

RHODOCOCCUS EQUI IN FOALS – EMERGING ANTIMICROBIAL RESISTANCE AND  
LIPOSOMAL GENTAMICIN AS A POTENTIAL NOVEL THERAPY

by

ALEXANDRA JANE BURTON

(Under the Direction of Steeve Giguère)

ABSTRACT

The facultative intracellular bacterium *Rhodococcus equi*, commonly causes pneumonia in foals. Mortality is 30% despite recommended combination antimicrobial therapy with a macrolide and rifampin. Over the past decade, the incidence of macrolide resistant *R. equi* in foals has increased. The aminoglycoside antibiotic gentamicin is highly active against *R. equi* but has limited clinical efficacy due to poor cellular penetration. Encapsulation of drugs in liposomes can enhance their cellular uptake. The broad objectives of this work were to 1) Assess the genotypic variation of macrolide resistance in *R. equi* isolates originating from a single farm, 2) compare the cellular uptake and efficacies of liposomal gentamicin (LG) and free gentamicin (FG) against *R. equi in vitro* and *in vivo*, and 3) to compare the pharmacokinetics and tolerability of LG and FG in healthy foals. Culture, sensitivity and genotyping by rep-PCR on tracheobronchial aspirates from foals and air samples indicated emergence of widespread macrolide and rifampin resistance in *R. equi* within one equine breeding farm. Resistant isolates formed 2 distinct genotypic clusters whereas there was more heterogeneity among susceptible isolates. Percentage of cultured equine alveolar macrophages containing intracellular LG was over 90% significantly greater than the % containing FG after 4, 24 and 48 hours incubation.

Athymic nude mice infected with *R. equi* 103S and treated with LG had significantly lower concentrations of bacteria in the spleen and liver compared to mice treated with FG or placebo. In healthy foals, after IV administration, LG had a significantly greater plasma half-life and volume of distribution compared with FG. Peak gentamicin concentrations in BAL cells were significantly higher for LG compared with FG when administered by either intravenous ( $5.27 \pm 2.67$  vs.  $2.98 \pm 1.67$   $\mu\text{g/ml}$ ) or nebulized ( $4.47 \pm 2.66$  vs.  $1.49 \pm 0.57$   $\mu\text{g/ml}$ ) routes. LG was well tolerated and indices of renal injury were not significantly different from those of foals administered FG. Liposomal encapsulation enables higher intracellular gentamicin concentrations to be achieved and as such, LG warrants further investigation for the treatment of infections in foals caused by intracellular pathogens such as *R. equi*.

INDEX WORDS: *Rhodococcus equi*, Liposome, Gentamicin, Horse, Foal, Antimicrobial resistance, Macrolide resistance.

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## DEDICATION

To the mares and foals

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## CHAPTER 1

### INTRODUCTION

Pneumonia is the leading cause of morbidity and mortality in foals in the United States (Cohen et al., 1994). *Rhodococcus equi*, a Gram-positive, facultative intracellular bacterium, is a common cause of severe pneumonia in 1 to 5 month-old foals. On *R. equi* endemic farms, morbidity rates can exceed 40 % (Chaffin et al., 2008, 2011) and despite recommended therapy with the combination of a macrolide and rifampin, the mortality rate of clinically affected foals is still around 30% (Ainsworth et al., 1998, Giguère et al., 2004). As such, *R. equi* exerts a major financial impact on the equine industry. Over the last 10 years, the incidence of macrolide and rifampin resistance has increased to the point where resistant isolates of *R. equi* are cultured from up to 40% of the foals on some farms. Foals infected with such resistant isolates are 7 times more likely to die than foals infected with susceptible isolates (Giguère et al., 2010).

All *R. equi* isolates from pneumonic foals, including macrolide-resistant isolates, are susceptible to the aminoglycoside gentamicin *in vitro* (Giguère et al., 2010, Carlson et al., 2010, Jacks et al., 2003). Additionally, gentamicin is one of the few antimicrobial agents that is bactericidal against *R. equi* rather than bacteriostatic (Giguère et al., 2012, Nordmann and Ronco, 1992). However, being a highly water-soluble drug, gentamicin has poor intracellular penetration (Gamzo et al., 2007). Although gentamicin is very effective against *R. equi in vitro*, historically, its efficacy *in vivo* has been limited (Sweeny et al., 1987). Liposomes are 0.08 – 5 µm vesicles composed of amphiphile bilayers surrounding an aqueous core. Following intravenous administration, liposomes rapidly localize in phagocytic cells, and thus can be used

as drug carriers for the treatment of intracellular infections (Gamzo et al., 2007, Drummond et al., 2008). Gentamicin encapsulated within liposomes (liposomal gentamicin) has been found to be significantly more efficacious than free (conventional) gentamicin against infections caused by other facultative intracellular bacteria including *Staphylococcus aureus*, *Listeria monocytogenes*, *Mycobacterium* spp., *Salmonella* spp., and *Brucella* spp (Bakker-Woudenberg et al., 1994, Gamzo et al., 2007, Klemens et al., 1990, Lutwyche et al., 1998, Vitas et al., 1996). Thus, liposomal gentamicin could be effective against *R. equi* also.

In summary, there is a need for more effective treatment for infections caused by *R. equi*; currently there is no adequate therapy for infection due to macrolide and rifampin resistant *R. equi* in foals. *In vitro*, gentamicin is the most active drug against *R. equi* and one of the few antimicrobial agents that is bactericidal against *R. equi* at therapeutic concentrations. Historically, gentamicin has not been effective for the treatment of pneumonia caused by *R. equi* due to its inability to penetrate cell membranes. Encapsulation of gentamicin in liposomes will carry the drug into *R. equi*-infected macrophages so that it can act on the pathogen at the site of infection.

The studies reported herein were undertaken to assess the incidence of macrolide and rifampin resistant *R. equi* in foals, to determine whether liposomal gentamicin is more effective to kill *R. equi* than free gentamicin or a macrolide rifampin combination, and to investigate the administration of liposomal gentamicin in foals in terms of pharmacokinetics and safety. The overall long-term goal behind this research is to develop a more effective treatment for infections caused by *R. equi* in foals, especially those due to macrolide and rifampin resistant isolates. This thesis reports the results of 4 major studies.

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

- 1- To determine the prevalence of macrolide and rifampin resistance on a horse breeding farm endemic for *R. equi* infections.
- 2- To document genotypic variation among macrolide-resistant and macrolide-susceptible isolates on the same farm.

Our *hypothesis* is that macrolide and rifampin resistance in *R. equi* is widespread on the farm and primary infection of foals with resistant isolates occurs, and that these resistant isolates have different genotypes from the sensitive isolates.

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

- 1- To determine the effect of age on the pharmacokinetics of a single, intravenous (IV) dose of gentamicin sulfate in foals.

Our *hypothesis* is that compared with adult horses, foals require a higher dose of gentamicin sulfate (12 mg/kg IV q 24 h) to achieve desired plasma concentrations.

The objectives and hypothesis of the third study (Chapter 5) are:

- 1- To compare the uptake of different formulations of liposomal gentamicin by J774.A1 murine macrophages *in vitro*.
- 2- To compare the activity and intracellular co-localization of liposomal gentamicin with that of free gentamicin in *R. equi* infected equine alveolar macrophages *in vitro*.
- 3- To compare the activity of two different formulations of liposomal gentamicin to that of free gentamicin and of other antimicrobials routinely used to treat *R. equi* in foals for the treatment of *R. equi* in a mouse chronic infection model.

Our *hypothesis* is that liposomal gentamicin will be more effective at killing *R. equi* in infected equine alveolar macrophages and mice than free gentamicin, rifampin, macrolides, or



macrolides-rifampin, and will co-localize with *R. equi* within cells.

The objectives and hypothesis of the fourth study (Chapter 6) are:

- 1- To compare the pharmacokinetics and pulmonary disposition of a single dose of IV or inhalational (nebulized) liposomal gentamicin to that of free gentamicin in foals.
- 2- To investigate of the safety and accumulation of liposomal gentamicin in foals after repeated administration at a dose of 6.6 mg/kg IV q 24 h for 7 days.

Our *hypotheses* are that administration of liposomal gentamicin will result in significantly higher concentrations of the drug in bronchoalveolar (BAL) macrophages and pulmonary epithelial lining fluid (PELF) than free gentamicin. In addition, that repeated administration of liposomal gentamicin a dose of 6.6 mg/kg IV q 24 h for 7 days will be well tolerated and result in reduced evidence of nephrotoxicity compared with free gentamicin.

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## CHAPTER 2

### LITERATURE REVIEW

#### The pathogen: *Rhodococcus equi*

*Rhodococcus equi* is a facultative intracellular Gram-positive bacterium (Genus [Rhodococcus]; Family, Nocardiaceae; Suborder, Corynebacterineae; Order; Actinomycetales; Phylum, Actinobacteria). Over the past 18 months, the taxonomy of this pathogen has been the subject of heated debate, because *R. equi* isolates appear to belong to a phyletic clade distinct from other species within the *Rhodococcus* genus, and thus should be in a genus of its own. The recent genus and species renaming proposals have included *Prescottia equi* (Jones et al., 2013<sup>a</sup>), which in turn was discarded in favor of *Prescottella equi* because the name Prescottia was already in use for a genus of orchid plant (Jones et al., 2013<sup>b</sup>), *Rhodococcus hoagii* (Kampfer et al., 2014), *Corynebacterium hoagii* (Tindall, 2014) and finally a call for preservation of the name *Rhodococcus equi* (Garrity, 2014). Due to this recent fluctuation and controversy with respect to official naming, for the purposes of this thesis, the pathogen will be referred to as *R. equi*, the name that has been used for the past 3 decades.

*Rhodococcus equi* is primarily a cause of pyogranulomatous bronchopneumonia in foals (Smith and Robinson, 1981, Zink et al., 1986, Giguère et al., 2011). It is also a common opportunistic pathogen of immunocompromised humans (Harvey and Sunstrum, 1991, Roda et al., 2009, Yamshchikov et al., 2010, Langer et al., 2010) and there are also a mounting number of reports of *R. equi* as a cause of abscessing infections and bacteriemia in immunocompetent adults and children (Herath et al., 2013, Dias et al., 2013, Langer et al., 2010, Roda et al., 2010,

Kamboj et al., 2005, Kedlaya et al., 2001). As a soil saprophyte, *R. equi* can live outside the host for prolonged periods of time and replicate in manure contaminated soil (Prescott et al., 1984). Biofilm formation by *R. equi* has been demonstrated recently, on intravenous catheters in cancer patients, and reproduced *in vitro*, highlighting another mechanism whereby this pathogen may persist outside the host (Al Akhrass et al., 2012). Although *R. equi* has a polysaccharide capsule, which likely contributes to biofilm formation, the capsule itself is not essential for virulence (Sydor et al., 2008).

Virulence in *R. equi* depends on the presence of a large plasmid, with plasmid cured derivatives being avirulent (Giguère et al., 1999). Sequencing of the plasmid showed a pathogenicity island (PAI) essential for virulence in foals. Coding sequences of the PAI include the virulence-associated protein (Vap) family, which is specific to *R. equi* (Takai et al., 2000<sup>a</sup>). There are 6 full-length vap genes, (*vapA*, *-C*, *-D*, *-E*, *-G*, *-H*) and 3-truncated vap pseudogenes (*vapF*, *-I*, and *-X*) (Letek et al., 2008). Of these, the *vapA* gene is essential for full virulence: *R. equi* expressing VapA are virulent and those that do not are avirulent (Jain et al., 2003). Strains of intermediate virulence expressing *vapB* have been isolated from pigs and their environments (Takai et al., 2000<sup>b</sup>). *Rhodococcus equi* expressing *vapB* is thought to be the most commonly found in humans, although *vapA* expressing strains are also isolated from human patients (Nicholson and Prescott, 1997, Ribeiro et al., 2011, Langer et al., 2010). Expression of the *vapA* gene is influenced by temperature and pH, which affect transcription of the *virR* and *virS* genes (also located on the PAI). VirR and VirS regulate *vapA* expression and *vapA* promoter ( $P_{vapA}$ ) activity respectively (Russell et al., 2004, Kakuda et al., 2014). Recently, it was demonstrated that the virulence plasmid containing the PAI is transferrable via conjugation between some *Rhodococcus* spp., and to the closely related species, *Dietzia* (Tripathi et al., 2012). Further, field

strains of virulent *R. equi* have been shown to transfer the virulence plasmid expressing *vapA* to avirulent *R. equi*, highlighting a means by which virulent *R. equi* can propagate in the environment (Stoughton et al., 2013).

Foals are mainly infected with *R. equi* by inhalation, after which the bacterium is phagocytosed by alveolar macrophages in the lungs (Johnson et al., 1983). Virulent *R. equi* persists via intracellular survival and replicates within the macrophages (Hondalus and Mosser, 1994). Avirulent *R. equi* are also phagocytosed by macrophages but are unable to survive because as they are enclosed within a phagosome, which matures into a phagolysosome. During maturation, a number of different signaling events and biochemical changes lead to bacterial killing mechanisms (Fernandez-Mora et al., 2005). Although initially enclosed within the macrophage phagosome, *R. equi* expressing *vapA* interrupts the normal progression of phagocytosis, and remains in an *R. equi* containing vacuole (RCV), which fails to mature normally (von Bargen et al., 2009, Fernandez-Mora et al., 2005). The RCV is held in suspended animation, failing to gain the enzymes cathepsin D, acid  $\beta$ -glucuronidase, proton-pumping ATPase and is unable to fuse with lysosomes to form a phagolysosome (Fernandez-Mora et al., 2005). Importantly, the vacuole does not become acidified for up to 48 hours and thus *R. equi* can replicate protected within the vacuole. Macrophage necrosis occurs by 24-48 hours after cell infection, releasing *R. equi* to invade further macrophages (Zink et al., 1987, Toyooka et al., 2005).

#### The problem: Clinical disease in foals

Although *R. equi* is an equine pathogen, clinical disease is usually only affects foals between 1 and 6 months of age (Giguère et al., 2011). Infection is via inhalation and *R. equi* can be cultured from the environment of virtually all horse farms, though the clinical disease in foals

is sporadic on some farms and endemic on others. The incubation period prior to clinical signs under experimental conditions following direct intrabronchial challenge depends upon infection dose and frequency, but has varied between 9 days (Giguère et al., 1999) and 28 days (Barton and Embury, 1987). Not all foals exposed to virulent *R. equi* develop clinical disease and, as with many pathogens, outcome following infection probably depends upon age and magnitude of challenge in combination with host factors such as genetics, concurrent disease, stress and concomitant flora. A recent study found that the challenge dose of *R. equi* was strongly associated with lung lesion severity and probability of death in foals infected experimentally at 1, 2 and 3 weeks of age (Sanz et al., 2014). Severity of lesion for a given dose was significantly less for 6-week old foals infected with a given dose (Sanz et al., 2014). Interestingly, a recent *in vitro* study showed that replication of *R. equi* was greatest in macrophages obtained from 3-month old foals as compared with foals of 3 days, 2 weeks, 1 month or with adult horses (Berghaus et al., 2014). It is possible that in natural field conditions, low dose infection occurs early in life and is an on-going process, but maximal replication does not occur until later, accounting for the high frequency of clinical disease at 2-5 months of age. Due to the formation of abscesses, which wall off infection, foals usually do not develop overt clinical signs until the *R. equi* lesions are fairly advanced. This necessitates lengthy treatment and has prompted research into methods of pre-clinical diagnosis, in particular pulmonary ultrasonography, in an attempt to circumvent development of advanced clinical disease (Slovis et al., 2005). However, foals with pulmonary ultrasonographic lesions frequently never develop overt clinical signs and frequently self-cure (Venner et al., 2012, 2013<sup>a,b</sup>, Sanz et al., 2014). Foals may also develop extra-pulmonary disease due to *R. equi*, either in addition to pneumonia or in the absence of pneumonia. Extra-pulmonary lesions include pyogranulomatous enterotyphlocolitis, abscessation of abdominal mesenteric or

colonic lymph nodes, osteomyelitis, immune-mediated polysynovitis and uveitis (Reuss et al., 2009).

*Rhodococcus equi* is one of the most common causes of foal pneumonia, which is the leading cause of morbidity and mortality in foals in the United States (Cohen et al., 1994). As such, it exerts a major financial impact on the equine industry. On premises where the disease is endemic, morbidity rates exceed 40 % (Chaffin et al., 2008, 2011) and costs associated with early diagnosis, veterinary care, long-term therapy, and animal mortality is exorbitant. In addition to significant immediate costs, *R. equi* pneumonia has a long-term detrimental effect on the equine industry because foals that recover from the disease are less likely to race as adults (Ainsworth et al., 1998). To date, there is no approved vaccine effective against *R. equi* and although administration of hyper-immune plasma to neonatal foals has some prophylactic value it is expensive, labor-intensive, and not uniformly effective (Martens et al., 1989, Hurley and Begg, 1995, Giguère et al., 2002).

#### Therapy and therapeutic challenges

A wide variety of antimicrobial agents are active against *R. equi in vitro*. However, many of these drugs are reported to be ineffective *in vivo*, likely because of poor cellular uptake and resulting low intracellular concentrations. The combination of a macrolide and rifampin has been the mainstay of therapy in foals infected with *R. equi* since the early 1980s (Hillidge, 1987) with only isolated reports of resistance. In recent years, clarithromycin or azithromycin, both newer generation macrolides, often replace erythromycin in the combination with rifampin (Giguère et al., 2004). Macrolides and rifampin are highly active and act synergistically against *R. equi in vitro* but only exert bacteriostatic activity (Giguère et al., 2012, Nordmann and Ronco, 1992). Despite recommended therapy with a macrolide and rifampin, the mortality rate of foals with



clinical signs of pneumonia caused by *R. equi* is approximately 30% (Ainsworth et al., 1998, Giguère et al., 2004). The mortality rate of foals with extrapulmonary disorders associated with *R. equi* is even worse at 57% (Reuss et al., 2009). Therefore, there is a need for more effective antimicrobial agents for the treatment of foals infected with *R. equi*. Because of the lack of effective preventative strategies, control of *R. equi* infections on many farms on which the disease is endemic relies on early detection of disease using pulmonary ultrasonography and initiation of treatment with antimicrobial agents prior to development of clinical signs (Slovis et al., 2005). Although controlled studies are lacking, this approach appears to have decreased mortality due to *R. equi* pneumonia on farms with endemic disease (McCracken and Slovis, 2009, Slovis et al., 2005, Venner et al., 2012). A limitation of this technique is that it does not allow imaging of the deeper lung tissue beyond the surface, such that large *R. equi* abscesses may be present in e.g. the mediastinal lymph nodes with minimal ultrasonographic change at the lung surface. However, in the authors' experience, this situation is comparatively rare.

#### Antimicrobial resistance, especially to macrolides and rifampin

Development of antimicrobial resistance in the face of selection pressure is not a recent phenomenon. It was first recognized very quickly after the first antibiotics (sulfonamides and penicillin) came into use (Abraham et al., 1941, Kirby and Rantz, 1942). Research specifically investigating this phenomenon followed shortly after (Kirby and Rantz, 1943, Waksman et al., 1945) with several other novel investigations being published during the 1940s.

There was a resurgence of interest with the discovery of multiple drug resistant bacteria in the 1960s (Watanabe and Fukasawa, 1961), and over the past 2 decades, antimicrobial resistance in bacteria has become of increasing concern due to increasing prevalence and emergence of bacterial strains resistant to all available therapies. Hauntingly, Alexander Fleming, the

discoverer of penicillin, forewarned about the potentials for development of bacterial resistance at the conclusion of his Noble Lecture in 1945: “There may be a danger, though, in under-dosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant. Here is a hypothetical illustration. Mr. X. has a sore throat. He buys some penicillin and gives himself, not enough to kill the streptococci but enough to educate them to resist penicillin. He then infects his wife. Mrs. X gets pneumonia and is treated with penicillin. As the streptococci are now resistant to penicillin the treatment fails. Mrs. X dies. Who is primarily responsible for Mrs. X’s death? Why Mr. X whose negligent use of penicillin changed the nature of the microbe. *Moral:* If you use penicillin, use enough”. Fleming was essentially describing the modern day mutant selection window or concentration hypothesis (Drlica, 2003).

A recent study has identified a significant increase in the cumulative incidence of macrolide and rifampin resistance over the last 10 years (Giguère et al., 2010). The temporal association between the widespread preventative/early therapeutic use of macrolides and rifampin and a perceived increase in the frequency of detection of resistant isolates in the last decade suggest that this practice may not be innocuous and that mass antimicrobial treatment of subclinically affected foals may have selected for antimicrobial resistance. The odds of death are 7 times higher in foals infected with resistant isolates (Giguère et al., 2010), and currently, there is no known effective treatment for foals infected with macrolide and rifampin resistant isolates. The problem of increasing resistance of bacteria to macrolides and rifampin extends beyond

equine rhodococcosis: these drugs are widely used in human medicine for the treatment of tuberculosis (rifampin) and of respiratory tract, skin, and genital tract infections (macrolides). Recently, accumulation of macrolide resistance in clinically important pathogens including *Shigella sonnei*, *Mycoplasma gallisepticum*, *Treponema pallidum*, *Campylobacter coli*, and *Nisseria gonorrhoeae* has been recognized in a wide variety of human and animal populations subjected to intensive macrolide treatment (Boumghar-Bourtchai et al., 2008, Chisholm et al., 2009, Gerchman et al., 2011, Juntunen et al., 2011, Stamm et al., 2010).

A cluster-randomized clinical trial in children demonstrated that resistance of nasopharyngeal *Streptococcus pneumoniae* to macrolides was significantly higher in communities randomized to mass azithromycin treatment compared to control communities not receiving antimicrobial agents (Skalet et al., 2010). Azithromycin sensitivity has been shown to increase again following cessation of the mass antimicrobial treatment for pneumococcal disease, but not until at least 24 months after treatments stopped (Haug et al., 2010). There is also evidence that sensitivity is much slower to return after macrolide exposure compared with exposure to other antibiotics, with the return also being significantly slower for azithromycin as compared with other macrolides (Kuster et al., 2014). This, along with the role of macrolides and rifampin in the treatment of various important bacterial pathogens of people, makes potential widespread macrolide- and rifampin-resistance on horse farms particularly concerning. Macrolide-resistant bacteria have been identified from air samples collected at least 150 m downwind from a swine facility (Gibbs et al., 2006) and multiple macrolide and tetracycline resistance genes found in pooled stored swine manure destined for fertilizer (Whitehead and Cotta, 2013). These serve as examples of how resistant bacteria may spread from the farm environment. With respect to horses, increasingly frequent transport of horses within the

continent and abroad, and the inherently close contact of horses with people underscore the risk of transmission of macrolide-resistant isolates. Macrolide resistance genes are typically located on mobile elements such as plasmids or transposons and can be transferred across bacterial genera (Roberts, 2011). These mobile elements may carry resistance to multiple classes of antimicrobial and to metal ions, heavy metals and quaternary ammonium compounds (Gullberg et al., 2014). Thus, resistance is likely to be present in other bacterial species as well at horse farms where mass therapy with macrolide and rifampin is common practice. Thus, the risk to human health may not only be related to the zoonotic potential of *R. equi* infection but also to emergence and propagation of other resistant bacterial pathogens.

The only viable long term approach to prevent emergence of resistant *R. equi* will be to decrease widespread use of antimicrobial in foals with subclinical pulmonary abscesses on endemic farms. In recent studies, conducted at a large horse breeding farm, it was demonstrated that many foals with subclinical pulmonary abscesses recover without therapy (Venner et al., 2012, 2013<sup>a,b</sup>). In the same studies, antimicrobial therapy did not accelerate spontaneous healing of subclinical pulmonary abscesses compared to administration of a placebo indicating that treatment of all foals with subclinical lesion is not necessary (Venner et al., 2012, 2013<sup>a,b</sup>). However, in the short to medium term, there is a tremendous need for a safe and effective antimicrobial agent for the treatment of foals infected with macrolide-resistant *R. equi* isolates. As stated above, there is also a need for more effective antimicrobial agents for the treatment of foals infected with macrolide-susceptible *R. equi*.

#### Gentamicin sulfate and its use in horses

Gentamicin sulfate is an aminoglycoside antibiotic that was discovered in the early 1960s as a product of the bacterium *Micromonospora purpureochromogenes* (Family,

Micromonospora; Suborder, Micromonosporaceae; Order, Actinomycetales; Phylum, Actinobacteria) (Br. Med J., 1967). Aminoglycosides exert concentration dependent bacterial killing characteristics. Their rate of killing increases as the drug concentration increases above the minimum inhibitory concentration (MIC) for a given pathogen with optimal maximum serum concentration ( $C_{max}$ ) to MIC ratio of 8-10:1 (Ebert et al., 1990, Moore et al., 1987). Based on the current guidelines from the Clinical and Laboratory Standards Institute (CLSI) bacterial isolates from horses with a MIC  $\leq 2 \mu\text{g/mL}$  are considered susceptible to gentamicin. As result, target peak gentamicin concentrations should be around 16-20  $\mu\text{g/mL}$ . Higher  $C_{max}$  also results in a longer post-antibiotic effect, and decreases selection of resistant bacterial mutants within a population (Jackson et al., 1990, Karlowsky et al., 1994). The adverse effect of gentamicin most recognized in horses is nephrotoxicity but because renal cellular uptake of gentamicin is saturable, nephrotoxicity is associated with insufficiently low trough levels, rather than excessively high peaks (Mingeot-Leclercq and Tulkens, 1999). The maximal trough concentration to minimize the risk of nephrotoxicity is unknown in horses. In human patients, trough gentamicin concentrations  $> 2 \mu\text{g/mL}$  have been associated with nephrotoxicity and troughs  $> 4 \mu\text{g/mL}$  for over 10 days with ototoxicity (Beringer and Winter, 2010, Dahlgren et al., 1975, Goodman et al., 1975). Thus, optimal trough concentration for minimizing nephrotoxicity after administration of gentamicin to human patients is controversial, with recommendations ranging from maintaining a trough  $< 2 \mu\text{g/mL}$  to maintaining a period of  $< 0.5 \mu\text{g/mL}$  for at least 4 hours (Touw et al., 2009, Nicolau et al., 1995, Pacifici et al., 2009). Maintaining a low trough concentration is not only important for reducing toxicity, but also in avoiding development of adaptive resistance. Adaptive resistance is a reversible down-regulation of the active transport of aminoglycoside into Gram-negative bacteria (Barclay and Begg, 2001). This resistance

phenomenon is induced rapidly after a dose of aminoglycosides but is reversible during drug free intervals. As a result, prolongation of the dose interval may enable time for the return of bacterial susceptibility before the subsequent dose (Barclay and Begg, 2001, Pagkalis, 2011).

By applying these principles, a dose of 6.6 mg/kg IV q 24 h has been recommended for gentamicin in adult horses (Magdesian et al., 1998, Santschi and Papich, 2000). However, this same dose may not be appropriate for immature horses, especially neonates. Because aminoglycosides are water-soluble and thus highly distributed in extracellular fluid (ECF), their clinical pharmacokinetics are strongly influenced by variations in patient ECF volume due to disease state or age (Beringer and Winter, 2010, Rea et al., 2008). For example, compared with adults, human neonates require a higher dose of gentamicin to achieve therapeutic peak concentrations because of their greater percentage of body water which leads to a significantly higher volume of distribution: 0.43 – 1.1 L/kg for neonates versus on average 0.25 L/kg for adults (Beringer and Winter, 2010, Touw et al., 2009). Although there are multiple studies reporting the pharmacokinetics of low dose multiple daily administration of gentamicin in foals (Abo El Sooud et al., 2003, Rasis et al., 1998, Riviere et al., 1983, Baggot et al., 1986, Cummings et al., 1990), there is little information on the pharmacokinetics of once-daily gentamicin. Therapeutic drug monitoring in a small number of critically ill foals, in various clinical settings, indicates that the standard adult dose of 6.6 mg/kg q 24 h for gentamicin is too low and a dose of 10-14 mg/kg has been proposed based upon these clinical observations (McKenzie and Furr, 2003, Geor and Papich, 2003).

Gentamicin is by far the most active drug against both macrolide-susceptible and macrolide-resistant isolates of *R. equi* in vitro (Giguère et al., 2010, Carlson et al., 2010, Jacks et al., 2003). In addition, gentamicin is one of the few antimicrobial agents that is bactericidal

(rather than bacteriostatic) against *R. equi* at therapeutic concentrations (Giguère et al., 2012, Nordmann and Ronco, 1992). However, being a highly water-soluble drug, gentamicin has poor intracellular penetration (Gamzo et al., 2007), which likely explains the reported lack of efficacy against *R. equi in vivo*. In a retrospective study, all 17 foals with *R. equi* pneumonia treated with the combination of penicillin and gentamicin died despite the fact that all isolates were sensitive to gentamicin (Sweeney et al., 1987). A delivery system that could enhance intracellular delivery of gentamicin would likely increase its *in vivo* efficacy against *R. equi* and prove more effective than conventional therapy with bacteriostatic agents.

#### Liposomes and liposomal gentamicin

Liposomes are 0.08 – 5 µm diameter lipid vesicles composed of one or several amphiphile bilayers surrounding an aqueous core (Gamzo et al., 2007, Drummond et al., 2008). After administration, liposomes rapidly localize in cells of the mononuclear phagocyte system via phagocytosis, and are enclosed in phagosomes within the cytoplasm (Raz et al., 1981). Due to this behavior, liposomes can be used as carriers of antibiotics for the treatment of infections caused by intracellular pathogens (Gamzo et al., 2007, Briones et al., 2008). They also extravasate at sites of increased vascular permeability and are readily taken up into sites of inflammation, enabling further drug targeting to organ specific infection or neoplasia (Bakker-Woudenberg et al., 1994, Drummond et al., 1999). Encapsulation in liposomes can also increase the therapeutic index of drugs with toxic side effects via more targeted drug delivery and altered drug metabolism. Specifically, liposomal aminoglycosides have altered pharmacokinetics such that drug accumulation in the kidney is reduced and nephrotoxicity decreased (Drummond et al., 2008). There are many different combinations of phospholipids that can be used to make liposomes. The specific lipid composition and method of formulation affect the rate at which

liposomes are taken up by phagocytes, the extent to which they localize in infected/neoplastic tissues and the encapsulation efficiency and stability of the internalized drug (Drummond et al., 2008, Vitas et al., 1996). At the most basic level, liposomes can be divided into two main categories: conventional liposomes, which are composed of phosphatidylcholine and cholesterol, and sterically stabilized liposomes, which have a hydrophilic polymer coating, usually polyethylene glycol (PEG). The PEG coating acts as a protective layer, reducing the rate of association with serum proteins and uptake by phagocytes, thus prolonging their systemic circulation time. A balance between stability in the circulation, uptake by the tissue in question, and optimal drug encapsulation needs to be achieved in order for liposomes to be effective drug carriers (Drummond et al., 1999).

Previous work has shown that liposomal gentamicin has significantly enhanced intracellular penetration and bacterial killing in macrophage cell cultures infected with other types of intracellular bacteria such as *Listeria monocytogenes*, *Mycobacterium avium*, *Salmonella* spp., and *Brucella abortus* (Lutwyche et al., 1998, Gamzo et al., 2007, Vitas et al., 1996). *In vivo*, liposomal gentamicin has already been used successfully in the treatment of infections caused by several species of intracellular bacteria in rodents, rabbits, guinea pigs, and humans (Bakker-Woudenberg et al., 1994, Gamzo et al., 2007, Klemens et al., 1990). Neutral phosphatidylcholine based liposomes have been shown to localize in phagosomes after engulfment by macrophages (Raz et al., 1981). Thus, co-localization of liposomal gentamicin with *R. equi* in the phagosome could allow early intracellular killing of *R. equi*. Free gentamicin will not enter the cell and only be able to kill *R. equi* once the macrophage cell membrane becomes damaged and permeable 24 hours into infection (Toyooka et al., 2005). Antibiotics encapsulated in liposomes can also be administered via inhalation (nebulization), which can



facilitate higher concentrations locally in the lung compared with systemic administration. For example, after direct intrabronchial administration of liposomal gentamicin in rabbits, concentrations of gentamicin in BAL cells were found to be significantly higher than following free gentamicin, where concentrations were undetectable (Demaeyer et al., 1993). For example, rifampin loaded liposomes have been nebulized for the treatment of the *Mycobacterium avium*, which survives and replicates within pulmonary macrophages (Zaru et al., 2009). Nebulized liposomal amikacin (Arikace) has been shown to be significantly more efficacious than nebulized free amikacin for the treatment of chronic *Pseudomonas aeruginosa* infection and has demonstrated efficacy and safety in human patients during Stage II and initial Stage III trials (Meers et al., 2008, Clancy et al., 2013, Ehsan et al., 2014). Aminoglycosides are often nebulized to foals with bacterial respiratory disease (McKenzie et al., 2000, 2004), and incorporation of the drug into liposomes may improve delivery to the infected alveolar macrophages and reduce non-specific systemic uptake. Recently, the safety and biodistribution of IV PEG-coated liposomes was examined *in vivo* in adult horses using radiolabeled PEG-coated liposomes. A single dose of 0.24  $\mu\text{mol/kg}$  radiolabelled  $^{99\text{m}}\text{Tc-25-PEG}$  liposomes and 2.4 $\mu\text{mol/kg}$  unlabeled PEG-liposomes was given to 10 horses with no adverse effects noted (Underwood et al., 2012). Furthermore, in a soft tissue abscess model in horses, PEG liposomes were seen to concentrate and persist at the site of infection (Underwood et al., 2011).

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## CHAPTER 3

### MACROLIDE- AND RIFAMPIN-RESISTANT *RHODOCOCCLUS EQUI* ON A HORSE BREEDING FARM, KENTUCKY, USA<sup>1</sup>

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## Abstract

Macrolide and rifampin resistance developed on a horse breeding farm after widespread use was instituted for treatment of subclinical pulmonary lesions in foals. Resistance occurred in 6 (24%) of 25 pretreatment and 8 (62%) of 13 (62%) post-treatment isolates from affected foals. Drug-resistant isolates formed 2 distinct genotypic clusters.

## Introduction

*Rhodococcus equi* is a major cause of pneumonia in young horses and a common opportunistic pathogen of immunocompromised humans<sup>1</sup>. Over the past decade, control of *R. equi* infections at many horse farms to which the disease is endemic has relied on early detection of subclinical pulmonary disease by use of thoracic ultrasonography and initiation of treatment with antimicrobial drugs before development of clinical signs<sup>2</sup>. This approach appears to have decreased deaths caused by *R. equi* pneumonia at some farms, although controlled studies are lacking<sup>2</sup>. However, the temporal association between widespread use of macrolides and rifampin and a perceived increase in the frequency of detection of drug-resistant isolates in the past decade<sup>3</sup> suggest that this practice may not be innocuous. We describe emergence of resistance to macrolides and rifampin among *R. equi* isolates obtained from a horse breeding farm. We conducted this study after initiation of an ultrasonographic screening program on the farm and resulting widespread use of these drugs in foals with subclinical pulmonary lesions.

## The Study

This study was conducted at a Thoroughbred horse breeding farm in Kentucky, USA. The farm initiated an ultrasonographic screening program in 2001 in an attempt to decrease deaths associated with pneumonia caused by *R. equi* through early diagnosis and treatment of foals with subclinical lesions. During March–July 2010, the farm reported 9 foals infected with

macrolide- and rifampin-resistant *R. equi* isolates. This finding led to a disease investigation consisting of retrospective data collection (Table 3.1), collection of air samples in September 2010 to determine the prevalence of drug-resistant *R. equi* in the environment, and prospective culture of pulmonary lesions from all foals in 2011 before initiation of antimicrobial drug therapy. A total of 124 air samples were collected from the 4 barns at the farm and from each of their respective surrounding paddocks by using a portable air-sampling device as described<sup>4</sup>. For the 2011 breeding season, a tracheobronchial aspirate was collected transendoscopically from each foal that had ultrasonographically detected pulmonary lesions before initiation of therapy by using a triple-guarded microbiological aspiration catheter. A second tracheobronchial aspirate was collected by using the same method 2 weeks after initiation of therapy. Decisions regarding the need for therapy and selection of antimicrobial agents were made by the farm veterinarian and manager.

After standard microbiological culture of air and tracheobronchial aspirate samples, confirmation of *R. equi* was accomplished by amplification of the *choE* gene and detection of the virulence plasmid by amplification of the *vapA* gene by using multiplex PCR<sup>5</sup>. For each isolate, the MICs of erythromycin, azithromycin, clarithromycin, and rifampin were determined from 3–5 isolated colonies by broth dilution in accordance with Clinical and Laboratory Standards Institute guidelines<sup>6</sup>. *R. equi* isolates with MIC values <2 µg/mL for azithromycin and clarithromycin, <0.5 µg/mL for erythromycin, and <1 µg/mL for rifampin were considered susceptible, and isolates with MIC values >8 µg/mL were considered resistant.

The similarity of *R. equi* isolates from air and tracheobronchial aspirate samples was determined by using a repetitive sequence-based PCR (DiversiLab; bioMérieux Inc., Durham, NC, USA) previously validated for *R. equi*<sup>7</sup>. Isolates were clustered as the same strain on the

basis of >95% similarity and a difference of <1 band<sup>8</sup>. The ultrasonographic screening program was introduced in 2001, and the first isolates of *R. equi* resistant to macrolides and rifampin were identified in 2008 (Table 3.1). There were no *R. equi* isolates that were resistant to either a macrolide or rifampin alone. Air sampling yielded 82 isolates of *R. equi*. Of these isolates, 15 (18%) contained the plasmid required for virulence in foals and 67 (82%) were avirulent. All 15 virulent isolates and 23 randomly selected avirulent isolates were used for in vitro antimicrobial drug susceptibility testing and genotyping. Two (5%) of 38 isolates tested were resistant to azithromycin, clarithromycin, erythromycin, and rifampin. One resistant isolate was virulent and came from an outdoor paddock location that had been used to house foals given a diagnosis of *R. equi* pneumonia caused by drug-resistant isolates. The other resistant isolate was avirulent and was isolated from an air sample obtained from an indoor barn location.

During the 2011 season, 132 foals were born on the farm. Thoracic ultrasonography showed evidence of pulmonary disease in 27 (20%) foals. Culture of a tracheobronchial aspirate before initiation of therapy yielded *R. equi* in 25 (93%) of the 27 foals sampled (Table 3.1). Of the 25 pretreatment *R. equi* isolates, 6 (24%) were resistant to macrolides and rifampin. Twenty-four foals were treated with clarithromycin and rifampin. Tracheobronchial aspirates were collected 2 weeks after initiation of therapy in 19 foals, and *R. equi* was cultured from 13 (68%) of the 19 samples. Of these 13 posttreatment isolates, 8 (62%) were resistant to macrolides and rifampin. After identification of resistant isolates, 3 foals had a third antimicrobial agent added to their therapy (gentamicin, n = 2; doxycycline, n = 1). All foals with a diagnosis of pneumonia caused by *R. equi* in 2011 survived.

Genotypes by repetitive sequence–based PCR from all 2010 isolates grouped into 1 main cluster consisting of resistant isolates from 5 foals and 1 air sample (Figure 3.1, cluster A).

Isolates resistant to macrolides and rifampin from the 2011 foals grouped closely, forming 4 clusters (B–E); the largest cluster (B) contained 7 isolates (Figure 3.2). Of the 11 foals for which pretreatment and posttreatment samples were collected in 2011, a total of 5 had similar pretreatment and posttreatment isolates and 6 had different pretreatment and posttreatment isolates (Table 3.2).

### Conclusions

Recently, development of macrolide resistance in clinically relevant pathogens has been recognized in human and animal populations that received intensive macrolide treatment<sup>9,10</sup>. We documented that macrolide- and rifampin-resistant isolates of *R. equi* occurred 7 years after initiation of an ultrasonographic screening program, which resulted in treatment of all foals with subclinical pulmonary lesions.

Compared with macrolide-susceptible *Campylobacter jejuni*, acquisition of macrolide resistance impairs the fitness and transmission of the pathogen in chickens, suggesting that the prevalence of macrolide-resistant *C. jejuni* would probably decrease in the absence of antimicrobial drug selection pressure<sup>11</sup>. Similarly, studies in humans have shown that macrolide resistance in *Streptococcus pneumoniae* decreased 2–5 years after use of azithromycin was stopped and selection pressure was abolished<sup>10</sup>. Although the fitness cost of macrolide resistance among *R. equi* isolates might be sufficient to ensure its eventual elimination, this elimination will take time and elimination of resistance will only occur in the absence of antimicrobial drug selection pressure<sup>10</sup>.

A recent study on a large horse farm indicated that the proportion of foals with ultrasonographic pulmonary lesions associated with *R. equi* infection that recovered was not different between foals given azithromycin and rifampin and foals given a placebo<sup>12</sup>. This

surprising finding, combined with the apparent increase in macrolide- and rifampin-resistance demonstrated in the present study, support the need to stop the practice of mass macrolide treatment for subclinical infection with *R. equi* in foals on horse breeding farms. The goal should be to more accurately identify, of the many subclinically infected foals, which few are likely to show development of disease and thus require treatment for it.

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Tables

**Table 3.1.** Table 1. Macrolide- and rifampin-resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA\*

Year	No. of foals born	No. of foals with lesions that were treated		No. of foals with positive <i>R. equi</i> culture	
		(% total foals)	No. of foals cultured	No. of foals with positive <i>R. equi</i> culture	No. of foals with macrolide and rifampin-resistant <i>R. equi</i> (%)
2001	95	30 (32)	30	30	0 (0)
2002	117	53 (45)	0	NA	NA
2003	148	58 (32)	2	?	?
2004	181	88 (49)	28	19	0 (0)
2005	168	70 (42)	30	?	?
2006	170	42 (41)	5	2	0 (0)
2007	181	93 (51)	4	?	?
2008	171	52 (30)	21	16	4 <sup>†</sup> (25)
2009	162	50 (31)	30	22	5 <sup>†</sup> (23)
2010	138	45 (33)	28	22	9 <sup>†</sup> (41)
2011	132	24 (18)	27	25	9 <sup>†</sup> (36)

\*NA, not applicable; ?, data missing from farm records. †Includes pre-treatment and post-treatment isolates.

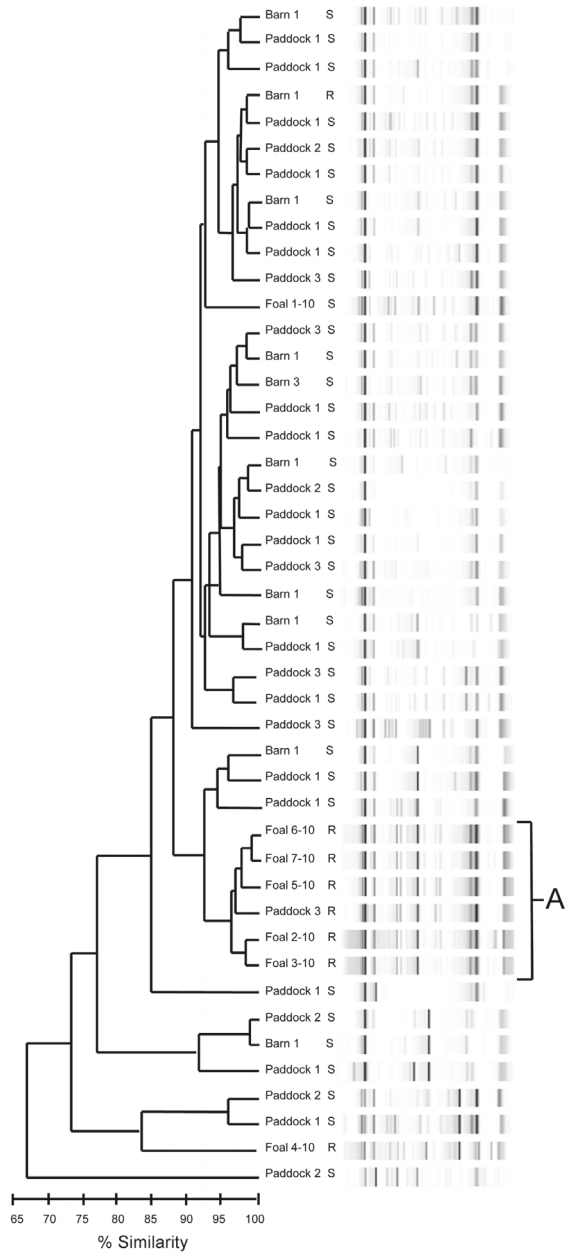
**Table 3.2.** Characteristics of pre-treatment and post-treatment *Rhodococcus equi* isolates obtained from foals on a horse breeding farm, Kentucky, USA, 2011\*.

2011 Foal ID	Pre-treatment		Post-treatment
	isolates (cluster)	Similarity (%)	isolates (cluster)
7-11	S	97.5	S
5-11	S	84.3	S
14-11	R (E)	96.4	R (E)
3-11	R (B)	96.5	R (B)
4-11	R (B)	97.4	R (B)
17-11	R (C)	98.5	R (C)
20-11	S	83.1	R (B)
18-11	S	78	R (C)
11-11	R (B)	87.2	S
15-11*	S	83.1	S
	-	73.2	R (D)
16-11*	S	78.8	R (D)
	R (B)	80.8	-

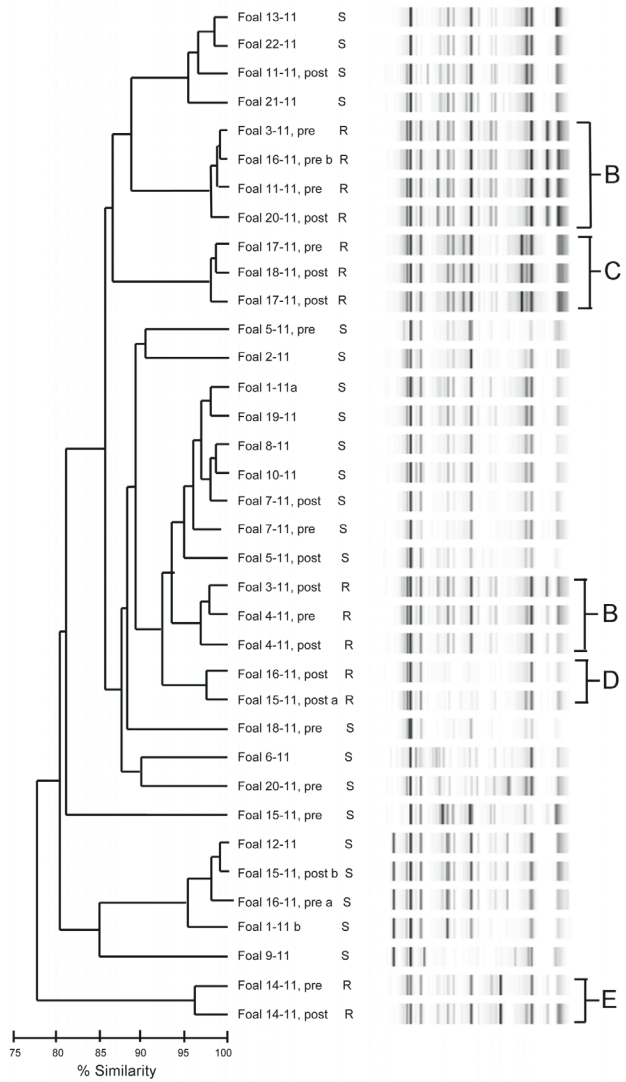
\*ID, identification; S, susceptible; R, resistant; NA, not applicable. †Determined using Pearson correlation coefficient and unweighted pair- group method with arithmetic means. ‡Two isolates were recovered from the pretreatment (foal ID 16-11) or posttreatment (foal ID 15-11) sampling.

## Figures

**Figure 3.1.** Dendrogram and virtual gel repetitive sequence–based PCR fingerprint patterns of foal and air (barn and paddock)–derived isolates of *Rhodococcus equi* on a horse breeding farm, Kentucky, USA, 2010. Macrolide and rifampin susceptibility (S) or resistance (R) are indicated. A indicates the main cluster of drug-resistant isolates (5 foal and 1 air).



**Figure 3.2.** Dendrogram and virtual gel repetitive sequence– based PCR fingerprint patterns of 36 *Rhodococcus equi* isolates obtained from foals on horse breeding farm, Kentucky, USA, 2011. Macrolide and rifampin susceptibility (S) and resistance (R) are indicated. B–E indicates clusters of drug-resistant isolates. Foals from which pretreatment (pre) and post-treatment (post) samples were obtained are indicated. a and b indicate samples from which 2 isolates were obtained.



CHAPTER 4  
EFFECT OF AGE ON THE PHARMACOKINETICS OF A SINGLE DAILY DOSE OF  
GENTAMICIN SULFATE IN FOALS<sup>2</sup>

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## Abstract

Therapeutic drug monitoring in a small number of foals of various ages indicates that the standard adult dose of 6.6 mg/kg bwt q. 24 h for gentamicin is too low and a dose of 12 mg/kg bwt has been proposed. The pharmacokinetics of this dosage in foals and the ages at which this higher dose should be used have not previously been investigated. The objective of this study was to determine the effect of age on the pharmacokinetics of a single 12 mg/kg bwt i.v. dose of gentamicin in foals. Six healthy foals were given a single i.v. dose of gentamicin at 1-3 days, 2, 4, 8 and 12 weeks of age. Plasma concentrations were measured using LC-MS/MS. Elimination half-life (mean  $\pm$  s.d.) was significantly longer in 1-3-day-old foals ( $8.2 \pm 2.0$  h) than in foals 4 weeks of age ( $3.7 \pm 1.5$  h) or older. Volume of distribution was significantly higher in 1-3-day-old foals ( $0.75 \pm 0.20$  l/kg bwt) than in 8- ( $0.27 \pm 0.10$  l/kg bwt) or 12-week-old foals ( $0.29 \pm 0.11$  l/kg bwt). Concentrations of gentamicin 1 h after administration were significantly lower in 1-3-day-old foals ( $20.52 \pm 2.07$   $\mu$ g/ml) than in all other age groups ( $>42.16 \pm 17.57$   $\mu$ g/ml). Concentrations of gentamicin 24 h after administration were significantly higher in the 1-3-day-old foals ( $1.97 \pm 0.90$   $\mu$ g/ml) than in all the other age groups ( $<0.85 \pm 0.46$   $\mu$ g/ml). The pharmacokinetics of gentamicin change considerably in the first 2 weeks of life. Intravenous administration of gentamicin at a dose of 12 mg/kg bwt q. 36 h would be required in foals less than 2 weeks of age. In foals 2 weeks of age or older, a lower dose of 6.6 mg/kg bwt given q. 24 h was predicted to be adequate.

## Introduction

Bacterial sepsis is the leading cause of morbidity and mortality in neonatal foals<sup>1-3</sup>. Gram-negative bacteria account for 70-95% of the microorganisms isolated from blood cultures with *Escherichia coli* being the most common isolate<sup>2-4</sup>. Given that enteric Gram-negative



microorganisms predominate, aminoglycosides such as gentamicin or amikacin are commonly used in conjunction with a beta-lactam antimicrobial to provide coverage against Gram-positive bacteria. Amikacin has traditionally been considered the agent of choice for the treatment of sepsis in equine neonates with normal renal function because of a slightly broader spectrum against Enterobacteriaceae<sup>2</sup>. However, in a recent study, amikacin and gentamicin were both active *in vitro* against approximately 84% of 554 bacterial isolates from 423 bacteremic foals<sup>4</sup>. Major advantages of gentamicin over amikacin include widespread availability and considerably lower cost.

Aminoglycosides exert concentration dependent bacterial killing characteristics. Their rate of killing increases as the drug concentration increases above the minimum inhibitory concentration (MIC) for a given pathogen with optimal maximum serum concentration ( $C_{max}$ ) to MIC ratio of 8-10:1<sup>5,6</sup>. Higher  $C_{max}$  also results in a longer post-antibiotic effect, and decreases selection of resistant bacterial mutants within a population<sup>7,8</sup>. By applying these principles, a dose of 6.6 mg/kg IV q 24 h has been recommended for gentamicin in adult horses<sup>9,10</sup>. Because aminoglycosides are water-soluble and thus highly distributed in extracellular fluid (ECF), their clinical pharmacokinetics are strongly influenced by variations in patient ECF volume due to disease state or age<sup>11,12</sup>. For example, compared with adults, human neonates require a higher dose of gentamicin to achieve therapeutic peak concentrations because of their greater percentage of body water which leads to a significantly higher volume of distribution: 0.43 – 1.1 L/kg for neonates versus on average 0.25 L/kg for adults<sup>11,13</sup>.

Although there are multiple studies reporting the pharmacokinetics of low dose multiple daily administration of gentamicin in foals<sup>14,17</sup>, there is little information on the pharmacokinetics of once-daily gentamicin. Therapeutic drug monitoring in a small number of

critically ill foals, in various clinical settings, indicates that the standard adult dose of 6.6 mg/kg q 24 h for gentamicin is too low and a dose of 10-14 mg/kg has been proposed based upon these clinical observations<sup>18,19</sup>. However, the pharmacokinetics of higher single daily doses of gentamicin in foals and the ages at which these higher dosages should be used have not been investigated. The objective of the present study was to determine the effect of age on the pharmacokinetics of a single IV 12 mg/kg dose of gentamicin in healthy foals.

## Materials and Methods

### *Animals*

Three male and 3 female Quarter Horse foals considered healthy on the basis of a thorough physical examination, complete blood count, plasma biochemical profile, and immunoglobulin G (IgG) concentrations were used. The foals ranged in weight from 45 to 63 kg at 1-3 days of age, 55 to 86 kg at 2 weeks of age, 76-143 kg at 4 weeks of age, 107-146 kg at 8 weeks of age, and from 133 to 177 kg at 12 weeks of age. The foals were kept with their dams in individual stalls during the experiment with *ad libitum* access to grass hay and water. The study was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

### *Experimental design and sample collection*

Gentamicin sulfate (Butler Schein Animal Health, Dublin, OH, USA) was administered intravenously (IV) at a dose of 12 mg/kg at 5 different ages. The first administration occurred when the foals were 1-3 days of age and was repeated on the same 6 foals at 2, 4, 8, and 12 weeks of age. Gentamicin sulfate was administered as a single IV bolus through a jugular vein catheter. Blood samples were obtained from a catheter placed in the opposite vein at 0 (prior to administration), 5, 10, 15, 20, 30, 45, 60, and 90 minutes as well as 2, 3, 4, 6, 8, 12, 16 and 24 hours after administration of the drug. Samples were centrifuged at  $1500 \times g$ , after which the

plasma was collected and stored frozen at -20°C until analysis. To monitor renal function, plasma creatinine was measured on heparinized blood samples collected before, and 24 hours after each dose of gentamicin sulfate.

*Drug analysis in plasma and body fluids by liquid chromatography tandem mass spectrometry (LC-MS/MS)*

The concentration of gentamicin sulfate in foal plasma was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS), adapted from previously described techniques<sup>20</sup>. Briefly, gentamicin was extracted from plasma (500 µL) using protein precipitation with an equal volume of ice-cold 90:10 acetonitrile: 0.2% formic acid (v/v). Extracted samples were centrifuged (2°C at 10,000 × g for 10 min), and the resultant supernatants were transferred to glass vials and capped. Calibration standards were prepared in drug-free foal plasma and extracted as described above. A concentration range of 0.09 –200 µg/mL of gentamicin sulfate was used to construct standard curves. The lower limit of quantification (LOQ) was 0.09 µg/mL. The inter-assay coefficient of variation was < 10% at concentrations 200-6.25 µg/ml and ≤ 15% at concentrations 6.25-0.09 µg/ml. The analytes were separated on an Agilent Eclipse XDB C18 column (100 x 4.6 mm i.d., 3.5 µm) equipped with a Phenomenex Security Guard C18 guard column (4.0 x 3.0 mm). Analytes were eluted from the column using isocratic mobile phase consisting of 0.2% formic acid and acetonitrile (1:1, v/v). LC-MS/MS measurement of gentamicin was performed on an Agilent 1100 HPLC system coupled to an Agilent XCT Ultra Plus ion-trap mass spectrometer (Santa Clara, CA, USA). The samples were introduced into the MS with a flow rate of 0.25 mL/min using an Electrospray Ionization (ESI) source. Injection volume was 20 µL with a total run-time of 5 minutes. Nitrogen gas was used as desolvation gas (flow rate of 8 L/min at 350 °C) and as nebulizer gas (30 psi), and helium was used as a

collision gas. Capillary voltage was set at 3500 V, skimmer was set at 40 V, and cap exit was set at 158.5 V. Mass spectra were acquired in positive-ion mode, and mass transitions were monitored using multiple-reaction monitoring (MRM). The MRM transition from  $m/z$  478  $\rightarrow$  322 was used for gentamicin quantification.

### *Pharmacokinetic analysis*

For each foal, 1-, 2-, and 3-compartment models were fitted to the plasma concentration versus time data by use of a computer software package (WinNonlin Professional v 5.1, Pharsight, Cary, NC). A linear two-compartment model with weighting by the inverse of the model predicted drug concentrations ( $1/y$ ) was most appropriate, based upon computer assisted examination of residual plots, goodness of fit, the Akaike's information criterion, and the sum of squares:  $C_t = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$ ; where  $C_t$  is the serum drug concentration at time  $t$ ;  $e$  is the base of the Napierian logarithm;  $A$  and  $\alpha$  are the intercept and slope, respectively, of the distribution phase;  $B$  and  $\beta$  are the intercept and slope, respectively, of the elimination phase. Elimination half-life ( $t_{1/2\beta}$ ) was calculated as the natural logarithm of 2 divided by  $\beta$ . The area under the concentration-time curve (AUC) and the area under the first moment of the concentration-time curve (AUMC) was calculated using the trapezoidal rule, with extrapolation to infinity using  $C_{\text{last}}/\beta$ , where  $C_{\text{last}}$  is the plasma gentamicin concentration at the last quantifiable time point. Mean residence time (MRT) was calculated as:  $\text{AUMC}/\text{AUC}$ . Apparent volume of distribution based on the AUC ( $V_{d_{\text{area}}}$ ) was calculated as:  $\text{dose}/\text{AUC} \cdot \beta$  and systemic clearance (CL) was calculated from:  $\text{dose}/\text{AUC}$ . Based on the model fit for the single dose data, the individual model parameters for each foal were fixed and used to simulate steady state gentamicin concentration-versus-time plots at various dosages (6.6, 9, 12, 15 and 18 mg/kg) and dosing intervals (24, 36 and 48 h) for each age group (WinNonlin Professional v 5.1, Cary, NC).

### *Statistical Analysis*

Normality and equality of variance of the data were assessed with use of the Shapiro-Wilk and Levene tests, respectively. Data that were not normally distributed were log transformed. A one-way ANOVA for repeated measures was used to determine the effect of age group (1-3 days, 2 weeks, 4 weeks, 8 weeks, or 12 weeks) on each measured and calculated pharmacokinetic variable. When appropriate, multiple pairwise comparisons were done by use of the Holm-Sidak test. For each day of gentamicin administration, plasma creatinine concentrations prior to administration of gentamicin were compared to plasma gentamicin concentrations 24 h after gentamicin administration using a paired t test. For all analyses, significance was set at  $P < 0.05$ .

### Results

Measured gentamicin concentrations at selected time points and calculated pharmacokinetic variables for the different age groups are presented in Table 4.1. Increasing age resulted in a significant decrease in  $t_{1/2\beta}$ ,  $V_{d_{area}}$ , clearance, AUMC, MRT and concentration 24 h after administration ( $C_{24h}$ ), and a significant increase in  $C_{0.5h}$ ,  $C_{1h}$  and intercepts of the distribution and elimination phases (Table 4.1). Elimination half-life was significantly longer in 1-3 day-old foals than in foals 4 weeks of age or older (Figure 4.1). Volume of distribution was significantly higher in 1-3 day-old foals ( $0.75 \pm 0.20$  L/kg) compared with 8- ( $0.27 \pm 0.10$  L/kg), or 12- ( $0.29 \pm 0.11$  L/kg) week-old foals. Concentrations of gentamicin 1 h after administration were significantly lower in 1-3 day-old foals ( $20.52 \pm 2.07$   $\mu\text{g/mL}$ ), compared with all other age groups. Concentrations of gentamicin 24 hours after administration were significantly higher in the 1-3 day-old foals ( $1.97 \pm 0.90$   $\mu\text{g/mL}$ ), compared with all the other age groups.

Predicted minimum plasma concentrations at steady state after administration of gentamicin at a dose of 12 mg/kg q 24 h were  $2.08 \pm 0.91$   $\mu\text{g/mL}$  for the 1-3 day-old foals, and  $< 1\mu\text{g/mL}$  for all other age groups (Table 4.2). Increasing the dosing interval to q 36 h in 1-3 day-old foals was predicted to reduce trough concentrations to  $0.72 \pm 0.34$   $\mu\text{g/mL}$  (Table 4.2). Based on modeling of the data, in foals 2 weeks of age or older, a dose of 6.6 mg/kg q 24 h was predicted to maintain mean concentrations of gentamicin 1 h after administration above 20  $\mu\text{g/mL}$  (Table 4.2).

None of the foals experienced any adverse effects during the course of the study. Plasma creatinine concentrations remained within the reference interval throughout the study. There was a significant decrease in plasma creatinine concentrations 24 h after administration of gentamicin in 1-3 day-old (from  $1.45 \pm 0.43$  to  $1.15 \pm 0.22$  mg/dL) and in 4 week-old (from  $1.42 \pm 0.29$  to  $1.28 \pm 0.25$  mg/dL) foals. Creatinine concentrations pre- and post-administration of gentamicin were not significantly different at 2 weeks, 8 weeks, and 12 weeks of age.

## Discussion

Over the past 30 years, in line with improved understanding of the pharmacodynamics of aminoglycosides<sup>5,6</sup>, dosing of gentamicin in adult horses has moved away from low doses (2.2-3.3 mg/kg) every 6-12 hours, to an extended dosing interval of 6.6 mg/kg q 24 h<sup>9,21</sup>. The present study demonstrates that age has a profound effect on the pharmacokinetics of gentamicin, particularly in foals less than 4 weeks of age. Therefore, extrapolation of the 6.6 mg/kg q 24 h dose to neonatal foals would not be appropriate.

As expected based upon the declining proportion of body water and extracellular fluid with increasing age, mean  $V_d$  decreased with age, being at its highest in 1-3 day-old foals ( $0.75 \pm 0.20$  L/kg). This value was higher than those previously reported for foals of the same age

receiving lower doses of gentamicin ( $0.31 \pm 0.03$  L/kg), but comparable to that reported in in healthy foals ( $0.588 \pm 119$  L/kg to  $0.862$  L/kg) receiving the aminoglycoside amikacin at 21-25 mg/kg IV<sup>17,22,23</sup>. By 8 weeks of age, the  $V_d$  of the foals in the present study ( $0.26 \pm 0.1$  L/kg) was similar to that reported in adult horses ( $0.14 \pm 0.06$  to  $0.21 \pm 0.05$ ) administered IV gentamicin at 6.6 mg/kg<sup>9,10</sup>. The decrease in  $V_d$  within the first few weeks of life is well documented in human patients<sup>13</sup>. In addition, wide variations in the  $V_d$  of aminoglycosides have been reported in human neonates of similar actual and gestational ages, with premature infants usually requiring a higher dose due to an increased  $V_d$ <sup>24</sup>. Differences in  $V_d$  for a given age and weight are especially likely in conditions where there is significant third-spacing such as diseases causing edema or cavity effusions, which would result in an increased  $V_d$ <sup>11</sup>.

Pharmacodynamic data gathered over recent decades show that administration of aminoglycoside by an extended dosing interval scheme maximizes efficacy with the goal of achieving a peak plasma drug concentration ( $C_{max}$ ) to MIC ratio of 8-10. Based on the current guidelines from the Clinical and Laboratory Standards Institute (CLSI) bacterial isolates from horses with a MIC  $\leq 2$   $\mu\text{g/mL}$  are considered susceptible to gentamicin. As result, target peak gentamicin concentrations should be around 16-20  $\mu\text{g/mL}$ . The  $C_{max}$  should be collected past the distribution phase to be representative of maximal tissue concentrations<sup>11</sup>. Unfortunately, the optimal time post IV gentamicin administration at which to sample for  $C_{max}$  in horses has been the source considerable debate with most authors recommending either 0.5 or 1 h after bolus administration<sup>18,19</sup>. Correct timing of peak sample collection is important because aminoglycosides have a short but important distribution phase. Drawing a peak sample prematurely during the distribution phase would result in unrepresentatively high concentrations and would lead to under dosing. Based on the results of the present study, 0.5 h post-

administration was still well within the distribution phase in all foals, regardless of age, thus sampling for peak concentration 1 h post-bolus administration would be more appropriate. Selection of a 1 hour peak sampling time is also supported by the fact that peak synovial and allantoic fluid concentrations occur 1 h after IV bolus administration of gentamicin to adult horses<sup>25,26</sup>.

In the present study, peak gentamicin concentrations 1 h after administration ( $C_{1h}$ ) to 1-3 day-old foals were right at the target concentration of 16-20  $\mu\text{g/mL}$  ( $20.52 \pm 2.07 \mu\text{g/mL}$ ; range 18.1 to 23.24  $\mu\text{g/mL}$ ). Corresponding with the higher  $V_d$ , peak plasma concentrations were significantly lower in 1-3 day-old foals compared with older foals. By 2 weeks of age,  $C_{1h}$  was considerably above the target of 20  $\mu\text{g/mL}$  in 5 of 6 foals ( $48.95 \pm 15.07 \mu\text{g/mL}$ ; range 19.71 to 60.71  $\mu\text{g/mL}$ ). Based on these results and modeling of the data, administration of a lower dose of 6.6 mg/kg, as recommended for adult horses, would be more appropriate in foals 2 weeks of age or older. However, due to the considerable inter-individual variation, predicted doses for particular age ranges should be taken as a starting point from which to tailor daily dose on a patient-by-patient basis to maintain target peak concentrations.

Elimination half-life was longer at 1-3 days of age and significantly decreased during the first month of life, which is consistent with maturation of renal function<sup>27</sup>. Although 1-3 day old foals had the lowest peak concentrations, they also had the highest trough concentrations ( $1.97 \pm 0.90 \mu\text{g/mL}$ ). Because renal cellular uptake of gentamicin is saturable, nephrotoxicity is associated with insufficiently low trough levels, rather than excessively high peaks<sup>28</sup>. The maximal trough concentration to minimize the risk of nephrotoxicity is unknown in horses. In human patients, trough gentamicin concentrations  $> 2 \mu\text{g/mL}$  have been associated with nephrotoxicity and troughs  $> 4 \mu\text{g/mL}$  for over 10 days with ototoxicity<sup>11,29,30</sup>. Optimal trough



concentration for minimizing nephrotoxicity after administration of gentamicin to human patients is controversial, with recommendations ranging from maintaining a trough  $< 2 \mu\text{g/mL}$  to maintaining a period of  $< 0.5 \mu\text{g/mL}$  for at least 4 hours<sup>13,31,32</sup>. Maintaining a low trough concentration is not only important for reducing toxicity, but also in avoiding development of adaptive resistance. Adaptive resistance is a reversible down-regulation of the active transport of aminoglycoside into Gram-negative bacteria<sup>33</sup>. This resistance phenomenon is induced rapidly after a dose of aminoglycosides but is reversible during drug free intervals. As a result, prolongation of the dose interval may enable time for the return of bacterial susceptibility before the subsequent dose<sup>33,34</sup>. Based on the afore mentioned findings and on the fact that 5 of 6 foals in the 1-3 day-old age group had trough concentrations  $> 2 \mu\text{g/mL}$  (range 0.24 – 2.78  $\mu\text{g/mL}$ ) a prolonged dosing interval of q 36 or 48 h would be recommended for foals less than 2 weeks of age. Large clinical data sets in children and neonates indicate that extending dosing intervals to q 36 h or q 48 h minimizes cost, simplifies administration, and results in more favorable peak and trough concentrations<sup>13,32</sup>. In addition, any disease resulting in renal insufficiency would also necessitate an increased dosing interval or use of an alternate antimicrobial.

Based on the present study, IV administration of gentamicin at a dose of 12 mg/kg q 36 h would be required to achieve adequate peak concentrations ( $\geq 20 \mu\text{g/mL}$ ) and to maintain trough concentrations  $< 2 \mu\text{g/mL}$  in healthy foals less than 2 weeks of age. In foals 2 weeks of age or older, a dose of 12 mg/kg was excessive and a dose of 6.6 mg/kg given q 24 h was predicted to be sufficient. Additional studies will be necessary to confirm this lower dosage. The effects of age on the pharmacokinetics of gentamicin in foals are likely complicated by the influence of disease, prematurity and concurrent administration of other medications. Thus, therapeutic drug monitoring is recommended to ensure that desired peak and trough concentrations are being

achieved, especially due to the inherent inter-individual variation in the pharmacokinetic parameters of gentamicin, even in healthy foals.

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## Tables

**Table 4.1.** Pharmacokinetic variables (mean  $\pm$  SD) for gentamicin after IV administration of gentamicin sulfate at a dosage of 12 mg/kg to 6 foals at 5 different ages (1-3 days, 2 weeks, 4 weeks, 8 weeks and 12 weeks).

Variable	Age Group					P value
	1 – 3 days	2 wks	4 wks	8 wks	12 wks	
A ( $\mu\text{g/mL}$ )	34.97 $\pm$ 15.09 <sup>a</sup>	94.64 $\pm$ 14.19 <sup>b</sup>	90.50 $\pm$ 44.83 <sup>ab</sup>	89.37 $\pm$ 16.01 <sup>b</sup>	261.40 $\pm$ 235.79 <sup>b</sup>	0.0003
$\alpha$ ( $\text{h}^{-1}$ )	1.32 $\pm$ 0.50	1.19 $\pm$ 0.36	1.76 $\pm$ 0.98	0.97 $\pm$ 0.35	1.61 $\pm$ 0.85	0.2975
$t_{1/2\alpha}$ ( $\text{h}^{-1}$ )	0.6 $\pm$ 0.3	0.7 $\pm$ 0.3	0.5 $\pm$ 0.2	0.8 $\pm$ 0.3	0.5 $\pm$ 0.2	0.3407
B ( $\mu\text{g/mL}$ )	13.36 $\pm$ 3.54 <sup>a</sup>	15.59 $\pm$ 6.47 <sup>ab</sup>	38.57 $\pm$ 24.93 <sup>b</sup>	33.91 $\pm$ 13.17 <sup>ab</sup>	22.50 $\pm$ 8.08 <sup>ab</sup>	0.0168
$\beta$ ( $\text{h}^{-1}$ )	0.09 $\pm$ 0.031 <sup>a</sup>	0.12 $\pm$ 0.034 <sup>ab</sup>	0.23 $\pm$ 0.11 <sup>b</sup>	0.18 $\pm$ 0.058 <sup>b</sup>	0.16 $\pm$ 0.032 <sup>ab</sup>	0.0028
$t_{1/2\beta}$ (h)	8.2 $\pm$ 2.0 <sup>a</sup>	5.8 $\pm$ 1.6 <sup>ab</sup>	3.7 $\pm$ 1.5 <sup>b</sup>	4.0 $\pm$ 1.0 <sup>b</sup>	4.4 $\pm$ 0.8 <sup>b</sup>	0.0010
Vd <sub>area</sub> (L/kg)	0.75 $\pm$ 0.21 <sup>a</sup>	0.76 $\pm$ 0.58 <sup>ab</sup>	0.37 $\pm$ 0.29 <sup>ab</sup>	0.26 $\pm$ 0.10 <sup>b</sup>	0.28 $\pm$ 0.10 <sup>b</sup>	0.0060
CL (ml/h/kg)	64.3 $\pm$ 10.4	63.7 $\pm$ 23.9	65.5 $\pm$ 34.8	44.9 $\pm$ 12.7	44.4 $\pm$ 11.8	0.0420
AUC <sub>0-∞</sub> (mg•h/ml)	191.5 $\pm$ 28.2	206.1 $\pm$ 59.5	210.8 $\pm$ 70.3	273.9 $\pm$ 66.2	283.5 $\pm$ 82.0	0.0649
AUMC <sub>0-∞</sub> (mg•h <sup>2</sup> /ml)	1971.2 $\pm$ 706.2 <sup>a</sup>	1049.3 $\pm$ 315.9 <sup>b</sup>	819.7 $\pm$ 376.0 <sup>b</sup>	1215.6 $\pm$ 518.8 <sup>ab</sup>	946.7 $\pm$ 132.6 <sup>b</sup>	0.0013
MRT (h)	10.5 $\pm$ 3.3 <sup>a</sup>	5.28 $\pm$ 0.83 <sup>b</sup>	4.03 $\pm$ 1.67 <sup>b</sup>	4.15 $\pm$ 1.08 <sup>b</sup>	3.4 $\pm$ 1.20 <sup>b</sup>	<0.0001
C <sub>0.5h</sub> ( $\mu\text{g/mL}$ )	30.37 $\pm$ 9.45 <sup>a</sup>	64.16 $\pm$ 12.63 <sup>b</sup>	71.22 $\pm$ 33.86 <sup>b</sup>	75.45 $\pm$ 26.22 <sup>b</sup>	96.08 $\pm$ 26.38 <sup>b</sup>	0.0100
C <sub>1h</sub> ( $\mu\text{g/mL}$ )	20.52 $\pm$ 2.07 <sup>a</sup>	48.95 $\pm$ 15.07 <sup>bc</sup>	42.16 $\pm$ 17.57 <sup>b</sup>	67.77 $\pm$ 12.28 <sup>c</sup>	65.05 $\pm$ 22.81 <sup>c</sup>	<0.0001
C <sub>24h</sub> ( $\mu\text{g/mL}$ )	1.97 $\pm$ 0.90 <sup>a</sup>	0.86 $\pm$ 0.46 <sup>b</sup>	0.37 $\pm$ 0.35 <sup>b</sup>	0.71 $\pm$ 0.61 <sup>b</sup>	0.67 $\pm$ 0.25 <sup>b</sup>	0.0036

A and  $\alpha$  = Intercept and slope, respectively of the distribution phase.  $t_{1/2\alpha}$  = Distribution half-life.

B and  $\beta$  = Intercept and slope, respectively of the elimination phase.  $t_{1/2\beta}$  = Elimination half-life.

Vd<sub>area</sub> = Volume of distribution based on AUC. CL = Clearance. AUC<sub>0-∞</sub> = Area under the plasma concentration versus time curve extrapolated to infinity. AUMC<sub>0-∞</sub> = Area under the

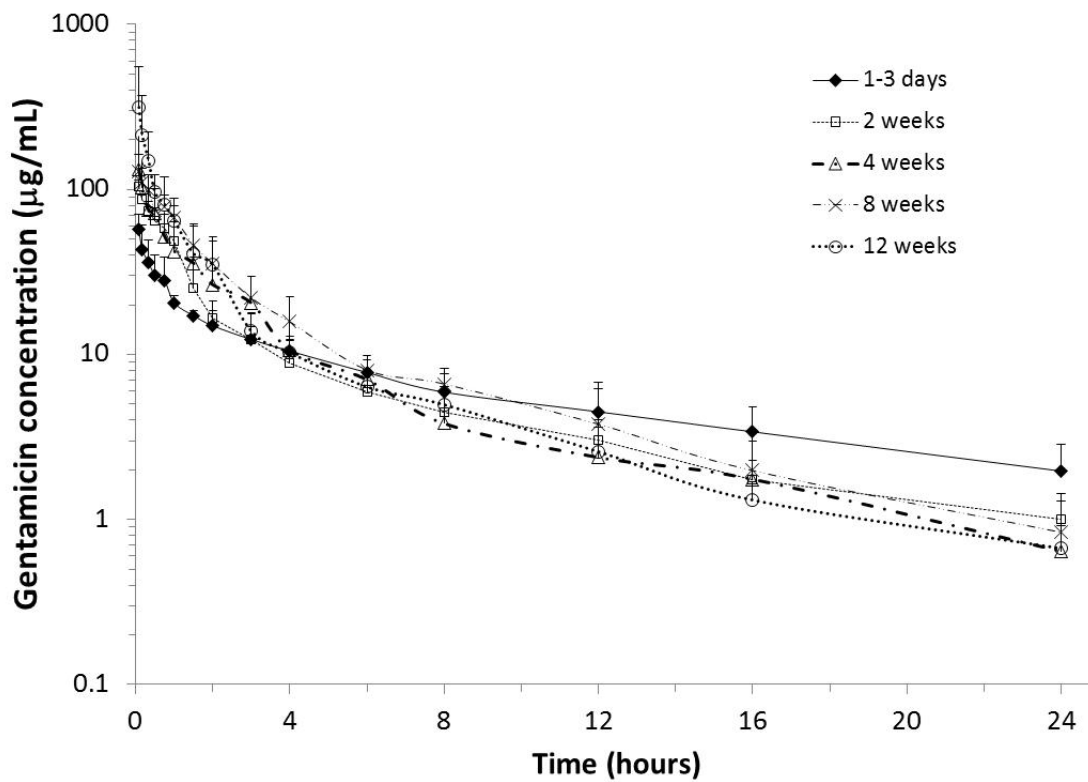
first moment of the concentration versus time curve extrapolated to infinity. MRT = Mean residence time.  $C_{30\text{min}}$  = Plasma concentrations of gentamicin 30 minutes after administration of a single IV dose.  $C_{1\text{h}}$  = Plasma concentrations of gentamicin 1 hour after administration of a single IV dose.  $C_{24\text{h}}$  = Plasma concentrations of gentamicin 24 hours after administration of a single IV dose. <sup>a,b,c</sup> Different letters within a given variable indicate a statistically significant difference between age groups ( $P < 0.05$ ).



**Table 4.2.** Predicted steady state peak (1 h post administration) and trough plasma concentrations of gentamicin (mean  $\pm$  SD) at different ages after IV administration of various dosages of gentamicin (q 24, q 36, or q 48 hours).

Age	Dose (mg/kg)	Dose interval (h)	Peak ( $\mu\text{g/mL}$ )	Trough ( $\mu\text{g/mL}$ )
1-3 days	12	24	25.23 $\pm$ 5.91	2.08 $\pm$ 0.91
		36	23.98 $\pm$ 6.40	0.72 $\pm$ 0.34
		48	23.56 $\pm$ 6.57	0.27 $\pm$ 0.14
	15	24	31.53 $\pm$ 7.40	2.60 $\pm$ 1.13
		36	29.97 $\pm$ 7.99	0.90 $\pm$ 0.43
		48	29.45 $\pm$ 8.22	0.34 $\pm$ 0.17
	18	24	37.85 $\pm$ 8.87	3.15 $\pm$ 1.36
		36	35.96 $\pm$ 9.60	1.08 $\pm$ 0.51
		48	35.34 $\pm$ 9.86	0.41 $\pm$ 0.21
2 weeks	6.6	24	24.64 $\pm$ 6.72	0.44 $\pm$ 0.15
	9	24	33.51 $\pm$ 9.00	0.60 $\pm$ 0.19
	12	24	44.68 $\pm$ 11.99	0.80 $\pm$ 0.26
4 weeks	6.6	24	25.27 $\pm$ 9.52	0.20 $\pm$ 0.22
	9	24	34.46 $\pm$ 12.98	0.27 $\pm$ 0.29
	12	24	45.95 $\pm$ 17.31	0.36 $\pm$ 0.39
8 weeks	6.6	24	34.55 $\pm$ 8.80	0.33 $\pm$ 0.25
	9	24	47.11 $\pm$ 12.00	0.44 $\pm$ 0.34
	12	24	62.81 $\pm$ 15.91	0.59 $\pm$ 0.46
12 weeks	6.6	24	34.52 $\pm$ 14.11	0.26 $\pm$ 0.11
	9	24	47.07 $\pm$ 19.24	0.36 $\pm$ 0.15
	12	24	62.75 $\pm$ 25.65	0.48 $\pm$ 0.2

Figures



**Figure 4.1.** Mean (+ SD) plasma concentrations of gentamicin after IV administration of gentamicin sulfate at a dosage of 12 mg/kg to 6 foals at 5 different ages (1-3 days, 2 weeks, 4 weeks, 8 weeks, and 12 weeks).

## CHAPTER 5

# EFFICACY OF LIPOSOMAL GENTAMICIN AGAINST RHODOCOCCLUS EQUI IN A MOUSE INFECTION MODEL AND COLOCALIZATION WITH R. EQUI IN EQUINE ALVEOLAR MACROPHAGES<sup>3</sup>

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<sup>3</sup> Burton, A.J., Giguère, S., Berghaus, L.J., Hondalus, M.K., and Arnold, R.D. To be submitted to *Veterinary Microbiology*.

## Abstract

*Rhodococcus equi*, a facultative intracellular pathogen and an important cause of pneumonia in foals, is highly susceptible to killing by gentamicin *in vitro*. However, gentamicin is not effective *in vivo*, due to its poor cellular penetration. Encapsulation of drugs in liposomes enhances cellular uptake. The objectives of this study were to compare liposomal gentamicin and free gentamicin with respect to their uptake by macrophages and intracellular colocalization with *R. equi* and to compare the efficacies of liposomal gentamicin, free gentamicin and clarithromycin with rifampin for the reduction of *R. equi* CFU in a mouse model of infection. After *ex vivo* exposure, a significantly higher mean ( $\pm$  SD) percentage of equine alveolar macrophages contained liposomal gentamicin ( $91.9 \pm 7.6\%$ ) as opposed to free gentamicin ( $16.8 \pm 12.5\%$ ). Intracellular colocalization of drug and *R. equi*, as assessed by confocal microscopy, occurred in a significantly higher proportion of cells exposed to liposomal gentamicin ( $81.2 \pm 17.8\%$ ) compared to those exposed to free gentamicin ( $10.4 \pm 8.7\%$ ). The number of *R. equi* CFU in the spleen was significantly lower in mice treated with liposomal gentamicin compared to that of mice treated with free gentamicin or to untreated control mice. Treatment with liposomal gentamicin also resulted in a significantly greater reduction in the number of *R. equi* CFU in the liver compared to treatment with clarithromycin in combination with rifampin. These results underscore the potential of liposomal gentamicin as a new treatment for infections caused by *R. equi*.

Keywords: Gentamicin sulfate, aminoglycoside, foal, horse, liposomes, macrophage.

## Introduction

*Rhodococcus equi*, a Gram-positive facultative intracellular bacterium, is a common cause of disease in 1 to 5 month-old foals. The most common manifestation of the disease is pyogranulomatous bronchopneumonia with abscessation but numerous extrapulmonary disorders also occur (Reuss et al., 2009). Despite recommended therapy with a macrolide and rifampin, the mortality rate of foals with clinical signs of pneumonia caused by *R. equi* is approximately 30% (Ainsworth et al., 1998; Giguere et al., 2004). The mortality rate of foals with extrapulmonary disorders associated with *R. equi* is even higher at 57% (Reuss et al., 2009). Over the last 10 years, the incidence of macrolide and rifampin resistance has increased (Giguere et al., 2010) and resistant isolates of *R. equi* are cultured from up to 40% of affected foals at some farms (Burton et al., 2013). Foals infected with *R. equi* isolates resistant to macrolides and rifampin are significantly more likely to die than foals infected with susceptible isolates (Giguere et al., 2010). Therefore, there is a need for more effective antimicrobial agents for the treatment of foals infected with *R. equi*.

All *R. equi* isolates from pneumonic foals, including macrolide-resistant isolates, are susceptible to the aminoglycoside antibiotic gentamicin *in vitro* (Giguere et al., 2010; Jacks et al., 2003; Riesenber et al., 2014). Additionally, gentamicin is one of the few antimicrobial agents that is bactericidal (rather than bacteriostatic) against *R. equi* (Berghaus et al., 2013; Nordmann and Ronco, 1992). Although gentamicin is highly active against *R. equi in vitro*, its efficacy *in vivo* has been limited (Sweeney et al., 1987) presumably because of poor cellular uptake due to its hydrophilic nature. A delivery system that could improve intracellular concentrations of gentamicin would likely increase its *in vivo* efficacy against *R. equi*.

Encapsulation in liposomes is one method by which the intracellular penetration of drugs might be enhanced. Liposomes are 0.08 – 5 µm in diameter lipid vesicles composed of one or several amphiphile bilayers surrounding an aqueous core (Drummond et al., 2008; Gamazo et al., 2007). Previous studies in a variety of species have shown that compared with free (conventional) gentamicin, liposomal gentamicin has significantly enhanced cellular penetration and activity against facultative intracellular bacteria such as *Listeria monocytogenes*, *Mycobacterium avium*, *Salmonella* spp., and *Brucella abortus* both *in vitro* and *in vivo* (Bakker-Woudenberg et al., 1994; Gamazo et al., 2007; Klemens et al., 1990; Lutwyche et al., 1998; Swenson et al., 1990; Vitas et al., 1996). Recently, it was shown that administration of liposomal gentamicin intravenously or via nebulization to foals results in significantly higher concentrations of gentamicin into bronchoalveolar cells compared with free gentamicin (Burton et al., 2014). However, to be effective clinically, liposomal gentamicin would have to colocalize with *R. equi* within the cell.

The main objectives of this study were to compare the cellular uptake of 2 different formulations of liposomal gentamicin, to compare uptake of liposomal gentamicin by equine alveolar macrophages and intracellular colocalization with *R. equi* to that of free gentamicin, and to compare the efficacy of liposomal gentamicin to that of free gentamicin or clarithromycin with rifampin for the reduction of *R. equi* burden in a mouse model of infection.

## Materials and Methods

### *Formulation of liposomes and liposomal gentamicin*

Liposomal gentamicin was formulated by aqueous capture using dipalmytolphosphatidycholine (Avanti Polar Lipids Inc., Alabaster, AL, USA) and cholesterol (Sigma-Aldrich, St-Louis, MO, USA)<sup>b</sup> with or without coating with 1,2-dipalmitoyl-sn-glycero-

3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG) in the following molar ratios: DPPC:Chol (9:5) [DPPC] and DPPC:Chol:DPPE-PEG (9:5:1) [DPPC-PEG]. The lipids were stored in chloroform at -80 °C were thawed and mixed in a glass round bottom flask. The chloroform was evaporated from the mixture using vacuum under a constant nitrogen stream (Rotavapor® R-210/R-215, Buchi Corporation, New Castle, DE, USA) and the resultant thin lipid film was rehydrated with 0.9% saline (control empty liposomes) or aqueous gentamicin sulfate (60 mg/ml) at a ratio of 21 mg gentamicin per  $\mu\text{mol}$  of lipid. After 5 freeze-thaw cycles in liquid nitrogen, the particles were sized to 100 nm using 3 passes through laser etched polycarbonate filters (0.08  $\mu\text{m}$ ) using a high-pressure extruder (Lipex, Northern Lipids Inc., Burnaby, BC, Canada). Non-encapsulated gentamicin was removed via three rounds of dialysis in 0.9% saline at 4°C using 10K MW cut-off dialysis cassettes (Slide-A-Lyzer, Thermo Fisher Scientific Inc., Rockford, IL, USA). The final concentration of gentamicin in the formulations was measured by HPLC-MS after dialysis, as described previously (Burton et al., 2014).

#### *Bacterial strains and MIC*

*R. equi* strain 103S, originally isolated from a pneumonic foal was used for infection of mice. This strain is known to be virulent based on VapA expression, its ability to replicate in macrophages and immunodeficient mice, and its ability to cause pneumonia in experimentally infected foals (Giguere et al., 1999). Equine alveolar macrophages were infected with green fluorescent protein (GFP)-expressing *R. equi*, which was created by transformation of virulent strain 103S with pGFPmut2, kindly provided by Russell Karls of the University of Georgia. pGFPmut2 is an *E. coli*-*Mycobacterium* spp. shuttle vector containing both an *oriE* and *oriM* as well as a hygromycin resistance cassette. GFP is expressed from the mycobacteriophage L5 promoter. Minimum inhibitory concentration (MIC) of gentamicin, clarithromycin and rifampin

were determined by broth macrodilution in glass tubes as described previously (Berghaus et al., 2013) and in accordance to the guidelines established by the CLSI (Clinical and Laboratory Standard Institute, 2011). The test medium was cation-adjusted Mueller-Hinton broth. Concentrations of antimicrobial agents tested represented two-fold dilutions between 256 and 0.031 µg/ml. MIC was defined as the first dilution with no bacterial growth after 24 h of incubation at 37°C.

#### *Uptake of liposomes by J774A.1 macrophages*

Murine monocyte-macrophage-like cells J774A.1 (American Type Culture Collection, Manassas, VA) were used initially to compare uptake of different liposomal formulations. These cells were selected because uptake and intracellular survival of *R. equi* in J774A.1 cells is similar to that observed in equine alveolar macrophages (Hondalus and Mosser, 1994). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, along with penicillin G and streptomycin (100 U/ml and 100 µg/ml, respectively). Prior to use in experiments, the cells were washed 3 times in antibiotic free media, resuspended at a concentration of  $5 \times 10^5$  cells/ml, and 1 ml of cell suspension was placed in each well of 24-well tissue culture plates and incubated (37°C, 5% CO<sub>2</sub>) for 3 h to allow adherence. Each formulation of liposome was labeled with the fluorochrome DiI (Life Technologies, Grand Island, NY, USA) as previously described (Claassen, 1992) and added to triplicate wells containing J774A.1 cells at lipid concentrations of 250 nM/ml. After an incubation period of 2 h to allow uptake, the media was removed, the monolayers were washed with phosphate buffered saline (PBS), and 1 ml of plain media (DMEM + 10% FBS) was added to each well. After incubation for an additional period of 2 h, monolayers were washed and the wells were scraped to dissociate the



cells. Cells were resuspended in 1ml of PBS with bovine serum albumin (2 mg/ml) and sodium azide (1 mg/ml) in a 3:2 ratio with 10% formalin. Uptake of the liposome formulations was quantified using flow cytometry (Accuri C6 Cytometer, Ann Arbor, MI). Forward scatter and side scatter were used to identify the cell population and to gate out cellular debris. Ten thousand events were counted per sample. For each liposome formulation, the percentage of cells exceeding the baseline fluorescence threshold was calculated. Negative controls were plain cells that had not been incubated with liposomal formulations. The results represent the mean  $\pm$  SD of 6 independent experiments.

#### *Mouse infection and treatment*

Six to eight week old male athymic nude (*nu/nu*) mice (Harlan Laboratories Inc., Indianapolis, IN, USA) were used in an *R. equi* infection model as previously described (Nordmann et al., 1992). For the infection of mice, frozen aliquots of *R. equi* strain 103S were thawed and grown for 1 h at 37°C in brain-heart infusion broth to induce expression of *vapA*. Bacteria were pelleted and resuspended in PBS. Mice (5 per group) were infected intravenously (IV) through the tail vein with approximately  $5 \times 10^6$  colony forming units (CFUs) per mouse. The total number of bacteria injected was confirmed retrospectively by dilution plating of the injection stock and counting CFUs. On day 4 after infection, treatment was started IV via the tail vein every 48 h, or subcutaneously (SC) every 24 h for 5 days. Treatments given IV included 2 different formulations of liposomal gentamicin (DPPC and DPPC-PEG) both at a gentamicin dose of 20 mg/kg of body weight, free gentamicin (20 mg/kg), an equivalent amount of control (gentamicin free) DPPC-PEG liposomes, and 0.9% NaCl (control). Treatments given SC were rifampin (10 mg/kg), clarithromycin (25 mg/kg), and 0.9% NaCl (control). The dosages and dosing intervals used were those known to result in plasma concentration similar to that achieved

with the use of these antimicrobial agents in horses and humans (Bermudez et al., 1990; Cynamon et al., 1992; Nordmann et al., 1992). All treatments were diluted to result in an injection volume of 0.1 ml. Mice were euthanized on day 8 post-infection, 1 hour after their drug treatment, by intra-peritoneal injection of pentobarbital (Euthasol, Virbac AH Inc., Fort Worth, TX, USA) followed by cervical dislocation. After euthanasia, the lungs, spleen, and liver were be aseptically harvested and homogenized in sterile PBS. The number of CFU of *R. equi* per organ (liver, spleen and lungs) were determined by plating serial 10-fold dilutions of organ homogenates onto trypticase soy agar (TSA) plates and incubating at 37°C for 48 h. Plasma for measurement of drug concentrations was collected from each mouse via cardiac puncture at the time of euthanasia. Concentrations of gentamicin were measured by HPLC-MS as described previously (Burton et al., 2014). Concentrations of clarithromycin and rifampin were measured using HPLC by a commercial laboratory (Infectious Disease Pharmacokinetics Laboratory, University of Florida, Gainesville, FL).

*Infection of equine alveolar macrophages for CFU counting and confocal microscopy*

Alveolar macrophages were collected from 6 healthy adult mares (3 Thoroughbreds, 2 Quarter Horses and 1 Appaloosa) via bronchoalveolar lavage (BAL) as described previously (Burton et al., 2014). Bronchoalveolar cells were washed 3 times with PBS, counted, and suspended at a concentration of  $1 \times 10^6$  macrophage/ml in Minimum Essential Medium-alpha (MEM $\alpha$ ) containing 10% horse serum and amphotericin B (25  $\mu$ g/ml). The bronchoalveolar macrophage cell suspension (1 ml) was added to each well of a 24 well plate (Nunc, ThermoFisher Scientific, Rochester NY) and of chamber slides (Lab-Tek, ThermoFisher Scientific Rochester NY) for subsequent CFU counting or confocal microscopy, respectively. Cells were incubated for 3 h at 37°C in 6% CO<sub>2</sub>. After incubation and subsequent washing,

approximately  $7 \times 10^5$  cells remained attached to each well. *R. equi* strain 103S expressing GFP was suspended in antimicrobial free MEM $\alpha$  supplemented with 10 % horse serum as a source of complement and used to infect the monolayers with a MOI of 5. Non-infected macrophage monolayers cultured under the same conditions were used as negative controls. The monolayers were then incubated for 45 min at 37°C in 6% CO<sub>2</sub> to allow phagocytosis. Media containing *R. equi* was removed carefully and each well was washed 3 times with PBS to remove the majority of extracellular *R. equi*. Cells were then treated with 1 ml of media containing either plain media (control), Texas Red-conjugated free gentamicin (20  $\mu$ g/ml), DPPC-PEG liposomal gentamicin labeled with the fluorochrome DiI (20  $\mu$ g/ml), or clarithromycin (0.9  $\mu$ g/ml) in combination with rifampin (6.7  $\mu$ g/ml), and incubated for up to 48 h. Labeling of free gentamicin and DPPC-PEG were performed as described previously (Claassen, 1992; Steyger et al., 2003). Concentrations of gentamicin, clarithromycin and rifampin were selected based on peak plasma concentrations achievable in foals (Burton et al., 2014; Castro et al., 1986; Womble et al., 2006).

The number of CFU within equine alveolar macrophages was determined at 4, 24 and 48 h post infection. At the given time point, media was removed, monolayers were washed 3 times with warm PBS, and the cells were lysed by adding 1 mL of sterile water prior to 40 minute of incubation. Individual wells were scraped with the tip of a pipet before the contents were transferred to a 1.5 ml tube. The lysates were vortexed for 5 minutes, followed by 5 minutes of sonication, and by an additional 5 minutes of vigorous vortexing. Dilutions of the lysate were plated onto TSA plates and the number of *R. equi* CFU were determined after 48 h incubation at 37°C. For each horse, the mean of duplicate wells was used for data analysis. Results were expressed as the reduction in the number of *R. equi* CFU ( $\log_{10}$ /well) relative to the uninfected control monolayers of each horse.

Chamber slides for confocal microscopy were processed at the same time points (4, 24, and 48 h post-infection) with the exception that no cells were treated with clarithromycin and rifampin for confocal microscopy. In half the wells, free gentamicin and DPPC-PEG containing media was removed at 4 h and replaced with plain media for further incubation until 24 and 48 h. For fixing, media was removed and cells were washed twice in PBS and 1 ml of 4% paraformaldehyde was added to each well and incubated for 20 minutes in the dark at 4 °C. The paraformaldehyde was then removed and the cells washed once with PBS. Slides were air-dried, treated with DAPI nuclear stain in adhesive (ProLong Gold®, Life Technologies, Grand Island, NY, USA) and a cover slip was placed. Slides were allowed to rest for 24 h at room temperature prior to examination by fluorescent laser scanning confocal microscopy. Images were captured on a Nikon A1R confocal microscope with a 60x lens (CFI Plan APO VC 60x Oil NA 1.4 WD 0.13mm) using NIS Elements AR imaging software (Nikon Instruments Inc., Melville, NY). Drug uptake and colocalization with *R. equi* was quantified at 60x magnification from 150 cells per slide. Colocalization was assessed manually because software designed for this purpose was found to be inconsistent due to the heterogeneity of the equine cells and variable intensity of the GFP *R. equi* depending upon clumping and stage of growth. Drug and *R. equi* uptake was expressed as percentage of total cells counted and colocalization (CL) or non-colocalization (NCL) as a percentage of the cells with *R. equi* uptake.

#### *Statistical analysis*

Normality of the data and equality of variances were assessed using Shapiro-Wilk's and Levene's tests, respectively. Variables that were not normally distributed were log- or rank-transformed prior to analysis. A paired t test was used to compare the uptake of the 2 liposome formulations in J774.A1 cells. A two-way ANOVA with one factor repetition was used to assess

the effect of drug (DPPC, DPPC-PEG, free gentamicin and plain liposomes), organ (spleen, liver, and lung) and their interactions on *R. equi* CFU. Preliminary data analysis using ANOVA revealed that organ CFU were significantly higher in control mice administered IV saline or IV plain liposomes compared to control mice administered SC saline. Therefore, for the comparison between DPPC-PEG and clarithromycin with rifampin, data was expressed as the  $\log_{10}$  reduction in CFU relative to their respective control group. For each organ, comparison of the reductions in the number of *R. equi* CFU between mice treated with DPPC-PEG and those treated with clarithromycin with rifampin was done using the Student t test. A two-way ANOVA with repeated measures was used to evaluate the effect of treatment, time, and their interactions on intracellular CFU, drug uptake, uptake of *R. equi* and percentage of colocalization. When indicated, multiple pairwise comparisons for ANOVAs were done using Tukey's method. For all analyses,  $P < 0.05$  was considered significant.

## **Results**

### *Uptake of liposomes by J774.A1 macrophages in vitro*

After 4 h, more than 90% of macrophages contained liposomes regardless of formulation. The mean ( $\pm$  SD) percentage of macrophages containing DPPC ( $99.1 \pm 0.8$  %) was significantly ( $P = 0.001$ ) higher than the percentage of macrophages containing DPPC-PEG ( $92.7 \pm 2.2$ %).

### *Efficacy of liposomal gentamicin (DPPC-PEG) in a mouse infection model*

The MICs of gentamicin, clarithromycin, and rifampin for strain 103S were 0.125  $\mu\text{g/ml}$ , 0.03  $\mu\text{g/ml}$ , and 0.06  $\mu\text{g/ml}$ , respectively. Mean ( $\pm$  SD) serum concentrations of gentamicin (DPPC:  $2.17 \pm 0.72$   $\mu\text{g/ml}$ ; DPPC-PEG:  $3.26 \pm 0.65$   $\mu\text{g/ml}$ ; free gentamicin:  $2.21 \pm 0.26$   $\mu\text{g/ml}$ ), clarithromycin ( $0.14 \pm 0.04$   $\mu\text{g/ml}$ ), and rifampin ( $0.29 \pm 0.14$   $\mu\text{g/ml}$ ) at the time of euthanasia were well above their respective MICs.

The efficacies of DPPC and DPPC-PEG, as determined by the number of *R. equi* CFU per organ in infected nude mice, were compared to that of free gentamicin. Mice treated with drug (gentamicin) free liposomes were used as controls. There were significant main effects of treatment ( $P < 0.001$ ) and of organ ( $P < 0.001$ ), and significant interactions between treatment and organ ( $P = 0.002$ ) on the number of *R. equi* CFU. The number of *R. equi* CFU in the liver was significantly lower in mice treated with DPPC, DPPC-PEG, or free gentamicin compared to that of control mice (Figure 5.1). The mean number of *R. equi* CFU in the spleen was significantly lower in mice treated with DPPC-PEG compared to that of mice treated with free gentamicin or control mice (Figure 5.1). The mean number of *R. equi* CFU in the lungs was not significantly different between treatment groups. Treatment with DPPC-PEG resulted in a significantly ( $P = 0.036$ ) greater reduction in the mean number of *R. equi* CFU in the liver relative to untreated controls compared to treatment with clarithromycin in combination with rifampin (Figure 5.2). There was a tendency ( $P = 0.058$ ) toward a greater reduction in the number of *R. equi* CFU in the lungs compared to treatment with clarithromycin in combination with rifampin (Figure 5.2). Mean reduction in the number of *R. equi* CFU in the spleen was not significantly different between the 2 treatment groups.

*Colocalization of R. equi and liposomal gentamicin (DPPC-PEG) in equine alveolar macrophages*

Quantification of comparative drug uptake and colocalization with *R. equi* in equine alveolar macrophages is summarized in Table 5.1. The mean mean percentage of equine alveolar macrophages containing DPPC-PEG was over 90% at each time point and was significantly greater than that of free gentamicin in all instances (Table 5.1). No significant differences in percentages of cells containing *R. equi* were observed, regardless of treatment or time point

(Table 5.1). *R. equi* was seen to colocalize with DPPC-PEG within the same 0.2  $\mu\text{m}$  3-D section at each time point (Figure 5.3). The percentage of cells exhibiting colocalization of *R. equi* and DPPC-PEG was over 80% in at each time point, increased over time, and was significantly greater for DPPC-PEG than for free gentamicin (Table 5.1). Mean percentage of cells with colocalization was significantly lower in cells treated with free gentamicin and subjected to media change ( $15.2 \pm 11.1$  %) compared to that of cells treated with free gentamicin and not subjected to a media change ( $57.0 \pm 25.0$ ). Correspondingly, more cells treated with free gentamicin exhibited non-colocalized *R. equi* than DPPC-PEG treated cells (Table 5.1). When media was replaced with drug-free media at 4 h, the mean percentage of cells with uptake of DPPC-PEG was not significantly different at 24 and 48 h compared with when the media containing DPPC-PEG was not removed. Conversely, for free gentamicin, mean percentage of cells with uptake was significantly lower at both 24 and 48 h when media was replaced (Table 5.1).

#### *Effects of liposomal gentamicin on R. equi CFUs in equine alveolar macrophages*

There were significant main effects of treatment ( $P = 0.013$ ) and time ( $P = 0.002$ ) on the mean number of *R. equi* CFU but the interactions between treatment and time were not statistically significant. The reduction in mean intracellular *R. equi* CFU relative to control (untreated) monolayers was significantly higher in monolayers treated with DPPC-PEG compared to monolayers treated with clarithromycin and rifampin, regardless of time points (Figure 4). The mean number of *R. equi* CFU in monolayers treated with free gentamicin were not significantly different from that of the other 2 treatment groups. Regardless of treatment, the reduction in *R. equi* CFU was significantly greater at 48 h than at 4 h or 24 h.

## Discussion

At the most basic level, liposomes can be divided into two main categories: conventional, short-circulating liposomes which are composed of natural or synthetic phospholipids  $\pm$  cholesterol, and long circulating liposomes sterically-stabilized with PEG coating. The PEG coating delays opsonization and uptake by mononuclear phagocytes relative to conventional liposomes, thus resulting in prolonged systemic circulation time and higher tissue concentrations (Bakker-Woudenberg et al., 1994). Although the uptake of both types of liposomes by J774A.1 cells was high ( $\geq 90\%$  of the cells), uptake of DPPC liposomes was significantly greater than that of DPPC-PEG liposomes. However, because a balance between uptake by phagocytic cells and stability in the circulation and at the site of infection must be achieved for therapeutic success *in vivo* (Drummond et al., 2008), both DPPC and DPPC-PEG liposomal gentamicin were tested in the mouse model of infection with *R. equi*. Although normal immunocompetent mice clear *R. equi* rapidly, T-cell immunodeficient nude mice cannot clear *R. equi* for at least 3 weeks after IV challenge, and remain persistently infected, thus providing a good animal model of chronic infection (Nordmann et al., 1992). This model has been used successfully to compare the efficacy of various antimicrobial agents against *R. equi* (Nordmann et al., 1992).

In the present study, treatment with DPPC-PEG liposomal gentamicin significantly decreased the number of *R. equi* CFU compared to treatment with free gentamicin (spleen) and to untreated controls (spleen and liver). The number of CFUs in each group followed the same pattern in the lungs. However, presumably as a result of the lower CFU, greater variation and small sample size, differences between treatment groups were not statistically significant. These data are consistent with previous work demonstrating that liposomal gentamicin has improved efficacy over free gentamicin in mouse models of *Mycobacterium avium* complex, *Salmonella*



spp. and *Brucella abortus* infection (Cordeiro et al., 2000; Fountain et al., 1985; Klemens et al., 1990). Although treatment with DPPC also resulted in significantly fewer *R. equi* CFUs in the spleen and liver compared to untreated controls, the number of CFUs were not significantly different from those obtained after treatment with free gentamicin in any organ. Therefore, DPPC-PEG was selected for comparison of liposomal gentamicin to standard therapy with a macrolide and rifampin in the mouse infection model and in equine alveolar macrophages.

Macrolides and rifampin are highly synergistic against *R. equi in vitro* (Giguere et al., 2012; Prescott and Nicholson, 1984) and erythromycin combined with rifampin is significantly more effective than either drug alone using the nude mouse model of *R. equi* infection (Nordmann et al., 1992). The present study used clarithromycin instead of erythromycin in combination with rifampin because it has greater activity against *R. equi in vitro* (Jacks et al., 2003) and greater efficacy in foals naturally infected with *R. equi* (Giguere et al., 2004). Treatment of mice with DPPC-PEG was significantly more effective than clarithromycin in combination with rifampin at decreasing *R. equi* CFUs in the liver and there was a tendency toward a greater reduction in *R. equi* CFUs with DPPC-PEG gentamicin in the lungs. These differences were observed despite administration of only 3 doses of DPPC-PEG compared to administration of 5 doses of clarithromycin and rifampin. A longer treatment period or daily administration of DPPC-PEG might have accentuated the differences between treatment groups.

Consistent with the results obtained in J774A.1 cells, uptake of DPPC-PEG by equine alveolar macrophages was rapid and efficient with more than 90% of the cells containing the drug as early as 4 h after exposure. When DPPC-PEG-containing media was replaced with drug-free media at 4 h, the percentage of cells containing the drug remained high for at least 48 h, indicating that the drug persists intracellularly as opposed to the high proportion of DPPC-PEG-

containing cells being only the result continuous uptake of new drug by the cells. In contrast, the uptake of free gentamicin was low ( $16.8 \pm 12.5\%$ ) at 4 h. Uptake of free gentamicin increased progressively (up to  $68.0 \pm 22.7\%$ ) in cells continuously exposed to the drug but not when the media was changed at 4 h. Once engulfed by resident macrophages, virulent *R. equi* avoid destruction by altering maturation and preventing acidification of the phagosomes, thereby preventing fusion with lysosomes (Fernandez-Mora et al., 2005; Toyooka et al., 2005; von et al., 2009; Zink et al., 1987). The present study demonstrated that DPPC-PEG colocalizes with *R. equi* within the same vacuole in up to 98% of infected equine alveolar macrophages. Consistent with this finding, the reduction in *R. equi* CFU in equine alveolar macrophages exposed to DPPC-PEG was significantly higher that observed in monolayers exposed to clarithromycin and rifampin. Although the proportion of cells with colocalization was significantly lower for free gentamicin, there was colocalization in approximately 45% of the cells. Uptake of free gentamicin by equine alveolar macrophages and colocalization with *R. equi* might be a contributing factor as to why explain why the number of *R. equi* CFU was not significantly different between monolayers exposed to DPPC-PEG and monolayers exposed to free gentamicin. It is common practice to use culture media containing gentamicin or other aminoglycosides to presumably kill extracellular *R. equi* without affecting its intracellular growth during intracellular survival and replication assays (Hondalus and Mosser, 1994). Although this assay has been used successfully to show clear differences in intracellular survival and replication between virulent and avirulent strains of *R. equi* (Coulson et al., 2010; Giguere et al., 1999), the result of the present study revealed a surprisingly high uptake of free gentamicin and colocalization of free gentamicin with intracellular *R. equi* in up to 45% of the cells. Furthermore, free gentamicin was as effective as the combination of clarithromycin with

rifampin in reducing *R. equi* CFU in equine alveolar macrophages. Although these data do not dispute the certain value of the aminoglycoside protection assay in screening the virulence of various *R. equi* strains, they indicate that intracellular replication of *R. equi* is likely dampened to some degree by the use of aminoglycosides.

Taken together, these results show that liposomal gentamicin is quickly engulfed by J774.A1 cells mouse and equine alveolar macrophages and that it colocalizes with *R. equi* within the same intracellular space, hence overcoming the previous limitation of free gentamicin as a sole treatment for *R. equi*. Furthermore DPPC-PEG was significantly more effective than free gentamicin or than clarithromycin in combination with rifampin in a mouse model of infection with *R. equi*. As such, these results underscore the potential of liposomal gentamicin as a new treatment for infections caused by *R. equi* and warrant its investigation in naturally or experimentally infected foals.

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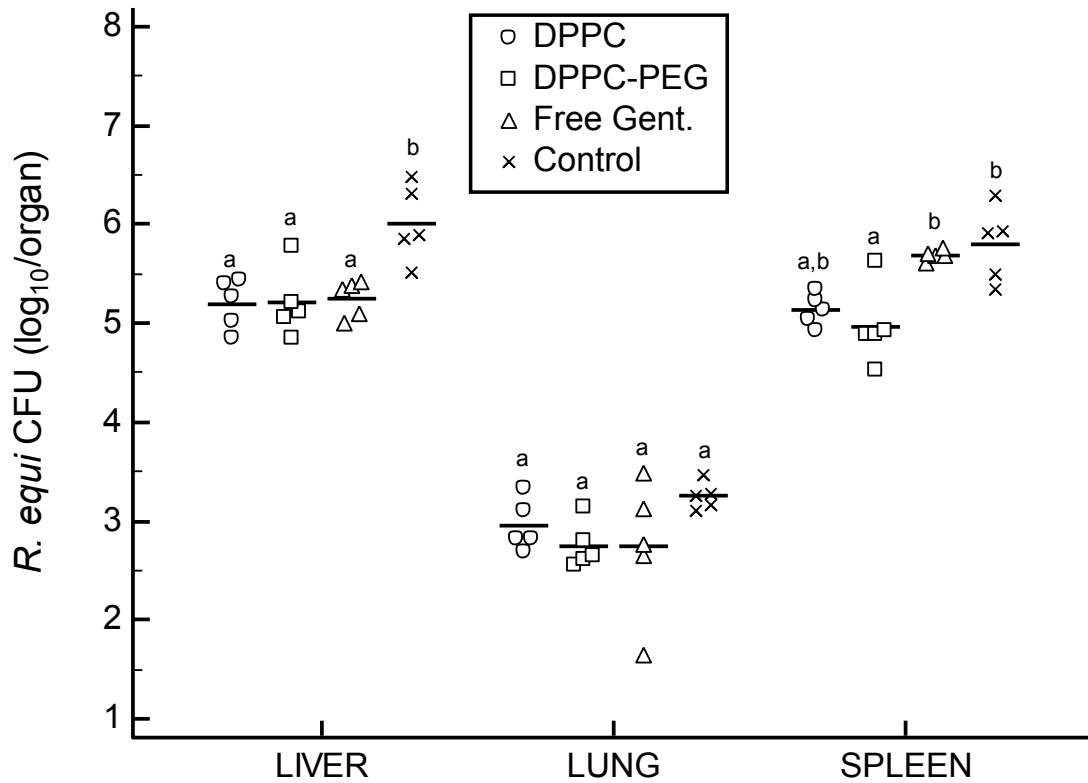


Tables

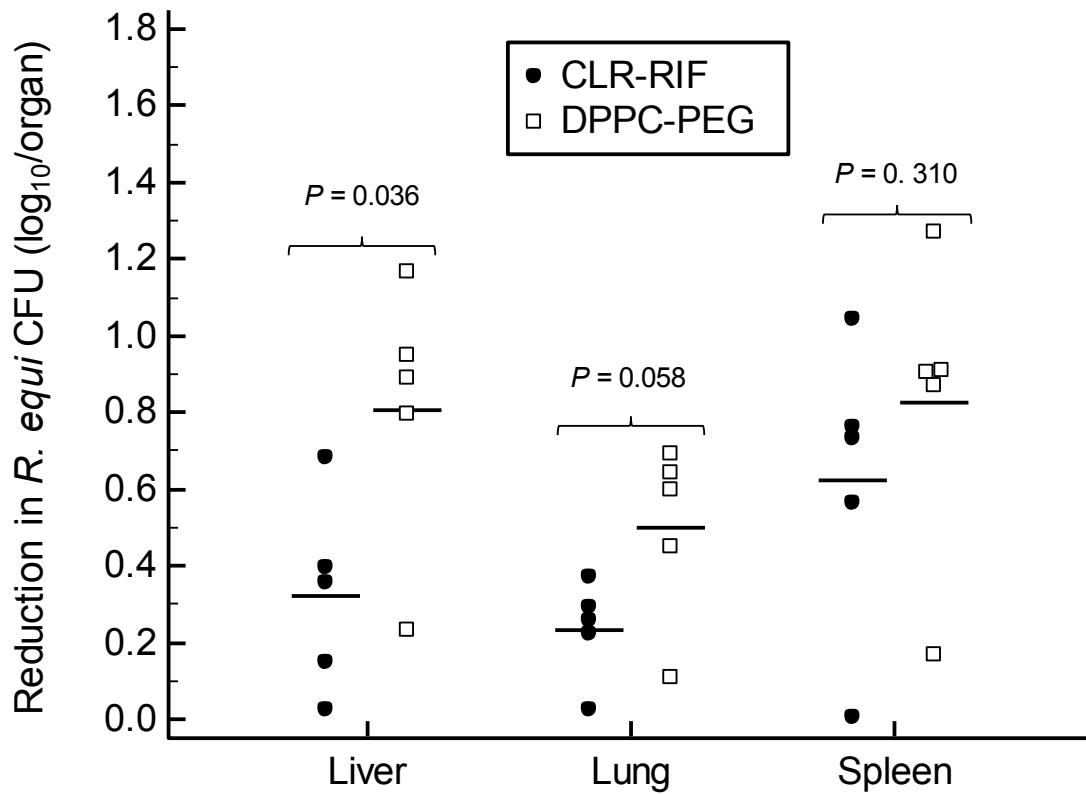
**Table 5.1.** Mean ( $\pm$  SD) percentage of equine alveolar macrophages with intracellular drug and *R. equi* and with intracellular colocalization drug and *R. equi*. Macrophages were infected with virulent *R. equi* expressing GFP and treated with fluorescently labeled liposomal gentamicin (DPPC-PEG) or free gentamicin (Free Gent.). Cells were imaged at 4, 24 and 48 hours post treatment by confocal microscopy. n = at least 150 cells per horse per time point, n = 6 horses.

Variable	Drug	Time			P value					
		4 h	24 h	48 h	Drug	Time	Drug $\times$ Time			
% total cells with intracellular drug*	DPPC-PEG	91.9 $\pm$ 7.5 <sup>a</sup>	92.5 $\pm$ 6.7 <sup>a</sup>	94.2 $\pm$ 7.8 <sup>a</sup>	< 0.001	0.008	0.024			
	Free Gent.	16.8 $\pm$ 12.5 <sup>b,1</sup>	56.9 $\pm$ 37.8 <sup>b,2</sup>	68.0 $\pm$ 22.7 <sup>b,2</sup>						
	DPPC-PEG $\Delta$	-	95.7 $\pm$ 6.9 <sup>a</sup>	94.5 $\pm$ 4.4 <sup>a</sup>						
	Free Gent. $\Delta$	-	11.0 $\pm$ 9.6 <sup>c</sup>	23.6 $\pm$ 13.9 <sup>b</sup>						
	<hr/>									
	% total cells with intracellular <i>R. equi</i> *	DPPC-PEG	56.6 $\pm$ 6.6	60.0 $\pm$ 9.9				71.4 $\pm$ 12.8	0.690	0.053
Free Gent.		58.6 $\pm$ 8.4	66.8 $\pm$ 8.3	57.0 $\pm$ 25.0						
DPPC-PEG $\Delta$		-	75.1 $\pm$ 17.3	63.9 $\pm$ 15.0						
Free Gent. $\Delta$		-	69.6 $\pm$ 11.4	66.7 $\pm$ 15.4						
<hr/>										
% cells with colocalization <sup>†</sup>		DPPC-PEG	81.2 $\pm$ 17.8 <sup>a,1</sup>	97.2 $\pm$ 3.1 <sup>a,2</sup>	98.1 $\pm$ 4.7 <sup>a,2</sup>	< 0.001	< 0.001	0.107		
	Free Gent.	10.4 $\pm$ 8.7 <sup>b,1</sup>	31.5 $\pm$ 20.3 <sup>b,2</sup>	44.5 $\pm$ 29.2 <sup>b,2</sup>						
	DPPC-PEG $\Delta$	-	94.3 $\pm$ 5.0 <sup>a</sup>	94.2 $\pm$ 5.0 <sup>a</sup>						
	Free gent. $\Delta$	-	6.8 $\pm$ 5.6 <sup>c</sup>	15.2 $\pm$ 11.1 <sup>c</sup>						

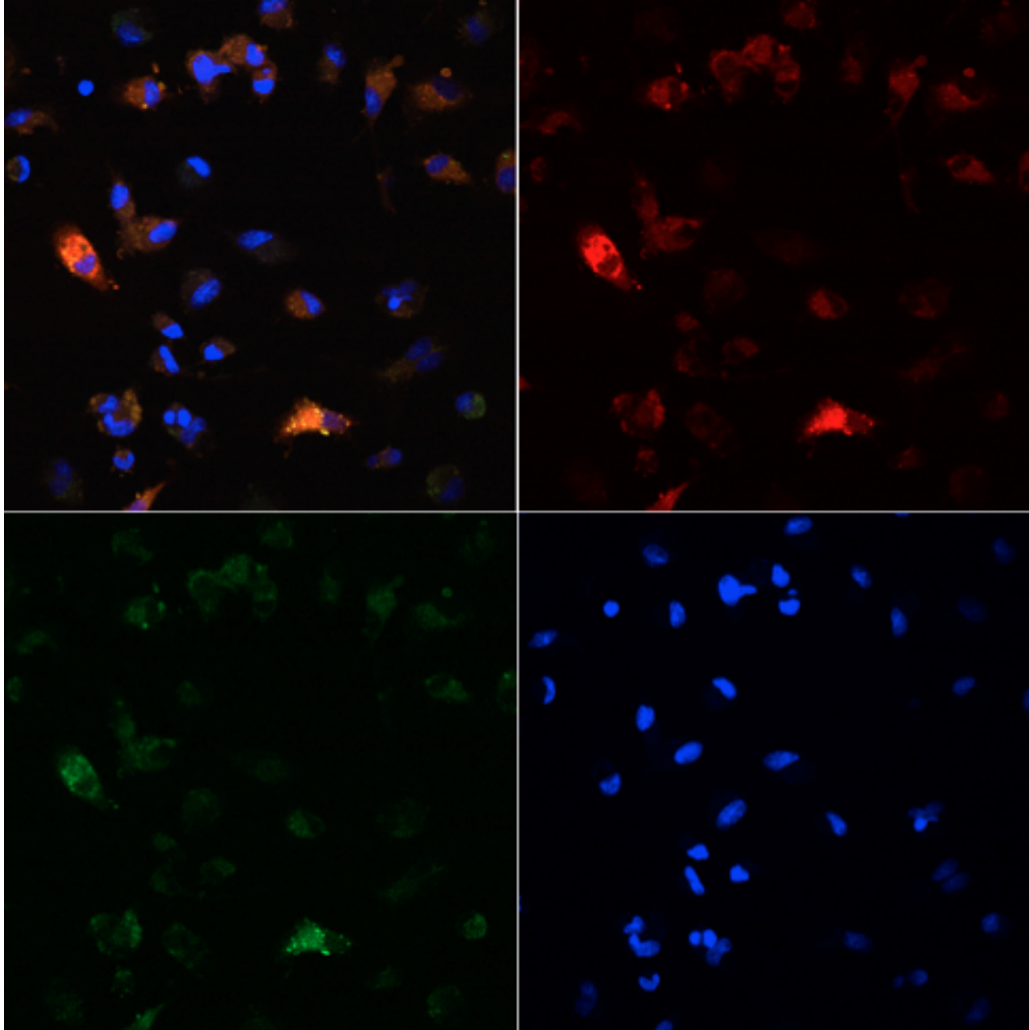
\*Percentage of all cells. <sup>†</sup>Percentage of cells containing *R. equi*.  $\Delta$  = media changed at T 4 h and replaced with drug-free media. <sup>a,b,c</sup> Different letters within a given time point (column) indicate a significant differences between drugs within a given variable ( $P < 0.05$ ). <sup>1,2,3</sup> Different numbers within a given time drug (row) indicate a significant differences time points ( $P < 0.05$ ).



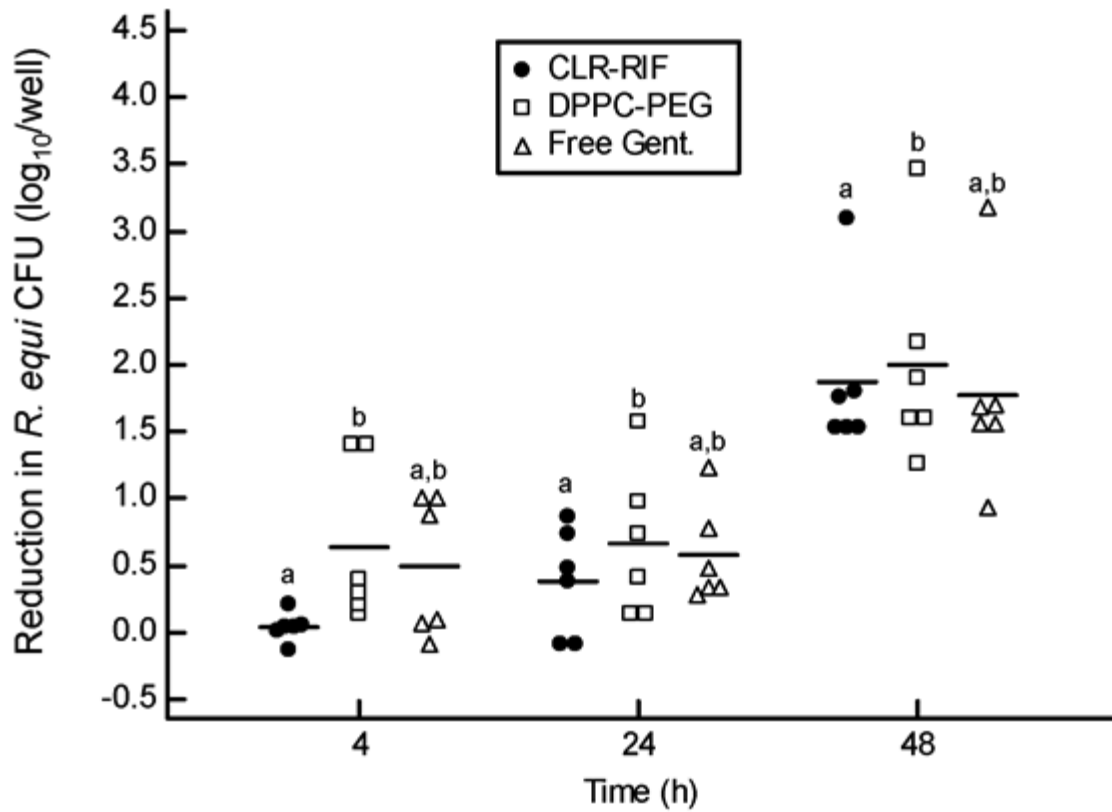
**Figure 5.1.** Mean ( $\pm$  SD)  $\log_{10}$  *R. equi* CFU per organ in nude mice infected intravenously with virulent *R. equi*. Four days after infection, mice (n=5 per group) were treated intravenously 3 times at 48 h intervals with drug-free liposomes (Control), free gentamicin (Free Gent.), liposomal gentamicin (DPPC), and PEG-coated liposomal gentamicin (DPPC-PEG). <sup>a,b</sup>Different letters within a given organ indicate a statistically significant differences between treatments ( $P < 0.05$ ).



**Figure 5.2.** Mean ( $\pm$ SD) reduction in the number of log<sub>10</sub> *R. equi* CFU relative to untreated controls in the spleens, livers, and lungs of nude mice (n=5 per group) infected intravenously with virulent *R. equi*. Four days after infection, mice were treated with PEG-coated liposomal gentamicin (DPPC-PEG) q 48 h IV or with clarithromycin-rifampin (CLR-RIF) q 24 h SC.



**Figure 5.3:** Confocal microscopy z-stack 0.2  $\mu\text{m}$  slice showing equine alveolar macrophages infected with GFP *R. equi* and treated with DiI-labeled liposomal gentamicin, T 4 h. Colocalization of *R. equi* (green, punctate) with liposomal gentamicin (red) is seen and results in yellow coloring in the merged image. Cell nuclei are blue.



**Figure 5.4:** Mean ( $\pm$  SD) reduction in the number of  $\log_{10}$  *R. equi* CFU relative to untreated control monolayers at 4, 24 and 48 h post-infection in equine alveolar macrophages (n = 6 horses) infected with *R. equi* 103+ and treated with either liposomal gentamicin (DPPC-PEG), free gentamicin (Free Gent.), or clarithromycin in combination with rifampin (CLR-RIF).  
<sup>a,b</sup>Different letters within a given time point indicate a statistically significant differences between treatments ( $P < 0.05$ ).

CHAPTER 6  
PHARMACOKINETICS, PULMONARY DISPOSITION AND TOLERABILITY OF  
LIPOSOMAL GENTAMICIN IN FOALS <sup>4</sup>

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<sup>4</sup> Burton, A.J., Giguère, S. and Arnold, R.D. 2014. *Equine Veterinary Journal*. Jun 18. doi:  
10.1111/evj.12309. [Epub ahead of print].

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## Abstract

Although gentamicin is highly active against *Rhodococcus equi in vitro*, its clinical efficacy has been limited presumably due to poor cellular uptake. Encapsulation of drugs in liposomes enhances their cellular uptake. The objective of this study was to compare the disposition of liposomal gentamicin (LG) and free gentamicin (FG) in the plasma, pulmonary epithelial lining fluid (PELF), and bronchoalveolar (BAL) cells of healthy foals after intravenous (IV) administration or by nebulization, and to assess the tolerability of the drug after repeated IV dosing. Eight healthy foals received a single IV or nebulized dose (6.6 mg/kg) of LG or FG in a balanced Latin square design, with a 14 day washout period between treatments. Subsequently, twelve healthy foals were administered either LG or FG at 6.6 mg/kg IV q 24h for 7 doses and urinary protein, creatinine,  $\gamma$ - glutamyltransferase and electrolytes were measured on days 0, 3 and 7 to quantify renal injury. Concentrations of gentamicin were measured using HPLC-MS. After IV administration, LG had a significantly higher mean ( $\pm$  SD) half-life ( $16.3 \pm 3.5$  vs.  $6.2 \pm 1.8$  h) and volume of distribution ( $2.00 \pm 1.03$  vs.  $0.72 \pm 0.32$  l/kg) compared with FG. Peak gentamicin concentrations in BAL cells were significantly higher for LG compared with FG after administration by both the IV ( $5.27 \pm 2.67$  vs.  $2.98 \pm 1.67$   $\mu\text{g/ml}$ ) and the nebulized ( $4.47 \pm 2.66$  vs.  $1.49 \pm 0.57$   $\mu\text{g/ml}$ ) routes. LG was well tolerated by all foals and indices of renal injury were not significantly different from those of foals administered FG. Administration of LG is well tolerated and results in higher intracellular drug concentrations than FG.

## Introduction

*Rhodococcus equi*, a Gram-positive facultative intracellular bacterium, is a common cause of pneumonia in 1 to 5 month-old foals. Despite recommended therapy with the combination of a macrolide and rifampin, the mortality rate of severely affected foals is still

around 30%<sup>1,2</sup>. Over the last 10 years, the incidence of macrolide and rifampin resistance has increased<sup>3</sup> and resistant isolates of *R. equi* are cultured from up to 40% of affected foals at some farms<sup>4</sup>. All *R. equi* isolates from pneumonic foals, including macrolide-resistant isolates, are susceptible to the aminoglycoside gentamicin *in vitro*<sup>3,5</sup>. Additionally, gentamicin is one of the few antimicrobial agents that are bactericidal against *R. equi*<sup>6,7</sup>. Although gentamicin is highly active against *R. equi in vitro*, its efficacy *in vivo* has been limited<sup>8</sup> presumably because of poor cellular uptake. A delivery system that could improve intracellular concentrations of gentamicin would likely increase its *in vivo* efficacy against *R. equi*.

Encapsulation in liposomes is one method by which the intracellular penetration of drugs might be enhanced. Liposomes are 0.08 – 5 µm in diameter lipid vesicles composed of one or several amphiphile bilayers surrounding an aqueous core<sup>9, 10</sup>. After administration, liposomes are phagocytized and rapidly localize in the phagosomes of mononuclear cells<sup>11</sup>. They also extravasate at sites of increased vascular permeability and are readily taken up into inflamed tissues, enabling targeted delivery to infected organs<sup>9, 12</sup>. Previous studies in a variety of species have shown that compared with free (conventional) gentamicin (FG), liposomal gentamicin (LG) has significantly enhanced cellular penetration and activity against facultative intracellular bacteria such as *Listeria monocytogenes*, *Mycobacterium avium*, *Salmonella* spp., and *Brucella abortus* both *in vitro* and *in vivo*<sup>10, 12-16</sup>. Recently, the safety and biodistribution of IV <sup>99m</sup>Tc-25 labelled liposomes was examined in adult horses<sup>17</sup>. Liposomes distributed primarily to the lungs, liver, spleen and kidneys and no adverse effects were noted<sup>17</sup>. In a soft tissue abscess model in horses, liposomes concentrated and persisted at the site of infection<sup>18</sup>.

We hypothesized that administration of LG to foals is well tolerated after intravenous (IV) administration or nebulization and results in increased concentrations of the drug in



bronchoalveolar (BAL) cells relative to FG. The objectives this study were to compare the disposition LG and FG in the plasma, pulmonary epithelial lining fluid (PELF) and BAL cells of healthy foals after administration by the intravenous route or by nebulization, and to assess the tolerability and accumulation of LG versus FG after repeated intravenous dosing.

## Materials and Methods

### *Formulation of liposomal gentamicin (LG)*

LG was formulated by aqueous capture using 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC),<sup>a</sup> cholesterol,<sup>b</sup> and 1,2-distearol-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG)<sup>a</sup> in chloroform in a molar ratio of 9:5:1. The DPPC, cholesterol and DSPE-PEG stored in chloroform at -80 °C were thawed and mixed in a 500 ml glass round bottom flask. The chloroform was evaporated from the mixture using vacuum under a constant nitrogen stream,<sup>c</sup> and the resultant thin lipid film was rehydrated with aqueous gentamicin sulfate<sup>d</sup> (250 mg/ml) at a ratio of 40.5 mg per  $\mu\text{mol}$  of lipid (resultant lipid concentration of 5  $\mu\text{mol}/\text{ml}$ ). After 5 freeze-thaw cycles in liquid nitrogen, the particles were sized using 3 passes through a high-pressure homogenizer.<sup>e</sup> Non-encapsulated gentamicin was removed via three rounds of dialysis<sup>f</sup> in 0.9% saline at 4°C. Mean percentage of initial gentamicin remaining encapsulated was 24.9% (range 20.1 – 32 %). Final particle size was verified using a dynamic light scattering particle sizer.<sup>g</sup> Median particle size diameter was 158 nm (range 139 – 178 nm) and mean ( $\pm$  SD) polydispersity index was  $0.114 \pm 0.020$ . LG was stored in the dark at 4°C and administered within 3 weeks of formulation.

### *Animals*

A total of 20 Quarter Horse foals ranging between 80 and 204 kg depending on age were used. Foals were considered healthy on the basis of physical examination, complete blood count,

and plasma biochemical profile. The foals were kept with their dams in individual stalls during the experiments and on pasture between experiments with *ad libitum* access to grass hay and water. The study was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

### *Experimental design and sample collection*

#### *Study 1: Single dose IV or nebulized liposomal (LG) or free gentamicin (FG)*

Eight foals received a single IV or nebulized dose (6.6 mg/kg bwt) of FG<sup>h</sup> or LG in a balanced Latin square design. Beginning at 5-7 weeks of age, every foal received each of the 4 possible drug-route combinations with a 14-day washout period between each administration. Intravenous FG and LG were diluted in 250 ml of sterile 0.9% saline and administered via a jugular catheter as a constant rate infusion over 15 min. Nebulized FG and LG were administered by inhalation over 15 min via a commercial equine nebulizer.<sup>i</sup> To ensure equivalent rate of delivery, FG was diluted to the same volume as LG with sterile 0.9% saline. Particle size of nebulized LG was verified using laser diffraction.<sup>j</sup> Blood samples for plasma separation were obtained from a catheter placed in the contralateral jugular vein prior to each drug administration, and at 5, 10, 20, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24 and 48 h after the end of the 15 min IV infusion or nebulization period. Bronchoalveolar lavage (BAL) fluid was collected at 2, 4, 8, 24, and 48 h. Foals were sedated with xylazine hydrochloride (0.5 mg/kg bwt IV) and butorphanol tartrate (0.07 mg/kg bwt IV) prior to collection of BAL fluid. Prior to analysis for gentamicin concentration, blood samples were centrifuged at 400 × g for 10 min and the resultant plasma frozen at -80°C until assayed.

### *Study 2: Repeated dose IV liposomal (LG) or free gentamicin (FG)*

Twelve 5- to 7-week-old foals were administered either LG (6 foals) or FG (6 foals) at 6.6 mg/kg bwt IV q 24 h for 7 doses. Each dose was diluted in 250 ml of sterile 0.9% saline and administered over 15 min via an indwelling 14 G catheter placed in a jugular vein. Blood samples for plasma separation and measurement of gentamicin concentrations were obtained on day 1 and on day 7 at the times listed for study 1. Bronchoalveolar lavage fluid was collected 2, 6 and 24 h after the end of infusion on day 7. Urine was collected the day prior to the first drug dose (day 0) and again 2 h after the end of the infusion on days 3 and 7. Foals were sedated with xylazine hydrochloride (1.1 mg/kg bwt IV) and anesthetized with ketamine (2.2 mg/kg bwt IV) to allow passage of a urinary catheter using aseptic techniques. Urinalysis was performed and urine and concurrently obtained plasma samples were submitted for measurement of creatinine,  $\gamma$ -glutamyltransferase (GGT), protein, calcium, chloride, magnesium, sodium and potassium concentrations. Fractional excretion of electrolytes, urinary GGT to creatinine ratio, and urinary protein to creatinine ratios were calculated. Prior to analysis for gentamicin concentration by LC-MS, urine and plasma samples were centrifuged at  $400 \times g$  for 10 min and the supernatant was frozen at  $-80^{\circ}\text{C}$  until assayed.

### *Bronchoalveolar lavage and processing*

A 10 mm diameter, 2.4 m BAL catheter<sup>k</sup> was passed via nasal approach until wedged into a bronchus. The lavage solution consisted of 4 aliquots of 60 ml 0.9% saline solution infused and instantly aspirated. Immediately upon collection, the total volume of BAL fluid recovered was measured and a 3 ml aliquot saved in an EDTA tube from which total nucleated cell count was determined by use of a cell counter.<sup>l</sup> BAL fluid was immediately centrifuged at  $400 \times g$  for 10 min. The BAL cells in the resultant pellet were washed, re-suspended in 500  $\mu\text{l}$  of acetonitrile :

0.2% formic acid (1:1, v/v), vortexed, and frozen at -80°C until assayed. Supernatant BAL fluid was also frozen at -80°C until assayed. Before assaying, the cell pellet samples were thawed, vortexed vigorously and sonicated for 10 min to ensure complete cell lysis. The resulting suspension was centrifuged at  $500 \times g$  for 10 min and the supernatant fluid was used to determine the intracellular concentrations of gentamicin.

*Drug analysis in plasma and body fluids by liquid chromatography tandem mass spectrometry (LC-MS/MS)*

The concentration of gentamicin sulfate in foal plasma was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described<sup>19</sup>. Briefly, gentamicin was extracted from plasma (250  $\mu$ l) and urine (500  $\mu$ l) using protein precipitation with an equal volume of ice-cold acetonitrile: 0.2% formic acid (9:1, v/v). Extracted samples were centrifuged (2°C at  $10,000 \times g$  for 10 min) twice. Two-hundred  $\mu$ l of the supernatants were transferred to polypropylene inserts for injection. The supernatant derived from the lysed BAL cell pellet was transferred to low-volume polypropylene inserts for direct injection without further processing. To measure gentamicin concentration in PELF, 20 ml of the initial BAL fluid supernatant was thawed, acidified with formic acid (99.9%) and centrifuged at  $1500 \times g$  for 10 min. An aliquot of the resultant supernatant was mixed with an equal volume of ice-cold acetonitrile, centrifuged (2°C at  $10,000 \times g$  for 10 min) and 200  $\mu$ l was transferred to polypropylene insert for injection. Calibration standards were prepared in drug-free foal plasma, BAL fluid or urine and then extracted as described above so that standard curves specific to each biologic matrix could be constructed. Concentration ranges of gentamicin sulfate used to construct standard curves and lower limits of quantification (LOQ) were as follows: plasma 0.045 –100  $\mu$ g/ml (LOQ, 0.045  $\mu$ g/ml), urine 3.125 - 100  $\mu$ g/ml (LOQ, 3.125  $\mu$ g/ml) and BAL

fluid 0.001 – 6.25 µg/ml (LOQ, 0.001 µg/ml). The inter-assay coefficient of variation was < 10% at concentrations 100-6.25 mg/ml and ≤ 20% at concentrations < 6.25 mg/ml. Analyte separation and LC-MS/MS measurement of gentamicin were performed exactly as described previously<sup>19</sup> except that BAL fluid samples were introduced in the MS with a flow rate of 0.18 ml/min and a total run time of 8 min.

#### *Calculation of gentamicin concentrations in PELF and BAL cells*

Estimation of the volume of PELF was determined by urea dilution method<sup>20</sup>. Urea nitrogen concentrations in BAL fluid (Urea<sub>BAL</sub>) and concurrent plasma samples (Urea<sub>PLASMA</sub>) were determined by use of a commercial quantitative colorimetric kit.<sup>m</sup> The volume of PELF (V<sub>PELF</sub>) in BAL fluid was derived from the following equation:  $V_{PELF} = V_{BAL} \times (Urea_{BAL}/Urea_{PLASMA})$ , where V<sub>BAL</sub> is the volume of recovered BAL fluid. The concentration of gentamicin in PELF (Gm<sub>PELF</sub>) was derived from the following relationship:  $Gm_{PELF} = Gm_{BAL} \times (V_{BAL}/V_{PELF})$ , where Gm<sub>BAL</sub> is the measured concentration of gentamicin in BAL fluid supernatant. The concentration of gentamicin in BAL cells (Gm<sub>CELLS</sub>) was calculated using the following relationship:  $Gm_{CELL} = (Gm_{PELLET}/V_{CELL})$  where Gm<sub>PELLET</sub> is the measured concentration of gentamicin in the cell pellet supernatant and V<sub>CELL</sub> is the mean volume of BAL cells. A volume of 1.20 µL per 10<sup>6</sup> BAL cells was used for calculations based on previous studies in foals<sup>21</sup>.

#### *Pharmacokinetic analysis*

For each foal, plasma, PELF, and BAL cell concentration versus time data were analyzed using commercial software.<sup>n</sup> Noncompartmental analysis was used for PELF and BAL cell data. A conventional linear two-compartment model with weighting by the inverse of the model (1/y) best predicted IV plasma gentamicin data based upon computer assisted examination of residual

plots, goodness of fit, and the sum of squares. The equation  $C_t = A \cdot e^{-a \cdot t} + B \cdot e^{-b \cdot t}$  was used where  $C_t$  is the serum drug concentration at time  $t$ ;  $e$  is the base of the Napierian logarithm;  $A$  and  $a$  are the intercept and rate constant, respectively, of the distribution phase;  $B$  and  $b$  are the intercept and rate constant, respectively, of the elimination phase. The rate constant of the elimination phase ( $\beta$ ) was determined by linear regression of the terminal phase of the logarithmic plasma concentration versus time curve using a minimum of 3 data points. Terminal half-life ( $t_{1/2\beta}$ ) was calculated as  $0.693/\beta$ . 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  $C_{24h}/\beta$ , where  $C_{24h}$  is the plasma concentration at the 24 h sampling time. Mean residence time (MRT) was calculated as:  $AUMC_{0-\infty}/AUC_{0-\infty}$ . Apparent volume of distribution based on the AUC ( $V_{d_{area}}$ ) was calculated as:  $dose / AUC_{0-\infty} \cdot \beta$ , apparent volume of distribution at steady state ( $V_{d_{ss}}$ ) was calculated as:  $dose \cdot AUMC_{0-\infty} / (AUC_{0-\infty})^2$ , and systemic clearance (CL) was calculated from:  $dose / AUC_{0-\infty}$ .

### *Statistical Analysis*

Normality and equality of variance of the data were assessed with use of the Shapiro-Wilk and Levene tests, respectively. Data that were not normally distributed were log or rank transformed. For study 1, the paired t test or the Wilcoxon rank sum test was used to compare IV pharmacokinetic variables between LF and FG. The effects of drug (LG vs FG), administration route (IV vs nebulized) and the interactions between drug and administration route on PELF and BAL cell pharmacokinetic variables were assessed using a two-way ANOVA for repeated measurement. For study 2, the effects of drug (LG vs FG), time (day 0, day 3, and day 7), and the interactions between drug and time on renal indices were assessed using a two-way ANOVA with one factor repetition (time). When warranted, multiple pairwise comparisons were done by

use of the Holm-Sidak test. The paired t test or the Wilcoxon rank sum test was used to compare IV pharmacokinetic variables obtained on day 1 to those obtained on day 7. Significance was set at  $P < 0.05$ .

## Results

### *Study 1: Single dose IV or nebulized LG versus FG*

Plasma concentration versus time data after IV administration of FG and LG are presented in Fig 6.1. Intravenous administration of LG resulted in significantly lower initial plasma concentrations but significantly higher  $V_d$ ,  $t_{1/2\beta}$ , MRT, and concentrations at 24 and 48 h compared with administration of FG (Table 6.1). The median particle size of nebulized LG was 3.1  $\mu\text{m}$  with 71% of the particles being  $< 5 \mu\text{m}$  and 92% being  $< 10 \mu\text{m}$ . Plasma concentrations of gentamicin after administration of nebulized LG or FG were  $\leq 0.78 \mu\text{g/ml}$  at all time points.

Regardless of route of administration, gentamicin concentrations in BAL cells,  $T_{\text{max}}$ , and  $\text{AUC}_{0-t}$  were significantly higher for LG than for FG (Table 6.2). Conversely,  $C_{\text{max}}$  in PELF was significantly higher after administration of FG compared with LG for both the IV and nebulized administration routes (Table 6.2). Similarly  $C_{\text{max}}$  in PELF was significantly higher after nebulization than after IV administration regardless of drug (Table 6.2).

### *Study 2: Repeated dose IV LG or FG*

Plasma pharmacokinetic variables obtained after administration of the first dose of LG or FG were not significantly different from those obtained in study 1 and not significantly different from those calculated after administration of the same formulation on day 7 (data not shown), indicating no accumulation of either LG or FG in plasma over 1 week of daily administration. Daily IV administration of LG resulted in significantly higher  $C_{\text{max}}$  ( $12.1 \pm 5.9$  vs.  $6.7 \pm 1.9 \mu\text{g/ml}$ ;  $P = 0.015$ ) and  $\text{AUC}_{0-t}$  ( $200 \pm 82.9$  vs.  $105 \pm 35.1 \mu\text{g}\cdot\text{h/ml}$ ;  $P = 0.007$ ) in BAL cells

compared to FG. Concentration in BAL cells at 24 hours ( $8.9 \pm 7.2$  vs.  $3.5 \pm 1.8$   $\mu\text{g/ml}$ ,  $P = 0.053$ ) and  $T_{\text{max}}$  (median = 6 h, range = 2 – 24 h for both groups;  $P = 0.86$ ) were not significantly different between LG and FG. There were no significant differences in gentamicin concentrations in urine between drug formulations or over time (Table 6.3). Indices of renal injury did not differ significantly between LG and FG. However, the mean fractional excretions of sodium and chloride were significantly greater on day 7 compared with day 0 or day 3 for both LG and FG (Table 6.3). Urinary pH and GGT:creatinine ratio were significantly different between treatment groups on day 0 (prior to drug administration). Therefore, these two parameters were expressed as a change from baseline (value on a given day – value on day 0) for data analysis (Table 6.4). For both LG and FG, the difference in GGT:creatinine ratio was significantly higher on day 7 compared with day 3. The difference in urine pH was not significantly different between day 3 and day 7 but was significantly higher in foals that received LG compared with foals that received FG. One foal from each treatment group had casts on urine sediment analysis on day 7. Three foals developed thrombophlebitis, 2 from the FG group and 1 from the LG group. One foal from each group developed mild self-limiting diarrhea during treatment.

### Discussion

In a prior study, age was found to have a profound effect on the pharmacokinetics of FG administered IV to foals<sup>19</sup>. The dose of 6.6 mg/kg bwt used in this study was based on simulations from data collected after administration of FG at a dose of 12 mg/kg bwt in the aforementioned study<sup>19</sup>. The mean ( $\pm$  SD) measured plasma concentration 1 h after IV administration of FG to 5-7 week-old foals in the present study ( $32.60 \pm 25.28$   $\mu\text{g/ml}$ ) was similar to predicted concentrations ( $25.27 \pm 9.52$   $\mu\text{g/ml}$  at 4 weeks of age and  $34.52 \pm 14.11$



$\mu\text{g/ml}$  at 12 weeks of age)<sup>19</sup>. Similarly, measured concentrations 24 h after administration in this study ( $0.25 \pm 0.19 \mu\text{g/ml}$ ) compared closely to predicted concentrations ( $0.20 \pm 0.22 \mu\text{g/ml}$  at 4 weeks of age and  $0.26 \pm 0.11 \mu\text{g/ml}$  at 12 weeks of age)<sup>19</sup>.

Aminoglycosides such as gentamicin are polycationic, highly polar, and have poor lipid solubility resulting in relatively low uptake by phagocytic cells<sup>22</sup>. Encapsulation in liposomes is one method by which the intracellular penetration of drugs might be enhanced. The *in vivo* disposition of liposomes varies dramatically depending upon their specific lipid composition, particle size, and method of formulation, all of which affect the rate at which liposomes are taken up by mononuclear phagocytes and the extent to which they localize in affected tissues<sup>9, 16</sup>. At the most basic level, liposomes can be divided into two main categories: conventional, short-circulating liposomes which are composed of natural or synthetic phospholipids  $\pm$  cholesterol, and long circulating liposomes sterically-stabilized with high-phase transition lipids, cholesterol and polyethylene glycol (PEG) coating which delay opsonization and uptake by mononuclear phagocytes relative to conventional liposomes, thus resulting in prolonged systemic circulation time and higher tissue concentrations. A balance between uptake by phagocytic cells and stability in the circulation and at site of infection must be achieved for therapeutic success. A sterically stabilized PEG-coated liposome formulation was developed for use in this study because of prior work showing significantly greater localization of PEG-coated over conventional liposomes in the lungs of pneumonic rats and because of higher or similar efficacy of PEG-coated liposomal antimicrobials in animal models of bacterial infection<sup>23,24</sup>.

The significantly longer plasma half-life exhibited by LG compared with FG after administration by the IV route in this study is consistent with the results of studies comparing liposomal versus free aminoglycosides in laboratory animals<sup>25</sup>. The significantly longer plasma

elimination half-life of LG in this study can be attributed to a significantly larger Vd because systemic clearance was almost identical for both formulations. The significantly lower initial plasma concentrations and higher Vd achieved after IV administration of LG are consistent with rapid uptake by phagocytes and distribution to tissues. The greater uptake of LG by phagocytes was confirmed by a significantly higher  $C_{max}$  and AUC in BAL cells after administration of LG compared with FG.

Aminoglycosides exert concentration dependent bacterial killing characteristics. Their rate of killing increases as the drug concentration increases above the minimum inhibitory concentration (MIC) for a given pathogen with optimal maximum plasma concentration ( $C_{max}$ ) to MIC ratio of 8-10:1<sup>26, 27</sup>. The MIC that inhibits at least 90% (MIC<sub>90</sub>) of *R. equi* isolates is 0.5  $\mu\text{g/ml}$ <sup>28</sup>. Although administration of both LG and FG resulted in peak concentrations of gentamicin in BAL cells above the MIC<sub>90</sub> of *R. equi*, only IV or nebulized LG reached the optimal  $C_{max}$  to MIC ratio of 8-10:1. The advantage of liposomal formulations of gentamicin over FG in the intracellular environment may not be related solely to differences in intracellular concentration. Liposome formulation similar to the one used in the present study, have been shown to concentrate in phagosomes after engulfment by macrophages<sup>11</sup>. Thus, co-localization of LG with bacteria in the phagosome could enhance intracellular killing of intracellular pathogens such as *R. equi*. Indeed, the LG formulation used in the present study was found to be superior to FG or to the combination of clarithromycin and rifampin to decrease tissue colony forming units of *R. equi* in a mouse infection model<sup>29</sup>. Similarly, LG has been shown to be more effective than FG in animal models of infection with other facultative intracellular pathogens such as *Listeria monocytogenes*, *Mycobacterium avium*, *Salmonella* spp., and *Brucella abortus*<sup>10, 12-16</sup>. The advantage of LG over FG may not only apply to the treatment of intracellular

pathogens. Infection models with extracellular pathogens such as *Klebsiella pneumoniae* have also shown an advantage of LG versus FG<sup>30</sup>.

Nebulized liposomal amikacin has been shown to be significantly more efficacious than nebulized free amikacin for the treatment of chronic *Pseudomonas aeruginosa* infection in rats and has been found to be safe and effective in people with cystic fibrosis during Stage II trials<sup>31, 32</sup>. In the present study, gentamicin concentrations in BAL cells were significantly higher after nebulization of LG than after nebulization of FG. Plasma concentrations of gentamicin were minimal after nebulization with LG despite concentrations in BAL cells similar to those achieved after IV administration. Therefore, nebulization of LG shows promise as an alternative to IV LG or concurrent administration by both routes could be used to further increase BAL cells and pulmonary concentrations of gentamicin with negligible contribution to systemic toxicity. Consistent with the greater cellular uptake of LG, concentrations of gentamicin in PELF were significantly higher after nebulization with FG than after IV FG or after administration of LG regardless of route.

Liposomal encapsulation of drugs can minimize non-target organ specific drug toxicity but this is dependent upon interactions between liposome formulation, drug encapsulated, as well as rate and location of drug release<sup>33</sup>. The main adverse effect of gentamicin recognized in horses is nephrotoxicity resulting from tubular necrosis. No adverse effects were encountered with single dose IV or nebulized LG, and the incidence of adverse events (diarrhea, thrombophlebitis) and indices of nephrotoxicity during repeated daily IV dosing were not significantly different between LG and FG. Urine GGT:creatinine ratio is a much more sensitive indicator of tubular damage than histopathology in adult horses with increases in urine GGT/creatinine ratio occurring after only 3-5 days of therapy with IV FG despite normal histopathology of the

kidney<sup>34, 35</sup>. Therefore, the increase in urine GGT/creatinine ratio observed after administration of LG or FG in the present study was not unexpected.

In conclusion, administration of LG to foals by the IV or nebulized route is well tolerated and results in significantly higher intracellular concentration of the drug compared to what is achieved after administration of FG. Additional studies will be needed to evaluate the efficacy of this LG formulation for the treatment of susceptible bacterial pathogens of horses.

#### Manufacturers' addresses

<sup>a</sup>Avanti PolarLipids Inc., Alabaster, AL, USA.

<sup>b</sup>Sigma-Aldrich, St-Louis, MO, USA.

<sup>c</sup>Rotavapor® R-210/R-215, Buchi Corporation, New Castle, DE, USA.

<sup>d</sup>PCCA, Houston, TX, USA.

<sup>e</sup>EmulsiFlex-C5®, Avestin Inc., Ottawa, ON, Canada.

<sup>f</sup>Slide-A-Lyzer dialysis cassettes, 10K MWCO, Thermo Fisher Scientific Inc. Rockford, IL, USA.

<sup>g</sup>90Plus Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, NY, USA.

<sup>h</sup>gentamicin sulfate Sparhawk Laboratories Inc., Lenexa, KS, USA.

<sup>i</sup>Flexineb™, Nortev, Galway, Ireland.

<sup>j</sup>Spraytec, Malvern Instrument Limited, Malvern, Worcestershire, UK.

<sup>k</sup>Jorgensen Laboratories, Inc., Loveland, CO, USA.

<sup>l</sup>Cellometer Auto T4, Nexcelom Bioscience, Lawrence, MA, USA.

<sup>m</sup>Biochain Urea Assay Kit, Hayward, CA, USA.

<sup>n</sup>PK Solutions 2.0, Summit Research Services, Montrose, CO, USA.

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## Tables

**Table 6.1.** Pharmacokinetic variables (mean  $\pm$  SD) for gentamicin in plasma after IV administration of free gentamicin sulfate (FG) or liposomal gentamicin sulfate (LG) at a dosage of 6.6 mg/kg to 8 foals (Study 1).

Variable	Drug		P value
	FG	LG	
A ( $\mu\text{g/ml}$ )	53.2 $\pm$ 25.8	28.8 $\pm$ 24.2	0.007
$\alpha$ ( $\text{h}^{-1}$ )	0.82 $\pm$ 0.28	0.76 $\pm$ 0.42	0.572
$t_{1/2\alpha}$ ( $\text{h}^{-1}$ )	0.97 $\pm$ 0.45	1.10 $\pm$ 0.44	0.602
B ( $\mu\text{g/ml}$ )	2.50 $\pm$ 1.50	1.49 $\pm$ 0.74	0.120
$\beta$ ( $\text{h}^{-1}$ )	0.12 $\pm$ 0.04	0.04 $\pm$ 0.01	<0.001
$t_{1/2\beta}$ (h)	6.20 $\pm$ 1.77	16.3 $\pm$ 3.5	<0.001
Vd <sub>area</sub> (l/kg)	0.72 $\pm$ 0.32	2.00 $\pm$ 1.03	0.010
Vd <sub>SS</sub> (l/kg)	0.24 $\pm$ 0.11	1.09 $\pm$ 0.71	0.012
CL (ml/h/kg)	85.2 $\pm$ 36.9	88.7 $\pm$ 45.5	0.822
AUC <sub>0-t</sub> ( $\mu\text{g} \cdot \text{h/ml}$ )	96.7 $\pm$ 63.8	66.1 $\pm$ 18.1	0.362
AUC <sub>0-∞</sub> ( $\mu\text{g} \cdot \text{h/ml}$ )	98.4 $\pm$ 64.3	71.9 $\pm$ 15.8	0.247
AUMC <sub>0-∞</sub> ( $\mu\text{g} \cdot \text{h}^2/\text{ml}$ )	360 $\pm$ 364	968 $\pm$ 574	0.016
MRT (h)	3.18 $\pm$ 1.25	13.0 $\pm$ 4.4	<0.001
C <sub>0.5h</sub> ( $\mu\text{g/ml}$ )	71.78 $\pm$ 92.14	19.14 $\pm$ 10.63	0.011
C <sub>1h</sub> ( $\mu\text{g/ml}$ )	32.60 $\pm$ 25.28	13.18 $\pm$ 4.40	0.040
C <sub>24h</sub> ( $\mu\text{g/ml}$ )	0.25 $\pm$ 0.19	0.50 $\pm$ 0.33	0.037
C <sub>48h</sub> ( $\mu\text{g/ml}$ )	0.09 $\pm$ 0.09	0.23 $\pm$ 0.16	0.012

A and  $\alpha$  = Intercept and rate constant, respectively of the distribution phase;  $t_{1/2\alpha}$  = Distribution half-life; B and  $\beta$  = Intercept and rate constant, respectively of the elimination phase;  $t_{1/2\beta}$  = Elimination half-life; Vd<sub>area</sub> = Apparent volume of distribution based on AUC; Vd<sub>SS</sub> = Apparent volume of distribution at steady state CL = Clearance; AUC<sub>0-∞</sub> = 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;  $C_{30min}$  = Plasma concentrations of gentamicin 30 minutes after administration;  $C_{1h}$  = Plasma concentrations of gentamicin 1 hour after administration;  $C_{24h}$  = Plasma concentrations of gentamicin 24 hours after administration;  $C_{48h}$  = Plasma concentrations of gentamicin 48 hours after administration.

**Table 6.2.** Pharmacokinetic variables (mean  $\pm$  SD unless otherwise specified\*) for gentamicin concentration in BAL cells and PELF after IV or nebulized (Neb) free gentamicin (FG) or liposomal gentamicin (LG) sulfate at a dosage of 6.6 mg/kg to 8 foals (Study 1).

Sample	Variable	Route	Drug		P value		
			FG	LG	drug	route	drug $\times$ route
BAL cells	C <sub>max</sub> ( $\mu$ g/ml)	IV	2.98 $\pm$ 1.67 <sup>a</sup>	5.27 $\pm$ 2.67 <sup>b</sup>	<0.001	0.076	0.472
		Neb	1.49 $\pm$ 0.57 <sup>a</sup>	4.47 $\pm$ 2.66 <sup>b</sup>			
	T <sub>max</sub> (h)*	IV	2 (2-8) <sup>a</sup>	24 (2-48) <sup>b</sup>	0.028	0.809	0.433
		Neb	3 (2-48) <sup>a</sup>	4 (2-24) <sup>b</sup>			
	AUC <sub>0-t</sub> ( $\mu$ g•h/ml)	IV	58.9 $\pm$ 41.5 <sup>a</sup>	145 $\pm$ 65 <sup>b</sup>	<0.001	0.102	0.860
		Neb	37.2 $\pm$ 19.0 <sup>a</sup>	114 $\pm$ 76 <sup>b</sup>			
	C <sub>24h</sub> ( $\mu$ g/ml)	IV	1.50 $\pm$ 1.23	4.27 $\pm$ 3.30	0.087	0.118	0.307
		Neb	1.01 $\pm$ 0.57	2.52 $\pm$ 2.26			
C <sub>48h</sub> ( $\mu$ g/ml)	IV	0.47 $\pm$ 0.62 <sup>a</sup>	2.09 $\pm$ 1.47 <sup>a</sup>	0.003	0.399	0.380	
	Neb	0.32 $\pm$ 0.23 <sup>a</sup>	1.26 $\pm$ 1.28 <sup>b</sup>				
PELF	C <sub>max</sub> ( $\mu$ g/ml)	IV	4.64 $\pm$ 1.99 <sup>a</sup>	1.21 $\pm$ 0.48 <sup>b</sup>	<0.001	0.007	0.273
		Neb	13.0 $\pm$ 6.7 <sup>c</sup>	2.05 $\pm$ 1.28 <sup>d</sup>			
	T <sub>max</sub> (h)*	IV	6 (4-8)	6 (2-24)	0.938	0.082	0.189
		Neb	2 (2-2)	4 (2-24)			
	AUC <sub>0-t</sub> ( $\mu$ g•h/ml)	IV	44.7 $\pm$ 21.0 <sup>a</sup>	12.9 $\pm$ 6.4 <sup>b</sup>	0.006	0.759	0.539
		Neb	41.0 $\pm$ 15.8 <sup>a</sup>	17.1 $\pm$ 13.5 <sup>b</sup>			
	C <sub>24h</sub> ( $\mu$ g/ml)	IV	0.84 $\pm$ 0.64	0.42 $\pm$ 0.60	0.371	0.119	0.457
		Neb	0.35 $\pm$ 0.34	0.74 $\pm$ 1.06			

\*Median and range. BAL = bronchoalveolar; PELF = pulmonary epithelial lining fluid. C<sub>max</sub> = Maximum concentration. T<sub>max</sub> = Time to maximum concentration. AUC<sub>0-t</sub> = Area under the plasma concentration versus time curve until the last measurable time point. C<sub>24h</sub> = Concentrations at 24 hours. C<sub>48h</sub> = Concentrations at 48 hours. <sup>a,b,c,d</sup>Different letters within a given variable indicate a statistically significant difference between drugs and/or administration route ( $P < 0.05$ ).

**Table 6.3.** Mean ( $\pm$  SD) urinary gentamicin concentrations and selected plasma and urinary indices of renal injury on days 0, 3 and 7 in foals receiving free gentamicin (FG; n=6) or liposomal gentamicin (LG; n=6) at a dose of 6.6 mg/kg IV q 24 h for 7 doses (Study 2).

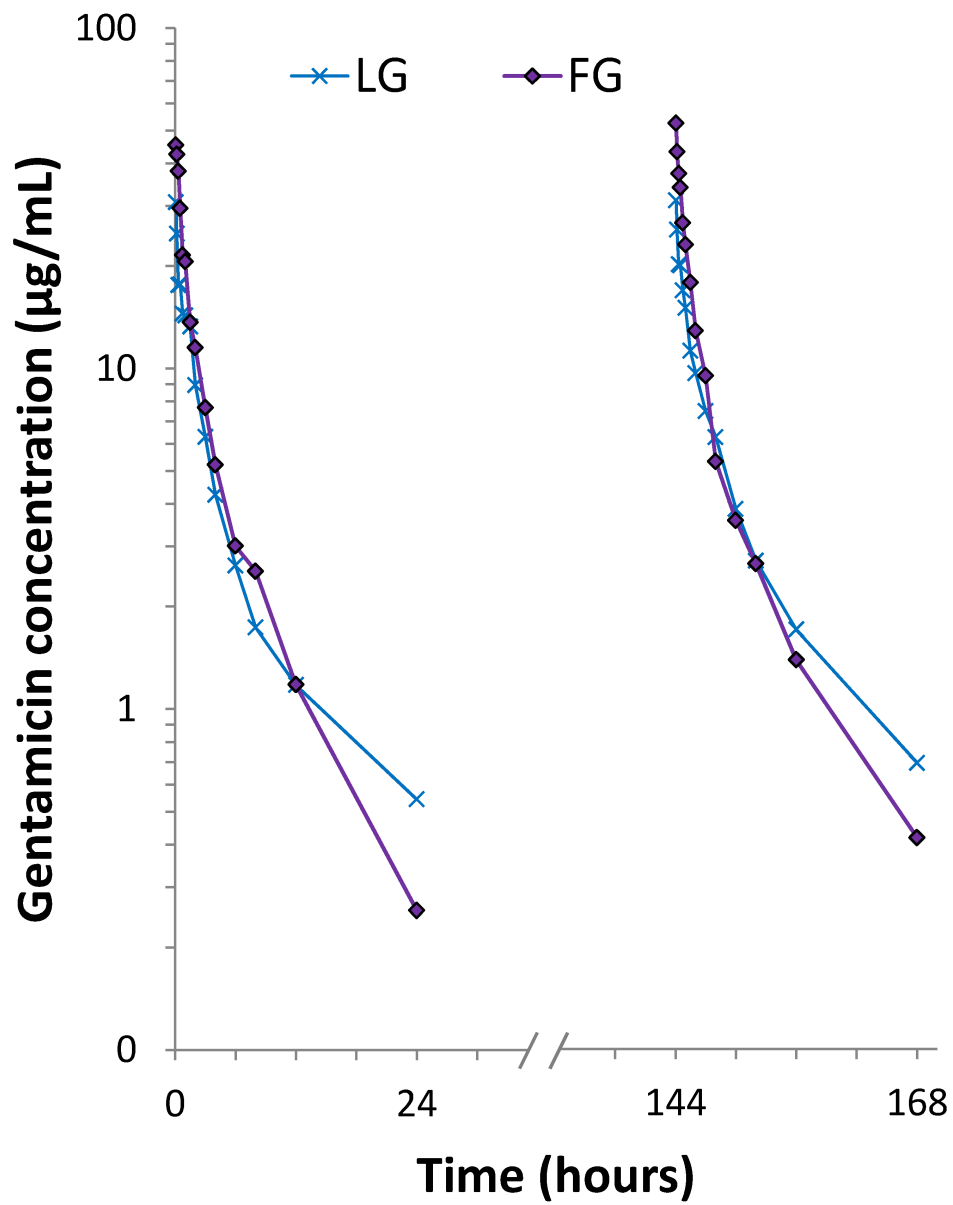
Variable	Dr ug	Time			P value		
		Day 0	Day 3	Day 7	Drug	Time	Drug $\times$ time
Urine gentamicin ( $\mu$ g/ml)	FG	-	94.8 $\pm$ 47.5	87.5 $\pm$ 5	0.130	0.376	0.746
	LG	-	66.2 $\pm$ 34.4	47.2 $\pm$ 53.0			
Plasma creatinine ( $\mu$ mol/l)	FG	116 $\pm$ 20	121 $\pm$ 21	118 $\pm$ 25	0.350	0.633	0.375
	LG	125 $\pm$ 7	122 $\pm$ 10	140 $\pm$ 36			
Urine protein/(creatinine x 0.001) ratio (g/mmol)	FG	23.9 $\pm$ 4.6	40.5 $\pm$ 48.5	34.7 $\pm$ 15.8	0.589	0.083	0.113
	LG	19.9 $\pm$ 9.5	20.5 $\pm$ 6.1	84.9 $\pm$ 91.3			
Urine GGT/(creatinine x 0.001) ratio (U/mmol)	FG	2.70 $\pm$ 1.62*	3.04 $\pm$ 1.26	8.75 $\pm$ 5.33	0.033	<0.001	0.376
	LG	1.38 $\pm$ 0.41*	2.26 $\pm$ 0.44	4.34 $\pm$ 1.86			
FE Na <sup>+</sup> (%)	FG	0.28 $\pm$ 0.4 <sup>a</sup>	0.12 $\pm$ 0.05 <sup>a</sup>	0.27 $\pm$ 0.12 <sup>b</sup>	0.796	0.011	0.614
	LG	0.17 $\pm$ 0.17 <sup>a</sup>	0.16 $\pm$ 0.07 <sup>a</sup>	0.30 $\pm$ 0.18 <sup>b</sup>			
FE K <sup>+</sup> (%)	FG	10.1 $\pm$ 4.9	18.4 $\pm$ 12.2	16.8 $\pm$ 10.6	0.721	0.067	0.632
	LG	13.0 $\pm$ 8.4	16.7 $\pm$ 8.9	20.6 $\pm$ 12.6			
FE Cl <sup>-</sup> (%)	FG	0.60 $\pm$ 0.29	0.6 $\pm$ 0.2	0.78 $\pm$ 0.19	0.699	0.003	0.357
	LG	0.5 $\pm$ 0.2	0.53 $\pm$ 0.15	0.88 $\pm$ 0.3			
FE Mg <sup>2+</sup> (%)	FG	13.9 $\pm$ 8.9	11.2 $\pm$ 6.5	9.8 $\pm$ 4.7	0.422	0.634	0.508
	LG	9.2 $\pm$ 4.4	9.5 $\pm$ 4.1	9.6 $\pm$ 6.4			
FE Ca <sup>2+</sup> (%)	FG	3.5 $\pm$ 3.4	1.9 $\pm$ 1.1	1.6 $\pm$ 1.4	0.419	0.168	0.990
	LG	1.5 $\pm$ 1.0	1.3 $\pm$ 0.9	1.3 $\pm$ 1.5			
USG	FG	1.003 $\pm$ 0.002	1.008 $\pm$ 0.008	1.002 $\pm$ 0.001	0.906	0.291	0.107
	LG	1.008 $\pm$ 0.009	1.003 $\pm$ 0.002	1.003 $\pm$ 0.001			
Urine pH	FG	7.2 $\pm$ 0.7*	6.3 $\pm$ 0.8	6.3 $\pm$ 0.3	0.589	0.047	0.026
	LG	6.4 $\pm$ 0.4*	6.4 $\pm$ 0.6	6.5 $\pm$ 0.4			

GGT =  $\gamma$ - glutamyltransferase. FE = fractional excretion. USG = Urine specific gravity. <sup>a,b</sup>Different letters within a given variable indicate a significant difference between days ( $P < 0.05$ ). \*Indicate a significant difference between LG and FG on day 0 ( $P < 0.05$ ).

**Table 6.4.** Mean ( $\pm$  SD) difference from baseline (day 0) in urine GGT:creatinine ratio and in urine pH in foals receiving free gentamicin (FG; n=6) or liposomal gentamicin (LG; n=6) at a dose of 6.6 mg/kg IV q 24 h for 7 doses (Study 2).

Variable	Drug	Time		P value		
		Day 3	Day 7	Drug	Time	Drug $\times$ time
Urine	FG	0.34 $\pm$ 1.62 <sup>a</sup>	6.05 $\pm$ 4.98 <sup>b</sup>	0.556	<0.001	0.214
GGT/(creatinine x 0.001) ratio (U/mmol)	LG	0.89 $\pm$ 0.38 <sup>a</sup>	2.96 $\pm$ 1.66 <sup>b</sup>			
Urine pH	FG	-0.83*	-1.67*	0.004	0.504	0.227
	LG	0.00*	0.25*			

GGT =  $\gamma$ - glutamyltransferase. <sup>a,b</sup>Different letters within a given variable indicate a significant difference between days ( $P < 0.05$ ). \*Indicates a significant difference between LG and FG ( $P < 0.05$ ).



**Figure 6.1.** Mean (+ SD) plasma concentrations of gentamicin after IV administration of free gentamicin or liposomal gentamicin at a dosage of 6.6 mg/kg to 8 foals (Study 1).

## CHAPTER 7

### CONCLUSION

This thesis encompasses the seminal work on the use of liposome encapsulated gentamicin sulfate as a novel antimicrobial treatment for disease caused by the bacterium *Rhodococcus equi* in foals. The catalyst for this work was the appearance in recent years of clinical *R. equi* isolates resistant to macrolides and rifampin; the sole therapy for disease caused by *R. equi* in foals. Pneumonia due to *R. equi* in foals is very common and in the absence of effective antimicrobial treatment, clinical disease almost invariably progresses to death. As such, it exerts a substantial financial and emotional toll within the equine breeding industry.

The first study described here (Chapter 3) aimed to verify, describe and characterize the emergence of resistance to macrolides and rifampin among *R. equi* isolates obtained from a typical large horse breeding farm in Kentucky, USA. In an attempt to decrease deaths associated with pneumonia caused by *R. equi* this farm initiated an ultrasonographic screening program in 2001 aimed at facilitation early diagnosis and treatment of foals with subclinical pulmonary lesions. In recent years, macrolide and rifampin resistant *R. equi* isolates had been identified from foals at this farm with clinical disease. The perception was that these isolates were occurring with increasing frequency from one year to the next, with concurrent escalation in treatment failures. The findings of our study demonstrated that macrolide- and rifampin-resistant isolates of *R. equi* began to emerge 7 years after initiation of this ultrasonographic screening program. Additionally, resistant isolates were found to be distinct



genotypes from sensitive isolates. These findings support the need for a viable alternative for treatment of foals with clinical disease due to *R. equi*, but also indicate that the practice of mass macrolide treatment for subclinical infection with *R. equi* in foals is not innocuous and should be discontinued.

The aim of the second study (Chapter 4) was to establish the appropriate dose of gentamicin sulfate in foals of different ages. This work had not been done before and it is well established that required aminoglycoside doses in neonates are often different from those in adults due to differences in total body water composition and metabolism. It was important to establish the pharmacokinetics of free gentamicin in foals prior to examining those of liposomal gentamicin. We established that the pharmacokinetics of gentamicin change considerably in the first 2 weeks of life. Intravenous administration of gentamicin at a dose of 12 mg/kg bwt q. 36 h would be required in foals less than 2 weeks of age. However, in foals 2 weeks of age or older, a lower dose of 6.6 mg/kg bwt given q. 24 h, which is the standard dose for adult horses, was predicted to be adequate.

The third study (Chapter 5) evaluated the uptake of liposomal gentamicin into macrophages, and also its efficacy against *R. equi* infection in comparison to free gentamicin and to rifampin and clarithromycin. Models used were J774A.1 murine macrophages and equine alveolar macrophages in cell culture and an established model of chronic *R. equi* infection in nude mice. Because *R. equi* preferentially survives and replicates within macrophages, and gentamicin is known to have relatively poor cellular penetration, it was vital to determine whether encapsulation in liposomes improved its cellular penetration. We found that uptake of liposomal gentamicin into J774A.1 and equine alveolar macrophages was significantly greater than for free gentamicin with over 90% of cells taking up drug within 4 hours. We also found

that compared with free gentamicin, liposomal gentamicin was significantly more effective against *R. equi* infection *in vitro* and *in vivo* and at least as effective compared with rifampin and clarithromycin.

The fourth study (Chapter 6) aimed to assess the pharmacokinetics, tolerability and toxicity of liposomal gentamicin as compared with free gentamicin in foals. Liposomal gentamicin, or free gentamicin was administered as a single IV or nebulized dose and also IV once daily. The pharmacokinetics of liposomal encapsulated drugs often differ from the free drug and as such, optimal dosing regimens may differ. Additionally, nephrotoxicity is the most important and limiting factor impacting administration of gentamicin to foals and thus assessment of this was important prior to embarking on further studies. We found that after IV administration, LG had a significantly higher mean ( $\pm$  SD) half-life ( $16.3 \pm 3.5$  vs.  $6.2 \pm 1.8$  h) and volume of distribution ( $2.00 \pm 1.03$  vs.  $0.72 \pm 0.32$  l/kg) compared with FG. Peak gentamicin concentrations in BAL cells were significantly higher for LG compared with FG after administration by both the IV ( $5.27 \pm 2.67$  vs.  $2.98 \pm 1.67$   $\mu\text{g/ml}$ ) and the nebulized ( $4.47 \pm 2.66$  vs.  $1.49 \pm 0.57$   $\mu\text{g/ml}$ ) routes. LG was well tolerated by all foals and indices of renal injury were not significantly different from those of foals administered FG. Administration of LG is well tolerated and results in higher intracellular drug concentrations than FG.

Taken together, these studies show that liposomal gentamicin is efficacious against *R. equi* *in vitro* and *in vivo* in a mouse model. It has also been established that in healthy foals, liposomal gentamicin is well tolerated via the IV or nebulized routes and reaches significantly higher intracellular concentrations compared with free gentamicin. Further, in healthy foals, liposomal gentamicin appears safe when administered once daily IV for 7 days at a dose of 6.6 mg/kg. These findings, together with the verification of increasing resistance to

macrolides and rifampin among clinically relevant *R. equi* isolates, strongly support further studies on liposomal gentamicin as a treatment in foals. The next logical step would be assessment of liposomal gentamicin as a treatment for *R. equi* in a disease model in foals.