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The use of streptolysin O (SLO) as an adjunct therapy for *Rhodococcus equi* pneumonia in foals

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ABSTRACT

Rhodococcus equi is a soil borne bacterium that causes severe morbidity and death in young foals. The economic costs of the disease include loss of life, treatment expenses, veterinary monitoring expenses and, perhaps most importantly, potential reduction in future athletic performance in horses that suffer severe lung abscessations caused by *R. equi*. Current standard of care for pneumonia caused by *R. equi* is treatment with a macrolide antimicrobial and rifampicin. However, the hallmark of pneumonia caused by *R. equi* is severe formation of pyogranulomas and a walling off effect that can prevent systemic antibiotics from reaching antimicrobial concentrations in lung tissues. It is hypothesized that streptolysin O (SLO) used as an adjunct therapy with antibiotics will reduce the duration and severity of disease caused by *R. equi* pneumonia compared to antibiotic therapy alone. Addition of SLO to the antibiotic enhanced clinical responses compared to the other groups, including the antibiotic alone group. Of particular significance were lower bacterial counts in the lungs and longer survival time in those foals treated with SLO and antibiotics.

1. Introduction

Infection with *Rhodococcus equi* is the most common cause of mortality in the young foal (Giguere and Prescott, 1997). Clinical signs most commonly include increased respiratory rate and fever (Takai et al., 2000). Auscultation of the lungs may reveal inspiratory and/or expiratory crackles and wheezes that occur most frequently in the cranioventral region (Lakritz et al., 1993). Diarrhea and other signs of gastrointestinal problems, such as colic, may occur. Infected foals may be found in severe respiratory distress or dead without previous clinical signs (Prescott and Hoffman, 1993). Foals that recover from infection can have a decreased chance of racing as adults (Ainsworth et al., 1998).

The severe pulmonary and abdominal granulomas that occur with *R. equi* infections present a formidable physical barrier to achieving bactericidal concentrations of antibiotics inside the lesions, let alone within the infected macrophages located in the granulomas (Schwab and Mandell, 1989; Womble et al., 2006; Wagner et al., 2006). The selection of lipid-soluble antibiotics capable of intracellular penetration is critical for the successful treatment. Historically, a combination of a macrolide antimicrobial (e.g. erythromycin 25 mg/kg three times daily) and rifampicin (5 mg/kg twice daily) has been used to treat infected foals (Giguere et al., 2010). Though effective, erythromycin is associated with significant adverse reactions including diarrhea, elevated liver enzymes and hyperthermia. While newer macrolide

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derivatives have reduced side effects, they nevertheless remain a concern. The emergence of macrolide antimicrobial resistant strains of *R. equi* is also a growing problem (Giguere et al., 2010). Enhancement of antibiotic therapy would have multiple benefits including a reduction in treatment duration, decrease in pathologic lesions, reduction of adverse reactions, and diminished clinical signs.

A number of adjunct approaches have been used in association with antibiotic treatments to enhance the overall clinical response to chronic bacterial infections of the lung (Welsh et al., 2010; Bansal and Chhibber, 2010). These adjunctive therapies have several goals including enhancement of antimicrobial efficacy and modulation of the immune response (Toossi, 1998). Streptolysin O (SLO) is a virulence factor of pyogenic streptococci which binds to cholesterol in the membranes of eukaryotic cells forming a pore (Bhakdi et al., 1996). While high concentrations of SLO can kill cells via the induction of caspases (Timmer et al., 2009), it has been reported that SLO and other bacteriolysins can facilitate the delivery of proteins and other molecules into living cells by permeabilizing the membrane (Walev et al., 2001; Naruse et al., 2009). The purpose of this study was to assess the effect of SLO on the response of *R. equi*-infected foals to antimicrobial therapy.

2. Materials and methods

2.1. Foals

A total of 24 foals (average age 4 ± 3 days) were used in this challenge model and two additional foals served as uninfected controls. The foals were obtained from mares raised and bred on the Department of Veterinary Science's North Farm. The foals were raised on the Veterinary Science farm and kept with their mares throughout the study. Foals were evaluated shortly after birth for inclusion in the study using the following criteria; normality in physical examination, absence of lung sounds on auscultation, normal temperature, and unremarkable ultrasound examination of the lungs. Transfer of maternal immunoglobulins was confirmed by SNAP test. None of foals received anti-rhodococcal plasma or other treatments prior to enrollment in the study. The average initial weight of the foals was 57.9 ± 8.5 kg and there was no difference between treatment groups. After birth, the foals were moved with their dams to individual box stalls. Baseline values for heart rate, respiratory rate, temperature, fibrinogen concentration, and leukocyte count were obtained the next day. All experimental protocols were approved by the University of Kentucky's Institutional Animal Care and Used Committee and the Institutional Biosafety Committee prior to the performance of this study.

2.2. Challenge

Foals were challenged with an intrabronchial instillation of *R. equi* (clinical isolate provided by Dr. M. Donahue, University of Kentucky Veterinary Diagnostic Laboratory). The isolate was confirmed to contain the virulence-associated plasmid by PCR (S. Giguere, University of Georgia). Bacterial cultures were grown in trypticase-soy broth overnight prior to inoculation. Bacterial concentrations were initially estimated based on OD readings of the overnight culture and then confirmed by serial dilution plate counting the following day. After sedation, a flexible fiber optic endoscope was used to deliver the bacteria suspended in 35 ml of sterile PBS into both main bronchi. The first six foals received $3.1 \pm 0.3 \times 10^8$ bacteria and the remaining foals received $1.4 \pm 0.1 \times 10^7$ bacteria. These dosages are similar to those used in other studies (Wada et al., 1997; Giguere et al., 1999; Hines et al., 2003) and can be expected to produce pneumonic disease in naïve foals (Wada et al., 1997). The foals were returned to their stalls with their dams and monitored daily for changes in rectal temperature, respiration, and pulse as determined by physical exam and auscultation of the lungs. Ultrasound scanning was performed weekly beginning one week after the inoculation and continuing throughout the time the foal remained on the study. Blood was obtained via jugular venipuncture on the day of each ultrasound scan for fibrinogen analysis. Fibrinogen levels were indirectly assessed by refractometry and ascertained by determining the difference between the serum and plasma total protein levels. The difference was multiplied by 1000 to give a result in mg/dl.

Clinical signs recorded included lung auscultations, overall attitude, appetite, resting respiratory rate, and pulse. Foals with suspect lesions (>2 cm) identified by ultrasound or with any clinical abnormalities consistent with rhodococcal pneumonia were assigned to one of the treatment groups (see below). Daily observation of attitude, appetite, and lung auscultation were scored separately (0–4; Table 1) and combined to generate an overall daily score for each foal. Twice daily temperatures were collected over the sampling period. If the temperature was normal (<39.2 °C) the temperature score for that day was scored "0", if >39.2, but less than 39.7, it was scored a "1", if >39.7 but less than 40.3, it was scored a "2", and temperatures greater than 40.3 where scored a "3". The average temperature score post-treatment was then calculated by summing the daily temperature scores after treatment began. An overall clinical score was calculated

Table 1
Daily clinical scoring rubric.

Score	Appetite	Attitude	Lung auscultation
0	Nursing frequently when observed	BAR (bright, alert and responsive)	Clear
1	Nursing occasionally	QAR (quiet, alert and responsive)	Increased noise or effort
2	Infrequently observed nursing	Quiet	Mild to moderate crackles and wheezes
3	Rarely observed nursing	Depressed	Severe crackles and wheezes
4	Not nursing	Moribund	Increased respiratory effort, abdominal breathing

by combining daily clinical score and daily temperature score for each foal beginning on the day of the onset of clinical signs and then dividing by the number of days of observation.

2.3. Treatments

Once a foal was determined to be clinically affected based on clinical signs (pyrexia, lung sounds and/or ultrasound findings) it was randomly assigned to one of the treatment regimens. The treatment consisted of either; (1) standard antibiotic therapy of clarithromycin (7.5 mg/kg) and rifampin (5 mg/kg) given *per os* twice daily with saline (vehicle), (2) antibiotic therapy with SLO, (3) SLO, alone or (4) vehicle, alone. The SLO treatment involved a 0.2 mL (2 IU in saline) administration by subcutaneous injection twice a day. The recombinant SLO was obtained from Capricorn Products (Portland, Maine) as a recombinant protein produced in *E. coli* and purified by metal affinity chromatography. All treatments were continued for 16 days unless symptoms progressed to the point where the foal needed to be euthanized. The foals were observed daily throughout the treatment period. Foals were treated symptomatically for fever and other clinical signs. In the later stages of the infection, those foals exhibiting flared nostrils and extreme difficulty in breathing, as well as severe depression, lethargy, and/or anorexia were euthanized early. All surviving foals were euthanized 16 days after the start of treatment.

2.4. Gross pathology

Complete necropsies were performed on all foals by a board-certified veterinary anatomic pathologist. Pneumonia and abscess scores were determined by modifying the previously described scoring system (Halbur et al., 1996) to correspond with equine pulmonary anatomy. All lung surfaces were assigned a percentage value based on total lung volume (dorsal aspect of right lung lobe = 24%, dorsal aspect of left lung lobe = 24%, ventral aspect of the right lung lobe = 24%, ventral aspect of left lung lobe = 24%, and accessory lung lobe = 4%). Scores were generated based on the percentage of pneumonic and abscessed lung. Digital images were taken at necropsy of all lung surfaces to determine the pneumonia scores. Adobe Photoshop CS3 was utilized to place a digital grid over each photographed lung surface and quantitate the amount of pneumonic and normal lung. The percentage of pneumonic vs. normal lung was then determined for each lung lobe. Pneumonia scores, from each lung lobe, were added together to form a cumulative pneumonia score for each animal. Abscess scores were directly assessed at necropsy by determining the percentage of abscessed lung in comparison to the total lung volume. Cumulative abscess scores were similarly determined for each animal by adding the abscess scores from each lung lobe together.

2.5. Bacteriology and histopathology

Lung tissue samples were obtained for bacteriological culturing and histopathology. For bacteriological cultures: 1 cm^2 pieces of lung tissue were obtained from the cranial apical, middle diaphragmatic, and dorsal diaphragmatic regions of the lung lobes. Tissue samples were homogenized in sterile saline and serially diluted before transferring 0.025 ml onto Tryptic Soy Agar Yeast Extract (TSAYE) plates. The resultant colonies were observed for overall consistency in appearance with respect to the known appearance of the organism, including development of red pigment over a 72 h period. Gram stain was used to confirm expected appearance of a gram positive coccobacillus.

Tissue samples for histopathology included: tracheobronchial lymph nodes, cranial segments of the right and left apical lung lobes, middle segments of the right and left diaphragmatic lung lobes, lateral basal segments of the left and right diaphragmatic lung lobes, accessory lung lobe, and a section of abscess wall (if present). Tissues were fixed for 24 h in 10% formalin, routinely processed, and stained with hematoxylin and eosin for microscopic evaluation. Each sample was histopathologically graded for lesion distribution (0 = none; 1 = focal; 2 = multifocal to coalescing; or 3 = diffuse), severity of pyogranulomatous inflammation (0 = none; 1 = mild; 2 = moderate; or 3 = severe), and distribution of parenchymal necrosis (0 = none; 1 = focal; 2 = multifocal to coalescing; or 3 = diffuse). A histopathologic pneumonia score was generated for each sample by adding the distribution, inflammation, and necrosis grades together. Total collagen estimation was performed by staining tissue sections from paraffin blocks with picrosirius red which preferentially stains collagen (Junqueira et al., 1979). The sections were viewed with a polarizing microscope to enhance the visualization of the collagen and rank ordered based on intensity of collagen staining by a viewer blinded to treatment.

2.6. Masson's trichrome histochemistry

Abscess walls were microscopically evaluated for fibrosis. Paraffin embedded sections of abscess wall were stained with Masson's trichrome (MT), according to the manufacture's (Dako) instructions, to accentuate fibrous connective tissue. The thickness of the fibrous connective tissue in the abscess wall was then measured in micrometers using an ocular measurement reticle. If abscesses were not grossly identified at necropsy, then the MT stain was performed on sections of the middle segment of the left diaphragmatic lung lobe. In these sections, fibrosis was measured around regions of microabssessation or necrosis.

2.7. Data analysis

The investigators and the veterinarian treating the foals were blinded to treatment. The data were analyzed using the group designations prior to decoding. Differences between treatment groups were determined using two way analysis of variance (Systat Software, Inc., San Jose, CA). Statistical significance was determined at $p < 0.05$. Since no treatment effect of SLO alone was observed, the results from these foals were combined with those of the vehicle only group. When no bacterial dose effect was observed, the data were combined together and bacterial

dose excluded from the model and the data analyzed using a one way analysis of variance.

3. Results

3.1. Clinical findings

Six foals (2 in each control group, SLO and None and 1 each in the antibiotic groups, antibiotic + vehicle and antibiotic + SLO) were initially challenged with $3.1 \pm 0.3 \times 10^8$ bacteria and the remaining foals received $1.4 \pm 0.1 \times 10^7$ bacteria. Those foals receiving the higher challenge dose exhibited clinical signs earlier than the lower challenge dose foals (8.25 ± 0.5 vs. 12.5 ± 0.9 days). There was a significant rise in fibrinogen levels post-infection peaking on day 12 in the high dose challenge group compared to day 19 for the low dose challenge group (Fig. 1). However, the fact that disease developed sooner did not equate with more severe clinical signs in the higher dose foals (Fig. 2). Those foals receiving antibiotic + SLO had lower clinical scores than the other treatment groups, though this was not statistically significant ($p < 0.190$). In regards to rectal temperature (Fig. 3), there was a significant ($p < 0.05$) treatment effect with those foals receiving antibiotic + SLO having lower average daily temperatures compared to the other treatment groups, which did not differ from one another. There was no effect of bacterial dose on the febrile response.

All foals were euthanized when clinical signs worsened (clinical score $\geq$10) or at 16 days post treatment initiation. Those foals receiving no treatment or SLO were euthanized significantly earlier than those receiving antibiotic + SLO (Fig. 4). While foals receiving antibiotic alone tended to survive longer than the none and SLO-treated groups, this was not statistically significant ($p = 0.057$ and $p = 0.467$, respectively). Though the antibiotic + SLO treated foals tended to survive longer than the antibiotic only foals, this was not significantly different ($p = 0.061$). There was no effect of bacterial dose on the time to necropsy.

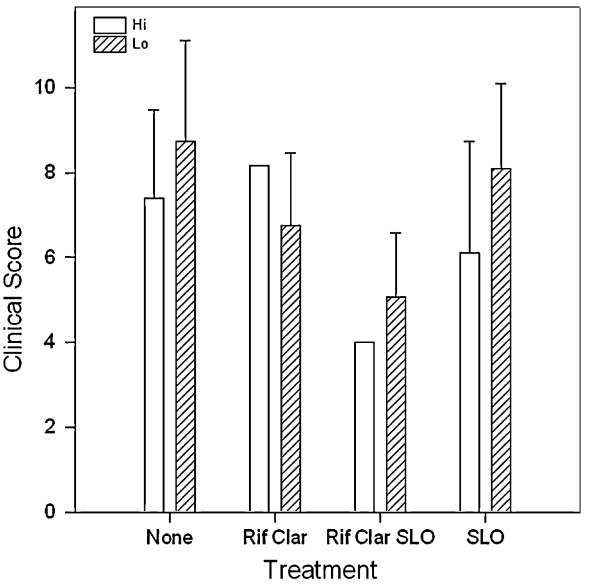

Fig. 2. Clinical scores post-challenge. Daily clinical scores were determined for each of the foals in the treatment groups and averaged. The open bar represent the average and SD of those foals receiving the higher bacterial dose, the hatched bar represent those foals receiving the lower dose.

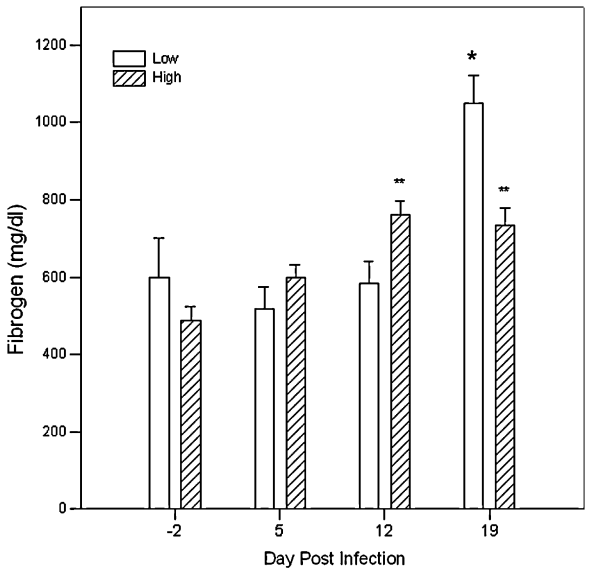

Fig. 1. Plasma fibrinogen post infection. Peripheral blood samples were collected on the days indicated and processed for the determination of fibrinogen. The open bar represent the average and SD of those foals receiving the higher bacterial dose, the hatched bar represent those foals receiving the lower dose. *: significantly more than the pre-challenge (d-2) sample for the low dose challenge. **: significantly more than the pre-challenge (d-2) sample for the high dose challenge.

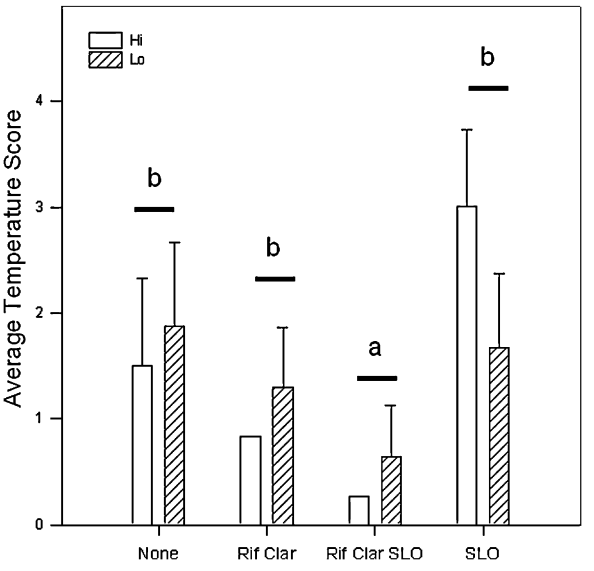

Fig. 3. Average temperature score post-treatment. Twice daily temperatures were collected from all foals and scored according to the method described in the text. All daily scores were averaged for the group and reported. The open bar represent the average and SD of those foals receiving the higher bacterial dose, the hatched bar represent those foals receiving the lower dose. Treatment groups with different letters were different from each other at $p < 0.05$. There was no effect of bacterial dose on average temperature scores.

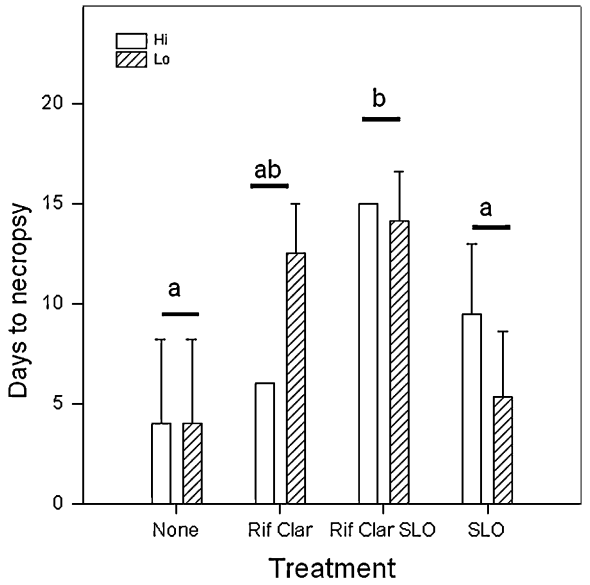

Fig. 4. Effect of treatment on post-treatment survival. Days from initiation of treatment to necropsy are reported for each treatment group. All foals were euthanized 15 days post treatment unless clinical signs necessitated early euthanasia for humane reasons. There was no effect of bacterial dose on days to necropsy. The open bar represent the average and SD of those foals receiving the higher bacterial dose, the hatched bar represent those foals receiving the lower dose. Treatment groups with different letters were different from each other at $p < 0.05$.

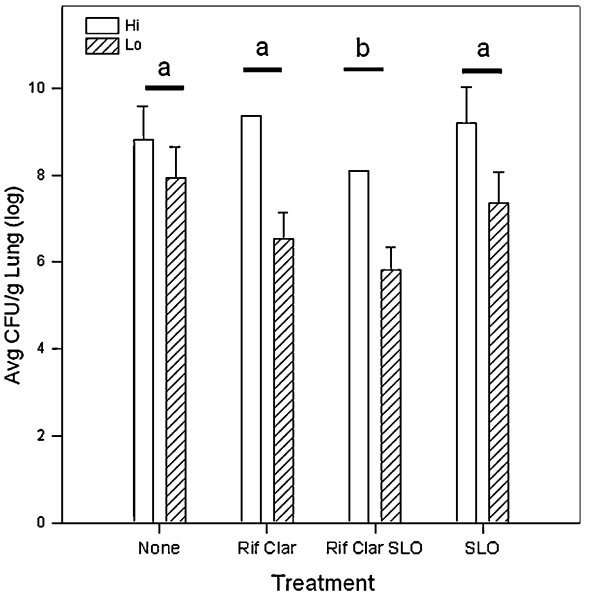

Fig. 6. Average number of colony forming units (CFU) obtained per gram of lung tissue. Tissue was obtained at necropsy and transported to the laboratory for bacterial culture. The open bar represent the average and SD of those foals receiving the higher bacterial dose, the hatched bar represent those foals receiving the lower dose. Treatment groups with different letters were different from each other at $p < 0.05$. There was an overall significant ($p < 0.05$) effect of bacterial dose on average CFU in the lungs.

3.2. Necropsy findings

At necropsy, there was no effect of bacterial dose on weight gain (weight at necropsy – weight at birth). While the weight gain in the antibiotic + SLO treated foals was higher than those of the other groups (Fig. 5), this failed to achieve statistical significance ($p = 0.062$). All foals exhibited multiple microgranulomas and pyogranulomas in their lungs at necropsy (see Supplemental figure). There was a significant effect ($p < 0.01$) of bacterial dose on the number of bacteria isolated from the lungs. Those foals treated with antibiotic + SLO had significantly ($p < 0.001$) lower bacterial counts than the other treatment groups (Fig. 6). However, there was no other effect of bacterial dose on any other necropsy findings. The gross pneumonia score for the antibiotic + SLO treatment group was significantly lower than those of the other treatment groups (Fig. 7, $p = 0.012$). There was a significant difference in total collagen in the histology sections for the antibiotic + SLO group (Table 2). The other pathological measures were not significantly different between the treatment groups.

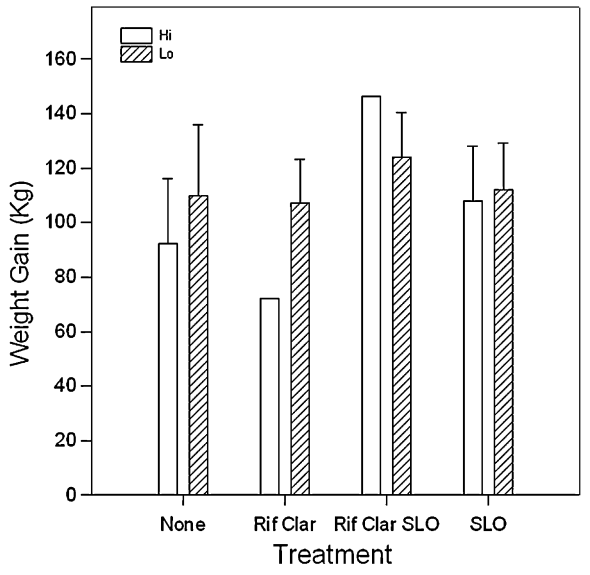

Fig. 5. Overall weight gain post-challenge. Final weights at necropsy were determined from each foal and its weight at the time of challenge was subtracted from this number to obtain overall weight gain. The open bar represent the average and SD of those foals receiving the higher bacterial dose, the hatched bar represent those foals receiving the lower dose. No effect of treatment or bacterial dose was observed.

4. Discussion

Our results indicate that a successful challenge model of acute rhodococcal pneumonia in the foals was employed. We initially utilized a challenge dose of $\sim 10^8$ CFU in order to obtain a 100% infection rate based on the literature (Wada et al., 1997), however, upon observing a rapid disease progression in those initial foals, we reduced the challenge dose 30-fold for the remainder of the study. All foals exhibited multiple microgranulomas in their lungs at necropsy and showed gross signs of pneumonia. *R. equi* was isolated from the lungs of the infected foals and we observed a dose-dependent relationship between the number of bacteria cultured from the lung and the dose of *R. equi* administered. While the higher dose led to more

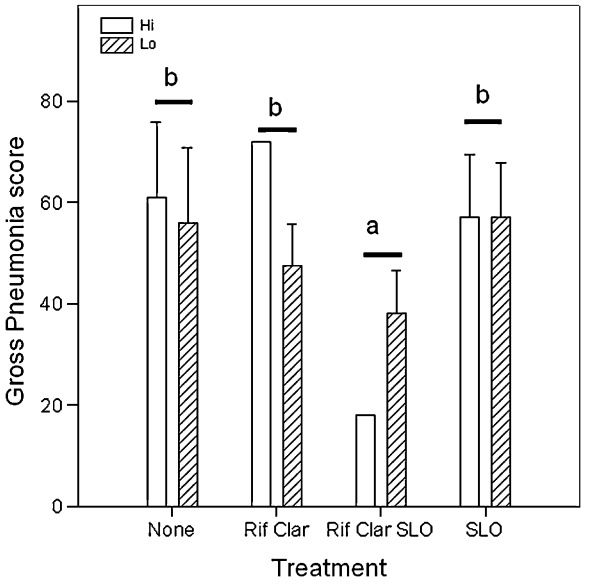

Fig. 7. Effect of treatment on pneumonia score. Pneumonia scores for each of the foals' lungs were determined after computational analysis of digital images, as described in the text. The open bar represent the average and SD of those foals receiving the higher bacterial dose, the hatched bar represent those foals receiving the lower dose. Treatment groups with different letters were different from each other at $p < 0.05$. There was no effect of bacterial dose on pneumonia scores.

rapid appearance of clinical signs post-challenge, this did not translate into more severe clinical signs or more severe pathologic findings at necropsy. This is consistent with the concept that once an inoculating dose overcomes the initial clearance capability of the lung, disease ensues (Bowles et al., 1989). However, it should be noted that the lesions were not typical of field cases which present as a chronic pyogranulomatous bronchopneumonia. While we did note pyogranulomas in the infected foals, this was not the predominant lesion. Had the dose been lower and the incubation period longer, we might have expected to see more typical lesions, as noted elsewhere (Wada et al., 1997).

The goal of this project was to determine if treatment of infected foals with SLO would enhance their response to standard antibiotic therapy. Treatment with clarithromycin (7.5 mg/kg), rifampin (5 mg/kg) and SLO given twice daily significantly reduced the bacterial load in the lung and lessened clinical signs. While antibiotic treatment alone was expected to similarly reduce bacterial load (Giguere et al., 2004), here it was not significantly different from the untreated and SLO only groups. This reduced efficacy of this standard antibiotic therapy was likely due to the severity of our challenge and the rapid progression of clinical signs. The overall reduction in CFU/g with antibiotic and SLO may be due to a direct antimicrobial effect, an indirect immunomodulatory effect, and/or a synergistic effect with the antibiotics, perhaps by enhancing their efficacy through increased penetration into the congested lung or individual granulomas. While we did not measure antibiotic levels in the lesions, it is known that SLO can facilitate the delivery of proteins and other molecules into living cells by binding to cholesterol in the membranes (Walev et al., 2001; Naruse et al., 2009). Alternatively, SLO may be contributing to the cytolysis of infected macrophages by interacting with the cholesterol oxidase of *R. equi* (Linder and Bernheimer, 1997). Cholesterol oxidase can cause the destabilization of the membrane through cholesterol oxidation, though its role in the pathogenicity of *R. equi* infections remains uncertain (Pei et al., 2006). Increased access to the membrane bound cholesterol by listeriolysin enhances this cytolytic potential (Fernanandez-Garayzabal et al., 1996). Here, SLO could play a similar role leading to increased lysis of infected macrophages and exposure of previously intracellular bacteria to the antimicrobials or other immune effector mechanisms in the extracellular environment. Further in vitro studies will be needed to identify the possible mechanism.

The addition of SLO to the antibiotic regimen also resulted in better clinical responses when compared to the antibiotic plus vehicle. Of particular interest were the lower temperature scores in the antibiotic + SLO group, a trend for a greater weight gain and overall longer survival. The gross pathological findings also supported an enhancement effect of the SLO treatment as the combined treatment group had significantly lower pneumonia scores, though there was more collagen deposited in the antibiotic + SLO group. Total collagen in the lung was also correlated with days of survival such that the longer the animal lived the more collagen there was in the lungs; as was the case for the antibiotic + SLO foals ($p = 0.061$). In mycobacterial infections, collagen synthesis and degradation are important events during granuloma or cavity formation (Gonzalez-Avila et al., 2009). While little is known regarding collagen deposition during *R. equi* infections, residual pulmonary scarring is typically not observed (Martens et al., 1989). The significance of increased total collagen staining in the histological samples from this study is unknown.

Table 2
Histopathology findings.*

	None	SLO	Antibiotic + vehicle	Antibiotic + SLO	*p*-value
Inflammation	14.8 ± 2.1	16.2 ± 1.6	17.0 ± 2.5	13.5 ± 3.1	0.163
Total collagen**	18.0 ± 4.2^{a}	19.7 ± 2.8^{a}	14.9 ± 5.3^{a}	5.4 ± 6.3^{b}	0.001
MT fibrosis score (avg thickness in μ)	329.8 ± 190.8	253.9 ± 118.7	205.0 ± 183.5	288.5 ± 202.6	0.836
Cumulative histopathology pneumonia score	35.7 ± 7.7	36.8 ± 4.9	37.5 ± 6.3	28.1 ± 9.5	0.329

Values with different superscripted letters are significantly different at the indicated *p*-value.
* Mean ± SD.
** Relative ranking where lower score equals more collagen present.

5. Conclusion

Adjunct therapy of Rhodococcal pneumonia with SLO enhanced the antimicrobial response of the foals, improved clinical responses, and reduced pathological signs of pneumonia.

Conflict of interest statement

Thomas Hatch and John McMichael are employees of Milkhaus Veterinary Products, Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.06.037.

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