PULMONARY DISPOSITION AND PHARMACOKINETICS OF MINOCYCLINE IN THE
ADULT HORSE

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THESIS
Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS - Veterinary Clinical Medicine
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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ABSTRACT

The purpose of this study was to determine the pharmacokinetics and pulmonary disposition of minocycline in horses after a single intravenous (IV) and intragastric (IG) dose and after multiple IG doses. The study hypotheses were that: minocycline would be present in the pulmonary epithelial lining fluid (PELF) and bronchoalveolar lavage (BAL) cells at concentrations exceeding those in plasma within 3 hours of IV or IG administration and achievable trough concentrations in the PELF and BAL cells after administration of IG minocycline would exceed a target concentration of 0.25 µg/mL.

Seven healthy adult horses from the resident teaching herd were used for the two part study. For part one of the study, 6 horses received IV (2.2 mg/kg) or IG (4 mg/kg) minocycline in a randomized cross-over design. Plasma samples were collected prior to minocycline administration and 16 times within 36 hours. Bronchoalveolar lavages were performed 4 times within 24 hours for collection of PELF and BAL cells. For part two of the study, minocycline (4 mg/kg) was administered IG every 12 hours for 5 doses to 6 horses. Plasma samples were collected before minocycline administration and 20 times within 96 hours. Bronchoalveolar lavages were performed 6 times within 72 hours for collection of PELF samples and BAL cells.

In study 1, mean bioavailability of minocycline was calculated at 48% (range 35-75%). In study 2, at steady state, mean ± SD maximum concentration (C_{max}) of minocycline in the plasma was 2.3 ± 1.3 µg/mL and the terminal half-life was 11.8 ± 0.5 hours. The median (25th and 75th percentiles) time to peak concentration (T_{max}) was 1.3 (1.0 – 1.5) hours. The C_{max} and T_{max} of minocycline in the PELF were 10.5 ± 12.8 µg/mL and 9.0 (5.5 – 12.0) hours, respectively. The C_{max} and T_{max} for BAL cells were 0.24 ± 0.1 µg/mL and 6.0 (0.0 – 6.0) hours, respectively.
Oral bioavailability (48%) varied considerably among adult horses (35 – 75%). While minocycline was detected in the PELF and BAL cells within 3 hours of IV or IG drug administration, only concentrations in the PELF exceeded those in plasma. As predicted, PELF trough concentrations exceeded the target concentration of 0.25 µg/mL at all measured time points. Contrary to the study hypothesis, minocycline BAL cell concentrations at all measured time points were well below concentrations detected in plasma and PELF and the 0.25 µg/mL target concentration. It was concluded that minocycline distributes into the PELF and BAL cells of adult horses.
ACKNOWLEDGEMENTS

I thank my graduate advisor, Dr. Kara Lascola, who organized the project and mentored me through the whole process. I truly appreciate her attention to detail and guidance step-by-step throughout the whole project from collecting the samples to writing the manuscript for publication. I would also like to thank my graduate committee members, Dr. Jonathan Foreman and Dr. Scott Austin, for their guidance during this study and assistance in writing the manuscript. Dr. Steeve Giguère was very helpful to this study in providing the pharmacokinetic and statistical analysis as well as assisting with any issues that came up and I am very appreciative of his contribution to this study. I am very grateful to Dr. Nidia Maradiage for her assistance in processing the bronchoalveolar lavage samples and being present at every bronchoalveolar lavage we performed. I would also like to thank the veterinary students who assisted with the project: Brooke Murphy Smith, Ben Rivard, Nicole Wettstein, Sadie Somers and Cassie Saufley.

This project was generously funded by the Illinois Equine Industry Research and Promotion Board, University of Illinois Companion Animal Memorial Fund, and the American Quarter Horse Association.
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INTRODUCTION

Oral antimicrobials are often the mainstay for the treatment of pneumonia in adult horses due to their relative ease of administration and reduced financial burden to the owner. Broad-spectrum antimicrobials available for oral administration to adult horses are limited to a few medications. Doxycycline, while demonstrating good distribution to the lung and a broad-spectrum of activity against many common bacterial respiratory pathogens including *Streptococcus* species (Bryant et al., 2000), underwent a pharmaceutical market shortage resulting in a marked increase in pricing. Doxycycline experienced a 2000% increase in average retail price between 2012 and 2013 (AARP, 2016). Although the cost and availability of doxycycline has improved recently, there remains a clinical need for additional oral antimicrobial treatment options in adult horses.

Minocycline, a tetracycline derivative, is a time-dependent bacteriostatic antimicrobial and as such its efficacy is influenced by the duration of time the antimicrobial concentration remains above the minimum inhibitory concentration (MIC). Minocycline is available off-label for oral administration to horses (Schnabel et al., 2012), and represents an attractive addition to the available antimicrobial options due to its broad-spectrum of antimicrobial activity and excellent tissue penetration (Agwuh and MacGowan, 2006; Bishburg and Bishburg, 2009), relatively low cost, and potential anti-inflammatory properties (Sapadin and Fleischmajer, 2006). Unfortunately, pharmacokinetic data on minocycline’s oral bioavailability and disposition into the lung is limited for adult horses. This information is important to more accurately predict the efficacy of minocycline in treating bacterial pneumonia in the adult horse.
CHAPTER 1: REVIEW OF THE LITERATURE

Bacterial pneumonia in adult horses

Respiratory disease in the adult horse can cause a significant financial loss to owners due to treatment costs and lost time when the horse is out of training and/or competition as well as possible loss due to death of some horses or diminished performance of others following recovery (Giguère, 2015). According to veterinarians nationwide, infectious respiratory tract diseases in horses have been identified as one of the most common medical entities (Pusterla, Watson and Wilson, 2006). Respiratory infections are the second most common reason for antimicrobial drug use in adult horses (Traub-Dargatz and Dargatz, 2009). A common cause of respiratory disease in the adult horse is bacterial pneumonia. Bacterial pneumonia in adult horses commonly occurs when bacterial pathogens from the nasal passages or oropharynx reach the lower airways and overwhelm the pulmonary defense system (Reuss and Giguère, 2015). The severity of bacterial pneumonia in adult horses can vary from mild-to-severe disease.

Although many bacterial pathogens can be associated with pneumonia in the adult horse, *Streptococcus equi* subspecies *zooepidemicus* is the most common bacterial pathogen recovered from transtracheal washes (Reuss and Giguère, 2015; Foreman, Hungerford and Folz, 1992; Sweeney et al., 1991). *Streptococcus equi* subspecies *zooepidemicus* is a commensal organism of the horse and is an opportunistic pathogen, which can cause respiratory compromise after viral pneumonia or other stressors such as transportation (McClure, Koenig and Hawkins, 2015; Oikawa et al., 1994; Foreman, Hungerford and Folz, 1992). A study investigating bacterial isolates from equine infections, not just respiratory infections, found that *Streptococcus equi* subspecies *zooepidemicus* was the most common isolate from all submission sites (accounting for 22% of all isolates) and was the most common bacterial isolate from the respiratory tract.
(Clark et al., 2008). As a commensal organism, *Streptococcus equi* subspecies *zooepidemicus* infections can occur whenever the host’s defense systems are compromised. Transportation is a known risk factor for causing pneumonia in adult horses. Bacterial contamination of the lower respiratory tract occurs as a routine consequence of transportation and is likely an important determinant in the development of transport-associated respiratory disease (Raidal, Bailey and Love, 1997). A study investigating the contamination of the lower respiratory tract and peripheral blood neutrophil function, found a peripheral blood neutrophilia and a reduction in neutrophil phagocytic function for at least 36 hours after transportation (Raidal, Bailey and Love, 1997). In a study using the long distance transport model of equine respiratory disease, it was found that in horses with positive culture results, 94.5% were identified as *Streptococcus equi* subspecies *zooepidemicus* (Davis et al., 2006). Adult horses commonly acquire bacterial pneumonia by aspiration of normal microorganisms of the upper respiratory tract (Giguère, 2015). Aspiration can occur secondary to esophageal obstruction or dysphagia in adult horses. Stress of transport also can affect the immune system and pulmonary defense system in the horse, which can result in the inability to effectively clear bacterial organisms especially *Streptococcus equi* subspecies *zooepidemicus* from the lower airway (Raidal, Bailey and Love, 1997; Oikawa et al., 1995).

Mixed bacterial infections can occur and may include both Gram positive and Gram negative bacteria as well as aerobic and anaerobic bacteria. In one study, the most common anaerobes isolated were *Bacteroides oralis* and *Bacteroides melaninogenicus* and horses with anaerobic infections had a lower survival rate than horses with aerobic infections (Sweeney, Divers and Benson, 1985).
Pneumonia and lung abscesses may result in structural changes in the lung parenchyma and potentially affect future athletic performance, but research suggests that horses that recover without complications have a favorable prognosis for return to athletic performance (Lavoie, Fiset and Laverty, 1994; Ainsworth et al., 2000; Seltzer and Byars, 1996). A study followed four adult horses that had lung abscesses and all returned to racing careers suggesting adult horses may perform successfully after appropriate treatment (Lavoie, Fiset and Laverty, 1994). A study found that based upon racing performance in horses that resumed racing after appropriate treatment for pulmonary abscesses, the horses did not develop long-term residual lung damage and racing performance for horses that raced prior to the illness was not significantly different after the illness (Ainsworth et al., 2000). Another study reviewing pleuropneumonia in Thoroughbred racehorses found that the prognosis for return to racing after recovery from uncomplicated pleuropneumonia appeared to be good (Seltzer and Byars, 1996). These studies suggest that appropriate and timely treatment of pneumonia in the adult horse is important and that uncomplicated cases of pneumonia in the adult horse have a favorable prognosis of return to an athletic career.

Pathophysiology of pneumonia

Pneumonia is an infectious process that can occur when the normal respiratory defense mechanisms are impaired which allows invasion and overgrowth of bacteria in the lung parenchyma and leads to intra-alveolar exudate (Alcon, Fàbregas and Torres, 2005). Many of the bacteria that cause pneumonia in the horse are commensal microorganisms of the upper respiratory tract and once these commensal microorganisms gain access to the lung parenchyma can cause inflammation and infection. The development of pneumonia requires that the pathogen reach the alveoli by overwhelming the defense system either by virulence or
overpopulation of the pathogen (Alcon, Fàbregas and Torres, 2005). Several factors can impede the normal respiratory defense mechanisms including underlying diseases, typically viral infections, use of sedatives, tracheal intubation, or antibiotics, all of which may alter the normal bacterial flora of the upper respiratory tract and lead to contamination of the lung parenchyma with bacterial pathogens (Alcon, Fàbregas and Torres, 2005). Pleuropneumonia occurs when the infection from the pulmonary parenchyma extends to the visceral pleural and pleural space (Giguère, 2015). The parietal pleural is highly innervated, and horses with pleuropneumonia often show signs of pleurodynia (pleural pain) (Giguère, 2015). Pleuropneumonia has similar etiologies compared to bronchopneumonia, and long distance transport appears to be a major inciting cause (Giguère, 2015; Austin, Forman and Hungerford, 1995) Pneumonia causes a local inflammatory response in the lung and either the immune system resolves the inflammation or it leads to lung injury and bacteremia. Figure 1 illustrates the systemic inflammatory response and the factors that modulate the response to pneumonia. Microbial, microenvironmental and host factors are all involved in the extent and severity of the lung injury and bacteremia. The lung injury and bacteremia lead to a systemic response which can lead to either resolution or sepsis. Sepsis is a negative feedback to the local inflammatory response and will continue the negative cascade of events.

The goal of the pulmonary defense system is to maintain the lower respiratory airways free of contaminating microorganisms that may initiate infection (Mason and Nelson, 2005). The status of the host’s immunity and the integrity of local pulmonary defense mechanisms are critical for effective antimicrobial therapy (Pennington, 1981). The pulmonary defense system includes layers of anatomic barriers as well as the respiratory tract immune system that can eliminate microbial pathogens from the respiratory tract before the establishment of infection.
**Figure 1:** Systemic inflammatory response to pneumonia. (From Deng JC and Standiford TJ. The systemic response to lung infection. *Clinics in Chest Medicine.* 2005; 26:1-9).
(Mason and Nelson, 2005). The pulmonary defense mechanisms can be grouped into two categories; physical defense mechanisms and cellular defense mechanisms (Harada and Repine, 1985). The upper airways have many physical defense mechanisms to protect the lower airways from contamination including the nasal turbinates that filter out large particles. The sneeze and cough reflexes help eliminate particles from the upper and lower respiratory tracts. The mucociliary escalator is an important defense mechanism that clears inhaled material. Optimal mucus volume and good ciliary function are necessary to maintain an effective mucociliary escalator (Harada and Repine, 1985). Viral infections and general anesthetics can decrease mucociliary clearance (Harada and Repine, 1985). Equine influenza and EHV-4 infections have demonstrated significant decrease in tracheal clearance rates for up to 30 days post infection (Willoughby et al., 1992).

Despite the physical defense mechanisms, many particulates and soluble material, such as microorganisms, make it down into the lower respiratory tract and are deposited in the alveoli. In response to this, cellular defense mechanisms are employed in an attempt to clear these pathogens. Alveolar macrophages play a central role by participating in phagocytic and immunologic reactions (Harada and Repine, 1985). The lower respiratory tract lining fluid includes many plasma proteins as well as secreted products from epithelial and inflammatory cells that have roles in the innate and adaptive immune systems (Mason and Nelson, 2005). The immune system, especially cytokines, is important for protective innate responses to bacterial pneumonia. Observations made in animal models with pneumonia have indicated that the inflammatory response with bacterial pneumonia is mostly compartmentalized to the lung (Deng and Standiford, 2005).
Pulmonary surfactant, which lines the alveolar surface, is composed of a complex of lipids and proteins (Chroneos, Sever-Chroneos and Shephard, 2010; Brogden, 1991). Pulmonary surfactant is important in respiratory function by decreasing surface tension at the air-liquid interface which prevents alveolar collapse at the end of expiration. It is an integral component of the respiratory system’s innate immune system assisting in controlling inflammation and preventing bacterial pathogen infections in the distal lung (Chroneos, Sever-Chroneos and Shepard, 2010). During pneumonia, bacteria cause changes in pulmonary surfactant and these changes are mediated by bacteria acting directly on secreted surfactant or indirectly through pulmonary type II epithelial cells that produce surfactant (Brogden, 1991). Immunoglobulins, specifically IgA and IgG, are important to the pulmonary defense system with IgA as the most predominant immunoglobulin in upper respiratory secretions and IgG as the most predominant immunoglobulin in lower respiratory secretions (Mason and Nelson, 2004).

Transportation and stress have been indicated as major causes of respiratory disease in the horse. Horses that are allowed to lower their heads after transportation have been shown to rapidly clear increased inflammatory airway secretions and increased numbers of bacteria (Raidal, Bailey and Love, 1997). Increases in cortisol concentrations, neutrophil counts and white blood cell counts were found in horses transported long distances compared to stabled horses. These results provide insights into the immunological mechanisms associated with long-term transport (Stull et al., 2004).

**Antimicrobial therapy**

Culture and sensitivity are important to diagnosing and treating bacterial pneumonia in the adult horse. Broad-spectrum antimicrobials are often initiated while culture and sensitivity results are pending because of the potential of polymicrobial infections (Ruess and Giguère,
2015). For suspected pneumonia cases, transtracheal washes are the preferred method for obtaining a sample for culture (Pusterla, Watson and Wilson, 2006; Reuss and Giguère, 2015). Organisms cultured from a transtracheal wash represent bacteria found in the distal trachea and lower airways (Pusterla, Watson and Wilson, 2006). Antimicrobial treatment for bacterial pneumonia, particularly when culture and sensitivity are unavailable should allow for broad-spectrum coverage as well as good sensitivity towards common pathogens, such as *Streptococcus equi* subspecies *zooepidemicus* at the site of infection (Reuss and Giguère, 2015; Sweeney et al, 1991).

With culture and sensitivity, only *in vitro* efficacy is evaluated. Bacterial susceptibility *in vitro* can be performed by disk diffusion, concentration-gradient agar diffusion, or broth dilution. Disk diffusion provides qualitative susceptibility data, whereas broth dilution and concentration/gradient tests express a quantitative minimum inhibitory concentration (MIC) (Giguère and Tessman, 2011). The MIC is the lowest concentration of an antimicrobial agent which inhibits the growth of the targeted bacteria (Giguère and Tessman, 2011). Susceptibility designations are established by comparing the antimicrobial’s MIC to clinical breakpoints instituted by the Clinical Laboratory Standards Institute. Breakpoints are the concentration above and below which bacterial isolates are categorized as susceptible, intermediate, or resistant (Giguère and Tessman, 2011). *In vitro* susceptibility of a specific pathogen does not guarantee clinical outcome because host factors such as age, immune status, site of the infection, distribution of the drug to the site of infection, severity of the infection and presence of mixed infection all contribute to the clinical response (Giguère and Tessman, 2011).

Antimicrobial efficacy *in vivo* is affected by how well the medication is transported and diffused into the area of infection. The activity of many antimicrobials is impaired by
unfavorable conditions that prevail at sites of infection (Rubinstein et al., 2000). These conditions include pH, anaerobiasis, the presence of purulent material and dead bacteria (Rubinstein et al, 2000; König, Simmen and Blaser, 1998). Many factors influence antimicrobial penetration into bronchial secretions, including physiochemical characteristics of the antimicrobial as well as host factors like bronchial inflammation and injury (Pennington, 1981). In bronchopneumonia, the increased local inflammation may enhance permeability to antimicrobial molecules (Pennington, 1981).

In the horse, antimicrobials are available for administration by many different routes including parenteral, oral, and aerosolized. Parenteral administration includes intravenous or intramuscular injections. Oral medications are administered by dosing syringe, nasogastric intubation, or as a top dressing on feed. Aerosolized antimicrobials are often used as adjunctive therapy along with systemic (parenteral or oral) antimicrobials. Aerosolized medications are most often administered via a nebulizer. There are many options for equine nebulizers and inhalers on the market. Aerosolized antimicrobials may achieve higher drug concentrations in the bronchial secretions and pulmonary epithelial lining fluid (PELF) than systemically administered antimicrobials (Reuss and Giguère, 2015; McKenzie and Murray, 2000). A study with gentamicin compared concentrations in the serum and bronchoalveolar lavage (BAL) fluid after intravenous and aerosol administration found that the gentamicin concentration in BAL fluid of healthy horses was significantly greater after aerosol administration versus intravenous administration (McKenzie and Murray, 2000). A major limitation of aerosolized gentamicin in the adult horse with bacterial pneumonia is the lack of activity gentamicin has against Streptococcus equi subspecies zooepidemicus (Giguère, 2015). Aerosol therapy is an effective method of drug delivery to the respiratory tract (Duvivier et al., 1997). However, some potential
limitations to aerosol therapy include impaired delivery of the antimicrobial to poorly ventilated areas of the lungs, time-consuming administration, pulmonary tissue irritation, and contamination of the antimicrobial solution with bacteria (McKenzie and Murray, 2000). Antimicrobials commonly used for aerosol therapy are cephalosporins, penicillins, and aminoglycosides (Duvivier et al., 1997). For severe pneumonia, especially in the early stages, intravenous antimicrobials are preferred because they can achieve higher plasma concentrations more quickly (Reuss and Giguère, 2015).

Many horses with mild-to-moderate pneumonia are treated at the home stables rather than in a referral hospital setting. Under these circumstances oral antimicrobials are favored because of the ease of administration, increased owner compliance, and the possibility for long-term treatment protocols (Winther, et al., 2010). Bioavailability in orally-administered antimicrobials varies considerably with the drug and the fed or fasted state of the horse. Food exerts complicated effects on the pharmacokinetics of a drug (Gu et al., 2007). Decreased oral bioavailability in fed versus fasted horses has been reported for several drugs (Bouckaert et al., 1994; Van Duijikere et al., 1995; Syker et al., 2015; Davis, Salmon and Papich, 2006) and may result from delayed gastric emptying or from a physical barrier to absorption with high roughage diets (Toothaker et al., 1980). In humans, food is not reported to effect tetracycline bioavailability (Agwuh and MacGovern, 2006; Bishburg and Bishburg, 2009). The tetracyclines are strong chelating agents (Chopra and Roberts, 2001). Chelation activity inhibits the biological role of metal-dependent proteins, leading to disturbances in microbial cell homeostasis and resulting in blockage of microbial nutrition, growth, and development (Santos et al., 2012). In adult horses, maximum concentration \( C_{\text{max}} \) decreased when doxycycline was administered as a top-dressing to grain (Davis, Salmon and Papich 2006) and feeding in dogs decreased \( C_{\text{max}} \) for
oral minocycline (Hnot et al., 2015). In horses provided *ab libitum* hay, the addition of grain did not result in decreased plasma minocycline concentrations (Schnabel et al., 2012). Cost, horse temperament, and owner/caretaker ability often dictate the antimicrobial and route of administration in adult horses with bacterial pneumonia.

Regarding oral antimicrobials there are only a few options for horses with bacterial pneumonia. Multiple antimicrobials may be administered especially in suspected polymicrobial or severe pulmonary infections. Trimethoprim-sulfonamide, a potentiated sulfonamide, is very affordable in the adult horse and is administered orally twice a day. Trimethoprim-sulfonamide includes two separate antimicrobials: Trimethoprim and sulfamethoxazole or sulfadiazine. When combined, these antimicrobials function synergistically and result in bactericidal activity (McClure, Koenig and Hawkins, 2015). Sulfonamides inhibit folate synthesis by inhibiting synthesis of bacterial DNA and trimethoprim inhibits folate synthesis by binding to dihydrofolate reductase and inhibiting the reduction of dihydrofolic acid to tetrahydrofolic acid (McClure, Koenig and Hawkins, 2015). However, trimethoprim-sulfonamide is generally not effective *in vivo* against *Streptococcus equi* subspecies *zooepidemicus*, the most common cause of bacterial pneumonia in the adult horse (Reuss and Giguère, 2015). Furthermore, in horses, potentiated sulfonamides do not achieve equal concentrations of both drugs in the lung (Winther et al., 2010) and their clinical utility for treating lower respiratory infections is limited due to their poor *in vivo* activity against *Streptococcus equi* subspecies *zooepidemicus* (Winther et al., 2010; Ensink, Smit and van Duijkeren, 2003; Ensink, Bosch and van Duijkeren, 2005; McClure, Koening and Hawkins, 2015).

Chloramphenicol is a broad-spectrum antimicrobial with good activity against aerobic and anaerobic bacteria as well as both Gram negative and Gram positive organisms (Kasten,
The mechanism of action for chloramphenicol is by inhibiting protein synthesis by binding to the 50S subunit of the 70S ribosome and preventing the bonding between amino acids and peptides (Smilack, Wilson and Cockerill, 1991; Kasten, 1999). Chloramphenicol is generally bacteriostatic, but has demonstrated bactericidal activity against meningitis-causing organisms (Smilack, Wilson and Cockerill, 1991; Kasten, 1999; Feder, Osier and Maderazo, 1981). Chloramphenicol distributes well in body fluids and reaches high concentrations in the brain and cerebral spinal fluid (Kasten, 1999; Smilack, Wilson and Cockerill, 1991). In humans, concerns of toxicity leading to reversible bone marrow depression and ‘grey syndrome’ in infants has decreased the popularity of the drug (Smilack, Wilson and Cockerill, 1991; Kasten, 1999; Feder, Osier and Maderazo, 1981). In the adult horse, chloramphenicol is expensive, is administered four times per day, and can be associated with human health risks (Giguère, 2015; Kasten, 1999). The human health risk for owners handling the drug is uncommon but can result in irreversible aplastic anemia in certain people that come into contact with the antimicrobial (Feder, Osier and Maderazo, 1981). Owners must be warned of the risk and advised to wear gloves when handling the medication.

Enrofloxacin, a fluoroquinolone, can be administered intravenously as well as orally to adult horses. Enrofloxacin has bactericidal activity and primarily targets Gram negative bacteria (Davis et al., 2006). The mechanism of action of enrofloxacin involves inhibition of bacterial DNA gyrase activity and interference with bacterial DNA packing, replication, and transcription (Magdesiam KG, 2015). In the horse, enrofloxacin has demonstrated good activity against Enterobacteriaceae, reliable penetration in tissue and phagocytic cells as well as respectable activity in purulent material (Reuss & Giguère, 2015). Enrofloxacin has poor activity against Streptococcus species as well as anaerobes and therefore its use as sole therapy is not
recommended in horses with bacterial pneumonia without documentation of the culture and sensitivity of the causative agent (Reuss and Giguère, 2015). A study that looked at the use of enrofloxacin in a long-distance transport model of equine respiratory disease concluded that solo therapy with enrofloxacin may not be appropriate for the treatment of Gram positive pathogens, specifically *Streptococcus equi* subspecies *zooepidemicus*, in equine respiratory disease (Davis et al., 2006). Enrofloxacin is administered once daily and is moderately expensive to treat an adult horse.

Metronidazole is a synthetic drug and is effective against anaerobic organisms as well as certain protozoa and parasites (Kasten, 1999; Smilack, Wilson and Cockerill, 1991). Metronidazole is one of the most useful antimicrobials for the treatment of serious anaerobic and polymicrobial infections (Kasten, 1999). The mechanism of action is damage to the bacterial DNA which results in cell death (Kasten, 1999; Smilack, Wilson and Cockerill, 1991). Metronidazole can be administered both orally and rectally to the horse. Clinical failures with metronidazole can occur when it is used alone to treat anaerobic pleuropulmonary infections probably because of the polymicrobial nature of these infections (Kasten, 1999).

Doxycycline, a second-generation tetracycline, has been used as an oral antimicrobial to treat bacterial pneumonia in the horse. Doxycycline is not administered intravenously in horses as it can cause collapse and sudden death, apparently secondary to cardiovascular effects (Riond et al., 1992). A study found that doxycycline at a dosage of 20 mg/kg every 24 hours orally would result in drug concentrations adequate for killing intracellular bacteria and bacteria with an MIC≤0.25 µg/mL (Davis, Salmon and Papich, 2006). Winter et al. (2010) examined the disposition of doxycycline in the PELF of horses and demonstrated that clinically relevant doxycycline concentrations were possible to achieve in PELF after intragastric administration.
Doxycycline administered intragastrically over multiple days to foals at a dose of 10 mg/kg every 12 hours corresponded to serum, PELF and BAL cell concentrations that were maintained above MIC for most bacterial pathogens commonly isolated from the lower respiratory tract of foals with pneumonia (Womble, Giguère and Lee, 2007). Under current recommendations, doxycycline is administered orally twice daily. In the past this drug was very affordable, but due to a pharmaceutical shortage, price increases over the past few years have made treating an adult horse with doxycycline prohibitively expensive.

Minocycline, also a second-generation tetracycline, has become more popular in veterinary medicine, especially with the increase in cost of doxycycline. Few studies have been performed in horses with minocycline and even fewer have examined the pulmonary pharmacokinetics. Adult horses achieved minocycline concentrations in synovial and cerebrospinal fluid that were adequate to treat infections caused by susceptible (MIC<0.25ug/ml) bacterial organisms after intragastric administration of 5 doses of minocycline (4 mg/kg) (Schnabel et al., 2012). In a separate study, the administration of a single intravenous dose of minocycline (2.2 mg/kg) resulted in minocycline concentrations in lung tissue homogenates that exceeded plasma concentrations at two hours post drug administration (Nagata et al., 2010). A recent publication demonstrated that in healthy 6 to 9 week-old foals, minocycline concentrations in PELF were significantly higher when compared to serum or synovial fluid concentrations (Giguère et al., 2016). The same study also calculated a bioavailability of 57% in foals and 32% in adult horses (Giguère et al., 2016).

**Tetracyclines**

Tetracyclines are broad-spectrum antimicrobials with activity against both Gram positive and Gram negative bacteria as well as intracellular chlamydiae, mycoplasmas and rickettsiae.
organisms (Roberts, 2003; Jonas and Cunha, 1982). Tetracyclines have been used in human medicine since the 1950s for treatment of a wide variety of bacterial infections. For humans, tetracyclines have been prescribed for prophylaxis and treatment of community-acquired infections especially respiratory infections (Roberts, 2003).

The mode of action of tetracyclines is through reversibly inhibiting bacterial protein synthesis by binding the ribosomal complex which prevents the association of aminoacyl-tRNA and messenger RNA within the bacterial ribosome (Roberts, 2003; Chopra and Roberts, 2001; Carris et al., 2015). The tetracycline molecules bind reversibly with the prokaryotic 30S of the bacterial ribosomal subunit, inhibiting protein synthesis (Roberts, 2003; Carris et al., 2015).

Tetracyclines also have non-antimicrobial effects, which are beneficial in disease states including anti-inflammatory properties, immunosuppression, inhibition of lipase and collagenase activity, and increased wound healing (Roberts, 2003). Tetracyclines reduce the concentration of a large variety of inflammatory mediators such as tumor necrosis factor, interleukin-1, matrix metalloproteinases (MMP), and reactive oxygen intermediates (Nieman and Zerler, 2001). One of the best characterized non-antimicrobial properties of the tetracyclines is their ability to inhibit members of the MMP family of endopeptidases. The MMPs are zinc-dependent proteases that are involved in many pathophysiological processes including inflammation and tissue invasion (Nieman and Zerler, 2001; Griffin et al., 2010). Horses with recurrent airway obstruction (RAO) have increased concentrations of MMP-8 and MMP-13 found in tracheal epithelial fluid compared to healthy horses (Raulo, Sorsa and Maisi, 2006). An in vitro study with RAO horses found decreased concentrations of MMP-8 and MMP-13 after administration of chemically modified tetracyclines (Raulo, Sorsa and Maisi, 2006). Another non-antimicrobial property of tetracyclines is their ability to scavenge reactive oxygen species (ROS) (Griffin et al, 2010;
Nieman and Zerler, 2001). Inflammatory processes produce excess ROS which can lead to oxidative destruction to cells and tissues (Griffin et al., 2010).

Minocycline is a second-generation tetracycline derivative and first appeared in scientific research in 1967 (Jonas and Cunha, 1982). Minocycline has the same basic four-ring structures as the other tetracyclines, but a substitution of a dimethyl amino group at C7 and the removal of a functional group at C6 allows for the unique chemical characteristics of minocycline (Jonas and Cunha, 1982). Compared to other tetracyclines, minocycline has superior lipophilic properties. These lipophilic properties are therapeutically significant as they result in increased penetration of tissues and enhanced gastrointestinal absorption (Jonas and Cunha, 1982). Minocycline also exerts anti-inflammatory properties that are completely separate from its antimicrobial actions (Yrânheikki et al., 1999). It has been reported in animals that the beneficial effect of minocycline is associated with reduction of COX-2 expression and prostaglandin production (Yrânheikki et al., 1999). Compared to doxycycline, minocycline has lower protein binding. Minocycline has reportedly had protein binding ranging from 68.1 ± 2.6% (Nagata et al., 2010) and 75-85% (Jonas & Cunha, 1982). Doxycycline was found to be less highly protein bound in horses than other species, with a mean percentage of 81.76 ± 2.43% (free drug 18.24%) (Davis, Salmon and Papich, 2006).

Pharmacokinetic principles

Pharmacokinetic/pharmacodynamic (PK/PD) studies currently play a crucial role in drug development, drug evaluation, and in establishing effective drug dosage regimens (Riviere, 2011; Derendorf and Meibohm, 1999). Pharmacokinetic/pharmacodynamic modeling links dose-concentration relationships (PK) and concentration-effect relationships (PD), which facilitate the description and prediction of the time course of the drug effects resulting from a certain dosing
Presently, the most commonly used PK/PD approaches for antimicrobial drugs depend on plasma concentrations as the PK input and MIC as the PD input (Giguère and Tessman, 2011). Minimum inhibitory concentration (MIC) is the lowest drug concentration that inhibits bacterial growth and minimum bactericidal concentration (MBC) is the lowest drug concentration that kills 99.9% of bacteria (Dowling and Davis, 2015). Minimum inhibitory concentrations are used to determine the antimicrobial dosage with the goal of achieving plasma and tissue concentrations above the *in vitro* MIC for the bacterial pathogen (Dowling and Davis, 2015).

Antimicrobials are commonly classified as bactericidal or bacteriostatic based on the MBC-to-MIC ratio. If the ratio is small, the antimicrobial is considered bactericidal, and drug concentrations are able to kill 99.9% of the organisms (Dowling and Davis, 2015). If the ratio is large, the antimicrobial is considered bacteriostatic. The distinction between bactericidal and bacteriostatic is not precise and depends on the antimicrobial concentration at the site of infection and the bacterial organisms involved (Dowling and Davis, 2015).

Dosage regimen is also calculated based on the PK and PD relationship between the antimicrobial and the bacterial pathogen. For PK parameters the area under the plasma concentration versus time curve for 24 hours is calculated and maximum plasma concentration ($C_{max}$) and the time the antimicrobial concentration exceeds a define PD threshold is time (T) (Dowling and Davis, 2015). Minimum inhibitory concentration is used commonly as the PD parameter. To relate PK and PD parameters to clinical efficacy, the action of the antimicrobial needs to be defined as concentration or time dependent. Concentration-dependent antimicrobials include aminoglycosides, fluoroquinolones and metronidazole. They exert concentration-dependent killing characteristics where bactericidal activity increases as plasma drug
concentrations increase above the MIC for that pathogen (Giguère and Tessman, 2011). Time-
dependent antimicrobials used in horses include β-lactams, tetracyclines, macrolides, potentiated
sulfonamides, and chloramphenicol. They are effective based on the length of time that the
pathogens are exposed to concentrations of the drugs above the MIC of the pathogen (Giguère
and Tessman, 2011).

Only free tissue concentrations of the antimicrobial at the target site are responsible for
the therapeutic effects (Liu, Muller and Derendorf, 2002). Many antimicrobials properties,
including protein binding, lipid solubility, and volume of distribution, affect how well an
antimicrobial may reach the infected tissue. Plasma-protein binding influences a drug’s ability to
penetrate tissue as only the unbound fraction is available to cross biologic membranes. Thus, the
higher the plasma-protein binding of the antimicrobial, the lower the ability to penetrate into the
tissue (Liu, Muller and Derendorf, 2002). Furthermore, only the free tissue concentration of an
antimicrobial at the site of infection, typically lower than the plasma concentration, is
responsible for the therapeutic effects of the drug (Liu, Muller and Derendorf, 2002). In the lung,
binding to protein within the PELF can further influence the amount of active drug that is
available (Kiem and Schentag, 2008) particularly during inflammatory conditions resulting in
changes in protein within the PELF. Protein concentrations in PELF have been reported to be
much lower than in the plasma in healthy humans and in humans with pulmonary disease
(Rennard et al., 1986). In healthy adult horses, the percentage of protein binding of doxycycline
in the PELF was much lower than protein binding in the plasma (Rodvold, George and Yoo,
2011). Lipid-soluble antimicrobials penetrate non-fenestrated capillary beds better than
antimicrobials that are more water soluble (Levison and Levison, 2007). Examples of lipid-
soluble antimicrobials are metronidazole and rifampin and examples of more water-soluble and
less lipid-soluble antimicrobials are aminoglycosides, glycopeptides, and β-lactams (Levison and Levison, 2009). Non-ionized antimicrobials tend to be more lipid soluble and can more easily diffuse across cell membranes. In contrast, ionized antimicrobials are less able to diffuse across lipid membranes because of low lipid solubility (Buxton and Benet, 2011; Levison and Levison, 2009).

The volume of distribution represents the relationship between the total amount of the antimicrobial in the body and the concentration of the antimicrobial measured in the plasma (Buxton and Benet, 2011). The volume of distribution of an antimicrobial reflects the extent to which the drug distributes from the plasma to the extravascular tissues (Buxton and Benet, 2011). Many host and antimicrobial properties can influence the volume of distribution including plasma and tissue volumes, drug protein binding, accumulation of drug in poorly perfused tissue as well as age, body mass, and disease state (Buxton and Benet, 2011).

Pharmacokinetic models are used to predict a drug’s behavior in the body. In order to apply mathematical principles, a model of the body must be selected (Spruill et al., 2014). The models are used to predict the time course of drug concentration throughout the body (Spruill et al., 2014). Pharmacokinetic data analysis utilizes either a model fitting using non-linear regression analysis or non-compartmental analysis (Reisfeld and Mayeno, 2012). Compartmental models are often used for describing concentration-time curves of drug plasma concentrations following drug administration. The compartmental model describes how long the drug remains available in the body and is used as a guide for determining dosage regimens, delivery of the drug, and expectations of its effect (Reisfeld and Mayeno, 2012). Non-compartmental modeling requires fewer assumptions than model-based approaches and describes the drugs clearance, elimination half-life, and $C_{\text{max}}$ (Reisfeld and Mayeno, 2012).
**Pulmonary pharmacokinetics**

Designing an optimal dosage regimen for the treatment of lower airway infections in the horse requires knowledge on the distribution of the antimicrobial in the equine lung. Several studies in the horse and other species have demonstrated that the pharmacokinetics of antimicrobial drugs in the lung cannot be predicted by the plasma concentrations (Winther, 2012; Giguère and Tessman, 2011; Kiem and Schentag, 2008). Most dosage regimens for equine antimicrobials have been established from pharmacokinetic studies, based on plasma concentrations over time and correlated with bacterial MICs (Winther, 2012). Using plasma concentrations frequently overestimates the target site concentrations and clinical efficacy (Liu, Müller and Derendorf, 2002). For pulmonary infections, antimicrobial concentrations in the PELF for extracellular pathogens and in alveolar macrophages cells for intracellular pathogen are thought to reflect antimicrobial activity in pneumonia (Kiem and Schentag, 2007). Figure 2 illustrates the blood-alveolar barrier which is composed of the capillary wall and alveolar wall, both of which are separated by the fluid filled interstitial space (Kiem and Schentag, 2007). The antimicrobial concentration measured in the PELF represents the proportion which diffuses readily across the alveolar capillary wall, the interstitial fluid, and the alveolar epithelial cells (Kiem and Schentag, 2007; Giguère and Tessman, 2011). There are several factors that affect the entry of an antimicrobial into the PELF including protein binding and lipophilicity. Only the free, unbound portion of the antimicrobial is considered microbiologically active and able to equilibrate between the serum and interstitial fluid, suggesting that highly protein-bound antimicrobials will have a lower concentration in the PELF (Kiem and Schentag, 2007; Giguère and Tessman, 2011). In a recent study, the properties of protein binding and lipophilicity, which are often cited as determinants of drug penetration into the PELF, did not have an influence on
Figure 2: Schematic diagram of the blood-alveolar antibiotic barrier. The blood-alveolar barrier is composed of two membranes, the capillary wall and alveolar wall, which are separated by a fluid-filled interstitial space. Antibiotics need to diffuse across the alveolar capillary wall, the interstitial fluid, and the alveolar epithelial cells to reach ELF. Cells can carry antibiotics to the ELF also. Kiem S, Schentag JJ. Antimicrobial Agents Chemotherapy. 2008; 52:24-36.
the penetration of the antimicrobials administered to the calves (Foster, Martin and Papich, 2016).

With inflammation and infection, the pulmonary membrane thickness may be increased, leading to potential diffusion impairments of antimicrobials diffusing into the PELF from the vascular space. However, in bronchopneumonia increased local inflammation may enhance permeability to antimicrobial molecules (Pennington, 1981). Only the free tissue concentrations of antimicrobials at the target site are responsible for the therapeutic effect (Liu, Müller and Derendorf, 2002). The more lipophilic the antimicrobial, the easier it can diffuse across membranes and enter the PELF (Winther, 2012). Lipophilic antimicrobials like minocycline enter most tissues of the body, penetrate cellular barriers, and then reach the infection foci (Baggot and Prescott, 1987). Because molecular weight for most antimicrobials is low, passive diffusion occurs across the membranes down the concentration gradient into the PELF (Winther, 2012). Passive diffusion is also influenced by the surface area and the membrane thickness.

There are a few different methods to measure antimicrobial concentration in the lung of the horse. Sampling methods for the equine lung include homogenized lung tissue, nasal and bronchial secretions, microdialysis, absorbent disc/cotton swab, and bronchoalveolar lavage. Tissue homogenates can often be misleading for pharmacokinetic purposes because the homogenate represents a mixture of all tissue compartments: intracellular, extracellular, blood, and interstitial drug concentrations (Winther, 2012; Giguère and Tessman, 2011). They also do not provide any information about active drug. The antimicrobial concentration can often be underestimated for drugs that equilibrate completely within the extracellular space and become diluted by cells and blood (Giguère and Tessman, 2011; Winther 2012). The antimicrobial concentration can also be overestimated in drugs that accumulate intracellularly (Giguère and
Tessman, 2011; Winther 2012). The use of antimicrobial concentrations from tissue homogenates to predict efficacy is misleading and is not recommended (Giguère and Tessman, 2011; Winther, 2012).

Nasal and bronchial secretions can be used to measure antimicrobial concentrations in respiratory secretions. However, the secretions obtained, especially nasal secretions, may not accurately represent the target site of the infection especially when it is in the lower respiratory tract (Giguère and Tessman, 2012). In human medicine, antimicrobial delivery to the respiratory tract is usually monitored by collecting bronchial secretions or sputum because it is much less invasive than bronchial biopsies. The bronchial secretions and sputum are thought to provide an approximation of local mucosal concentrations of the antimicrobial (Pennington, 1981). It was found that the correlation between antimicrobial serum concentration and bronchial secretions or sputum was variable (Pennington, 1981). Measurement of antimicrobial concentrations in secretions can have some of the same issues as with tissue homogenates, especially with intracellular antimicrobials and potentially overestimating the antimicrobial concentration (Giguère and Tessman, 2011).

Microdialysis is a more recent technique that allows real time continuous monitoring of unbound or free antimicrobial concentrations directly in the extracellular fluid of the tissue of interest using a minimally invasive dialysis probe (Giguère and Tessman, 2011; Winther, 2012). Advantages are that long-term continuous monitoring can occur in secluded sites such as inside vessel, tissue, and bone, and it causes relatively minor tissue trauma (Winther, 2012). The main disadvantages are the need to perform a thoracotomy to insert the probe. In addition, this technique cannot measure lipophilic drugs or intracellular concentrations of antimicrobials (Giguère and Tessman, 2011). Microdialysis has been performed experimentally on pulmonary
tissue in anaesthetized humans and laboratory animals. In horses, microdialysis has been performed on muscle tissue, placenta, and hoof lamellar tissue but not on pulmonary tissue (Winther, 2012).

Absorbent disc or cotton swabs have been used to sample bronchial secretions and PELF in large animals (Winther, 2012). A guarded swab is passed through the nares or mouth and trachea into the bronchus where the swab is then used to absorb the PELF (Foster, Martin and Papich, 2016). This sampling technique retrieves a very small amount of undiluted PELF secretion for analysis. The very small amount may restrict how much analysis may be performed on one sample. Also, repeated sampling may increase inflammation in the lower airways which may increase inflammatory exudate which may influence an influx of drug into the secretion (Winther, 2012). This technique is a relatively simple procedure to collect undiluted PELF samples, repeatable and does not require specialized equipment or a dilution method calculation (Winther, 2012; Foster, Martin and Papich, 2016).

Bronchoalveolar lavage allows for measurement of antimicrobial concentration in PELF and BAL cells. The antimicrobial measured in the PELF represents the portion of the antimicrobial that diffuses across the blood-alveolar barrier (Giguère and Tessman, 2012). Bronchoalveolar lavages are a standard procedure used in equine medicine for diagnostic procedures mainly to evaluate cytology and microbiology in the equine lower respiratory tract (Winther, 2012). Bronchoalveolar lavages are relatively simple to perform in the standing sedated horse and require little equipment. An endoscope or tubing such as an equine BAL catheter are passed through the nares, down the trachea, past the carina, and wedged in a terminal pulmonary bronchus. Sterile isotonic fluid is infused rapidly into the lung and subsequently aspirated (Pusterla, Watson and Wilson, 2006). Advantages of this technique are that it is a simple and
repeatable procedure and is relatively non-invasive causing little iatrogenic damage (Winther, 2012). The disadvantages are the quantity of PELF aspirated from BAL fluid can be small and variable and an endogenous dilution factor must be used to accurately correct for the amount of dilution in the aspirated BAL fluid (Winther, 2012). The urea dilution method is commonly used to calculate PELF concentrations.

**Urea dilution method**

Urea can be used as an endogenous marker of PELF because it is small in size and relatively non-polar, allowing it to travel freely and easily across membranes (Giguère and Tessman, 2011; Rennard et al., 1985; Mills and Litser, 2005; Kirschvink et al., 2001; van de Graaf et al., 1991; Rennard et al., 1986; McGorum et al., 1993). The assumption is that the urea concentration in the PELF should be equal to the urea concentration in the plasma because of complete diffusion (Giguère and Tessman, 2011; Kirschvink et al., 2001; van de Graaf et al., 1991; Rennard et al., 1986; McGorum et al., 1993). Therefore, the volume of PELF can be adjusted for the dilution with saline by a simple mathematical equation and then the concentration of the drug in the PELF can be calculated.

The concentration of urea in the BAL fluid and plasma can be used to calculate the volume of PELF ($\text{Vol}_{\text{PELF}}$) as follows:

$$\text{Vol}_{\text{PELF}} = \text{Vol}_{\text{BAL}} \times \left( \frac{\text{Urea}_{\text{BAL}}}{\text{Urea}_{\text{Plasma}}} \right)$$

$\text{Vol}_{\text{BAL}}$ represents the volume of BAL measured at the time of collection and $\text{Urea}_{\text{BAL}}$ and $\text{Urea}_{\text{Plasma}}$ represent the concentration of urea in BAL fluid and plasma, respectively.

Once the volume of PELF is known, concentrations of the drug within PELF ($\text{Drug}_{\text{PELF}}$) can be calculated as follows:

$$\text{Drug}_{\text{PELF}} = \text{Drug}_{\text{BAL}} \times \left( \frac{\text{Volume}_{\text{BAL}}}{\text{Volume}_{\text{PELF}}} \right)$$
Drug_{BAL} represents the concentration of drug measured in BAL fluid.

A study estimating the volume of PELF recovered by lavage using urea as a marker of dilution concluded that the volume of recovered PELF could be easily quantified with reasonable accuracy (Rennard et al., 1985). A study performed in cats found the estimation of PELF fraction in lavage fluid using standardized lavage technique and correction for urea dilution resulted in a more accurate measure of cellular and non-cellular components in PELF (Mills and Lister, 2005). Even though Mills and Lister (2005) were not investigating drug concentrations in PELF they concluded that in measuring cellular components in the PELF, the urea dilution method resulted in more accurate measurements. Other studies have compared urea with other markers such as albumin and inulin (McGorum et al., 1993; Kirschvink et al., 2001). McGorum et al. (1993) evaluated urea and albumin as endogenous markers of dilution of equine BAL and concluded that both techniques proved satisfactory and were of equal accuracy. Albumin is a larger molecule and has reduced permeability compared to urea and therefore the urea dilution technique is considered superior for determining the concentration of PELF in BAL samples (McGorum et al., 1993). Inulin is an exogenous marker and has been studied as exogenous markers of BAL fluid dilution. An ideal external marker for BAL fluid dilution should be an inert marker for which the only source of the marker is the instilled lavage fluid. Additionally, the marker should not be metabolized by cells or cross the pulmonary epithelium, and can mix completely with the PELF in the lavaged lung segment (Kirschvink et al., 2001). A study performed in horses determined there was no difference in the inulin or urea method in healthy versus heaves-affected horses and that inulin did not present any major advantages over urea, but the combined use of both inulin and urea improved standardization of studies comparing PELF compounds (Kirschvink et al., 2001).
There is concern that an influx of urea from blood either by increased respiratory membrane permeability or increased dwell time of infused fluid during BAL may lead to overestimation of the amount of PELF in BAL fluid and thus result in an underestimation of the concentration of drugs, proteins and cells in the PELF (van de Graaf et al., 1991; Giguère et al., 2011). A clinical study in humans determined that urea influx during lavage was increased in patients with a high respiratory membrane permeability and this led to a relative underestimation of solutes in PELF (van de Graaf, et al., 1991). Respiratory membrane permeability may be increased with inflammation or infection. Because urea passes rapidly from blood and tissue spaces into the instilled lavage fluid, the dwell time of fluid in the lungs during lavage should be minimal (Haslam and Baughman, 1999). If the dwell time is over 1 minute, PELF volume is expected to be overestimated by 100 to 300% (Kiem and Schentag, 2008; Rennard et al., 1986). Potential blood contamination during the BAL can also artificially increase the urea concentration in the PELF which can decrease the calculation of the drug concentration in the PELF (Kiem and Schnetag, 2008).

**BAL vs bronchial swab**

Recently there has been increased research in determining if BAL sampling or bronchial swab sampling is more accurate at accessing antimicrobial pulmonary pharmacokinetics in animals. The bronchial swab technique is advantageous over BAL sampling, because it allows for direct measurement of antimicrobial concentration without the need to correct for dilution and it can be repeated in the same animal without concern for the volume of fluid required for sampling (Foster, Martin and Papich, 2016). Direct PELF sampling using a bronchial swab technique has also been described for large animals and offers the advantage of not requiring volume correction (Foster, Martin and Papich, 2016). The bronchial swab technique measures
drug concentrations at the level of the trachea or bronchi and thus may not be directly comparable to BAL derived measurements which sample the lower airways and alveoli (Kiem and Schentag, 2008). Many studies in humans and animals have employed the BAL technique to measure antimicrobial concentrations in the lower airways to predict antimicrobial action against pulmonary bacterial pathogens (Giguère and Tessman, 2011; Giguère et al., 2011; Tynan et al., 2016; Kuti and Nicolau, 2015; Mzyk et al., 2016; Zeitlinger et al., 2016). To the author’s knowledge a direct comparison between these methods has not been performed.
CHAPTER 2: PULMONARY DISPOSITION AND PHARMACOKINETICS OF MINOCYCLINE IN THE ADULT HORSE

Introduction

Bacterial pneumonia is an important cause of illness in the adult horse (Giguère, 2015). Owners can incur significant financial losses due to pneumonia especially in the competition or race horse for whom transport associated pneumonia is not uncommon (Austin, Foreman and Hungerford, 1995; Raidal, Bailey and Love, 1997). Prompt and effective treatment carries a favorable prognosis for return to competition in uncomplicated pneumonia cases (Ainsworth et al., 2000; Seltzer and Byars, 1996).

Oral antimicrobials are often the mainstay for the treatment of pneumonia in adult horses due to their relative ease of administration and often reduced financial burden to the owner. Broad-spectrum antimicrobials available for oral administration to adult horses are limited to chloramphenicol, potentiated sulfonamides (sulfadiazine or sulfamethoxazole, combined with trimethoprim), enrofloxacin, metronidazole, and doxycycline. Doxycycline, while demonstrating good distribution to the lung and a broad-spectrum of activity against many common bacterial respiratory pathogens including Streptococcus species (Bryant et al., 2000), underwent a drug market shortage resulting in marked increases in pricing (AARP, 2016). Although the cost and availability of doxycycline has improved recently, there remains a clinical need for additional oral antimicrobial treatment options in adult horses.

Minocycline, a tetracycline derivative, is a time dependent bacteriostatic antimicrobial and as such its efficacy is influenced by the duration of time the drug concentration remains above MIC. Minocycline is available off-label for oral administration to horses (Schnabel et al., 2012), and represents an attractive addition to the antimicrobial options due to its broad-spectrum of antimicrobial activity and excellent tissue penetration (Agwu and MacGowan, 2006;
Bishburg and Bishburg, 2009), relatively low cost, and potential anti-inflammatory properties (Sapadin and Fleischmajer, 2006). Pharmacokinetic and pulmonary disposition data on minocycline in the adult horse is lacking and is important information in order to more accurately predict the efficacy of minocycline in treating bacterial pneumonia in the adult horse.

There have been limited studies performed with minocycline in the adult horse. Schnabel et al. (2012) demonstrated that in adult horses minocycline achieved concentrations in synovial and cerebrospinal fluid that were adequate to treat infections caused by susceptible (MIC <0.25 ug/ml) bacterial organisms after intragastric (IG) administration of 5 doses of minocycline (4 mg/kg) (Schnabel et al., 2012). In a separate study, the administration of a single intravenous (IV) dose of minocycline (2.2 mg/kg) resulted in minocycline concentrations in lung tissue homogenates that exceeded plasma concentrations at two hours post drug administration (Nagata et al., 2010). While these findings suggest drug distribution into the lung, tissue homogenate concentrations are often inaccurate as they do not allow for differentiation between free (active) drug and drug bound to biological material (Giguère and Tessman, 2011; Liu, Muller and Derendorf, 2002; Mouton et al., 2008). Measurement of drug concentrations in the PELF and BAL cells is thought to provide superior information regarding intrapulmonary antimicrobial activity (Giguère and Tessman, 2011). Measurement of antimicrobial concentrations in the PELF and BAL cells has been described in multiple species including adult horses and foals (Rennard et al., 1986; Kiem and Schentag, 2008; Conte et al., 1996).

The objectives of this study were to determine the pharmacokinetics of minocycline after a single IV and single IG dose and to determine the pharmacokinetics and pulmonary disposition of minocycline after multiple IG doses in adult horses. Our study hypotheses were that minocycline would be present in the PELF and BAL cells at concentrations exceeding those in
plasma within 3 hours of IV or IG administration and achievable trough concentrations in the PELF and BAL cells after administration of IG minocycline would exceed a target concentration of 0.25 µg/mL.

**Material and methods**

**Animals**

Three non-pregnant mares and four geldings between 10 and 31 years of age (mean of 15 years of age and a median of 13 years of age) and weighing between 438 and 622 kilograms (mean weight of 527 kilograms and median weight of 543 kilograms) were used in this study. Study inclusion was determined based on normal physical and rebreathing examinations, and a normal routine plasma biochemistry profile performed prior to study onset. Horses were housed in individual stalls for 24 hours prior to and for the duration of each experimental period and were maintained on pasture between experimental periods. Horses were fed 2 flakes of mixed grass and alfalfa hay twice daily and had access to water *ad libitum*. Horses were monitored for drug reactions by performing and assessing physical examinations every 12 hours during the study and for 48 hours after completion of the study. This study was approved by the University of Illinois Institutional Animal Care and Use Committee (protocol No. #14261).

**Experimental design**

Study 1: Single dose IV or IG minocycline – Minocycline hydrochloride was administered IV once at a dose of 2.2 mg/kg or IG via nasogastric intubation once at a dose of 4 mg/kg with a 7-day washout period between administrations in a randomized cross-over experimental design. Hay was provided the afternoon prior to drug administration according to the standard feeding schedule at the Veterinary Teaching Hospital and two hours after IV or IG drug administration the morning of the study. Twelve hours prior to IV or IG drug administration
horses were instrumented with a 14-gauge extended use over-the-needle IV catheter placed aseptically in the upper third of the left jugular vein for the purpose of serial collection of whole blood. For the purpose of IV drug administration, a second catheter was placed in the right jugular vein as described above. The right catheter was removed immediately after drug administration. The left catheter was removed 24 hours after IV or IG drug administration. Whole blood samples were collected immediately prior to drug administration, time (T) 0, and at T = 0.08, 0.17, 0.25, 0.33, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 and 48 hours after drug administration. BAL was performed at T = 2, 4, 8, and 24 hours after drug administration.

Study 2: Multiple dose IG minocycline – Minocycline was administered IG via nasogastric intubation at a dose of 4 mg/kg every 12 hours for a total of 5 doses. Horses were maintained on a standard morning and afternoon feeding schedule with the morning feeding offered two hours after the morning drug administration and the afternoon feeding offered approximately four hours prior to the evening drug administration. Whole blood samples were collected via direct venipuncture from the left jugular vein immediately prior to administration of the first dose, T = 0, and at T = 0.5, 1, 1.5, 2, 6, 12, 14, 24, 26, 36, 38, 48, 48.5, 49, 49.5, 50, 54, 60, 72 and 96 hours after administration of the first dose of minocycline. BAL was performed at T = 24, 36, 48, 54, 60, and 72 hours after administration of the first dose of minocycline. Whole blood sample collection at T = 12, 24, 36, and 48 hours and BAL collection at T = 24, 36, and 48 hours were performed immediately prior to drug administration and were defined as trough concentrations for the purpose of this study.

Minocycline hydrochloride preparation and administration

Intragastric drug: Minocycline hydrochloride powder was suspended in 500 mL of water and administered via nasogastric intubation immediately after resuspension. The nasogastric tube
was flushed with 1500 mL of water following drug administration and the tube immediately removed.

Intravenous drug: Minocycline hydrochloride powder was dissolved in sterile water\(^d\) to a 5 mg/mL solution under sterile conditions. The solution was then filtered through a 25 mm syringe filter with a 0.2 micron Supor\(^\circledR\) membrane\(^e\) placed in light-protective sterile bottles and kept out of direct light until administration within 2 hours of preparation. Intravenous drug delivery was via dedicated IV catheter, extension set and syringes and was timed to occur over 5 minutes. Heart and respiratory rates were monitored every 1 minute during drug administration and every 2.5 minutes for 15 minutes post drug administration.

**Bronchoalveolar lavage procedure and sample processing**

Prior to the BAL, horses were sedated with 150 mg of xylazine hydrochloride\(^f\) IV into the jugular vein opposite to that used for whole blood sample collection. BAL fluid was collected through the biopsy channel of a sterilized 3m endoscope\(^g\) to ensure sampling of the distal airways. BAL sample collection alternated between the right or left lung, the order of which was randomly assigned prior to the initial BAL. Instillation of a dilute lidocaine solution (5 mL of 2\% lidocaine diluted in 20 mL of saline) was used in passing the endoscope into the trachea and past the level of the carina. Once the endoscope was wedged in the most distal bronchus, 300 mL of sterile isotonic saline\(^h\) was infused in 60 mL aliquots through the endoscope biopsy channel followed by gentle aspiration into sterile 60 mL syringes immediately after infusion of the last aliquot. The total volume of fluid recovered and side of lung sampled (right or left) was recorded and the individual samples pooled for immediate sample processing. Upon collection BAL fluid was pooled, 5 mL reserved for determination of total nucleated cell count and the remainder immediately centrifuged (3504 rpm x 10 minutes at 4\(^\circ\)C) for separation of BAL cells from BAL
supernatant containing the PELF. The cell pellet was washed with PBS and re-suspended in 500 µL acetonitrile: 0.2% formic acid. BAL supernatant and cells were stored at -80°C until batch analysis at a later date.

Whole blood collection and sample processing

Whole blood collected by catheter aspiration or direct venipuncture was placed into Na-heparin tubes and centrifuged (3504 rpm x 10 minutes at 4°C) within 15 minutes of collection. Plasma was then aliquoted and frozen at -80°C until batch analysis at a later date.

Minocycline analysis via liquid chromatography/mass spectrometry (LC/MS/MS)

Plasma and BAL cell and supernatant samples were thawed immediately prior to preparation for LC/MS/MS analysis. For metabolite extraction of plasma samples, 50 µL of plasma was mixed with 70 µL of methanol and spiked with 2 µL of internal standard (demeclocycline; 13.8 µg/mL) and followed by vortexing and centrifugation. For metabolite extraction of supernatant, 50 µL of supernatant was mixed with 70 µL of methanol and spiked with 1 µL of internal standard and followed by vortexing. The liquid was then loaded into the sample vial for LC/MS/MS injection. For BAL cell samples, cell pellets were subject to controlled sonication at the following settings: 4°C, amplitude 95, 10 minutes process time, pulse-on time 30 seconds, pulse-off time 45 seconds. The cell extracts were subject to centrifugation (14,800 rpm x 10 minutes at 4°C). The supernatant was subject to rapid drying and then resuspended into 150 µL 80% methanol, spiked with internal standard, and vortexed. The liquid was then loaded into the sample vial for LC/MS/MS injection.

LC-MS/MS analysis was performed according to standard laboratory procedures in the Metabolomics Laboratory using a hybrid triple Quadrupole-Linear Accelerator trap mass spectrometer designed for LC-MS/MS analysis and specialized software for data acquisition.
The LC separation was performed with the following specifications: column 4.6 x 50mm, 5µm; mobile phase A 0.1% formic acid in water; mobile phase B 0.1% formic acid in acetonitrile; flow rate 0.35 mL/minute; linear gradient at 0-2 minute 100%A, 7-10.5 minute 0%A, 11-15.5 minute 100%A, autosampler at 15°C, injection volume 15 µL. Mass spectra were acquired under positive electrospray ionization with the ion spray voltage at +5500 V. The source temperature was 450°C, the curtain gas, ion source gas 1 and ion source gas 2 were 32, 50, and 65 psi, respectively. Multiple reaction monitoring was used for quantitation of minocycline (m/z 458.2 -> m/z 441.1) with demeclocycline as the internal standard (m/z 465.2 -> m/z 448.2). Aliquots of drug free equine plasma and BAL fluid and cell suspension were provided for construction of standard calibration curves prior to sample analysis. Extraction efficiency for minocycline and the internal standard, and within and between run accuracy and precision for minocycline detection in plasma and BAL supernatant and cell suspension were performed.

**Determination of minocycline concentration within the PELF and BAL cells**

The estimation of volume of PELF was performed via the urea dilution method as previously described (Sapadin and Fleischmajer, 2006; Liu, Muller and Derendorf, 2002). Urea concentrations within BAL fluid and time-matched plasma samples were measured by use of a commercially available quantitative colorimetric urea determination kit. The kit used the improved Jung method which utilizes a chromogenic reagent that forms a colored complex specifically with urea. The intensity of the color, measured at 520 nm, is directly proportional to the urea concentration in the sample. The concentration of urea in the BAL fluid and plasma was used to calculate the volume of PELF (VolPELF) as follows:

\[
\text{Vol}_{\text{PELF}} = \text{Vol}_{\text{BAL}} \times \left( \frac{\text{Urea}_{\text{BAL}}}{\text{Urea}_{\text{Plasma}}} \right)
\]
Vol_{BAL} represented the volume of BAL measured at collection and Urea_{BAL} and Urea_{Plasma} represented the concentration of urea in BAL fluid and plasma, respectively.

Once the volume of PELF was known, concentrations of minocycline within PELF (Mino_{PELF}) was calculated as follows:

\[ \text{Mino}_{\text{PELF}} = \text{Mino}_{\text{BAL}} \times \left( \frac{\text{Volume}_{\text{BAL}}}{\text{Volume}_{\text{PELF}}} \right) \]

Mino_{BAL} represented the concentration of minocycline measured in BAL fluid by LC-MS/MS.

**Pharmacokinetic and statistical analysis**

For each horse, plasma minocycline concentration versus time data were analyzed based on non-compartmental pharmacokinetics using commercially available software\(^9\). Maximum plasma concentration (C_{max}) and time to achieve maximum plasma concentration (T_{max}) were obtained directly from the data. The rate constant of the terminal phase (\(\lambda z\)) was determined by linear regression of the logarithmic plasma concentration versus time curve using a minimum of 4 data points. Half-life of the terminal phase (t_{1/2\lambda z}) was calculated as ln 2 divided by \(\lambda z\). 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 \(C_{\text{min}}/\lambda z\), where \(C_{\text{min}}\) is the plasma concentration at the last measurable time point. Mean residence time (MRT) was calculated as: AUMC/AUC. Bioavailability was calculated as (AUC_{IG}/AUC_{IV}) x (DOSE_{IV}/DOSE_{IG}). Apparent volume of distribution based on the AUC (Vd_{area}) was calculated as: IV dose /AUC \(\times \lambda z\), apparent volume of distribution at steady state (Vd_{ss}) was calculated as (IV dose/AUC)/(AUMC/AUC), and systemic clearance (CL) was calculated from: IV dose/AUC.

Normality of the data and equality of variances was assessed using Shapiro-Wilk’s and Levene's tests, respectively. A paired t test was used for comparisons of C_{max} in PELF and BAL.
cells after IG and IV minocycline administration and for comparison of AUC for determination of steady state. Comparison of $C_{\text{max}}$ between sampling sites (plasma, PELF, BAL cells) was done using a Friedman repeated measures ANOVA. When necessary, multiple pairwise comparisons were done using the Student-Newman-Keuls method. Comparison of plasma and PELF minocycline trough concentrations were performed using a linear mixed-effects model with horse modeled as random effect and sample type, time and interaction between time and sample type modeled as fixed nominal effects. Model fit was assessed using Akaike information criterion values. Significance was set at $P < 0.05$.

Results

Horses used for study 1 and study 2 were identical except for one gelding that was lost to the study for an unrelated reason after study 1 and was replaced with another gelding for study 2. All horses tolerated IV and IG minocycline administration and BAL procedures well for both study 1 and study 2 and adverse clinical signs were not noted during drug administration. One horse developed a mild fever (103°Fahrenheit rectal temperature) at 36 hours into study 2. All other physical examination findings were within normal limits and a single dose of flunixin meglumine (1.1 mg/kg, IV) was administered to that horse. The 36 hour BAL was not performed, but the 36 hour drug dose was administered. All subsequent physical examinations were normal through 96 hour post drug administration and the horse was included in the remainder of the study.

Study 1

Mean ± SD $C_{\text{max}}$ and oral bioavailability (F%), and median (range) $T_{\text{max}}$ after IG administration of minocycline were $1.584 ± 0.9 \mu g/mL$, $48 ± 15\%$, and $1.0$ (range 0.5-1.0) hours, respectively. The plasma pharmacokinetic variables after IV or IG administration of
minocycline are summarized in Table 1. The mean plasma concentration-time curves for minocycline for IV and IG administration were constructed (Figure 3). Area under the plasma concentration time curves (AUC) were 14 ± 5.2 (IV) and 12 ± 5.1 (oral) µg • h/mL.

Concentrations of minocycline detected in PELF and BAL cells after a single IV or IG dose are summarized in Table 2. Mean ± SD $C_{\text{max}}$ and median (range) $T_{\text{max}}$ in the PELF after IG and IV minocycline administration were 5.032 ± 1.26 µg/mL and 3 (range 2.0-4.0) hours, and 8.65 ± 8.29 µg/mL and 2.0 (range 2.0-4.0) hours, respectively. Mean ± SD $C_{\text{max}}$ and median (range) $T_{\text{max}}$ in BAL cells after IG and IV minocycline administration were 0.006 ± 0.008 µg/mL and 2 (range 2.0-8.0) hours, and 0.007 ± 0.007 µg/mL and 6.0 (range 2.0-8.0) hours, respectively. Differences in $C_{\text{max}}$ between IG and IV minocycline were not statistically significant for the PELF (P=0.314) or BAL cells (P=0.799). $C_{\text{max}}$ in the PELF was significantly greater than $C_{\text{max}}$ in plasma and BAL cells for both the IG and IV minocycline (P < 0.001 all). $C_{\text{max}}$ in BAL cells was significantly lower than $C_{\text{max}}$ in plasma and PELF for both IV and IG minocycline (P < 0.001 all).

**Study 2**

Plasma pharmacokinetic parameters measured after the first and fifth IG dose of minocycline are summarized in Table 3. Mean (SEM) plasma minocycline concentrations while dosing to steady state are illustrated in Figure 4. The $\text{AUC}_{\text{0–}\infty}$ after administration of a single 4 mg/kg dose (15.2 ± 5.5 µg•h/mL) was not significantly different (P = 0.613) from the $\text{AUC}$ after the fifth dose, between 48 and 60 hours (13.9 ± 6.4 µg•h/mL), indicating that steady state was reached. At steady state mean plasma $C_{\text{max}}$ was 2.3 ± 1.3 µg/mL, terminal half-life ($t_{1/2}$) was 11.8 ± 0.5 hours and $T_{\text{max}}$ was 1.3 (1.0-1.5) hours.
Minocycline was detected in PELF and BAL cells at all measured time points, but concentrations in the PELF were much higher than those measured in BAL cells, and changes in PELF or BAL cell concentrations were not noted while dosing to plasma steady state (Figure 5 & 6). PELF, BAL cell, and time matched plasma pharmacokinetic variables measured after IG administration of five doses of minocycline are presented in Table 4. PELF $C_{\text{max}}$ of minocycline was greater when compared to both plasma and BAL cell $C_{\text{max}}$, and BAL cell $C_{\text{max}}$ was lower when compared to both plasma and PELF $C_{\text{max}}$ ($P < 0.05$ all). Minocycline concentrations measured in PELF were significantly greater than plasma concentrations at all trough time points ($P < 0.001$; Figure 7). Mean plasma trough concentrations were all > 0.55 $\mu$g/mL and for each horse, the minimum plasma trough concentrations were > 0.25 $\mu$g/mL. Median PELF concentrations at all time points were > 2.0 $\mu$g/mL and for each horse, the minimum PELF concentrations were > 0.68 $\mu$g/mL (Figure 7).
Table 1. Plasma pharmacokinetic variables (mean ± SD unless otherwise specified) after intravenous (2.2 mg/kg) or intragastric (4.0 mg/kg) administration of minocycline to 6 adult horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Intravenous</th>
<th>Intragastric</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_z$ (h$^{-1}$)</td>
<td>0.067 ± 0.008</td>
<td>NA</td>
</tr>
<tr>
<td>$t_{1/2\lambda z}$ (h)</td>
<td>10.5 ± 1.3</td>
<td>NA</td>
</tr>
<tr>
<td>C$_{initial}$ (µg/mL)</td>
<td>3.862 ± 1.378</td>
<td>NA</td>
</tr>
<tr>
<td>Vd$_{area}$ (L/kg)</td>
<td>2.73 ± 1.24</td>
<td>NA</td>
</tr>
<tr>
<td>Vd$_{ss}$ (L/kg)</td>
<td>2.16 ± 0.84</td>
<td>NA</td>
</tr>
<tr>
<td>CL (ml/h/kg)</td>
<td>176.2 ± 65.2</td>
<td>NA</td>
</tr>
<tr>
<td>AUC$_{0-24h}$ (µg • h/mL)</td>
<td>13.5 ± 5.0</td>
<td>10.8 ± 4.0</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (µg • h/mL)</td>
<td>14.0 ± 5.2</td>
<td>11.9 ± 5.1</td>
</tr>
<tr>
<td>AUMC$_{0-\infty}$ (µg • h$^2$/mL)</td>
<td>170.3 ± 60.6</td>
<td>NA</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>12.2 ± 0.9</td>
<td>NA</td>
</tr>
<tr>
<td>C$_{12h}$ (µg/mL)</td>
<td>0.294 ± 0.197</td>
<td>0.248 ± 0.073</td>
</tr>
<tr>
<td>C$_{max}$ (µg/mL)</td>
<td>NA</td>
<td>1.584 ± 0.899</td>
</tr>
<tr>
<td>T$_{max}$ (h)*</td>
<td>NA</td>
<td>1.0 (0.5 – 1.0)</td>
</tr>
<tr>
<td>F (%)</td>
<td>NA</td>
<td>48.2 ± 15.2</td>
</tr>
</tbody>
</table>

*Median and range; NA = not applicable

$\lambda_z$ = rate constant of the terminal phase. $t_{1/2\lambda z}$ = half-life of the terminal phase. C$_{initial}$ = Initial measured plasma concentration (at 5 min). Vd$_{area}$ = Apparent volume of distribution based on AUC. Vd$_{ss}$ = Apparent volume of distribution at steady state. CL = Systemic clearance. AUC$_{0-24h}$ = Area under the plasma concentration versus time curve from time 0 to 24 h. AUC$_{0-\infty}$ = Area under the plasma concentration versus time curve extrapolated to infinity. AUMC$_{0-\infty}$ = Area under the first moment of the concentration versus time curve extrapolated to infinity. MRT = Mean residence time. C$_{max}$ = Maximum plasma concentration (observed) after the first dose. T$_{max}$ = Time to maximum plasma concentration (observed) after the first dose. C$_{12h}$ = Plasma concentration 12 h after administration of the first dose. F = Oral bioavailability.
Table 2. Mean ± SD PELF and BAL cells minocycline concentrations (µg/mL) after IV (2.2 mg/kg) or IG (4.0 mg/kg) administration of minocycline to 6 adult horses.

<table>
<thead>
<tr>
<th>TIME (h)</th>
<th>PELF</th>
<th>BAL cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IV</td>
<td>IG</td>
</tr>
<tr>
<td>2</td>
<td>8.53 ± 8.4</td>
<td>4.48 ± 1.6</td>
</tr>
<tr>
<td>4</td>
<td>3.31 ± 1.3</td>
<td>3.96 ± 1.4</td>
</tr>
<tr>
<td>8</td>
<td>2.29 ± 1.9</td>
<td>1.21 ± 0.71</td>
</tr>
<tr>
<td>24</td>
<td>0.7 ± 0.4</td>
<td>0.72 ± 0.5</td>
</tr>
</tbody>
</table>

IV = intravenous; IG = intragastric

Table 3. Plasma pharmacokinetic variables (mean ± SD unless otherwise specified) after IG (4.0 mg/kg) administration of a single dose of minocycline (1st dose) and at steady state (after 5th dose) in 6 adult horses.

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>1st Dose</th>
<th>5th Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( C_{max} ) (µg/mL)</td>
<td>1.73 ± 1.05</td>
<td>2.33 ± 1.27</td>
</tr>
<tr>
<td>( T_{max} ) (h)</td>
<td>1.0 (1.0 – 1.5)</td>
<td>49.3 (49.0 – 49.5)</td>
</tr>
<tr>
<td>AUC(_{0\rightarrow\infty}) (µg•h/mL)</td>
<td>15.2 ± 5.5</td>
<td>13.9 ± 6.4</td>
</tr>
<tr>
<td>AUC(_{0\rightarrow t}) (µg•h/mL)</td>
<td>9.11 ± 3.84</td>
<td>38.55 ± 15.74</td>
</tr>
<tr>
<td>( t_{1/2\lambda} ) (h)</td>
<td>NA</td>
<td>11.8 ± 0.53</td>
</tr>
</tbody>
</table>

*Median and range; NA = not applicable

Table 4 – Median (25th and 75th percentiles) plasma, PELF, and BAL cells pharmacokinetic variables for minocycline after oral administration of 5 doses at 4 mg/kg of body weight q 12 h to 6 adult horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Plasma*</th>
<th>PELF</th>
<th>BAL cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( C_{max} ) (µg/mL)</td>
<td>0.98 (0.62 – 1.14)(^a)</td>
<td>5.23 (2.99 – 17.60)(^b)</td>
<td>0.007 (0.004 – 0.017)(^c)</td>
</tr>
<tr>
<td>( T_{max} ) (h)</td>
<td>6.0 (6.0 – 6.0)</td>
<td>9.0 (4.5 – 12.0)</td>
<td>3.0 (0 – 6.0)</td>
</tr>
<tr>
<td>AUC(_{48-96h}) (µg · h/mL)</td>
<td>15.40 (9.73 – 17.70)(^a)</td>
<td>66.34 (42.46 – 220.66)(^b)</td>
<td>0.067 (0.031 – 0.182)(^c)</td>
</tr>
</tbody>
</table>

*Only plasma samples obtained at the time of BAL collection were considered for calculation of \( T_{max} \), \( C_{max} \) and AUC
\(^a,b,c\) Different letters within a given row indicate statistically significant (P < 0.05) differences between fluids with Friedman’s repeated measures analysis on ranks. When necessary, multiple pairwise comparisons were done using the Student-Newman-Keuls method.
Figure 3: Concentration vs time curve after single dose oral (4 mg/kg) and IV (2 mg/kg) minocycline. Area under the plasma concentration time curves (AUC) were 14 ± 5.2 (IV) and 12 ± 5.1 (oral) µg • h/mL.
Figure 4: Mean (SEM) plasma minocycline concentrations while dosing to steady state. Time points circled in blue represent trough concentrations obtained immediately prior to drug administration.
Figure 5: Median, IQR and min-max minocycline concentrations in PELF measured prior to drug administration (24, 36, 48 h) and at 6, 12 and 24 h after final minocycline dose.
Figure 6: Median, IQR and min-max minocycline concentrations in BAL cells (b) measured prior to drug administration (24, 36, 48 h) and at 6, 12 and 24 h after final minocycline dose.
Figure 7: Mean plasma and median PELF minocycline trough concentrations. Bars represent minimum and maximum concentrations. Stars denote significant differences between PELF and plasma (P<0.001). The dashed line is placed at 0.25 µg/mL.
**Discussion**

In the present report, oral bioavailability (48%) varied considerably among adult horses (35 – 75%). While minocycline was detected in the PELF and BAL cells within 3 hours of IV or IG drug administration, only concentrations in the PELF exceeded those in plasma. As predicted, PELF trough concentrations exceeded the target concentration of 0.25 µg/mL at all measured time points. Contrary to our study hypothesis, minocycline BAL cell concentrations at all measured time points were well below concentrations detected in plasma and PELF and the 0.25 µg/mL target concentration.

The reported oral bioavailability of minocycline varies considerably across species (Maalnad, Guardabassi and Papich, 2014; Savin and Houin, 1988; Tynan et al., 2916). Schnabel et al. (2012) estimated the oral bioavailability of minocycline at 23% in non-fasted adult horses although IV minocycline administration was not performed. Recently, Giguère et al (2016) determined oral bioavailability of minocycline to be 32.0 + 18.0% in adult horses and 57.8 + 19.3% in foals (Giguère et al., 2016). In study 1 of this report, bioavailability was greater than reported for adult horses in both previous studies and greatly exceeded estimates of bioavailability of oral doxycycline reported in adult horses (Bryant et al., 2000; Savin and Houin, 1988; Womble, Giguère and Lee, 2007). In this study and those of Giguère et al. (2016) and Schnabel et al. (2011), minocycline was administered as a single 4 mg/kg dose via nasogastric intubation, but it is not known whether differences in feeding schedules between this study (hay twice daily) and the other studies (hay ad libitum) may have influenced drug absorption and thus measurement of bioavailability. In study 1, oral bioavailability was determined to be 48% but with considerable variation among adult horses (35 – 75%). Figure 8 illustrates the plasma minocycline concentration time curve for each horse in study 1 and shows the variability
between individual horses. Horse 1 which had the lowest minocycline plasma concentrations was also the oldest horse in the study at age 31 years old. We did not investigate the effect horse age might have on oral absorption of minocycline, but that would be something to determine in the future.

Feeding can decrease oral bioavailability for a variety of drugs (Gu et al., 2007; Sykes et al., 2015; Davis, Salmon and Papich, 2006; Hnot et al., 2015). While the impact of feeding on minocycline bioavailability has not been investigated in adult horses, significant reductions in \( C_{\text{max}} \) of minocycline have been identified in fed versus fasted dogs (Hnot et al., 2015) and \( C_{\text{max}} \) of doxycycline was significantly reduced in horses when the drug was administered concurrently with feed as opposed to 2 hours after feeding (Davis, Salmon and Papich, 2006). During study 2 of this report, horses were fed twice daily according to the standard Veterinary Teaching Hospital feeding schedule. Due to time constraints associated with BAL sample collection, horses were fed two hours after the morning drug administration and three to four hours prior to the evening drug administration. Interestingly, minocycline plasma concentrations measured two hours after morning drug administration were consistently higher than those measured two hours after evening drug administration, a result which suggests that feeding may have impacted drug bioavailability. Figure 9 illustrates the difference in plasma concentrations after morning drug administration as compared to the evening drug administration in Study 2. Plasma drug concentrations were significantly greater after the morning administration (\( P < 0.05 \)). Horses in study 2 were fed twice daily according to a standard feeding schedule and therefore drug administration in the morning occurred approximately 14 hours after feeding while drug administration in the evening occurred approximately 5 hours after feeding. This suggested that feeding may impact minocycline absorption. We performed a separate study with the objective to
compare the pharmacokinetics of minocycline after administration of a single oral dose in horses with feed withheld and with feed provided at the time of drug administration. Six healthy adult horses were administered intravenous (2.2 mg/kg) and oral minocycline (4 mg/kg) with access to hay at the time of oral drug administration (fed) and with access to hay delayed for 2 hours after oral drug administration (fasted), with a 7-day washout between treatments. Plasma concentration versus time data were analyzed based on non-compartmental pharmacokinetics (Figure 10). Mean ± SD bioavailability (fasted: 38.6% ± 4.6; fed: 15.7% ± 2.3) and C\textsubscript{max} (fasted: 1.343 ± 0.418 µg/mL; fed: 0.281 ± 0.157 µg/mL) were greater in fasted horses compared to fed horses (P < 0.05 both). Median (range) T\textsubscript{max} (h) in fasted horses was 2.0 (1.5 – 3.5) and in fed horses was 5.0 (1.0 – 8.0), and was not significantly different between groups (Table 5).

Delaying feeding hay 2 hours after oral minocycline administration improves drug bioavailability and thus plasma concentrations. This feeding study determined feeding did have an effect on the oral bioavailability of minocycline in the adult horse.

Another concern we had was the possible effect sedation might have on drug absorption. Each time a BAL was performed, the horse was sedated with 150 mg of xylazine to help restrain the horse for the procedure. Sutton et al. (2012) investigated the effects of xylazine, detomidine, acepromazine, and butorphanol on equine solid phase gastric emptying and concluded that in cases presenting with nasogastric reflux or gastric impaction, low doses of xylazine would be recommended to facilitate diagnostic procedures and did not significantly affect gastric emptying in the horse. Another study did find that xylazine produced a significant delay in gastric emptying, but the influence was transient and unlikely to be of clinical significance in healthy ponies (Doherty et al., 1999). We were concerned there could be an effect on minocycline absorption and plasma concentration due to the xylazine administration so we performed a
randomized cross over study with 2 horses and administered oral minocycline (4 mg/kg) via nasogastric intubation with 150 mg of xylazine intravenous administration and without sedation with a 7-day washout period between treatments. We found no difference in minocycline plasma concentrations between the sedated and unsedated horses with a p value greater than 0.05.

In study 1 and study 2 reported here, PELF minocycline concentrations exceeded plasma concentrations at all time points with a mean PELF $C_{\text{max}}$ that was 5 times plasma $C_{\text{max}}$ at steady state plasma concentration. Penetration into the PELF is influenced by drug lipophilicity and protein binding (Rodvold, George and Yoo, 2011). The lipophilic properties of group 2 tetracyclines (minocycline and doxycycline) may explain the high minocycline concentrations in PELF in this study (Agwuh and MacGowan, 2006; Sapadin and Fleischmajer, 2006; Maaland et al., 2014) as well as similar findings reported for minocycline in foals (Giguère et al., 2016) and for doxycycline in adult horses and foals (Womble, Giguère and Lee, 2007; Davis, Salmon and Papich, 2006).

Plasma protein binding influences a drug’s ability to penetrate tissue because only the unbound fraction is available to cross biologic membranes. The percentage of plasma protein binding of minocycline was not determined in this study but has been reported to be approximately 68% in adult horses (Nagata et al., 2010) which is similar to previously reported percentages in dogs and humans (Agwuh and MacGowan, 2006; Maaland et al., 2014) and lower than estimated protein binding of doxycycline (Womble, Giguère and Lee, 2007). Binding to protein within the PELF can further influence the amount of active drug that is available (Kiem and Schentag, 2008) particularly during inflammatory conditions resulting in changes in protein within the PELF. Protein concentrations in PELF have been reported to be much lower than in the plasma in healthy humans and in humans with pulmonary disease (Rennard et al., 1986) and
in healthy adult horses the percent protein binding of doxycycline in the PELF was much lower than protein binding in the plasma (Rodvold, George and Yoo, 2011).

Pulmonary pharmacokinetic analysis in this study was based on measurement of minocycline concentrations in the PELF and BAL cells using BAL accompanied by volume correction with the urea dilution method. The PELF represents secretions on the interior alveolar wall and within the small bronchi and is often a site where bacterial organisms reside (Rodvold, George and Yoo, 2011). Thus, measurement of drug concentrations in the PELF, and in alveolar cells, is thought to provide superior information on intrapulmonary antimicrobial activity when compared to measurement of plasma or tissue homogenate drug concentrations (Giguère and Tessman, 2011; Rodvold, George and Yoo, 2011; Winther, 2012).

While BAL with urea dilution has been widely described in horses and other species (Winther et al., 2010b; Giguère et al., 2016; Womble, Giguère and Lee, 2007; Rodvold, George and Yoo, 2011; Winther, 2012; Cox et al., 2010; Giguère et al., 2011), potential limitations associated with urea dilution that may result in over- or under-estimation of active drug concentrations within the PELF include cell lysis during BAL collection, blood contamination, increased BAL fluid dwell time, and measurement of both protein bound and unbound drug (Giguère and Tessman, 2011; Kiem and Schentag, 2008; Villarino et al., 2013; Foster, Martin and Papich, 2016). Standardization of the BAL technique, as done in this study, is believed to minimize some of these limitations (Kiern and Schentag, 2008). Direct PELF sampling using a bronchial swab technique has also been described for large animals and offers the advantage of not requiring volume correction (Foster, Martin and Papich, 2016). This technique measures drug concentrations at the level of the trachea or bronchi and thus may not be directly
comparable to BAL derived measurements which sample the lower airways and alveoli (Kiem and Schentag, 2008).

Bronchoalveolar lavage in the standing sedated horse is a repeatable and relatively non-invasive procedure that typically causes little iatrogenic damage (Tynan et al., 2016; Ensink et al., 1993; Tee et al., 2012). The possibility exists that multiple BAL procedures within the same horse could influence PELF drug concentrations due to residual fluid remaining in the lung from a previous BAL or due to the potential that multiple BAL procedures could cause inflammation within the lung. Tee et al. (2012) investigated the effects of serial BAL samples in healthy Standardbred horses and found that there was no significant change in neutrophil percentages in the BAL fluid at any collection point. Visual evidence of residual fluid was not noted during the bronchoscopic-guided BAL procedures. Cytology on BAL samples did identify increases in cell count and neutrophils (Table 6) which suggests some degree of inflammation associated with the procedure. Inflammation is thought to improve pulmonary drug absorption by facilitating drug transport into the PELF (Kiem and Schentag, 2008; Tynan et al., 2016). Whether the degree of transient inflammation associated with the BAL procedure was sufficient to influence PELF minocycline concentrations measured in this study is unknown.

In this study, minocycline C\textsubscript{max} in BAL cells represented only 0.4% of plasma C\textsubscript{max} and 0.1% of PELF C\textsubscript{max} after single and multiple oral doses. This finding was surprising as higher intracellular concentrations were expected. High intracellular concentrations are reported for tetracyclines (Davis, Salmon and Papich, 2006; Gabler, 1991; Forsgren and Bellahsene, 1985) with cellular-to-intracellular concentration ratios in polymorphonuclear leukocytes of 13 and 17 reported in humans (Forsgren and Bellahsene, 1985) and adult horses (Davis, Salmon and Papich, 2006), respectively. PELF and BAL cell minocycline concentrations reported by Giguère
et al (2016) for foals were similar to our study. While it is possible that intracellular drug concentration in BAL cells is less than that of peripheral cells given that the drug must first penetrate into the lung, in foals administered doxycycline, drug concentrations achieved in BAL cells were similar to serum concentrations (Womble, Giguère and Lee, 2007). The exact reason for decreased minocycline concentrations in BAL cells in this study is unknown. Lysis of BAL cells during sample processing can result in loss of drug for analysis and it is possible that lysis may have occurred during preparation of the cell pellet for analysis. If poor BAL cell minocycline activity truly exists, it would be less likely to be of a clinical concern in adult horses as bacterial pathogens typically associated with pneumonia are extracellular and would thus be susceptible to the high minocycline concentrations in the PELF.

Tetracyclines are reported to have good antimicrobial activity against many Streptococcus species (Schnabel et al., 2012). In order to accurately predict the efficacy of an antimicrobial for the treatment of bacterial pneumonia, knowledge of specific MICs for common bacterial pathogens in relation to achievable PELF concentrations is important. An MIC <0.25µg/mL is often used to predict efficacy of doxycycline and minocycline for the treatment of equine pneumonia and other bacterial infections (Sweeney et al., 1991) and was therefore chosen as the target drug concentration in the PELF and BAL cells for this study. Information on Streptococcus equi subspecies zooepidemicus MIC values for minocycline in horses is limited to two studies. MIC$_{50}$ values for minocycline are reported to be between 0.06 µg/mL and 0.12 µg/mL (Schnabel et al., 2012; Forsgren and Bellahsene, 1985) while MIC$_{90}$ values for minocycline are reported to be between 0.12 µg/mL and 8 µg/mL respectively (Schnabel et al., 2012; Ensink et al., 1993). While geographic differences in antimicrobial susceptibility may exist between these studies, these results suggest that achievable minocycline concentrations in
the PELF would be sufficient to treat most *Streptococcus equi* subspecies *zooepidemicus* bacterial pneumonias.

The results of this study suggest that while minocycline distributes into the PELF and BAL cells of healthy adult horses, only minocycline concentrations in PELF would be sufficient for the treatment of bacterial pneumonia. To better predict drug bioavailability and pulmonary disposition in clinical cases, evaluation of minocycline pulmonary pharmacokinetics in horses with pulmonary disease should be further investigated.
Figure 8: Plasma minocycline concentration versus time curve for each individual horse in study 2. Each color represents a different horse.
Figure 9: Minocycline plasma concentration versus time at “trough” meaning the plasma concentration prior to next minocycline administration and the plasma concentration 2 hours post minocycline administration.
Figure 10: Mean plasma minocycline concentrations (± SD) after IV (2.2 mg/kg) or oral (4.0 mg/kg) administration of minocycline to six adult horses. Horses treated orally were either fasted overnight and fed hay 2 h after drug administration (Oral-fasted) or fed hay prior to drug administration (Oral-fed).
Table 5: Plasma pharmacokinetic variables (mean ± SD unless otherwise specified) after IV (2.2 mg/kg) or oral (4.0 mg/kg) administration of minocycline to 6 adult horses. Horses treated orally were either fasted overnight and fed hay 2 h after drug administration (fasted) or fed hay prior to drug administration (fed).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Intravenous</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fasted</td>
</tr>
<tr>
<td>$\lambda_z$ (h$^{-1}$)</td>
<td>0.060 ± 0.009$^a$</td>
<td>0.062 ± 0.008$^a$</td>
</tr>
<tr>
<td>$t_{\frac{1}{2}\lambda_z}$ (h)</td>
<td>11.8 ± 1.9$^a$</td>
<td>11.3 ± 1.2$^a$</td>
</tr>
<tr>
<td>AUC$_{0-24h}$ (µg • h/mL)</td>
<td>22.2 ± 7.5</td>
<td>16.0 ± 4.5</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (µg • h/mL)</td>
<td>23.3 ± 7.7</td>
<td>17.0 ± 4.8</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>13.9 ± 1.6$^a$</td>
<td>15.4 ± 4.5$^a$</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>NA</td>
<td>1.343 ± 0.418</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)*</td>
<td>NA</td>
<td>2.0 (1.5 – 3.5)</td>
</tr>
<tr>
<td>F (%)</td>
<td>NA</td>
<td>38.6 ± 4.6</td>
</tr>
<tr>
<td>Vd$_{\text{area}}$ (L/kg)</td>
<td>1.78 ± 0.75</td>
<td>NA</td>
</tr>
<tr>
<td>CL (ml/h/kg)</td>
<td>104.4 ± 37.3</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Median and range; NA = not applicable

$\lambda_z$ = rate constant of the terminal phase. $t_{\frac{1}{2}\lambda_z}$ = half-life of the terminal phase. AUC$_{0-24h}$ = Area under the plasma concentration versus time curve from time 0 to 24 h. AUC$_{0-\infty}$ = Area under the plasma concentration versus time curve extrapolated to infinity. MRT = Mean residence time. $C_{\text{max}}$ = Maximum plasma concentration (observed) after the first dose. $T_{\text{max}}$ = Time to maximum plasma concentration (observed) after the first dose. F = Oral bioavailability. Vd$_{\text{area}}$ = Apparent volume of distribution based on AUC. CL = Systemic clearance.

$^a,b$ Different superscript letters within a row indicate statistically significantly differences between the 3 methods of administration (P < 0.05).

$^\dagger$Indicates a statistically significant difference between fed and fasted (P < 0.05).
Table 6: Mean ± SD total white blood cell count (WBC) and percent cell differentials measured in BAL fluid in Study 1 and Study 2. Mac = macrophage; lymph = lymphocyte; neut = neutrophil; eos = eosinophil; mast = mast cell.

<table>
<thead>
<tr>
<th></th>
<th>STUDY 1</th>
<th></th>
<th>STUDY 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2h</td>
<td>4h</td>
<td>8h</td>
</tr>
<tr>
<td>WBC</td>
<td>154 ± 95</td>
<td>197 ± 97</td>
<td>171 ± 54</td>
</tr>
<tr>
<td>% Mac</td>
<td>37 ± 4</td>
<td>37 ± 13</td>
<td>37 ± 15</td>
</tr>
<tr>
<td>% lymph</td>
<td>54 ± 7</td>
<td>53 ± 17</td>
<td>53 ± 16</td>
</tr>
<tr>
<td>% neut</td>
<td>5 ± 2</td>
<td>9 ± 7</td>
<td>17 ± 24</td>
</tr>
<tr>
<td>% eos</td>
<td>0.3 ± 0.5</td>
<td>0.1 ± 0.2</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>% mast</td>
<td>3 ± 4</td>
<td>3 ± 4</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>
CONCLUSION

This study demonstrated that minocycline distributes into the PELF and BAL cells of healthy adult horses. It was determined that only minocycline concentrations in PELF would be sufficient for the treatment of bacterial pneumonia in adult horses. Most pathogens causing bacterial pneumonia in the adult horse are extracellular pathogens and therefore the concentrations achieved in the PELF in this study suggest that minocycline would be suitable for the treatment of bacterial pneumonia.

The effect of feeding on minocycline bioavailability was evaluated in a follow-up study performed after the results from the initial pulmonary pharmacokinetic study suggested that time of feeding prior to drug administration influenced minocycline plasma concentrations. The results of the feeding study were presented in the discussion and demonstrate that feeding hay reduces oral bioavailability of minocycline in the adult horse and delaying feeding hay for 2 hours after oral minocycline administration improves the drug bioavailability and thus plasma concentrations.

The possible effect of sedation on the bioavailability of minocycline was also briefly investigated in a small study population and concluded not to have a significant effect on minocycline plasma concentrations.

The bioavailability for minocycline in the adult horse calculated in study 1 was determined to be 48% but with considerable variation among adult horses (35 – 75%). Previous research had estimated the oral bioavailability of minocycline at 23% in non-fasted adult horses (Schnabel et al., 2012) and 32.0 ± 18.0% in adult horses and 57.8 ± 19.3% in foals (Giguère et al., 2016). In study 1 of this report, bioavailability was greater than reported for adult horses in
both previous studies. It is not known whether differences in feeding schedules between this study (hay twice daily) and the other studies (hay *ad libitum*) may have influenced drug absorption and thus measurement of bioavailability. Interestingly, in the feeding study we performed we estimated minocycline bioavailability to be 38.6% ± 4.6 in fasted horses and 15.7% ± 2.3 in fed horses. The bioavailability estimated from the feeding study was more consistent with the bioavailability findings of the previous studies (Schnabel et al., 2012; Giguère et al., 2016). Bioavailability is variable among study subjects which was shown in Figure 8 as well as the in the large range from study 1 and large standard deviation in Giguère et al. (2016) study.

For the study, healthy adult horses were used. As discussed in the literature review, inflammation associated with bacterial pneumonia may alter antimicrobial transport and action at the site of infection. To better predict pulmonary disposition in clinical cases, minocycline pulmonary distribution in horses with pulmonary disease should be evaluated.

Minocycline is an attractive antimicrobial option to treat bacterial pneumonia in the adult horse. This study demonstrated the pharmacokinetics and pulmonary disposition in the adult horse. Minocycline is an orally-administered antimicrobial twice daily and is reasonably affordable in the adult horse. Based on the feeding study performed, we recommend withholding feed for 2 hours post minocycline administration for optimal minocycline plasma concentrations.
FOOTNOTES


f. AnaSed® 100 injection manufactured by Lloyd, Shenandoah, Iowa.

g. Olympus corporation of the America, Center Valley, Pennsylvania.


i. Covidien Ltd., Dublin Ireland.

j. Sigma-Aldrich Corp, Atlanta, Georgia.

k. QSonica LLC, Newton, Connecticut.


m. 5500 QTrap LC/MS/MS system, Sciex, Framingham Massachusetts.

n. 1200 series HPLC system, Agilent Technologies, Santa Clara, California.

o. Analyst 1.6.2 software. AB Sciex LP, Concord, Ontario, Canada.


q. PK Solutions 2.0, Summit Research Services, Montrose, Colorado.
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Davis JL, Salmon JH, Papich MG. Pharmacokinetics and tissue distribution of doxycycline after oral administration of single and multiple doses in horses. *AJVR.* 2006; 67:310-316.


Foster DM, Martin LG, Papich MG. Comparison of active drug concentrations in the pulmonary epithelial lining fluid and interstitial fluid of calves injected with enrofloxacin, florfenicol, ceftiofur, or tulathromycin. *Plos One*. 2016; Feb 12; doi:10.1371/journal.pone.014900


Giguère S. Bacterial Pneumonia and pleuropneumonia in adult horses. In: Smith BP (5th ed.) Large Animal Internal Medicine (pp.417-480) St. Louis, MO: Mosby Elsevier.


