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Pulmonary disposition of tilmicosin in foals and *in vitro* activity against *Rhodococcus equi* and other common equine bacterial pathogens

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The objectives of this study were to determine the serum and pulmonary disposition of tilmicosin in foals and to investigate the *in vitro* activity of the drug against *Rhodococcus equi* and other common bacterial pathogens of horses. A single dose of a new fatty acid salt formulation of tilmicosin (10 mg/kg of body weight) was administered to seven healthy 5- to 8-week-old foals by the intramuscular route. Concentrations of tilmicosin were measured in serum, lung tissue, pulmonary epithelial lining fluid (PELF), bronchoalveolar lavage (BAL) cells, and blood neutrophils. Mean peak tilmicosin concentrations were significantly different between sampling sites with highest concentrations measured in blood neutrophils (66.01 ± 15.97 μg/mL) followed by BAL cells (20.1 ± 5.1 μg/mL), PELF (2.91 ± 1.15 μg/mL), lung tissue (1.90 ± 0.65 μg/mL), and serum (0.19 ± 0.09 μg/mL). Harmonic mean terminal half-life in lung tissue (193.3 h) was significantly longer than that of PELF (73.3 h), bronchoalveolar cells (62.2 h), neutrophils (47.9 h), and serum (18.4 h). The MIC_{90} of 56 *R. equi* isolates was 32 μg/mL. Tilmicosin was active *in vitro* against most streptococci, *Staphylococcus* spp., *Actinobacillus* spp., and *Pasteurella* spp. The drug was not active against *Enterococcus* spp., *Pseudomonas* spp., and *Enterobacteriaceae*.

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INTRODUCTION

Tilmicosin is a semi-synthetic 16-membered lactone ring macrolide chemically derived from tylosin (Ose, 1987). Tilmicosin is approved for subcutaneous administration in the therapy or control of pneumonia caused by *Mannheimia haemolytica* in cattle and sheep. It is also approved for use in feed for the control of swine respiratory disease associated with *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*. In addition, the drug is active *in vitro* against a variety of pathogens of cattle and swine including *Histophilus somni*, *Haemophilus parasuis*, *Actinobacillus suis*, *Arcanobacterium pyogenes*, *Erysipelothrix rhusiopathiae*, *Staphylococcus* spp., some *Streptococcus* spp., and many *Mycoplasma* spp. (Ose, 1987; DeRosa *et al.*, 2000; Prescott, 2000). The pharmacokinetic properties of tilmicosin are similar to that of macrolides in general, and are characterized by low serum concentrations but large volumes of distribution, with accumulation and persistence in many tissues including the lung, which may concentrate the drug 60-fold compared with serum (Ziv *et al.*, 1995; Scorneaux & Shryock, 1999; Clark *et al.*, 2004). Despite low extracellular concentrations, tilmicosin accumulates substantially in phagocytic cells of cattle and swine (Scorneaux & Shryock, 1998, 1999).

Pneumonia is a leading cause of morbidity and mortality in foals (Cohen, 1994). Gram-positive bacteria such as *Streptococcus equi* subspecies *zooepidemicus* and *Rhodococcus equi* are the most common causes of pneumonia in foals between 1 and 6 months of age (Hoffman *et al.*, 1993; Giguère *et al.*, 2002). Gram-negative bacteria such as *Pasteurella* spp., *Actinobacillus* spp., *Bordetella bronchiseptica*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* may also be cultured from tracheobronchial aspirates of affected foals. Macrolide antimicrobial agents are commonly used in equine medicine for treatment of foal pneumonia, particularly when infection with *R. equi* is suspected or confirmed (Hillidge, 1987). Tilmicosin may be a useful alternative to currently used antimicrobial agents owing to its accumulation in lung tissue and phagocytic cells, as well as *in vitro* activity against many gram-positive and gram-negative

bacterial species. In addition, availability of a long-acting antimicrobial agent providing sustained therapeutic concentrations at the site of infection would result in less frequent administration, which in turn may improve client compliance. However, the lack of pharmacokinetic studies and *in vitro* susceptibility data with bacterial pathogens of horses precludes the rational use of this antimicrobial agent in foals.

The objectives of the study reported here were to determine the serum and pulmonary disposition of tilmicosin in foals and to investigate the *in vitro* activity of the drug against *R. equi* and other common bacterial pathogens of horses.

MATERIALS AND METHODS

Animals

Four male and three female Thoroughbred foals between 5 and 8 weeks of age and weighing between 80 and 135 kg were selected for this study. The foals were considered healthy on the basis of history, physical examination, complete blood count and plasma biochemical profile. The foals were kept with their dams in individual stalls during the experiment with *ad libitum* access to grass hay and water. The study was approved by the Institutional Animal Care and Use Committee at the University of Florida.

Experimental design and sample collection

A proprietary fatty acid salt formulation of tilmicosin (250 mg/mL; IDEXX Pharmaceuticals, Durham, NC, USA) was administered as a single dose of 10 mg/kg of body weight via the intramuscular route in the semimembranosus/semitendinosus muscles. Blood samples for serum separation were obtained from a jugular catheter at 3, 6, 10, 20, 30, 60, and 90 min; and at 2, 3, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 168, and 288 h after the drug was administered. Blood (30 mL) was collected in heparinized tubes for neutrophil separation at 4, 24, 48, 72, 168, and 288 h.

Bronchoalveolar lavage (BAL) was performed at 24, 48, 72, 168, and 288 h. Samples of cerebrospinal fluid (CSF) and peritoneal fluid were collected aseptically 4, 24, and 72 h after administration of tilmicosin. Lung tissue was obtained 24, 72, 168, and 288 h after administration of the drug. Foals were sedated by administration of xylazine hydrochloride (1 mg/kg, i.v.) and butorphanol tartrate (0.07 mg/kg, i.v.) prior to collection of BAL fluid, lung tissue, and body fluids. Immediately after the collection of BAL fluid, general anesthesia was induced by i.v. administration of diazepam (0.1 mg/kg) and ketamine (2.5 mg/kg) for collection of lung tissue, CSF, and peritoneal fluid. Samples of CSF were collected from the atlantooccipital space by use of a 3.5-inch, 20-gauge spinal needle. Abdominal fluid was collected by use of an 18-gauge needle. Lung tissue was obtained aseptically from the 8th intercostal space at the level of the point of the shoulder using a 16-gauge spring activated biopsy instrument with a 20 mm specimen notch (J528a; Jorgenson Laboratories, Loveland, CO, USA). Blood, CSF, and peritoneal fluid samples were centrifuged and serum, body fluid supernatants, and lung tissue samples were stored at −80 °C until analysis.

Bronchoalveolar lavage

A 10 mm in diameter, 2.4 m BAL catheter (Jorgenson Laboratories) was passed via nasal approach until wedged into a bronchus. The lavage solution consisted of four aliquots of 50 mL physiologic saline (0.9% NaCl) solution infused and aspirated immediately. Total nucleated cell count in BAL fluid was determined by use of a hemacytometer. Bronchoalveolar fluid was centrifuged at 200 ***g*** for 10 min. Bronchoalveolar cells were washed, re-suspended in 500 μL of phosphate-buffered solution (PBS), vortexed, and frozen at −80 °C until assayed. Supernatant BAL fluid was also frozen at −80 °C until assayed. Before assaying, the cell pellet samples were thawed, vortexed vigorously, and sonicated for 3 min to ensure complete cell lysis. The resulting suspension was centrifuged at 500 ***g*** for 10 min and the supernatant fluid was used to determine the intracellular concentrations of tilmicosin.

Blood neutrophils

Whole blood was collected in sodium heparin vacutainer tubes and allowed to separate at room temperature for 45 min. The plasma layer was removed and PBS was added until a combined volume of 30 mL was reached. Blood neutrophils were separated by density gradient centrifugation using Ficoll-hypaque. Supernatant layers were removed, the pellet was washed twice with PBS and gently resuspended in 500 μL of PBS. The cell count of the resulting suspension was determined by use of a hemacytometer. The suspension was vortexed and frozen at −80 °C until assayed. Before assaying, the samples were thawed, vortexed vigorously, and sonicated for 3 min to ensure complete neutrophil lysis. The resulting suspension was centrifuged at 500 ***g*** for 10 min and the supernatant fluid was used to determine the concentrations of tilmicosin in neutrophils.

Drug analysis in serum

Concentrations of tilmicosin in serum were measured by high performance liquid chromatography (HPLC) with UV detection. The HPLC method was based on a previously established procedure reported by Stobba-Wiley *et al.* (2000). The HPLC system consisted of a 126 solvent delivery system, 508 autosampler, and 126 diode array detector (Beckman Coulter, Fullerton, CA, USA). Sample extracts were injected onto a SphereClone phenyl column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA) and separated using a multi-gradient mobile phase at a flow rate of 1.5 mL/min at room temperature. Mobile phase components were water (solvent A), 20 mM dibutyl-ammonium-trifluoroacetate (solvent B), and acetonitrile (solvent C). Initial conditions of 50% A and 50% C were maintained for the initial 4 min. Thereafter, linear gradient

profiles were ran as follows: 4–5 min, 80% A, and 20% C; 5–5.1 min, 80% B, and 20% C; 5.1–25 min, 65% B, and 35% C; 25–25.1 min return to initial conditions. The retention time for tilmicosin was 20.4 min with a total run time of 50 min. The standard curve for tilmicosin in serum was linear in the range ($r^2 > 0.99$) of 0.125–4 μg/mL with a limit of detection (LOD) of 0.048 μg/mL and a limit of quantification (LOQ) of 0.070 μg/mL. The interday coefficients of variation for replicate serum samples ($n = 4$) within this concentration range varied from 4.4% to 8.1%. The amount of tilmicosin recovered from the serum by a C18 solid phase extraction cartridge (Water, Milford, MA, USA) was 98% on each day of analysis.

Drug analysis in lung tissue, cells, and body fluids

Lung tissues were weighed and placed in 500 μL of 10% chloroform in methanol. The samples were then sonicated for 60 min and centrifuged at 13 000 ***g*** at 4 °C for 15 min. The supernatant was decanted and 300 μL of water was added prior to analysis. Neutrophil and BAL cell suspensions (200 μL) were transferred to microfuge tubes containing 400 μL of methanol. This mixture was vortexed for 10 sec and centrifuged at 13 000 ***g*** at 4 °C for 15 min. Peritoneal, CSF, and BAL fluid were centrifuged at 4 °C for 15 min.

Concentrations of tilmicosin were measured by HPLC assay with mass spectrometry MS/MS Q Trap detection. Briefly, The HPLC system consisted of an Agilent 1100 binary pump, degasser, and autosampler (Model numbers G1312A, G1379A and G1313A, respectively; Agilent Technologies, Palo Alto, CA, USA). The detector used was a Qtrap LC/MS/MS system with an electrospray ionization (ESI) source and switch valve (Applied Biosystems MDS SCIEX, Foster City, CA, USA). The mobile phase consisted of an initial condition of 100% of 10 mM ammonium acetate in water on pump A (%A) and 0% 10 mM ammonium acetate in methanol on pump B (%B). At 2 min the %B was gradually increased to 100% over 5 min. At 7 min the initial condition of 100% A was re-established and the system was re-equilibrated for 8 min. Prepared lung tissues, BAL cells, and peripheral blood neutrophil samples were pumped through a Gemini 5 micron C18 110A, 30 × 4.60 mm column (Phenomenex) at a flow rate of 1.0 mL/min at room temperature. The first 2 min of solvent flow was diverted to waste through the switch valve and then to the mass spectrometer after 2 min. BAL, CSF, and peritoneal fluid samples were first passed through a LiChrospher RP-18 ADS restricted access material cartridge (25 micron 25 × 4 mm, 60A; Merck KGaA, Darmstadt, Germany) with the first 2 min of solvent being diverted to waste through the switch valve. At 2 min the switch valve diverted the solvent to a Gemini C18 column that was connected to the ESI source of the mass spectrometer. The MS/MS multireaction monitoring parameters were monitored as follows: ionization mode, electrospray positive ion mode; capillary voltage, 5 kV; source temperature, 400 °C; curtain gas setting, 35; ion source gas 1 setting, 55; ion source gas 2 setting, 85; collision gas, medium; interface heater, on; Q1 resolution, unit; and Q3 resolution, unit. MS parameters were as follows: precursor ion (amu):869.4; product ion (amu):174; declustering potential, 80 V; exit potential, 10 V; collision cell entrance potential, 30 V; collision energy, 50 V; and collision cell exit potential, 10 V.

The standard curves for tilmicosin in lung tissue were linear ($r^2 > 0.99$) in the range of 1–25 ng/mL with a LOD of 0.2 ng/mL and a LOQ of 0.5 ng/mL. Tilmicosin concentrations in lung are reported as μg of drug per gram of tissue based on the sample wet weight. The intraday coefficients of variation for replicate quality control samples ($n = 3$) within the concentration ranges varied from 2.3% to 8.7%. The standard curves for tilmicosin in BAL cell and neutrophil suspension were linear ($r^2 > 0.99$) in the range of 5–5000 ng/mL with an LOD of 0.6 ng/mL and a LOQ of 1.8 ng/mL. The intraday coefficients of variation for replicate quality control samples ($n = 3$) within the concentration ranges varied from 2.4% to 6.5%. The standard curves for tilmicosin in BAL, CSF, and peritoneal fluid were linear ($r^2 > 0.99$) in the range of 1–200 ng/mL with a LOD of 1.4 ng/mL and a LOQ of 3.9 ng/mL. The intraday coefficients of variation for replicate quality control samples ($n = 3$) within the concentration range varied from 2.4% to 5.0%.

Calculation of tilmicosin concentrations in PELF, BAL Cells, and neutrophils

Pulmonary distribution of tilmicosin was determined as reported (Baldwin *et al.*, 1992). Estimation of the volume of pulmonary epithelial lining fluid (PELF) was done by urea dilution method (Rennard *et al.*, 1986; Conte *et al.*, 1996). Serum urea nitrogen concentrations ($Urea_{SERUM}$) were determined by use of enzymatic methodology (Labsco Laboratory Supply Company; Louisville, KY, USA) on a chemistry analyzer (Hitachi 911 analyzer, Boehringer Mannheim Inc., Indianapolis, IN, USA). For measurement of urea concentration in BAL fluid, the proportions of reagents to specimen were changed from 300 μL of reagent/3 μL of serum to 225 μL of reagent/50 μL of BAL fluid. The volume of PELF (V_{PELF}) in BAL fluid was derived from the following equation: $V_{PELF} = V_{BAL}(Urea_{BAL}/Urea_{SERUM})$, where V_{BAL} is the volume of recovered BAL fluid. The concentration of tilmicosin PELF (TIL_{PELF}) was derived from the following relationship: $TIL_{PELF} = TIL_{BAL}(V_{BAL}/V_{PELF})$, where TIL_{BAL} is the measured concentration of tilmicosin in BAL fluid.

The concentration of tilmicosin in BAL cells and blood neutrophils (TIL_{CELLS}) was calculated using the following relationship: $TIL_{CELL} = (TIL_{PELLET}/V_{CELL})$ where TIL_{PELLET} is the concentration of antimicrobial in the cell pellet supernatant and V_{CELL} is the mean volume of foal BAL cells or neutrophils, respectively. Volumes of 1.20 μL/10^6 BAL cells and 0.450 μL/10^6 neutrophils were used for calculations based on previous studies in foals (Jacks *et al.*, 2001; Davis *et al.*, 2002).

Pharmacokinetic analysis

For each foal, serum, lung tissue, PELF, BAL cells, or neutrophils tilmicosin concentration vs. time data were analyzed based on noncompartmental pharmacokinetics using computer software (PK Solutions 2.0; Summit Research Services, Montrose, CO,

USA). The elimination rate constant (K_{el}) was determined by linear regression of the terminal phase of the logarithmic concentration vs. time curve using a minimum of three data points. Terminal half-life ($t_{1/2}$) was calculated as the natural logarithm of 2 divided by K_{el}. Pharmacokinetic values were calculated as reported by Gibaldi and Perrier (1982). The area under the concentration–time curve (*AUC*) and the area under the first moment of the concentration–time curve (*AUMC*) were calculated using the trapezoidal rule, with extrapolation to infinity using C_{min}/K_{el}, where C_{min} was the final measurable tilmicosin concentration. Mean residence time was calculated as: *AUMC*/*AUC*.

Statistical analysis

Normality of the data and equality of variances were assessed using the Kolmogorov–Smirnov and Levene's tests, respectively. A one-way repeated measure ANOVA was used to compare each pharmacokinetic parameter between sampling sites (serum, lung tissue, PELF, BAL cells, and neutrophils). In rare instances when the assumptions of the ANOVA were not met, a Friedman repeated measure ANOVA on ranks was used. When indicated, multiple pairwise comparisons were done using the Student–Newman–Keuls test. Differences were considered significant at $P < 0.05$.

Determination of MIC and MBC of tilmicosin against Rhodococcus equi

Rhodococcus equi isolates ($n = 56$) were obtained from tracheobronchial aspirates or postmortem specimens from pneumonic foals. For each isolate, minimum inhibitory concentration (*MIC*) and minimum bactericidal concentrations (MBC) were determined by a macrodilution broth dilution technique in glass tubes in accordance to the guidelines established by the Clinical and Laboratory Standard Institute (CLSI; formerly NCCLS) (National Committee for Clinical Laboratory Standards, 1999a,b, 2002). A standard inoculum of 5×10^5 was used for each isolate. Concentrations of tilmicosin tested represented twofold dilutions between 256 and 0.03 μg/mL. All *MIC* and MBC determinations were performed in triplicate for each isolate. *MIC* was determined as the first dilution with no bacterial growth after 24 h of incubation at 37 °C. MBC was calculated as the lower concentration of drug resulting in a 99.9% reduction of the original inoculum. Control strains used to validate the assay were *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 (Odland *et al.*, 2000). The *MIC* required to inhibit growth of 50% of isolates (MIC_{50}) and the *MIC* required to inhibit growth of 90% of isolates (MIC_{90}) were determined.

Checkerboard assay

Activity of tilmicosin in combination with rifampin, gentamicin, amikacin, doxycycline, enrofloxacin, trimethoprim-sulfa, vancomycin, imipenem, or ceftiofur against *R. equi* was assessed using the modified checkerboard technique as previously described (Pillai *et al.*, 2005). Three isolates of *R. equi* were randomly selected for this assay. The three isolates had a *MIC* of 32 μg/mL. All experiments were performed in triplicate for each of the isolate. For each antimicrobial agent, concentrations of 64-, 16-, 4-, 1-, and 0.5-times the *MIC* were used to study antibiotic combinations. An inoculum of 5×10^5 was used for each *R. equi* isolate. For each combination, the fractional inhibitory concentration (FIC) index after 24 h of incubation was calculated using the following formula: FIC index = FIC A + FIC B = (*MIC* of A in combination/*MIC* of A alone) + (*MIC* of B in combination/*MIC* of B alone). A FIC index of ≤ 0.5 indicates synergism, a FIC index >0.5–4 indicates indifference and a FIC index >4 indicates antagonism (Pillai *et al.*, 2005).

Time-kill curve assay

A time-kill curve assay was used to evaluate the effect of time and tilmicosin concentration on *in vitro* survival of *R. equi*. All experiments were performed in triplicate using the same three *R. equi* isolates used for the checkerboard assay. An inoculum of 5×10^5 CFU/mL was used for each isolate. All experiments were performed with 4 mL of Mueller–Hinton broth in glass tubes. Concentrations of tilmicosin of 64-, 16-, 4-, 1-, and 0.5-times the *MIC* were used. A tube without tilmicosin was used as control. After 0, 2, 6, and 24 h of incubation, aliquots were collected from each tube. The aliquots were centrifuged, the bacterial pellets were washed twice to prevent antimicrobial carry over, and the colony-forming units (CFU) was counted.

In vitro activity of tilmicosin against equine bacterial pathogens

A total of 183 bacterial isolates from various equine clinical samples were examined. Isolates were obtained from clinical samples submitted to the microbiology laboratory of the University of Florida Veterinary Medical Center from July 2005 to January 2006. Susceptibility testing was performed using the disk diffusion method. Briefly, fresh isolates were grown on blood agar plates, and colonies were suspended in sterile water to achieve turbidity equal to that of a 0.5 McFarland standard (final bacterial concentration of approximately 1×10^5 CFU/mL). A sterile swab was dipped into the inoculum suspension and used to inoculate the entire surface of 100-mm Mueller–Hinton plates three times by rotating the plate approximately 60 ° for each inoculation to ensure an even distribution. After allowing the excess moisture to dry (approximately 10–15 min), 15 μg tilmicosin disks (BBL Sensi-Disc, Hardy Diagnostics, Santa Maria, CA, USA) were applied to the agar. The plates were incubated for 18–24 h at 37 °C. A test was considered valid only when there was adequate growth on the plate. The zone diameter was measured to the nearest millimeter. Control strains used weekly to validate the assay were *S. aureus* ATCC 29213, *E. coli* ATCC 25922, and *E. faecalis* ATCC 29212. Results were considered valid only when zone diameters obtained with the control stains were within the reference range proposed (Odland *et al.*, 2000). According to CLSI guidelines, isolates with a zone diameter

≥14 mm (corresponding to a *MIC* ≤ 8 μg/mL) were considered susceptible (Shryock *et al.*, 1996).

RESULTS

Serum and pulmonary disposition of tilmicosin in foals

Quantifiable tilmicosin concentrations were found in two of seven foals at 3 min after i.m. injection and in five of seven foals at 10 min postinjection. Serum concentrations remained below the LOQ throughout the sampling period in two foals. Concentrations below the LOQ were reported as zero for calculation of mean ± SD (Fig. 1). Serum pharmacokinetic parameters were derived from the five foals with quantifiable serum concentrations (Table 1). Maximum tilmicosin concentrations (C_{max}) and *AUC* were significantly higher for neutrophils than for BAL cells, PELF, lung tissue, and serum. Similarly, C_{max} and *AUC* were significantly higher for BAL cells than for PELF, lung tissue, and serum. Terminal half-life in lung tissue (193.3 h) was significantly longer than that of other sampling sites. Concentrations of tilmicosin in CSF and peritoneal fluid were similar to concurrent serum concentrations (Table 2).

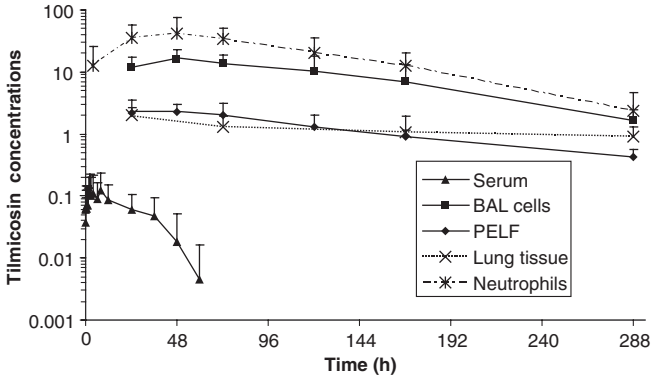

Fig. 1. Mean ± SD tilmicosin concentrations in serum, bronchoalveolar lavage cells, pulmonary epithelial lining fluid (μg/mL), and lung tissue (μg/g) of seven foals following a single i.m. dose of tilmicosin (10 mg/kg of body weight).

Table 2. Mean ± SD tilmicosin concentrations (μg/mL) measured in serum, CSF, and peritoneal fluid following administration of a single i.m. dose of tilmicosin (10 mg/kg of body weight) to seven foals

	Time after tilmicosin administration (h)		
Sample	4	24	72
Serum	0.11 ± 0.10	0.06 ± 0.04	ND
CSF	0.10 ± 0.03	0.10 ± 0.02	0.06 ± 0.01
Peritoneal fluid	0.10 ± 0.05	0.10 ± 0.03	0.02 ± 0.01

CSF, cerebrospinal fluid; ND, not detectable; SD, standard deviation.

One foal died as a result of hemothorax within minutes of collection of the 72 h lung biopsy. One foal developed tachypnea and profuse sweating approximately 2 h after injection of tilmicosin. The clinical signs persisted for approximately 45 min. Two foals developed a 10–15 cm in diameter area of painful swelling at the injection site within 12–24 h of injection. In one foal, the lesion was associated with hind limb lameness that persisted for 48 h. Three foals developed a small 1–2 cm in diameter hard nodule at the injection site. Four foals developed watery diarrhea 36–48 h after administration of tilmicosin. Diarrhea resolved without therapy within 48 h of onset.

In vitro susceptibility testing and antimicrobial drug combinations

Both the MIC_{50} and MIC_{90} of 56 *R. equi* isolates were 32 μg/mL (range 16–64 μg/mL). Tilmicosin was not bactericidal against *R. equi* at concentrations up to 256 μg/mL. Combination of tilmicosin with rifampin, gentamicin, amikacin, doxycycline, enrofloxacin, trimethoprim-sulfa, vancomycin, imipenem, or ceftiofur did not result in synergistic or antagonistic activity with median FIC indices ranging between 0.53 and 1.5. The time-kill experiment revealed that tilmicosin is a time-dependent antimicrobial agent *in vitro* with no benefit from increasing drug concentrations above four times the *MIC* (Fig. 2). Tilmicosin was active *in vitro* against most streptococci, *Staphylococcus* spp., *Actinobacillus* spp., and *Pasteurella* spp. (Table 3). Tilmicosin was not active against *Enterococcus* spp., *Pseudomonas* spp., and *Enterobacteraceae*.

Table 1. Serum, neutrophil, and pulmonary pharmacokinetic variables (mean ± SD unless otherwise specified) for tilmicosin after i.m. administration to seven foals at a dose of 10 mg/kg of body weight

Variable	Serum*	Lung†	PELF‡	BAL cells‡	Neutrophils‡
K_{el} (/h)	0.038 ± 0.018[a]	0.004 ± 0.001[b]	0.009 ± 0.004[b]	0.011 ± 0.003[b]	0.015 ± 0.007[b]
$AUC_{0-\infty}$ (μg·h/mL)	5.76 ± 1.87[a]	711 ± 351[b]	461 ± 115[b]	2342 ± 1006[c]	5982 ± 1017[d]
MRT (h)	34.5 ± 18.0[a]	323 ± 91.0[b]	180 ± 48.9[c]	117 ± 29.6[c]	98.7 ± 27.3[d]
$t_{1/2}$ (h)§	18.4 ± 10.7[a]	193.3 ± 71.37[b]	73.1 ± 47.8[a]	62.2 ± 26.9[a]	47.9 ± 34.1[a]
T_{max} (h)	5.50 ± 3.43[a]	30.8 ± 18.1[b]	52.0 ± 18.1[b]	54.9 ± 33.1[b]	41.1 ± 18.1[b]
C_{max} (μg/mL or μg/g)	0.19 ± 0.09[a]	1.90 ± 0.65[b]	2.91 ± 1.15[b]	20.1 ± 5.1[c]	66.01 ± 15.97[d]

$AUC_{0-\infty}$, area under the serum concentration vs. time curve from time 0 to infinity; C_{max}, peak tilmicosin concentration; K_{el}, elimination rate constant; *MRT*, mean residence time; SD, standard deviation; $t_{1/2}$, terminal half-life; T_{max}, time to peak tilmicosin concentration. *$n = 5$ because two foals had serum tilmicosin concentrations below the limit of quantification. †$n = 5$ because one foal died after the 72 h sample and lung samples were too small for drug analysis in one foal. ‡$n = 6$ because one foal died after the 72 h sample. §harmonic mean ± pseudo SD. [a,b,c,d]Different letters within a row indicate a statistically significant difference between sampling sites ($P < 0.05$).

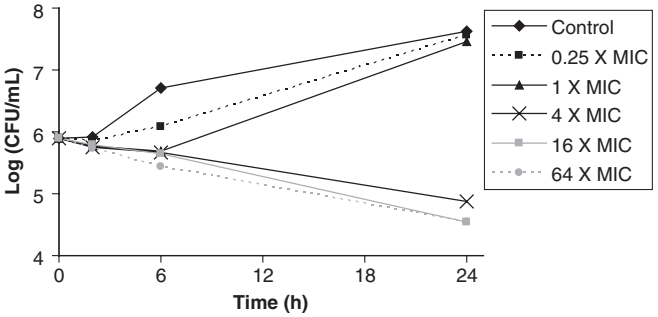

Fig. 2. Effect of time and tilmicosin concentration on *in vitro* survival of a clinical isolate of *Rhodococcus equi*. The isolate was grown in tubes containing concentrations of tilmicosin of 64-, 16-, 4-, 1-, and 0.5-times its minimum inhibitory concentration. A tube without tilmicosin was used as control. After 0, 2, 6, and 24 h of incubation, aliquots were collected from each tube to determine colony-forming units. Identical results were obtained with two additional isolates.

DISCUSSION

A safe antimicrobial agent providing high and sustained drug concentrations in the lungs would be a useful addition to currently available antimicrobial agents for the treatment or prevention of pneumonia in foals. Tilmicosin has been approved for the control and treatment of respiratory disease in cattle, sheep, and swine. Tilmicosin has also been shown to be effective for the treatment of mastitis in cattle and sheep, pasteurellosis in rabbits, and *Mycoplasma gallisepticum* infections in chicken (McKay *et al.*, 1996; Kempf *et al.*, 1997; Croft *et al.*, 2000; Dingwell *et al.*, 2003). The currently available injectable tilmicosin formulation has been advocated as potentially fatal when administered to horses, swine, and goats (Elanco Animal Health, 1995a). The cardiovascular system is the target of toxicity in laboratory and domestic animals with tachycardia and decreased cardiac contractility being reported following parenteral administration of tilmicosin (Main *et al.*, 1996). To minimize the risk of toxicity, a fatty acid salt formulation of tilmicosin newly developed as a safer and convenient formulation for use in cats (Kordick *et al.*, 2003) was used in the present study.

Mean peak serum concentrations and *AUC* achieved in the present study (0.19 μg/mL) were considerably lower that that achieved after subcutaneous administration of the same dose to cattle (0.87 μg/mL), sheep (0.82 μg/mL), and goats (1.56 μg/mL) (Ramadan, 1997; Modric *et al.*, 1998). Peak serum concentrations in foals were also lower than that observed after administration of the same fatty acid salt formulation administered at a dose of 10 mg/kg s.c. to cats (0.73 μg/mL) (Kordick *et al.*, 2003). Tilmicosin serum terminal half-life in the present study (18.4 h) was slightly shorter than that reported after s.c. administration to cattle (29.4 h), sheep (34.6 h), and goats (29.3 h) (Ramadan, 1997; Modric *et al.*, 1998).

Recent data suggest that traditional pharmacodynamic parameters based on plasma concentrations of macrolides may not best apply to the treatment of pulmonary infections and infections caused by facultative intracellular pathogens such as *R. equi* (Drusano, 2005). Serum concentrations of tilmicosin in cattle and swine are much lower than its *MICs* for common respiratory tract pathogens. Nevertheless, multiple studies have demonstrated the efficacy of tilmicosin in the treatment of respiratory disease in these species (Musser *et al.*, 1996; Paradis *et al.*, 2004). Lung concentrations of tilmicosin remain above the *MIC* of *M. haemolytica* (3.15 μg/mL) for at least 72 h following a single s.c. injection at a dose of 10 mg/kg (Elanco Animal Health, 1995a). In cats, maximum lung concentrations of tilmicosin of 5.62 μg/mL are achieved on day two following administration of the fatty acid salt formulation and measurable concentrations are still present in the lungs on day 21 (Kordick *et al.*, 2003).

While drug concentration in plasma is clearly a driving force for penetration to the site of infection, the actual drug-concentration time profile at a peripheral site may be quite different from that of plasma. Macrolides cross the cellular membranes primarily by passive diffusion (Fietta *et al.*, 1997). Tilmicosin, like other macrolides, is a potent weak base that becomes ion-trapped within acidic intracellular compartments

Table 3. *In vitro* activity of tilmicosin against 183 bacterial isolates obtained from horses as assessed by the disk susceptibility method

Micro-organism (*n*)	Zone diameter (mm)			Susceptibility (%)
	Median	25th percentile	Range	
Gram-positives				
α-hemolytic streptococci (7)	19	11	0–20	71
β-hemolytic streptococci (37)	19	18	15–26	100
Enterococcus spp. (5)	0	0	0	0
Rhodococcus equi (9)	0	0	0	0
Staphylococcus spp. (25)	18	16	0–28	96
Gram-negatives				
Actinobacillus spp. (9)	16	12	0–19	67
Enterobacter spp. (9)	0	0	0	0
Escherichia coli (11)	0	0	0–14	9
Klebsiella spp. (5)	0	0	0	0
Pasteurella spp. (6)	23	18	18–34	100
Pseudomonas spp. (12)	0	0	0–13	0
Salmonella enterica (48)	0	0	0–11	0

such as lysosomes (Scorneaux & Shryock, 1999). The ratio of cellular to extracellular concentration of tilmicosin is 193, 43, and 13, respectively, in bovine alveolar macrophages, monocyte-derived macrophages, and mammary epithelial cells (Scorneaux & Shryock, 1999). Consistent with these findings, peak tilmicosin concentrations in BAL cells of foals were approximately 107 times higher than peak serum concentrations. A number of *in vitro* and *in vivo* studies support the notion that white blood cells act as carriers for the delivery of macrolides to the site of infection (Retsema *et al.*, 1993; Mandell & Coleman, 2001). Studies with tilmicosin in rats support this concept as drug concentrations in the lung of rats inoculated with *Mycoplasma pulmonis* were significantly higher that those of noninfected controls (Modric *et al.*, 1999).

Macrolides inhibit protein synthesis by reversibly binding to 50S subunits of the ribosome. Macrolides are generally bacteriostatic agents but they may be bactericidal at high concentrations (Prescott, 2000). In the present study, tilmicosin was only bacteriostatic against *R. equi* at concentrations up to 256 μg/mL. The MIC_{90} of tilmicosin against foal isolates of *R. equi* (32 μg/mL) in the present study was similar to that of a previous study looking at a combination of human and equine isolates (>32 μg/mL) (Bowersock *et al.*, 2000). Consistent with a bacteriostatic antimicrobial agent, tilmicosin exerted time-dependent activity against *R. equi in vitro*. Peak tilmicosin concentrations in blood neutrophils were above the MIC_{90} of *R. equi* isolates but only for a period of 24–48 h. Even if tilmicosin concentrated more than 100-fold in BAL cells of foals, drug-concentrations achieved in lung tissue, PELF, and BAL cells were consistently below the MIC_{90} of *R. equi*. Tilmicosin was active *in vitro* against all β-hemolytic streptococci and *Pasteurella* spp., and most α-hemolytic streptococci, *Staphylococcus* spp., and *Actinobacillus* spp. Additional studies will be required to determine the clinical efficacy of this fatty acid salt formulation of tilmicosin against susceptible bacterial pathogens in foals.

Adverse effects observed in the present study consisted mainly of swelling at the injection site in five foals and self-limiting diarrhea in four foals. One foal developed tachypnea and profuse sweating approximately 2 h after injection. In swine, i.m. administration of the commercially available formulation at a dose of 10 mg/kg has resulted in tachypnea, and convulsions, and death occurs with dosages ≥20 mg/kg (Elanco Animal Health, 1995a). Tilmicosin included in the diet of horses at concentrations of 400, 1200, and 2000 ppm has resulted gastrointestinal disturbance in all groups and death of one horse consuming the 2000 ppm diet (Elanco Animal Health, 1995b). In another study, s.c. administration of the commercially available formulation of tilmicosin to foals at a dose of 10 mg/kg resulted in immediate loss of the normal fecal streptococcal population and a corresponding massive overgrowth of coliform bacteria (Clark & Dowling, 2005). The fecal flora slowly recovered over the next 7 days. Mild self-limiting diarrhea was observed in one foal (Clark & Dowling, 2005). Diarrhea is a well recognized adverse effect in foals treated with macrolides such as erythromycin, azithromycin, or clarithromycin (Stratton-Phelps *et al.*, 2000; Giguère *et al.*, 2004).

In conclusion, the fatty acid salt formulation of tilmicosin investigated in the present study resulted in high and sustained concentrations of tilmicosin in the lung, PELF, and BAL cells of foals following a single i.m. administration. The drug was active *in vitro* against a variety of bacterial pathogens. These data warrant further investigations into the clinical efficacy of this formulation of tilmicosin in foals with respiratory disease.

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REFERENCES

Baldwin, D.R., Honeybourne, D. & Wise, R. (1992) Pulmonary disposition of antimicrobial agents: methodological considerations. *Antimicrobial Agents and Chemotherapy*, **36,** 1171–1175.

Bowersock, T.L., Salmon, S.A., Portis, E.S., Prescott, J.F., Robison, D.A., Ford, C.W. & Watts, J.L. (2000) MICs of oxazolidinones for *Rhodococcus equi* strains isolated from humans and animals. *Antimicrobial Agents and Chemotherapy*, **44,** 1367–1369.

Clark, C. & Dowling, P.M. (2005) Antimicrobial-associated diarrhea in horses. Proceedings of the 23rd ACVIM Forum. pp. 169–171. Baltimore, MD.

Clark, C., Woodbury, M., Dowling, P., Ross, S. & Boison, J.O. (2004) A preliminary investigation of the disposition of tilmicosin residues in elk tissues and serum. *Journal of Veterinary Pharmacology and Therapeutics*, **27,** 385–387.

Cohen, N.D. (1994) Causes of and farm management factors associated with disease and death in foals. *Journal of the American Veterinary Medical Association*, **204,** 1644–1651.

Conte, J.E., Golden, J., Duncan, S., McKenna, E., Lin, E. & Zurlinden, E. (1996) Single-dose intrapulmonary pharmacokinetics of azithromycin, clarithromycin, ciprofloxacin, and cefuroxime in volunteer subjects. *Antimicrobial Agents and Chemotherapy*, **40,** 1617–1622.

Croft, A., Duffield, T., Menzies, P., Leslie, K., Bagg, R. & Dick, P. (2000) The effect of tilmicosin administered to ewes prior to lambing on incidence of clinical mastitis and subsequent lamb performance. *Canadian Veterinary Journal*, **41,** 306–311.

Davis, J.L., Gardner, S.Y., Jones, S.L., Schwabenton, B.A. & Papich, M.G. (2002) Pharmacokinetics of azithromycin in foals after i.v. and oral dose and disposition into phagocytes. *Journal of Veterinary Pharmacology and Therapeutics*, **25,** 99–104.

DeRosa, D.C., Veenhuizen, M.F., Bade, D.J. & Shryock, T.R. (2000) *In vitro* susceptibility of porcine respiratory pathogens to tilmicosin. *Journal of Veterinary Diagnostic Investigation*, **12,** 541–546.

Dingwell, R.T., Leslie, K.E., Duffield, T.F. *et al.* (2003) Efficacy of intramammary tilmicosin and risk factors for cure of *Staphylococcus aureus* infection in the dry period. *Journal of Dairy Science*, **86,** 159–168.

Drusano, G.L. (2005) Infection site concentrations: their therapeutic importance and the macrolide and macrolide-like class of antibiotics. *Pharmacotherapy*, **25,** 150S–158S.

Elanco Animal Health (1995a) *Micotil 300 Injection. Tilmicosin Phosphate*. Manufacturer's drug insert (revised October, 1995), Indianapolis, IN.

Elanco Animal Health (1995b) *Pulmotil 90 Feed Additive*. Manufacturer's drug insert (revised February, 1995), Indianapolis, IN.

Fietta, A., Merlini, C. & Gialdroni, G.G. (1997) Requirements for intracellular accumulation and release of clarithromycin and

azithromycin by human phagocytes. *Journal of Chemotherapy*, **9,** 23–31.

Gibaldi, M. & Perrier, D. (1982) Noncompartmental analysis based on statistical moment theory. In *Pharmacokinetics*, 2nd edn. Eds Gibaldi, M. & Perrier, D. pp. 409–417. Marcel Dekker, New York.

Giguère, S., Gaskin, J.M., Miller, C. & Bowman, J.L. (2002) Evaluation of a commercially available hyperimmune plasma product for prevention of naturally acquired pneumonia caused by *Rhodococcus equi* in foals. *Journal of the American Veterinary Medical Association*, **220,** 59–63.

Giguère, S., Jacks, S., Roberts, G.D., Hernandez, J., Long, M.T. & Ellis, C. (2004) Retrospective comparison of azithromycin, clarithromycin, and erythromycin for the treatment of foals with *Rhodococcus equi* pneumonia. *Journal of Veterinary Internal Medicine*, **18,** 568–573.

Hillidge, C.J. (1987) Use of erythromycin–rifampin combination in treatment of *Rhodococcus equi* pneumonia. *Veterinary Microbiology*, **14,** 337–342.

Hoffman, A.M., Viel, L., Prescott, J.F., Rosendal, S. & Thorsen, J. (1993) Association of microbiologic flora with clinical, endoscopic, and pulmonary cytologic findings in foals with distal respiratory tract infection. *American Journal of Veterinary Research*, **54,** 1615–1622.

Jacks, S., Giguère, S., Gronwall, R.R., Brown, M.P. & Merritt, K.A. (2001) Pharmacokinetics of azithromycin and concentration in body fluids and bronchoalveolar cells in foals. *American Journal of Veterinary Research*, **62,** 1870–1875.

Kempf, I., Reeve-Johnson, L., Gesbert, F. & Guittet, M. (1997) Efficacy of tilmicosin in the control of experimental *Mycoplasma gallisepticum* infection in chickens. *Avian Disease*, **41,** 802–807.

Kordick, D.L., Murthy, Y. & Henley, K. (2003) *Biodistribution of a Novel Formulation of Tilmicosin in Cats*, American Society for Microbiology, 103rd general meeting, Washington D.C. Abstract Z014, pp. 94.

Main, B.W., Means, J.R., Rinkema, L., Smith, W.C. & Sarazan, R.D. (1996) Cardiovascular effects of the macrolide antibiotic tilmicosin, administered alone and in combination with propranolol or dobutamine, in conscious unrestrained dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **19,** 225–232.

Mandell, G.L. & Coleman, E. (2001) Uptake, transport, and delivery of antimicrobial agents by human polymorphonuclear neutrophils. *Antimicrobial Agents and Chemotherapy*, **45,** 1794–1798.

McKay, S.G., Morck, D.W., Merrill, J.K., Olson, M.E., Chan, S.C. & Pap, K.M. (1996) Use of tilmicosin for treatment of pasteurellosis in rabbits. *American Journal of Veterinary Research*, **57,** 1180–1184.

Modric, S., Webb, A.I. & Derendorf, H. (1998) Pharmacokinetics and pharmacodynamics of tilmicosin in sheep and cattle. *Journal of Veterinary Pharmacology and Therapeutics*, **21,** 444–452.

Modric, S., Webb, A.I. & Davidson, M. (1999) Effect of respiratory tract disease on pharmacokinetics of tilmicosin in rats. *Laboratory Animal Science*, **49,** 248–253.

Musser, J., Mechor, G.D., Grohn, Y.T., Dubovi, E.J. & Shin, S. (1996) Comparison of tilmicosin with long-acting oxytetracycline for treatment of respiratory tract disease in calves. *Journal of the American Veterinary Medical Association*, **208,** 102–106.

National Committee for Clinical Laboratory Standards (1999a) *Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guidelines*. NCCLS document M26-A, Wayne, PA.

National Committee for Clinical Laboratory Standards (1999b) *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standards*. NCCLS document M31-A, Wayne, PA.

National Committee for Clinical Laboratory Standards (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement; Approved Standards*. NCCLS document M100-S12, Wayne, PA.

Odland, B.A., Erwin, M.E. & Jones, R.N. (2000) Quality control guidelines for disk diffusion and broth microdilution antimicrobial susceptibility tests with seven drugs for veterinary applications. *Journal of Clinical Microbiology*, **38,** 453–455.

Ose, E.E. (1987) In vitro antibacterial properties of EL-870, a new semisynthetic macrolide antibiotic. *Journal of Antibiotics (Tokyo)*, **40,** 190–194.

Paradis, M.A., Vessie, G.H., Merrill, J.K. *et al.* (2004) Efficacy of tilmicosin in the control of experimentally induced *Actinobacillus pleuropneumoniae* infection in swine. *Canadian Journal of Veterinary Research*, **68,** 7–11.

Pillai, S.K., Moellering, R.C. & Eliopoulos, G.M. (2005) Antimicrobial combinations. In *Antibiotics in Laboratory Medicine*, 5th edn. Ed. Lorian, V. pp. 365–440. Lippincott Williams & Wilkins, Philadelphia.

Prescott, J.F. (2000) Lincosamides, macrolides, and pleuromutilins. In *Antimicrobial Therapy in Veterinary Medicine*, 3rd edn. Eds Prescott, J.F., Baggot, D.J. & Walker, R.D. pp. 229–262. Iowa State University Press, Ames.

Ramadan, A. (1997) Pharmacokinetics of tilmicosin in serum and milk of goats. *Research in Veterinary Science*, **62,** 48–50.

Rennard, S.I., Basset, G., Lecossier, D., O'Donnell, K.M., Pinkston, P., Martin, P.G. & Crystal, R.G. (1986) Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *Journal of Applied Physiology*, **60,** 532–538.

Retsema, J.A., Bergeron, J.M., Girard, D., Milisen, W.B. & Girard, A.E. (1993) Preferential concentration of azithromycin in an infected mouse thigh model. *Journal of Antimicrobial Chemotherapy*, **31** (Suppl. E), 5–16.

Scorneaux, B. & Shryock, T.R. (1998) Intracellular accumulation, subcellular distribution and efflux of tilmicosin in swine phagocytes. *Journal of Veterinary Pharmacology and Therapeutics*, **21,** 257–268.

Scorneaux, B. & Shryock, T.R. (1999) Intracellular accumulation, subcellular distribution, and efflux of tilmicosin in bovine mammary, blood, and lung cells. *Journal of Dairy Science*, **82,** 1202–1212.

Shryock, T.R., White, D.W., Staples, J.M. & Werner, C. (1996) Minimum inhibitory concentration breakpoints and disk diffusion inhibitory zone interpretive criteria for tilmicosin susceptibility testing against *Pasteurella* spp. associated with bovine respiratory disease. *Journal of Veterinary Diagnostic and Investigation*, **8,** 337–344.

Stobba-Wiley, C.M., Chang, J.P., Elsbury, D.T., Moran, J.W., Turner, J.M. & Readnour, R.S. (2000) Determination of tilmicosin residues in chicken, cattle, swine and sheep tissue by liquid chromatography. *Journal of AOAC International*, **83,** 837–845.

Stratton-Phelps, M., Wilson, W.D. & Gardner, I.A. (2000) Risk of adverse effects in pneumonic foals treated with erythromycin versus other antibiotics: 143 cases (1986–1996). *Journal of the American Veterinary Medical Association*, **217,** 68–73.

Ziv, G., Shem-Tov, M., Glickman, A., Winkler, M. & Saran, A. (1995) Tilmicosin antibacterial activity and pharmacokinetics in cows. *Journal of Veterinary Pharmacology and Therapeutics*, **18,** 340–345.