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ORIGINAL ARTICLE

Streptolysin-O/antibiotics adjunct therapy modulates site-specific expression of extracellular matrix and inflammatory genes in lungs of *Rhodococcus equi* infected foals

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Abstract The addition of streptolysin-O (SLO) to the standard antibiotics regimen was shown to be superior to antibiotics alone after experimental infection of foals with *Rhodoccocus equi* (*R. equi*). The objective of this study is to investigate this response by determining the site-specific expression of extracellular matrix (ECM) and inflammatory response genes in biopsy samples taken from three distinct lung regions of the infected foals. Twenty-four foals were challenged by intrabronchial instillation of *R. equi* and assigned to four treatment groups: SLO/antibiotics adjunct therapy, antibiotics-only therapy (7.5 mg/kg clarithromycin and 5 mg/kg rifampin), SLO-only, and saline-only treatments. Treatments were administered twice daily for 16 days unless symptoms progressed to the point where the foals needed to be euthanized. Gene expressions were determined using custom-designed equine real-time qPCR arrays containing forty-eight genes from ECM remodeling and inflammation pathways. A non-parametric Wilcoxon signed-rank test for independent samples was applied to two pairs of time-matched comparison groups, SLO/antibiotics vs. antibiotics-only and SLO-only vs. saline-only, to document the significant differences in gene expressions within these groups. Several genes, MMP9, MMP2, TIMP2, COL1A1, COL12A1, ITGAL, ITGB1, FN1, CCL2, CCL3, CXCL9, TNFα, SMAD7, CD40, IL10, TGFB1, and TLR2, were significantly regulated compared to the unchallenged/untreated control foals. The results of this study demonstrate that enhancement of clinical responses by SLO is consistent with the changes in expression of critical genes in ECM remodeling and inflammatory response pathways.

Keywords *Rhodococcus equi* · Streptolysin-O · Adjunct therapy · Gene expression · Extracellular matrix · Autoimmunity

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Introduction

An age-dependent susceptibility to *Rhodoccocus equi* (*R. equi*) has been well documented in foals under six months of age (Meijer and Prescott 2004; Prescott 1987). *R. equi* infection commonly manifests as a severe pyogranulomatous pneumonia in foals, while adult horses are largely unaffected by the disease. *R. equi* can also cause extra-pulmonary diseases (EPDs) such as septic arthritis, neonatal diarrhea, abdominal and spinal cord abscesses, and immune-mediated diseases,

such as polysynovitis (Reuss et al. 2009). As an opportunistic pathogen in humans, *R. equi* can cause severe pyrogranulomatous pneumonia in immunocomprimised individuals (Weinstock and Brown 2002; Yamshchikov et al. 2010), but no effective vaccine currently exists for its prevention.

Streptolysin-O (SLO) is a hemolytic exotoxin produced by *Streptococcus* species of groups A, C, and G. As a thiol-activated, cholesterol-binding agent, this single-chain protein (60–70 kDa) can form pores in cell membranes in its reduced state, but does not readily form them post-oxidation. Structurally, the SLO primary gene product is comprised of 571 amino acids with several structural and functional domains, including a cholesterol-binding domain. Similar toxins are produced by other species of gram-positive bacteria (Alouf et al. 1984; Bhakdi et al. 1985; Harris et al. 1998; Johnson et al. 1980; Palmer 2001).

Previous studies have reported that in an ex vivo human skin organ culture wound model, SLO markedly promoted wound reepithelialization, and an in vitro wound healing scratch assay demonstrated that SLO could enhance keratinocyte migration and proliferation (Tomic-Canic et al. 2007). SLO has also been shown to modulate the excessive production of collagen in two scleroderma murine models (Mamber et al. 2004). In another study, SLO selectively activated ErbB1 kinase, resulting in the inhibition of matrigel invasion of human breast cancer cells, suggesting a potential new therapy for the inhibition of breast cancer metastasis (Hall et al. 2011). Finally, SLO has been examined as an adjunct therapy to standard antibiotic treatment in foals challenged with *R. equi*. (Horohov et al. 2011). This study demonstrated that SLO adjunct therapy enhanced the clinical responses of foals by reducing the duration and severity of the disease, as well as by lowering the bacterial counts in the lungs, compared to foals treated with antibiotics alone.

The pathology of *R. equi* infections manifests a complex interaction between inflammatory cells and ECM molecules and is a fertile area of investigation. Therefore, the observed clinical improvement of foals may have been attributed to SLO's ability to modulate ECM and inflammatory gene expression in the lungs of infected animals. To gain better insight into the role of SLO as an adjunct therapy in the treatment of *R. equi* infections, we have obtained three distinct biopsy samples from the lungs of foals: samples adjacent to the granulomas, samples between the granulomas and the disease-free tissue (intermediate), and samples distal to the granulomas (disease-free tissue). Accordingly, the aim of this study was to evaluate the expression of a select number of ECM and inflammatory genes by utilizing custom-designed equine-specific qPCR arrays in three distinct lung biopsy samples from the foals challenged with *R. equi* and treated with SLO/antibiotics adjunct therapy, antibiotics-only, SLO-only, and saline-only control.

Materials and methods

Foals

A total of twenty-six (average age 4±3 days) mix breed foals were selected for this study. Twenty-four foals were allocated for challenge and two foals served as unchallenged and untreated controls. The foals were obtained from mares raised and bred on the Department of Veterinary Science's North Farm at the University of Kentucky. The average initial weight of the foals was 57.9±8.5 kg; there were no differences between the treatment groups and the untreated controls. All foals were kept with their dams throughout the study and examined for eligibility for the study according to the following criteria: normality in physical examination, absence of abnormal lung sounds on auscultation, normal temperature, and unremarkable ultrasound examination of the lungs. Twenty-four hours after birth, baseline values for heart rate, respiratory rate, temperature, fibrinogen concentration, and leukocyte count were obtained. Transfer of maternal immunoglobulins was confirmed by SNAP test (IDEXX, Westbrook, Maine) and none of the foals received anti-rhodococcal plasma or other treatments prior to enrollment in the study. All procedures involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky in compliance with the guidelines established by the National Institute of Health.

Challenge and treatments

A clinical isolate of *R. equi*, UKVDL 206, was provided by Dr. Mike Donahue at the University of Kentucky Veterinary Diagnostic Laboratory. This low passage strain contained the virulence-associated protein A gene, confirmed by a PCR assay (Dr. Steeve Giguère, University of Georgia), and was isolated from a clinical case of equine rhodococcosis. Bacterial cultures were grown in trypticase-soy broth overnight, and the concentrations were initially estimated based on OD readings of the overnight cultures, confirmed by serial dilution plate counting the following day. Foals were challenged with an intrabronchial instillation of *R. equi* after sedation. A flexible fiberoptic endoscope was used to deliver the bacteria, suspended in 35 ml of sterile PBS, into both primary bronchi. The first six foals received $3.1\pm0.3\times10^{8}$ bacteria and the remaining foals received $1.4\pm0.1\times10^{7}$ bacteria. These dosages were similar to those used in other studies that produced pneumonic disease in foals (Hines et al. 2003; Giguère et al. 1999; Wada et al. 1997). The foals that received the higher dosage of bacteria developed symptoms of the disease sooner, but this did not equate with more severe

clinical symptoms in those foals (Horohov et al. 2011). After intrabronchial instillation of *R. equi*, the foals were returned to their stalls and dams, where they were monitored daily for changes in temperature, respiration, and pulse, as determined by physical exam and auscultation of the lungs. Starting one week after the inoculation, weekly ultrasound scanning was performed and continued throughout the study. Clinical signs recorded included lung auscultations, overall attitude, appetite, resting respiratory rate, and pulse. Foals with lesions (>2 cm) determined by ultrasound, or with any clinical signs consistent with rhodococcal pneumonia, were assigned to one of the treatment groups. A scoring system from 1 to 4 was developed in order to generate an overall daily score for each foal (Table 1). Over the sampling period, temperatures were collected twice daily. If the temperature was normal (<39.2 °C), the temperature score for that day was "0"; if >39.2 °C but< 39.7 °C, the temperature score was "1"; if >39.7 °C but< 40.3 °C, the temperature score was "2"; and if greater than 40.3 °C, the temperature score was "3". A post-treatment score was determined by taking the average of the daily temperature scores after treatment began. An overall clinical score was calculated by combining the daily score and the daily temperature score for each foal beginning on the day of the onset of clinical signs and dividing by the number of days of observation.

The recombinant SLO, obtained from Capricorn (Portland, Maine), was produced in *E. coli* and purified by metal affinity chromatography. Once a foal was determined to be affected based on clinical signs (pyrexia, lung sounds and/or ultrasound findings), it was randomly assigned to one of the treatment groups. The following treatments were given twice daily: (1) antibiotics therapy (7.5 mg/kg clarithromycin and 5 mg/kg rifampin) given *per os* with saline, (2) antibiotics therapy with SLO, (3) SLO-only, and (4) saline-only (vehicle). SLO was administered by subcutaneous injections in 0.2 ml (2 IU in saline) doses. All treatments were continued for 16 days unless symptoms progressed to the point where the foal needed to be euthanized. Those foals exhibiting flared nostrils and extreme difficulty in breathing, severe depression, lethargy, and/or anorexia were euthanized early. All surviving foals were euthanized 16 days after the start of the treatment.

Tissue collection and RNA isolation

Necropsies were performed by a board-certified veterinary anatomic pathologist, and pneumonia and abscess were determined by modifying the previously described scoring system (Halbur et al. 1996) to correspond with equine pulmonary anatomy. For RNA isolation, three biopsy samples were collected from each animal and preserved in RNAlater® (Ambion, cat#AM7020) solution immediately after euthanasia. The first tissue sample was taken adjacent to the granuloma (abscess). The second tissue sample was taken from tissue between the first sample and the disease-free section of the lung. The third tissue sample was taken from the disease-free section of the lung. Samples were kept in RNAlater® at 4 °C for a week then stored in a −80 °C freezer. Approximately 50 to 100 mg of tissue per sample were homogenized in 1 ml TRIZOL® Reagent (Invitrogen, Carlsbad, CA). An additional isolation step was included in order to remove the insoluble material from the homogenate by centrifugation at 12,000×g for 10 min at 2 to 8 °C. Resulting pellets were removed while supernatants were transferred to new tubes. Per 1 ml of supernatant, 0.2 ml chloroform was added to the tubes and vortexed for 15 s. Following incubation at room temperature for 2 to 3 min, the samples were centrifuged at 12,000×g for 15 min at 2 to 8 °C. The colorless upper aqueous phases were then transferred to new tubes and the RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. The samples were then incubated at room temperature for 10 min and centrifuged at 12,000×g for 10 min at 2 to 8 °C. Next, RNA pellets were washed twice with 75 % ethanol by centrifuging at 7,500×g for 5 min at 2 to 8 °C. RNA pellets were air dried for 5 to 10 min and dissolved in nuclease-free water. Assessment of RNA integrity was performed using an RNA LabChips® kit and Agilent 2100 bio-analyzer (Agilent Technologies, Santa Clara, CA) at Brown University's Genomics Core Facility. This analysis yielded results indicating that the RNA extracted from the lung samples was of good quality and suitable for qPCR array technology.

Table 1 Daily clinical scoring system

Score	Appetite	Attitude	Lung auscultation
0	Nursing frequently	Bright, alert & responsive	Clear
1	Nursing occasionally	Quiet, alert & responsive	Increased noise or effort
2	Infrequently nursing	Quiet	Mild/moderate crackles/wheezes
3	Rarely nursing	Depressed	Severe crackles/wheezes
4	Not nursing	Moribund	Increased respiratory effort and abdominal breathing

RT-PCR and PCR

RNA samples (0.5 μg) were treated with gDNA elimination buffer and incubated in a 42 °C thermocycler (Labnet, Multigene mini) for 5 min in order to digest possible gDNA contamination. Complementary DNA was synthesized using High Capacity RNA to cDNA Master Mix in accordance with the protocol provided by the manufacturer (Applied Biosystems, Foster City, CA). The cDNA synthesized from 0.5 μg total RNA was combined with SYBR Green® Master Mix provided by the supplier and loaded into 384 well TaqMan Array Cards. Forty-four inventoried equine assays were selected from five different molecular pathways (Extracellular Matrix, Immune Response, Inflammation, Cytokines and Chemokines) (Table 2) and 384 well TaqMan Array Cards were constructed (Life Technologies, Carlsbad, CA) to determine the changes in gene expression. Four genes, HPRT1, GAPDH, GUSB and 18S, were allocated as the internal housekeeping controls for the study. The amplification efficiencies of the genes were verified by control reactions built into each array by the manufacturer. TaqMan Array Cards were processed according to the manufacturer's protocol, and the reactions were executed by utilizing the ABI 7900 Real-Time PCR machine at Brown University's Center for Genomics and Proteomics Core Facility.

Data analysis

For each sample, the cycle threshold (Ct) values obtained from the qPCR reactions were normalized by dividing the expression levels (2^{-Ct}) of the gene of interest by the average expression level of the housekeeping genes. The housekeeping genes used for normalization were HPRT1 (Hypoxanthine-guanine phosphoribosyl transferase), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), GUSB (Beta glucuronidase), and 18S (small ribosomal subunit). The same normalization was performed with the unchallenged/untreated samples. In order to calculate the relative fold-change at the mRNA level for each gene, the normalized expression of the gene of interest in the experimental samples was divided by the normalized expression of the same gene of interest in the unchallenged/untreated control samples. A non-parametric Wilcoxon signed-rank test for independent samples was used to determine the p-values (Kruskal-Wallis method produced similar p-values as well). The statistical method was applied to compare the gene expressions between the SLO/antibiotics and the antibiotics-only groups, as well as the gene expressions between the SLO-only and the saline-only treatment groups. Since the statistical analysis was independently applied to each pair of time-matched treatment groups, cross-comparison of the independently analyzed groups was avoided while interpreting the results. In our genomics analysis, each treatment group contains one animal that received the high dosage of *R. equi* (except the saline-only treatment group). The rationale of keeping these animals in the final statistical analysis was: (1) higher dosage did not result in difference in clinical and pathological signs, and (2) any of the gene expressions between the animal that received the high dose and the animals that received the low dose did not show statistical difference within the treatment groups. Genes that generated p-values of ≤ 0.05 were considered statistically significant in this study.

Results

Comparison of gene expressions between SLO/antibiotics and antibiotics-only treatment groups

Initially, eight animals were allocated for the SLO/antibiotics group and seven animals for the antibiotics-alone treatment group. For some animals, severe disease manifestation resulted in euthanasia between days three and eight. One animal in the SLO/antibiotics group and three animals in the antibiotics-only group were euthanized prematurely before the predetermined necropsy, day sixteen. Animals that were euthanized prematurely were excluded from the genomics analysis in order to time-match the gene expressions between the comparisons. Exclusions of one animal from the SLO/antibiotics treatment group and three animals from the antibiotics-only treatment group reduced the numbers of animals to seven and four for these groups, respectively. The SLO/antibiotics vs. antibiotics-only comparison yielded a total of ten genes that were uniquely regulated by SLO in tissue biopsies between the granulomas and disease-free sections (intermediate). Neither disease-free sections nor biopsy sites adjacent to granulomas showed significant gene regulation in this comparison group (Table 3). Overall levels of mRNA transcripts for ITGB1, CCL3, CXCL9, TNF, TLR2 and TGFB1 were elevated while MMP2, COL12A1, FN1 and SMAD7 transcript levels were down regulated as compared to the unchallenged/untreated control animals. In the absence of SLO, expression levels of CCL3, CXCL9, TNF, TLR2 and TGFB1 were notably high in foals that received only antibiotics, while MMP2, COL12A1, FN1, SMAD7 and ITGB1 were further down regulated ($p \leq 0.05$). SLO addition to the antibiotics regimen resulted in distinctive changes in gene expression levels.

Comparison of gene expressions between SLO-only and saline-only treatment groups

Five animals were allocated for the SLO-only control group and four animals for the saline-only control group. Due to the severity of the clinical symptoms of *R. equi* infection,

Table 2 Gene symbols, gene names and NCBI gene reference numbers for the genes screened by the qPCR arrays

Gene symbol	Gene name	NCBI gene reference
HPRT1	Hypoxanthine phosphoribosyltransferase 1	AY372182.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001163856.1
GUSB	Glucuronidase, beta	EF485029.1
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	NM_001111302.1
COL1A1	Collagen, type I, alpha 1	AF034691.1
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	NM_001122762.1
COL12A1	Collagen, type XII, alpha 1	AB070841.1
COL14A1	Collagen, type XIV, alpha 1	NM_001163870.1
PECAM1	Platelet/endothelial cell adhesion molecule	NM_001101655.1
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	AB266841.1
18S	18S small ribosomal subunit	X03205.1
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	AB266840.1
TIMP2	TIMP metallopeptidase inhibitor 2	AJ010315.1
MMP13	Matrix metallopeptidase 13 (collagenase 3)	NM_001081804.1
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	NM_001081847.1
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	AJ010314.1
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	NM_001081802.1
ITGAL	Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	EU881921.1
FN1	Fibronectin 1	U52107.1
CCL11	Chemokine (C-C motif) ligand 11	NM_001081871.1
CCL2	Chemokine (C-C motif) ligand 2	NM_001081931.1
CCL3	Chemokine (C-C motif) ligand 3	NM_001114941.1
CCR2	Chemokine (C-C motif) receptor 2	NM_001097606.1
CXCL10	Chemokine (C-X-C motif) ligand 10	NM_001114940.1
CXCL9	Chemokine (C-X-C motif) ligand 9	NM_001130078.1
CSF3	Colony stimulating factor 3 (granulocyte)	NM_001081860.1
IFNG	Interferon, gamma	NM_001081949.1
IL1A	Interleukin 1, alpha	NM_001082500.1
IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	NM_001082511.1
IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	NM_001082516.1
IL17A	Interleukin 17A	NM_001143792.1
IL2	Interleukin 2	NM_001085433.1
IL4	Interleukin 4	NM_001082519.1
IL6	Interleukin 6 (interferon, beta 2)	NM_001082496.1
IL8	Interleukin 8	NM_001083951.1
TLR4	Toll-like receptor 4	NM_001099769.1
TNF	Tumor necrosis factor (TNF superfamily, member 2)	NM_001081819.1
CD28	CD28 molecule	NM_001100179.1
CD40	CD40 molecule, TNF receptor superfamily member 5	NM_001081902.1
IL1R1	Interleukin 1 receptor, type I	NM_001081794.1
IL10	Interleukin 10	NM_001082490.1
IL18	Interleukin 18	NM_001082512.1
IL2RA	Interleukin 2 receptor, alpha	NM_001111342.1
PTGER2	Prostaglandin E receptor 2 (subtype EP2), 53 kDa	NM_001127352.1
SMAD3	SMAD family member 3	AB076029.1
SMAD7	SMAD family member 7	AB106116.1
TLR2	Toll-like receptor 2	NM_001081796.1
TGFB1	Transforming growth factor, beta 1	NM_001081849.1

Table 3 Fold up or down regulation from the unchallenged, untreated, normal lung biopsies for the animals that received SLO/antibiotics and antibiotics-only treatments at day sixteen. Table shows the fold up or down regulations for the genes that had statistical significance at p-value ≤0.05. * indicates that the foal received the high dosage of *R. equi*

Tissue	Genes	SLO/antibiotics treatment							Antibiotics-only				p-value
		1*	2	3	4	5	6	7	A*	B	C	D	
Intermediate	MMP2	−1.11	−1.80	−2.54	−1.40	−1.77	−1.19	−2.52	−3.46	−1.91	−2.71	−1.91	0.05
	COL12A1	1.76	−2.62	−2.13	1.32	−2.52	−1.35	−1.91	−3.45	−2.53	−3.01	−2.09	0.05
	ITGB1	2.19	1.25	1.51	2.74	1.19	1.04	1.12	−1.98	−1.07	−1.36	1.50	0.05
	FN1	1.44	−2.87	−1.87	1.04	−1.40	1.15	−2.17	−4.31	−2.21	−3.74	−3.31	0.01
	CCL3	2.53	4.09	3.28	5.24	10.47	2.59	9.51	18.65	7.74	13.25	8.40	0.05
	CXCL9	7.51	6.99	11.62	9.94	15.18	2.56	4.86	22.21	12.45	13.99	12.11	0.03
	TNFα	1.73	−1.05	1.15	2.18	3.30	2.51	2.04	7.31	3.23	2.63	2.68	0.03
	SMAD7	1.65	−1.02	−1.73	1.44	1.23	1.12	−1.58	−1.14	−1.61	−1.64	−1.76	0.05
	TLR2	3.37	3.98	3.63	8.03	10.44	−1.13	6.82	21.86	7.74	7.85	11.32	0.05
	TGFB1	1.73	3.07	3.03	6.22	9.61	1.43	5.66	10.39	9.08	4.23	11.12	0.05

four animals were prematurely euthanized in the SLO-only group and all animals were euthanized in the vehicle-only group on treatment day five. Therefore, four animals from the SLO-only group and all animals from the saline-only group were chosen to compare the gene expression profiles between the two treatment groups.

Real-time PCR analysis of the tissue adjacent to the granulomas and from the disease-free biopsies identified a total of nine differentially expressed genes between the SLO-only and saline-only control groups ($p \leq 0.05$). Levels of mRNA for MMP9, CCL3, CCL2 and IL10 were up regulated while COL1A1, TIMP2, ITGAL, CD40 and TGFB1 were reduced as compared to the unchallenged/untreated control animals. SLO was able to either down regulate or suppress the elevation of the mRNA levels for MMP9, COL1A1, TIMP2, ITGAL, CCL2, CCL3, TGFB1 in biopsy samples adjacent to granulomas. In distal tissues, SLO-only treated foals showed relatively higher CCL3, CD40 and IL10 levels than that of saline-only treated animals (Table 4). Unlike the SLO/antibiotics vs. antibiotics-only comparison group, no significant regulation in gene expression was observed in biopsy samples between the granulomas and the disease-free tissue.

Discussion

The expression levels of select genes that are involved in ECM remodeling and inflammatory response were assessed in three distinct lung regions of foals treated with SLO/antibiotics adjunct therapy, antibiotics-only, SLO-only or saline-only regimens after *R. equi* challenge. Following euthanasia, biopsy samples were taken from regions adjacent to the granulomas, between the granulomas and the disease-free sections, and from disease-free sections (distal to granulomas) of the lungs. Genomics experiments employing pathway-specific qPCR arrays were utilized to determine the effects of the treatments on specific genes in a site specific manner. The expression level of each gene was normalized to its counterpart in unchallenged and untreated control animals. Statistical analyses were independently applied to two pairs of treatment groups: (1) SLO/antibiotics vs. antibiotics-only treatment groups and (2) SLO-only vs. saline-only treatment groups.

Determination of site-specific expression of critical genes might potentially facilitate development of new therapies or improve the treatments that are currently in use for *R. equi* infections. We attempted to define the site-specific gene expression patterns for some of the major constituents of inflammation and ECM remodeling pathways. Animals that were not treated with antibiotics did not complete the 16 day study period. The majority of these foals showed severe clinical symptoms, thus, were prematurely euthanized at day five of the study. Genomics results from these animals reflect their severe clinical status early in the disease progression with no conventional intervention. Therefore, we claim that site-specific expression profiles of these genes in severely afflicted foals can be exploited as molecular therapeutic targets. Here, we demonstrate that in SLO-only or saline-only treated foals, the number of responsive genes is numerous and the amplitude of gene expression is more robustly regulated in sites adjacent to granulomas as compared to biopsy samples derived from the distal tissue. This erratic expression pattern is concentrated around granulomas; this is most likely due to the severity of the disease and the lesions in these lungs. In contrast, animals in the SLO/antibiotics and antibiotics-only groups showed less severe clinical symptoms and completed the 16 day study period. No significant changes in gene expression were detectable in tissue adjacent to the granulomas while

Table 4 Fold up or down regulation from the unchallenged, untreated, normal lung biopsies for the animals that received SLO-only and saline-only treatments at day five. Table shows the fold up or down regulations for the genes that had statistical significance at p-value ≤ 0.05. * indicates that the foal received the high dosage of *R. equi*

Tissue	Genes	SLO-only				Saline-only				p-value
		1*	2	3	4	A*	B*	C	D	
Adjacent	MMP9	18.38	36.95	21.63	20.94	40.75	49	71.49	82.09	0.02
	COL1A1	−30.4	−134	−40.7	−87.14	−6.24	−6.28	−1.45	−11.24	0.02
	TIMP2	−3.6	−11.61	−6.23	−6.07	−3.43	−2.17	−1.29	−6.02	0.04
	ITGAL	−1.64	−5.01	−1.65	−1.53	−1.44	−1.07	1.97	−1.47	0.02
	CCL2	1.53	−1.29	1.66	−1.17	2.73	2.2	6.54	1.53	0.04
	CCL3	20.29	42.03	29.65	26.6	70.62	41.85	71.45	92.48	0.04
	TGFB1	−1.87	−1.53	1.07	1.19	2.53	1.93	7.89	3.52	0.02
Distal	CCL3	2.89	5.16	6.78	12.01	1.48	2.33	3.92	2.31	0.04
	CD40	−1.08	1.83	1.86	1.81	−1.17	−1.47	1.15	−1.58	0.04
	IL10	12.52	13.51	15.49	24.9	5.14	5.43	9.8	10.96	0.02

samples from intermediate sites displayed near basal levels and mild changes in gene expression, confirming tissue stabilization and recovery.

R. equi infections share some pathological similarities to tuberculosis infections, in which granulomas are the hallmark of the disease. MMP9 matrix-modifying protease (gelatinase), has been implicated in the pathogenesis of various inflammatory conditions and is highly expressed in both human tuberculosis and in the mouse model of tuberculosis (Greenlee et al. 2007; Park et al. 2005; Price et al. 2001; Sheen et al. 2009; Taylor et al. 2006; Van den Steen et al. 2002). Moreover, a study using *Mycobacterium marinum* in a zebrafish model demonstrated that MMP9 enhanced recruitment of macrophages, which contributed to nascent granuloma maturation and bacterial growth. Reducing MMP9 expression attenuated granuloma formation and bacterial growth (Volkman et al. 2010). Thus, interception of MMP9 production could hold promise as a host-targeting therapy. Our real-time PCR array data revealed that the MMP9 gene transcription was lower in the treatment groups that received SLO/antibiotics ($p \leq 0.08$, data not shown) and SLO-only ($p \leq 0.05$), as compared to those that did not receive SLO. Furthermore, MMP2 mRNA levels were reduced in both the SLO/antibiotics and the antibiotics-only treatment groups ($p \leq 0.05$), while a similar regulation was also observed in SLO-only animals but not in saline-treated foals ($p \leq 0.08$, data not shown). In agreement with previous in vitro studies, SLO has displayed a unique property of preventing a surge and down-regulating MMP expression (Hall et al. 2011; Mamber et al. 2011). These observations suggest that by suppressing levels of MMPs in the SLO/antibiotics treatment group, SLO contributed to the improved clinical outcomes of the foals by limiting granuloma maturation and bacterial growth (Chang et al. 1996; Hernandez-Pando et al. 2000; Hrabec et al. 2002; Izzo et al. 2004; Taylor et al. 2006; Volkman et al. 2010).

Foals that did not receive SLO/antibiotics or antibiotics-only treatments exhibited particularly low mRNA for COL1A1, a major component of type 1 collagen. This profoundly low level is a representation of the severe disease state at day five of the treatments. On the other hand, COL1A1 mRNA levels were stabilized in intermediate tissue biopsies by the end of day sixteen, suggesting that the foals that received the SLO/antibiotics adjunct therapy began restoring normal tissue structure in the lung as part of the recovery/proliferative phase (Horohov et al. 2011). Addition of SLO to the antibiotics therapy clearly enhanced the restoration of COL1A1 mRNA near normal levels in SLO/antibiotics treated foals, while antibiotics-only treated foals still had relatively low levels of COL1A1 transcript. Interestingly, we had previously found that SLO treatment in vitro up regulates various isoforms of collagen genes in normal human keratinocytes (Mamber et al. 2011). Similarly, ITGB1 mRNA was higher in the SLO/antibiotics treatment group while the antibiotics-only group showed lower transcript levels, suggesting that adjunct therapy is promoting better tissue adhesion and integrity by modulating integrin mRNA levels in the lungs of recovering foals. Perhaps, this could also explain the low bacterial count and the better clinical scores obtained from the foals that received the adjunct therapy.

Low levels of FN1 in three- to four-week-old foals were found to be the limiting factor for phagocytosis as compared to the adult horses. Fibronectin has been suggested to be the limiting opsonic factor in foals, and supplementation of fibronectin has been shown to improve phagocytotic activity of foal neutrophils (Demmers et al. 2001; Gröndahl et al. 1997, 1999; McTaggart et al. 2001). Adjunct therapy with

SLO provided an advantage to foals by normalizing FN1 mRNA to near basal levels while these levels, were significantly lower in foals that received antibiotics-only treatment. Lower FN1 transcription in antibiotics-only treated foals might be contributing to a less clinically favorable outcome, suggesting a further mechanistic explanation for the SLO-associated improvement in foals that received adjunct therapy.

High inflammatory chemokine levels are associated with disease severity in tuberculosis models, and low plasma chemokine levels after anti-tuberculosis treatment promote disease recovery and better outcome (Almeida et al. 2009; Lyadova et al. 2010; Hasan et al. 2009). Especially, CCL3 has been proposed to be a critical agent for the clearance of various bacterial infections (Huffnagle et al. 1997). We found that SLO/antibiotics treated animals had lower CCL3 mRNA expression than antibiotics-only treated animals, suggesting that animals that received the adjunct therapy were experiencing less bacterial burden and recovering from the disease at the time of necropsy. CCL3 levels were notably high in control groups. Biopsy samples adjacent to and distal from granulomas had significant induction of CCL3 mRNA, implying that *R. equi* infection is not localized and progressively spreading to disease-free regions of the lungs. Overall, low chemokine expression patterns show consistency with the better clinical response obtained by SLO/antibiotics adjunct therapy (Horohov et al 2011), and this correlation suggests that CCL3 levels can be used as a good indicator to monitor the disease state and recovery.

Two pro-inflammatory mediators, TNF and TGFB1, are known to be produced in both epithelial cells and macrophages and recruit inflammatory cells during bacterial infections (Bettelli et al. 2007; Korn et al. 2007). While they are necessary components of host defense, their production results in the destruction of host tissue. The foals that received SLO adjunct therapy had lower TNF and TGFB1 transcript levels as compared to the antibiotics-only treated foals. This finding indicates that SLO possesses an immunomodulatory property. Interestingly, SLO-only treated foals had higher levels of anti-inflammatory IL10 and lower levels of TGFB1 mRNA than saline-only controls, demonstrating further proof of the immunomodulatory function of streptolysin-O. Nevertheless, expression of these two genes in the favorable direction did not change the course of the disease for these animals. The absence of IL10 mRNA expression in other biopsy regions or SLO/antibiotics and antibiotics-only groups might be attributed to the spatial and temporal regulation of this gene during the disease process (Hedrich and Bream 2010). The expression of two genes, SMAD7 and CD40, were also altered in both comparison groups. However, the magnitudes of the associated fold-changes were small, such that any interpretation regarding the physiological consequences of these minute changes would be tenuous.

Toll-like receptors (TLRs) are critical mediators of the innate immune response, and many of the pro-inflammatory cytokines are produced following activation of TLRs, resulting in the activation of host defenses. On the other hand, the prolonged expression of TLRs leads to tissue destruction and unwanted side effects (Henneke et al. 2002; Means et al. 1999). SLO/antibiotics treated foals displayed lower levels of TLR2 mRNA than in antibiotics-only treated foals. Unlike the more pronounced induction of TLR2 in antibiotics-only foals, modest TLR2 mRNA expression was nicely correlated with the small induction of downstream inflammatory mediators, such as CCL3, CXCL9, TNF and TGFB1, in foals treated with the adjunct therapy. Therefore, SLO might initially interact with TLR2, resulting in down regulation of a cascade of pro-inflammatory mediators.

In conclusion, the results presented here suggest that the SLO altered expressions of several critical ECM and inflammatory response genes, and these modulations in gene expressions, resulted in a better disease outcome in *R. equi* challenged foals. The therapeutic benefit of SLO as an adjunct therapy has been shown in a previous study (Horohov et al. 2011). This study aimed to examine the expression of a panel of genes in order to understand the improved clinical outcomes obtained following SLO/antibiotics adjunct therapy. The data presented here will serve as the reference point for future studies as the levels of proteins associated with these genes will need to be confirmed, and the cell type-specific gene expression in each biopsy site will be elucidated by laser capture microdissection in a larger study.

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Conflict of Interest Volkan Gurel, Ph.D, Kristyn Lambert and John McMichael, Ph.D are paid employees of Beech Tree Labs, Inc.

Ethical Standards Experiments comply with current laws of the United States.

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