

Aerosol delivery of *Rhodococcus equi* specific IgG to the lungs of adult ponies.

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ABSTRACT

The objective of this study was to determine if *R. equi* IgG purified from commercially available hyperimmune *R. equi* plasma and delivered to the lungs of adult ponies would cause a local inflammatory response and if increases in total and *R. equi* specific IgG occurred post administration. IgG was purified and concentrated from plasma via protein G affinity chromatography. A cross over study was performed. Eight healthy adult ponies were randomly assigned to two groups of four; each pony acted as its own control. Either the IgG product or 0.9% Saline was delivered via a vibrating mesh nebulizer during the first treatment phase. During the second treatment phase ponies received the opposite treatment. A 4 week washout period was allowed between phases. Bronchoalveolar fluid was recovered using a low volume endoscopic technique prior to aerosolization (time 0), and at 1 hr, 6 hrs, and 24 hours post administration. The BAL fluid total IgG concentration and *R. equi* specific IgG titers were determined via ELISA and cytologic analysis was performed. No clinically significant local inflammatory response was identified in response to IgG treatment. While total IgG concentrations were increased at T1 compared to T0, no significant effects of time were found (P=0.19). However, overall significantly higher concentrations of total IgG were found after administration of saline when compared to IgG administration (P=0.023). While the *R. equi* specific titer increased at T1 after IgG administration, no significant difference was identified between treatment or time (P=0.261). Overall the individual response to IgG was variable. It is possible that the protein rich IgG acted as a relatively hypertonic solution and caused

fluid influx from the pulmonary parenchyma after treatment thereby diluting the total IgG present when compared to saline administration. This conclusion cannot be verified as BAL dilution correction was not performed. However, it is unknown what titer or level of increased IgG is necessary to assist with prevention of disease. Future research should focus on the effect of *R. equi* specific IgG on pulmonary cells to determine if administration of local *R. equi* specific IgG would alter intrapulmonary immune responses to *R. equi*.

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List of Abbreviations

α	alpha
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
C	centigrade
C3	complement 3
CD4+	cluster of differentiation 4 T-lymphocyte
CD8+	cluster of differentiation 8 T-lymphocytes
CFU	colony forming units
cm	centimeter
CpG	CpG oligodeoxynucleotide
CTL	cytotoxic T- lymphocyte
dl	deciliter
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease 1
DPI	dry powder inhaler
ELISA	enzyme linked immunosorbent assay
Fe ³⁺	ferric iron
FPLC	fast performance liquid chromatography
g	gram
HIP	hyperimmune plasma
IFN- γ	interferon gamma
IgA	immunoglobulin A
IgG	immunoglobulin G
IgG1	immunoglobulin G 1
IL-1 α	interleukin 1 alpha
IL-4	interleukin 4
IL-5	interleukin 5
IL-10	interleukin 10
IL-12	interleukin 12
IV	intravenous
kb	kilobase
kDa	kilodalton
kg	kilogram
LAM	lipoarabinomannin
LTTR	LysR- type transcriptional regulator
MDI	metered dose inhaler
mg	milligram
MHC	major histocompatibility complex
MHC II	major histocompatibility complex type 2
MIC ₉₀	minimum inhibitory concentration 50%
ml	milliliter
mm	millimeter
mM	micromole
MMAD	mass median aerodynamic diameter

MOI	multiplicity of infection
NaCl	sodium chloride
<i>nar G</i>	nitrate reductase G
ng	nanogram
nm	nanometer
OR	odds ratio
<i>orf 8</i>	open reading frame 8
PAI	pathogenicity island
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PELF	pulmonary epithelial lining fluid
ReqLAM	<i>rhodococcus equi</i> lipoarabinomannan
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Th1	T-lymphocyte helper cell subset 1
Th2	T-lymphocyte helper cell subset 2
<i>tra A</i>	transfer gene A
µg	microgram
µl	microliter
µm	micrometer
<i>vapA</i>	virulence associated protein A
<i>vap C</i>	virulence associated protein C
WBC	white blood cell

Chapter 1. *Rhodococcus equi* pathophysiology

Microbiology of *rhodococcus equi*

Rhodococcus equi is a gram-positive, facultative, intracellular coccobacillus that survives and replicates within macrophages causing granulomatous inflammation¹⁻⁴. It is a soil saprophyte with a coprophilic-soil life cycle^{5,6}. *Rhodococcus* species are aerobic, catalase positive, partially acid fast and are non-motile and non-sporulating⁷. *R. equi* belongs to the genera Actinomycete and the taxon Mycolata that also includes *Nocardia*, *Corynebacterium* and *Mycobacterium*³. A unique cell envelope of mycolic acids that are linked to arabinogalactan wall polysaccharide and glycolipids characterize these bacteria³. This unique cell envelope forms a permeability barrier to hydrophilic compounds resulting in the formation of a periplasmic space⁸. The cell envelope likely plays a role in resistance of *R. equi* to harsh environmental conditions such as environmental stress, oxidative stress, and low pH and may be a factor in its ability to survive within the macrophage⁹. However the bacterial capsule of *R. equi* has been shown not to be an essential virulence factor¹⁰.

Epidemiology

***Rhodococcus equi* and the environment: soil**

R. equi is considered to be ubiquitous in the environment on most horse farms. It is ingested and survives transit through the gastrointestinal tract of herbivores and then is shed back into the environment in feces where it replicates and persists in the soil^{5,6}. Persistence of *R. equi* within the environment increases herd exposure and allows for dissemination of the bacterium throughout the local habitat². In one study the virulent strains of *R. equi* excreted in adult equine feces comprised less than 10 – 15% of the total

concentration of <2000 *R. equi* cfu/g feces¹¹. Normal foal excretion of *R. equi* in feces peaks between 3 and 12 weeks of age, and they shed up to 10-40% virulent *R. equi* in their feces with a total concentration of 1×10^3 to 1×10^4 cfu/g feces^{12,13}. Fecal shedding of virulent *R. equi* is the highest in foals with rhodococcal pneumonia and can comprise as much as 80% or more of the total concentration of 10^6 to 10^8 of *R. equi* cfu/g feces^{14,15}. *R. equi* multiplies more rapidly in soils that contain horse feces as bacterial growth is enhanced by the volatile fatty acids acetate and propionate which are present in horse feces^{16,17}. *R. equi* is vulnerable to treatment at temperatures greater than 45°C and its growth is hindered at extremes of pH (pH>9 or <5)^{16,18}. Despite these vulnerabilities no selective factors have been identified to modify concentrations of virulent *R. equi* within the soil^{19,20}. Currently, frequent removal of feces is the only recommendation to decrease the environmental burden of *R. equi* in the soil^{21,22}. Multiple studies evaluating the association between the soil burden of virulent *R. equi* and the incidence of rhodococcal pneumonia on farms have found that neither the total concentration of *R. equi*, nor the proportion of virulent *R. equi* in the soil, are accurate indicators of prevalence or risk of foals developing *R. equi* pneumonia on the premises²³⁻²⁵.

Rhodococcus equi and the environment: air

Inhalation of virulent *R. equi* is considered the primary route of pulmonary infection in the foal^{25,26}. At the farm level a correlation between the concentration of airborne, virulent, *R. equi* and the cumulative incidence of *R. equi* pneumonia was found on Australian Thoroughbred breeding farms²⁵. Also in Australia, the concentration of airborne, virulent *R. equi* was found to be higher during drier months²⁵. Whereas on Kentucky breeding farms higher concentrations of virulent *R. equi* was found in the

months of January and February²⁷. Recently, exposure to greater than 2 CFU counts of airborne, virulent *R. equi* during the first two weeks of life was significantly and causally associated with the development of *R. equi* pneumonia in foals (OR 3.9)²⁸. In the same study the odds of developing *R. equi* pneumonia was increased in March (OR 2.3) compared to other months²⁸. This was correlated to the time when a greater mare/foal density was present on the farm. Based on these studies it is possible that attempts to decrease airborne, virulent *R. equi* during the first few weeks of life might be able to decrease the incidence of disease on farms.

Rhodococcus equi and the environment: farm environment

Farm environmental and management practices have been evaluated to identify any risk factors and areas for possible intervention to decrease or prevent *R. equi* pneumonia. Studies in Ontario, Canada and 33 states from the United States of America showed a significantly increased risk of *R. equi* pneumonia was associated with increased number of years the facility was used as an equine breeding facility^{29,30}. A study of Texas horse farms revealed that farms affected with *R. equi* were significantly more likely to be >200 acres in size³¹. This same study found a significantly increased risk for *R. equi* pneumonia at large farms with more mare/foal pairs and higher concentration of foals (>0.25 foals/acre) as well as farms with mares that were present transiently for breeding purposes³². Areas of relatively higher exposure to *R. equi* on farms are believed to be areas of increased risk of development of *R. equi* infection in foals^{1-4,25,29-31}. Exposure to dusty environments and increased length of stabling have been associated with increased risk of *R. equi* pneumonia^{5,6,29,31}. Increased number of airborne *R. equi* were found on dry and windy days in Japan^{7,33}. Approximately two times the concentration of airborne,

virulent *R. equi* was found in high traffic areas when compared to grass paddocks on Australian Thoroughbred farms^{3,25}. A study that evaluated farm management practices found that common desirable management practices that were successful in disease prevention were not successful in preventing the development of *R. equi* pneumonia on farms^{3,29}. The same study also found that larger farms with greater than 15 foals had a significantly increased odds of having *R. equi* pneumonia on the premises^{8,29}. The cumulative knowledge amassed from these studies turned the focus of prevention of *R. equi* from management and farm factors to why foals are uniquely susceptible to *R. equi*.

Pathogenesis

The main route of pulmonary infection is via inhalation of virulent *R. equi*^{9,34}. While the true incubation period of naturally occurring disease is unknown, Sartwell's model has been used to determine time of exposure of foals to *R. equi*^{10,35}. Sartwell's model reveals that disease occurring from a point source exposure or a well-defined genetic cause have a lognormal distribution to their time of onset. Horowitz, et al. investigated the age of onset of clinical signs and age to death for *R. equi* pneumonia in foals from Japan and Argentina and found that this followed a logarithmic distribution, this finding was different than the control foals that were affected with colic^{5,6,35}. This evidence supports the theory that foals are exposed and become infected with *R. equi* soon after birth. Horowitz et al. also were able to propose an incubation period of 49 days from birth to onset of clinical signs and an incubation period of 65 days from birth to death based upon the Sartwell model and their raw data^{2,35}. This information strengthens the idea that prophylactic interventions against *R. equi* pneumonia in foals need to occur shortly after birth.

The incubation period in experimentally induced disease has been shown to depend upon the inoculum dose. High dose intra-bronchially challenged foals developed disease approximately 9 days post inoculum; whereas those that received a low dose had an approximately 2-4 week incubation period^{6,11,36}. Environmental concentration of virulent bacteria, foal age and defense mechanisms all are likely to play a role in the incubation period in any individual foal. Another important means of exposure to the bacterium is via ingestion, however in experimental studies the hematogenous acquisition of pneumonia rarely occurs after ingestion of multiple, large doses of virulent *R. equi*^{12,13,37}.

Infection likely occurs early in life as foals housed on endemic farms had a median age at first diagnosis of 35 – 50 days^{14,15,35,38,39}. A study by Wada et al, showed that older foals are also susceptible to rhodococcal infection as foals aged 27 – 67 days all developed rhodococcal pneumonia after experimental exposure to the bacterium regardless of inoculum size^{16,17,40}. Interestingly, in an experimental model foals less than 14 days of age exposed to intra-bronchial aerosolization of *R. equi* developed fatal rhodococcal pneumonia, whereas foals exposed at 14 days or older spontaneously recovered after development of pneumonia^{16,18,41}.

Virulence

Virulence of *R. equi* in foals is associated with an indigenous 80 – 90 kb plasmid^{19,20,42,43}. Intracellular replication of the bacterium is enabled by the presence of the plasmid^{4,21,22,36}. Curing or loss of the plasmid rendered a strain of the bacteria unable to infect either foals or mice^{23-25,36,40,44}. The virulence plasmid has a narrow host range but can be transferred to other *Rhodococcus spp* and related organisms via conjugation^{25,26,45}. Conjugation was found to be dependent upon the relaxase-encoding gene *traA*^{25,45}. The

implications of this discovery are that infections diagnosed by identification of the virulence plasmid alone must be viewed with caution as conjugal transfer can allow it to be present within other related organisms^{25,45}.

A pathogenicity island (PAI) that is approximately 27.5 kb in size has been identified within the plasmid and houses the virulence genes^{27,46}. Within the PAI three specific genes have been shown to play key roles in virulence^{28,47,48}. Virulence associated protein A (*vapA*) is encoded on the PAI along with two other genes that positively regulate it, *virR* and *orf8*, and these are essential for resistance of innate macrophage defenses and *in vivo* bacterial multiplication^{28,46,47,49}. *VapA* is surface-expressed, highly immunogenic and lipid modified^{29,30,46,50}. *VapA* expression is dependent upon multiple environmental factors reflective of conditions encountered by the bacterium upon entry into host macrophages: temperature (37°C), pH (6.5), oxidative stress, and low concentrations of calcium, iron and magnesium^{1,20,31,51,52}. The *virR* gene encodes a LysR-type transcriptional regulator (LTTR) required for *vapA* transcription^{32,49}. Based upon observation of binding activity it is speculated that *virR* activates *vapA* transcription via a direct interaction with RNA polymerase that is bound to the *vapA* promoter⁴⁹. The *orf8* gene encodes a response regulator for a cognate sensor kinase protein that itself is not located on the virulence plasmid⁴⁶. Decreased *vapA* transcription and attenuation of the bacterium occurs if either *virR* or *orf8* genes are not expressed^{49,53}. Expression of a gene that encodes nitrate reductase G (*narG*) has also been shown to be necessary for expression of virulence⁵⁴. This finding indicates that anaerobic or microaerophilic milieus, such that may be present inside abscesses, may be important for growth of *R. equi* during infection^{34,55}.

Cellular entry and survival

After inhalation, virulent *R. equi* is taken up by alveolar macrophages. Complement fixation via the alternate pathway is required for bacterial adhesion and cell entry, as the Mac-1 (C3) receptor must be present for *R. equi* to enter the cell⁵⁶. Lipoarabinomannan's (LAM) are lipoglycans that are complex mycobacterial cell envelope components and have been identified as putative virulence factors for *Mycobacterium tuberculosis*^{57,58}. A novel lipoarabinomannan, ReqLAM, has been identified within the cell envelope of *R. equi*⁵⁹. *R. equi* may use ReqLAM to gain entry into macrophages via the mannose receptor allowing it to circumvent the normal macrophage antimicrobial response⁵⁹. Once *R. equi* has been engulfed by the macrophage virulent strains are able to modify the phagocytic vacuole to prevent acidification and lysosomal fusion⁶⁰. Avirulent strains, however, are unable to make these modifications and are killed in the acidic phagolysosome⁶⁰. Once *R. equi* establishes an infection it is able to modify iron concentrations and resist host reactive oxygen species in order to persist intracellularly^{20,51}. Unchecked replication of the bacterium within macrophages leads to cellular necrosis and death, followed by dispersion of the intracellular bacteria.

Immunity

If virulent *R. equi* is opsonized with specific antibodies then the bacterium will enter the cell via the macrophage Fc receptor, with phagolysosomal fusion and acidification occurring, thus leading to bacterial death⁶¹. In murine models killing of virulent *R. equi* is dependent upon the production of interferon gamma (IFN γ) by macrophages⁶². Peroxynitrite formation from reactive oxygen and nitrogen intermediates is triggered by IFN γ , and peroxynitrite is needed to kill *R. equi*⁶². The reactive oxygen and nitrogen

intermediates alone are not able to kill the bacterium⁶², as tumor necrosis factor alpha (TNF) is also required to clear virulent *R. equi* infections in murine models, so other cytokines may play a role in clearance of the bacterium as well⁶³. Unlike the macrophage, the neutrophils of both foals and adults are fully capable of killing virulent *R. equi*⁶⁴⁻⁶⁷.

Cell mediated immunity

Due to its intracellular nature, it is thought that the cell-mediated immune response is necessary for protection against *R. equi*. Functional T lymphocytes are required for mice to clear virulent *R. equi* from the lungs⁶⁸. However, plasmid cured avirulent strains are cleared from the lungs of mice lacking functional T lymphocytes within one week, suggesting that clearance of avirulent strains depend on innate defense mechanisms⁶⁸. T lymphocyte clearance of virulent *R. equi* requires both cytokine secretion and direct cytotoxicity. While both CD4+ and CD8+ T lymphocytes are involved in clearance of *R. equi* in mice, CD4+ T lymphocytes are required for complete pulmonary clearance of the bacterium⁶⁹⁻⁷¹. In mice a Th1 response that leads to expression of IFN γ results in pulmonary clearance of virulent *R. equi*, while a type 2 response with secretion of IL-4 is detrimental to clearance^{72,73}. Adult horses are resistant to infection with *R. equi* and have been used to study the immune response that is necessary for protection and clearance of infection. A lymphoproliferative response to *R. equi* antigens with the development of *R. equi* specific cytotoxic T lymphocytes (CTL) and production of IFN γ occurs in adults for clearance of the bacterium⁷⁴⁻⁷⁶. The *R. equi* specific CTL's in immune adult horses are unrestricted major histocompatibility complex type one and recognize lipids from the bacterial cell wall^{77,78}.

Foal immune factors

All newborn mammals are immunologically immature. This immaturity includes decreased innate immune responses, decreased antigen presenting cell function and a decreased ability to mount Th1 immune responses⁷⁹. Age related deficiencies exist in the foal's ability to respond to infection appropriately and can contribute to a bias towards a Th2 type response rather than a Th1 response that is necessary to clear intracellular pathogens, and specifically *R. equi* infection. These deficiencies include: deficient CTL production, decreased cytokine expression by antigen presenting cells, and decreased capacity of lymphocytes and neutrophils to produce and up-regulate cytokines^{77,79-84}. Patton et al. demonstrated that foals 3 weeks of age were unable to mount sufficient *R. equi* specific CTL responses to clear *R. equi*, however this was improved at 6 weeks of age and was found to be similar to adults by 8 weeks of age⁷⁷. Foals also displayed a significantly decreased expression of CD1 and MHC II by their antigen presenting cells when compared to that of adults^{79,80}. Age was also a significant factor when the capacity of foal lymphocytes and neutrophils to produce and up-regulate cytokines was compared to that of adults^{80-83,85}. Young foals have also been shown to be deficient in both Th2 (IL-4) and Th1 (IFN γ) cytokines when responding to mitogens; leaving the basis of the bias towards a Th2 type immune response of foals elusive^{81,82,86-89}. Further complicating the understanding of foal immunity to *R. equi* infections is the finding that concentrations of IFN γ and IL-4 secreted by blood mononuclear cells at birth were not correlated with the subsequent development of *R. equi* pneumonia and when experimentally infected with virulent *R. equi* foals exhibited similar IFN γ and antibody responses to that of adult horses^{89,90}. Combined with the finding that spontaneous resolution of disease has been

observed after experimental infection of foals supports the acknowledgment that much is still to be elucidated in regards to foal immunity to *R. equi* infections^{41,91,92}.

Clinical manifestations and diagnosis of rhodococcal infections in foals

Pneumonia

Rhodococcal pneumonia is a devastating cause of severe pneumonia in foals, and is characterized by pyogranulomatous inflammation and subsequent abscess formation of lung tissue and mediastinal lymph nodes^{21,34,93,94}. The slow progression of *R. equi* pneumonia combined with the foal's ability to compensate for gradual loss of functional pulmonary parenchyma can make early clinical diagnosis difficult. Initial clinical signs include mild tachypnea and fever, if these are missed the disease continues to progress to a chronic form until loss of functional pulmonary parenchyma no longer allows compensation and disease becomes apparent. The clinical signs of rhodococcal pneumonia typically include lethargy, fever, cough, nasal discharge, and tachypnea that can progress to dyspnea with increased severity of disease³⁴. Histopathologic evaluation of affected pulmonary parenchyma reveals pyogranulomatous pneumonia characterized by edema and cellular proliferation of alveolar septa and pleura, terminal bronchioles and alveolar spaces fill with neutrophils, macrophages, necrotic debris and multinucleated giant cells; multifocal areas of necrosis is not uncommon⁹⁵. Immunohistochemical labeling of sections of lung from affected foals revealed *R. equi* antigen present in the cytoplasm of alveolar macrophages, neutrophils and multinucleated giant cells⁹⁵. Except in areas of fibrosis, positive staining of the cytoplasm of histiocytes, fibroblasts and endothelial cells of veins also have been observed⁹⁵. Occasionally, interstitial pneumonia with diffuse miliary pyogranulomatous lesions can occur; affected foals present with

acute tachypnea and fever or can be found dead, while foals that survive to be treated often die hours to days after initial signs appear⁹⁶. A retrospective study of 19 foals with interstitial pneumonia found seven to have positive *R. equi* cultures, two of these were associated with abscess formation within the pulmonary parenchyma⁹⁷. Histopathologic evaluation of pulmonary parenchyma affected by interstitial pneumonia revealed epithelial necrosis of alveoli and terminal bronchioles, prominent intra-alveolar fibrin, alveolar lumens that contained epithelioid cells, macrophages and multinucleated giant cells with syncytial cell formation in sixteen of the nineteen affected foals⁹⁷.

Extra-pulmonary disease

Extra-pulmonary disease in foals can occur concurrently with rhodococcal pneumonia or alone. Extra pulmonary involvement is relatively common in foals with rhodococcal pneumonia, and was found to be present in up to 74% of foals diagnosed with *R. equi* pulmonary infections⁹⁸. The intestinal form of *R. equi* was found to be significantly more common in foals with a history of infection for greater than 3 weeks (59%) compared to foals with an acute history of disease (30%)⁹⁹. Necropsy of 125 foals found 50% to have intestinal lesions: 18% involved the small intestine, 30% involved the cecum, 48% had colonic lesions and 72% had mesenteric and/or colonic lymph node involvement⁹⁹. These lesions can consist of ulcerative enterocolitis or typhlitis, granulomatous/suppurative inflammation of mesenteric lymph nodes, and often abscessation of the abdominal lymph nodes⁹⁹.

Immune-mediated polysynovitis occurs in 25-30% of foals affected with rhodococcal disease^{98,100}. Evaluation of synovial fluid and synovium from affected joints reveals a non-septic mononuclear pleocytosis and lymphoplasmacytic synovitis respectively¹⁰⁰⁻¹⁰².

Immunoglobulin deposition within the synovium was detected using fluorescein-labeled anti-equine IgG in three affected foals¹⁰¹. Antibodies directed against autologous or heterologous Fc portion of immunoglobulin, called rheumatoid factors, were identified in the synovial fluid of another affected foal¹⁰². Other immune-mediated processes such as uveitis, anemia, and thrombocytopenia have also been documented^{98,103}. Overall, foals with extra pulmonary involvement have a significantly decreased survival rate (43%) when compared to foals without extra pulmonary disease (82%)⁹⁹.

Diagnosis

The diagnosis of rhodococcal infections in foals relies upon clinical signs, clinicopathologic evaluation, and ancillary diagnostic tests including ultrasonographic and radiographic studies, tracheal wash culture and PCR of tracheal secretions. No single test exists that can alone definitively diagnose rhodococcal infections. Due to the ability of foals to compensate and conceal outward evidence of disease until it is severe, routine screening tests are recommended for foals that reside on endemic farms. Twice weekly auscultation of foals from birth to 14 weeks of age on a Canadian farm with endemic *R. equi* was successful at preventing mortality and aiding in the early identification of disease¹⁰⁴. However, on large farms this amount of scrutiny may be difficult to accomplish. Giguere et al. evaluated the value of different screening tests to detect subclinical *R. equi* infections and suggested that foals housed on endemic farms have monthly complete blood counts performed and that foals with white blood cell counts \geq 13,000 cells/ul should be subjected to veterinary examination¹⁰⁵. They also recommended that foals with no clinical signs consistent with pneumonia and white blood cell counts \geq 14,000 cells/ul have additional diagnostic testing to more completely rule out sub-clinical

rhodococcal infections¹⁰⁵. When evaluating white blood cell count alone as a diagnostic test 13,000 WBC/ul had 95% sensitivity and 61.5% specificity as a predictor for *R. equi* infection and a 15,000 WBC/ul had 78% sensitivity and 90% specificity¹⁰⁵. Serum fibrinogen alone was found to be an ineffective screening test as a serum concentration of 400 mg/dl had a sensitivity of 90% and a specificity of 51%¹⁰⁵. As fibrinogen increased its sensitivity as a diagnostic test decreased and its specificity increased¹⁰⁵. Seventy percent of foals with white blood cell counts of $\geq 14,000$ cells/ul and seventy-eight percent of foals with white blood cell counts $\geq 15,000$ cells/ul developed *R. equi* pneumonia over the 6 month study period¹⁰⁵.

Leclere et al, evaluated diagnostic tools for foals with clinical rhodococcal infections compared to foals with pneumonia caused by organisms other than rhodococcus and as a diagnostic tool, either a white blood cell count of $\geq 20,000$ cells/ul or a fibrinogen concentration of >700 mg/dl had $>85\%$ specificity for *R. equi* infection¹⁰⁶. Pneumonic foals with white blood cell counts $<6,400$ cells/ul or fibrinogen <400 mg/dl were deemed unlikely to be affected with *R. equi*.¹⁰⁶ Serum *R. equi* antibody and serum amyloid A concentrations have also been proven to be ineffective screening tools¹⁰⁷⁻¹⁰⁹.

Thoracic ultrasonography has not been evaluated in a controlled study as a screening tool. When ultrasound examinations were performed on endemic farms it became apparent that some foals without clinical evidence of disease had subclinical disease evidenced by small areas of pulmonary consolidation without apparent clinical signs and some of these foals cleared disease without any treatment¹¹⁰. Currently it is recommended to serially monitor foals at 1 – 2 week intervals if small areas of pulmonary consolidation (<1 -2cm diameter) are identified¹¹¹. A proportion of these foals may self-clear without treatment,

however lesions that progress in size or fail to regress after several scans warrant antimicrobial treatment.

Definitive diagnosis of *R. equi* pneumonia in foals occurs when the organism is identified via culture or PCR of the *vapA* gene from tracheobronchial aspirate of a foal that displays any of the following: clinical signs of lower respiratory tract disease, radiographic or ultrasonographic evidence of pulmonary abscessation or septic inflammation of the lower respiratory tract⁹³. Concurrent documentation of disease is required as foals may inhale *R. equi* from contaminated environments but be either disease free or have subclinical disease. On an endemic farm, 35% of foals with positive tracheobronchial aspirate cultures for *R. equi* failed to show clinical signs of respiratory disease¹¹².

Treatment of rhodococcal infections in foals

The intracellular nature of *R. equi* in conjunction with both the pyogranulomatous response and abscess formation greatly limits the antimicrobials that are effective against the bacterium. In vitro, a variety of antimicrobials are effective against the bacterium; however only antimicrobials that are lipophilic and capable to penetrate macrophages and purulent debris should be utilized for therapy of rhodococcal diseases¹. Antimicrobial therapy of rhodococcal infections relies upon the combination of a macrolide (erythromycin, azithromycin, or clarithromycin) with rifampin. Macrolides and rifampin exert time dependent, bacteriostatic activity against *R. equi* in vitro¹¹³. Synergism occurs both in vitro and in vivo when a macrolide is combined with rifampin, and their combined use reduces the probability of resistance developing to either drug^{113,114}. In the 1980's the shift from the treatment of *R. equi* pneumonia with antimicrobials that are not lipophilic, such as penicillin and gentamicin, to the use of erythromycin in combination

with rifampin lead to a significant decrease in foal mortality¹⁰⁰. Survival rates of foals treated with the combination of erythromycin and rifampin have been reported to be as high as 88%¹¹⁵, however the adverse effects of erythromycin use include idiosyncratic severe hyperthermia and tachypnea, as well as self-limiting diarrhea in the foal¹¹⁶. Fatal colitis in mares associated with treatment of their accompanying foals with erythromycin has also been reported¹¹⁰. While the bioavailability of erythromycin is poor in foals that are not fasted (8%) it is improved in foals that are fasted (26%)¹¹⁷. The variable absorption of erythromycin in the foal in combination with its potential side effects has lead to the use of more recently developed macrolides, primarily azithromycin and clarithromycin. Azithromycin and clarithromycin are more bioavailable to foals when administered orally, have prolonged half-lives, and significantly increased concentrations in both bronchoalveolar cells and pulmonary epithelial lining fluid when compared to erythromycin¹¹⁸⁻¹²⁰. Azithromycin administered at 10 mg/kg orally surpasses the 90% minimum inhibitory concentration (MIC₉₀) for *R. equi* and persists within pulmonary cells at high concentrations for 48 hours after administration, allowing for extended interval dosing^{118,120}. When compared to azithromycin, clarithromycin administered at a dose of 7.5 mg/kg orally, reaches higher concentrations within both bronchoalveolar cells and pulmonary epithelial lining fluid, however it has a shorter period of persistence within the pulmonary cells and epithelial lining fluid, necessitating a dosing interval of 12 hours^{121,122}. Clarithromycin-rifampin was demonstrated to be significantly more effective when compared to erythromycin-rifampin and azithromycin-rifampin in a retrospective study¹²³, however there have been no prospective controlled studies to compare the efficacy of the different macrolides against rhodococcal infections. Concurrent treatment

with rifampin administered at 5 mg/kg orally every 12 hours, has been shown to decrease plasma, pulmonary epithelial lining fluid and bronchoalveolar cell concentrations of clarithromycin and tulathromycin^{124,125}. Despite this finding, it is evident that the combination of a macrolide with rifampin effectively treats rhodococcal infections of foals, and administering the medications at different times may offset this effect.

Two long-acting macrolides, tulathromycin and tilmicosin, have been investigated for therapy of rhodococcal infections of foals as they would potentially provide sustained therapeutic concentrations and require less frequent dosing. Tulathromycin is a semi-synthetic macrolide that is approved for use in both cattle and swine. After intramuscular injection at a dose of 2.5 mg/kg, tulathromycin was shown to concentrate in the bronchoalveolar cells of foals¹²⁶. However, tulathromycin has been shown to exhibit poor activity against *R. equi* in vitro with a MIC₉₀ > 64 µg/mL; this concentration is over 100 fold higher than concentrations reached via currently recommended dosages^{126,127}. This was supported by the finding that one week after treatment with tulathromycin pulmonary abscesses were significantly larger and duration of treatment was significantly longer when compared to foals treated with azithromycin¹²⁸. Tilmicosin is another long-acting macrolide approved for use in cattle and swine. Tilmicosin also exhibits poor activity against *R. equi* when administered at 10 mg/kg intramuscularly, with an MIC₉₀ of 32 µg/mL, and injection site swelling has been noted to occur at sites of administration on occasion in foals¹²⁹. Neither tulathromycin nor tilmicosin are currently recommended for therapy of rhodococcal infections in foals. However, gamithromycin, a long-acting macrolide that is approved for treatment and prevention of respiratory disease in non-lactating dairy cattle does show excellent in vitro activity against *R. equi*, when

administered at 6 mg/kg intramuscularly, with a MIC₉₀ of 1.0 µg/mL¹³⁰. Safety and clinical efficacy of gamithromycin have not been established, and as a result it is not currently recommended for the treatment of rhodococcal infections of foals. A single intramuscular injection maintained bronchoalveolar lavage cell concentrations above the MIC₉₀ of *R. equi* for approximately 7 days¹³⁰.

Prevention and control of rhodococcal infection in foals

Investigations regarding the prevention of *R. equi* infections in foals have been the subject of numerous studies. Three main areas have been evaluated: farm management practices, chemoprophylaxis and immunoprophylaxis. As previously discussed no specific farm management practices were found to be associated with *R. equi* pneumonia meaning that good management practices that are useful at preventing other foal diseases are not effective against *R. equi*. Therefore, the focus on prevention of *R. equi* infections of foals has been directed towards chemoprophylaxis and immunoprophylaxis.

Environmental prophylaxis

Recently, viruses that infect and replicate within bacteria, called bacteriophages, have been identified against several rhodococcal species and specifically *R. equi*¹³¹⁻¹³⁵. Phages of the families *siphoviridae* and *mycoviridae* have been found to infect *R. equi*¹³¹⁻¹³⁵. Virulent bacteriophages are highly specific as to the strains of bacteria that they infect; bacterial killing via inducing cell lysis allows for release of progeny phages¹³⁶. Phage therapy is beneficial as they are self-replicating within the bacterial host and continue to amplify at the site of infection as long as sensitive bacteria are present¹³⁶. Shibayama and Dobbs found that bacteriophages encode early proteins that alter or inactivate indispensable proteins to the bacteria upon infection and identified ten inhibitory genes

for proteins of unknown function that inhibited the host bacteria when introduced on a plasmid¹³¹.

In relation to *R. equi*, phage therapy is being investigated, as a possible method of environmental prophylaxis to decrease the environmental burden of *R. equi* with the supposition that decreasing this burden will decrease infection rates. The majority of the current literature regarding bacteriophages and *R. equi* involve the discovery and genotyping of phages that are capable of infecting *Rhodococcus* spp, and these have been recovered from both soil samples, and waste water treatment sludge¹³²⁻¹³⁵. One study investigated the application of the *R. equi* bacteriophage DocB7 to soil samples inoculated with *R. equi*¹³⁵. Application of 1×10^5 CFU/gram of *R. equi* to the sterile soil samples yielded a concentration of 7.4×10^7 CFU/gram of *R. equi* after 48 hours of incubation. Bacteriophage doses are calculated by multiplicity of infection (MOI), which is the ratio of infectious agents to the amount of infectious targets. When a MOI of ten was applied to sterile soil, the lowest subsequent recovery of *R. equi* 6.0×10^4 CFU/gram was obtained after 48 hours of incubation¹³⁵. The response seen to different MOI was not linear, and this corresponds with the belief that there is an optimal dosage of bacteriophages, such that if too high a dose is applied initially there are no bacteria left for adequate replication of the phages^{135,137}. This is an interesting area of investigation and clinical studies in the environment are warranted to further determine their utility.

Chemoprophylaxis

Chemoprophylaxis is the administration of a medication prior to development of a disease with the purpose of preventing occurrence of the disease. Several anti-microbial agents have been investigated for use as chemoprophylactic agents against rhodococcal

infections of foals. The first example of this approach was the use of the macrolide antimicrobial azithromycin as a chemoprophylactic agent on endemic farms, but unfortunately this approach has yielded divergent results. When administered at 10mg/kg orally every 48 hours for the first 14 days of life, a significant reduction in the incidence of *R. equi* pneumonia was observed in treated foals (5.3%) versus control foals (20.8%)¹³⁸. However, in a study subsequently conducted on an endemic farm in Germany prophylactic azithromycin administered at a dose of 10 mg/kg every 24 hours for 4 weeks did not reduce the incidence of rhodococcal pneumonia, although the onset of disease was delayed¹³⁹. While adverse effects were not observed due to azithromycin administration in either study, the use of azithromycin as a chemoprophylactic agent is not recommended due to concerns that this approach may contribute to the development of antimicrobial resistance, particularly within *R. equi* organisms themselves^{138,139}. This is particularly concerning as cross resistance may develop between different macrolides, and there are no effective drugs available to treat foals suffering from infections with resistant strains of *R. equi*.

Another approach involved the trivalent semi-metallic element gallium, which mimics ferric iron (Fe^{3+}) and interferes with microbial replication and growth. Ferric iron is integral for metabolic and DNA-synthetic pathways of *R. equi*^{140,141}. It is targeted by the host's innate immune system via sequestration by the iron transport protein, transferrin. However, *R. equi* can circumvent this defense mechanism as it has the ability to utilize transferrin-bound iron¹⁴¹. Gallium binds to transferrin ferric sites, can be attained from transferrin by some bacteria, and in sites of inflammation is favorably taken up by mononuclear phagocytes^{142,143}. Gallium's antimicrobial activity is due to its inability to

be reduced to a divalent molecule, thus preventing its use by bacteria in iron-dependent DNA-synthetic pathways. Gallium has been shown to suppress the growth of *R. equi* in vitro by interfering with iron uptake and utilization¹⁴⁴.

Gallium maltolate has been extensively investigated as a possible chemoprophylactic agent against *R. equi*. When administered prophylactically to murine macrophage-like (J774A.1) cells it was shown to cause significant, dose-dependent decreases in intracellular concentrations of *R. equi*¹⁴⁰. Gallium maltolate was readily absorbed after oral administration and decreased tissue burdens of *R. equi* in experimentally infected mice¹⁴⁴. In a group of neonatal foals gallium maltolate administered intragastrically at 20 mg/kg yielded adequate *R. equi* inhibitory concentrations in most, but not all foals¹⁴⁵. However, oral administration of a methylcellulose form of gallium maltolate to neonatal foals at a dose of 40mg/kg was shown to achieve adequate serum concentrations¹⁴⁶. Disappointingly, gallium maltolate given orally at a dose of 30mg/kg every 24 hours during the first two weeks of life did not reduce the incidence of rhodococcal pneumonia in foals on an endemic farm³⁸. As a result, gallium maltolate is not currently recommended as a chemoprophylactic agent against rhodococcal infections of foals.

Immunoprophylaxis

Vaccination

Immunization with protective *R. equi* antigen of both mares and foals has been unrewarding. Despite significant increases in the colostral antibodies of immunized mare, in both field and experimental challenges the vaccination of mares did not protect their foals against development of disease^{147,148}. Vaccination of mares against *vapA* with a nanoparticle, resulted in high anti *vapA* IgG titers in both mares and foals and may have conferred protection against natural challenge but the study was performed in only a very small group of animals¹⁴⁹. The bacterium, *Salmonella enterica* Typhimurium, which was modified to express *vapA* antigen was used to orally immunize mice against *R. equi* and conferred protection against infection¹⁵⁰.

Intra-gastric administration of live, virulent, *R. equi* to newborn foals was shown to induce accelerated development of *R. equi* specific CTL's and elicited complete protection against subsequent heavy intra-bronchial challenge¹⁵¹⁻¹⁵³. Murine models suggest that DNA immunization with *vapA* confers protection against infection and the IgG subisotype developed is consistent with a Th1 based immune response¹⁵⁴. When a similar DNA vaccine was administered to adult horses it produce a cell mediated response, whereas the response to vaccination in foals was poor¹⁵⁵. Intra-bronchial administration of a live fully attenuated strain of *R. equi* to foals did not result in protection against development of disease¹⁵⁶. When a strain of *R. equi* that had been genetically altered to remove isocitrate lysate and cholesterol oxidase genes was given intrabronchially it protected three foals that it was administered to, however two of the foals developed pneumonia from the vaccinate strain⁵⁵. It appears that avirulent, plasmid

cured strains are not able to elicit a adaptive immune response and replication is required for cell mediated immune response induction⁷⁴.

Immunostimulants

Immunostimulants have been investigated to see if they could enhance the foal's immune response to virulent *R. equi* and confer protection against the development of disease.

Enhanced *ex vivo* or *in vitro* phagocytic cell function or cytokine induction occurred in foals after administration of inactivated *Parapoxvirus ovis*, *Propionibacterium acnes*, and unmethylated CpG's^{84,157,158}. However, *Propionibacterium acnes* failed to decrease the incidence of *R. equi* pneumonia on an endemic farm even though it successfully increased IFN γ production⁹⁰.

Hyperimmune plasma

Administration of *Rhodococcus equi* specific hyperimmune plasma is the only currently recommended prophylactic treatment to prevent the development of disease⁹³. Difficulty exists in comparing the various studies involving hyperimmune plasma administration for *R. equi* prophylaxis. When comparing studies no consistent, universal process was utilized to produce the hyperimmune plasma and this could account for some variations in the results. Also while some studies evaluated the ability of hyperimmune plasma to decrease the incidence, or risk of developing new cases of disease over a period of time, other studies evaluated its effect on prevalence, or the effect of administration on the total number of cases in the study population. Hyperimmune plasma given prior to both experimental and naturally occurring disease has been shown to have protective effects, generally decreasing prevalence and severity of *R. equi* pneumonia^{159,160}. Overall, most studies showed reduced incidence of *R. equi* pneumonia on endemic farms in foals

administered hyperimmune plasma when compared to control foals^{39,147,160,161}. However, within the studies that evaluated the effect of hyperimmune plasma on incidence of disease, not all of them have shown a statistically significant decrease in incidence of *R. equi* pneumonia following hyperimmune plasma administration^{39,92,162}. Despite the inter- and intra-study variations and differing outcomes after administration of hyperimmune plasma the general consensus is that it confers some protective benefits and is an imperfect, yet viable prophylactic treatment. The specific protective components of hyperimmune plasma are incompletely understood. *Vap* protein antibodies, especially those directed against *Vap A* and *Vap C*, are important, as are non-specific factors such as complement, cytokines and fibronectin^{75,163}. The timing of administration and dose of hyperimmune plasma is currently unknown. However, one study found that foals administered two liters of plasma had persistence of antibodies for 90 days when compared to only 30 days in foals that were administered one liter of plasma¹⁶⁰. It is generally recognized that administration of hyperimmune plasma should occur prior to exposure as its administration 9 days after aerosol infection of foals with *R. equi* was not protective against development of disease¹⁶⁴. Currently, recommendations are for foals to be administered one liter of hyperimmune plasma no later than 2 days of age with another liter administered between 2 – 4 weeks of age⁹³. Disadvantages of treatment with hyperimmune plasma include: expense, amount of labor required, incomplete protection against disease, and reactions to transfusion^{139,147,159,160}. The range of patient reaction to transfusion can be as mild as urticaria or severe as anaphylaxis with the risk of death¹⁶⁵. However, a retrospective study evaluating transfusion reactions to commercial equine plasma found the incidence in neonates less than 7 days of age to be 9.7%¹⁶⁶. Reactions

observed in the six affected foals included fever, tachycardia, tachypnea and colic; none of the reactions proved fatal in the reported cases¹⁶⁶.

Principles of aerosol delivery and therapy

Aerosol delivery of pharmaceuticals has been successfully used to treat various conditions such as pneumonia, inflammatory airway disease and neoplasia in multiple species including horses¹⁶⁷⁻¹⁷³. Advantages associated with aerosol drug delivery include: rapid onset of action, high concentrations of drug delivered to the lower airways with decreased volume of systemically administered dose, avoidance of adverse systemic effects, and ease of administration¹⁷⁴.

Effective drug delivery is heavily dependent upon the characteristics of the substance being delivered, the size of the individual particles when aerosolized and patient respiratory characteristics¹⁷⁴. Deposition of aerosols within the respiratory tract is dependent upon the size of the individual aerosol particles. Large particles, greater than 10 μm in size are unable to change direction easily and are deposited via inertial impaction into the nasopharynx¹⁷⁴. Particles that reach the small airways are deposited via sedimentation as the airflow becomes too slow to effectively move them. Particles $<2 \mu\text{m}$ in size are deposited within the alveoli and are taken up via diffusion¹⁷⁴. Particle diameter of a solution when aerosolized depends upon the density of the substance, its surface tension, and adherence of particles¹⁷⁴.

Tidal volume, minute ventilation, and the presence or absence of pulmonary disease all impacts the respiratory physiology of the patient and the distribution of particles into the lower airways¹⁷². The unique properties of the equine lung include its large tidal volume,

potentials for high rates of flow, and the large surface pulmonary surface area make them an ideal candidate for aerosol therapies¹⁷².

Once macromolecules are delivered to the lung, several things determine their fate.

Absorption of the molecules can occur by yet unknown mechanisms via the blood / air barrier. Proteins can be degraded by peptidases or proteases. Cognate receptors are present for albumin, transferrin, IgG and IgA. These receptors are involved with the absorption and secretion of those substances across the epithelial barrier¹⁶⁷. Also, alveolar macrophages have been shown to take up macromolecules in sheep, rodents and monkeys¹⁶⁷.

Many different delivery devices have been utilized and described in the literature for drug aerosolization. Jet nebulizers, metered dose inhalers (MDIs), dry powdered inhalers (DPIs), ultrasonic and vibrating mesh nebulizers have all been used to deliver substances to the pulmonary parenchyma each with it's own advantages and disadvantages that can vary based upon the drug delivered and condition being treated¹⁷⁴. Solutions that are nebulized to equines are administered via a fitted facemask that collects the aerosol and allows for it to be inspired and delivered into the airways.

Dry powder and metered inhalers are pre-filled containers that generate aerosol upon manual activation. Metered inhalers suspend the drug in a propellant while dry powder inhalers rely upon active inspiration of the patient to deliver the drug. Metered and dry powder inhalers have been used to treat equine respiratory diseases, but their primary utilization has been directed toward therapy of lower airway inflammation¹⁶⁹. However, both delivery devices have their disadvantages. Metered inhalers release environmental contaminants, drug delivery can be inexact, and the volume of drug that reaches the lower

airway can be small. Dry powdered inhalers rely upon the patient's ability to generate adequate tidal volume via inspiration to deliver drug to the airways and the volume of drug delivered can also be small. Many different types of nebulizers have been used to deliver aerosols to equine patients. Jet nebulizers create aerosol from liquid suspensions by utilizing gas from a compressor or compressed gas¹⁷⁴. When using jet nebulizers, particle size can be adjusted based upon the rate of flow of the gas used to create the aerosol allowing generation of particle sizes within the 0.2 – 5 μm range. Jet nebulizers are cumbersome to use for equine aerosol delivery as the excessive noise of the compressed gas can startle the patient and overall slow rate of drug delivery¹⁷⁴. Another disadvantage of jet nebulizers when aerosolizing protein is the force of the gas can act to denature the protein itself.

Ultrasonic nebulizers create aerosols via the vibration of piezoelectric crystals that are driven by alternating electric current; these nebulizers are quiet, and produce a very concentrated aerosol¹⁷⁴. The main disadvantage of ultrasonic nebulizers is that while generating the aerosol they give off heat that can denature the drug, particularly proteins that are nebulized.

Vibrating mesh nebulizers are a recent technology for generation of aerosols and create their aerosol via either active or passive devices. Active devices use a micro-pump that has a domed aperture plate with laser-perforated holes of specific diameter¹⁷⁵. Electric current generated across the aperture plate causes it to expand vertically and create aerosol when the solution comes into contact with the plate. Passive devices have a perforated plate that is connected to a piezoelectric crystal that is then coupled to a transducer horn that induces the passive vibrations causing the solution to be extruded

through the perforations thereby generating the aerosol¹⁷⁵. Advantages of vibrating mesh nebulizers include their lack of heat generation, efficiency and almost silent operation as well as their compact size. Until the advent of vibrating mesh nebulizers, proteinaceous solutions were problematic to deliver as most of the delivery devices denatured or damaged the protein to be delivered.

Local treatment of the respiratory tract via nebulization of protein has been described previously in several other species^{168,176-178}. Proteins that have been successfully nebulized include recombinant human DNase I as therapy for cystic fibrosis, cyclosporine A to treat chronic rejection of transplant organs, interleukin – 5 as therapy for human asthma, and IgG1 for the treatment of neoplasia; all these products have been delivered successfully to the airway without generating systemic or local adverse reactions^{168,176-180}.

Local delivery of *R. equi* specific IgG to the lungs of foals is an attractive option for prophylaxis of *R. equi* infections. If it were effective aerosolized *R. equi* hyperimmune plasma would represent a method to deliver IgG to the site of primary infection and would be faster and easier to administer when compared to intravenous hyperimmune plasma.

Chapter 2. Aerosol delivery of *rhodococcus equi* specific IgG to the lungs of ponies.

Introduction

Rhodococcus equi is a facultative, intracellular gram-positive coccobacillus that is found ubiquitously in the environment. It is the most significant cause of severe and sometimes fatal chronic, suppurative bronchopneumonia and a myriad of extra-pulmonary diseases in foals from 3 weeks to 5 months of age. Early diagnosis can be challenging leading to the presence of severe infection before clinical signs are evident in the affected foal. Morbidity rates of up to 40% have been documented on endemic farms. Strains of *R. equi* that are resistant to macrolide antibiotics are associated with worse prognosis. A multitude of studies have evaluated farm management, chemoprophylaxis, and immunoprophylaxis methods in an effort to develop strategies to prevent disease without ample success. Intravenously administered *R. equi* specific hyperimmune plasma is the most effective prophylactic agent to date. However, it is not completely effective in eliminating *R. equi* pneumonia; moreover, its administration is invasive, time consuming and costly. Previous studies examining the effectiveness of aerosolized therapies administered to horses for treatment of pulmonary diseases have met with great success and the delivery of aerosolized proteins to the lungs has been validated in other species. The purification and quantitative description of *R. equi* IgG for aerosolization was previously performed¹⁸¹. This preparation of *R. equi* IgG had a MMAD value of 4.7 microns and was also shown to be present in the lungs of adult horses after delivery¹⁸¹. The aim of the current study was to investigate if local delivery of *R. equi* specific IgG to the lungs of ponies elicited a significant inflammatory response and determine how it affected the total quantitative IgG

and *R. equi* specific IgG titers so that it can be further investigated as a novel prophylactic treatment for *R. equi* pneumonia in foals.

Materials and methods

Animals

Eight adult ponies aged from 5 – 21 years, including six mares and two geldings were leased from their owners for the duration of the study. Ponies were randomly allotted into two groups of four; each pony was used as its own control in a crossover design. Each group contained one gelding and 3 mares. One pony was identified to have pediculosis caused by *Wernerckiella equi* at the start of the study. Treatment consisted of topical fipronil^a on the affected pony and three other in-contact ponies. The other four ponies were treated with topical permethrin spray^b. After treatment no further evidence of pediculosis was found in the affected pony, none of the other ponies developed pediculosis.

Ponies were housed in paddocks in their respective groups during treatment periods. They received ad libitum grass hay and water. During sampling periods they were housed in a 12 x 12 box stall with pine shavings and received ad libitum access to grass hay and water with the exception of the 30 minutes immediately post sedation when they were muzzled. During the washout period the ponies were all maintained on grass pasture and fed additional grass hay with access to free choice water at all times.

Each pony was confirmed to be free of historic, chronic or active respiratory disease based upon clinical history and absence of nasal discharge, dyspnea, tachypnea, and cough. Owners were questioned prior to inclusion in the study for any prior history of respiratory disease. Physical examinations were performed including re-breathing

procedures to determine if underlying respiratory disease was present. Any animal with a prior history of respiratory disease or evidence of respiratory disease on physical examination was excluded from the study group. Complete physical examinations occurred at admission, and a minimum of every twenty-four hours prior to treatment or sample collection. Ponies were visually inspected continuously during treatment and for the first 12 hours post treatment or sample collection. The Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee approved the study protocol.

IgG purification and concentration

Horse IgG was purified and concentrated by Jianzhong Hu in the laboratory of Dr. Mike Zhang, using commercially available *Rhodococcus equi* equine hyperimmune plasma^c by protein G affinity chromatography using an AKTA explorer 100 fast performance liquid chromatography (FPLC) system^d 10 ml of protein G Sepharose 4 fast flow resin^e was packed in XK-16 column for purification. The column was equilibrated with 10 column volumes of binding buffer (20mM sodium phosphate, pH 7.0). 10 ml of hyper immune equine plasma was then loaded onto the column. After washing the column with 3 column volumes of binding buffer, the bound IgG was eluted in 100mM glycine, 100 mM arginine, and pH 2.7. The eluates were pooled and concentrated to a final concentration of 30 mg/mL IgG using a Millipore LabScaleTM TFF system equipped with a Biomax 30 ultrafiltration cassette^f Purified protein product was tested via both total and *R. equi* specific ELISA.

Fig 1. Protein G affinity chromatography for IgG purification

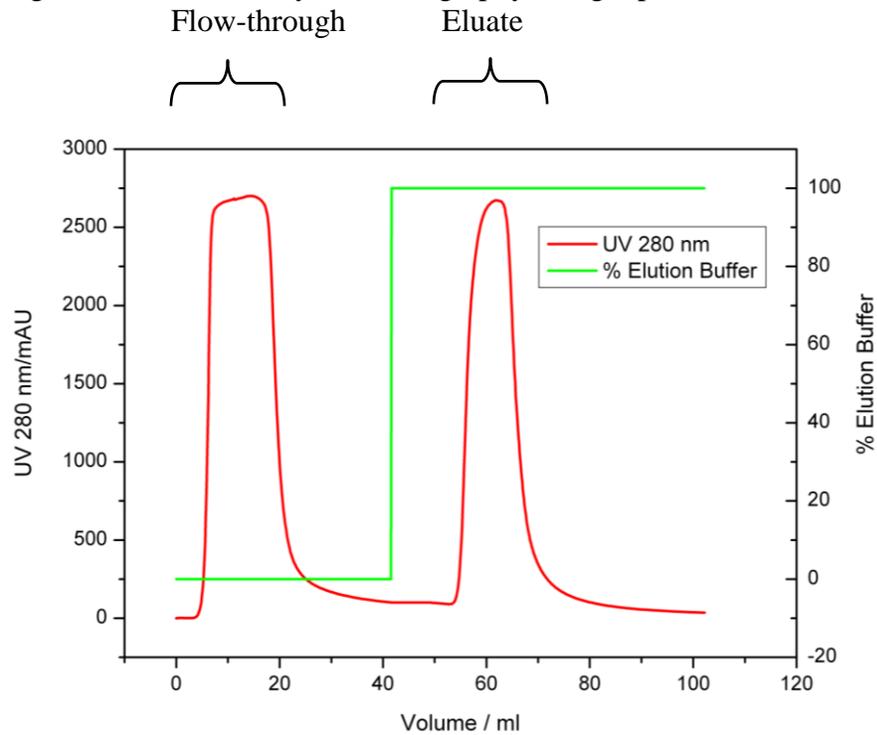
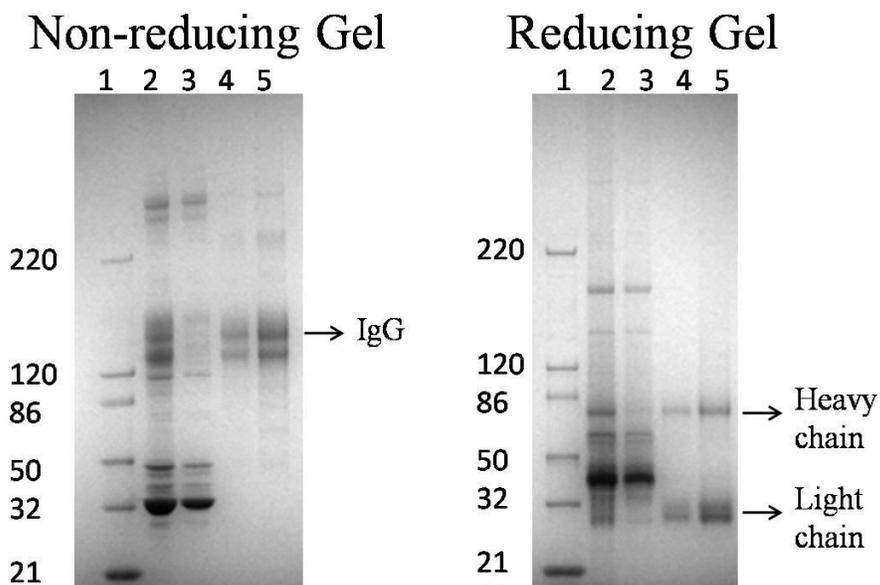


Fig 2. SDS-PAGE analysis of horse IgG purification process



1: Marker; 2: Hyperimmune horse plasma; 3: Chromatography flow-through; 4: Chromatography eluate; 5: Concentrated horse IgG

Table 1. Summary of the purification process

	Plasma	Flow-through	Eluate	Concentrated IgG
Total protein concentration (mg/ml)	90.6	36.9	8.5 mg/ml	35.2 mg/ml
IgG concentration (mg/ml)	17.6	1.7	7.5 mg/ml	30.8 mg/ml
Step Recovery	NA	NA	81.0%	95.2 %
Purity	NA	NA	88.2%	87.5%

NA: Not available.

Aerosol administration

R. equi IgG was thawed to room temperature and filtered through a sterile 0.22 μ m polyethersulfone, protein-sparing filter^g to remove particulate matter or potential bacterial or viral pathogens. The total volume of IgG administered was 8 ml, or 240 mg (30mg/ml). The control solution consisted of 8ml of sterile 0.9% sodium chloride^h solution. The solution to be administered was placed into the reservoir of the vibrating mesh nebulizerⁱ, and the device was run until the solution was completely aerosolized. Nebulization time was recorded for each treatment. After use any residual protein film was removed from the nebulizer cup with a dry gauze 4x4; this step was not necessary when saline was administered. Following each use 30 ml of sterile saline^h was added to the nebulizer reservoir and aerosolized to cleanse the nebulizer. The associated mask and reservoir chamber were cleaned according to manufacturers recommendations with warm water and dish soap and allowed to air dry between ponies. Four ponies received treatment with aerosolized IgG during the first treatment period (A, B, C, D) and the

remaining ponies (E, F, G, H) received treatment with the control, aerosolized saline, during the first treatment period. The first treatment period was followed by a four-week washout period. In the second treatment period of the crossover study, all ponies received the opposing treatment type.

Bronchoalveolar lavage

Ponies were sedated with a combination of detomidine^j (0.01mg/kg IV) and butorphanol^k (0.007 mg/kg IV) prior to nebulization and administered inhaled albuterol^l (450µg/pony) five minutes prior to bronchoalveolar lavage. Prior to subsequent bronchoalveolar lavage procedures ponies were sedated with xylazine^m (0.33 – 0.65 mg/kg IV). Bronchoalveolar lavage was performed with a three-meter video endoscopeⁿ at predetermined time periods, which included an initial lavage prior to nebulization (Time 0) and at 1 hour (Time 1), four hours (Time 4) and 24 hours (Time 24) post nebulization. In order to minimize dilution of IgG and to determine uniform distribution of IgG within the lungs, low-volume BAL was performed in four different sites within the lung at corresponding times: right caudoventral lung at Time 0, left caudoventral lung at Time 1, right craniodorsal lung at Time 4, and left craniodorsal lung at Time 24, as previously described^{171,173}. After an adequate plane of sedation was achieved, the endoscope was passed through the upper airway into the lower airway; 20 ml of 2% mepivacaine^o was infused selectively via the endoscope during passage to reduce bronchospasm and cough. Once the endoscope was well seated within the bronchus, sterile saline^h (30 ml 0.9% NACL) was delivered into the airway via the endoscope followed by 30 ml of air to ensure complete delivery. The saline was then immediately aspirated out of the bronchus. The recovered lavage fluid was used to make multiple cytocentrifuge slides within 15

minutes of fluid collection. The remaining fluid was then divided into 3-4ml aliquots that were stored at -20C for future testing. Each sample was thawed and filtered through a sterile 0.22µm polyethersulfone, protein-sparing filter^g prior to ELISA testing.

Cytologic evaluation

Differential cell counts were performed on all BALF samples. Slides were prepared by adding 100 microliters of BALF to a single use cytocentrifuge chamber^p positioned adjacent to a single glass microscope slide. Slides were generated using the Cytopro[®] centrifuge model 7620^p and cytocentrifugation was performed at 1,250 x g for four minutes. Slides were then air dried at room temperature prior to staining. A minimum of two slides were made for each sample; one was catalogued and the other was stained for evaluation. Slides were prepared using a modified Romanowsky stain^q and evaluated for appropriate distribution of cells. If slides were determined to be of inadequate quality then subsequent slides were made. Differential cell counts were performed by the same investigator (AF). A total of 400 cells per slide were counted to determine total neutrophil, macrophage, lymphocyte and eosinophil percentages. For any single sample that had a neutrophil percentage greater than 15% the second slide was stained and cell counts were verified by repeat analysis.

Total IgG quantitation

Total IgG was measured using a commercial quantitative equine IgG enzyme linked immunosorbent assay ELISA^f Concentrations were obtained in ng/ml. Equine IgG standards were prepared by adding 500 µl of diluent reagent provided in the kit to eppendorf tubes^f that were labeled for their final concentrations of standard (125, ng/ml, 62.5ng/ml, 31.25ng/ml, 15.6ng/ml, 7.8ng/ml and 0 ng/ml). The high standard of 125

ng/ml had no diluent added, and 1,000 μ l of horse IgG standard provided in the kit was added to the tube labeled 125 ng/ml. Then, 500 μ l of this solution was added to the tube labeled 62.5 ng/ml. All tubes were vortexed for 30 seconds to allow for adequate mixing prior to use. Doubling dilutions were to obtain the required, labeled concentrations. The tube labeled 0 ng/ml contained only diluent.

This test kit was designed for use with equine serum. It was optimized for use with BALF by performing serial dilutions of BALF with starting concentrations: neat, 1:1, 1:2, 1:5, 1:10, 1:1000, 1:62,500, 1:12,500, 1:250,000 and 1:500,000. A dilution of 1:5000 was determined to be the optimum dilution to achieve an OD within the working range of the ELISA. All samples were diluted 1:5000 using the sample diluent provided in the kit^f (PBS, Triton X-100® and 2-chloroacetamide). Dilution was performed as follows: 1 μ l of each BALF sample was added to 4999 μ l of diluent.

The principle of the ELISA is as follows: the micro plate wells are coated with polyclonal antibodies to horse IgG forming the capture phase of the ELISA. The antibodies present bind uniformly to all subclasses of horse IgG. Captured horse IgG reacts with detector antibody, a polyclonal anti-horse IgG conjugated with horseradish peroxidase. This reagent also uniformly reacts with all subclasses of horse IgG. Enzyme activity in the wells is quantified using tetra methyl benzidine as a substrate which results in a color change from colorless to blue.

Total IgG quantification of BALF was completed as follows. One well of each plate was left empty and served as the substrate blank. Standards (200 μ l) were duplicated and added to rows 11 and 12 with highest concentration in well A (11 &12) and each subsequent lower concentration in the well below. Diluted BALF (200 μ l) was added to

test wells. Each sample was tested in duplicate. The micro plates were then sealed with provided plate sealers^f and incubated at 37°C for 30 minutes. A micro plate washer^s was used to aspirate the contents of each well and wash each well four times with 1X plate wash buffer^f (PBS, Tween 20® and 2-chloroacetamide). After the final wash cycle the contents of each well were aspirated by the plate washer and any remaining fluid was removed by carefully striking the plate on a pad of absorbent paper. Then 100 µl of provided detector antibody^f (conjugated goat anti-horse IgG peroxidase) was added into each well with the exclusion of the blank well. The plate was again covered with provided plate sealers and incubated for 30 minutes at 37°C. The plate was then washed as previously described. Then 100 µl of substrate (containing tetra methyl benzidine) was pipetted into each well including the substrate blank well. The plate was then incubated at room temperature (23°C) for 30 minutes. A blue color developed in the wells containing horse IgG. After incubation 100 µl of, a proprietary stop solution was added to each well, causing all blue colored wells to change to yellow. Spectrophotometric optical density was measured with the Hidex Chameleon®^t at 450nm. Results were calculated and reported using a software program, Microsoft Excel®^u.

The IgG product used for nebulization was also tested to determine the total IgG concentration. Dilutions of 1:6250, 1:12500, 1:250000 and 1:500,000 were made to test the solution. Dilutions were made as follows. A stock solution of 1:1000 was made by adding 10 µl of the IgG product to 9.90ml of diluent. Then 1:62500 concentration was made by adding 160 µl of stock solution to 9.84ml of diluent. The 1:12500 concentration was made by adding 80 µl of the stock solution to 9.92ml of diluent. The 1:250000 concentration was made by adding 40 µl of the stock solution to 9.96ml of diluent. The

1:500000 concentration was made by adding 20 µl of the stock solution to 9.98 ml of diluent. Testing was performed by adding 200 µl of the neat concentrations of each diluent to the A rows and performing doubling dilutions down the columns. Testing was performed in duplicate. The remainder of the ELISA was performed as described above.

***R. equi* specific IgG titer**

An *R. equi* specific IgG ELISA was developed based upon that described by Takai et al¹⁸². The *R. equi* specific ELISA was used to determine sample titers of *R. equi* IgG and optimized using a checkerboard technique for use on BALF. Ninety-six well micro-titration plates^v were coated with 1µg/ml *R. equi* soluble antigen in carbonate-bicarbonate buffer (pH 9.6, total volume 100ul/well) and stored overnight at 4C. An automated plate washer^s was used to wash plates with four wash cycles with phosphate buffered saline with 5% Tween (PBST)^w. 100ul 1% non-fat milk was then applied to the plate for one hour at room temperature (23°C) as a blocking agent. Plates were washed as previously described. 200 ul BALF was applied neat, in triplicate, to the A row of wells and doubling dilutions were performed down the columns. The plate was incubated for one hour at room temperature (23°C). Plates were washed as previously described. Alkaline phosphate conjugated rabbit anti-horse IgG heavy and light chain antibody^x was diluted at 1:25,000 by adding 4 ul of antibody to 9.96 ml of carbonate bicarbonate buffer. 50ul was added to each well and allowed to incubate for one hour at room temperature (23°C). Plates were washed as previously described. 100ul of substrate (alkaline phosphatase p-nitrophenyl phosphate (PNPP))^y was applied to each well and allowed to incubate for 120 minutes. The reaction was stopped with 50ul of 2N sodium hydroxide and absorbance was read immediately at 405nm with the Hidex Chameleon^{®t}.

Statistical analysis

Descriptive statistics were generated for all data and mean values were reported. A one-way analysis of variance (ANOVA) with repeated measures were used to compare median values of BAL fluid IgG concentrations between treatments, time and horse. For data that was non-normally distributed a Wilcoxon signed rank test for paired data was used to determine significance. Significance was pre-set at $P < 0.05$.

Results

Nebulization times

Nebulization times were similar for both treatments. The average time for saline administration was 11 minutes (Table 1) while the average time for IgG administration was 15 minutes (Table 2).

Table 2.

Saline Treatment	Nebulization Time (min)
A	14
B	11
C	8
D	14
E	13
F	9
G	10
H	10
Average Time	11.12

Table 3.

IgG Treatment	Nebulization Time (min)
A	15
B	11
C	11
D	12
E	13
F	20
G	23
H	16
Average Time	15.12

BAL recovery volumes in milliliters

The low volume BAL technique overall yielded consistent results between animals and treatments. The average percentage of recovery for saline treatment was 41 – 47% (12.37 – 14.25 mls); which is similar to the average percentage of recovery for the IgG treatment

group 41-48% (12.37 – 14.37 mls). The samples were of adequate cellularity and quantity with ample sample volume for all testing that was performed.

Table 4. BAL recovery in milliliters: saline treatment

Saline Treatment	T0	T1	T6	T24
A	14	14	11	13
B	15	17	12	13
C	8	12	11	16
D	13	13	13	14
E	11	12	15	12
F	12	15	14	16
G	18	18	10	11
H	12	13	13	14
Average	12.87	14.25	12.37	13.62
Average % Recovered	43	47	41	45

Table 5. BAL recovery in milliliters: IgG treatment

IgG Treatment	T0	T1	T6	T24
A	11	13	13	11
B	12	20	14	15
C	16	11	13	15
D	6	12	11	10
E	12	11	12	12
F	11	14	16	10
G	17	20	12	13
H	14	14	12	15
Average	12.37	14.37	12.87	12.62
Average % Recovered	41	48	43	42

Cytologic evaluation

Overall no clinically significant differences in the cytology profiles were found between treatments or time points. The neutrophil percentages did not vary significantly over time or between treatments. A single pony had neutrophilia present at times 6 and 24 post

administration of IgG. Macrophage percentages failed to vary significantly over time or by treatment. Overall, eosinophils were not frequently identified in the BALF. However, a significant difference was found over time in eosinophil percentage in the IgG treatment group ($P = 0.03$), although this was not likely clinically significant. A single pony did tend to have eosinophils identified in its BALF regardless of treatment or time. In the saline treatment group the lymphocyte percentage was found to be significantly different over time with the percentage decreasing over time ($P = 0.02$). However, this was not a clinically significant finding.

Table 6. Neutrophil cytology results expressed as percentage (%)

Neutrophil	IgG T0	IgG T1	IgG T6	IgG T24	NACL T0	NACL T1	NACL T6	NACL T24
A	5	3	5	4	1	2	3	3
B	4	4	11	9	5	2	3	6
C	9	9	17	9	8	3	8	6
D	6	5	6	3	1	1	2	4
E	3	1	3	5	4	1	6	4
F	4	2	6	4	10	6	10	8
G	3	7	62	66	10	15	18	37
H	3	1	6	7	6	5	8	6
Mean	4.625	4	14.5	13.375	5.625	4.375	7.25	9.25

Table 7. Macrophage cytology results expressed as percentage (%)

Macrophage	IgG T0	IgG T1	IgG T6	IgG T24	NACL T0	NACL T1	NACL T6	NACL T24
A	71	62	67	64	65	65	68	78
B	57	53	63	68	45	53	72	73
C	55	73	68	54	52	64	61	70
D	61	56	82	64	62	66	54	68
E	73	73	84	69	70	67	71	71
F	70	80	71	73	55	69	70	61
G	59	54	28	15	64	47	55	38
H	69	71	61	71	54	79	70	85
Mean	64.375	65.25	65.5	59.75	58.375	63.75	65.125	68

Table 8. Lymphocyte cytology results expressed as percentage (%)

Lymphocyte	IgG T0	IgG T1	IgG T6	IgG T24	NACL T0	NACL T1	NACL T6	NACL T24
A	24	34	27	31	34	32	28	18
B	39	43	26	22	49	45	24	21
C	34	17	15	35	40	32	31	24
D	32	39	11	33	36	33	43	27
E	24	26	13	25	26	56	23	24
F	26	19	20	23	35	25	20	31
G	37	37	10	19	27	36	27	24
H	26	27	33	21	39	15	20	8
Mean	30.25	30.25	19.375	26.125	35.75	34.25	27	22.125

Table 9. Eosinophil cytology results expressed as percentage (%)

Eosinophil	IgG T0	IgG T1	IgG T6	IgG T24	NACL T0	NACL T1	NACL T6	NACL T24
A	0.5	0	0.5	0.5	0	0	0.5	0
B	0.5	0	0	0.5	0	0	0	0
C	0.5	0.5	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0
E	0	0	0	0.5	0	0	0	0
F	0.5	0	0.5	0.5	0	0.5	0	0
G	0.5	0	0	0	0	0	0	0.5
H	2	0	0	0.5	0.5	0.5	2	0.5
Mean	0.5625	0.0625	0.125	0.3125	0.0625	0.125	0.3125	0.125

Total IgG ELISA

Overall the concentration of total IgG was variable between individuals, treatment and time point. The mean concentrations of IgG increased after both IgG treatment (mean 87,163 ng/ml) and saline treatment (mean 183,028 ng/ml) at T1. While total IgG concentrations were increased at T1 compared to T0, no significant effects of time were found ($P=0.19$). However, overall the NACL treatment group had significantly higher concentrations of total IgG than the IgG treatment group ($P=0.023$).

Total IgG concentrations

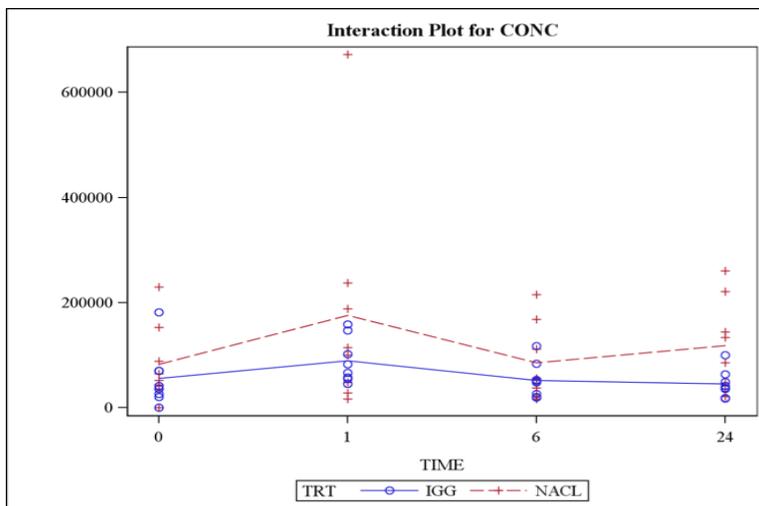
Table 10. Total IgG concentrations: saline treatment (ng/ml)

NACL Treatment	T0	T1	T6	T24
A	152,576	237,804	204,428	114,879
B	15,496	47,680	37,995	36,207
C	61,984	15,943	16,092	93,572
D	225,288	197,276	54,385	46,935
E	29,353	26,969	171,052	124,266
F	84,036	184,463	55,428	277,438
G	51,405	97,446	117,412	236,612
H	43,359	656,643	21,605	21,307
Mean	82,937	183,028	84,800	118,902

Table 11. Total IgG concentrations: IgG treatment (ng/ml)

IgG Treatment	T0	T1	T6	T24
A	0	83,622	81,516	105,580
B	19,702	64,070	26,620	40,307
C	40,607	56,550	20,153	17,897
D	25,417	97,760	53,090	38,051
E	168,447	46,473	51,887	20,604
F	67,228	44,217	50,233	66,476
G	66,627	158,421	21,055	50,383
H	34,140	146,188	118,966	38,051
Mean	52,771	87,163	52,940	47,169

Figure 3. Graphical results of total IgG ELISA (ng/ml)



***R. equi* specific IgG ELISA**

The titer of the *R. equi* product was 1:10,000. Results in tables are the average of the triplicate titer results of the ELISA. On average after treatment with IgG the *R. equi* specific titer increased 2 to 4 fold at T1. This response was short-lived and was decreased at T6, but most ponies had persistence of an increased titer at T 24. While the *R. equi* specific titer after IgG treatment increased at T1, no significant difference was identified between treatment or time (P=0.261). Overall the individual response to IgG was variable.

Figure 4. *R. equi* specific IgG titer: IgG treatment

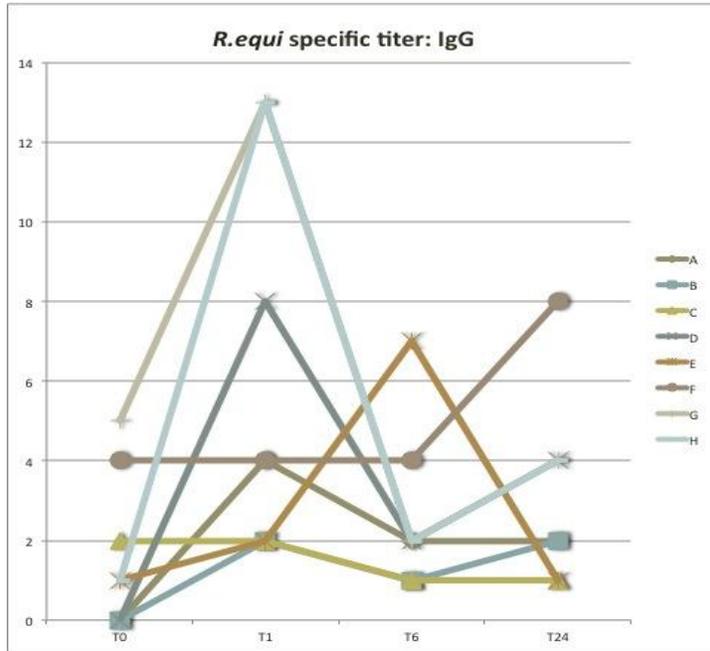


Figure 5. *R. equi* specific IgG titer: saline treatment

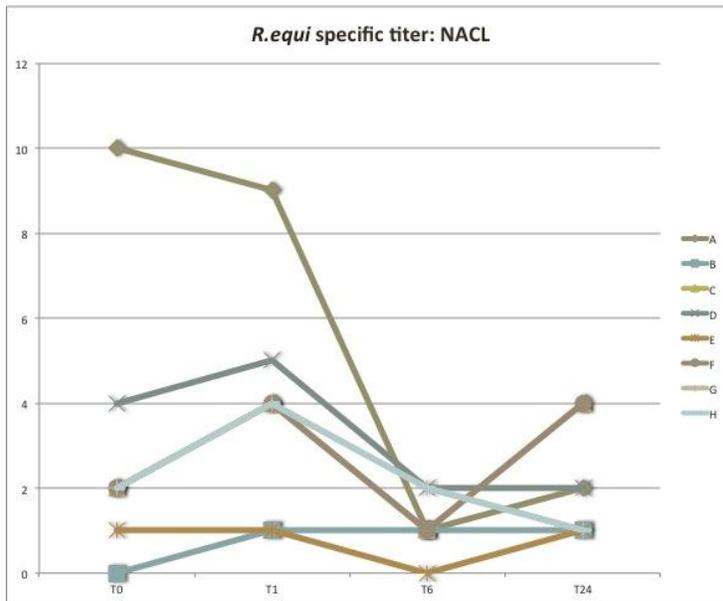


Table 12. R. equi specific ELISA results expressed as a titer (e.g. 10 = 1:10)

NACL Treatment	T0	T1	T6	T24	IgG Treatment	T0	T1	T6	T24
A	10	9	1	2	A	0	4	2	2
B	0	1	1	1	B	0	2	1	2
C	2	4	1	4	C	2	2	1	1
D	4	5	2	2	D	0	8	2	4
E	1	1	0	1	E	1	2	7	1
F	2	4	1	4	F	4	4	4	8
G	2	4	2	1	G	5	13	2	4
H	2	4	2	1	H	1	13	2	4
Mean	3	4	1	2	Mean	2	6	3	3

Discussion

Administration of the aerosolized *R. equi* specific IgG protein product to the lungs of ponies was not time consuming, taking on average 15 minutes. The IgG nebulization time data could have been mildly skewed due to the nebulizer becoming clogged for the pony with the longest nebulization time. It seemed that the protein built up on the mesh nebulizer and there was a trend for increased nebulization times as the equipment was used more. However, the time of nebulization was still less than would be required for intravenous administration of plasma.

Small ponies (less than 13 hands) were chosen for the experiment over adult horses as the lung volume of the ponies more closely approximates that of foals. Aerosol administration was uncomplicated and required only one person to perform as the nebulization device is self contained. No loss of the IgG product occurred into the environment during nebulization as the device used was easily adjustable and fit the ponies snugly to allow for complete delivery of the amount of solution to be aerosolized.

While the IgG product was not tested directly for particle size, a similar solution was tested by the authors previously with a vibrating mesh nebulizer, and yielded an average MMAD of 4.7 microns, which is well within the range of acceptable size for deposition into the deep lung parenchyma. However, it is possible that some of the IgG product particles were too large when aerosolized and did not become delivered to the deep lung being lost to impaction in the upper airways. The ponies tolerated the procedure well with neither immediate or long-term adverse effects nor local inflammation being induced by the procedure.

The low-volume BAL technique obtained fluid with a good cellular yield, and all samples were useful for characterization of the cellular response of the lower airway to treatments. Based upon the cytology results it does not appear that administration of the IgG product induced a local inflammatory response in the lungs of the ponies after nebulization. A statistically significant difference in eosinophil concentration over time was observed during IgG treatment. This is likely due to the very low numbers of eosinophils detected and was not a clinically significant finding. During saline treatment the lymphocyte percentage decreased significantly over time. The decrease was not clinically significant, as the percentages of all cell types remained within accepted normal ranges. However, it is unknown why this was observed only after saline administration. The single pony that developed BALF neutrophilia post administration of IgG had no changes in physical examination and tended to have higher neutrophil counts after saline administration as well. It is possible that this particular pony had more airway reactivity at baseline without having active inflammation present.

While the *R. equi* specific IgG titer was increased after administration of the IgG product, this increase did not reach statistical significance. This was likely due to the large range of variability between individuals in response to the IgG product. It also could be possible that the aerosolized antibody particles were distributed unevenly within the lung, contributing to the variable IgG concentrations observed among individual horses and time points. A method to decrease the potential effect of sampling site would have been to randomize the site of collection for each pony at each time point to remove the potential influence of this on the statistical analysis.

Another explanation for the interindividual BAL fluid IgG concentrations could be due to absorption or uptake of the IgG into local sites such as alveolar macrophages, respiratory epithelium or absorbed into systemic circulation. Investigation of the specific concentration of total or *R. equi* specific IgG within the pulmonary epithelial lining fluid would have required the use of an endogenous or exogenous dilutional marker. Commonly used markers include urea, inulin, methylene blue, technetium 99m Tc-pentate and $^{51}\text{Cr-EDTA}$ ¹⁸³⁻¹⁸⁷. While it is not uncommon for these techniques to be used, they have been shown to introduce additional errors within the reported concentrations of pulmonary epithelial lining fluid^{188,189}. Therefore, determination of the IgG concentration within the pulmonary epithelial lining fluid was not performed.

The curious finding of a statistically significant increase in total IgG in the saline treatment group, but not after IgG administration is difficult to explain. It is possible that fluid shifts within the lung parenchyma after administration of aerosolized IgG caused dilution of the total IgG concentration. The concentrated protein product could have acted as a relatively hypertonic solution causing an osmotic shift of fluid from the lung

parynchma into the bronchioles. Alternatively the IgG particles could have been taken up by macrophages, or sequestered within the pulmonary epithelium. It is also possible that some of the IgG present in the samples were sequestered within pulmonary cells and that this IgG was removed from the samples when the cells were filtered out prior to ELISA testing. The filters used were specifically selected for their protein sparing nature, but if the IgG was trapped within the cells themselves it would have been removed with the cells during filtration. Cell-associated IgG could be further investigated via flow cytometry. The IgG product could be labeled and then flow cytometry could be performed on the BALF to identify if the cells are taking up the IgG.

The major criticism of the study design is that a dilution marker such as urea was not used to be able to determine if fluid shifts occurred within the lung that may have concentrated or diluted the total IgG within the airway lumen. Further investigation is needed to determine the fate of inhaled IgG, as well its impact upon intrapulmonary immune responses or the dose that would be protective against the development of *R. equi* pneumonia. It is possible that an alternate sample would be necessary, such as biopsy of lung tissue with special staining for the presence of IgG. However, a rational next step in this research would be to collect alveolar macrophages, and co-incubate them with the *R. equi* specific IgG product. These cells could then be exposed to virulent *R. equi* and it could be determined if this pre-exposure aided in phagocytosis and clearance of the bacterium.

Conclusion

Administration of *R. equi* specific IgG to the lungs of ponies via aerosolization was uncomplicated and well tolerated. It proved to be of benefit in for aiding phagocytosis

and clearance of *R. equi* it could be an appropriate, easily administered alternative to intravenous hyperimmune plasma. However, it would be necessary to determine the optimal dose of *R. equi* IgG to be delivered locally. Based upon the Sartwell's model it would be necessary that it be administered to foals in the first few days of life. Potentially, multiple administrations could be necessary as is currently the case with hyperimmune plasma given intravenously. A potential disadvantage of its administration could be if a foal experienced a local anaphylactic response to the product. No evidence of such a response was seen with administration to the adult ponies, however with the overall high incidence of transfusion reactions seen to intravenously administered plasma, it could be possible that a reaction to the aerosolized protein product could prove catastrophic. Due to the nature of the bacterium it is unlikely that a single prophylactic treatment will be universally effective in its prevention. As such, it is imperative that continued research into multimodal approaches at prophylaxis occur to enable veterinarians to prevent this devastating disease.

Footnotes:

- a. Frontline® spray Merial, LTD. Duluth, GA, USA
- b. Absorbine Ultrashield®, W.F Young, Inc, Long Meadow, MA, USA
- c. Pneumomune-Re, Lake Immunogenicis, Ontario, NY, USA
- d. ATKA Explorer, G.E. Healthcare, Uppsalla, Sweden
- e. Sepharose resin, G.E. Healthcare, Uppsalla, Sweden
- f. EMD Millipore corporation, Billuria, MA, USA
- g. Millex®, GVPVDF Filter Unit, Millipore, Carrigtwohill, County Cork, Ireland
- h. Baxter, Deerfield, IL, USA

- i. Flexineb™, Jiffy Steamer, Equine Division, Union City, TN, USA
- j. Dormeosedan®, Intervet/Schering-Plough, Summit, NJ, USA
- k. Torbugesic®, Zoetis, Kalamazoo, MI, USA
- l. ProAir®HFA, Teva Respiratory LLC, Waterford, Ireland
- m. Anased®, Lloyd Inc, Shenandoah, IA, USA
- n. Model VQ- 8303A, Olympus Medical Systems Corporation, Tokyo, Japan
- o. Carbocaine®-V, Pfizer, New York, NY, USA
- p. Cytopro® Model 7620, Wescor, Inc, Logan, UT, USA
- q. Diff Quick®, Allan Scientific, Kalamazoo, MI, USA
- r. Immunotek, Zeptometrix Corporation, Buffalo, NY, USA
- s. Bio-Rad Model 1575 ImmunoWash, Bio-Rad Laboratories, Hercules, CA, USA
- t. Hidex Chameleon®, Hidex corporation, Turku, Finland
- u. Excel®, Microsoft corporation, Redmond, WA, USA
- v. Thermo Fisher, Waltham, MD, USA
- w. PBST, Sigma-aldrich, St. Louis, MO, USA
- x. Bethyl, Montgomery, TX, USA
- y. One-step Pnpp, Pierce, Rockford, IL, USA

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