation, manuscript preparation, manuscript reviewing and editing, visualization, and project administration, and CM contributed to the investigation. QC, XS, and HD were provided resources. HD contributed to supervision and funding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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