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Efficacy of liposomal gentamicin against *Rhodococcus equi* in a mouse infection model and colocalization with *R. equi* in equine alveolar macrophages

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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 equine 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 (±SD) percentage of equine alveolar macrophages contained liposomal gentamicin (91.9 ± 7.6%) as opposed to free gentamicin (16.8 ± 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 ± 17.8%) compared to those exposed to free gentamicin (10.4 ± 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 support further investigation of liposomal gentamicin as a new treatment for infections caused by *R. equi*.

1. Introduction

Rhodococcus equi, a Gram-positive facultative intracellular bacterium, is a common cause of disease in 1–5 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; Giguère 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 (Giguère et al., 2010) and resistant isolates of *R. equi* are cultured from up to 40% of affected foals at some farms (Burton

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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 (Giguère 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* (Giguère et al., 2010; Jacks et al., 2003; Riesenberg 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 two 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.

2. Materials and methods

2.1. 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) with or without coating with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] 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, 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 μmol of lipid. After five freeze–thaw cycles in liquid nitrogen, the particles were sized to 100 nm using three passes through laser etched polycarbonate filters (0.08 μm) 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 10 kDa MW cut-off dialysis cassettes (Slide-A-Lyzer, Thermo Fisher Scientific Inc., Rockford, IL, USA). The final concentration of gentamicin in the liposome formulations was measured after dialysis by HPLC–MS as described previously (Burton et al., 2014).

2.2. Bacterial strains and MIC

R. equi strain 103^+, 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 (Giguère et al., 1999). Green fluorescent protein (GFP)-expressing *R. equi* was created by transformation of virulent strain 103^+ 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. Strain 103^+ expressing GFP was used to infect equine alveolar macrophages.

Minimum inhibitory concentration (MIC) of gentamicin, clarithromycin and rifampin was determined by broth macrodilution 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 2-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 35 ± 2 °C.

2.3. 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