PURIFICATION AND QUANTITATIVE DESCRIPTION OF
RHODOCOCCUS EQUI IgG DESIGNED FOR AEROSOL
NEBULIZATION TO FOALS

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Master of Science
In Biomedical and Veterinary Sciences

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The objective of this study was to purify IgG from commercially available hyperimmune *Rhodococcus equi* plasma and to assess the delivery of IgG as an aerosol to the equine lung. IgG was purified from plasma, and the IgG concentration of both the plasma and the purified IgG was determined by ELISA. The purified IgG was aerosolized using a vibrating mesh nebulizer and aerosol characterization was performed using cascade impaction. The purified IgG was nebulized to six healthy adult horses in order to assess the efficacy of pulmonary delivery and safety of administration. Bronchoalveolar fluid was retrieved endoscopically using a low volume technique prior to aerosolization (time 0) and at 0.5, 4 and 24 hours post aerosolization. The BAL fluid IgG concentration was determined and cytologic analysis was performed.

The IgG concentrations of the plasma and purified IgG were 2,175 mg/dL and 1,145 mg/dL, respectively. The MMAD of the purified IgG aerosol was 4.7 microns. The mean BAL fluid IgG concentration increased 61% from 19.33 µg/dL at time 0 to 31.5 µg/dL at 0.5 hours, but this increase
was not significant (P=0.603). No significant change was observed in inflammatory cell numbers over time or at any time point during the study. This study demonstrated that IgG antibodies were purified at a concentration acceptable for nebulization, and that the nebulization unit generated aerosol particles from the IgG solution of appropriate size for pulmonary delivery. Nebulization of purified IgG to adult horses was well tolerated and caused no local or systemic adverse effects.
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<tr>
<td>α</td>
<td>alpha</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>C</td>
<td>centigrade</td>
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<tr>
<td>CD4+</td>
<td>cluster of differentiation 4 T-lymphocyte</td>
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<td>CD8+</td>
<td>cluster of differentiation 8 T-lymphocyte</td>
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<td>CV</td>
<td>coefficient of variation</td>
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<td>deciliter</td>
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<td>deoxyribonuclease 1</td>
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<td>DPI</td>
<td>dry powder inhaler</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>Ga</td>
<td>gallium</td>
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<td>Fe^{3+}</td>
<td>ferric iron</td>
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<td>HIP</td>
<td>hyperimmune plasma</td>
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<td>IFN-γ</td>
<td>interferon gamma</td>
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<td>MDI</td>
<td>metered dose inhaler</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MIC_{50}</td>
<td>minimum inhibitory concentration 50%</td>
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<tr>
<td>MIC_{90}</td>
<td>minimum inhibitory concentration 90%</td>
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<td>milliliter</td>
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MMAD  mass median aerodynamic diameter
NaCl  sodium chloride
ng    nanogram
nm    nanometer
PBS   phosphate buffered saline
PELF  pulmonary epithelial lining fluid
Th1   T-lymphocyte helper cell subset 1
Th2   T-lymphocyte helper cell subset 2
µg    microgram
µm    micrometer
vapA  virulence associated protein A
Chapter 1. *Rhodococcus equi* pathophysiology

**Microbiology of *Rhodococcus equi***

*Rhodococcus equi* is a gram-positive, facultative intracellular bacterium.\(^1\)-\(^4\) It is an aerobic, non-hemolytic, non-motile, non-sporulating, catalase positive, encapsulated coccobacillus that is weakly acid-fast and appears with a red pigmentation in its natural form.\(^1\),\(^2\),\(^5\),\(^6\) It is a member of the phylogenetic group nocardioform actinomycetes which includes *Mycobacterium*, *Corynebacterium* and *Nocardia* among other genera of bacteria.\(^1\),\(^3\),\(^7\),\(^8\) *Rhodococcus equi* has a near-worldwide distribution, appearing on six of seven continents.\(^9\) The organism is abundantly distributed in the soil and has been isolated from both freshwater and marine habitats.\(^2\),\(^10\),\(^11\)

Characteristically, the bacterium is capable of surviving within alveolar macrophages and does so by prevention of phagosome-lysosome fusion and acidification of the phagosome.\(^8\),\(^12\),\(^13\) Once established within the macrophage, the bacterium is able to proliferate intracellularly and this can result in a fivefold increase in its population within 48 hours.\(^13\) Proliferation of the bacterium ultimately leads to necrosis and death of the infected macrophage and release of viable organisms into the surrounding tissue. The release of inflammatory mediators and recruitment of inflammatory cells follow, which in turn causes
massive destruction to the surrounding pulmonary tissue with attendant formation of cavitary abscesses and granulomas.\textsuperscript{5}

**Pathogenicity of *Rhodococcus equi***

The pathogenicity of *Rhodococcus equi* has been attributed to the presence of virulence associated plasmids.\textsuperscript{5,14,15} A total of twelve equine virulence plasmids have been identified and range between 80 – 90 kb in size.\textsuperscript{5,7,11} A 27.5 kb pathogenicity island has been identified within these plasmids and contains a family of nine virulence associated protein (\textit{vap}) genes.\textsuperscript{5} Disease is primarily mediated through virulence associated plasmid A (\textit{vapA}) which is a 15 – 17 kDa protein encoded within the pathogenicity island.\textsuperscript{15-17} Multiple studies have shown that removal of the \textit{vapA} protein from *Rhodococcus equi* strains results in the bacteria losing the ability to survive and replicate within alveolar macrophages.\textsuperscript{7,13,14}

The bacterium has been shown to colonize the intestine of foals beginning within the first week of life.\textsuperscript{18,19} Bacterial multiplication occurs through the first eight weeks following exposure and colonization of the pulmonary and gastrointestinal tracts, ceasing roughly by 12 weeks of age.\textsuperscript{18,20} Bacterial multiplication within exposed foals typically occurs within alveolar macrophages and macrophages residing within the lamina propria of the intestine. Thus, *Rhodococcus equi* is ingested by horses of any age, passed through the
gastrointestinal tract and shed into the surrounding environment by fecal elimination.\textsuperscript{2,18,21} Establishment of the bacterium within the environment serves to increase herd exposure, leading to bacterial population within individuals and continued distribution throughout the local habitat.\textsuperscript{16} This cycle has the potential to continue in perpetuity. \textit{Rhodococcus equi} grows more readily in soils enriched with horse manure than those without suggesting that the concentration of the organism within the equine environment is amplified over time.\textsuperscript{10,20} The presence of the volatile fatty acids acetate and propionate within horse manure supports growth of the organism.\textsuperscript{20,21} The overall incidence of \textit{Rhodococcus equi} recovered from horse manure has been reported to be between 40.4\% and 70.9\%.\textsuperscript{10,22} The recovery of \textit{Rhodococcus equi} from soil (14.9\%) and cobweb (6.1\%) samples, comparatively, was much lower.\textsuperscript{10} Interestingly, hospitalized horses have been shown to have a significantly higher incidence (80.9\%) of \textit{Rhodococcus equi} recovered from their manure as compared to \textit{Rhodococcus equi} recovered in manure from horses on the farm (31.2\%).\textsuperscript{10}

The prevalence and severity of rhodococcal disease varies between individual foals and is influenced by local farm and environmental conditions.\textsuperscript{5,7,16,23-25} Multiple studies have investigated specific links between the development of rhodococcal pneumonia in foals and equine breeding farm characteristics, farm management, preventative health care practices and foal-
related variables. Foals residing on farms larger than 200 acres in size were 18 times more likely to be affected by *Rhodococcus equi*. Additionally, foals residing on those farms with ten or more resident dam-foal pairs were nearly three times more likely to be affected with rhodococcal pneumonia. In the same study, other factors that showed significance in the bivariate analyses, but not the final multivariate analyses, included the presence of more than 160 horses on the farm, more than 17 foals present on the farm, the stocking density of the foals (>0.25 foals/acre) and the presence of transient mares on the property. Foals unaffected by *Rhodococcus equi* pneumonia were found to be significantly more likely to have been housed in paddocks or pastures in small groups (≤5 mare-foal pairs) compared with larger groups (>5 mare-foal pairs) that were affected by rhodococcal pneumonia. This finding supported the suggestion that an increased density of mare-foal pairs on any given paddock or pasture area was a risk factor for developing pulmonary infection from *Rhodococcus equi*. Farms with an elevated airborne concentration of virulent *Rhodococcus equi* have also been reported to have an increased prevalence of *Rhodococcus equi* associated disease. Additional conditions commonly identified as farm risk factors include high environmental temperature, low environmental humidity, increased dust concentration, sparse grass covering and neutral to alkaline soil with poor moisture.
Due to its ubiquity within the equine environment, it is thought that virtually all foals are exposed to *Rhodococcus equi* within the first few weeks of life.\textsuperscript{4,32,33} While not all foals present within the same environment or similar environmental conditions develop rhodococcal infections, those that do appear to become infected via bacterial inhalation or ingestion within days following birth.\textsuperscript{12,32} Combined environmental exposure and a poor immune response may lead to active infection in susceptible animals.\textsuperscript{34} Morbidity rates among foals from birth through six months are reported to be between 5-17%.\textsuperscript{35,36} The pneumonic form of the disease occurs chiefly from the inhalational route with exposure occurring between one to six months of age. Disease manifestation most commonly is reported between two to four months of age.\textsuperscript{11,16,32} The time between exposure, infection and the development of clinical signs may be delayed for several weeks.\textsuperscript{11,12} Fully immunocompetent adult horses are capable of mounting effective immunological responses to challenges with *Rhodococcus equi*.\textsuperscript{4} Therefore, those foals that do not develop rhodococcal infections within the first six months and survive to adulthood with an intact immune system appear to acquire lifelong immunity against the bacterium.\textsuperscript{23}

**Host factors**

Development of disease in affected foals is regulated not only by the virulence of the organism but also by the susceptibility of the foal.\textsuperscript{37} Equids
naturally establish an epitheliochorial placentation during gestation; therefore, all protective antibodies must be obtained through ingestion of colostrum from their dam. The foal relies upon this passive transfer of immunity for its defense against a host of infectious agents over the course of several weeks until its own immune system has developed to a full capacity.\textsuperscript{38} It has been observed that development of rhodococcal disease coincides at the time when these maternal antibodies have declined, usually around five to ten weeks after birth.\textsuperscript{11,39} Factors such as poor colostral production by the mare, inadequate intake by the foal or poor intestinal absorption may limit the total antibody available to respond to a pathogenic challenge.\textsuperscript{40,41} These limiting factors may arise from disease or debilitation in either or both the mare and foal. The incidence of partial or complete failure of passive transfer has been reported to be between 9.6 – 18\%.\textsuperscript{40,42-45} Traditionally, foal susceptibility to \textit{Rhodococcus equi} has been thought to relate to concurrent increase in aerosol concentration of virulent \textit{Rhodococcus equi} during the period of decreased protective antibody levels in the foal.\textsuperscript{11} Recent evidence has shown that infection and subsequent death of foals due to \textit{Rhodococcus equi} pneumonia follows a lognormal distribution.\textsuperscript{19} As described by Sartwell’s statistical model, the onset of disease among individuals follows a predictable pattern of distribution that originates from a point source infection. Development of disease as described by this model allows an accurate estimation of the time of the initial infection
based upon the normal distribution of the observed disease among individuals within a group. Results from the Horowitz study supported that the original time of infection from *Rhodococcus equi* occurred within the first week of life based upon the observation of the median age of death at 65 days following birth among all 107 affected foals and the log-normal nature of the distribution.\textsuperscript{19}

The principle immunoglobulin produced in colostrum is IgG, which also comprises 80\% of the total serum immunoglobulins.\textsuperscript{37} While antibodies such as IgA and IgM play an active role in foal immunity, it is the IgG immunoglobulin that is reported to be most active in immune defense against commonly encountered pathogens, including respiratory pathogens such as *Rhodococcus equi*.\textsuperscript{46,47} Transfer of maternal antibodies and possibly other humoral components significantly improves the opsonic capacity and phagocytic activity of peripheral blood polymorphonuclear leukocytes.\textsuperscript{48-50} Peripheral blood polymorphonuclear leukocytes have been shown to exhibit reduced chemotactic and phagocytic activity prior to the ingestion of colostrum compared to phagocytic activity following colostral ingestion.\textsuperscript{48} *In vitro* opsonization and oxidative burst activity of peripheral blood leukocytes are also significantly improved when compared to the activity of leukocytes not in the presence of colostral antibodies.\textsuperscript{51} Most importantly, opsonizing IgG antibodies have been shown to enhance alveolar macrophage phagocytosis and destruction of *Rhodococcus equi* by improved
phagosome-lysosome fusion.\textsuperscript{52-54} When challenged with virulent strains of \textit{Rhodococcus equi}, the production of IgG antibodies was greatly increased and this increase was associated with clearance of the bacteria in adult horses.\textsuperscript{55}

Maternal immunoglobulin concentrations begin to decline in correspondence with an increase in endogenous immunoglobulin production in the foal, usually at three to four weeks following birth.\textsuperscript{56-58} Evidence shows that with successful passive transfer of immunity combined with the foal’s innate production of immunoglobulin, the humoral immune system is fully established by three months of age with the serum concentration of IgG antibody comparable to that of adults.\textsuperscript{38,59} However, a potential period of susceptibility exists during this period of immune constitution between the decline of maternal antibodies and the establishment of protective endogenous immunoglobulin concentrations.\textsuperscript{60} In healthy foals, the half-life of colostral IgG is approximately 28 days.\textsuperscript{11,38} Foals with evidence of sepsis, partial or complete failure of passive transfer may be more at risk for development of rhodococcal pneumonia.\textsuperscript{61} Additionally, the function of opsonization factors (i.e. complement, fibronectin, etc.) may be dependent upon a month long maturation period following birth. This can lead to decreased neutrophil activity during this time with subsequent potential for increased susceptibility to pathogens.\textsuperscript{54,61}
While transfer of immunity is vital to establish the foal’s immune defense, interdependency exists between the foal’s humoral and cell-mediated immune responses. Healthy foals are born with a full complement of functional leukocytes; however, the total number available to participate in immune defense is limited. The finite number of leukocytes available to participate in host defense may be quickly overwhelmed from a robust pathogen challenge. Despite their reduced population, cellular expansion in healthy foals occurs steadily. Significant increase in the total number of leukocytes, CD4+, CD8+ T-lymphocytes and MHC Class II molecules within peripheral blood and bronchoalveolar lavage fluid is observed between one to four months of age in healthy foals. The increase in total leukocyte number coincides with a period of increased environmental pathogen challenge. Multiple studies in mice have confirmed that CD8+ and especially CD4+ T-lymphocytes play crucial roles in eliminating *Rhodococcus equi* from the lungs. Similar findings have been described in adult horses when challenged intrabronchially with *Rhodococcus equi*. Additional research has demonstrated that healthy, two to four week old foals with a CD4+ to CD8+ T-lymphocyte ratio of less than three were significantly more likely to develop rhodococcal pneumonia (OR = 7.14; 95% CI 1.96-14.3). While impractical as a diagnostic methodology, this finding strongly
suggests that the CD4+ T-lymphocyte’s role is paramount to the host’s successful immune defense against a challenge from *Rhodococcus equi*.

Susceptibility of foals to *Rhodococcus equi* may result from an inappropriate Th2 lymphocyte response. It has been recognized that the Th1, cell-mediated immune response results in macrophage activation and is generally desired to resist rhodococcal infections; however, foals exhibit a bias towards a Th2 response.\(^{37,60,69}\) This occurs in large part from the activity of cytokines such as IL-4, IL-5 and IL-10 produced by feto-placental tissues which favor the Th2 humoral response and inhibits the Th1 response.\(^{60,70,71}\) Secondary placental factors, such as prostaglandins and progesterone down regulate IL-12 and up regulate IL-10 production.\(^{72,73}\) These changes are a typical occurrence during gestation and are thought to arise as a protective mechanism to avoid immunological rejection of the fetus.\(^{37}\) However, within the first month of life, an increase in the Th1 promoting cytokines, interferon-\(\gamma\), IL-1\(\alpha\) and transforming growth factor-\(\beta1\) is observed and may indicate a maturation process of the foal’s immune system.\(^{69}\) Thus, cytokine influence over naïve helper T-lymphocytes plays a predominant role in cellular differentiation into either Th1 or Th2 subsets.\(^{69}\) Virulent strains of *Rhodococcus equi* have been shown to alter host cytokine expression to favor its survival. Due to the intracellular presence of *Rhodococcus equi* within macrophages, interferon-\(\gamma\) is needed to eliminate the bacterium.\(^{74}\) Interferon-\(\gamma\) mRNA expression by CD4+
T-lymphocytes is down regulated by *Rhodococcus equi* while pulmonary IL-10 is increased, which furthers the influence of the biased Th2 response.\textsuperscript{75}

**Management of *Rhodococcus equi* infections**

Resolution of clinical disease has relied heavily on antimicrobial therapy, typically using a combination of rifampin with a macrolide antibiotic. Reported survival rates for foals treated with erythromycin and rifampin are as high as 88\%.\textsuperscript{76} Unfortunately, erythromycin has variable absorption in the foal, requires frequent oral dosing and carries a risk of potentially fatal side effects including diarrhea and hyperthermia. Fatal colitis has also been reported in mares accompanying foals being treated with erythromycin.\textsuperscript{77-79} Alternatives to erythromycin have been developed; the most commonly used include azithromycin and clarithromycin. Azithromycin has moderate bioavailability and a large volume of distribution within horses.\textsuperscript{80,81} Most importantly, it has excellent uptake within pulmonary macrophages and pulmonary epithelial lining fluid.\textsuperscript{80,81} Further advantages to azithromycin include once daily oral dosing and an improved safety profile with minimal side effects.\textsuperscript{80,81} Azithromycin exceeds the 90\% minimum inhibitory concentration (MIC\textsubscript{90}) for *Rhodococcus equi* and remains at high concentrations within pulmonary cells for 48 hours.\textsuperscript{81,82} Clarithromycin quickly reaches peak serum concentration and has a bioavailability similar to azithromycin.\textsuperscript{83,84} Clarithromycin concentration within bronchoalveolar cells and
pulmonary epithelial lining fluid is superior to azithromycin, but its duration is reported to be only 12 hours.\textsuperscript{82,84} This concentration is adequate to achieve the MIC\textsubscript{90} for \textit{Rhodococcus equi} isolates and maintains a very good safety profile.\textsuperscript{84} Clarithromycin has been shown to be more effective against \textit{Rhodococcus equi} \textit{in vitro} than either erythromycin or azithromycin.\textsuperscript{85} When combined with rifampin, clarithromycin was shown to be more effective \textit{in vivo} for the treatment of rhodococcal pneumonia in foals compared to azithromycin or erythromycin combined with rifampin.\textsuperscript{86}

Telithromycin, a newer generation of macrolide has been reported to have good peak serum activity and concentration within bronchoalveolar lavage cells and pulmonary epithelial lining fluid.\textsuperscript{87} However, the terminal serum half-life was much shorter (4 hours) compared to azithromycin and clarithromycin.\textsuperscript{87} The reported MIC\textsubscript{90} of telithromycin for macrolide-susceptible \textit{Rhodococcus equi} strains is significantly lower than the MIC\textsubscript{90} for macrolide-resistant strains. Yet, the telithromycin MIC\textsubscript{50} for macrolide resistant strains was significantly lower compared to clarithromycin, azithromycin and erythromycin.\textsuperscript{87} More research and development on new macrolide antimicrobials is needed. Until such a time, treatment will rely upon the continued use of either azithromycin or clarithromycin combined with rifampin.
Unfortunately, there is mounting evidence that supports an increased development of antimicrobial resistance to macrolide antimicrobials amongst *Rhodococcus equi* isolates.\(^8^8-^9^2\) One study showed that 63\% of macrolide-resistant *Rhodococcus equi* isolates were resistant to multiple macrolide antimicrobials.\(^8^8\) Overall prevalence of antimicrobial-resistant *Rhodococcus equi* isolates submitted to nine veterinary diagnostic laboratories was 3.7\%.\(^8^8\) While that number may appear low, this finding is concerning as few classes of antimicrobials other than the macrolides are clinically useful in the treatment of rhodococcal infections. This is due to the fact that while many antimicrobial drugs have been shown to be effective *in vitro*, most show poor results *in vivo*.\(^8^5,^9^3,^9^4\) Antimicrobial treatment for *Rhodococcus equi* in foals can last as long as eight weeks. This prolonged treatment has the potential for development of antimicrobial resistance, adverse antimicrobial reactions, can be cost prohibitive for clients and carries no guarantee for resolution of the foal’s clinical condition.\(^2^4,^3^1,^7^6,^9^3,^9^5-^9^7\)

**Prevention of *Rhodococcus equi* infections**

Measures preventing the establishment of rhodococcal infections within foal populations are preferred over treatment of clinical infections. Several methods of prevention have been employed, the most complex and difficult of which is altering the environment to minimize exposure, thereby preventing the establishment of infection. These preventive means include regular removal of
manure, decreasing stocking density of foals, judicious pasture management, promotion of grass growth and covering, limiting excessive foal exposure to dust and ensuring adequate colostral intake by the foal.$^{11,29}$ While these efforts to limit foal exposure to *Rhodococcus equi* have resulted in decreased morbidity, they can never eliminate infections or reduce their severity.

**Immunization**

Vaccination represents the best means to increase systemic antibody levels against *Rhodococcus equi* antigens, specifically the *vapA* protein. Vaccination strategies against *Rhodococcus equi* have focused on immunization of either or both the pregnant mare and the young foal. Immunization of the mare within the last two months of gestation has been employed to increase *Rhodococcus equi*-specific colostral IgG antibody available to the foal at birth. One study showed that 86% of foals born to vaccinated mares were seropositive for *Rhodococcus equi*-specific antibodies after passive immunoglobulin transfer.$^{98}$ It was noted that foal mortality within the vaccine study population decreased by half after the vaccination program was implemented.$^{98}$ However, several mares had a poor immunological response to vaccination and failed to produce *Rhodococcus equi*-specific antibody in their colostrum.$^{98}$ In the same study, a group of foals received both colostral immunoglobulin transfer from immunized mares and direct immunization of the *Rhodococcus equi* vaccine. Foals in this group showed a
positive response to immunization, but a reduction in their mortality due to \textit{Rhodococcus equi} was not achieved.\textsuperscript{98} A similar study was performed using a \textit{Rhodococcus equi vapA} vaccine in mares. Results showed elevated titers specific for \textit{Rhodococcus equi vapA} protein in vaccinated mares as compared to unvaccinated mares.\textsuperscript{99} Nevertheless, a combination of passively acquired immunity and direct immunization with the \textit{Rhodococcus equi vapA} vaccine failed to provide protective immunity against natural \textit{Rhodococcus equi} exposure in their foals, with half of those foals succumbing to the resultant disease.\textsuperscript{99} Multiple studies have documented similar results of adequate systemic and pulmonary immune response in mares and foals to \textit{Rhodococcus equi} vaccination, but failure of the foal to gain protective immunity after direct challenge with \textit{Rhodococcus equi}.\textsuperscript{100-102}

A limitation of the \textit{Rhodococcus equi} vaccination in foals is the lack of a robust response to immunization. This common observation is thought to stem from maternal antibody interference producing an inadequate immune response to vaccination from the foal’s immune system.\textsuperscript{47,60,103,104} In addition, humoral and lymphoproliferative immune responses to a killed adjuvanted vaccine have been shown to be dampened in foals as compared to adults.\textsuperscript{105} The immune response to vaccination appears to be age-related as three month old foals show improved
responses when compared with three day old foals; however, both have attenuated immune responses when compared with adults.\textsuperscript{105}

An alternative vaccination method is an oral vaccine consisting of virulent strains of \textit{Rhodococcus equi}. When given to foals, protective effects have been achieved as early as three weeks of age.\textsuperscript{106,107} When challenged with \textit{Rhodococcus equi}, foals have survived and developed minimal pulmonary effects while non-immunized foals have succumbed to rhodococcal pneumonia and developed characteristic pathological changes.\textsuperscript{106,107} Post-mortem changes were described as severe in the non-immunized foals, but were limited in vaccinated foals with little to no appreciable gross or microscopic lesions.\textsuperscript{106,107} While these results are encouraging, the use of virulent \textit{Rhodococcus equi} oral vaccines is imprudent as it would lead to wide spread dissemination and contamination with virulent organisms throughout the environment.

\textbf{Passive immunization}

To date, the most successful preventative method has been the prophylactic administration of \textit{Rhodococcus equi}-specific hyperimmune plasma. This plasma is collected from adult donor horses previously immunized against \textit{Rhodococcus equi}. Thus, the collected donor plasma given to susceptible foals has an elevated concentration of \textit{Rhodococcus equi}-specific antibodies and potentially other immune modulators. This immunoprophylactic method is predicated upon the
premise that this excess antibody concentration will provide an increased level and duration of protection to the foal thereby preventing establishment of the infection in the weeks following birth until the foal’s immunity is fully constituted. Traditionally, hyperimmune plasma has been given within the first few days following birth, although more recently, many farms are repeating administration between 30 to 60 days later. The success of this strategy relies heavily upon the volume and antibody concentration given, the timing of administration, the concentration of virulent bacteria in the environment, farm management factors and the foal’s immunocompetence. Conflicting reports regarding the efficacy of *Rhodococcus equi* hyperimmune plasma exist, with several studies providing evidence supporting a reduction of morbidity and mortality while other studies found no benefit.\textsuperscript{98,101,108-114} Despite the division within the evidence, the use of *Rhodococcus equi* hyperimmune plasma remains a common standard of practice within the equine industry, especially on *Rhodococcus equi* endemic farms. Potential disadvantages include the expense of treatment, the degree of labor involved, treatment failures and possible transfusion reactions.\textsuperscript{101,108,110,112,115} The incidence of reactions to plasma transfusion in one study was reported to be 10%, with none of the cases being fatal.\textsuperscript{116} In the five horses in which plasma transfusion reactions occurred, two were reported to have developed tachycardia
and pyrexia and one case each of urticaria, tachypnea and severe pruritus with swollen eyes.\textsuperscript{116}

**Chemoprophylaxis**

More recent research studies have examined novel and diverse methods specifically focused on chemoprophylactic interventions for reducing the development of *Rhodococcus equi* pneumonia in foals. The use of the macrolide antimicrobial azithromycin was examined for its chemoprophylactic effect against *Rhodococcus equi* pneumonia on endemically infected equine breeding farms.\textsuperscript{33,115} Azithromycin (10 mg/kg) given orally once every 48 hours in the first two weeks after birth for a total of seven treatments to a group of 170 foals resulted in a decreased overall incidence of *Rhodococcus equi* pneumonia (5.3\%) as compared to 168 control foals (20.8\%).\textsuperscript{33} Control foals were nearly five times more likely to have clinical evidence of pneumonia attributed to *Rhodococcus equi* when compared with azithromycin treated foals.\textsuperscript{33}

A second study examined the use of azithromycin (10 mg/kg) given orally once daily for 28 days to a group of 25 foals.\textsuperscript{115} Comparing one treatment group with two control groups, morbidity from pulmonary abscesses secondary to *Rhodococcus equi* were comparable between groups (60\% in the azithromycin treated group, 68\% in the first control group and 70\% in the second control group).\textsuperscript{115} The results raise doubt that the application of azithromycin to young
foals in this manner was protective for preventing the development of *Rhodococcus equi* pneumonia. Adverse effects generated by the prophylactic administration of azithromycin were not observed in either study.\textsuperscript{33,115}

The primary concern related to this chemoprophylactic strategy is the development of bacterial resistance of both target and nontarget bacteria.\textsuperscript{33} Additionally, the documented prevalence of macrolide resistance to isolates of *Rhodococcus equi* from affected foals has been reported to be nearly 4%.\textsuperscript{88} With evidence of macrolide resistance already established, the consequences of further development of resistance to this class of anti-microbials has rendered their prophylactic use impractical and unwise.

Gallium maltolate is an antimicrobial formulation of the trivalent, semi-metal gallium (Ga). Gallium is chemically similar to ferric iron (Fe\textsuperscript{3+}) which has been described as crucial for survival and replication of most bacterial pathogens, including *Rhodococcus equi*.\textsuperscript{117} Like ferric iron, gallium binds to plasma transferrin and is readily taken up by bacteria. However, instead of being used for the purpose of bacterial DNA synthesis and replication, gallium inactivates bacterial enzymes, such as ribonucleotide reductase. Without these necessary enzymes, interference with the normal bacterial replication process occurs and bacterial cell death ensues.\textsuperscript{118} Gallium has a particular affinity for concentrating at sites of infection and inflammation, especially within macrophages.\textsuperscript{119-121} *In vitro*
studies of gallium have shown that it suppresses the growth of *Rhodococcus equi*, likely by interfering with iron uptake and utilization.\textsuperscript{117} Both macrolide susceptible and macrolide resistant strains of *Rhodococcus equi* were similarly susceptible to gallium at concentrations of 558 ng/ml at 24 hours and 2,230 ng/ml at 48 hours after *in vitro* examination.\textsuperscript{122} Gallium maltolate has been shown to have excellent bioavailability following oral administration in a host of species, including foals.\textsuperscript{123-125} Orally administered gallium maltolate is capable of achieving serum concentrations that would inhibit growth and destroy *Rhodococcus equi* at a dose of 40 mg/kg.\textsuperscript{125} Unfortunately, clinical evidence does not support the beneficial effect of chemoprophylactic gallium maltolate as prevention against *Rhodococcus equi* pneumonia. When supplied at 30 mg/kg orally within the first two weeks of life to foals at an endemic breeding farm, no reduction in the incidence of rhodococcal pneumonia infections were observed between treated and control groups of foals.\textsuperscript{126}

**Immune stimulation**

Immunostimulants serve to enhance either or both the innate or adaptive arms of the immune system. The use of these products is thought to increase IFN-\(\gamma\) production, leading to activation of neutrophils and macrophages necessary to prevent the establishment of *Rhodococcus equi* within neonatal foals. Activation of the neonatal immune system using two commercially available
immunostimulants has been recently described with good effect in foals challenged with exogenous *Rhodococcus equi*. Treatment in a group of foals with *Propionibacterium acnes* within the first eight days following birth resulted in significantly reduced intracellular proliferation of *Rhodococcus equi* within macrophages 12 days after birth. The same study reported similar findings in a group of foals treated with the immunostimulant parapoxvirus ovis within eight days of birth. In this group, peripheral blood neutrophils had a significantly greater ability to phagocytize opsonized *Rhodococcus equi* and an improved oxidative burst response was observed 12 and 24 days following birth when compared to the day of birth. Adverse effects from administration of either immunostimulant were not observed. While promising as a chemoprophylactic strategy, no large scale prospective clinical trial has been performed to examine the use of immunostimulants against endemic *Rhodococcus equi* pneumonia in foals.

**Aerosol passive immunization**

The ideal prophylaxis strategy would completely avoid or limit any deleterious effects to the patient, be inexpensive, easily administered and act locally within the lungs for a reasonable period of time in order to deny the establishment of *Rhodococcus equi*. Systemically administered pharmaceutics may have poor bioavailability, a wide distribution, poor penetrance into the target organ or poor concentration within the target organ or a combination of these.
An archetypal method of treating established pulmonary disease is the administration of pharmaceutics via aerosol nebulization. Reports from a multitude of species, including horses, have shown success in treating a variety of conditions such as pneumonia, lower airway inflammatory disease and neoplasia with aerosolized drug delivered via nebulization.\textsuperscript{128-136} The noted advantages of aerosol therapy include the ease of administration, high concentration within the lower respiratory tract, rapid onset of action, decreased volume of the systemically administered dose and avoidance of potential adverse systemic effects.\textsuperscript{137}

The effectiveness of the aerosol administered to patients is dependent upon the characteristics of the substance being delivered, the characteristics of the individual aerosol particles, the collective properties of the aerosol itself and respiratory characteristics of the patient.\textsuperscript{136,137} The pattern of distribution of aerosols through the respiratory tract is dependent upon the size of the individual particles themselves. As a condition of their mass, larger particles are distributed almost exclusively within the upper respiratory tract and therefore, the ideal particle size for distribution within the bronchial tree lies between 0.2 – 5 µm. Aerosols generated from commercially available nebulizers are not monodispersed, but instead contain particles of various sizes. The mass median aerodynamic diameter (MMAD) describes the average particle size of these heterodispersed
aerosols and is widely accepted as a representative value relating the likely region of deposition of aerosols within the lungs.

Two primary physical properties act upon the aerosol as a whole and influence the deposition of individual particles within the airways.\textsuperscript{138} The first is inertial impaction which primarily influences large particles and deposits them chiefly within the upper and central airways. The second physical property of aerosols is sedimentation which influences smaller particles through gravity. These small particles are deposited into deeper airways. Finally, physiological properties of the patient’s respiratory system such as tidal flow volume, minute volume and the presence of respiratory disease influence the particle distribution to the lower airway.\textsuperscript{136} The horse’s unique respiratory system including its large tidal volume, high flow rate and expansive pulmonary surface area make it the ideal model to examine the effects of a variety of nebulized solutions.\textsuperscript{136}

Many aerosol delivery devices have been described in the medical literature. Among these are jet nebulizers, metered dose inhalers (MDIs), dry powder inhalers (DPIs), ultrasonic nebulizers and vibrating mesh nebulizers.\textsuperscript{137,139} As expected, each device has advantages and disadvantages compared with the others and may be more or less appropriate for therapy depending upon the patient’s specific condition or the characteristics of the drug being delivered. In the case of our equine patients, once any nebulizer has generated the aerosol, it is conducted from
the unit to a tightly fitted facemask where it is inspired via the nares into the upper and lower airways.

Metered and dry powder inhalers deliver their drug through prefilled containers, with aerosol generated upon manual activation. In the case of the MDIs, the drug is suspended in a propellant. Dry powder inhalers do not use a propellant, but instead rely upon the patient actively inspiring the drug in order to actuate its release from a capsule. Both MDIs and DPIs have been used in the treatment of equine respiratory disease, primarily for the treatment of lower airway inflammation.\textsuperscript{132} Disadvantages of MDIs include the release of environmental contaminants, the imprecise delivery of the drug and the small volume of drug delivered to the lower airway. Disadvantages of the DPIs include the reliability of the patient generating an adequate tidal volume to inspire and deliver the drug to the lower airways and the small volume of drug delivered to the lower airway.

Jet nebulizers generate an aerosol from liquids using gas from either a compressor or compressed gas.\textsuperscript{137} An advantage of this technology is that particle size can be adjusted based upon the rate of gas flow. This allows for generation of selected particle sizes within the desired MMAD range of 0.2 – 5 µm. The main disadvantages of using jet nebulizers in horses is the noise generated by the compressed gas which tends to startle the patients and the slow rate of drug delivery.\textsuperscript{137}
Ultrasonic nebulizers generate aerosol through the vibrations of a piezoelectric crystal driven by an alternating electric current. These nebulizers are capable of producing a highly concentrated aerosol and are relatively quiet. Their primary disadvantages are that they generate heat and may denature the solution intended for aerosolization. This is especially appreciated with proteinaceous solutions. These nebulizers are also expensive, which may be a limitation.

Vibrating mesh nebulizers represent the most recent aerosol generation technology (Fig. 1). These nebulizers aerosolize solutions utilizing either passive or active devices. Passive devices utilize a perforated plate attached to a piezoelectric crystal coupled to a transducer horn that induces “passive” vibrations resulting in extrusion of the solution through the perforations and generation of the aerosol. Active devices utilize a “micropump” which contains a domed aperture plate with laser perforated holes of specific diameter. An electric current is generated across the aperture plate causing it to expand in a vertical plane. The aerosol is generated by the action of the vibrating plate in contact with the solution. These nebulizers are quiet, efficient and do not generate heat and subsequent denaturation of the solution. These nebulizers are also compact and convenient to use, but cannot be repaired when they reach the end of their service.
Aerosolization of protein for the purposes of local treatment of the pulmonary tract via nebulization has been previously described in other species.\textsuperscript{129,141-146} Each report has either used or compared one or several of the nebulizer technologies available, all of which have shown success in achieving particle distribution of proteins to the lower airways. A variety of proteins including IgG1 for the treatment of neoplasia, recombinant human DNase I for the treatment of cystic fibrosis, interferon-\(\gamma\) in the treatment of IgE mediated lower airway sensitization, interleukin-5 delivery in the treatment of human asthma and the use of activated protein C in the treatment of acute respiratory distress syndrome have all been successfully used with intended effect and without evidence systemic or local adverse reactions.\textsuperscript{129,141-145}

**Figure 1: Vibrating mesh nebulizer**
Chapter 2. Purification and quantitative description of *Rhodococcus equi* IgG designed for aerosol nebulization to foals

**Introduction**

First isolated in 1923, *Rhodococcus equi* remains a major cause of disease and death in foals from one to six months of age.\(^{14,35,147}\) Published worldwide mortality rates from rhodococcal infections are estimated to average 3% annually, but have been historically reported to be as high as 80% in severely affected areas.\(^{16,148}\) Mortality rates at veterinary teaching hospitals vary widely, but are reported to be between 12% for foals receiving appropriate treatment to 58% of foals presenting without previous medical therapy.\(^{26,42,76,93,95,149}\) *Rhodococcus equi* primarily causes a subacute to chronic, suppurative to pyogranulomatous bronchopneumonia with severe pulmonary abscessation in many affected foals.\(^{7,11,39}\) Additionally, a host of extrapulmonary disorders including enteritis, uveitis and osteomyelitis have been reported.\(^{5,11,35}\) The first case of human rhodococcal infection was reported in 1967.\(^{150}\) While not as equally affected as foals, twelve cases of rhodococcal infections were reported in humans between the years 1967 and 1983.\(^9\) The incidence of rhodococcal infections in immunocompromised human patients has steadily increased since that time.\(^{2,5,7,9,11}\)

Pneumonia caused by *Rhodococcus equi* is widespread throughout a variety of habitats, can carry a high morbidity in foals and may lead to death in severe
cases. While successful, treatment may be expensive, prolonged and is not without hazards. Preventative therapy, specifically chemoprophylaxis is a promising method of limiting the severity or preventing the establishment of the *Rhodococcus equi* organism within the host. Despite its potential, many chemoprophylaxis studies have shown limited success. The purpose of this study was to purify IgG from commercially available hyperimmune *Rhodococcus equi* plasma and assess its delivery to the lungs.

**Materials and Methods**

**Antibody purification**

Antibody purification was performed to extract IgG by gravity flow of commercial *Rhodococcus equi* hyperimmune plasma through a commercial gel filtration system. Briefly, ten mls of a proprietary, 20% gel solution was placed in a 12.3 ml polypropylene column and allowed to settle into the gel form. Once settled, the solution created a four ml volume of gel. Twenty mls of purification buffer (pH 6.5, 1:100 concentration) was added to the column and allowed to filter through the gel-bed. A total of four mls of the *Rhodococcus equi* hyperimmune plasma was then filtered through each column. The four mls of plasma was followed by four mls of the purification buffer to fully extrude the purified IgG from the gel bed by gravity. Light absorbance was measured via spectrophotometer at 280 nm for each ml of filtered plasma from a randomly
selected filtration column. The absorbance for each ml of purified IgG was recorded and only that portion of purified IgG with an optical density ≥3.000 was collected. The purified IgG was stored in 2.5 ml aliquots and frozen at -20°C. At the end of each filtration cycle, the gel-bed was regenerated by first adding 20 ml of provided regenerant solution immediately followed by 20 ml of deionized water. Finally, 20 mls of purification buffer solution was allowed to filter through the gel-bed. The filter columns were regenerated no more than three times before being discarded.

**Aerosol characterization**

The particle size distribution of the aerosolized purified IgG was assessed by multistage cascade impaction. Ten milliliters of the purified IgG was placed into the reservoir of a vibrating mesh nebulization unit. The nebulization unit was connected to the cascade impactor by a 33 cm section of flexible, corrugated plastic tubing with a 32 mm internal diameter. The aerosol was conducted into the cascade impactor under negative pressure at 28.3 liters per minute (1 actual cubic foot per minute, ACFM) using a manufacturer calibrated vacuum pump. The time to aerosolize the ten mls of purified IgG was recorded. Upon completion of aerosolization, all eight stages were weighed separately using a calibrated analytical balance and the results were recorded. Each stage of the cascade impactor corresponds with a pre-determined particle size distribution (Fig. 2).
Mass median aerodynamic diameter (MMAD) for the aerosol was represented by the single stage representing the cutoff below which resided 50% of the total aerosol mass. This was derived from the difference in mass of the individual stages prior to aerosolization and the mass of the individual stages following nebulization. The proportion of particles capable of being inspired into the lower airways was defined as the respirable range, which consisted of particles with an aerosol diameter of \( \leq 5 \) microns. Between each testing cycle, the stages of the cascade impactor were rinsed with tap water, placed in a cleaning solution for ten minutes and then copiously rinsed again with tap water. The stages were hand dried and inspected for residual material prior to the next testing cycle. The reservoir and T-port adapter of the vibrating mesh nebulization unit was similarly cleaned to remove residual IgG extract that may have remained from the aerosol sampling procedure. In order to cleanse the nebulizer, ten mls of sterile saline was aerosolized over 30 minutes. The nebulization unit was then allowed to dry at room temperature between testing cycles, minimally 30 minutes.
Figure 2: Eight stage cascade impactor with specific Stage particle diameter capture size

A: 90° aerosol intake column
Stage 0: 9.0 – 10 µm
Stage 1: 5.8 – 9.0 µm
Stage 2: 4.7 – 5.8 µm
Stage 3: 3.3 – 4.7 µm
Stage 4: 2.1 – 3.3 µm
Stage 5: 1.1 – 2.1 µm
Stage 6: 0.65 – 1.1 µm
Stage 7: 0.43 – 0.65 µm
Animals

Six healthy adult horses (one gelding & five mares) between four and twenty-one years of age were used for the purposes of this study. Three horses belonged to the research herd of the Virginia Tech Marion duPont-Scott Equine Medical Center (VTMDSEMC) while the remaining three horses were privately owned and volunteered by their owners for the study upon their permission. Breeds of the horses included three Thoroughbreds, one Oldenburg, one Quarter Horse and one Thoroughbred-Connemara cross. Horses were housed in 12 x 12 box stalls bedded with pine shavings during the study period. Water and hay were available to the research subjects at all times with the exception being after sedation for the BAL procedure, during which times they were muzzled for approximately 30 minutes.

Each animal was confirmed to be free of historic, chronic or active respiratory disease based upon their clinical history and the absence of clinical signs such as nasal discharge, labored respiration or coughing. Each horse received a complete physical examination including a rebreathing examination prior to inclusion in the study. Whole blood was collected into sterile EDTA vacutainer tubes from each horse and a complete blood count was performed in order to assess for signs of acute or chronic inflammation. A complete physical examination was repeated during the study immediately prior to purified IgG
nebulization (Time 0) and at predetermined time points after nebulization (0.5 hours, 4 hours, 12 hours and 24 hours). The study protocol was approved by the Virginia Polytechnic and State University Animal Care and Use Committee.

**Aerosol administration of purified IgG**

Prior to nebulization, ten mls of purified IgG was thawed to room temperature, centrifuged at 1,250 x gravity and filtered through a sterile single use, 0.22 µm polyethersulfone filter in order to remove any particulate material or potential pathogens. The sterilized purified IgG was placed into the reservoir of a vibrating mesh nebulization unit. The aerosol was inhaled directly by each patient through a close-fitting, equine-specific face mask, connected to the nebulization unit with the same section of flexible, corrugated plastic tubing as previously described. A one way inhalational valve was positioned upstream of the nebulization unit in order to decrease environmental loss of the aerosol, as the inhalation valve integral to the facemask was not entirely effective at preventing backflow into the nebulizer device. The entire ten mls of purified IgG was aerosolized and the total nebulization time was recorded for each horse. The reservoir, T-port adapter, the flexible corrugated plastic tubing and the face mask were cleaned using tap water between uses. Ten mls of sterile saline was added to the nebulizer reservoir and was aerosolized over 30 minutes to cleanse the
nebulizer. The corrugated tubing, face mask and the nebulization unit were then allowed to air dry at room temperature for 30 minutes between horses.

**Bronchoalveolar lavage**

Bronchoalveolar lavage (BAL) was performed with a three meter-long, video endoscope² for all horses at predetermined time periods which included an initial lavage prior to nebulization (Time 0) and at 30 minutes (Time 0.5), four hours (Time 4) and 24 hours (Time 24) following nebulization. In order to assess uniform distribution of IgG within the lungs, lavage fluid was sampled from four specific sites at corresponding times: right caudoventral lung at Time 0, left caudoventral lung at Time 0.5, left caudodorsal lung at Time 4 and right caudodorsal lung at Time 24, as previously described elsewhere.¹³⁴,¹³⁵ A small volume lavage technique was used, as previously described.¹³⁴,¹³⁵ Horses were sedated with a combination of detomidine² (0.01 mg/kg, IV) and butorphanol¹⁴ (0.007 mg/kg, IV) prior to the procedure. Once sedated, a total volume of 20 mls of 2% mepivicaine⁵ was selectively infused to reduce bronchospasm as the endoscope was passed through the upper and lower airways. Once the bronchoscope was well seated within the bronchus, 30 mls of sterile saline (0.9% NaCl) was delivered into the specific airway via the endoscope. The saline was immediately followed by 30 mls of air to ensure complete saline infusion into the bronchus. The saline was then immediately aspirated using the 60 ml syringe that
was used for saline and air infusion, the lavage fluid was recovered and the sample submitted for cytological analysis. Ten mls of the lavage sample was centrifuged at 1,250 x g for ten minutes and the supernatant was divided into 2.5 milliliter aliquots which were stored at -20°C for future determination of the IgG concentration.

**Total IgG quantification**

Total IgG concentrations of the plasma product, purified IgG and bronchoalveolar lavage samples were determined by means of a commercial equine IgG enzyme linked immunosorbent assay (ELISA). Equine IgG standards were prepared by first adding 500 microliters of provided diluent reagent to five test tubes labeled for their respective concentrations (62.5 ng/ml, 31.25 ng/ml, 15.6 ng/ml, 7.8 ng/ml and 0 ng/ml). Diluent reagent was not added to the 125 ng/ml standard test tube. Next, 1,000 microliters of the provided horse IgG standard was added to the test tube labeled 125 ng/ml. Five-hundred microliters from this test tube was added to the test tube labeled 62.5 ng/ml. The test tube was vortexed and the process of adding 500 microliters and vortexing was repeated sequentially for the remainder of the standard concentration test tubes with the exception of the last test tube, labeled 0 ng/ml to which only diluent was added.

Based upon preliminary trials, the plasma product was prepared to a final dilution of 1:250,000 while the purified IgG solution was prepared to a final
dilution of 1:125,000 due to the lower concentration of IgG in the purified IgG solution. Dilution was performed as follows: One microliter of either plasma or purified IgG was combined with 500 microliters of diluent reagent in a test tube labeled Tube A to make a dilution of 1:500. Next, ten microliters from Tube A was combined with 90 microliters of diluent reagent in a test tube labeled Tube B to make a dilution of 1:5,000. Forty microliters from Tube B was added to 960 microliters of diluent reagent in a test tube labeled Tube C to make a dilution of 1:125,000. One further dilution was performed for the plasma product only: 500 microliters of the plasma sample from Tube C was combined with 500 microliters of the diluent reagent in a test tube labeled Tube D for a final dilution of 1:250,000. All test tubes were gently vortexed after addition of the sample to ensure equal distribution throughout the solution. These solutions were utilized to validate the performance of the IgG assay for equine plasma and the purified IgG solution.

Determination of the total IgG concentration of the BAL fluid samples was performed for each sample at each designated time period (Time 0, Time 0.5, Time 4 and Time 24). Based upon preliminary trials, the BAL fluid samples were prepared to a final dilution of 1:500 by adding two microliters of BAL fluid to 1,000 microliters of assay diluent. All samples were gently vortexed to allow for equal distribution throughout the solution.
When performing the ELISA, the appropriate fluid samples were pipetted into their respective duplicate wells of a 96 well microplate coated with purified goat anti-horse IgG. One well was left empty and served as a substrate control, designated as “blank”. The samples were then incubated for 30 minutes at room temperature (23°C). A four-cycle wash phase using a combination of PBS, Tween 20® and 2-chloroacetamide was performed on each of the sample wells with an automated washer. One-hundred microliters of detector antibody containing conjugated goat anti-horse IgG peroxidase was added to each sample well with the exception of the “blank” well. The plate was incubated again for 30 minutes at room temperature and the sample wells were once again washed using a four-cycle wash phase. One hundred microliters of substrate containing tetramethyl benzidine was added to each sample well and the blank well. The wells were incubated at room temperature for 30 minutes. One hundred microliters of stop solution was added to each well after color change was noted. The optical density of each well was read at 450 nm with a microtiter plate reader within 15 minutes of adding the stop solution. Results were calculated and reported using a software program (Microplate Manager®, Version 4.0).

**Cytologic analysis**

A differential cell count was performed on BAL fluid samples for all sample time periods (Time 0, Time 0.5, Time 4 and Time 24) for all horses. Slide
preparation was performed by adding 0.08 mls of the lavage sample to a single use, cytocentrifuge chamber positioned adjacent to a single, glass microscope slide. Cytology slides were generated by cytocentrifugation at 1,250 x g for four minutes and were allowed to air dry at room temperature prior to staining. Two slides were made for each collection time period. One slide was catalogued while the other was prepared with a modified Romanowsky staining method. Differential cell counts were determined microscopically from evaluation of 200 nucleated cells for each sample.

**Statistical analysis**

Descriptive statistics were generated for all data and mean values were reported. A one-way analysis of variance (ANOVA) with repeated measures was used to compare the median values of the BAL fluid IgG concentration for each horse over time. Mean values of nucleated cell counts for each horse were compared over time using an ANOVA. Posthoc comparisons were made using Tukey’s procedure. Significance was pre-set at (P<0.05).

**Results**

**Antibody purification**

By measuring the absorbance of each milliliter of purified IgG, it was determined that the highest IgG concentration was consistently observed in the second through the fifth milliliter of purified IgG. Therefore, the first milliliter of
purified IgG was discarded and the second through fifth milliliter were retained for further characterization and use. A total of 300 mls of *Rhodococcus equi* IgG plasma was filtered through the antibody purification columns. Two-hundred and twenty-eight milliliters of purified IgG was collected, representing a 76% yield of purified *Rhodococcus equi*-specific IgG antibody from the original plasma. The purified IgG was aliquoted and stored as previously described. The concentration of IgG determined in the *Rhodococcus equi* plasma was 2,175 mg/dL whereas the concentration of IgG determined in the purified IgG was 1,145 mg/dL. The actual IgG gram yield for 300 mls of plasma and for 228 mls of purified IgG was 6.5 grams and 2.6 grams, respectively.

**Aerosol characterization**

The mass median aerodynamic diameter for both the saline control and the purified IgG was between 3.3 – 4.7 µm (Table 1). The total purified IgG aerosolized within the respirable range available to the lower airway (<4.7 µm) was 0.7816 grams or 53% of the total purified IgG aerosol that was deposited within the impactor (Fig. 3). By comparison, the total saline control aerosolized within the respirable range available to the lower airway was 1.076 grams or 55% of the total aerosolized saline control. The average time to completely aerosolize ten mls of saline control and purified IgG was 19.2 and 16.9 minutes, respectively.
Table 1. Cascade impactor deposition data for aerosolized saline control and purified IgG solution

<table>
<thead>
<tr>
<th>Particle Diameter Range</th>
<th>Saline Control (grams)</th>
<th>Standard Deviation</th>
<th>Purified IgG (grams)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0: 9.0 – 10 µm</td>
<td>0.2011</td>
<td>0.0332</td>
<td>0.1553</td>
<td>0.0135</td>
</tr>
<tr>
<td>Stage 1: 5.8 – 9.0 µm</td>
<td>0.4119</td>
<td>0.1016</td>
<td>0.3649</td>
<td>0.0507</td>
</tr>
<tr>
<td>Stage 2: 4.7 – 5.8 µm</td>
<td>0.2784</td>
<td>0.1223</td>
<td>0.1779</td>
<td>0.0563</td>
</tr>
<tr>
<td>Stage 3: 3.3 – 4.7 µm</td>
<td>0.5703</td>
<td>0.1876</td>
<td>0.4145</td>
<td>0.0365</td>
</tr>
<tr>
<td>Stage 4: 2.1 – 3.3 µm</td>
<td>0.318</td>
<td>0.0343</td>
<td>0.2446</td>
<td>0.0658</td>
</tr>
<tr>
<td>Stage 5: 1.1 – 2.1 µm</td>
<td>0.1638</td>
<td>0.0478</td>
<td>0.1090</td>
<td>0.05</td>
</tr>
<tr>
<td>Stage 6: 0.65 – 1.1 µm</td>
<td>0.0191</td>
<td>0.0107</td>
<td>0.0108</td>
<td>0.007</td>
</tr>
<tr>
<td>Stage 7: 0.43 – 0.65 µm</td>
<td>0.0048</td>
<td>0.0036</td>
<td>0.0027</td>
<td>0.0026</td>
</tr>
</tbody>
</table>
Figure 3: Cumulative mass percentage of aerosolized purified IgG as deposited in cascade impactor
Patient data prior to and following nebulization protocol

All test subjects were determined to be clinically healthy at the beginning of the study. Horse 2 had a regularly irregular cardiac arrhythmia identified as second degree atrioventricular block determined by auscultation. This commonly ausculted equine cardiac arrhythmia was not indicative of respiratory disease and the horse was retained in the study. Horse 4 had a mild leukopenia (4,000 cells/µL) with an appropriate distribution of cellular populations. The remainder of horse 4’s hematology, vital signs, physical examination and pulmonary auscultation were unremarkable and he was retained in the study. Horse 5 was persistently tachycardic (40 – 52 beats per minute) from the initial examination until the conclusion of the final BAL collection. This horse suffered previous orthopedic and soft tissue injuries as a weanling that may have contributed to a chronic, underlying musculoskeletal pain. Cardiac and pulmonary auscultation was unremarkable and physical examination revealed no explainable causes for the persistent tachycardia. The mare was retained for the purposes of the study. Results of physical examination, hematology and careful pulmonary auscultation for the remainder of the test subjects were unremarkable. Nebulization with ten mls of purified IgG was performed routinely. All horses tolerated the procedure well, the entire volume of the purified IgG was delivered and very little aerosol
was lost into the environment. The mean time for complete nebulization of the purified IgG to the horses was 25.2 minutes.

The BAL procedure was performed routinely after adequate sedation in all horses at all time points. The volume of BAL fluid retrieved from the lower airway at each time point is listed for each horse (Table 2). The mean BAL fluid recovery was 13.8 ml (range 10 – 22 ml), representing 46% recovery of the infused volume. A minimal amount of exudative material was observed at the larynx of horse 5 at the 24 hour BAL collection period. The trachea and lower airways appeared absent from exudative drainage in this horse. The remainder of the test subjects had no evidence of either airway hyperreactivity or exudate at any time points.

Table 2: BAL fluid volume (ml) retrieved from research subjects

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time 0</th>
<th>Time 0.5</th>
<th>Time 4</th>
<th>Time 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse 1</td>
<td>12 mls</td>
<td>15 mls</td>
<td>11 mls</td>
<td>11 mls</td>
</tr>
<tr>
<td>Horse 2</td>
<td>14 mls</td>
<td>12 mls</td>
<td>12 mls</td>
<td>13 mls</td>
</tr>
<tr>
<td>Horse 3</td>
<td>14 mls</td>
<td>16 mls</td>
<td>14 mls</td>
<td>14 mls</td>
</tr>
<tr>
<td>Horse 4</td>
<td>22 mls</td>
<td>11 mls</td>
<td>12 mls</td>
<td>15 mls</td>
</tr>
<tr>
<td>Horse 5</td>
<td>12 mls</td>
<td>10 mls</td>
<td>16 mls</td>
<td>18 mls</td>
</tr>
<tr>
<td>Horse 6</td>
<td>12 mls</td>
<td>14 mls</td>
<td>15 mls</td>
<td>16 mls</td>
</tr>
</tbody>
</table>
Total IgG quantification

The standard control curve for plasma and IgG ELISA testing protocol was performed and determined to have an excellent linear curve fit with a high correlation coefficient (r=0.99) and intra-assay precision (CV = 6.5%) (Fig. 4). However, the intra-assay precision between the plasma and plasma duplicates (CV = 23%) and IgG and IgG duplicates (CV = 21%) was less. The standard curve for the BAL IgG ELISA testing protocol also had an excellent linear curve fit with a high correlation coefficient (r=0.991) and intra-assay precision (CV = 1.8%). The overall intra-assay precision for all horses and all time points was accurate as well (CV = 3.5%).

The mean IgG concentrations of the BAL fluid for each horse at all time points are summarized below (Table 3, Fig. 5). The mean increase in BAL fluid IgG concentration for all horses observed between initial sampling and 30 minutes post-nebulization was 61%. Overall, the mean BAL fluid IgG concentration remained elevated at 4 hours, but had returned to baseline at 24 hours. Statistical analysis using a one-way repeated measures ANOVA failed to show a significant difference (P=0.603) when comparing BAL IgG concentration over all time points.

Table 3: IgG concentration (µg/ml) of BAL fluid

<table>
<thead>
<tr>
<th>Time</th>
<th>Mean +/- SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 0</td>
<td>19.33 +/- 16.44</td>
</tr>
<tr>
<td>Time 0.5</td>
<td>31.50 +/- 18.77</td>
</tr>
<tr>
<td>Time 4</td>
<td>33.83 +/- 43.00</td>
</tr>
<tr>
<td>Time 24</td>
<td>15.53 +/- 24.26</td>
</tr>
</tbody>
</table>
Figure 4: Standard IgG concentration curve determined for plasma and purified IgG concentrations
Figure 5: Mean IgG concentration of BAL fluid from research subjects at studied time intervals

![Mean BAL IgG concentration vs. time](image-url)
**BAL cytology**

Cell populations of lymphocytes, macrophages and neutrophils are expressed as cells/200 cells for each horse for all time points (Table 4). Descriptive statistics are reported for all three cell populations for each horse at each time point. Generally, no significant differences were observed collectively in either the macrophage (P=0.385), lymphocyte (P=0.636) or neutrophil (P=0.182) populations over time as determined by one-way repeated measures ANOVA.

**Table 4: Median (range) of nucleated cell counts (cells/200 cells) determined for all horses compared with time**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Time 0</th>
<th>Time 0.5</th>
<th>Time 4</th>
<th>Time 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>103.5 (22 – 142)</td>
<td>44.5 (22 – 142)</td>
<td>34.5 (19 – 94)</td>
<td>36 (25 – 122)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>90 (52 – 177)</td>
<td>150.5 (49 – 171)</td>
<td>137 (95 – 167)</td>
<td>128.5 (51 – 167)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.5 (1 – 14)</td>
<td>5.5 (1 – 13)</td>
<td>7.5 (3 – 58)</td>
<td>20 (2 – 46)</td>
</tr>
</tbody>
</table>

**Discussion**

IgG was purified from the commercial hyperimmune plasma in order to minimize the delivery of extraneous material to the lower respiratory tract via nebulization and to enhance the aerosolization characteristics of the test solution. The antibody purification procedure was relatively straightforward due to use of a commercial system. Preparation of the gravity flow columns was carried out per manufacturer’s instructions. The 20% gel slurry provided to make the gel bed was
added easily to the gravity columns; however, the uniformity of the volume of settled gel within the column subjectively appeared variable upon visual inspection. The uniformity of the settled gel bed within the column did not appear to have a negative effect on the rate of flow through the filtration column. Proper preparation and maintenance of the columns throughout the filtration procedure and refrigerated storage was necessary. The gel bed had a tendency to separate away from the porous disc added to the top of the gel bed after the columns returned to room temperature after 30 minutes or after plasma filtration. In these cases, the porous disc was manually readjusted to the manufacturer’s suggested 5 mm separation. It was observed that if the settled gel bed was separated from the porous disc by more than 5 mm, the rate of flow through the filtration column was noticeably slower. Simple readjustment of the porous discs allowed those columns to return to a proper rate of flow. Regeneration of the filtration column was performed per manufacturer’s instructions and was uncomplicated. Batch filtration was performed with all filtration columns and each was discarded after the fourth filtration.

Light absorbance was read for each one ml portion of purified IgG. This was a quality control measure to guarantee that only the highest concentration of IgG antibody would be harvested and utilized for the total IgG quantification, aerosol characterization and nebulization portions of the study. Results from the
absorbance measurement consistently showed that the highest concentrations were observed between two and five mls for all batches including those columns that were regenerated for a second, third and fourth use. Verification of absorbance for each batch was performed routinely on a randomly selected filtration column. Seventy-six percent of the total volume of *Rhodococcus equi* plasma was retained as purified IgG for the purposes of the study. This modest yield is encouraging as only a small portion of the plasma was discarded, thus keeping expenses to a minimum while retaining a large proportion of concentrated IgG available for use in several patients. Possible improvement can be made in the yield if further research is conducted to improve technology used to concentrate antibody from plasma. However, the IgG concentration of the purified IgG was approximately half that of the plasma from which it was collected, indicating that the extraction procedure was not highly efficient.

Total IgG concentration for both the commercially available plasma and the purified IgG was determined by enzyme linked immunosorbent assay (ELISA). Because the working range of the assay was far below the concentration of IgG in the solutions of interest, several preliminary ELISA trials were performed for each solution in order to determine the ideal dilution factor that most accurately reported their respective concentrations. The final dilution factors for the plasma and purified IgG were determined to be 1:250,000 and 1:125,000, respectively. The
standard IgG concentration curve generated for both the plasma and purified IgG had an excellent correlation coefficient and a coefficient of variation of <10%, thus validated the results of the standard concentrations (Fig. 4). However, the intra-assay coefficients of variation were increased for both the plasma and purified IgG at >20%, indicating their results were imprecise. Several factors can be offered as explanations for the high coefficients of variation observed in the results. These factors include poor pipetting technique, inconsistent rinse of the individual wells or contamination from adjacent wells. All of these laboratory errors may have contributed individually or cumulatively to the individual result reported. A second explanation may be the variability in the aliquoted samples used for testing. This is less likely; however, given that the aliquoted samples were thoroughly mixed both prior to storage and testing. It was generally accepted by the researchers that the uniformity of IgG concentration for both the plasma product and the purified IgG was unlikely to be affected by the process of dividing the samples. Finally, degradation of the IgG antibody molecule could have occurred, but this was unlikely as plasma and antibody products are routinely stored frozen for extended periods of time and their immunogenic effects in patients are not degraded by the process.

The vibrating mesh nebulizer functioned very well to aerosolize both the saline control and the purified IgG. Both the saline control and purified IgG were
aerosolized completely and in a timely manner. The corrugated plastic tubing which served as a connection between the vibrating mesh nebulizer unit to the 90 degree intake column of the cascade impactor fit snugly between both units and showed no evidence of leakage. A small volume of condensate was noticed to collect within the corrugated tubing, but did not appear to affect the total volume of aerosolized material and is to be expected.

The cascade impaction unit sampled aerosol with good consistency throughout all eight stages for both the saline control and purified IgG for multiple testing cycles. Particle size as determined by MMAD was consistently found to be between 3.3 – 4.7 µm for both aerosols on multiple testing cycles. The reported MMAD for the Aeroneb Pro as reported by Aerogen is 2.1 µm, so it was surprising to learn that the MMAD for both aerosol solutions was greater in size. Although performance of these particular nebulization or impaction units may be responsible for the difference between the reported MMAD of the unit and the MMAD of our experiment, several other possibilities may exist for the observed discrepancy. Design methodologies including the volume of solution aerosolized, the connection components between the nebulization unit and cascade impactor, the design of the cascade impactor including the rate of air flow, environmental factors such as temperature and barometric pressure and finally the number of testing iterations may possibly contribute to the differences between our data and the
average MMAD reported for the unit used in our experiments. The design methodology for determination of MMAD in this experiment directly addressed these variables and only minor differences, particularly with the temperature and barometric pressure were noted. Another possible explanation may be inherent to the medication itself. Characteristics affecting nebulization of solutions may include particle density, molecular weight, particle charge and velocity of the particles generated in the aerosol. Any of these factors may account for the differences observed between the reported MMAD for the nebulization unit and the results from this experiment.

Particles within the respirable range have the ability to penetrate to the lower airways. Both the saline control and the purified IgG performed similarly in this vein, with MMAD values of 4.7 microns for each. Further studies should be performed to better define and compare the respirable range of commonly aerosolized medications nebulized to both human and animal patients. These future studies may provide insightful information regarding the volume of medication needed for effective aerosolization to nebulized patients.

All patients were determined to be systemically healthy without evidence of respiratory disease prior to the start of the study. The arrhythmia identified in horse 2 is a commonly described arrhythmia found in athletically fit horses that normalizes with excitement or exercise, as was the case in this horse. As this did
not affect the horse’s respiratory or systemic health, the horse was retained for the experiment. The leukopenia found in horse 4 was curious as he was otherwise systemically healthy. This horse was donated for the purposes of the research conducted for this experiment and for a second research project following the completion of this study. The horse’s history was reviewed and apart from a long standing, non-infectious, bilateral suspensory ligament desmitis, he was otherwise clinically healthy. The complete blood count cell population was appropriately distributed and indicated no systemic response to infection. Furthermore, his physical and respiratory examinations were normal. After finding no infectious cause for the leukopenia, systemic illness or active respiratory disease, it was decided to include the horse in the study. Finally, it was presumed that the persistent tachycardia from the initial physical examination through the end of the study observed in horse 5 was due to chronic discomfort from a multitude of previous orthopedic and soft tissue injuries. The mare was otherwise clinically healthy and had no evidence of chronic respiratory infection or inflammation that would lead to the observed clinical findings. She too was included in the study.

Prior to nebulization, the purified IgG was removed from freezer storage and allowed to thaw to room temperature. Ten milliliters of solution were combined and then passed through a 0.22 micron anti-bacterial and anti-viral filter. Potential unintentional deleterious errors could have been made and might have included
degradation of the antibody created by the heat of centrifugation or possible introduction of bacterial pathogens due to the amount of handling prior to nebulization; however, painstaking caution was taken specifically to mitigate and avoid these errors.

Endoscopic collection of the BAL fluid at each time point for all horses was routine and uncomplicated. Sedation was complete in all horses and the procedure was performed with ease in all patients. The small volume BAL technique using 30 mls of sterile 0.9% sodium chloride had been validated for use in previous studies and was selected for its relative ease of use, minimal dilution of the pulmonary epithelial lining and high cellular yield.\textsuperscript{134,135} While the endoscopy procedure was primarily used to collect samples, the upper and lower airways were assessed during the procedure. Horse 5 at time point 24 hours was observed to have a small volume of thickened mucus within her larynx. The lower airways were assessed and appeared normal. The mare showed no evidence of lower airway infection, but a mild neutrophilia (13\%) was recorded for her BAL cytology at Time 24. She showed no other clinical evidence of upper or lower airway disease at any point throughout the study. The mare was trailered 44 miles from a private farm to the clinic on the day that the mare participated in the research. The mare showed no signs of clinical respiratory infection at any time before, during or after the research trial. It is possible that the mare inhaled particulate matter from
the trailering process and had inflammation localized to the upper airway that contributed to the production of the thickened mucus observed endoscopically the following day.

Close examination of the data show variable BAL fluid IgG concentrations between individual horses at all time points. All patients with the exception of horses 4 and 6 had detectable baseline IgG concentrations within their BAL fluid at Time 0. Additionally, with the exception of horse 4, all research subjects showed an increase in overall BAL fluid IgG concentration between Time 0 and Time 0.5. All patients with the exception of horses 4 and 5 had a decrease in BAL fluid IgG concentration between Time 0.5 and Time 4. Conversely, horses four and five had an increase in their BAL fluid IgG concentration between those time periods. It should be stressed that in most of these cases, the increases were modest. While the increase in BAL fluid IgG concentrations between Time 0 and Time 0.5 was not statistically significant, it is encouraging that the total IgG concentration within the lower airway did appear to be influenced by exogenous delivery of IgG antibody. Future research is needed to determine both the total amount of IgG needed for nebulization necessary to significantly increase the pulmonary concentration of IgG in equine patients. Secondly, more research is needed to determine what protective immunological effect if any, exogenous delivery of IgG antibody can have in horses, specifically juveniles. The wide variation in IgG
concentration between patients is difficult to explain, but may be caused by administration, collection, patient or testing variables. Pulmonary delivery of the aerosol is determined by several factors in the patient, including tidal volume and minute ventilation. It may be possible that the respiratory rates, inspiratory tidal volume and thus minute ventilation of the patients varied and led to unequal nebulization of the aerosol. The rate of aerosol generation and waste into the environment may have contributed by reducing the total volume of aerosolized IgG available for inspiratory introduction into the lower airway. This is unlikely given that the environmental waste of the aerosol was minimal and the respiratory rates of the six patients during nebulization were closely matched. While unmeasured, the tidal volume of the average size, 454 kilogram horse is 6.5 liters per breath for a minute volume of 77 liters per minute.\textsuperscript{151} It was assumed that tidal volume, respiratory rate, minute ventilation, pulmonary surface area and volume of aerosol distribution within the lower airways would be equivalent among the horses. Thus it was assumed that nebulized IgG would deposit itself on the surface evenly within the healthy lower airways.

The BAL fluid collection was performed as previously described.\textsuperscript{134,135} The collection was performed in this method to characterize the representative distribution of IgG concentration within the entire airway, rather than a solitary pulmonary region. Ventral sites were collected first in order to avoid the potential
dilutional effect of saline administered more dorsally in the lung. The dorsal sites sampled later are unlikely to have been influenced by any residual saline present in the ventral aspects of the lungs. It may be possible that the distribution of the aerosolized antibody particles was uneven and contributed to the variable IgG concentrations observed among individual horses and individual time points. Future research studies may be needed to establish the best methodology for BAL fluid collection or valid other means of collecting pulmonary epithelial lining fluid samples.

An alternative explanation for the high degree of variation in the BAL fluid IgG concentrations may be due to the absorption or uptake of the IgG particles into local sites (i.e. pulmonary epithelial lining fluid, alveolar macrophages, respiratory epithelium) or the systemic circulation. Several methodologies have been described to determine the volume of pulmonary epithelial lining fluid within BAL fluid. These techniques rely upon either endogenous or exogenous dilutional markers such as urea, inulin, methylene blue, technetium 99m Tc-pentatate and $^{51}$Cr-EDTA.\textsuperscript{152-157} Although commonly used, these correction techniques have been shown to introduce additional errors within in the reported concentrations of pulmonary epithelial lining fluid.\textsuperscript{157,158} Therefore, it was decided not to attempt the determination of IgG concentration within the actual pulmonary epithelial lining fluid. However, further research is needed to describe the distribution of the
nebulized IgG particles within the lower airway or systemic circulation which may include more direct means of measurement of the IgG concentration within the pulmonary epithelial lining fluid. Finally, possible laboratory errors may have been committed in the determination of the BAL fluid IgG concentration as those similarly described in determination of the plasma and purified IgG concentrations. While great caution was taken during the testing, random errors may have been committed which contributed to the variability of the values reported.

Each sampling of BAL fluid analyzed for cytology showed a very good cellular yield, useful for diagnostic characterization of the lower airway cellular response. No evidence of chronic lower airway disease (i.e. reactive inflammatory cells, mucus, Curschmann’s spirals) was observed in any horse on cytology at Time 0. A decrease in the mean number of macrophages and an increase in the mean number of lymphocytes was observed from lower airway cytology samples following Time 0. The relevance and meaning of this observation as it applies to the immune response to exogenously delivered IgG is unknown at this time. Overall, no significant changes were observed in macrophage or lymphocyte cell populations compared over time for all horses. The neutrophil population showed no significant change in the median neutrophil population from the subject group compared over time. However, a slight increase was observed to trend in the neutrophil population across all time points. The best explanation for the observed
trend is a normal lower airway inflammatory response due to the frequency of bronchoalveolar lavage sampling that occurred in the study population. This phenomenon has been described in previous studies utilizing the same lower airway endoscopic BAL methodology.\textsuperscript{134,135}

**Conclusion**

Results from this research demonstrated an excellent concentration of IgG within commercially available plasma. While the antibody purification process extracted only approximately half of the IgG from the commercially available plasma, the concentration that remained was adequate for nebulization to the lower airways of the research subjects. The average particle size of the aerosolized purified IgG was appropriate for nebulization into the lower airway. The nebulization procedure including the use of the vibrating mesh nebulizer was effective, efficient and well tolerated in all horses. Nebulization of the purified IgG showed no short term evidence of adverse reactions or lower airway irritation in any individual. The IgG concentration within the lower airway of individual horses was highly variable, but a non-significant increase in IgG concentration was observed between baseline as compared to 30 minutes and 4 hours post-nebulization. Further research is necessary to identify the factors that lead to the observed variation in lower airway IgG concentrations as well as to determine the
best concentration or volume needed to uniformly increase the concentration of IgG within the lower airway.
Footnotes:

a. EqStim®, Neogen Corporation, Lexington, KY, USA
b. Zylexis®, Pfizer Animal Health, New York, NY, USA
c. Pneumomune-Re, Lake Immunogenics, Ontario, NY, USA
d. Melon™ Gel, Thermo Scientific, Rockford, IL, USA
e. BioPhotometer, Eppendorf, Hamburg, Germany
f. Andersen Cascade Impactor, Thermo Scientific, Franklin, MA, USA
g. Aeroneb® Pro, Aerogen, Dublin, Ireland
h. 32 mm internal diameter corrugated plastic tubing, GlobalMed Inc, Trenton, Ontario, Canada
i. Model 1531-107B-G557X, Gast Manufacturing, Inc, Benton Harbor, MI, USA
j. Sartorius BP 110S, Pegasus Scientific, Inc, Burtonsville, MD, USA
k. Endozime®, Ruhof, Mineola, NY, USA
l. BD Vacutainer®, BD, Franklin Lakes, NJ, USA
m. Millex®, GP Filter Unit, Millipore, Carrigtwohill, County Cork, Ireland
n. AeroMASK™, Trudell Medical International, London, Ontario, Canada
o. Model VQ-8303A, Olympus Medical Systems Corporation, Tokyo, Japan
p. Dormosedan®, Pfizer, New York, NY, USA
q. Dolorex®, Intervet/Schering-Plough, Summit, NJ, USA
r. Carbocaine®-V, Pfizer, New York, NY, USA
s. Immunotek, Zeptometrix Corporation, Buffalo, NY, USA

t. Bio-Rad Model 1575 ImmunoWash, Bio-Rad Laboratories, Hercules, CA, USA

u. Model 550 Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA

v. Cytopro®, Wescor, Inc, Logan, UT, USA
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