PHARMACOKINETICS AND PULMONARY DISPOSITION OF CLARITHROMYCIN AND TILMICOSIN IN FOALS.

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2006

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ACKNOWLEDGMENTS

I would like to thank my family for their continuous support while I pursue my goals in veterinary medicine. Their unwavering confidence in my abilities makes the accomplishment of this thesis even more valuable to me. I also want to thank my boyfriend Michael who pushed me to go further, never doubting that I would make it.

I especially want to thank Dr. Steeve Giguère for giving me this incredible opportunity. He opened the door to an experience that has forever shaped me. I have learned more than I imagined I would, not only about science and veterinary medicine but about myself. I greatly appreciate the support, guidance, and mentorship that he provided to me. I will be forever grateful.

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of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

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August 2006

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Major Department: Veterinary Medicine

Bronchopneumonia is the leading cause of morbidity and mortality in foals aged between 1 and 6 months. Gram-positive bacteria such as Streptococcus equi subspecies zooepidemicus and Rhodococcus equi are the most common causes of pneumonia in foals. Erythromycin, a macrolide antimicrobial agent, is commonly used in equine medicine for treatment of foal pneumonia, especially in foals infected with Rhodococcus equi. Two other macrolides, clarithromycin and tilmicosin, may be useful alternatives to currently used antimicrobial agents owing to their accumulation in lung tissue and phagocytic cells, as well as their broad spectrum in vitro activity. The objectives of this study were to determine the pharmacokinetics and pulmonary distribution of clarithromycin and tilmicosin in foals, and to investigate the in vitro activity of tilmicosin against common bacterial pathogens of horses. Clarithromycin (7.5 mg/kg) was administered to six foals via intravenous (IV) and intragastric (IG) routes, in a cross-over design. Concentrations of clarithromycin and its 14-hydroxy-metabolite in serum were

measured by HPLC. A microbiologic assay was used to measure clarithromycin activity in serum, urine, peritoneal fluid, synovial fluid, cerebrospinal (CSF), pulmonary epithelial lining fluid (PELF), and bronchoalveolar (BAL) cells. Following IV administration, clarithromycin had a t½ of 5.4 hours, a body clearance of 1.27 L/h/kg, and an apparent volume of distribution at steady state of 10.4 ± 2.1 L/kg. Oral bioavailability of clarithromycin was 57.3 ± 12.0 %. In a separate study, a single dose of a fatty acid salt formulation of tilmicosin (10 mg/kg) was administered by the intramuscular route to 7 healthy 5- to 8-week-old foals. Concentrations of tilmicosin in serum were measured by HPLC and concentrations in lung tissue, PELF, and BAL cells were measured by mass spectrometry. Mean peak tilmicosin concentrations were significantly higher in BAL cells (20.1 ± 5.1 □g/mL) than in lung tissue (1.90 ± 0.65 □g/mL), PELF (2.91 ± 1.15 □g/mL), and serum (0.19 ± 0.09 □g/mL). Harmonic mean elimination half life in lung tissue (193.3 h) was significantly longer than that of serum (18.4 h). Elimination half lives in BAL cells and PELF were 62.2 h and 73.3 h, respectively. Tilmicosin was active in vitro against most streptococci, Staphylococcus spp., Actinobacillus spp., and Pasteurella spp. The drug was not active against Rhodococcus equi, Pseudomonas spp., and Enterobacteraceae. In conclusion, oral administration of clarithromycin at a dosage of 7.5 mg/kg every 12 hours would maintain serum, PELF, and BAL cell concentrations above the minimum inhibitory concentration for R. equi and S. zooepidemicus isolates for the entire dosing interval. The formulation of tilmicosin investigated in the present study resulted in high and sustained concentrations in the lung, PELF, and BAL cells of foals and may be appropriate for the treatment of susceptible bacterial infections.

CHAPTER 1
INTRODUCTION

Bacterial pneumonia is the leading cause of morbidity and mortality in foals aged between 1 and 6 months. Gram-positive bacteria such as *Streptococcus equi* subspecies *zooepidemicus* and *Rhodococcus equi* are the most common causes of pneumonia in foals. Gram-negative bacteria such as *Pasteurella* spp., *Actinobacillus* spp., *Escherichia coli*, and *Klebsiella pneumoniae* may also occasionally be cultured from tracheobronchial aspirates of affected foals. Administration of antimicrobial agents is the most important part of the therapeutic plan. When *R. equi* is suspected or confirmed, therapy has historically consisted of administration of the macrolide erythromycin in combination with rifampin. This combination has dramatically reduced foal mortality since its introduction. However, this treatment regimen is not without problems. Erythromycin has poor and variable oral bioavailability in foals, r*equi*res multiple daily dosing, and most importantly, has a high incidence of potentially fatal adverse effects. Therefore, there is a tremendous need for other effective and potentially safer antimicrobial agents to combat infection caused by this devastating pathogen. Two other macrolides, clarithromycin and tilmicosin, may be useful alternatives to currently used antimicrobial agents.

The documented pharmacokinetic advantages of clarithromycin over erythromycin in humans include higher oral bioavailability, longer elimination half-life, larger volume of distribution, and improved tissue and phagocytic cell uptake. Tilmicosin may also be a useful alternative to the current antimicrobial agents used in horses 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 such as tilmicosin would result in less frequent administration, which in turn may improve client compliance. The overall goal of the work presented in this thesis is to determine the pharmacokinetics and pulmonary distribution of clarithromycin and tilmicosin in foals. This thesis includes two studies.

The objectives and hypotheses of the first study (Chapter 3) are:

1- To determine the pharmacokinetics of clarithromycin and its metabolite in foals.
   Our *hypothesis* is that oral clarithromycin is well absorbed in foals and is metabolized to 14-hydroxy clarithromycin.
2- To determine concentrations of clarithromycin in body fluids and bronchoalveolar cells.
   Our *hypothesis* is that oral clarithromycin provides serum and pulmonary drug concentrations above the minimum inhibitory concentration of *R. equi*.

The objectives and hypotheses of the second study (Chapter 4) are:

1- To determine the pulmonary disposition of tilmicosin in foals.
   Our *hypothesis* is that a new fatty acid salt formulation of tilmicosin provides high and sustained concentrations in the lungs of foals.
2- To investigate the *in vitro* activity of tilmicosin against *R. equi* and other common bacterial pathogens of horses.
   Our *hypothesis* is that tilmicosin is active *in vitro* against common equine bacterial pathogens of the respiratory tract.

CHAPTER 2
LITERATURE REVIEW

Foal Pneumonia

Lower respiratory tract infection is the leading cause of both morbidity and mortality in foals aged between 1 and 6 months (Cohen, 1994). The morbidity rate is approximately 6% across the United States. It is likely, however, that the true incidence of infection is much higher and that many cases of infection go unrecognized and resolve spontaneously. Indeed, careful weekly physical examination and cytologic examination of the lower respiratory tract in more than 200 Thoroughbred foals on 10 farms in Ontario, Canada demonstrated an average morbidity from bacterial infection of the distal respiratory tract of 82% (Hoffman *et al.*, 1993a). Increased susceptibility to disease in this age group may result from delay in the establishment of a competent immune system and environmental factors such as overcrowding, shipping, and sales (Wilson, 1992). The disease may be subclinical initially; however, as the infection progresses, clinical signs may include depression, inappetence, coughing, nasal discharge, and tachypnea. Fever is a common finding as well. Severely affected foals may develop respiratory distress. Common laboratory abnormalities in foals with bacterial pneumonia include leukocytosis, hyperfibrinogenemia, and hyperglobulinemia (Barr, 2003). Mild anemia may develop in chronic cases. Radiography and ultrasonographic examination of the thorax are useful diagnostic imaging tools to detect and assess the severity of lung lesions. Culture of a tracheobronchial aspirate is necessary to determine the causative microorganism. The vast majority of cases of foal pneumonia are bacterial in origin.

Viral agents such as influenza, equine herpesvirus-1 (EHV-1), EHV-2, EHV-4, rhinovirus, and adenovirus may cause primary lung disease or predispose to secondary bacterial pneumonia (Wilson, 1992). However, in most cases of foal pneumonia, viral agents cannot be isolated at the time of presentation (Hoffman *et al.*, 1993b). Most bacteria associated with pneumonia are ubiquitous in the foal's environment. The pathophysiology of bacterial pneumonia begins with either inhalation of environmental microbes or aspiration of oropharygeal bacteria. The bacteria become pathogenic only when the pulmonary defense mechanisms are compromised or are overwhelmed by a large number of bacteria (Wilson, 1992). The inflammatory response induced by bacterial invasion will result in infiltration with neutrophils and other inflammatory cells into the airways and pulmonary parenchyma. Inflammatory cells and their mediators cause damage to the airway epithelium and capillary endothelium, leading to flooding of the terminal airways with inflammatory cells, serum cellular debris and fibrin. This process is generally more severe in the cranioventral portions of the lung. These lesions interfere with gas exchange and, if severe enough, the resulting ventilation-perfusion mismatch leads to hypoxemia and clinical signs of respiratory disease.

Gram-positive bacteria such as Streptococcus equi subsp. zooepidemicus and Rhodococcus equi are the most common causes of pneumonia in foals (Hoffman et al., 1993b; Giguère et al., 2002; Barr, 2003). Gram-negative bacteria such as Pasteurella spp., Actinobacillus spp., Escherichia coli, Klebsiella pneumoniae, Salmonella enterica, and Bordetella bronchiseptica may also be cultured from tracheobronchial aspirates of affected foals. Mixed bacterial infections are common as well (Wilson, 1992; Hoffman et al., 1993b). Administration of antimicrobial agents is the most important part of the

therapeutic plan. The choice of the antimicrobial agent depends on the results of culture and susceptibility testing of tracheobronchial aspirates, severity of the clinical signs, cost, ease of administration, and history of response to therapy within the herd. Since a high percentage of pneumonia in foals older than 1 month is due to penicillin-sensitive bacteria such as S. equi subsp. zooepidemicus, penicillin is often used for initial therapy, pending culture results. Ceftiofur, a third generation cephalosporin, has a broad spectrum of activity that includes most of the etiologic agents of foal pneumonia, except R. equi. If resistant Gram-negative organisms are present, an aminoglycoside (gentamicin or amikacin) is often combined with penicillin (Wilson, 1992). When R. equi is suspected or confirmed, therapy consists of administration of a macrolide in combination with rifampin. It is common practice to use a combination of a macrolide and rifampin as the first line of therapy on farms where R. equi is endemic as this combination is also active against streptococci.

Rhodococcus Equi

R. equi is a facultative intracellular pathogen that has the ability to survive and even replicate within macrophages (Zink et al., 1987). *R. equi* is closely related to Mycobacterium tuberculosis. Both *R. equ*i and *M. tuberculosis* are members of a phyogenetically distinct group called Mycolata, which are characterized by a unique cell envelope that consists of mycolic acids (Sutcliffe, 1997). This unique envelope forms a permeability barrier to hydrophilic compounds and promotes granuloma formation so the organism is able to multiply in and destroy macrophages (Sutcliffe, 1997). The similarity between *R. equi* and *M. tuberculosis* is further emphasized by the degree of homology of their genome sequence (Rahman *et al.*, 2003).

The most common manifestation of *R. equi* infections in foals is a chronic suppurative bronchopneumonia with extensive abscessation and associated suppurative lymphadenitis. Other, less-common clinical manifestations of *R. equi* infections in foals include ulcerative enterocolitis, colonic or mesenteric lymphadenopathy, immune-mediated synovitis and uveitis, osteomyelitis, and septic arthritis (Giguère and Prescott, 1997). *R. equi* has also been increasingly recognized as an important cause of pneumonia in immunosuppressed people, especially those infected with HIV. *R. equi* may also cause disease in other animal species such as cattle, sheep, goats, dogs, and cats; however infection is rare and usually associated with immunosuppresion (Prescott, 1991). The reasons for the peculiar susceptibility of young foals are not entirely clear.

R. equi is a saprophytic inhabitant of soil. Although all horse farms are infected to various degrees with *R. equi*, the clinical disease is enzootic and devastating on some farms, sporadic on others, and unrecognized on most farms. On farms where the disease is enzootic, costs associated with veterinary care, early diagnosis, long-term therapy, and mortality of foals may be very high. In addition to significant immediate costs, *R. equi* pneumonia has a long-term detrimental effect on the equine industry because foals that recover from the disease are less likely to race as adults (Ainsworth *et al.*, 1998).

The ability of *R. equi* to induce disease in foals likely depends on both host and microbial factors. The key to the pathogenesis of *R. equi* is its ability to replicate within pulmonary macrophages apparently by inhibiting phagosome-lysosome fusion (Zink *et al.*, 1987). Unlike most environmental *R. equi*, isolates from pneumonic foals typically contain 80-90 kb plasmids. The plasmid encodes seven related virulence-associated proteins designated VapA and VapC through VapH (Takai *et al.*, 2000). Plasmid-cured

derivatives of virulent *R. equi* strains lose their ability to replicate and survive in macrophages (Giguère *et al.*, 1999). Plasmid-cured derivatives also fail to induce pneumonia and are completely cleared from the lungs of foals two weeks following heavy intrabronchial challenge, confirming the absolute necessity of the large plasmid for the virulence of *R. equi* (Giguère *et al.*, 1999). Vap A is highly immunogenic, lipid-modified protein expressed on the surface of *R. equi* (Tan *et al.*, 1995). An *R. equi* mutant lacking a 7.9 kb DNA region spanning 5 vap genes (*vapA, -C, -D, -E, -F*) was attenuated for virulence in mice and failed to replicate in macrophages (Jain *et al.*, 2003). Complementation with *vapA* alone could restore full virulence, whereas complementation with *vapC*, *vapD* or *vapE* could not (Jain *et al.*, 2003). More recently, attenuation of 2 other plasmid-encoded genes was also found to decrease virulence in mice despite enhanced transcription of *vapA* (Ren and Prescott, 2004). These findings suggest that other plasmid-encoded genes besides *vapA* contribute to the virulence of *R. equi*.

Inhalation of virulent *R. equi* is the major route of pneumonic infection. Ingestion of the organism is a significant route of exposure, and likely also of immunization, but rarely leads to hematogenously acquired pneumonia unless the foal has multiple exposures to large numbers of bacteria (Johnson *et al.*, 1983). A majority of foals may be exposed by ingesting the bacterium; however, they probably develop a strong immune response and are protected against subsequent intrabronchial challenge (Hooper-McGrevy *et al.*, 2005).

Control of *R. equi* infections on farms where the disease is enzootic is difficult. Attempts at actively immunizing foals against *R. equi* infections have consistently failed. Intravenous administration of hyperimmune plasma obtained from horses vaccinated

against *R. equi* has given contradictory results. Therefore, screening strategies promoting early recognition of *R. equi* cases with treatment of infected foals will reduce losses, decrease the spread of virulent organisms and limit the cost of therapy on farms where the disease is endemic.

A wide variety of antimicrobial agents are effective against *R. equi in vitro* (Jacks *et al.*, 2003). However, many of these drugs are ineffective *in vivo*. The discrepancy in results is likely due to the intracellular nature of this bacterium and the fact that it causes abscesses where diffusion and activity of many antimicrobial agents is not optimal. In one study, all 17 foals with *R. equi* pneumonia treated with the combination of penicillin and gentamicin died despite all isolates being susceptible to gentamicin *in vitro* (Sweeney *et al.*, 1987). In the mid 1980s, the combination of erythromycin and rifampin became the treatment of choice (Hillidge, 1987). The combination of erythromycin and rifampin has become the prevalent treatment for *R. equi* infections in foals and has dramatically reduced foal mortality since its introduction (Hillidge, 1987; Sweeney *et al.*, 1987). Although both erythromycin and rifampin are bacteriostatic against *R. equi* (Nordmann and Ronco, 1992), they are highly effective *in vitro*. The combination of these two antimicrobials is synergistic as well, both *in vitro* and *in vivo* and when used in combination reduces the likelihood of resistance to either drug (Prescott and Nicholson 1984; Nordmann *et al.*, 1992; Nordmann and Ronco, 1992). Rifampin and, to a lesser extent, erythromycin are lipid soluble, allowing them to penetrate caseous material. Although combined therapy with erythromycin and rifampin has dramatically improved the survival rate of foals infected with *R. equi*, this treatment regimen is not without problems. Erythromycin has poor and variable oral bioavailability in foals, requires

multiple daily dosing, and most importantly, has a high incidence of potentially fatal adverse effects (Lakritz *et al.*, 2000a; Lakritz *et al.*, 2000b; Stratton-Phelps *et al.*, 2000). The use of erythromycin to treat foals with pneumonia results in an increased risk of diarrhea, hyperthermia, and respiratory distress compared with pneumonic foals treated with either penicillin or trimethoprim sulfa (Stratton-Phelps *et al.*, 2000). *Clostridium difficile* enterocolitis has also been observed occasionally in the dams of nursing foals while the foals are being treated with oral erythromycin presumably because of sufficient active erythromycin to perturb the intestinal flora (Baverud *et al.*, 1998).

Macrolides

Macrolide antimicrobial agents are chemically comprised of a lactone ring with 14 to 16 carbons attached to 2 sugar moieties. Macrolide antimicrobials are typically classified according to the size of their macrocyclic lactone ring (Figure 1.1). Macrolides inhibit protein synthesis by reversibly binding to 50S subunits of the ribosome. Their binding sites on the 23S rRNA of the 50S ribosomal subunit overlap with that of clindamycin but are different from those of chloramphenicol (Prescott, 2000). Macrolides are generally bacteriostatic agents. They may be bactericidal at high concentrations and against a low inoculum of highly susceptible bacteria. Their spectrum of activity includes mostly Gram-positive microorganisms, most *Mycoplasma* spp., some Chalmydiae as well as some Gram-negative pathogens such as *Haemophilus influenzae*, *Campylobacter jejuni*, *Bordetella* spp., and *Mannheimia haemolytica* (Alvarez-Elcoro and Enzler, 1999). This class of antimicrobials has been important in the treatment of respiratory tract, skin, and soft tissue infections as well as venereal disease in humans. Some macrolides (tilmicosin, tulathromycin) are also approved for the treatment of bronchopneumonia in cattle.

Macrolide antimicrobial agents other than erythromycin may provide a suitable alternative for the treatment of *Rhodococcus equi* infections in foals. Azithromycin and clarithromycin are used with increasing frequency in human medicine. Compared with erythromycin, azithromycin and clarithromycin have a higher oral bioavailability, longer elimination half lives, larger volumes of distribution, as well as improved tissue and phagocytic cell uptakes (Whitman and Tunkel, 1992; Conte *et al.*, 1995; Rodvold, 1999). In humans, the incidence and the severity of adverse reactions for azithromycin and clarithromycin are also considerably decreased compared with erythromycin (Whitman and Tunkel, 1992). Another macrolide, tilmicosin, is not currently approved for use in horses but may provide a suitable alternative for the treatment of *R. equi*. The pharmacokinetics and pulmonary distribution of azithromycin have been studied extensively in foals (Jacks *et al.*, 2001; Davis *et al.*, 2002). However, there is no information on the pulmonary distribution of clarithromycin and tilmicosin in foals.

Clarithromycin

Clarithromycin is a semi-synthetic antimicrobial agent that is derived from erythromycin. Clarithromycin has an O-methyl ether substitution instead of the C-6 hydroxyl group of erythromycin at position 6 of the macrolide ring (Rodvold, 1999). This modification provides greater stability than erythromycin in gastric content, thus improving oral bioavailability. The documented pharmacokinetic advantages of clarithromycin over erythromycin in humans include higher oral bioavailability, longer elimination half-life, larger volume of distribution, and improved tissue and phagocytic cell uptake (Conte *et al.*, 1995; Rodvold, 1999).

Clarithromycin undergoes hepatic metabolism as well as elimination by secretion into the intestinal lumen. In humans, clarithromycin is metabolized in the liver by

cytochrome P-450 enzymes to the active metabolite 14-hydroxy-clarithromycin (Ferrero *et al.*, 1990). This active metabolite contributes approximately 50% of the biological activity of clarithromycin and has synergistic effects with clarithromycin (Fernandes *et al.*, 1988). Other species including rats, mice or desert tortoise do not produce the 14-hydroxy metabolite (Ferrero *et al.*, 1990; Bedos *et al.*, 1992; Wimsatt *et al.*, 1999).

Microorganisms with MIC $\leq$ 2 μg/ml are generally regarded as susceptible and $\geq$ 8 μg/ml as resistant to clarithromycin and erythromycin. The efficacy of clarithromycin *in vitro* is greatest against the aerobic and facultative anaerobic non-spore-forming, Gram-positive bacteria. It also has *in vitro* activity against several microorganisms such as, *Mycoplasma* spp., *Chlamydia* spp., and some Mycobacteria. The lack of activity against most Gram-negative bacteria is likely due to its inability to penetrate the bacterial cell wall. Clarithromycin is active against many Gram-positive bacterial pathogens of horses (Jacks *et al.*, 2003). Of the macrolides tested so far, clarithromycin has the greatest *in vitro* activity against *R. equi* isolates cultured from pneumonic foals (Table 1.1).

Approved indications for the use of clarithromycin in people include the treatment of: pharyngitis/tonsillitis due to *Streptococcus pyogenes*; acute maxillary sinusitis due to *Haemophilus influenzae, Moraxella catarrhalis,* or *Streptococcus pneumoniae*; community acquired pneumonia or bronchitis due to *Haemophilus influenzae, Mycoplasma pneumoniae, Streptococcus pneumoniae, Moraxella catarrhalis,* or *Chlamydia pneumoniae*; uncomplicated skin infections due to *Staphylococcus aureus,* or *Streptococcus pyogenes*; and disseminated mycobacterial infections due to *Mycobacterium avium,* or *Mycobacterium intracellulare.* Finally, clarithromycin

combined with amoxicillin and a proton pump inhibitor is also approved for the treatment of gastric and duodenal ulceration caused by *Helicobacter pylori*.

Clarithromycin is a macrolide antimicrobial agent which achieves low plasma concentrations relative to the minimum inhibitory concentration (MIC) of the pathogens it is used to treat. The physiochemical properties of clarithromycin, including its lipophilicity indicate that the drug concentrations at a peripheral site would be greater than concurrent serum concentrations (Drusano, 2005). The disposition of clarithromycin in pulmonary epithelial lining fluid (PELF) and alveolar macrophages (AM) has been investigated extensively in healthy human volunteers. In humans, clarithromycin achieves considerably greater concentrations in pulmonary epithelial lining fluid and alveolar macrophages than either erythromycin or azithromycin (Conte *et al.*, 1995;Conte *et al.*, 1996; Patel *et al.*, 1996; Rodvold *et al.*, 1997). However, the half-life of clarithromycin at these sites is much shorter than that of azithromycin.

A recent preliminary study confirmed that therapeutic concentrations are achieved in serum following oral administration of clarithromycin to foals (Jacks *et al*., 2002). In a retrospective study of foals presented to a referral institution, the combination of clarithromycin-rifampin was found to be superior to azithromycin-rifampin or erythromycin-rifampin for the treatment of pneumonia caused by *R. equi* (Giguère *et al.*, 2004). However, concentrations of the drug in body fluids, pulmonary epithelial lining fluid (PELF) and bronchoalveolar (BAL) cells have not been measured. Recent studies demonstrate that the concentration of macrolides at the site of infection may be a better indicator of clinical efficacy than serum concentrations alone (Drusano, 2005).

Tilmicosin

Tilmicosin is a semi-synthetic derivative of tylosin. Typical of macrolides, it inhibits Gram-positive bacteria including *Clostridium* spp., *Staphylococcus* spp., and *Streptococcus* spp., some Gram-negative bacteria including *Actinobacillus* spp., *Campylobacter* spp., *Histophilus* spp., and Mannheimia/*Pasteurella* spp. (Prescott, 2000). All Enterobacteriaceae are resistant to tilmicosin. *Mycoplasma* susceptibility can be quite variable because of resistance (Vicca *et al.*, 2004). *Mannheimia*/*Pasteurella* spp. isolated from cattle are regarded as susceptible if their MIC is $\leq$ 8 µg/ml, intermediate if MIC is 16 µg/ml, and resistant if their MIC is $\geq$ 32 µg/ml (Shryock *et al.*, 1996).

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 (> 2 L/kg), with accumulation and persistence in tissues including the lung, which may concentrate drug 20-60 fold compared to serum (Ziv *et al.*, 1995; Clark *et al.*, 2004). Intracellular concentrations have been shown to be 40 times greater than that of serum (Ziv *et al.*, 1995; Scorneaux and Shryock, 1999).

Tilmicosin has been developed as a long-acting formulation for use in bovine and ovine respiratory disease. A single SC dose of 10 mg/kg results in lung concentrations exceeding the MIC of *M. haemolytica* for 72 hours (Ziv *et al.*, 1995; Scorneaux and Shryock, 1999). Experimental and field data support the value of a single-dose SC prophylaxis on arrival of cattle in feedlots and in the treatment in pneumonia of cattle (Ose and Tonkinson, 1988; Musser *et al.*, 1996; Morck *et al.*, 1997; Hoar *et al.*, 1998). Repeat injections after three days are necessary in some animals (Laven and Andrews, 1991; Scott, 1994). Tilmicosin is not approved for use in lactating cattle because of the prolonged period (two to three weeks) during which milk residues can be detected.

Intramammary tilmicosin at drying-off have been shown to be efficacious in curing some existing *S. aureus* infection (Dingwell *et al.*, 2003).

Tilmicosin is also approved as an oral medication for the control of *Actinobacillus* spp. or *P. multocida* pneumonia in swine (Paradis *et al.*, 2004). It may also be useful in the control of atrophic rhinitis. In-feed, treatment with 400 ppm of tilmicosin phosphate significantly reduced the presence of *A. pleuropneumoniae* on the surface of tonsils but was unable to completely eliminate the organism from deeper tonsillar tissues and to prevent bacterial shedding by carrier animals (Fittipaldi *et al.*, 2005).

Macrolides have immunomodulatory effects that are beneficial for humans suffering from many inflammatory pulmonary diseases. These effects are independent of the antibacterial activity of these drugs. Neutrophils play an important role in the destruction and elimination of bacterial invaders. However, they also release lipid mediators such as leukotriene B_4 (LTB_4) which induce a local inflammatory response. Lesions of the lung contain viable as well as necrotic neutrophils which add to the tissue damage caused by invading microorganisms. Apoptotic cell death is less damaging because the cells maintain cellular membranes, preventing further release of damaging LTB_4 (Nerland *et al.*, 2005). Tilmicosin has been shown to induce apoptosis and reduce LTB_4 as well as prostaglandin E2 concentrations in pulmonary fluid of cattle and swine with pneumonia (Lakritz *et al.*, 2002; Nerland *et al.*, 2005). These anti-inflammatory effects may contribute to the therapeutic efficacy of tilmicosin.

Tilmicosin is potentially toxic to the cardiovascular system, which varies to some extent with species. According to the product insert, the drug is fatal to swine when administered by IM injection at doses ranging between 10-20 mg/kg. The toxic dose for

goats is only about 30 mg/kg SC. The toxic effects of tilmicosin are mediated through its effects on the heart, possibly by causing rapid depletion of calcium (Main *et al.*, 1996). There are no published reports on the safety of tilmicosin in horses. The product insert suggests that the currently available tilmicosin formulation may be fatal in the equine species. This formulation is also toxic when used in cats. A tilmicosin-fatty acid salt has been developed as a safe and convenient formulation for cats (Kordick *et al.*, 2003). Tilmicosin may be a useful alternative to the current antimicrobial agents used in horses 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.

Table 1.1 - MIC_{90} (μg/mL) of azithromycin, clarithromycin, and erythromycin against common equine bacterial pathogens.

Organism (n)	Azithromycin	Clarithromycin	Erythromycin
Rhodococcus equi (60)	1.0	0.12	0.25
Streptococci (45)	<0.12	<0.06	<0.25
Staphylococcus spp. (18)	0.5	0.25	0.25
Pasteurella spp. (10)	0.25	1.0	1.0
Klebsiella spp. (9)	>8.0	>4.0	>4.0
Escherichia coli (16)	>8.0	>4.0	>4.0
Salmonella enterica (11)	4.0	>4.0	>4.0

Adapted from Jacks and Giguère 2003

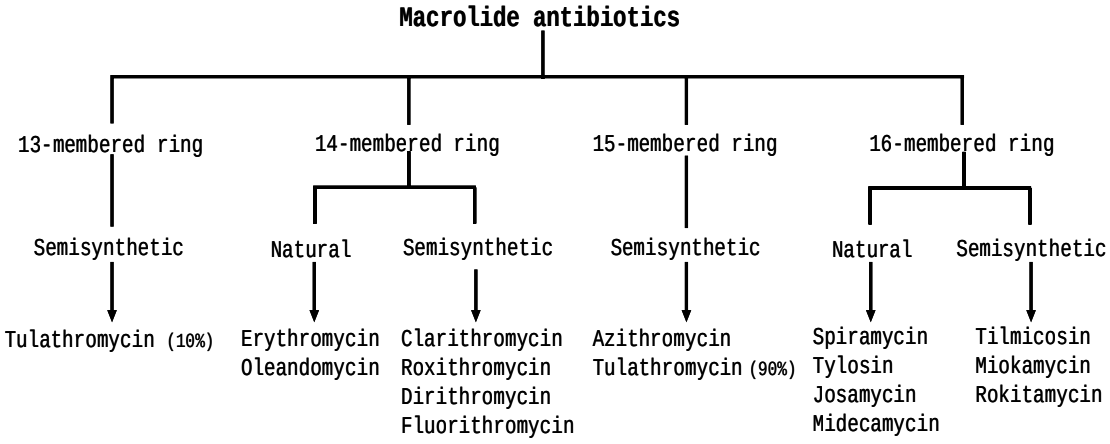

Figure 1.1 - Classification of macrolides according to the number of atoms comprising the lactone ring.

CHAPTER 3
PHARMACOKINETICS OF CLARITHROMYCIN AND CONCENTRATION IN BODY FLUIDS AND BRONCHOALVEOLAR CELLS IN FOALS

Abstract

The objective of this research was to determine pharmacokinetics of clarithromycin and the concentrations achieved in body fluids and bronchoalveolar cells in foals. Six healthy 2- to 3-week-old foals were used in this project. Clarithromycin (7.5 mg/kg of body weight) was administered to each foal via intravenous (IV) and intragastric (IG) routes, in a cross-over design. After the first IG dose, 5 additional doses were administered at 12-hour intervals. Concentrations of clarithromycin and its 14-hydroxy metabolite in serum were measured by HPLC. A microbiologic assay was used to measure clarithromycin activity in serum, urine, peritoneal fluid, synovial fluid, cerebrospinal (CSF), pulmonary epithelial lining fluid (PELF), and bronchoalveolar (BAL) cells. Following IV administration, clarithromycin had a $t_{½}$ of 5.4 hours, a body clearance of 1.27 L/h/kg, and an apparent volume of distribution at steady state of 10.4 ± 2.1 L/kg. Detection of 14-hydroxy-clarithromycin was achieved in all 6 foals by 1 h post-administration. Oral bioavailability of clarithromycin was 57.3 ± 12.0 %. Peak serum clarithromycin concentration following multiple IG administration was 0.88 ± 0.19 μg/mL. After multiple IG doses, peritoneal fluid, CSF, and synovial fluid clarithromycin concentrations were similar to or lower than serum concentrations whereas urine, PELF, and BAL cell concentrations were significantly higher than concurrent serum concentrations. Oral administration at a dosage of 7.5 mg/kg every 12 hours would

maintain serum, PELF, and BAL cell concentrations above the minimum inhibitory concentrations of *Rhodococcus equi* isolates for the entire dosing interval.

Introduction

Clarithromycin is a semi-synthetic macrolide antimicrobial agent chemically derived from erythromycin A. It differs from erythromycin A by having an O-methyl ether substitution at position 6 of the macrolide ring. This change provides greater stability in gastric acid resulting in enhanced absorption by the oral route. This structural difference also results in a longer elimination half life, a larger volume of distribution, as well as improved tissue and phagocytic cell uptake compared to erythromycin (Conte *et al*., 1995; Rodvold, 1999). Clarithromycin undergoes extensive hepatic metabolism in people and is primarily metabolized to 14-hydroxy-clarithromycin (Ferrero *et al*., 1990). This metabolite is responsible for approximately 50% of the total biological activity of clarithromycin and has an additive or synergistic effect with the parent compound (Fernandes *et al*., 1988; Martin *et al*., 2001).

Macrolide antimicrobial agents in combination with rifampin are commonly used in equine medicine for treatment of *Rhodococcus equi* infections in foals. *R. equi,* a Gram-positive facultative intracellular pathogen surviving in macrophages, is a common cause of pneumonia in foals between 3 weeks and 5 months of age. Combined therapy with erythromycin and rifampin has dramatically improved the historical survival rate of affected foals (Hillidge, 1987). However, recent evidences indicate that clarithromycin may be superior to erythromycin for the treatment of *R. equi* pneumonia in foals. Clarithromycin is more active against *R. equi in vitro* than either erythromycin or azithromycin (Jacks *et al*., 2003). In addition, in a retrospective study of foals presented to a referral institution, the combination of clarithromycin-rifampin was found to be

superior to azithromycin-rifampin or erythromycin-rifampin for the treatment of pneumonia caused by *R. equi* (Giguère *et al*., 2004).

A recent preliminary study confirmed that therapeutic concentrations are achieved in serum following oral administration of clarithromycin to foals (Jacks *et al*., 2002). However, a single oral dose was given precluding accurate determination of steady state drug concentrations and calculation of important pharmacokinetic parameters such as oral bioavailability, clearance, and apparent volume of distribution. In addition, concentrations of the drug in body fluids, pulmonary epithelial lining fluid and bronchoalveolar (BAL) cells were not measured. Recent studies demonstrate that the concentration of macrolides at the site of infection may be a better indicator of clinical efficacy than serum concentrations alone (Drusano, 2005). Finally, the methodology used to measure drug concentration in the preliminary study did not allow detection of metabolites such as 14-hydroxy-clarithromycin.

The objectives of the present study were to determine the pharmacokinetics and oral bioavailability of clarithromycin in foals as well as to measure drug concentrations in body fluids and BAL cells after a multi-dose intragastric (IG) regimen. An additional objective was to determine if clarithromycin is converted to the 14-hydroxy metabolite in foals.

Materials and Methods

Horses and Experimental Design

Four male and two female foals (5 Thoroughbred and 1 Quarter Horse) between 2 and 3 weeks of age and weighing between 71 and 100 kg were selected for use in the 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.

Clarithromycin was administered at a dose of 7.5 mg/kg of body weight via the IV and the IG routes, using a cross-over design. For the IV study, purified clarithromycin powder (Courtesy of Franks Pharmacy, Ocala, FL, USA) was dissolved in sterile water (100mg/ml) and administered as a single bolus through a catheter placed into the left jugular vein. Blood samples were obtained from a catheter placed in the right jugular vein at 0 (prior to administration), 3, 6, 10, 20, 30, 60, 90 minutes and at 2, 3, 4, 6, 8, 12, and 24 hours after the drug was administered.

For the IG route, clarithromycin tablets (250 mg tablets; Biaxin, Abbott Laboratories, Chicago, IL, USA) were dissolved in 50 ml of water and administered by nasogastric tube. For the first 24 hours, blood samples were collected as described for the IV study. Afterwards, 5 additional doses were administered at 12 hour intervals (24, 36, 48, 60, 72 hours after the initial dose). Blood samples were collected immediately before each additional dose and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 hours after dose 2, 4, and 6.

Bronchoalveolar lavage was performed and samples of synovial fluid, peritoneal fluid, cerebrospinal fluid (CSF), and urine were collected aseptically 2 and 12 hours after administration of the last IG dose. Foals were sedated by administration of xylazine hydrochloride (1.0 mg/kg, IV), and butorphanol tartrate (0.07 mg/kg, IV). Immediately after collection of BAL fluid (see below), general anesthesia was induced by IV administration of diazepam (0.1 mg/kg, IV) and ketamine (2.5 mg/kg). Samples of synovial fluid were obtained from the intercarpal or radiocarpal joint by use of a 20-

gauge needle. 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. A flexible 8-F Foley catheter was used to collect urine directly from the bladder. Samples were centrifuged and the supernatants were stored at –80°C until analysis.

Bronchoalveolar Lavage

A 10 mm diameter, 1.8 m bronchoscope (Pentax, Welch Allen, Orangeburg, NY, USA) was passed via nasal approach into either the left or right lung until wedged in a fourth to sixth generation bronchus. The lavage solution consisted of 4 aliquots of 50 ml physiologic saline (0.9% NaCl) solution infused and aspirated immediately. The bronchoscope was passed alternating into either the left or right lung to prevent the effect of repeated bronchoalveolar lavages on differential cell counts. Total nucleated cell count in BAL fluid was determined by use of a hemacytometer. Bronchoalveolar fluid was centrifuged at 200 X g for 10 minutes. Bronchoalveolar cells were washed, re-suspended in 1 mL of phosphate-buffered solution, vortexed and frozen at -80ºC until assayed. Supernatant BAL fluid was also frozen at -80ºC until assayed. Before assaying, the cell pellet samples were thawed, vortexed vigorously, and sonicated for 2 minutes to ensure complete cell lysis. The resulting suspension was centrifuged at 500 *g* for 10 minutes and the supernatant fluid was used for determination of intracellular clarithromycin concentrations.

Drug Analysis by High Performance Liquid Chromatography (HPLC)

Serum samples underwent a two-step extraction procedure prior to analysis by HPLC. Samples (500 µl) of serum were thawed and mixed with an equal volume of internal standard roxithromycin (Sigma, St-Louis, MO, USA) (4 µg/ml in 10 mM NaH_2PO4 buffer, pH 3) and acidified with 2 N HCl to a final pH of 3. Each acidified

sample was mixed briefly and transferred quantitatively onto a solid phase extraction column (Varian Bond Elut C18, 500 mg. Varian, Inc. Palo Alto, CA, USA) that had been pre-conditioned with 5 ml of methanol and 10 mM phosphate buffer (pH 3). Following sample loading, each column was rinsed with 5 ml of 10 mM phosphate buffer (pH 3) prior to elution of drugs with 5 ml of alkalinized methanol (99:1 mix of methanol: 1 N NaOH). Methanolic eluates were collected and evaporated to dryness in a vacuum concentrator at ambient temperature. Dried samples were reconstituted in 4 N NaOH (500 µl) by incubation at room temperature for 30 min with intermittent vortex mixing. Thereafter, 3 ml of hexane:ethyl acetate (50:50) was added and samples mixed vigorously. Aqueous and organic layers were separated by centrifugation (4 min at 4000 g) and a portion of the organic layer was removed and evaporated to dryness. Dried samples were reconstituted in mobile phase and analyzed immediately by HPLC (Beckman System Gold; Beckman Coulter, Inc. Fullerton, CA, USA) with electrochemical detection (HPLC-EC). Samples were injected and separated on a reversed phase column (Supelco Discovery C18, 150 x 5.6 mm, 5 µm particle size) using a filtered (0.2 µm) degassed mobile phase containing a 55:45 mixture (v/v) of 1 mM sodium phosphate (pH 7.0) and acetonitrile (final adjusted pH 7.5) at a flow rate of 1ml/min. Concentrations of the three macrolide antibiotics were measured by amperometric detection using an LC-4C electrochemical detector (BAS, Lafayette, IN, USA) with a platinum electrode set at +1100mV potential (1nA full scale). Peak areas for all three compounds exhibited a linear relationship versus drug concentration over the ranges of 0.25 – 5.00 µg/ml for 14-hydroxy-clarithromycin (Courtesy of Abbott Laboratories, Abbott Park, IL, USA) and 0.50 -5.00 µg/ml for clarithromycin (US

Pharmacopeia, Rockville, MD, USA) and roxithromycin with a correlation coefficient (*r*) value ≥ 0.99. Each sample was run in duplicate and drug concentrations were estimated by comparison of peak areas against linear standard curves for each analyte. Therefore, 0.5 μg/ml was used as the lowest limit of quantification for clarithromycin. Average retention times were 3.8 (14-hydroxy-clarithromycin), 9.0 (clarithromycin) and 11.0 min (roxithromycin). In spiked serum samples, drug extraction yields of clarithromycin (76 ± 3.2 %) and the internal standard roxithromycin (77 ± 3.9 %) were highly correlated (r = 0.98). In contrast, extraction yield for 14-hydroxy-clarithromycin was greater but more variable 92 ± 11.4 % and was poorly correlated (r = 0.78) with internal standard and, for that reason, exact concentrations of the metabolite are not reported here.

Measurement of Clarithromycin Activity Using a Microbiologic Assay

Concentrations of clarithromycin were determined in serum, synovial fluid, peritoneal fluid, CSF, BAL fluid, and BAL cells, using an agar well diffusion microbiologic assay with *Micrococcus luteus* (ATCC 9341, American Type Culture Collection, Rockville, MA, USA) as the assay organism. One milliliter of a bacterial suspension was grown overnight in trypticase soy broth and adjusted to an optical density of 0.5 at 550 nm. This suspension was added to tempered neomycin assay agar (Neomycin assay agar, Fischer Scientific Inc, Pittsburgh, PA, USA) and distributed evenly over the assay plates. The plates were allowed to solidify for 45 minutes, and 0.5 mm wells were punched and filled with 50 μl of samples or clarithromycin standards (US Pharmacopeia, Rockville, MD, USA) ranging in concentrations from 0.02 to 5.0 μg/ml. Known amount of purified clarithromycin were added to equine serum, synovial fluid, and urine to produce standard curves for each type of substrate. Bronchoalveolar cells,

BAL fluid, CSF, and peritoneal fluid were assayed with standards diluted in phosphate-buffered saline. The agar plates were incubated for 36 hours at 30 C. Zones of bacterial inhibition were measured to the nearest 0.1 cm. Each sample or standard was assayed in triplicate and mean values for 3 measurements of the zone diameters were determined. The lower limit of quantification of the assay was 0.02 μg/ml for serum, BAL cells, and body fluid samples. Negative control samples did not cause bacterial inhibition, which indicated no antibacterial activity of equine serum, or body fluids, or BAL cell supernatants. Plots of zone diameters versus standard clarithromycin concentrations were linear between 0.02 and 5 μg/ml with *r* values ranging between 0.993 and 0.998. The coefficients of variation for repeatedly assayed samples at concentrations > 0.1 μg/ml and < 0.1 μg/ml were < 5% and < 10%, respectively.

Estimation of PELF and BAL Cell Volumes and Determination of Clarithromycin Concentrations in PELF and BAL Cells

Pulmonary distribution of clarithromycin was determined as reported (Baldwin et al., 1992). Estimation of the volume of PELF was done by urea dilution method (Conte *et al*., 1996; Jacks *et al*., 2001). 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 ($Urea_{BAL}$), the proportion of reagents to specimen was changed from 300 μl/3 μl in serum to 225 μl/50 μl. The volume of PELF (V_{PELF}) in BAL fluid was derived from the following equation: $V_{PELF} = V_{BAL} X (Urea_{BAL}/Urea_{SERUM})$, where V_{BAL} is the volume of recovered BAL fluid. The concentration of clarithromycin in PELF (CLR_{PELF}) was derived form the following

relationship: $CLR_{PELF} = CLR_{BAL}$ X (V_{BAL}/ V_{PELF}), where CLR_{BAL} is the measured concentration of clarithromycin in BAL fluid.

The concentration of clarithromycin in BAL cells (CLR_{BAL}) was calculated using the following relationship: CLRBAL = (CLRPELLET/VBALC) where CLRPELLET is the concentration of antimicrobial in the BAL cell pellet supernatant and V_{BALC} is the mean volume of foal BAL cells. A V_{BALC} of 1.20 μl/10^6 cells was used for calculations based on a previous study in foals (Jacks *et al.*, 2001).

Pharmacokinetic Analysis

For each foal, the plasma concentration versus 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 plasma concentration versus time curve using a minimum of 3 data points. Elimination half-life ($t_{½}$) 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 plasma concentration. Mean residence time (MRT) was calculated as: AUMC/AUC. Apparent volume of distribution based on the AUC (Vd_{area}) was calculated as: dose /AUC• K_{el}, apparent volume of distribution at steady state (VD_{ss}) was calculated as (dose/AUC)/(AUMC/AUC), and systemic clearance (CL) was calculated from: dose/AUC. Bioavailability was calculated as (AUC_{IG}/AUC_{IV}) x ($dose_{IV}$/$dose_{IG}$).

Statistical Analysis

Pharmacokinetic-derived data are presented as mean ± SD unless otherwise specified. The paired *t*-test was used to compare differences in K_{el} between IV and IG administration as well as peak serum concentration after the first dose ($C_{max\ 0\text{-}24h}$) and peak serum concentrations after the last dose ($C_{max\ 72\text{-}84h}$). The Friedman repeated measures ANOVA on ranks was used to compare clarithromycin concentrations between sampling sites (serum, synovial fluid, peritoneal fluid, CSF, urine, PELF, BAL cells). When indicated, multiple pairwise comparisons were done using the Student-Newman-Keuls test. Differences were considered significant at $P < 0.05$.

Results

Following IV administration of clarithromycin (7.5 mg/kg), serum drug concentrations were similar when measured by HPLC or the microbiologic assay; although, HPLC-based measurements tended to be higher immediately following drug administration (Figure 2.1). Pharmacokinetic parameters were calculated based on data obtained with the microbiologic assay because the high limit of quantification of the HPLC method did not allow accurate evaluation of the terminal elimination phase of the drug. Clarithromycin had a $t_{½}$ of 5.4 hours (harmonic mean), a body clearance of 1.27 L/h/kg, and a Vd_{ss} of 10.4 ± 2.1 L/kg (Table 1). Detection of 14-hydroxy-clarithromycin was first achieved 0.5 h after IV administration in 3 foals and by 1 h post-administration in all 6 foals. Time to maximum concentration (T_{max}) of 14-hydroxy-clarithromycin following IV administration was 1.7 ± 1.2 h.

After IG administration, quantifiable clarithromycin activity was found in 4 of 6 foals at 10 minutes and in all 6 foals at 15 minutes. The time to peak serum clarithromycin concentration (T_{max}) was 1.6 ± 0.4 h and F was 57.3 ± 12.0 % (Table 1).

Peak serum clarithromycin concentration following multiple IG administration ($C_{max\ 72\text{-}84h}$: 0.88 ± 0.19 μg/mL) was significantly higher ($P = 0.011$) that that achieved after the first IG dose ($C_{max\ 0\text{-}24h}$: 0.52 ± 0.17 μg/mL) (Figure 2). Differences between K_{el} after IV and IG administration were not significant ($P = 0.617$). After multiple IG doses, peritoneal fluid, CSF, and synovial fluid clarithromycin concentrations were similar to or lower than serum concentrations whereas urine, PELF, and BAL cell concentrations were significantly higher than concurrent serum concentrations (Table 2). Detection of 14-hydroxy-clarithromycin was first achieved 0.5 h after IG administration in 2 foals and by 2 h post-administration in all 6 foals with a T_{max} of 1.7 ± 1.2 h.

One foal developed transient tachypnea and profuse sweating 5 min after administration of the IV bolus. One foal developed diarrhea after the third IG dose and another foal developed diarrhea after the last intragastric dose. In both foals, the diarrhea resolved without therapy within 36 h.

Discussion

Clarithromycin undergoes extensive metabolism in people. Of the 8 metabolites that have been identified, 14-hydroxy-clarithromycin is the most abundant and the only one with substantial antimicrobial activity (Fernandes *et al*., 1988; Ferrero *et al*., 1990). The metabolism of clarithromycin is unique in people since it is the only 14-membered macrolide to demonstrate 14-hydroxylation. The 14-hydroxy metabolite of clarithromycin is also produced in monkeys but not in rats, mice, or desert tortoises (Ferrero *et al*., 1990; Bedos *et al*., 1992; Wimsatt *et al*., 1999). The present study confirms the production of 14-hydroxy-clarithromycin in foals with peak concentrations detected approximately 1.7 h following IV or IG administration. In people, peak 14-

hydroxy-clarithromycin concentrations at approximately 1.3 μg/ml were detected 3 h following oral administration of a dose of 7.5 mg/kg of body weight (Gan *et al*., 1992). Unfortunately, exact concentrations of 14-hydroxy-clarithromycin could not be determined in the present study due to the inability to find an internal standard exhibiting parallel recovery efficiency.

The microbiological assay used to calculate pharmacokinetic parameters in the present study only allows an approximation of the drug disposition because it does not differentiate between clarithromycin and its 14-hydroxy metabolite. However, in a clinical situation, the total antimicrobial activity measured by the microbiological assay is adequate to determine a dosage regimen. The oral bioavailability of clarithromycin in the present study (57%) is similar to that reported in people (55%) but lower than the 70-75% reported in dogs (Chu *et al*., 1992; Vilmanyi *et al*., 1996). The oral bioavailability of clarithromycin in the present study is similar to that of azithromycin (38-56%) and much higher than that of erythromycin (14%) in foals (Lakritz *et al*., 2000:1011-1015; Jacks *et al*., 2001; Davis *et al*., 2002). Clarithromycin elimination half-life in the present study (5.4 h) was slightly longer than that reported after oral administration to dogs (3.9 h) (Vilmanyi *et al*., 1996). Elimination half-lives reported in people range between 3 to 5 h for clarithromycin and 4 to 9 h for 14-hydroxy-clarithromycin (Rodvold, 1999). The elimination half-life of clarithromycin in the present study is longer than that reported for erythromycin (1 h) but considerably shorter than that of azithromycin (16-20 h) in foals (Prescott et al., 1983; Lakritz et al., 1999; Lakritz *et al*., 2000:914-919; Jacks *et al*., 2001; Davis *et al*., 2002).

The optimal dosing of antimicrobial agents is dependent not only on the pharmacokinetics, but also on the pharmacodynamics of the drug. The pharmacodynamic properties of a drug address the relationship between drug concentration and antimicrobial activity. Much confusion exists over the pharmacodynamics of macrolides and azalides because their concentration-time profile is low relative to the minimum inhibitory concentrations of the pathogens for which they are used typically. An important factor in determining the efficacy of many macrolides in animal models of infection with extracellular bacteria is the length of time that serum concentrations exceed the MIC of the pathogen (T > MIC) (Rodvold, 1999). In a mouse thigh model of *Streptococcus pneumoniae* infection, T > MIC for at least 60% of the dosage interval with clarithromycin was the best predictor of efficacy (Craig, 1997). In a murine model of pneumococcal pneumonia, T > MIC of 50-70%, C_{max}/MIC of 3-7, and AUC_{0-24}/MIC 40-100 were all comparable in predicting efficacy (Tessier *et al*., 2002). 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).

While drug concentration in plasma is clearly a driving force for penetration to the site of infection, the actual drug-concentration time profile in a peripheral site may be quite different from that of plasma (Drusano, 2005). Macrolides cross the cellular membranes primarily by passive diffusion (Fietta *et al*., 1997). They are potent weak bases that become ion-trapped within acidic intracellular compartments such as lysosomes and phagosomes. 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). However, the white blood cell delivery theory does not explain the very high concentrations of these drugs in PELF, as this was demonstrated in healthy subjects where trafficking of white blood cells to the PELF should have been minimal (Conte *et al*., 1995; Rodvold *et al*., 1997). A high concentration of macrolides in PELF has long been proposed as a key factor in their efficacy against respiratory pathogens in people. The preferential activity of clarithromycin in the lung was recently demonstrated in mice infected with *S. pneumoniae* isolates with efflux-mediated macrolide resistance. Consistent bacterial kill was observed in the lung model whereas no drug effect was seen in the thigh model (Maglio *et al*., 2004). These differences in bacterial activity between sites were explained by the higher concentrations in PELF than in serum (Maglio *et al*., 2004).

In the present study, administration of clarithromycin at 7.5 mg/kg every 12 h resulted in serum concentrations above the MIC inhibiting 90% of *R. equi* isolates (MIC_{90} =0.12 μg/ml) throughout the entire dosing interval, a mean C_{max}/MIC_{90} ratio of 7, and mean $AUC_{0\text{-}24}/MIC_{90}$ ratio of 57. Because serum concentrations alone should not be used to determine the likelihood of clinical efficacy in the treatment of *R. equi* pneumonia of foals, clarithromycin concentrations were also measured in PELF and BAL cells. Estimation of PELF volume by use of the urea dilution method may result in falsely increased BAL fluid urea concentration by diffusion of urea from the interstitium and blood if BAL fluid dwell-time is prolonged (Baldwin *et al*., 1992). Prolonged BAL fluid dwell-time was minimized in our study by use of rapid infusion of 100 ml of saline solution followed by immediate aspiration. Overestimation of urea concentrations in

BAL fluid would falsely increase the volume of PELF, which would in turn result in an underestimation of clarithromycin concentrations in PELF (Baldwin *et al*., 1992). Concentrations of clarithromycin in PELF and BAL cells in the present study considerably exceeded the MIC_{90} of *R. equi* isolates obtained from foals with pneumonia. Clarithromycin concentrations in PELF and BAL cells in the present study were also considerably higher than concentrations reported following multiple daily administration of azithromycin to foals. In the present study, clarithromycin concentrations in BAL cells and PELF had decreased considerably 12 h following administration. This is in contrast to azithromycin concentrations in PELF and BAL cells which do not decrease for at least 48 h following administration to foals (Jacks *et al*., 2001). Collectively, these findings in foals are consistent with studies in people showing much higher peak clarithromycin concentrations in PELF and BAL cells compared to azithromycin, but much longer persistence of azithromycin than clarithromycin at these sites (Conte *et al*., 1996; Patel *et al*., 1996; Rodvold *et al*., 1997). Following administration of a single oral dose to people, clarithromycin is no longer detectable in PELF after 24 h and in BAL cells after 48 h (Conte *et al*., 1996). The release of azithromycin from cells is much slower than that of erythromycin and clarithromycin, resulting in sustained concentrations of azithromycin in tissues for days following discontinuation of therapy (Fietta *et al*., 1997). Clarithromycin concentrations in peritoneal fluid, synovial fluid, and CSF were significantly lower than PELF concentrations in the present study indicating preferential diffusion of clarithromycin into pulmonary fluid.

Adverse effects in humans receiving clarithromycin are rare and usually related to the gastrointestinal tract with diarrhea, nausea, and abdominal pain being the most

frequently reported (Alvarez-Elcoro & Enzler, 1999). Two of 6 foals in the present study developed mild self-limiting diarrhea. The incidence of diarrhea in the present study was similar to that of a retrospective study in which 5 of 18 foals (28%) with *R. equi* pneumonia treated with clarithromycin and rifampin also developed diarrhea (Giguère *et al*., 2004). This is similar to the incidence of diarrhea reported in foals being treated with erythromycin-rifampin (17 to 36%) (Stratton-Phelps *et al*., 2000; Giguère *et al*., 2004). In contrast, the incidence of gastrointestinal adverse effects in people is significantly lower during clarithromycin (4%) than during erythromycin (19%) therapy (Anderson *et al*., 1991).

Table 3.1 - Pharmacokinetic variables (mean ± SD unless otherwise specified) for clarithromycin after IV or intragastric administration to 6 foals at dose of 7.5 mg/kg of body weight.

Variable	**IV**	**Intragastric**
K_{el} (h^{-1})	0.129 ± 0.022	0.141 ± 0.05
$AUC_{0-\infty}$ (μg•h/mL)	6.2 ± 1.5	3.4 ± 1.1
$AUMC_{0-\infty}$ (μg•h^2/mL)	51.1 ± 16.2	24.4 ± 9.7
MRT (h)	8.25±0.989	7.1±1.70
$t_{½}$ (h)	5.4*	NA
Vd_{area} (L/kg)	9.9 ± 1.8	NA
Vd_{ss} (L/kg)	10.4 ± 2.1	NA
Clearance (L/h/kg)	1.27 ± 0.25	NA
T_{max} (h)	NA	1.6 ± 0.4
$C_{max\ 0\text{-}24h}$ (μg/mL)	NA	0.52 ± 0.17
$C_{max\ 72\text{-}84h}$ (μg/mL)	NA	0.88 ± 0.19
C_{84h}	NA	0.20 ± 0.06
F (%)	NA	57.3 ± 12.0

NA = Not applicable

*Harmonic mean.

K_{el} = Elimination rate constant. $t_{½}$ = Elimination half-life. AUC = Area under the serum concentration versus time curve. AUMC = Area under the first moment of the concentration versus time curve. MRT = Mean residence time. Vd_{area} = Apparent volume of distribution (area) Vd_{ss} = Apparent volume of distribution (steady-state) $t_{½abs}$ = Absorption half-life. T_{max} = Time to peak serum concentration. $C_{max\ 0\text{-}24h}$ = Peak serum concentration after the first dose. $C_{max\ 72\text{-}84h}$ = Peak serum concentration after repeated doses. C_{84h} = Minimum serum concentration 12 h after the last dose. F = Oral bioavailability.

Table 3.2 - Mean ± SD clarithromycin activity in body fluids and BAL cells of six foals after 6 intragastric administrations (7.5 mg /kg every 12 hours).

	Time after administration (h)	
Sample	2	12
Serum (μg/mL)	0.83 ± 0.18^{a}	0.20 ± 0.06^{a}
Synovial fluid (μg/mL)	0.27 ± 0.06^{b}	0.08 ± 0.02^{a}
Peritoneal fluid (μg/mL)*	0.43 ± 0.32^{b}	0.11 ± 0.06^{a}
Urine (μg/mL)	36.8 ± 46.4^{c}	2.53 ± 0.82^{b}
CSF (μg/mL)	0.22 ± 0.09^{b}	0.13 ± 0.09^{a}
Pulmonary epithelial lining fluid (μg/mL)	76.2 ± 59.4^{c}	21.4 ± 20.5^{c}
Bronchoalveolar cells (μg/mL)†	269 ± 232^{d}	117 ± 107^{d}

*n=3 at 2 h and n=5 at 12 h

†Drug concentrations are in μg/ml of bronchoalveolar cell volume

[a,b,c,d]Different letters within a column indicate statistically significant difference in clarithromycin concentrations ($P < 0.05$)

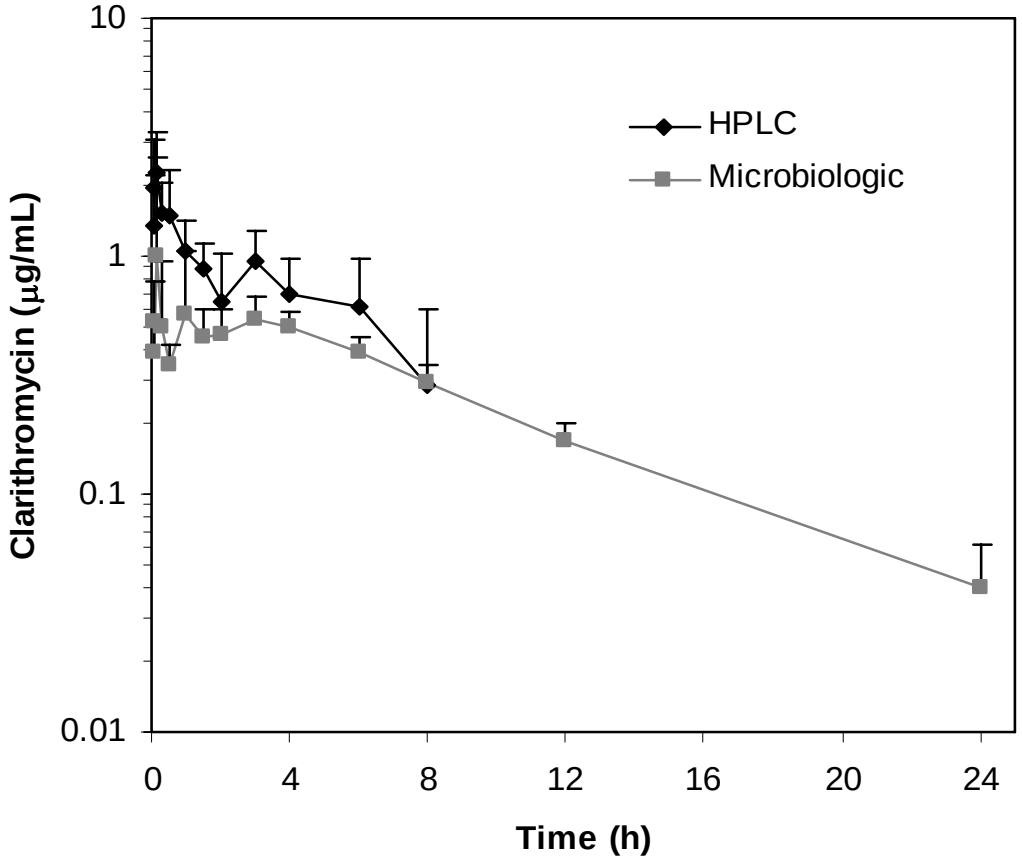

Figure 3.1—Mean (+ SD) serum clarithromycin concentration as measured by HPLC method or microbiologic assay in 6 foals administered a single IV dose of 7.5 mg/kg.

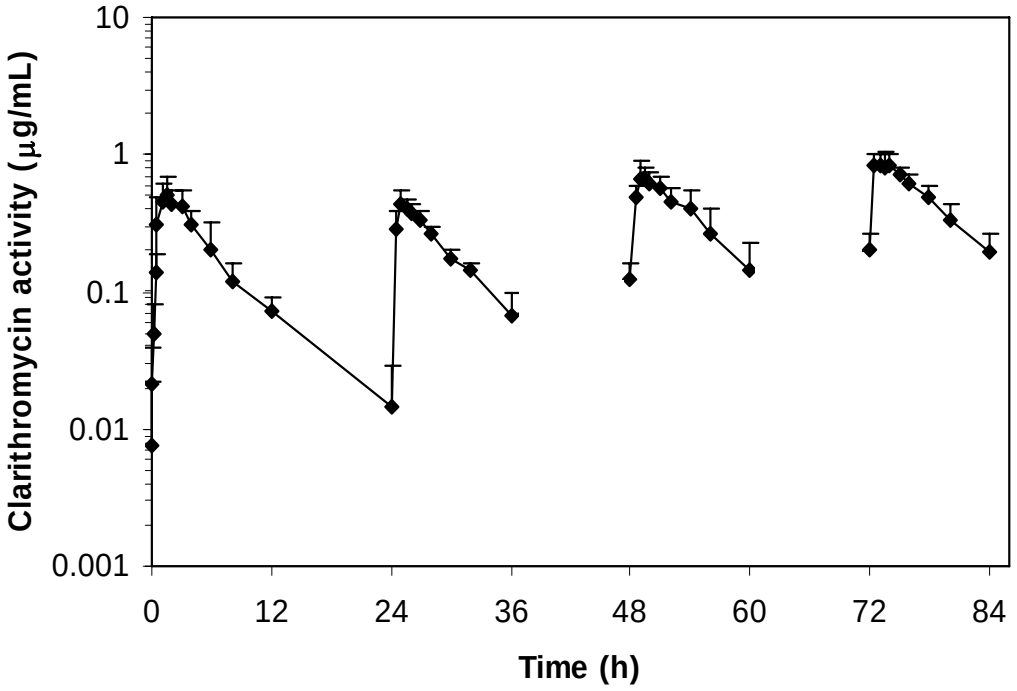

Figure 3.2—Mean (+ SD) serum clarithromycin activity (µg/ml) in 6 foals following intragastric clarithromycin (7.5 mg/kg) administration at 0, 24, 36, 48, 60, and 72 hours. Results are based on measurements with the microbiologic assay.

CHAPTER 4
PULMONARY DISPOSITION OF TILMICOSIN IN FOALS AND *IN VITRO* ACTIVITY AGAINST *RHODOCOCCUS EQUI* AND OTHER COMMON EQUINE BACTERIAL PATHOGENS

Abstract

Tilmicosin is a long-acting macrolide currently approved for treatment of respiratory disease in cattle, sheep and swine. 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 *R. 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 7 healthy 5- to 8-week-old foals by the intramuscular route. Concentrations of tilmicosin in serum were measured by HPLC and concentrations in lung tissue, pulmonary epithelial lining fluid (PELF), and bronchoalveolar (BAL) cells were measured by mass spectrometry. Mean peak tilmicosin concentrations were significantly higher in BAL cells (20.1 ± 5.1 μg/mL) than in lung tissue (1.90 ± 0.65 μg/mL), PELF (2.91 ± 1.15 μg/mL), and serum (0.19 ± 0.09 μg/mL). Harmonic mean elimination half life in lung tissue (193.3 h) was significantly longer than that of serum (18.4 h). Elimination half lives in BAL cells and PELF were 62.2 h and 73.3 h, respectively. 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 *Enterobacteraceae*. In conclusion, the 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.

Introduction

Tilmicosin is a semi-synthetic 16-membered lactone ring macrolide chemically derived from tylosin (Prescott, 2000). Tilmicosin is approved as a suspension 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. (Watts *et al.*, 1994; 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 to serum (Ziv *et al.*, 1995; Scorneaux and Shryock, 1999; Clark *et al.*, 2004). Despite low extracellular concentrations, tilmicosin accumulates substantially in phagocytic cells of cattle and swine (Scorneaux and Shryock, 1998; Scorneaux and Shryock, 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 (Wilson, 1992). Macrolide antimicrobial agents are commonly used in equine medicine for treatment of foal pneumonia, particularly when infection with *Rhodococcus equi* is suspected or confirmed. 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 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.

Material and Methods

Horses and experimental design

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) was administered as a single dose of 10 mg/kg of body weight via the intramuscular route in the semimembranosus/semitendinosus muscles. Blood samples (8 mL) were obtained from a jugular catheter at 3, 6, 10, 20, 30, 60, 90 minutes and at 2, 3, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 168, and 288 hours after the drug was administered. Bronchoalveolar lavage (BAL) was performed 24, 48, 72, 168, and 288 hours and samples of cerebrospinal fluid (CSF) were collected aseptically 4, 24, and 72 hours after administration of tilmicosin. Lung tissue was obtained 24, 72, 168, and 288 hours after administration of the drug. Prior to collection of BAL, lung tissue, and CSF, foals were sedated by administration of xylazine hydrochloride (1 mg/kg, IV) and butorphanol tartrate (0.07 mg/kg, IV). Immediately after collection of BAL fluid, general anesthesia was induced by IV administration of diazepam (0.1 mg/kg) and ketamine (2.5 mg/kg) for collection of lung tissue and CSF fluid. Using sterile techniques, CSF was collected from the atlantooccipital space by use of a 3.5 inch, 20-gauge spinal needle. Blood and CSF samples were centrifuged and serum and CSF supernatants were stored at -80ºC until analysis. 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).

Bronchoalveolar lavage

A 10 mm diameter, 2.4 m bronchoalveolar lavage catheter (Jorgenson laboratories, Loveland, CO) was passed via nasal approach until wedged into a bronchus. The lavage solution consisted of 4 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 X g for 10 minutes. Bronchoalveolar cells were washed, re-suspended in 500 µl of phosphate-buffered solution, vortexed and frozen at -80ºC until assayed. Supernatant BAL fluid was also frozen at -80ºC until assayed. Before assaying, the cell pellet samples were thawed, vortexed vigorously and sonicated for 3 minutes to ensure complete cell lysis. The resulting suspension was centrifuged at 500 X g for 10 minutes and the supernatant fluid was used to determine the intracellular concentrations of tilmicosin.

Drug analysis

The serum and other tissue samples were analyzed by validated methods at Idexx Pharmaceuticals (Durham, NC). Serum concentrations of tilmicosin were determined by HPLC analysis. The extraction efficiency from serum was 98%. The limit of quantification (LOQ) was 0.08 □g/mL. The tilmicosin concentrations in all other body fluids or tissues were determined by mass spectrometry. The LOQ were 0.5 ng/mL, 1.44 ng/mL, 1.9 ng/mL, and 0.6 ng/mL for lung tissue, BAL fluid, CSF, and BAL cells, respectively.

Estimation of PELF and BAL Cell Volumes and Determination of Tilmicosin Concentrations in PELF and BAL Cells

Pulmonary distribution of tilmicosin was determined as reported (Baldwin *et al.*, 1992). Estimation of the volume of 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 ($Urea_{BAL}$), the proportion of reagents to specimen was changed from 300 µl/3 µl in serum to 225 µl/50 µl. The volume of PELF (V_{PELF}) in BAL fluid was derived from the following equation: $V_{PELF} = V_{BAL} X (Urea_{BAL}/Urea_{SERUM})$, where V_{BAL} is the volume of recovered BAL fluid. The concentration of tilmicosin PELF (TIL_{PELF}) was derived form the following relationship: $TIL_{PELF} = TIL_{BAL} X (V_{BAL}/ V_{PELF})$, where TIL_{BAL} is the measured concentration of tilmicosin in BAL fluid.

The concentration of tilmicosin in BAL cells (TIL_{BAL}) was calculated using the following relationship: $TIL_{BAL} = (TIL_{PELLET}/V_{BALC})$ where TIL_{PELLET} is the concentration of antimicrobial in the BAL cell pellet supernatant and V_{BALC} is the mean volume of foal BAL cells. A V_{BALC} of 1.20 µl/10^6 cells was used for calculations based on a previous study in foals (Jacks *et al.*, 2001).

Pharmacokinetic Analysis

For each foal, serum, lung tissue, PELF, or BAL cells tilmicosin concentration versus 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 versus time curve using a minimum of 3 data points. Elimination half-life ($t_{½}$) 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 (MRT) 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). 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 minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of tilmicosin against R. equi

R. equi isolates (n =56) were obtained from tracheobronchial aspirates or post-mortem specimens from pneumonic foals. For each isolate, MIC and 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 (formerly NCCLS) (NCCLS, 1999a; NCCLS, 1999b; NCCLS, 2000) . A standard inoculum of 5 x 10^5 was used for each isolate. Concentrations of tilmicosin tested ranged 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 37ºC (National Committee for Clinical Laboratory Standards, 2000). MBC was calculated as the lower concentration of drug resulting in a 99.9% reduction of the original inoculum(National Committee for Clinical Laboratory Standards, 1999a). Control strains used to validate the assay were *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 (Odland *et al.*,

2000). The MIC r*equi*red to inhibit growth of 50% of isolates (MIC_{50}) and the MIC r*equi*red 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. 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 3 *R. equi* isolates as 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. After 0, 2, 6 and 24 hours 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 CFU was counted.

In vitro activity of tilmicosin against equine bacterial pathogens

A total of 183 bacterial isolates from various *equi*ne clinical samples were examined. Isolates were obtained from clinical samples submitted to the microbiology laboratory at 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 X 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 3 times by rotating the plate approximately 60^o for each inoculation to ensure an even distribution. After allowing the excess moisture to dry (approx 10 to 15 minutes), 15 μg tilmicosin disks (BBL Sensi-Disc, Hardy Diagnostics, Santa Maria, CA) were applied to the agar. The plates were incubated for 18 to 24 hours at 37^oC. 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 *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Enterococcus 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 2 of 7 foals at 3 minutes after IM injection and in 5 of 7 foals at 10 minutes post-injection. Serum concentrations remained below the limit of quantification throughout the sampling period in 2 foals. Concentrations below the limit of quantification were reported as 0 for calculation of mean ± SD (Figure 3.1). Serum pharmacokinetic parameters were derived from the 5 foals with quantifiable serum concentrations (Table 3.1). Maximum tilmicosin concentrations (C_{max}) and AUC were significantly higher in BAL cells than in serum, lung tissue, and PELF (Table 3.1). Similarly, C_{max} and AUC were significantly higher in PELF and lung tissue than in serum. Elimination half life in lung tissue (193.3 h) was significantly longer than that of serum (18.4 h).

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. 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 with no benefit from increasing drug concentrations above 4 times the MIC (Figure 3.2). Tilmicosin was active *in vitro* against most streptococci, *Staphylococcus* spp., *Actinobacillus* spp., and *Pasteurella* spp. (Table 3.2).

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 (Micotil 300 package insert, 1995). 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 that that observed after administration of the same fatty acid salt formulation administered at a dose of 10 mg/kg SC to cats (0.73 μg/mL) (Kordick *et al.*, 2003). Tilmicosin serum elimination half-life in the present study (18.4 h) was slightly shorter than that reported after SC 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 *Mannheimia haemolytica* (3.15 μg/mL) for at least 72 hours following a single SC injection at a dose of 10 mg/kg (Micotil 300 package insert, 1995). 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 such as lysosomes (Scorneaux and 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 and 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 and 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*. 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 these pathogens in foals.

Adverse effects observed in the present study consisted mainly of swelling at the injection site in 5 foals and self limiting diarrhea in 4 foals. One foal developed tachypnea and profuse sweating approximately 2 h after injection. In swine, IM 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 (Micotil 300 package insert, 1995). Tilmicosin included in the diet of horses at concentrations of 400, 1200, and 2000 ppm has resulted in gastrointestinal disturbance in all groups and death of 1 horse consuming the 2000 ppm diet (Pulmotil 90 package insert, 1995). In another study, SC 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 and Dowling, 2004). The fecal flora slowly recovered over the next 7 days. Mild self-limiting diarrhea was observed in one foal (Clark and Dowling, 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 IM 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.

Table 4.1 – Serum and pulmonary pharmacokinetic variables (mean ± SD unless otherwise specified) for tilmicosin after IM administration to seven foals at a dose of 10 mg/kg of body weight.

Variable	**Serum**[1]	**Lung**[2]	**PELF**	**BAL cells**
K_{el} (h^{-1})	0.04 ± 0.02^{a}	0.004 ± 0.001^{b}	0.009 ± 0.004^{b}	0.01 ± 0.003^{b}
$AUC_{0-\infty}$ (μg•h/mL)	5.76 ± 1.87^{a}	711 ± 351^{b}	461 ± 115^{b}	2342 ± 1006^{c}
MRT (h)	34.5 ± 18.0^{a}	323 ± 91.0^{b}	180 ± 48.9^{c}	117 ± 29.6^{c}
$t_{½}$ (h)*	18.4^{a}	193.3^{b}	$73.1^{a,b}$	$62.2^{a,b}$
T_{max} (h)	5.50 ± 3.43^{a}	$30.8 \pm 18.1^{a,b}$	52.0 ± 18.1^{b}	54.9 ± 33.1^{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}

[1]n=5 because 2 foals had serum tilmicosin concentrations below the limit of quantification.
[2]n=5 because one foal died after the 72 h sample and lung samples were too small for drug analysis in one foal.
*harmonic mean.
[a,b,c,d]Different letters within a row indicate a statistically significant difference between sampling sites ($P < 0.05$).
K_{el} = Elimination rate constant. AUC = Area under the serum concentration versus time curve. MRT = Mean residence time. $t_{½}$ = Elimination half-life. T_{max} = Time to peak serum concentration. C_{max} = peak serum concentration.

Table 4.2 – Tilmicosin *in vitro* susceptibility of 183 bacterial isolates obtained from horses.

Microorganism (n)	Zone diameter (mm)			Susceptibility (%)
	Median	25th percentile	Range	
Gram positives				
□-hemolytic streptococci (7)	19	11	0-20	71
□-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
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

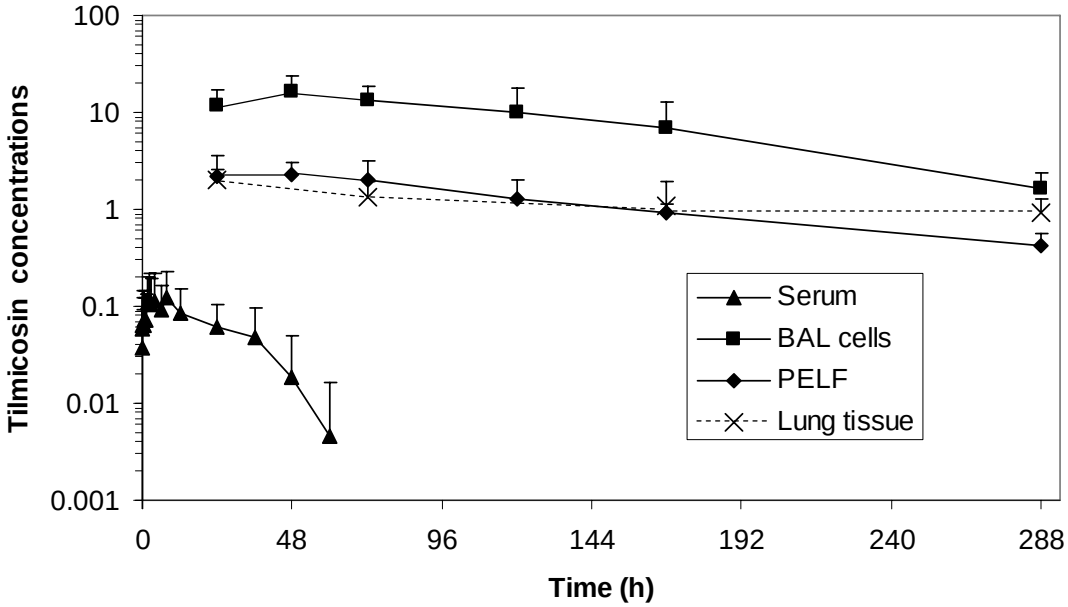

Figure 4.1 – Mean ± SD tilmicosin concentrations in serum, BAL cells, PELF (μg/mL), and lung tissue (μg/g) of 7 foals following a single IM dose of tilmicosin (10 mg/kg of body weight).

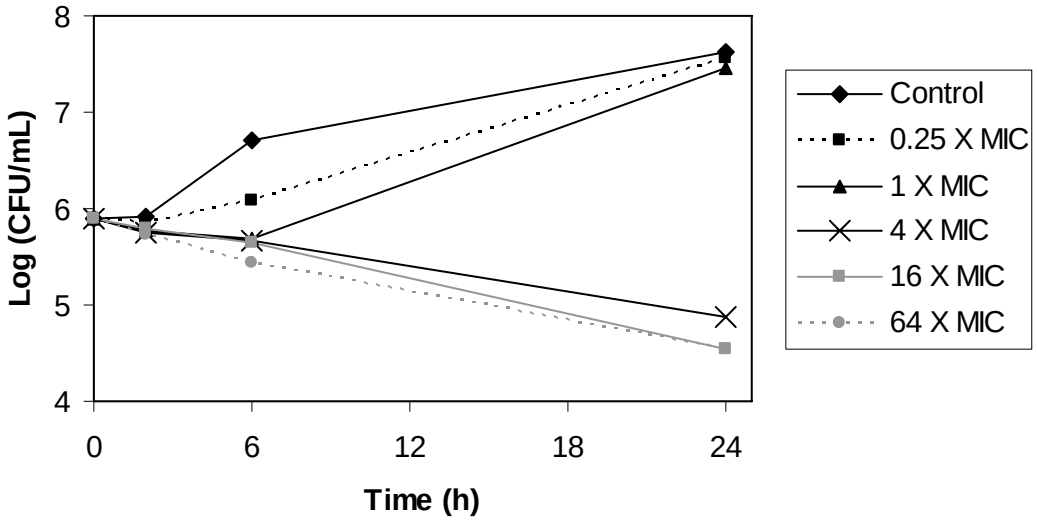

Figure 4.2 – Effect of time and tilmicosin concentration on *in vitro* survival of a clinical isolate of *R. equi*. Identical results were obtained with 2 additional isolates.

CHAPTER 5
SUMMARY AND CONCLUSIONS

The present study investigated the pharmacokinetics and pulmonary disposition of clarithromycin and tilmicosin in foals. The optimal dosing of antimicrobial agents is dependent not only on their pharmacokinetics, but also on the pharmacodynamics of the drug. The pharmacodynamic properties of a drug address the relationship between drug concentration and antimicrobial activity. Much confusion exists over the pharmacodynamics of macrolides. An important factor in determining the efficacy of many macrolides in animal models of infection with extracellular bacteria is the length of time that serum concentrations exceed the MIC of a given pathogen (T > MIC) (Rodvold, 1999). However, 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* and that concentrations at the site of infection are more important in predicting efficacy (Drusano, 2005). Macrolides enter phagocytic cells by passive diffusion and they accumulate in acidic intracellular compartments such as lysosomes and phagosomes. 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 (Mandell et al., 2001).

To provide a better assessment of the potential usefulness of clarithromycin and tilmicosin for the treatment of bronchopneumonia in foals, we measured drug concentrations in PELF, BAL cells, and lung tissue. Oral clarithromycin was wellabsorbed in foals and resulted in mean PELF concentrations approximately 95 times

higher and mean BAL cell concentrations approximately 335 times higher than concurrent serum concentrations. Clarithromycin undergoes extensive metabolism in people. Of the 8 metabolites that have been identified, 14-hydroxy-clarithromycin is the most abundant and the only one with substantial antimicrobial activity (Fernandes *et al*., 1988; Ferrero *et al*., 1990). Although clarithromycin is not converted to14-hydroxy clarithromycin in rodents and reptiles, the present study confirmed production of 14-hydroxy-clarithromycin in foals.

A new fatty acid salt formulation of tilmicosin, developed as a safer and convenient formulation for use in cats (Kordick *et al.*, 2003), was investigated in the present study. 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 because the drug concentrates in lung tissue and phagocytic cells (Musser *et al.*, 1996; Paradis *et al.*, 2004). Tilmicosin accumulated in the lungs of foals. BAL cells achieved the highest concentrations with C_{max} approximately 100 times higher than that achieved in plasma. Tilmicosin concentrations in PELF were almost identical to that of lung concentrations, indicating that measurement of drugs in PELF may represent a less invasive alternative to lung biopsies. Lung tissue and PELF tilmicosin concentrations were approximately 10-15 times higher than peak serum concentrations. However, tilmicosin concentrations at all of the times sampled remained considerably below the MIC_{90} of *R. equi*. Therefore, tilmicosin, at the dose used in the present study, would not be adequate for the treatment of *R. equi* infections in foals.

In conclusion, oral administration of clarithromycin at a dosage of 7.5 mg/kg every 12 hours would maintain serum, PELF, and BAL cell concentrations above the minimum inhibitory concentration for *R. equi* and *S. zooepidemicus* isolates for the entire dosing interval. The formulation of tilmicosin investigated in the present study resulted in high and sustained concentrations in the lung, PELF, and BAL cells of foals and may be appropriate for the treatment of susceptible bacterial infections. Additional studies will be required to establish the safety and determine the efficacy of these drugs in a clinical setting.

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BIOGRAPHICAL SKETCH

Ariel Womble was born in Charlotte, N.C., to David Womble and Connie Harris. She moved to Palm Harbor, Florida when she was eight years old where she lived and attended high school. While in high school she earned a scholarship to attend the University of Florida where she earned a Bachelor of Science in animal science. During her years as an undergraduate she worked part-time at the Veterinary Teaching Hospital where she met Dr. Steeve Giguère. Through this meeting she became interested in research and pursuing a Master of Science degree in veterinary medical science. After graduation she began work in graduate studies.