# PASSIVE IMMUNIZATION WITH HYPERIMMUNE PLASMA TO PROTECT FOALS AGAINST *RHODOCOCCUS EQUI* PNEUMONIA

A Dissertation

by

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## ABSTRACT

*Rhodococcus equi* (*R. equi*) is a common cause of pneumonia in foals. The only product licensed by the United States Department of Agriculture (USDA) for reducing the incidence of *R. equi* pneumonia in foals is transfusion of hyperimmune plasma (HIP) derived from donors immunized against *R. equi*. The reported effectiveness of *R. equi* HIP (RE HIP) transfusion to prevent pneumonia in foals is variable. This variability is likely related to differences among studies in design and plasma products used. Thus, the first objective of this dissertation is to provide a literature review with a summary of the studies to better understand the clinical evidence of the efficacy of transfusing RE HIP to prevent pneumonia. The following chapters describe studies we conducted to investigate questions pertaining to the efficacy of HIP. First, we conducted a retrospective cohort study among foals transfused with either 2 L or $\leq$ 1 L of RE HIP to investigate the impact of the volume of plasma transfused for reducing the incidence of *R. equi* pneumonia. The proportion of the foals receiving $\leq$ 1 L RE HIP that developed subclinical pneumonia (32%; 26/82) was significantly (P= 0.0068) greater than that among foals transfused with 2 L of RE HIP (12%; 8/68). This led us to investigate whether pneumonia was associated with either serum antibody activities against the virulence-associated protein A (VapA) among foals transfused with 2 L of RE HIP, or with serum antibody activities against β-1→6-poly-*N*-acetyl glucosamine (PNAG, a polysaccharide expressed on the surface of *R. equi* and other bacteria) and deposition of complement component 1q (C´1q)

onto PNAG among foals transfused with PNAG HIP. This study demonstrated that the amount of antibody activity targeting *R. equi* antigens (VapA or PNAG) was positively associated with protection against *R. equi* pneumonia. Last, based on results of *in vitro* experiments, we tested the hypothesis that transfusion with PNAG HIP would be superior to RE HIP in foals for protecting against *R. equi* pneumonia in a randomized, controlled, blinded clinical trial. We found that there was no difference in protection between the plasma types.

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## Contributors

This work was supervised by a dissertation committee consisting of Dr. Noah Cohen (chair of committee) of the Department of Large Animal Clinical Sciences, Dr. Angela Bordin of the Department of Large Animal Clinical Sciences, Dr. Michelle Coleman of the Department of Large Animal Clinical Sciences, Dr. James Heird of the Department of Animal Science, Dr. Thomas Welsh, Jr. of the Department of Animal Science and Department of Integrative Biosciences.

The data for all projects were recorded by the farm veterinarians and staff to be sent to our laboratory for analysis. The veterinarians included Dr. Glenn Blodgett, Dr. Nathan Canaday, Dr. Carly Turner-Garcia, Dr. Patricia Flores-Ahlschwede, Dr. Laurie L. Metcalfe, and Dr. Mark Nevill. The data analyzed for Chapter II were conducted by Dr. Noah Cohen of the Department of Large Animal Clinical Sciences. In Chapter III and Chapter IV Department of Medicine, Brigham & Women's Hospital, Harvard Medical School performed the ELISA, C´1q deposition onto PNAG, for plasma or serum samples respectively. In Chapter III, Jocelyne Bray and Sophia Cortez from the Equine Infectious Disease Laboratory, Texas A&M University, helped perform the VapA and PNAG ELISAs on serum samples collected. MG Biologics Inc., performed the VapA ELISA test on serum samples for Chapter IV. All other work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

| | |
|---|---|
| BWT | Body weight |
| C´1q | Complement component 1q |
| CBC | Complete blood count |
| CFU | Colony forming units |
| CI | Confidence interval |
| EIA | Equine infectious anemia |
| ELISA | Enzyme-linked immunosorbent assay |
| EPV-H | Equine parvovirus hepatitis |
| glm | Generalized linear model |
| HIG | Hyper immunoglobulins |
| HIP | Hyperimmune plasma |
| IgG | Immunoglobulin G |
| IM | Intramuscular |
| IVIG | Intravenous immunoglobulin |
| mAb | Monoclonal antibody |
| mRNA | Messenger RNA |
| NA | Not applicable |
| ND | Not determined |
| OD | Optical density |
| OR | Odds ratio |
| PNAG | β-1→6-poly-*N*-acetyl glucosamine |

| | |
|---|---|
| PNAG HIP | β-1→6-poly-*N*-acetyl glucosamine hyperimmune plasma |
| PMNs | Polymorphonuclear leukocytes |
| *R. equi* | *Rhodococcus equi* |
| RE HIP | *Rhodococcus equi* hyperimmune plasma |
| SD | Standard deviation |
| T-TBA | Trans-endoscopic tracheobronchial aspirate |
| TACO | Transfusion-associated circulatory overload |
| Th1 | T helper-1 |
| TRALI | Transfusion-associated lung injury |
| US | United States |
| USDA | United States Department of Agriculture |
| VapA | Virulence-associated protein A |
| VapC | Virulence-associated protein C |
| VapD | Virulence-associated protein D |
| VapE | Virulence-associated protein E |
| VapG | Virulence-associated protein G |
| WBC | White blood count |
| WKRE | Whole formaldehyde-killed *R. equi* preparation |

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# 1. INTRODUCTION: TRANSFUSION OF HYPERIMMUNE PLASMA TO FOALS TO PROTECT AGAINST *RHODOCOCCUS EQUI* PNEUMONIA[1*]

## 1.1. *Rhodococcus equi* Pneumonia

*Rhodococcus equi* (***R. equi***) is a gram-positive, facultative, intracellular pathogen of macrophages and a soil saprophytic bacterium.[1,2] Inhalation of airborne *R. equi* can cause a severe form of pneumonia in foals between ages 1 and 6 months that is prevalent at large breeding farms worldwide.[3-21] Although pyogranulomatous bronchopneumonia is the most common clinical manifestation of *R. equi* infection in foals, extrapulmonary disorders such as uveitis, polysynovitis, and intra-abdominal abscesses also occur.[22] This disease is important to the equine industry because the costs for treatment and lost income from mortality can be very high.[23] Adverse effects of treatment such as diarrhea[24] and hyperthermia[25] further complicate management. Additionally, foals that recover from the disease are less likely to race as adults than their birth-cohort.[26]

Preventing disease is generally preferable to treating affected cases to control infectious diseases, particularly for an insidiously-progressive diseases such as *R. equi* pneumonia. Unfortunately, a licensed vaccine for preventing *R. equi* does not exist. Chemoprophylaxis of *R. equi* pneumonia with macrolides

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[1*] This chapter is under revision: Kahn SK, Cohen ND, Bordin AI, Coleman MC, Herid JC, Welsh Th. Transfusion of hyperimmune plasma for protecting foals against *Rhodococcus equi* pneumonia. Equine Vet J. Submitted 04 May 2022.

(with or without combination with rifampicin) has been investigated, but evidence of efficacy is conflicting.[27,28] More importantly, chemoprophylaxis is considered unacceptable because overuse of macrolides has been linked to the emergence of macrolide- and rifampicin-resistant *R. equi* in the United States (US).[29-33] The only product licensed by the US Department of Agriculture (USDA) for reducing the incidence of *R. equi* pneumonia in foals is hyperimmune plasma (**HIP**) derived from donors hyperimmunized against *R. equi* (**RE HIP**). The purpose of this review is to summarize current knowledge regarding transfusion with HIP to control *R. equi* pneumonia in foals.

### 1.2. Foundational Studies of Humoral Immunity to *R. equi* and RE HIP

Several foundational studies were conducted that provided evidence for using RE HIP to reduce the incidence of *R. equi* pneumonia at farms. The scientific basis for RE HIP transfusion preventing foals from developing *R. equi* pneumonia is that *R. equi*-specific antibodies are essential for mediating protection. To the authors' knowledge, the first report evaluating antibody activity against *R. equi* used an enzyme-linked immunosorbent assay (**ELISA**) to examine serum samples from experimentally- and naturally-infected horses.[34] Horses experimentally infected with $3.4 \times 10^8$ colony forming units (CFU) *R. equi* lacked detectable antibody activities before infection but seroconverted after infection, with first detectable antibody activities 11 days after infection.[34] Seroprevalence, however, was highly variable among horses that were naturally exposed to *R. equi*

in their environment, including foals with *R. equi* pneumonia (n=15), dams of these foals (n=15), and healthy weanlings from the same farm that did not develop pneumonia (n=15).[34] This variability likely reflects differences among individual horses in the timing and magnitude of exposure to *R. equi*. Although this study demonstrated that antibodies recognizing *R. equi* were generated after exposure to the bacterium in many horses and foals, the functional activity of these antibodies against *R. equi* was not examined.[34]

Hietala and Ardans reported that killing of *R. equi* was associated with the extent of phagosome-lysosome fusion in individual macrophages.[2] Phagosome-lysosome fusion was more frequent in alveolar macrophages from *R. equi*-exposed horses than in macrophages from non-exposed horses.[2] Subsequently, Martens *et al*. demonstrated that the function of polymorphonuclear leukocytes (**PMNs**) was greater when exposed to *R. equi* opsonized with serum antibody activity against *R. equi* than with serum lacking activity against *R. equi*.[35] Collectively, these findings suggested humoral immunity contributes to killing of *R. equi in vitro*, providing a scientific basis for exploring the role of RE HIP to protect foals against *R. equi* pneumonia.

The efficacy of RE HIP was first reported in an experiment in which 6 pony foals were transfused twice at ages 3 and 5 days with 20 ml/kg body weight (**BWT**) of RE HIP (total dose = 40 ml/kg BWT) and another 6 pony foals were intravenously infused at ages 3 and 5 days with lactated Ringer's solution as

controls.[36] The RE HIP was harvested from 2 donors inoculated 4 times with a 10-ml suspension containing an estimated 5.7 x $10^8$ CFU of viable *R. equi* isolated from the lungs of a pneumonic foal. The 12 pony foals were then infected at age 7 days by nebulizing 1.425 x $10^8$ CFU of the same strain of *R. equi* used to immunize the plasma donor horses through a cuffed bronchial tube wedged in a distal bronchus. Approximately 3 weeks after infection, 5 of 6 control foals developed severe pneumonia and were euthanized, whereas all foals transfused with RE HIP developed radiographic evidence of pulmonary consolidations but did not develop clinical signs of pneumonia.[36]

The seminal experimental study by Martens *et al*. was followed by pioneering studies from California evaluating the efficacy of RE HIP under field conditions. Madigan *et al*. reported results of a 2-year field trial of the effectiveness of RE HIP to prevent pneumonia at a farm in northern California with endemic *R. equi* pneumonia.[37] During the first year of the study, all foals at the farm (n=68) were transfused with 1 L of RE HIP, but the timing of transfusion varied by birth-month among foals: foals born in January or February received plasma on March 1, whereas foals born after March 1 received plasma prior to age 30 days. The numbers of foals born before or after March 1 were not reported. ELISA antibody activities for *R. equi* were increased from a mean optical density (OD) ratio (*i.e.*, sample OD to positive control OD) of 29.8 (standard deviation [SD], $\pm$ 21.6) to a mean OD ratio of 69.1 (SD, $\pm$ 22.5); OD ratios remained >50 for

all foals for approximately 60 days following transfusion of RE HIP (when endogenous antibody activities began to rise among all foals), and none of these foals developed pneumonia. In contrast during the 5-year period prior to the study during which RE HIP was not administered, the farm experienced cases of pneumonia and death from *R. equi* infections.

During the second year of the study, foals were assigned to 2 study groups on the basis of the preferences of individual mare owners regarding immunization of mares against *R. equi* and transfusion of foals with RE HIP (*i.e.,* foals were not randomly assigned to study groups). Group 1 included 85 foals born to mares vaccinated with an autogenous *R. equi* bacterin at 90, 60, and 30 days prior to foaling, and 16 foals born to non-vaccinated mares; all 101 foals in Group 1 were transfused with 1 L of RE HIP. Group 2 consisted of 14 foals born to mares immunized with the *R. equi* bacterin during pregnancy as described above; none of the Group 2 foals were transfused with RE HIP (or other plasma product). The cumulative incidence of *R. equi* pneumonia was significantly ($P<0.05$) lower among foals in Group 1 (3%; 3/101 foals transfused with RE HIP) than in Group 2 (43%; 6/14 foals not transfused with RE HIP).[37] The vaccination status of the dams of the 3 foals in Group 1 that developed pneumonia was not specified in the report.

Muller and Madigan subsequently reported significant ($P<0.05$) efficacy of transfusing foals with 1 L of RE HIP for protecting foals against pneumonia in a 5-

year field trial conducted between 1988 and 1992[38] at the same endemic farm studied previously and included results from their initial 2-year study.[37] In this report, the method of treatment assignment was not reported, RE HIP was transfused to foals at various ages (**Supplemental Table 1**), and the timing of transfusions was based on the authors' clinical observation that *R. equi* pneumonia at this farm was most often diagnosed in the month of May.[38]

These initial field studies at this farm in northern California were important and strongly influenced equine veterinary practice, but they had important limitations. The design of the first year of the study did not include controls. Although increased *R. equi* antibody activities were documented after RE HIP transfusion relative to before transfusion, interpretation of these antibody activity levels is difficult in the absence of controls because of natural exposure to *R. equi* in the environment.[6-8] Historical controls were used to compare the cumulative incidence of *R. equi* pneumonia. Historical controls are problematic for evaluating the efficacy of RE HIP because of the year-to-year variation in cumulative incidence of *R. equi* pneumonia at endemic farms.[8,39] The number of controls was small relative to treated foals (*e.g.*, n=14 controls versus n=101 transfused foals in year 2), and the assignment to treatment group was not randomized. Because the majority of the mares in year 2 were immunized against *R. equi* vaccine (84%; 85/101), it was impossible to separate the effects of active immunization of mares

from passive immunization of foals using RE HIP. A wide range of serum *R. equi*-specific antibody activity by ELISA were observed among foals after transfusion.

The aforementioned pivotal field studies pointed the importance of humoral immunity and were the impetus for other investigators to determine the effectiveness of RE HIP to protect against both natural infection with *R. equi* under field conditions and experimental infection of foals.[10,40-48] The next section of our narrative review summarizes the results of these studies to provide an overview of current knowledge of this topic.

### 1.3. Subsequent Evaluations of RE HIP Efficacy

#### 1.3.1. Field Studies

Subsequent to experimental studies of efficacy of RE HIP to protect foals against intrapulmonary infection,[36] several field studies were conducted to evaluate the clinical efficacy of RE HIP (**Table 1**).[10,37,38,44-47] Evidence of the effectiveness of RE HIP has been variable, and includes both evidence of significant protection against *R. equi* pneumonia[10,37,38,44,45] and evidence of absence of protection.[46,47] The initial field studies conducted in California (described above) demonstrated a protective effect.[37,38] Soon after, Hurley and Begg reported failure of RE HIP to protect foals against *R. equi* pneumonia at a farm in Australia[47] where foals were either transfused with 1 L of RE HIP within 48 hours of birth (n=34) or not transfused (n=57); the method of RE HIP assignment was not reported.[47] Diagnosis of *R. equi* pneumonia was based on

clinical signs and isolation of *R. equi* from tracheo-bronchial aspirates of pneumonic foals. The proportion of foals that developed pneumonia did not differ significantly (P>0.05) between RE HIP-transfused foals (27%; 9/34) and the control foals (21%; 12/57).[47] Giguère *et al*. conducted a randomized, controlled field trial which failed to detect a significant difference in the cumulative incidence of disease (19%; 13/68) in foals transfused with 1 L of RE HIP at age 1 to 10 days followed by a second liter of RE HIP between ages 30 to 50 days and that of control foals at the same farm that were not transfused (30%; 24/80).[46] Strengths of this study included use of a randomized, controlled study design and confirmation of the diagnosis of *R. equi* pneumonia for all foals with clinical signs of pneumonia by microbiologic culture and cytologic examination of tracheo-bronchial aspirate fluid.[46]

Becu *et al*. reported the results of a field trial (conducted at 3 farms in Argentina between 1993 to 1995) that entailed immunizing 380 mares with soluble antigens to *R. equi* either 2 or 3 times during the last 2 months of pregnancy.[10] Foals at Farm 1 were tested for evidence of passive transfer of *R. equi* antibodies from their dams as determined by an agar gel immunodiffusion test. If the foals were deemed to have inadequate levels of passive transfer of *R. equi* antibodies, they were transfused with RE HIP at ages 4 and 25 days; if the foals were deemed to have adequate passive transfer of *R. equi* antibodies they were transfused once at age 25 days. At Farms 2 and 3, all foals were transfused with RE HIP at ages

4 and 25 days, and foals at Farm 2 were transfused a third time at age 60 days. The volume of RE HIP transfused to foals was determined on the basis of results from complement fixation assay for anti-*R. equi* antibody activity in the different lots of RE HIP: the volume transfused ranged from 300 to 1200 ml of plasma per foal. At the 3 farms, mortality decreased significantly ($P<0.05$) to 0.2% (1/380) during the 3-year period of the study from a historical cumulative incidence of 5.8% (the numbers of foals from which the historical cumulative incidence of mortality was calculated were not provided). The cumulative incidence of disease (*i.e.*, morbidity) did not differ from historical controls at Farm 1, remaining around 15 to 22% each year. Cumulative incidence at Farm 2 dropped from approximately 33% in 1992 to 0% in 1993 and 1994; however, the incidence rose to 12% in 1995.[10] At Farm 3, the incidence of pneumonia dropped from 30% in 1992 to <1% in the years from 1993 to 1995.[10] This study had 2 important limitations for assessing the efficacy of RE HIP for preventing *R. equi* pneumonia. First, use of historical controls was undesirable. Second, the protocol for transfusion varied between farms which made it difficult to separate effects of farm from effects of timing of transfusion.

Higuchi *et al*. conducted a 2-year field study of the efficacy of RE HIP at 3 farms in Japan.[44] Sixteen foals were transfused with 1 to 2 L of RE HIP between ages 10 and 39 days, and 19 foals that were not transfused served as controls; the method of treatment assignment was not reported.[44] Although the incidence

of pneumonia was lower among transfused foals (7%;1/16) than non-transfused control foals (26%; 5/19), this difference was not significant (P>0.05).[44] The small sample size, limited statistical power, and the varying age and volume at which foals were transfused limited methodological consistency.

In a randomized, controlled trial designed to evaluate the efficacy of gallium maltolate to prevent *R. equi* pneumonia in foals, Chaffin *et al*. observed a significant reduction in the cumulative incidence of *R. equi* pneumonia among foals transfused with RE HIP.[45] The study included 483 foals from 12 farms located in 4 states (Illinois, Iowa, Oklahoma, and Texas), including 355 foals transfused with RE HIP and 128 foals that were not transfused with RE HIP. All 355 transfused foals received 1 L of RE HIP soon after birth (median age at transfusion, 1 day; range 1 to 4 days); 267 of the study foals were transfused with a second L of RE HIP at various ages ranging from 2 to 32 days (median, 14 days). The manufacturer of the RE HIP transfused to foals varied among farms but not within farms. The cumulative incidence of pneumonia among foals transfused with RE HIP (29%;103/355) was significantly (P = 0.001) lower than that for the controls (45%; 58/128).[45] These results should be interpreted with caution because this study was not designed specifically to assess the efficacy of RE HIP such that foals were not randomly assigned to receive RE HIP. Moreover, age(s) of transfusion, diagnosis of *R. equi* pneumonia, disease monitoring, and other parameters varied or might have varied within and among farms.

### 1.3.2. Experimental Studies

Similar to field studies, experimental studies of the efficacy of RE HIP subsequent to the initial report by Martens *et al*.[36] have also yielded conflicting results (**Table 2**).[40,41,43,48] Perkins *et al*. investigated the efficacy of RE HIP relative to a standard commercial equine plasma using 16 colostrum-deprived foals.[48] All foals were transfused with standard equine plasma (*i.e.*, commercial equine plasma with low antibody activity against *R. equi* and virulence-associated protein A [**VapA**]; 15 ml/kg) within 24 hours of birth. At age 14 days, 6 foals were transfused with the standard equine plasma (15 ml/kg) and 10 foals were transfused with commercial RE HIP. At age 21 days, all 16 foals were infected intrabronchially with virulent *R. equi*, and foals were monitored by investigators blinded to the treatment group of foals for clinical signs of disease (daily examinations) and for thoracic radiographic evidence of pneumonia (performed 3 times weekly). All 16 foals developed clinical signs of pneumonia, and 5 foals either died or were euthanized because of pneumonia. However, there were no differences in the proportion of deaths, or clinical, clinicopathological, or radiographic findings between the 2 treatment groups.[48] These findings indicated that RE HIP was not superior to non-hyperimmune plasma. This study, however, had limitations that complicate interpretation of the results. The dose of plasma administered was relatively low (15 ml/kg versus the 40 ml/kg used in the first study demonstrating efficacy[36]). An untreated control group was not included to

document efficacy of either the RE HIP or the standard plasma products. Foals in the RE HIP group received standard plasma rather than RE HIP at the first transfusion.[48] The immunological consequences of colostral deprivation beyond restricting antibodies are ill-defined and could have impacted results.

In another experimental study,[41] foals age 2 days were either transfused with 1 L of RE HIP (n=15) or infused intravenously with 1 L of 0.9% saline solution (n=15). Treatment assignment was randomized and investigators evaluating clinical outcomes were blinded to which treatment group the foals were assigned. Foals were then challenged at age 7 days with 1 x $10^8$ CFU of *R. equi* delivered to the right mainstream bronchus through an endoscope. At age 28 days, the frequency of pulmonary consolidations in the control foals (80%; 12/15) was significantly ($P<0.05$) greater than that for the foals transfused with RE HIP (29%; 4/15), indicating a protective effect of RE HIP.[41]

Erganis *et al*. immunized pregnant mares with an adjuvanted *R. equi* bacterin at 8, 9, and 10 months of gestation and subsequently administered RE HIP to foals (n=4) of vaccinated mares at approximately 1 month of age; control foals (n=4) born to unvaccinated mares were not administered RE HIP.[43] Treated foals received 150 ml of RE HIP intravenously 2 days before infection with 1 x$10^5$ CFU of *R. equi* injected intercostally into the left lung. After infection, 50 ml of RE HIP was administered subcutaneously on post-infection days 1, 5, 9, 13, and 17. The lesion scores of lungs and other organs observed at necropsy were 3.5-fold

less in the RE HIP-treated foals than the control foals, and this difference was statistically significant ($P<0.05$).[43] Although these results suggest salutary effects of RE HIP, important limitations of this study include inability to assess the impact of maternal vaccination on foals independent of RE HIP transfusion and the small number of foals studied.

Sanz *et al*. conducted a randomized, controlled experiment in which foals were assigned either to be transfused with 1 L of RE HIP between ages 8 and 48 hours (n=9) or to serve as non-transfused controls (n=9).[40] All foals were infected intratracheally at age 4 days with $6 \times 10^3$ CFU of virulent *R. equi*. Although the proportion of foals that developed pneumonia among foals transfused with RE HIP (11%; 1/9) was lower than that of control foals (44%; 4/9), this difference was not significant. However, foals transfused with RE HIP had significantly ($P<0.05$) lower weekly and cumulative scores of thoracic ultrasonographic lesions and white blood cell concentrations, indicating that RE HIP attenuated the severity of pneumonia.[40]

### 1.3.3. Interpreting the Varying Results Among Studies

Multiple explanations exist for the variability in effectiveness of RE HIP among reported studies, including the type/source of the plasma, activity of total IgG or IgG sub-isotypes recognizing *R. equi* in the plasma, complement activity in the RE HIP or the foals, volume of plasma transfused, age at which foals were

transfused, and the *R. equi* antigens against which the donor horses were hyperimmunized.

Although data are sparse, varying antibody activity against *R. equi* likely contributes to varying efficacy of RE HIP for preventing *R*. *equi* pneumonia. Virulent isolates of *R. equi* express the virulence-associated protein (Vap) A (VapA), which is encoded on an 85- to 90-kilobase plasmid that is necessary to cause disease in foals.[49-53] In the US, antibody activity of VapA is used to assess the potency of products licensed by the USDA for preventing *R. equi* pneumonia.[54] Not all plasma products evaluated in studies of effectiveness of RE HIP for preventing pneumonia were tested for activity of antibodies against VapA.[36-38,43,44,47] The plasma products used in the studies summarized here varied both in terms of whether they were commercially manufactured and by commercial manufacturer. Anti-VapA immunoglobulin G (**IgG**) activity was reported to vary among US manufacturers of RE HIP and also among batches or lots from the same manufacturer.[54] In a recent study from our laboratory foals were transfused with 2 L of either RE HIP or plasma hyperimmunized against β-1→6 poly-*N*-acetyl glucosamine (**PNAG HIP**) within 24 hours of birth. Results showed that the odds of pneumonia were significantly higher for foals transfused with RE HIP that had lower antibody activity against VapA and and for foals transfused with PNAG HIP that had either lower activity of PNAG or less deposition of complement component 1q (C'1q) onto PNAG.[55]

The role of the relative or absolute amount of IgG sub-isotype(s) recognizing *R. equi* antigens in the efficacy of RE HIP is unknown. Conflicting evidence exists regarding the association of IgG sub-isotypes with *R. equi* infection.[54,56-59] Hooper-McGrevy *et al*. examined sub-isotypes of IgG among 3 groups of horses and foals: 1) adult horses (presumably immune to *R. equi* infection); 2) apparently healthy foals exposed to *R. equi* (*i.e.*, healthy foals at a farm with recurrent cases of *R. equi* pneumonia); and 3) foals from the same farm with clinical signs of pneumonia attributed to *R. equi*.[58] Adult horses had higher serum activity of IgGa ($IgG_1$) sub-isotype recognizing VapA, VapC, and VapG compared to the pneumonic foals, whereas the pneumonic foals had higher serum activities of sub-isotypes IgGb ($IgG_{4/7}$) and IgG(T) ($IgG_{3/5}$) recognizing VapA, VapC, VapD, VapE, and VapG compared to adult horses. Further, the pneumonic foals had significantly greater IgGb ($IgG_{4/7}$) recognizing VapC, VapE, and VapG than the healthy foal group. The authors concluded that the higher IgGa in the adult horses and healthy foals reflected a T helper-1 (Th1)-biased immune response whereas the pneumonic foals had a Th2-biased immune response that rendered them more susceptible to infection.[58] However, Hooper-McGrevy *et al*. subsequently reported conflicting results from a study in which foals were either immunized against *R. equi* by intragastric administration of live virulent *R. equi* (n=4) or were administered saline solution intragastrically (n=3 control foals).[57] All foals were subsequently infected intrabronchially with virulent *R. equi*. The foals

immunized by intragastric administration of *R. equi* were protected against pneumonia whereas the 3 saline-gavaged control foals developed *R. equi* pneumonia. The foals immunized intragastrically had higher serum IgG(T) activity and lower IgGa and IgGb activity against *R. equi* antigens from the day of challenge infection through 14 days post-challenge than did the control foals that developed pneumonia.[57]

Activities of IgG and sub-isotypes IgGa, IgGb, and IgG(T) against VapA were compared between 4 RE HIP products from 3 different manufacturers.[54] One RE HIP product had significantly ($P<0.05$) more VapA-specific IgGa than the other RE HIP products, whereas the IgGb and IgG(T) activities against VapA did not differ significantly among the RE HIP products.[54] The RE HIP product with the high VapA-specific IgGa activity is the only RE HIP documented to reduce the severity of *R. equi* pneumonia in transfused foals.[40,41] In a study demonstrating efficacy of maternal vaccination against PNAG to protect foals against *R. equi* pneumonia, activity of IgGa recognizing PNAG was significantly higher in foals born to mares vaccinated against PNAG from ages 2 to 56 days compared to foals born to unvaccinated mares and that developed pneumonia, whereas IgGb activity against PNAG was of lesser relative magnitude and remained significantly different from the control group only through age 42 days.[56] This finding was supported by an *in vitro* study in which anti-PNAG IgGa derived from PNAG HIP was demonstrated to mediate significantly greater ($P<0.05$) complement

deposition onto PNAG and opsonophagocytic killing of *R. equi* by neutrophils than did IgGb derived from the same PNAG HIP.[59] Further evaluation of the role of IgG sub-isotypes recognizing *R. equi* antigens in RE HIP is warranted.

Complement is an important mediator of opsonophagocytic killing of *R. equi*,[56,60] and opsonization of *R. equi* is important for intracellular killing of *R. equi*.[61,62] Individual plasma donors with similar IgG concentrations can vary in their complement-mediated opsonizing capacity.[62] Foals have been reported to have decreased opsonic capacity relative to adults,[62,63] although evidence is conflicting.[64] Consequently, it is possible that variation in complement activity between plasma products or among foals[62-65] contributes to the variable clinical efficacy of RE HIP observed under field and experimental conditions.

The volume of plasma transfused to foals likely influences effectiveness of RE HIP. The original report documenting efficacy of RE HIP to protect foals against experimental intrapulmonary infection used 1 L of RE HIP to pony foals that weighed approximately 25 kg, corresponding to a dose of approximately 40 ml of RE HIP/kg of BWT.[36] The volume of 1 L was subsequently adapted for field use;[8,37,38,44,47] however, because foals of most breeds of horses weigh approximately 50 kg at birth, transfusion of 1 L of RE HIP corresponds to a dose of only 20 ml/kg BWT. Serum concentrations of antibody activity against *R. equi* of foals that were transfused between 10 and 39 days of age with 2 L of RE HIP remained high until age 90 days, whereas foals transfused with 1 L maintained

similarly high serum reactivity only until age 60 days.[44] At some farms, a portion of the foals are transfused with 2 L of RE HIP (approximately 40 ml plasma/kg BWT) soon after birth while others are transfused with only 1 L of RE HIP (approximately 20 ml/kg BWT). Two observational epidemiological studies conducted at such farms indicated that 2 L was superior to 1 L for preventing either clinical pneumonia[66] or subclinical pneumonia (*i.e.*, thoracic ultrasonographic and clinicopathological abnormalities consistent with pneumonia in the absence of clinical signs of pneumonia).[67] ***To date, a randomized, controlled clinical trial comparing the protective effects of transfusing 2 L of RE HIP versus 1 L of RE HIP has not been conducted***. Such a study is greatly needed for the equine breeding industry to provide strong evidence of the benefits (and risks) of transfusing 2 L of RE HIP relative to the common practice of transfusing only 1 L of RE HIP. In the absence of results of well-designed clinical trials, the 2 observational epidemiological studies indicate that transfusion of 2 L of RE HIP is superior to transfusing 1 L of RE HIP.[66,67]

The age at which plasma is first transfused to foals has varied greatly among studies, ranging from ages 8 hours to 60 days.[8,10,36-38,40-42,44,46-48] Foals are exposed to *R. equi* from birth,[6,68-70] and epidemiological evidence suggests that foals are infected soon after birth,[27,71] when they are more susceptible to infection.[40] These data indicate that administering RE HIP shortly after birth would be superior because antibodies are generally more effective for preventing than

for treating infections.[72] For example, RE HIP failed to improve survival among 6 pony foals that were infected intrabronchially at age 7 days and transfused 7 and 9 days later with RE HIP relative to 4 control pony foals that were not transfused with RE HIP,[73] whereas using the same challenge model and source of plasma, transfusion of RE HIP protected foals against pneumonia.[36] Administration of RE HIP or PNAG HIP prior to experimental infection at a dose of 40 ml/kg protected foals against subsequent challenge infection at ages ranging from 4 days to 28 days.[36,42,56]

Most foals develop clinical signs of pneumonia caused by *R. equi* between 1 and 3 months of ages. Because the nadir of foal serum IgG concentrations occurs around age 6 to 8 weeks, some farms administer 1 L of RE HIP soon after birth and a second liter at age 3 to 4 weeks.[8,46] To our knowledge, only 1 study has examined the efficacy of sequential transfusion compared with a single transfusion within 48 hours of birth. That study was a retrospective cohort study with historical controls: during 2009 and 2011, most foals at the farm were transfused with 2 L of RE HIP within 24 hours of birth, and in 2010 foals were transfused with 1 L of RE HIP within 24 hours of birth and with a second liter at age 3 weeks. The incidence of *R. equi* pneumonia was significantly lower during the years in which foals received 2 L of RE HIP within 24 hours of birth than during the year in which 1 L of RE HIP was administered within 24 hours of birth followed by a second transfusion of RE HIP at age 3 weeks.[8] Unfortunately, the incidence

of *R. equi* can vary between years at individual farms,[8,39] and the data from this study do not permit differentiation between effects of year and plasma volume. In a clinical trial of 165 foals at a single farm in Florida, foals were randomly assigned either to no transfusion or to transfusion with 1 L of RE HIP between 1 and 10 days of age and again between 30 and 50 days of age.[46] Although the incidence of pneumonia was lower among transfused foals (19%; 13/68) than non-transfused foals (30%; 24/80), this difference was not significant (P=0.09).[46] Collectively, existing data are insufficient to conclude that transfusion of a second liter of RE HIP at 3 to 4 weeks of age provides protection beyond that conferred by transfusion of 1 L soon after birth.

It is plausible that efficacy of transfused plasma to protect foals against *R. equi* varies by the antigen(s) targeted by hyperimmunization of donors. In the US, commercially available plasma is produced against *R. equi* and potency is assessed by relative activity against VapA.[54] Experimentally, PNAG HIP has been demonstrated to protect against experimental intrabronchial infection with *R. equi*.[56] *In vitro*, PNAG HIP mediated significantly greater opsonophagocytic killing of *R. equi* than RE HIP or non-hyperimmune plasma.[74] In a randomized, controlled, blinded, multi-farm clinical trial, however, the efficacy of transfusion of 2 L of either PNAG HIP or RE HIP had equivalent efficacy, although results varied significantly among individual farms.[75] Results of this clinical trial illustrate the limitations of translating *in vitro* findings to patients, and how small-scale, single-

farm studies can result in irreproducible results. Nevertheless, it is possible that the antigen(s) used to immunize donor horses could enhance efficacy of RE HIP products.

In addition to reducing the incidence of clinical and subclinical pneumonia, transfusion of RE HIP may aid in controlling *R. equi* by reducing environmental exposure.[76,77] Airborne concentrations of virulent *R. equi* have been associated with increased odds of pneumonia at the level of farm[16] and individual foal.[6,70] As noted above, foals are exposed to *R. equi* in their environment from birth.[6,68-70] The organism appears to be ubiquitous in soil at horse farms,[4,78-80] and fecal shedding by mares and foals is prevalent.[76,81,82] Foals with *R. equi* pneumonia shed higher concentrations of *R. equi* in their feces thus putting more in the environment which can potentially infect more foals,[77] and this shedding is reduced when foals are treated with macrolide antibiotics.[27] Transfusion of RE HIP was recently demonstrated to decrease the fecal shedding of virulent *R. equi* in a group of foals.[76] Among 6 foals that developed pneumonia following experimental infection, 2 that received RE HIP did not shed any *R. equi* in their feces whereas 4 foals that were not transfused with RE HIP shed virulent *R. equi* for up to 2 weeks after infection.[76] Although caution is warranted in interpreting the results of this study because of the small sample size, this finding merits further evaluation because it could represent an indirect but important means by

which RE HIP transfusion contributes to reducing the incidence of *R. equi* pneumonia at farms.

Despite the varying results and limitations of the evidence in the studies cited above, the authors recommend transfusion of 2 L within 24 hours of birth to foals to reduce the cumulative incidence of *R. equi* pneumonia for the following reasons. Evidence exists that transfusion of 2 L at birth can lead to relatively high antibody activities through 3 months of age,[44] providing highest antibody activity when foals are most susceptible to infection.[40] Transfusion of 2 L of RE HIP soon after birth appears to be safe[8,66,67] and requires only a single transfusion, whereas a second transfusion after 3 or more weeks increases the likelihood of immune reactions to plasma products and entails a second procedure requiring restraint and intravenous catheterization that carries some risk for foals.

### 1.4. Mechanism of RE HIP-Mediated Protection

How RE HIP protects foals against *R. equi* pneumonia remains unclear. Antibodies recognizing *R. equi* have received most attention because equine polyclonal antiserum has been used for over a century to treat and prevent infectious diseases,[72,83] foals lack serum antibodies at birth because the equine placenta is impermeable to immunoglobulins,[84] and plasma products are either acellular or hypocellular, rendering transfer of antigen-specific lymphocytes highly improbable. Several lines of evidence suggest that antibodies play a protective role in *R. equi* pneumonia. In particular, antibodies against VapA have received

considerable attention because VapA is surface-expressed and immunodominant.[42,50,51,85,86]

The protective effect of VapA antibodies in plasma was first investigated in mice using plasma harvested from horses immunized against VapA.[87] The plasma from immunized horses was precipitated twice with 95% ethanol, followed by diethylaminoethyl cellulose chromatographic separation to recover IgG. Mice were divided into 4 groups for intraperitoneal administration of treatments including, 0.9 mg of IgG (Group A, n=50), 1.8 mg of IgG (Group B, n=50), control mice receiving saline (n=10), and control mice that received normal nonimmune equine IgG (n=10). The mice were then challenged intraperitoneally with *R. equi*. The mice in both control groups died within 3 to 7 days after infection, but the mice that received the higher amount of IgG (Group B) were completely protected and the mice that received the lower dose (Group A) were partially protected.[87] This study demonstrated the importance of humoral immunity to VapA in mice and substantiated that a dose-response relationship for anti-VapA IgG exists.[87] To investigate the role of antibodies in mediating protection by RE HIP, Hooper-McGrevy *et al*. conducted an experiment in which pony foals received intravenously either 1 L of RE HIP (n=7), 1 L of immunoglobulins specific for VapA and VapC purified from donors hyperimmunized with recombinant VapA and VapC (n=7), or no treatment (n=11); foals were treated at age 3 weeks and infected 1 day after treatment.[42] Foals receiving either RE HIP or the VapA/VapC

immunoglobulin preparation had significantly ($P<0.05$) later onset of clinical signs of pneumonia, less severe pulmonary lesion scores determined post mortem, and significantly fewer *R. equi* recovered from the lungs than control foals; however, clinical, pathological, and microbiological findings were similar for foals treated with RE HIP and the VapA/VapC immunoglobulin preparation,[42] indicating that protection from transfusion of RE HIP was consistent with that from IV administration of purified immunoglobulins targeting VapA and VapC. Hooper-McGrevy *et al*. compared IgG responses to VapA between 4 foals immunized intragastrically with $1 \times 10^8$ CFU/ml of virulent *R. equi* with those of 3 control foals that were gavaged with saline.[57] Foals that were immunized intragastrically were protected against subsequent intrabronchial infection with the same strain of virulent *R. equi*, whereas the control foals all developed clinical signs of pneumonia.[57] Activity levels of total IgG against VapA and VapC were significantly higher in the immunized than the control group, indicating that VapA and VapC were highly immunogenic and correlated with protection.[57]

Studies in which maternal vaccination has protected foals against *R. equi* pneumonia suggest a role for antibodies in mediating protection by transfer of maternal antibodies to foals postnatally via colostrum.[10,56,88] Maternal vaccination with a VapA-rich supernatant from cultured *R. equi* or with whole formaldehyde-killed *R. equi* preparation (WKRE) prepared from an isolate obtained from an infected foal was evaluated at a farm with endemic *R. equi* pneumonia.[88] The

study included 3 groups of mares and their foals: 1) mares vaccinated intramuscularly (IM) with *R. equi* VapA protein antigen at 9, 6, and 3 weeks before their expected due date (n=24); 2) mares vaccinated IM with WKRE at 24, 12, and 4 weeks before their expected due date (n=8); and, 3) unvaccinated mares (negative control group; n=15).[88] Results indicated that both vaccines administered to mares protected their foals against natural infection: 0 of 32 foals born to vaccinated mares developed pneumonia whereas 4 of 15 (27%) foals born to unvaccinated mares developed pneumonia, a difference which was significant (P=0.02). The IgG activity against VapA in Group 1 foals at age 30 days was higher than that in Group 2 (P<0.0001) and control foals. At age 24 hours, foals in Group 1 had a non-significant (P>0.05) increase in IgG activity against *R. equi* than foals in Group 2; however, activity for Group 1 foals was significantly (P<0.05) higher than that in the control foals (P<0.05).[88] Similarly, antibody activity against PNAG in foals born to dams vaccinated with a PNAG conjugate vaccine was significantly (P<0.05) higher than that for foals born to unvaccinated mares, and serum activities against PNAG of total IgG were significantly (P<0.05) associated with protection against intra-bronchial infection of foals with virulent *R. equi* at age 28 days.[56]

Although findings of the aforementioned studies indicated that antibodies (particularly against VapA) appear to mediate protection of mice and foals from *R. equi* infection, reports of the failure of maternal vaccination to protect foals against

*R. equi* despite significant increases in serum of vaccinated mares and their foals and colostral antibody activities against *R. equi* or VapA[37,89,90] suggest that other factors might mediate protection against pneumonia by RE HIP. As noted above, complement activity might be lower in foals,[62,63] and complement is important for mediating killing *R. equi* by antibodies.[2,56,62] Plasma contains functional complement, other opsonins such as fibronectin, and cytokines that might mediate protection against bacterial infections. *In vitro*, opsonization with either RE HIP or non-*R. equi*-hyperimmune plasma increased killing of *R. equi* by equine alveolar macrophages, but RE HIP was not superior to standard plasma at mediating opsonophagocytic killing of *R. equi* by alveolar macrophages.[61] Thus, it is possible that non-*R. equi*-hyperimmune plasma products with opsonic activity might enhance immune responses in foals and have similar clinical efficacy to RE HIP. Although results of a small-scale study support this hypothesis,[48] results of another small-scale study using PNAG HIP indicate that specificity of antibody activity was necessary for protection against intrabronchial infection with *R. equi*.[56]

Plasma contains cytokines and opsonins, and also may contain other immunomodulatory components such as microparticles or residual cells.[91] To our knowledge, the effects of plasma transfusion on innate immune activation have not been investigated in foals. It is possible that plasma transfusion stimulates trained innate immune memory resulting in protection against intracellular pathogens such as *R. equi*.[62,65,92]

### 1.5. Risk of HIP Transfusion

Although several studies[8,40,41,48,55,66,67,75] indicate that transfusion of HIP to foals at age ≤24 hours is safe, inherent risks associated with plasma transfusion including hypersensitivity reactions,[93-96] transfusion-related lung injury (TRALI),[97-100] transfusion-associated circulatory overload (TACO),[100] virus transmission,[101,102] intravenous catheterization of foals, and handling foals. In a retrospective study reporting the incidence of transfusion reactions between 2003 and 2005 of 107 client-owned horses referred to the University of Wisconsin teaching hospital transfused with plasma, 6% (6/107) of horses had an adverse reaction and all were in foals <7 days of age.[94] The reactions identified were fever (4/6), tachycardia (2/6), tachypnea (2/6), colic (2/6), and muscle fasciculations (1/6). It is worth noting that all of these foals were referred to the hospital for high risk of sepsis. The plasma dose transfused to the foals was 1 L for foals <25 kg BWT and 2 L for foals >25 kg BWT.[94] This study is important because it reports the potential risk of transfusion of plasma; however, as an observational study it has the limitation of selection bias. All of the foals enrolled in the study were unhealthy and had other underlying problems, such that the foals studied do not reflect the general foal population transfused with RE HIP at breeding farms. The syndrome of TRALI is an uncommon sequela of transfusion and usually presents as shortness of breath, fever, and hypotension.[99,100] In contrast, TACO typically presents as tachycardia, caused by having fluid imbalance or cardiogenic shock

that can potentially lead to heart failure.[100] To the authors' knowledge, neither TRALI nor TACO have been reported in foals transfused with RE HIP, and adverse reactions have not been reported among foals transfused with RE HIP soon after birth.[8,40,41,48,55,66,67,75]

Transmission of viruses such as equine parvovirus hepatitis (the presumed cause of Theiler's disease),[101,102] is another risk associated with transfusion. To help mitigate this risk, manufacturers of plasma and serum products in the US are required by the USDA to follow testing protocols for their donor herd for equine infectious anemia (EIA), piroplasmosis, dourine, glanders and brucellosis.[103] Recently, testing for equine parvovirus hepatitis (EPV-H) has been added to the list of viruses for which manufacturers are required to test their donor herd.[104] To the authors' knowledge, Theiler's disease in foals has only been described in 1 foal with subclinical evidence of hepatopathy after exposure to a biologic other than HIP,[105] indicating that the risk is very low for transfused foals to develop viral hepatitis following transfusion with RE HIP. Handling foals and attendant procedures for transfusion carry risks. Anecdotally, catheters may be lost into the vasculature when they fail (break off) at the hub or are inadvertently transected with scissors or scalpels used to remove sutures used to secure the catheters; these catheters may become lodged in the heart, lung, or other organs. Any time a foal is restrained, they may exhibit flight behavior that results in physical injury that is self-induced or caused by the restrainer. Although the risks associated with

transfusion are rare, it is important that veterinarians clearly convey these risks to those who own and care for foals so that the risks may be weighed against the benefits, and so that efforts to identify and mitigate these risks are taken by those transfusing foals.

### 1.6. Limitations of Current Knowledge

Although numerous studies evaluating RE HIP (and other plasma products such as PNAG HIP) have been conducted, our current knowledge of the efficacy of RE HIP remains somewhat impoverished. Randomized, controlled, blinded, multicenter clinical trials to demonstrate strong evidence of the efficacy of RE HIP are lacking. A number of hurdles exist to conducting such trials. First and foremost, the costs for such studies would be relatively high, and the authors' experience has been that funding for such studies is difficult to attain. Second, ideally a non-transfused control group is necessary to document clinical efficacy of RE HIP, but it has been the authors' experience that many veterinarians and horse breeding-farm managers are unwilling to withhold RE HIP from foals because they consider the data from experimental and observational studies of the efficacy of RE HIP sufficiently convincing. Although non-*R. equi* hyperimmune plasma could be administered as a control plasma, this would only enable answering the question of whether specificity of antibodies is essential for protection. Novel study designs might be implemented to facilitate trials of the efficacy of RE HIP to ensure that the fewest number of foals receive a product

inferior to RE HIP, such as the use of adaptive trial study designs.[106] Another challenge for conducting clinical trials to prevent or treat *R. equi* pneumonia is the changing diagnostic criteria for *R. equi* pneumonia used at farms. The advent approximately 20 years ago of thoracic ultrasonographic screening at endemic farms to detect pulmonary lesions consistent with *R. equi* pneumonia in foals prior to the onset of clinical signs has transformed the case definition of this disease.[107,108] Prior to the widespread use of ultrasonographic screening, pneumonia caused by *R. equi* was an insidious disease in which pathological changes in the lungs were far advanced by the time clinical signs were manifested and diagnostic testing was initiated.[4,5,22] Within the past 10 to 15 years, the disease has been recognized in its earlier stages, including many subclinical cases that would recover without treatment.[20,107,109,110] Encouragingly, some evidence exists that RE HIP might be effective for controlling sub-clinical pneumonia,[67] although the strength of this evidence is weak because it is based on a single observational study that lacked an untreated control group.

Similarly, randomized, controlled, blinded, multi-farm clinical trials comparing the dose (2 L [≈40 ml/kg] vs 1 L [≈20 ml/kg]) and timing (*e.g.*, within 24 hours of birth versus within 24 hours of birth and again at age 3 to 4 weeks) are lacking.[66,67] The magnitudes of expected effects and variations among farms in diagnostic and prophylactic practices will require large sample sizes for studies to establish either non-inferiority or superiority of different doses and timing

schedules of RE HIP. Answering these questions about efficacy, dose, and timing are of considerable importance to the equine industry. It is our belief and hope that the industry will choose to support well-designed studies needed to address these clinical questions.

It also remains unclear by what mechanism(s) RE HIP might mediate protection against *R. equi* pneumonia. Understanding whether specific antibodies are essential and whether a specific sub-isotype of IgG is most effective for mediating protection would be important for designing successful novel approaches. Transfusing a 50 kg foal with 40 ml/kg of RE HIP results in expanding blood volume by approximately 40%. Although this procedure has been performed in hundreds of foals without reports of adverse effects,[8,55,66,67,75] TACO is recognized in human beings after transfusions.[100] Thus, alternative approaches for delivering antibodies are warranted. Our laboratory is investigating the potential of using mRNA encoding monoclonal antibodies to protect foals against *R. equi* pneumonia. This approach might also be used to design mRNAs to investigate the effects of specific sub-isotypes of IgG and assess functional differences among monoclonal antibodies. In addition to antibodies, further investigation of the role of complement and other aspects of innate immunity and the effects of RE HIP on adaptive immune responses such as T lymphocyte-mediated immunity are warranted.

## 1.7. Conclusions

Current evidence supports transfusion of RE HIP to protect foals against *R. equi* pneumonia, but findings are far from conclusive. Based on current evidence from experimental[36] and observational[55,66,67] studies described in this narrative review, the authors recommend transfusion of 2 L of RE HIP that has high activity against VapA to foals within 24 hours of birth.[40,54,87] Although specific antibodies appear to mediate protection provided by RE HIP,[42,57,88] other mechanisms such as complement supplementation or innate immune memory might also contribute to the efficacy of RE HIP.[2,48,56,61,62,65,92] Great need exists to provide more robust evidence about the efficacy, dose, and timing of RE HIP transfusion for protecting foals against *R. equi* pneumonia. Alternative approaches to passive immunization such as mRNA-encoded antibodies also merit investigation to avoid some of the risks associated with transfusing foals.

| Study ID | Study Design | Outcome Measure | Outcome Measure Principals | Outcome Measure Controls | Results Statistically Significant (P<0.05) | Relative Risk Reduction |
|---|---|---|---|---|---|---|
| Madigan 1991[37] | Randomized- X<br>Controlled-✓<br>Blinded-X | Clinical signs of pneumonia | 1988: 0% (0/68)<br>1989: 3% (3/101) | 1988: No controls<br>1989: 43% (6/14) | Yes | 1988: NA*<br>1989: -93% |
| Muller 1992[38] | Randomized- X<br>Controlled-✓<br>Blinded-X | Clinical signs of pneumonia | 1988: 0% (0/68)<br>1989: 3% (3/101)<br>1990: 8% (10/120)<br>1991: 3% (4/126) | 1988: None<br>1989: 43% (6/14)<br>1990: 50% (4/8)<br>1991: 60% (3/5) | Yes | 1988: NA*<br>1989: -93%<br>1990: -84%<br>1991: -95% |
| Hurley 1995[47] | Randomized- X<br>Controlled-✓<br>Blinded-X | Clinical signs of pneumonia | 27% (9/34) | 21% (12/57) | No | +29% |
| Becu 1997[10] | Randomized- X<br>Controlled-✓<br>Blinded-X | Mortality from *R. equi* pneumonia | 0.2% (1/380) | 5.8% (Historical Controls) | Yes | -96% |
| Higuchi 1999[44] | Randomized- X<br>Controlled-✓<br>Blinded-X | Clinical signs of pneumonia | 7% (1/16) | 26% (5/19) | No | -73% |
| Giguere 2002[46] | Randomized- ✓<br>Controlled-✓<br>Blinded-X | Clinical signs of pneumonia | 19% (13/68) | 30% (24/80) | No | -37% |
| Chaffin 2011[45] | Randomized- X<br>Controlled-✓<br>Blinded-X | Clinical signs of pneumonia | 29% (103/355) | 45% (58/128) | Yes | -36% |

**Table 1.1** Summary of field studies of the efficacy of *Rhodococcus equi* hyperimmune plasma (RE HIP) among client-owned foals. Relative risk reduction was calculated using the following formula and indicates the extent to which RE HIP reduced the incidence of pneumonia in transfused foals relative to that in controls: [(Outcome measure principals – Outcome measure control) / Outcome measure control].

*NA (Not Applicable)

| Study ID | Study Design | Outcome Measure | Outcome Measure Principals | Outcome Measure Controls | Results Statistically Significant (P<0.05) | Relative Risk Reduction |
|---|---|---|---|---|---|---|
| Martens 1989[36] | Randomized- ✓ Controlled-✓ Blinded-X | Clinical signs of pneumonia | 0% (0/6) | 83% (5/6) | Yes | -100% |
| Perkins 2002[48] | Randomized- ✓ Controlled-✓ Blinded-✓ | Mortality from pneumonia | 30% (3/10) | 33% (2/6) | No | -9% |
| Caston 2006[41] | Randomized- ✓ Controlled-✓ Blinded-✓ | Radiographic detection of lesions | 29% (4/15) | 80% (12/15) | Yes | -64% |
| Erganis 2014[43] | Randomized- ✓ Controlled-✓ Blinded-X | Lesion scores of lungs and other organs at necropsy, higher score = more lesions | Mean lesion score 5.5, (n=4) | Mean lesion score 19.5, (n=4) | Yes | -72% |
| Sanz 2016[40] | Randomized- ✓ Controlled-✓ Blinded-X | Clinical signs of pneumonia | 11% (1/9) | 44% (4/9) | No | -75% |

**Table 1.2** Summary of experimental studies evaluating the efficacy of *Rhodococcus equi* hyperimmune plasma (RE HIP) to reduce the incidence of pneumonia in mares and foals. Relative risk reduction was calculated using the following formula and indicates the extent to which RE HIP reduced the incidence of pneumonia in transfused foals relative to that in controls: [(Outcome measure principals – Outcome measure control) / Outcome measure control].

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# 2. TRANSFUSION WITH 2 LITERS OF HYPERIMMUNE PLASMA IS SUPERIOR TO TRANSFUSION OF 1 LITER OR LESS FOR PROTECTING FOALS AGAINST SUB-CLINICAL PNEUMONIA ATTRIBUTED TO *RHODOCOCCUS EQUI* [2]*

## 2.1. Introduction

Pneumonia is a common cause of disease and death in foals,[1,2] and *Rhodococcus equi* (*R. equi*, a.k.a., *Prescottella equi* [*P. equi*]) is considered the most common cause of severe pneumonia.[3-5] Pneumonia caused by *R. equi* is endemic at many horse-breeding farms with cumulative incidence often exceeding 20% to 40% of the foal population.[6-8] At farms where the disease is endemic, the costs can be very high for veterinary care, long-term therapy, and lost revenue from mortality of some foals. In addition to significant direct costs, *R. equi* pneumonia has a long-term detrimental effect on the equine industry because foals that recover from the disease are less likely to race as adults.[9]

Methods for preventing *R. equi* pneumonia include chemoprophylaxis, vaccination, and transfusion of hyperimmune plasma.[10-26] Chemo-prophylaxis with macrolides is not acceptable because of concerns for increasing antimicrobial

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resistance in *R. equi* strains[13,27,28] (and possibly other bacteria), and because evidence of effectiveness has been conflicting.[10,14] Currently, there is no licensed vaccine available in North America. Although transfusion of plasma derived from donors hyperimmunized against *R. equi* (RE HIP) is incompletely effective,[11-13,15,29] transfusion of RE HIP remains the only USDA-approved, well-established, and clinically-acceptable approach for reducing the incidence of clinical pneumonia in foals caused by *R. equi*. The practice of thoracic ultrasonographic screening to identify foals with pulmonary lesions prior to the onset of clinical signs of pneumonia has led many farms to implement therapy of subclinical pneumonia, such that at many horse-breeding farms subclinical pneumonia is the most common manifestation of *R. equi* pneumonia.[30-33] The effectiveness of transfusion of RE HIP for subclinical pneumonia is unknown, however.

A number of explanations for the variability in efficacy of RE HIP among farms exist. First, evidence exists that immunity to *R. equi* and other intracellular pathogens is cell mediated,[34-36] such that antibody-mediated protection might be ineffective. Second, commercial plasma products vary within and among manufacturers,[37] including the concentration of antibodies against the virulence-associated protein A (VapA) which is found on the surface of virulent *R. equi*. This is substantiated by the finding that clinical efficacy has been demonstrated for some sources of plasma[38,39] but not others.[13] Third, the dose (volume) of plasma transfused might impact efficacy. It is common for foals at endemic farms to be transfused with 1 liter (L) of RE HIP.[3,6,7] Considering that the weight of the average

foal is around 50 kg, this dose of RE HIP is approximately 20 ml/kg. The study first documenting evidence of protection of transfusion of RE HIP against experimental infection of foals with *R. equi*,[11] however, was conducted using 1 L of RE HIP administered to pony foals weighing approximately 25 kg, which is a dose of approximately 40 ml/kg. Thus, the standard volume transfused at breeding farms could contribute to incomplete efficacy. Because the impact of transfusion of RE HIP and the dose of RE HIP on subclinical pneumonia are unknown, the purpose of this observational study conducted at a horse-breeding ranch in Texas was to determine whether transfusion with 2 L of RE HIP was superior to transfusion of 1 L for protecting foals against subclinical pneumonia attributed to *R. equi*.

## 2.2. Materials and Methods

### 2.2.1. Study Population

A retrospective cohort study was conducted using data from foals that were born at a large breeding farm in Texas from January 2017 to May 2017 and that resided at the ranch through weaning. This ranch has had history of multiple foals with *R. equi* pneumonia recurring annually for at least 10 years, as identified by microbiologic cultures of samples from foals. Foaling records from the ranch indicated there were 153 eligible mares that foaled at the ranch, but 2 of their foals died or were euthanized for reasons other than *R. equi* pneumonia. Thus, there were 151 foals born that remained at the ranch whose foals could be followed through weaning to determine the cumulative incidence of *R. equi* pneumonia.

Data were extracted from paper and electronic records at the ranch by 3 investigators (KB, JNR, NDC) who visited the ranch, extracted data from these records using a data collection form, and then entered these data into a spreadsheet for data analysis. The study was approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP# 2017-0043 CA), which included review and approval by the Clinical Research Review Committee of the Texas A&M University College of Veterinary Medicine & Biomedical Sciences. Identity of individual mares and foals remained confidential. Foals at the ranch were transfused under the supervision of a farm veterinarian and monitored for reactions by visual inspection for signs of tremors, tachypnea, and collapse, and by monitoring the heart rate for excessively low or high heart rate. All foals were transfused within 24 hours of birth and received product from a single manufacturer (Mg Biologics, Inc., Ames, IA). Foals at the ranch were routinely screened using thoracic ultrasonography by the farm veterinarians (GPB; NMC) at 5, 7, and 9 weeks of age to detect areas of pulmonary abscess formation or consolidation. These lesions were considered evidence of pneumonia and were attributed to *R. equi* on the basis of farm history. Foals that developed these lesions in the absence of clinical signs were defined as foals with **subclinical pneumonia**, and were routinely treated for *R. equi* pneumonia using either gamithromycin (6 mg/kg; intramuscularly; q 7 days), or azithromycin (10 mg/kg; per os; q 24 hours) combined with rifampin (5 to 10 mg/kg; per os; q 24 hours).

Foals diagnosed with *R. equi* pneumonia also had blood collected for white blood cell (WBC) concentration and fibrinogen concentration on the day of diagnosis.

### 2.2.2. Data Collection

Information collected from mares and foals included the following: 1) mare identifier; 2) date of foaling; 3) whether mare was a recipient for a transferred embryo; 4) whether the breeding of the foal was for use in racing (race-bred) or ranch activities (ranch-bred); 5) whether the foal developed 1 or more pulmonary lesions (*i.e.*, areas of pulmonary abscess formation or consolidation $\geq$ 2 cm in maximal diameter that were detected during thoracic ultrasonography); 6) the size of the largest maximal diameter of a lung lesion observed ultrasonographically in the foal (mm); 7) results of semi-quantitative total IgG in foal serum (evidence of adequate transfer of passive immunity); 8) the volume of *R. equi* hyperimmune plasma (Mg Biologics, Inc., Ames, IA) transfused to foals (0, 1, or 2 liters, corresponding to an estimated dose of 0, 20, or 40 ml/kg, respectively, assuming that foals weighed approximately 50 kg); 9) whether the foal was treated for *R. equi* pneumonia; and, 10) whether the foal was treated for *R. equi* pneumonia using gamithromycin (standard treatment) or azithromycin and rifampin (used for foals considered by the farm veterinarians to be more severely affected, such as foals that had larger or more pulmonary lesions detected). Race-bred foals were managed differently than ranch-bred foals. Race-bred foals typically were foaled in a large stall in which they were housed for the first 3 days of life, whereas ranch-bred foals were predominantly foaled in pasture, but brought in within 24 hours of

foaling for evaluation by the farm veterinarians and transfusion of RE HIP. Ranch-bred foals were transfused exclusively with 1 L of RE HIP because of financial considerations, whereas most race-bred foals were transfused with 2 L of RE HIP.

### 2.2.3. Data Analysis

Descriptive and inferential data analysis was performed. Categorical data were cross-tabulated and analyzed using chi-squared or, when any expected cell had fewer than 5 observations, Fisher's exact test. Continuous data were summarized as medians and ranges, and compared using Wilcoxon rank-sum or Kruskal-Wallis tests. For multivariable analysis, multivariable logistic regression was used. The outcome (dependent) binary variable was whether or not the foal developed thoracic ultrasonographic lesions attributed to *R. equi* pneumonia, and association with independent variables was summarized using the odds ratios resulting from logistic regression analysis (and 95% confidence intervals [CIs]) estimated using maximum likelihood methods. A purposive, backward step-wise model was fit, wherein variables with the smallest *t* value for the Wald test were excluded, and all pair-wise interaction terms were included at each stage. Variables were retained if the *P* value for the Wald test was < 0.05. Goodness-of-fit was assessed by inspection of diagnostic residual plots.

## 2.3. Results

### 2.3.1. Univariable Associations with Subclinical *R. equi* Pneumonia

Of the 151 foals included in the study, 34 (23%) developed subclinical pneumonia attributed to *R. equi*. All 34 foals were treated with either

gamithromycin (n=16) or azithromycin plus rifampin (n=18). None of the treated foals developed clinical pneumonia or died. Month of birth was available from the records for 144 of the 151 foals (Table 2.1). There was a significant (P = 0.0098; Fisher's exact test) difference in the distribution of birth-months between foals that did not have subclinical *R. equi* pneumonia lesions and those that did: 71% of the foals with lesions were born in April and May, whereas only 36% of the foals without lesions were born during April and May. Birth-month was also considered as a binary variable of whether or not foals were born in April or May (Table 2.2). The proportion of foals that were born in April or May was significantly (P = 0.0006; chi-squared test) greater for foals with subclinical pneumonia (71%; 24/34) than for foals without thoracic ultrasonographic lesions (35%; 39/110).

Whether foals were ranch- or race-bred was determined for 150 foals (Table 2.2). The proportion of ranch-bred foals that developed subclinical pneumonia (32%; 26/82) was significantly (P = 0.0068; chi-squared test) greater than that for race-bred foals (12%; 8/68). Whether the mare was an embryo transfer recipient was not significantly associated with subclinical pneumonia (Table 2.2). Results of semi-quantitative IgG testing of foals' sera were available from the records of 67 foals. Of these foals, 65 (97%) had IgG concentration > 800 mg/dl, and 2 had concentrations of IgG > 400 mg/dl but $\leq$ 800 mg/dl. This difference was not significant (Table 2.2).

Of the 151 foals, data were available from 149 regarding the volume of RE HIP transfused to the foals: 2 foals received no RE HIP, 85 foals were transfused

with 1 L, and 62 received 2 L. For purposes of analysis, plasma transfusion was considered as the binary variable of transfusion of 2 L versus transfusion of < 2 L. Transfusion of < 2 L was significantly associated with developing subclinical *R. equi* pneumonia (Table 2.2).

As noted above, race/ranch breeding was significantly ($P < 0.0001$; Fisher's exact test) associated with plasma volume: all (100%) of the ranch-bred foals received 1 L of RE HIP, whereas of the 67 race-bred foals for which data were available, 62 (93%) received 2 L RE HIP, 3 received 1 L RE HIP, and 2 received no RE HIP. The 2 race-bred foals that did not receive RE HIP did not develop subclinical *R. equi* pneumonia. There were no reports of adverse reactions to transfusion in the records. Although the proportion of race-bred foals that were born in April or May (34%; 21/62) was less than that of ranch-bred foals (52%; 42/82), this difference was not significant ($P = 0.0564$).

The largest maximum diameter of thoracic ultrasonographic lesions observed among foals ranged from 8 mm to 42 mm. The lesion sizes of foals transfused with 2 L (median, 25 mm; range, 17 to 41 mm) did not differ significantly ($P = 0.3709$; Wilcoxon rank-sum test) than that of foals transfused with < 2 L (median, 28 mm; range, 8 to 42 mm).

**Table 2.1.** Month of birth of foals by whether or not they developed subclinical *R. equi* pneumonia from a ranch in Texas.

| | ***Subclinical Pneumonia*** | |
|---|---|---|
| ***Month*** | ***No*** | ***Yes*** |
| January | 5 (5%) | 0 (0%) |
| February | 18 (16%) | 3 (9%) |
| March | 48 (44%) | 7 (21%) |
| April | 27 (25%) | 17 (50%) |
| May | 12 (11%) | 7 (21%) |
| Total | 110 (100%) | 34 (100%) |

**Table 2.2.** Variables significantly associated with development of subclinical *R. equi* pneumonia among 151 foals born at a ranch in Texas.

| | ***Subclinical Pneumonia*** | | |
|---|---|---|---|
| ***Variable*** | ***No*** | ***Yes*** | ***P value[#]*** |
| Born in April or May | 39 (35%) | 24 (71%) | 0.0006 |
| Born before April | 71 (65%) | 10 (29%) | |
| | | | |
| Race-bred | 60 (88%) | 8 (12%) | 0.0068 |
| Ranch-bred | 56 (68% | 26 (32%) | |
| | | | |
| Embryo recipient | 52 (81%) | 12 (19%) | 0.4513 |
| Not a recipient | 65 (75%) | 22 (25%) | |
| | | | |
| Foal IgG > 800 mg/dl | 59 (91%) | 6 (9%) | 0.1995[*] |
| Foal IgG 400 to 800 mg/dl | 1 (50%) | 1 (50%) | |
| | | | |
| 2 L RE HIP | 55 (89%) | 7 (11%) | 0.0126 |
| < 2 L RE HIP | 61 (70%) | 26 (30%) | |

[#]P values from chi-squared tests unless indicated by [*] = Fisher's exact test

### 2.3.2. Multivariable Associations with Subclinical *R. equi* Pneumonia

Multivariable logistic regression was performed to identify variables that were significantly associated with development of subclinical pneumonia while simultaneously adjusting for other variables in the model. The best-fitting model included effects of birth-month of April or May (relative to earlier than April) and transfusion of 2 L of RE HIP versus transfusion of < 2 L RE HIP (Table 2.3). Because of the strong association between volume of RE HIP transfused and being race- or ranch-bred, it was impossible to dissociate these variables, and inclusion of both variables in multivariable modeling resulted in neither variable being significantly associated with *R. equi* pneumonia.

**Table 2.3.** Summary of multivariable logistic regression modeling for association of variables with odds of developing subclinical pneumonia among 151 foals born at a ranch in Texas.

| Variable | Odds Ratio (95% CI) | P value |
|---|---|---|
| Foaled in April or May | 4.6 (2.0 to 11.0) | 0.0007 |
| Not receiving 2 L plasma | 2.8 (1.1 to 7.1) | 0.0363 |

### 2.3.3. Comparison of Clinical and Clinicopathologic Findings in Foals with Subclinical *R. equi* Pneumonia

Among foals diagnosed with subclinical pneumonia, there were no significant differences between foals transfused with 2 L or foals transfused with < 2 L in the age (days), screening week, maximal diameter of lesions, month of birth, WBC and fibrinogen concentration at time of diagnosis, or duration of lesions (Table 2.4). These results should be interpreted with caution because of the small sample size and because these comparisons were not a primary study aim.

**Table 2.4.** Comparison of variables between foals with subclinical pneumonia by whether they were transfused with 2 L of RE HIP or <2 L RE HIP at a ranch in Texas.

***4.a. Categorical variables:*** Number of foals (%).

| | ***Transfused with 2 L RE HIP*** | | |
|---|---|---|---|
| ***Variable*** | ***No*** | ***Yes*** | ***P value**** |
| Born in April or May | 19 (73%) | 5 (62%) | 0.6664 |
| Born before April | 7( 27%) | 3 (38%) | |
| February | 2 (8%) | 1 (12%) | 0.4190 |
| March | 5 (19%) | 2 (25%) | |
| April | 12 (46%) | 5 (63%) | |
| May | 7 (27%) | 0 (0%) | |
| Age-week of diagnosis | | | |
| 5 | 14 (54%) | 3 (38%) | 0.2820 |
| 7 | 9 (35%) | 2 (25%) | |
| 9 | 3 (12%) | 3 (38%) | |

*P values from Fisher's exact test

***2.4.b. Continuous variables:*** Median (Range)

| | ***Transfused with 2 L RE HIP*** | | |
|---|---|---|---|
| ***Variable*** | ***No (n=26 foals)*** | ***Yes (n=8 foals)*** | ***P value**** |
| Age at diagnosis (days) | 41 (33 to 65 ) | 46 (34 to 65) | 0.5022 |
| WBC (cells/µl x $10^3$) | 14.1 (7.6 to 23.8) | 12.8 (9.4 to 16.1) | 0.9208 |
| Fibrinogen (mg/dl) | 615 (370 to 1,070) | 650 (310 to 700) | 0.8550 |
| Largest diameter of any 1 ultrasound lesion (mm) | 27.5 (8.0 to 42.0) | 23.5 (17.0 to 41.0) | 0.3709 |

## 2.4. Discussion

Our findings indicate that transfusion of 2 L of RE HIP was associated with significantly lower cumulative incidence of subclinical pneumonia attributed to *R. equi* than transfusion of < 2 L of RE HIP. This finding is consistent with evidence that antibodies can protect against *R. equi* pneumonia,[40,41] and suggests that transfusion of RE HIP can reduce the incidence of subclinical pneumonia in foals. Although evidence exists that not all foals with subclinical pneumonia require treatment with antimicrobials,[31-33] many farms treat all foals with subclinical lesions. Thus, reducing subclinical pneumonia at farms that use a screen-and-treat approach would reduce antimicrobial use by reducing incidence of subclinical pneumonia. This finding indicates the need to compare the effects of transfusion of 2 L versus 1 L of RE HIP on clinical *R. equi* pneumonia at farms that do not perform screening for detecting subclinical pneumonia to determine the extent to which clinical pneumonia is reduced, thereby reducing the number of foals requiring antimicrobial treatment. It is plausible that the variable clinical efficacy of transfusion of RE HIP is in part explained by a volume of 1 L being lower than optimal for preventing subclinical or clinical pneumonia. Because foals are likely infected early in life[42] when they are more susceptible to infection[43] with *R. equi*, transfusing 2 L early in life rather than 1 L shortly after birth and 1 L at 3 to 4 weeks of age might be a better strategy.

Despite the plausibility of the association of greater protection with a larger dose of RE HIP, a major limitation of this observational study is that we were

unable to differentiate the effects of RE HIP dose (*i.e.*, volume transfused) from whether the foals were race- or ranch-bred. Thus, the observed difference in cumulative incidence of subclinical pneumonia could be attributable to management differences between race- and ranch-bred foals. We consider this to be a less probable explanation for our findings for the following reasons. First, a previous study of several hundred foals from the same ranch during a proximate time-period found no significant difference in the incidence of *R. equi* pneumonia between race- and ranch-bred foals.[44] Second, the principal difference between race- and ranch-bred foals was that the latter were born at pasture. Evidence exists that foals born at pasture such as the ranch-bred foals are less likely to develop *R. equi* pneumonia.[6,45] Thus, the difference in management between race- and ranch-bred foals would have been expected to reduce the incidence of *R. equi* pneumonia (and presumably subclinical pneumonia as well).

It is also plausible that genetic differences between race- and ranch-bred foals resulted in the observed difference in the cumulative incidence of *R. equi* pneumonia between these 2 groups of foals. The genetic polymorphisms associated with *R. equi*, however, have generally been very modest in magnitude,[46] but it would require a very strong genetic association to explain confounding that would result in an association with an odds ratio of nearly 3 (Table 2.3). Thus, we believe the protective effects of transfusion of 2 L of RE HIP relative to transfusion < 2 L of plasma is the more likely explanation for the difference in incidence of subclinical pneumonia than is the effects of race- versus

ranch-breeding. A randomized, controlled study design would provide superior evidence to that from our observational study, and such a study is warranted in light of the findings of this study.

The association of birth-month with subclinical pneumonia was unexpected. Although the reasons for this observation could not be determined, there are a number of plausible explanations. First, it has been suggested that the disease is more prevalent when warmer, drier conditions prevail.[3,5] Second, density of horses is known to be a risk factor for *R. equi* pneumonia.[6,7] Conceivably, greater density of mares and foals later in the breeding season might increase conditions favoring dissemination and inhalation of *R. equi*, including increased airborne concentrations of *R. equi*. The association of birth-months with subclinical *R. equi* pneumonia was not confounded by volume of RE HIP transfused or race- or ranch-breeding, but we cannot exclude confounding by other factors not measured.

This study had other limitations in addition to being unable to differentiate effects of plasma volume from breeding-use. We elected to include the 2 foals that were not transfused. Because neither of these foals developed *R. equi* pneumonia, their inclusion could have only biased our study towards the null (*i.e.*, away from a protective effect for transfusion of 2 L vs < 2 L). Analyses were repeated excluding data for these 2 foals and neither the magnitude (odds ratios increased by < 5%) nor statistical significance of findings were changed. The overall incidence of subclinical pneumonia was lower than expected on the basis

of experiences at this ranch (unpublished data) and other farms,[30-33] but year-to-year variation in endemic farms is expected.[3,28,42] This limited the power of the study. The study was conducted only at a single ranch during a single year; extrapolating results to other farms/ranches or even the same ranch in different years should be made with caution. The diagnosis of *R. equi* pneumonia was presumptive and not based on results of microbiologic culture. Consequently, other causes of pneumonia cannot be excluded. Some farms transfuse foals during the first day or 2 of life and then again between ages 3 and 4 weeks. This practice was not evaluated in this study.

To our knowledge, this is the first report describing potential benefits of RE HIP for reducing the severity of subclinical pneumonia. If true, this is important because reducing the proportion of foals with subclinical pneumonia will reduce the proportion of foals treated with macrolides. Such mass treatment with antimicrobials has been linked to emergence of macrolide resistance.[27,28] Despite the limitations of this observational study, it provides impetus for additional investigations regarding the impact of dose/volume of RE HIP for controlling *R. equi* pneumonia, and the need for randomized, controlled studies to evaluate the impact of transfusion of RE HIP on subclinical pneumonia.

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# 3. ANTIBODY ACTIVITIES IN HYPERIMMUNE PLASMA AGAINST THE *RHODOCOCCUS EQUI* VIRULENCE- ASSOCIATED PROTEIN A OR POLY-*N*-ACETYL GLUCOSAMINE ARE ASSOCIATED WITH PROTECTION OF FOALS AGAINST RHODOCOCCAL PNEUMONIA[3]*

## 3.1. Introduction

*Rhodococcus equi* (*R. equi*) is a common cause of severe pneumonia in foals.[1-5] Virulent strains of this facultative, intracellular pathogen contain a plasmid that encodes for the virulence-associated protein A (VapA) that is necessary for bacterial replication in macrophages.[6] Pneumonia caused by *R. equi* is endemic at many horse-breeding farms, with annual cumulative incidence at farms often affecting 20% to 40% of the foal population.[7-9] At endemic farms, costs can be high for treatment, veterinary care, long-term therapy, and lost revenue from deaths of foals infected with *R. equi*. In addition to these immediate costs, *R. equi* pneumonia has a long-term detrimental effect to the equine industry because foals that recover from the disease are less likely to race as adults.[10]

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Pneumonia caused by *R. equi* is recognized as either clinical or subclinical forms.[11,12] The clinical form of *R. equi* pneumonia has an insidious progression: pathological changes in the lungs are well-advanced by the time clinical signs develop.[3,4,6] The subclinical form of *R. equi* pneumonia is characterized by the presence of pulmonary consolidations or abscesses identified by thoracic ultrasonography performed as a screening test at endemic farms in the absence of overt clinical signs of pneumonia.[11,12] Foals with ultrasonographically-identified pulmonary lesions greater than a certain threshold of a maximal diameter (*e.g.*, ≥ 2 cm of maximum diameter) but lacking other clinical signs are often treated with antimicrobials.[12] The rationale for this screen-and-treat approach is that it will reduce mortality and duration of treatment of foals at endemic farms.[12]

Methods for preventing *R. equi* pneumonia include chemoprophylaxis, vaccination, and administration of hyperimmune plasma (HIP).[13-30] Of these, the only USDA-approved and well-established method for reducing the incidence of *R. equi* pneumonia is transfusion of HIP from equine donors hyperimmunized against *R. equi* (RE HIP).[13-15,17] In addition to RE HIP, we recently demonstrated that transfusing foals between 12 to 24 hours after birth with plasma from donors hyperimmunized against the bacterial capsular polysaccharide β-1→6-poly-*N*-acetyl glucosamine (PNAG) prevented *R. equi* pneumonia following intra-bronchial infection at age ~28 days, whereas transfusion with commercial plasma from donor horses that were not hyperimmunized against either PNAG or *R. equi* and that had only background levels of antibody activity against PNAG and VapA

failed to protect foals similarly infected.[31] Additionally, our laboratory has shown that PNAG HIP is superior to both RE HIP and standard plasma at mediating opsonophagocytic killing of *R. equi* by equine neutrophils.[32] Fixation of the complement component 1q (C´1q) to the PNAG antigen with vaccination-derived antibodies is considered essential to the functional activity of these antibodies both *in vitro* and within sera of foals receiving anti-PNAG antibodies via passive transfer from vaccinated dams.[31,32]

Evidence of the effectiveness of HIP for reducing the incidence of *R. equi* pneumonia under field conditions, however, remains variable and conflicting,[12-16,28,33] and in the case of PNAG HIP is lacking. One possible explanation for the irregular effectiveness of RE HIP under field conditions is variable dosing. Results of observational studies indicate that administration of 2 L of RE HIP to foals is superior to administration of 1 L for reducing the cumulative incidence of clinical or subclinical pneumonia.[29,30] Moreover, the activity of *R. equi*-specific antibody varies among manufacturers and among lots/batches within manufacturers.[34] Collectively, these findings indicate that variation in the amount of antibody transfused to a foal is inversely related to the risk of pneumonia developing in that foal. Specific evidence of an association between antibody activities in transfused foals and protection against pneumonia, however, is limited. Thus, we conducted a randomized, controlled, double-masked field trial to examine the association between disease outcome and relative antibody activities (*i.e.*, ratio of optical density [OD] of sample to OD of positive control) to the virulence associated

protein A (VapA) of *R. equi* and PNAG, and activity of deposition of C′1q onto PNAG among foals randomly assigned to be transfused with 2 L of either RE HIP or PNAG HIP at 2 large breeding farms where *R. equi* pneumonia is endemic and where transfusion of RE HIP was historically used to control *R. equi* pneumonia. These farms differed in their diagnostic approach: Farm A did not use screening to identify foals prior to the onset of clinical signs (*i.e.*, diagnosis of *R. equi* pneumonia was based on detecting clinical signs of pneumonia), whereas Farm B used a combination of results of thoracic ultrasonographic screening and complete blood counts (CBC) to identify foals with pulmonary lesions and abnormal findings of CBC for presumptive diagnosis of subclinical *R. equi* pneumonia. We hypothesized that the cumulative incidence of clinical and subclinical *R. equi* pneumonia would be significantly lower either among foals transfused with RE HIP that had higher relative antibody activities to VapA, or among foals transfused with PNAG HIP that had higher antibody activities to PNAG or C′1q deposition onto PNAG.

## 3.2. Materials and Methods

### 3.2.1. Study Population

The study was approved by Texas A&M University's Institutional Animal Care and Use Committee and the Clinical Research Review Committee of the Texas A&M University's College of Veterinary Medicine & Biomedical Sciences (Animal Use Protocol 2018-0429), and included signed informed consent from either the owner or agent of the owner for all study foals. The study was

conducted during the 2019 foaling season and included foals from 2 large breeding farms (Farm A and Farm B) that had a history of cumulative incidence of *R. equi* pneumonia $\geq$ 20% per foaling season over the preceding 5 years, and at which RE HIP was used historically to control pneumonia caused by *R. equi*. Each farm was known to have >150 foals born annually that resided through weaning at the farm. Diagnosis of presumed *R. equi* pneumonia among foals at Farm A was made on the basis of clinical signs (*i.e.*, **clinical pneumonia**), whereas diagnosis of presumed *R. equi* pneumonia at Farm B was made on the basis of results of thoracic ultrasonographic screening and specific abnormal findings of CBCs (*i.e.*, **subclinical pneumonia**). These farms were intentionally selected to allow us to examine the association of serum activity against antigens of interest with pneumonia among foals diagnosed with either clinical or subclinical pneumonia because both approaches are commonly used in private equine practice.[35] To be eligible for inclusion in the study, foals were required to have been healthy at birth and to have evidence of adequate passive transfer of immunoglobulins based on a commercial test kit (SNAP Foal IgG test, IDEXX, Inc.). At each farm, a total of 120 healthy Quarter Horse foals (n = 240 total foals) were randomly assigned in equal numbers using a blocked design to 1 of 2 groups: Group 1 received PNAG HIP (n = 60 per farm) and Group 2 received RE HIP (n = 60 per farm). This sample size was based on the number of foals available at each farm, and the number of liters of PNAG HIP available to the investigators. To the authors' knowledge, published data regarding the distribution

of specific antibody levels immediately post-transfusion among foals transfused with either RE HIP or PNAG HIP were not available for *a priori* sample size calculations.

### 3.2.2. Transfusions and Clinical Evaluation

Each foal was transfused with 2 L (approximately 40 mL/kg of body weight) of plasma within 24 hours of birth by trained veterinary technical staff or veterinarians at each farm. Foals were transfused with either RE HIP or PNAG HIP from a single manufacturer (Mg Biologics, Inc., Ames, IA). The RE HIP was derived from donor horses hyperimmunized using a propriety method against *R. equi* and the PNAG HIP was derived from donor horses hyperimmunized, using a propriety method, with a conjugate vaccine composed of pentamers of β-1-6-linked glucosamine covalently linked to tetanus toxoid as a carrier protein ($5GlcNH_2$-TT).[31] Plasma was labeled by the manufacturer as either 1 or 0 in order to mask the identity of the plasma both to those individuals transfusing foals at farms and to those performing data analysis. Treatment order (*i.e.*, allocation sequence) at each farm was pre-assigned randomly by investigators at Texas A&M University prior to the foaling season based on expected foaling dates of mares, and this treatment order was sent to the farm veterinarians prior to initiation of the study. Serum samples (4 mL) were collected immediately post-transfusion from the jugular vein contralateral to the jugular vein used for transfusion. These sera were used to determine relative antibody activities in the foals' sera as described below.

At Farm A, foals were monitored by the farm veterinary medical and veterinary technical staff at least twice daily for signs of clinical pneumonia. These signs included lethargy, coughing, depressed attitude, increased respiratory rate (> 60 breaths/minute) or effort (abdominal lift, flaring nostrils), and extra-pulmonary manifestations of *R. equi* infection such as polysynovitis or uveitis. Foals were diagnosed with presumed *R. equi* pneumonia if they had all of the following: 1) cough; 2) fever (rectal temperature > 39.4°C); 3) lethargy or tachypnea or dyspnea; and 4) ultrasonographic evidence of pulmonary abscess(es) or consolidation(s) ≥ 2 cm in maximal diameter. Any foals found to have clinical signs of pneumonia were tested by complete blood count (CBC) and thoracic ultrasonography performed by the farm veterinarians. As noted previously, the veterinarians diagnosing the foals were masked to the identity of the plasma transfused to individual foals. Foals that developed pneumonia were treated with clarithromycin (7.5 mg/kg; orally; q 12 h) and rifampicin (5 mg/kg; orally; q 12 h) until resolution of clinical signs and thoracic ultrasonographic lesions. Medical records, including reports of all findings and treatments, were maintained daily for each individual foal.

At Farm B, thoracic ultrasonography was performed on all foals at ages 5, 7, and 9 weeks by farm veterinarians to examine the lungs for pulmonary abscesses or consolidations. If foals had consolidations or abscesses ≥ 2 cm in maximal diameter and increased concentrations of white blood cells, neutrophils, or fibrinogen detected from results of CBCs performed concurrently with thoracic

ultrasound screening examinations, they were treated for presumed subclinical *R. equi* pneumonia with azithromycin (10 mg/kg; orally; q 24 h) and rifampicin (5 mg/kg; orally; q 12 h) until resolution of thoracic ultrasonographic lesions. Medical records, including reports of all findings and treatments, were maintained daily for each individual foal.

At the end of the season, the medical records from both farms were reviewed, and the proportion of foals that developed pneumonia attributed to *R. equi* (either clinical at Farm A or subclinical at Farm B) was determined. Diagnosis of *R. equi* pneumonia was determined prior to data analysis, and data analysis was performed prior to the unmasking of plasma type. In addition to the health data, the following information was collected for each foal: 1) date of birth; 2) sex; 3) results of semiquantitative blood concentration of immunoglobulin G; 4) whether there was a reaction to transfusion with hyperimmune plasma.

### 3.2.3. Immunoglobulin ELISA

Serum samples from study foals were tested by enzyme-linked immunoassay (ELISA) for relative activities of antibodies against PNAG and the VapA protein of *R. equi*. ELISA plates (Maxisorp, Thermo Scientific, Rochester, NY, USA) were coated with either 0.6 µg/ml of purified PNAG or 0.5 µg/ml purified VapA diluted in sensitization buffer (0.04M $PO_4$, pH 7.2) overnight at 4 ℃.[31] Plates were washed 3 times with PBS containing 0.05% Tween 20, blocked with 120 µl of PBS containing 1% skim milk for 1 hour at 37 ℃, and washed again. Foal serum samples (100 µl) were added in duplicate to wells of the ELISA plate and

incubated for 1 hour at 37℃. Serum samples were initially diluted in the incubation buffer (PBS with 1% skim milk and 0.05% Tween 20) to 1:100. A sample each of PNAG HIP and of RE HIP were included in each ELISA plate as controls: for the PNAG ELISA, the PNAG HIP was the positive control and the RE HIP was the negative control, and for the VapA ELISA the RE HIP was the positive control and the PNAG HIP was the negative control. Plates were washed again, then 100 µl per well of anti-horse IgG conjugated to HRP (Bethyl Laboratories, Montgomery, TX, USA, diluted at 1:30,000) was added to the wells. Plates were incubated for 1 hour at room temperature and then washed again. SureBlue Reserve One Component TMB Microwell Peroxidase Substrate (SeraCare, Gaithersburg, MD, USA) was added to the wells for 2 minutes. The reaction was stopped by adding sulfuric acid solution to the wells. Optical densities (ODs) were determined at a wavelength of 450 nm by using a microplate reader. The relative activity of antibody was calculated by dividing the individual sample OD values by that of the respective positive control from the same plate, which we defined as the **OD ratio**.

### 3.2.4. C´1q deposition assays

The rationale for testing deposition of C´1q onto PNAG is that it is a functional assay: anti-PNAG antibodies require complement deposition to mediate their opsonic killing, and not all antibodies to PNAG fix complement.[31,32] An ELISA targeting the C´1q component of C´1 (C´1q) was used to determine the serum endpoint activities for deposition of equine C´1 onto purified PNAG. ELISA plates

were sensitized with 0.6 µg PNAG/ml and blocked with skim milk as described above. Dilutions of different foal sera were added in 50-µl volumes, after which 50 µl of 10% intact, normal horse serum were added as a source of C´1q. After 60 minutes of incubation at 37℃, plates were washed and 100 µl of goat anti-human C´1q, which also binds to equine C´1q, diluted 1:1,000 in incubation buffer was added and plates were incubated at room temperature for 60 minutes. After washing, 100 µl of rabbit anti-goat IgG whole molecule conjugated to alkaline phosphatase and diluted 1:1,000 in incubation buffer was added, and then incubated for 1 hour at room temperature. Washing and developing of the color indicator was then performed by adding p-nitrophenyl phosphate substrate and color development determined after 60 min at room temperature. $OD_{405}$nm values of this highest serum dilution tested were used to determine relative activity. Negative OD values after background subtraction (no primary antibody added) indicated sera with less activity than this control.[31]

**3.2.5. Data analysis**

We first compared the OD ratios of VapA, and PNAG and OD activities for C´1q deposition onto PNAG between foals transfused with RE HIP and those transfused with PNAG HIP using the generalized linear modeling (glm) function in R (version 3.6.1) and an identity link, with OD ratios (for VapA and PNAG) or relative OD (for C´1q) as the dependent (outcome) variable and plasma group as the independent variable. The purpose of this analysis was to ensure that antibody activities differed significantly between groups as expected (*e.g.*,

significantly higher VapA antibody OD ratios among foals transfused with RE HIP than among foals transfused with PNAG HIP), as a measure of internal validity of the study. The primary questions of interest for this study were whether the OD ratios of VapA *among RE HIP-transfused foals* were significantly lower among foals that developed pneumonia, and whether the OD ratios against PNAG and or relative OD of C′1q *among PNAG HIP-transfused foals* were significantly lower among foals that developed pneumonia. Data were analyzed using multivariable logistic regression within the glm function in R using a logit link, with pneumonia as the binary outcome variable and relative antibody activity level for a given antigen and farm as dependent variables. For purposes of analysis, antibody activities were analyzed as a binary variable using the median OD ratio/relative OD among foals transfused with a given plasma (*e.g.*, median VapA OD ratio among foals transfused with RE HIP). The median was used for purposes of simplicity of analysis, and was not selected as a diagnostic cut-point for protection against pneumonia. Farm was included in the models to account for the potential effects of differences between farms in the method of diagnosis. Although not a primary aim of the study, we also compared the 2 different plasmas (RE HIP and PNAG HIP) for protection against pneumonia using multivariable logistic regression within the glm function in R with logit link, with pneumonia as the binary outcome and plasma type and farm as dependent variables. All data analysis was performed using the R program (Version 3.6.1, R Core Team, Vienna, Austria).

Significance for all analyses was set at $P < 0.05$, and 95% confidence intervals (95% CI) were estimated using maximum likelihood methods.

### 3.3. Results

#### 3.3.1. Study Population

Farm A had a total of 119 foals included in the project; 60 foals were transfused with RE HIP and 59 were transfused with PNAG HIP. One foal at Farm A was lost to follow up due to complications associated with neonatal isoerythrolysis. Data from this foal was excluded from analysis. Farm B had a total of 114 foals included; 57 foals were transfused with RE HIP (plasma 0), and 57 foals were transfused with PNAG HIP (plasma 1). Six of the 120 eligible foals from Farm B were excluded because of an unanticipated shortfall of plasma production by the manufacturer. Among foals at both farms transfused with RE HIP, the median OD ratio of VapA antibodies was 0.88 (range, 0.64 to 1.09). Among foals at both farms transfused with PNAG HIP, the median OD ratio of PNAG antibodies was 0.54 (range, 0.29 to 0.75) and the median relative $OD_{405}$nm for C′1q deposition was 1.01 (range, 0.07 to 2.99). These median values were used as cut-points to create binary variables (*i.e.*, high versus low) for antibody levels for use in logistic regression modeling. At both farms, OD ratios of VapA antibodies were significantly ($P < 0.05$) higher among foals transfused with RE HIP than with PNAG HIP (Figure 3.1). Similarly, the OD ratios for PNAG antibodies and relative OD values for C′1q deposition were significantly ($P < 0.05$) higher among foals transfused with PNAG HIP than RE HIP (Figures 3.2 and 3.3).

None of the foals at either farm was noted to have an adverse reaction to transfusion. No deaths of foals at either farm were attributed to *R. equi* pneumonia.

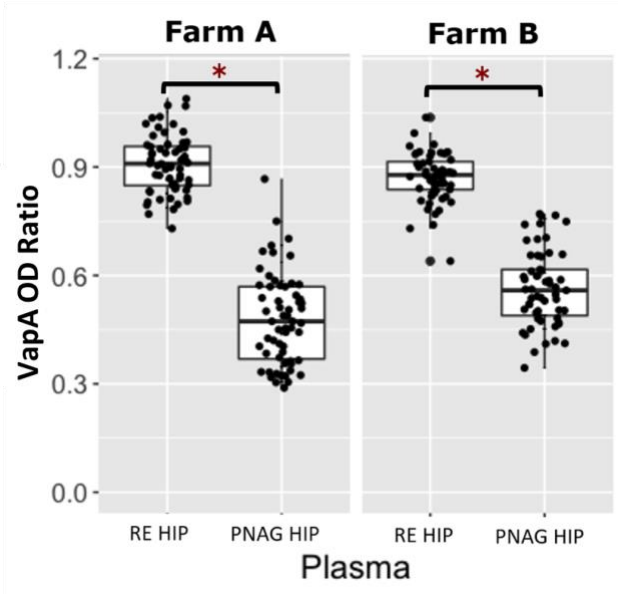


**Figure 3.1. Comparison of VapA optical density ratios between *R. equi* hyperimmune plasma and PNAG hyperimmune plasma.**

Boxplots of optical density (OD) ratios from ELISAs measuring deposition onto purified VapA protein by serum antibodies in 119 foals from Farm A and 114 foals from Farm B stratified by plasma type, faceted by farm. Foals transfused with *R. equi* hyperimmune plasma (RE HIP) at both farms had significantly higher (asterisks represent P<0.05) VapA OD ratios than foals that were transfused with β-1→6 poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP). The mean (95% CI) OD ratio of anti-VapA antibodies among foals at Farm A and B were 0.91 (0.89 to 0.93) and 0.87 (0.85 to 0.89), respectively among foals transfused with RE HIP and were 0.48 (0.45 to 0.52) and 0.56 (0.54 to 0.59), respectively, among foals transfused with PNAG HIP.

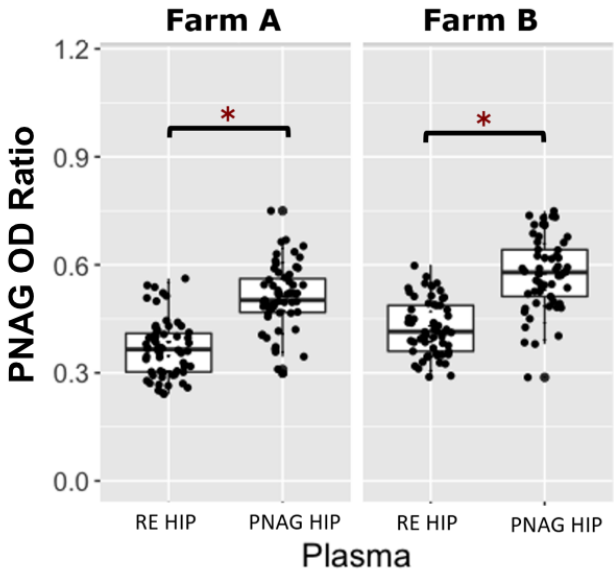


**Figure 3.2. Comparison of PNAG optical density ratios between *R. equi* hyperimmune plasma and PNAG hyperimmune plasma.**

Boxplots of optical density (OD) ratios from ELISAs measuring deposition onto β-1→6 poly-*N*-acetyl glucosamine (PNAG) by serum antibodies in 119 foals from Farm A and 114 foals from Farm B, stratified by plasma type, faceted by farm. Foals transfused with PNAG hyperimmune plasma (PNAG HIP) at both farms had significantly higher (asterisks represent $P < 0.05$) OD ratios for PNAG than foals that were transfused with *R. equi* hyperimmune plasma (RE HIP). Statistical significance is indicated by asterisks. The mean (95% CI) OD ratio of anti-PNAG antibodies among foals at Farm A and B were 0.51 (0.49 to 0.53) and 0.58 (0.55 to 0.60), respectively among foals transfused with PNAG HIP and were 0.37 (0.35 to 0.39) and 0.43 (0.40 to 0.43), respectively, among foals transfused with PNAG HIP.

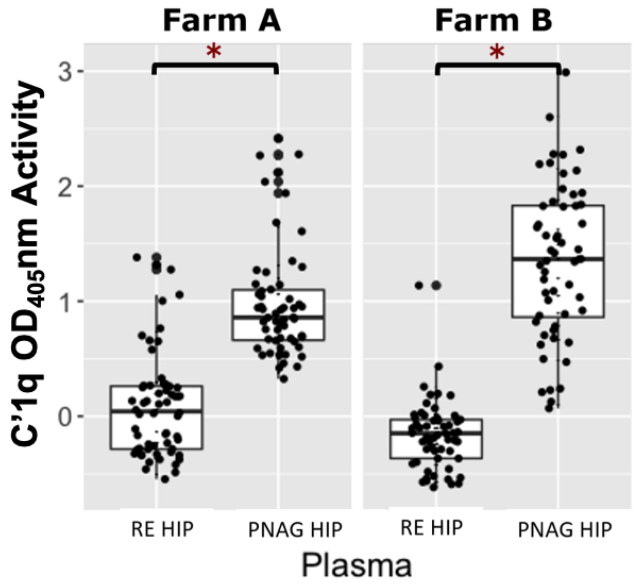


**Figure 3.3. Comparison of C′1q relative optical density between *R. equi* hyperimmune plasma and PNAG hyperimmune plasma.**

Boxplot of relative optical density ($OD_{405}$nm) activities for deposition of complement component 1 (C′1q) onto β-1→6 poly-*N*-acetyl glucosamine (PNAG) by serum antibodies in 119 foals from Farm A and 114 foals from Farm B, stratified by plasma type, faceted by farm. Foals transfused with PNAG hyperimmune plasma (PNAG HIP) at both farms had significantly higher (asterisks represent $P < 0.05$) C′1q $OD_{405}$nm activities than foals that were transfused with *R. equi* hyperimmune plasma (RE HIP). The mean (95% CI) OD activity of C′1q antibodies among foals at Farm A and B were 0.98 (0.85 to 1.11) and 1.35 (1.17 to 1.53), respectively among foals transfused with PNAG HIP and were 0.09 (-0.03 to 0.21) and -0.17 (-0.24 to 0.09), respectively, among foals transfused with PNAG HIP.

### 3.3.2. VapA Antibody OD Ratios Following RE HIP Transfusion

Of the 233 foals from both farms, 117 foals were transfused with RE HIP. Of those 117 foals transfused with RE HIP, 29% (34/117) developed either clinical or subclinical pneumonia and 71% (84/117) remained healthy. For logistic regression modeling, a low level of antibody activity against VapA was defined as an OD ratio ≤ 0.89 (the population median) and a high level of antibody activity against VapA was defined as OD ratio > 0.89. The proportion of foals that developed pneumonia among foals with a low level of VapA activity was 40% (24/60), whereas the proportion of foals that developed pneumonia among foals with a high level of VapA activity was 18% (10/57). The proportion of foals transfused with RE HIP that developed pneumonia at Farm A was 42% (25/60) whereas the proportion that developed pneumonia at Farm B was only 16% (9/57). Using multivariable logistic regression analysis of the RE HIP-transfused foals, the odds of pneumonia were approximately 6-fold higher (P = 0.0005) among foals with a low level of VapA antibody activity relative to foals with a high level of VapA antibody activity, accounting for effects of farm (Figure 3.4, Table 3.1). Using multivariable logistic regression, the odds of pneumonia among foals transfused with RE HIP were approximately 7-fold higher (P = 0.0002) for foals at Farm A than for Farm B, accounting for effects of VapA antibody activity (Fig 3.4, Table 3.1).

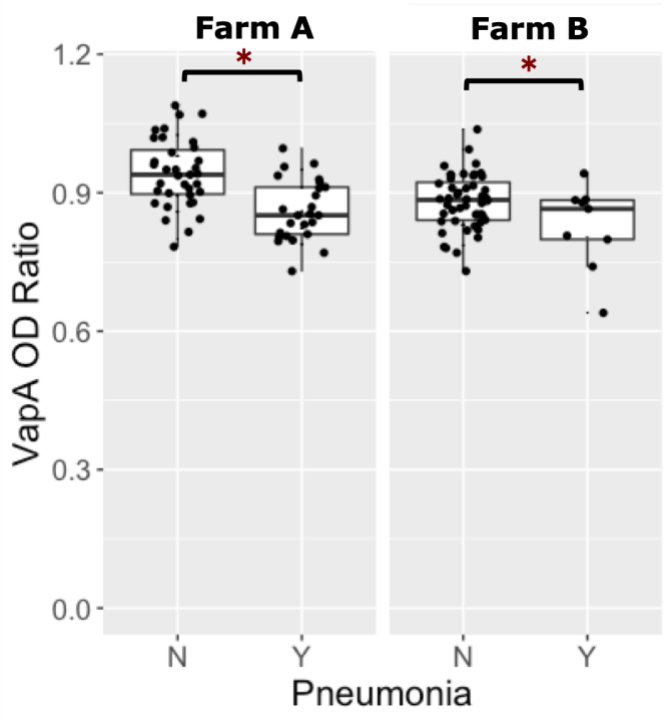


**Figure 3.4. Foals transfused with *R. equi* hyperimmune plasma and their comparison of VapA optical density ratios with whether they developed pneumonia or remained healthy**

Boxplots of optical density (OD) ratios from ELISAs measuring deposition onto purified VapA protein by serum antibodies in 117 foals transfused with *R. equi* hyperimmune plasma (RE HIP) stratified by pneumonia status, faceted by farm. Foals transfused with RE HIP at both farms that remained healthy (no pneumonia = 'N') had significantly higher (asterisks represent $P<0.05$) VapA OD ratios than foals that developed pneumonia (pneumonia = 'Y').

**Table 3.1. Odds ratios for the outcome of pneumonia associated with VapA activity-level and farm**

Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *R. equi* among 117 foals transfused with *R. equi* hyperimmune plasma (RE HIP) at both Farm A and Farm B. A binary VapA activity-level variable was created using the median value of VapA of the optical density (OD) ratio for the group of foals was included in the model as well as a variable of farm to account for the potential effects of differences between farms in the method of diagnosis.

| Variable | Odds Ratio (95% CI) | P Value |
|---|---|---|
| VapA OD ratio | | |
| High (OD ratio > 0.89) | 1 (NA) | NA |
| Low (OD ratio ≤ 0.89) | 5.95 (2.17-16.13) | 0.000524 |
| | | |
| Farm | | |
| Farm B | 1 (NA) | NA |
| Farm A | 6.94 (2.49- 19.23) | 0.000201 |

### 3.3.3. PNAG Antibody OD Ratios Following PNAG HIP Transfusion

Of the 233 foals from both farms, 116 were transfused with PNAG HIP. Of the 116 foals transfused with PNAG HIP, 22% (25/116) developed pneumonia. For logistic regression modeling, a low level of antibody activity against PNAG was defined as an OD ratio ≤ 0.54 (the population median) and a high level of antibody activity against PNAG was defined as an OD ratio > 0.54. The proportion of foals that developed pneumonia among foals with low PNAG antibody activity was 31% (18/58), whereas the proportion of foals with pneumonia among foals with high level of antibody activity against PNAG OD ratios was 12% (7/58). Among foals transfused with PNAG HIP, at Farm A 27% (16/59) developed pneumonia compared with 16% (9/57) of foals at Farm B. Using multivariable logistic regression analysis of the PNAG HIP-transfused foals, the odds of pneumonia were approximately 3-fold higher (P = 0.0005) among foals with a low level of antibody activity against PNAG relative to foals with a high level of antibody activity against PNAG, accounting for effects of farm; the odds of pneumonia, however, did not differ significantly (P = 0.4174) between farms (Figure 3.5, Table 3.2).

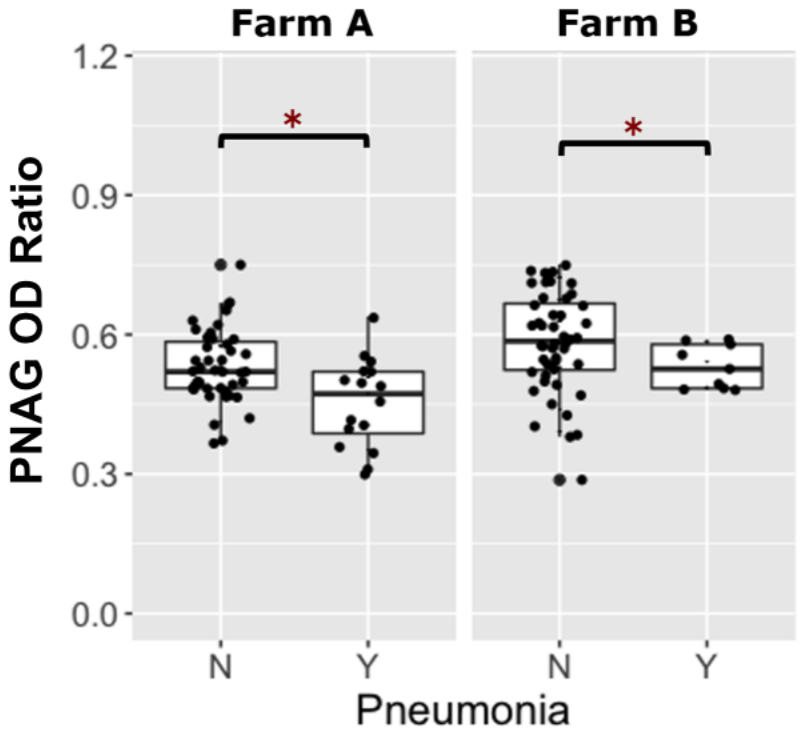


**Figure 3.5. Foals transfused with PNAG hyperimmune plasma and their comparison of PNAG optical density ratios with whether they developed pneumonia or remained healthy**

Boxplots of optical density (OD) ratios from ELISAs measuring deposition onto purified β-1→6 poly-*N*-acetyl glucosamine (PNAG) by serum antibodies in 116 foals transfused with PNAG hyperimmune plasma (PNAG HIP) stratified by pneumonia status, faceted by farm. Foals transfused with PNAG HIP at both farms that remained healthy (no pneumonia = 'N') had significantly higher (asterisks represent $P<0.05$) PNAG OD ratios than foals that developed pneumonia (pneumonia = 'Y').

**Table 3.2. Odds ratios for the outcome of pneumonia associated with PNAG activity-level and farm**

Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *R. equi* among 116 foals transfused with β-1→6 poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP) at both Farm A and Farm B. A binary PNAG activity-level variable was created using the median value of PNAG optical density (OD) ratio for the group of foals was included in the model as well as a variable of farm to account for the potential effects of differences between farms in the method of diagnosis.

| Variable | Odds Ratio (95% CI) | P Value |
|---|---|---|
| PNAG OD ratio | | |
| High (OD ratio > 0.54) | 1 (NA) | NA |
| Low (OD ratio ≤ 0.54) | 2.94 (1.08 - 8.65) | 0.0347 |
| | | |
| Farm | | |
| Farm B | 1 (NA) | NA |
| Farm A | 1.49 (0.57 - 3.91) | 0.4174 |

### 3.3.4. C´1q Activity

For logistic regression modeling, a low level of C´1q deposition activity was defined as a relative $OD_{405}$nm activity of ≤ 1.01 (the population median) and a high activity level was defined as a relative $OD_{405}$nm activity > 1.01. The proportion of foals that developed pneumonia among those with low C´1q activities was 39% (22/57), whereas the proportion of foals with pneumonia among foals with high C´1q activities was 5% (3/57). As noted above, at Farm A, 27% (16/59) of foals transfused with PNAG HIP developed pneumonia compared with 16% (9/57) of foals receiving PNAG HIP at Farm B. Using multivariable logistic regression analysis of the PNAG HIP-transfused foals, the odds of pneumonia were approximately 11-fold higher (P = 0.0003) among foals with low C´1q activities (*i.e.*, ≤ 1.01) relative to foals with high activities for C´1q deposition; the odds of pneumonia, however, did not differ significantly (P = 0.9777) between farms (Figure 3.6, Table 3.3).

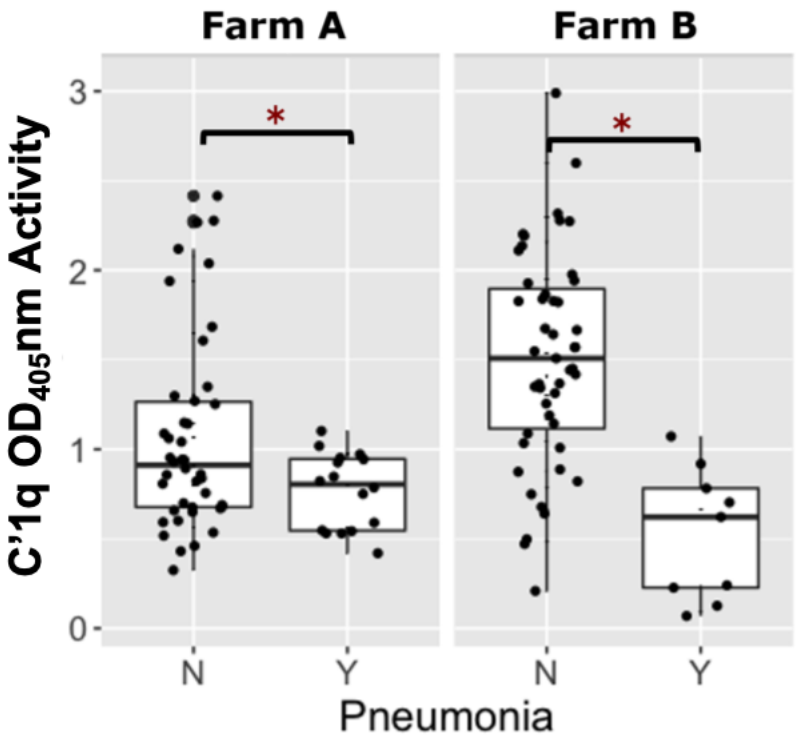


**Figure 3.6. Foals transfused with PNAG hyperimmune plasma and the comparison of C´1q activity with whether they developed pneumonia or remained healthy**

Boxplot of optical density ($OD_{405}$nm) activities for complement component 1q (C´1q) deposition onto PNAG by serum antibodies in 116 foals transfused with β-1→6 poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP) stratified by pneumonia status, faceted by farm. Foals transfused with PNAG HIP at both farms that remained healthy (no pneumonia = 'N') had significantly higher (asterisks indicate $P < 0.05$) C´1q deposition activities than foals that developed pneumonia (pneumonia = 'Y').

**Table 3.3. Odds ratios for the outcome of pneumonia associated with C´1q activity-level and farm**

Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *R. equi* among 114 foals transfused with β-1→6 poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP) at both Farm A and Farm B. A binary variable for C´1q $OD_{405}$nm relative activity that was created using the median value of C´1q $OD_{405}$nm relative activity for the population of foals as a cut-point was included in the model, as well as a variable of farm to account for the potential effects of differences between farms in the method of diagnosis.

| Variable | Odds Ratio (95% CI) | P Value |
|---|---|---|
| C´1q $OD_{405}$nm activity Level | | |
| High (OD ratio >1.01) | 1 (NA) | NA |
| Low (OD ratio ≤ 1.01) | 11.37 (3.00 - 43.48) | 0.0003 |
| | | |
| Farm | | |
| Farm B | 1 (NA) | NA |
| Farm A | 0.99 (0.35 - 2.79) | 0.9777 |

### 3.3.5. Association of Plasma Type with Pneumonia

Of the 233 foals from both farms, 117 were transfused with RE HIP and 116 were transfused with PNAG HIP. The proportion of foals that developed pneumonia was 29% (34/117) among foals transfused with RE HIP and 21% (25/116) among foals transfused with PNAG HIP (Figure 3.7). Using multivariable logistic regression with pneumonia as the binary outcome and plasma type and farm as dependent variables, the odds of pneumonia in foals transfused with RE HIP were not significantly higher among foals transfused with PNAG HIP (OR= 1.5, 95% CI, 0.82 to 2.79; P = 0.1832) relative to foals transfused with RE HIP, adjusted for effects of farm. However, the odds of pneumonia were approximately 2.8-fold higher (P = 0.0013) for foals at Farm A than for foals at Farm B (Table 3.4): 34% (41/119) of foals at Farm A developed pneumonia compared to 16% (18/114) at Farm B (Figure 3.8).

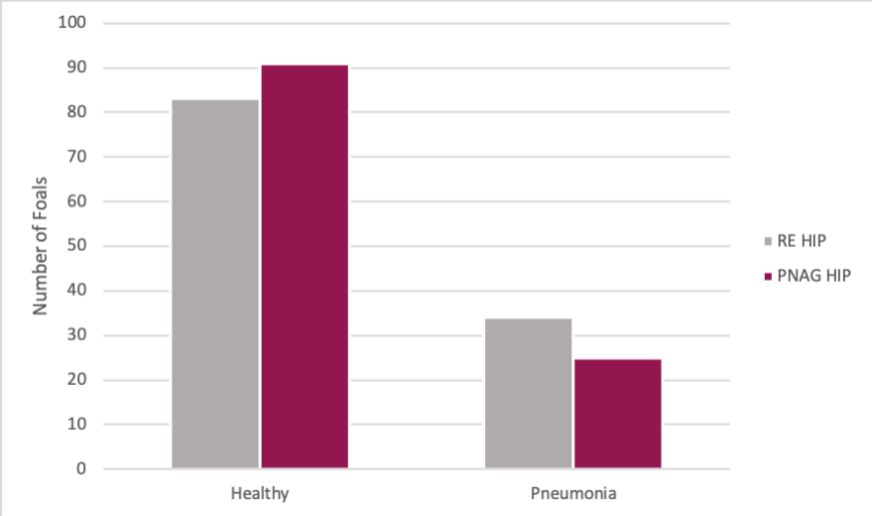


**Figure 3.7. Distribution of foals that were transfused with *R. equi* hyperimmune plasma or PNAG hyperimmune plasma and whether they developed pneumonia or remained healthy**

The distribution of foals transfused with either *R. equi* hyperimmune plasma (RE HIP), n=117 foals in grey, and β-1→6-linked poly-*N*-acetyl-glucosamine hyperimmune plasma (PNAG HIP), n=116 foals in maroon. On the x axis is whether they remained healthy or developed pneumonia. In the RE HIP transfused foals 29% (34/117) foals developed pneumonia compared to the PNAG HIP transfused foals 21% (25/116) foals developed pneumonia.

**Table 3.4. Odds ratios of developing pneumonia associated with type of plasma foal was transfused with and farm**

Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *R. equi* among 233 foals transfused with either *R. equi* hyperimmune plasma (RE HIP) or β-1→6 poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP) at both Farm A and Farm B.

| Variable | Odds Ratio (95% CI) | P Value |
|---|---|---|
| Plasma Type | | |
| PNAG HIP | 1 (NA) | NA |
| RE HIP | 1.51 (0.82 - 2.79) | 0.1832 |
| | | |
| Farm | | |
| Farm B | 1 (NA) | NA |
| Farm A | 2.82 (1.50 - 5.32) | 0.0013 |

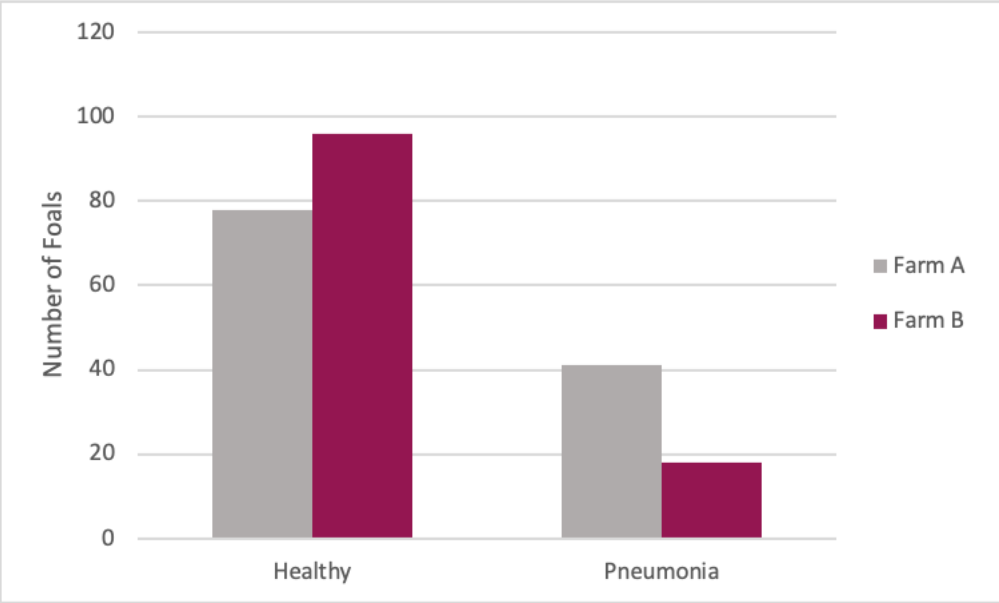


**Figure 3.8. Distribution of foals that developed pneumonia by farm of origin**

The distribution of 233 foals that remained healthy or developed pneumonia stratified by Farm on the X axis. At Farm A in grey, 34% (41/119) of foals developed pneumonia and Farm B in maroon, 16% (18/114) of foals developed pneumonia.

### 3.4. Discussion

The odds of developing either clinical or subclinical *R. equi* pneumonia were inversely associated with antibody activities against VapA among foals transfused with RE HIP, and with antibody activities against PNAG and C´1q among foals transfused with PNAG HIP. The validity of these results is supported by prior studies demonstrating that antibodies to PNAG protect against

experimental intrabronchial infection of foals with *R. equi*,[31] and evidence that plasma with high relative antibody activity against VapA is protective against *R. equi* infection.[13, 36, 37] These findings are important for equine veterinarians and equine farm managers because they provide further evidence of the effectiveness of transfusion of RE HIP and PNAG HIP to protect foals against *R. equi* pneumonia and because HIP remains the only USDA-approved method for controlling *R. equi* pneumonia at endemic equine breeding farms.

The finding that VapA and PNAG antibody levels and the relative deposition of C´1q onto PNAG appears to be a useful correlate of protective immunity and could be an important guide for plasma production, ideally leading to improved consistency and quality of plasma produced by manufacturers. Variation in IgG antibody activity in RE HIP both among and within lots of products from each of 3 different commercial manufacturers has been documented;[34] the coefficient of variation was as high as 107% for VapA-specific IgGa among lots.[34] In this study, we collected serum samples from transfused foals, but regrettably we did not have samples from the plasma lots post-thawing for transfusion for antibody measurements. Consequently, we cannot differentiate how much of the variability among foals in relative activity of antibodies against VapA, PNAG, and C´1q deposition was attributable to variation among lots of plasma or to other factors such as plasma handling prior to transfusion, timing of serum sample collection relative to transfusion, and foal-level factors. For example, thawing plasma at too high of a temperature could result in denaturing immunoglobulins.

Although we asked farms to collect serum samples immediately post-transfusion, it is possible that there was some variation among foals in the timing of collection that contributed to the observed variation in activity of antibodies among foals. Finally, variability among individual foals in volume of distribution and the background activity of antibodies against VapA or PNAG transferred from mares to foals via colostrum could have contributed to the varying OD ratios among foals. This variability in antibody activities among foals, however, enabled us to document that higher values of activity were positively associated with protection against pneumonia in foals.

The finding that antibodies delivered by transfusion can protect foals in an activity-dependent manner suggests that maternal vaccination that results in high colostral levels can be effective for protecting foals against *R. equi*.[21, 28] Plasma transfusion will, however, remain an important and commonly practiced method for preventing *R. equi* pneumonia even if a vaccine for *R. equi* pneumonia is developed because not all pregnant mares will be vaccinated or produce high-quality colostrum, and not all foals of vaccinated mares will absorb adequate colostrum.

Among PNAG-transfused foals, activities for C´1q deposition were a stronger predictor of pneumonia than antibody activities against PNAG. C´1 is the initiating protein of the classical complement cascade,[38] and it is activated when the immunoglobulins specific to PNAG bind to this portion of the C1qrs molecule.[31,38] Thus, C´1q deposition reflects not merely the amount but the

functionality of antibodies. Our findings suggest that the relative levels of functional antibodies measured by assaying C′1q deposition onto PNAG is a better indicator of the potency of plasma than simply measuring anti-PNAG binding activity. It is unclear whether a similar relationship between functional antibody activities versus total antibody activities exists for antibodies against VapA. Interestingly, there was a cluster of foals in the RE HIP group that had relatively high C′1q deposition activities onto the PNAG antigen (Figure 3.3). Careful review of farm records and comparison of the OD ratios of VapA to C′1q OD activity indicated that these higher-than-expected C′1q OD activities in the RE HIP group were not attributable to labeling or other technical errors in plasma transfusion. None of the RE HIP donors had been vaccinated with PNAG. Because PNAG is found on the surface of many different bacteria, it is possible that this finding is the result of some mares producing functional antibodies against PNAG as a result of infection or natural exposure that were transferred to their foals via colostrum.

The association of a higher activity of antibodies against either VapA or PNAG with reduced odds of pneumonia was observed even after accounting for effects of farm, indicating that transfusion of either plasma protected against both *clinical* and *subclinical* pneumonia. This is consistent with results of a previous observational study,[29] and indicates that plasma transfusion has clinical benefits for foals at farms that use ultrasonography or other methods to screen foals for detection of subclinical pneumonia.

The significant effect of farm among foals transfused with RE HIP was attributed to a higher cumulative incidence of pneumonia at Farm A among foals transfused with RE HIP (42%; 25/60) than among foals transfused with PNAG HIP (27%; 16/59), whereas at Farm B the proportion of foals with pneumonia was identical for foals transfused with either RE HIP or PNAG HIP (19%; 9/48). Because pneumonia at Farm A was based on clinical signs whereas pneumonia at Farm B was subclinical, it is possible that PNAG HIP was more effective for protection against clinical than subclinical pneumonia. Further study is needed to substantiate the validity of this observation through replication and to identify any possible mechanism(s) of superior protection against clinical disease for PNAG HIP. Of note, in the study demonstrating protective efficacy of the PNAG vaccine given to mares whose foals were challenged at 4 weeks of life,[31] most of the PNAG-immune foals developed ultrasonographic lesions, but only 1 of 12 developed clinical *R. equi* pneumonia. This indicates the antibody to PNAG is highly effective at protecting against disease but not necessarily against subclinical infection based on ultrasonography.

We did not find a significant difference between the 2 plasma products in protection against *R. equi* pneumonia. These results should be interpreted with caution, however, because this study was not designed to test the hypotheses either of superiority or of non-inferiority between these plasma products. Although *in vitro* data indicate PNAG HIP is superior to RE HIP for mediating killing of *R. equi*,[32] the study reported here was not designed to compare protection

between the 2 plasma types and our absence of evidence of a difference should not be construed as evidence of absence of an effect.

Despite the randomized and masked design, this study had limitations. Not all foals had a trans-endoscopic tracheobronchial aspirate (T-TBA) performed to confirm *R. equi* pneumonia, such that most cases were presumptively diagnosed. However, we do not know of any large breeding farms that perform T-TBA on all foals with suspected *R. equi* pneumonia, and it is unlikely that large breeding farms would consent to this procedure for all foals suspected of *R. equi* pneumonia. Another limitation was that we lacked a placebo or other control group in this study, such as foals that were not transfused or were transfused with plasma from donors that were not hyperimmunized. This was not feasible as the participating farms were unwilling to forego plasma transfusion to foals.

In summary, antibody activities for VapA, PNAG, C´1q are important indicators of protection against *R. equi* pneumonia, and plasma with a higher activity of antibodies against either VapA or PNAG appears more effective for preventing *R. equi* pneumonia.

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# 4. RANDOMIZED, CONTROLLED TRIAL COMPARING *RHODOCOCCUS EQUI* AND POLY-*N*-ACETYL GLUCOSAMINE HYPERIMMUNE PLASMA TO PREVENT *R. EQUI* PNEUMONIA IN FOALS[4*]

## 4.1. Introduction

Although many organisms cause respiratory disease in foals, *Rhodococcus equi* (*R. equi*) is considered the most common cause of severe pneumonia.[1-3] Rhodococcal pneumonia is important to the equine industry because it is endemic at many horse-breeding farms (with cumulative incidence often exceeding 20%-40% of the foal population),[4-7] where the costs resulting from veterinary care, long-term treatment, and mortality of some foals can be very high.[8] In addition to substantial immediate costs, *R. equi* pneumonia has a long-term detrimental effect on the equine industry because foals that recover from the disease are less likely to race as adults.[9]

Methods for preventing *R. equi* pneumonia include chemoprophylaxis, vaccination, and transfusion of commercial plasma prepared from donors

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hyperimmunized against *R. equi*.[10-30] Chemoprophylaxis using macrolides is not acceptable because of concerns for increasing antimicrobial resistance in *R. equi* strains,[13,31] and because evidence of effectiveness has been conflicting.[10,14] Despite efforts to develop a vaccine against *R. equi*, [12,16-26] a licensed vaccine is not available. Moreover, vaccines are neither completely effective nor universally administered. Consequently, alternative approaches for prevention will be needed even if an effective vaccine for *R. equi* is developed and approved for commercial use.

In the United States (US), the only approach for decreasing the incidence of *R. equi* pneumonia at horse breeding farms that is licensed by the United States Department of Agriculture is transfusion of *R. equi* hyperimmune plasma (RE HIP). The use of RE HIP for preventing *R. equi* pneumonia is well established.[11-13,15,29,30] Nevertheless, evidence of effectiveness under field conditions remains variable and conflicting.[15,27,29-31] This variable clinical efficacy is likely explained in part by variation in the activity of antibodies recognizing *R. equi* among plasma products,[32] differences in volume of plasma transfused to foals,[29,30] interindividual variability in susceptibility to infection, and variation among veterinarians in criteria for diagnosis of *R. equi* pneumonia.[2,4,7-10,12,14,15,19,27,29,30,33]

Recently, it was determined that transfusion of foals within 36 hours of birth with 2 L of plasma from donors hyperimmunized against the conserved microbial polysaccharide β-1→6 poly-*N*-acetyl glucosamine (PNAG) protected these foals against experimental intrabronchial infection with virulent *R. equi* at 28 days of

age, whereas foals transfused at the same age with 2 L of plasma from donors not hyperimmunized against PNAG or *R. equi* were not protected.[28] Moreover, PNAG hyperimmune plasma (PNAG HIP) was superior to RE HIP for mediating opsonophagocytic killing of *R. equi* by neutrophils in vitro.[34] These in vitro findings suggested PNAG HIP might be more effective than RE HIP for protecting foals against rhodococcal pneumonia. Results of in vitro or small-scale experimental studies, however, might not be reproducible or representative of clinical efficacy. Thus, we conducted a randomized, controlled, blinded clinical trial at farms in several US states to compare the effectiveness of transfusing foals with either 2 L of PNAG HIP or 2 L of RE HIP for decreasing the cumulative incidence of pneumonia attributed to *R. equi* infection. Our objective was to determine whether PNAG HIP was superior to RE HIP for protecting foals against *R. equi* under field conditions.

## 4.2. Materials and Methods

### 4.2.1. Study Population

The participating farms were selected because of excellent record-keeping, expert and committed staff, and expressed willingness to record data for the project. Our goal was to include 400 foals from farms in Kentucky (n = 1 farm), New York (n = 1 farm), Oklahoma (n = 1 farm), and Texas (n = 2 farms). This sample size was calculated using the following assumptions: (a) statistical power = 90%; (b) significance level $P < 0.05$; (c) cumulative incidence in RE HIP-transfused foals = 30%; and (d) cumulative incidence in PNAG HIP-transfused

foals = 15%. Calculations indicated 322 foals would be needed (161 foals per group). We targeted a population of 400 foals to account for losses of foals to follow-up occurring for reasons such as transfer to other farms or unexpected deaths. Each of the participating farms was a large breeding farm that had a cumulative incidence of *R. equi* pneumonia over the past 3 years of approximately 25%, and had at least 50 foals residing at the farm from birth through weaning. Eligible farms were willing to randomly assign at least 50 foals born consecutively (*i.e.*, no selection of which foals were included in the study) to be transfused with 2 L of either PNAG HIP or RE HIP and to record data using study forms provided by the investigators.

Eligible foals appeared healthy at birth and had evidence of adequate transfer of passive immunity based on a commercial test kit (*e.g.*, SNAP Foal IgG test, IDEXX, Inc), and resided at the farm from birth through weaning. Foals that developed clinical signs of sepsis, diarrhea other than so-called “foal-heat diarrhea,” or infectious disease other than pneumonia attributed to *R. equi* were excluded from the study. Foals with evidence of other perinatal disorders (*e.g.,* perinatal asphyxia/hypoxic-ischemic encephalopathy) were excluded.

### 4.2.2. Transfusion

Each participating foal was transfused with 2 L (approximately 40 mL/kg of body weight) within 24 hours of birth. Plasma was labeled as either 0 or 1 by the collaborating manufacturer (Mg Biologics, Inc, Ames, Iowa) (Appendix B). Treatment order was preassigned randomly based on expected foaling date of

mares using a blocked design to ensure that equal numbers of foals were assigned to receive each type of plasma. Treatment assignment was made in blocks of 10 with equal distribution of each plasma within 10-foal blocks (*e.g*., if the first 5 foals were randomly assigned to plasma “1” then the next 5 foals would receive plasma “0”). The purpose of this blocking was to ensure that no seasonal bias occurred in the distribution of assigned plasma to obviate potential confounding effects of birthdate on the association of plasma type with development of pneumonia. Investigators (including data analysts) and farm personnel were blinded to the identity of the 2 plasma types until final data analysis was completed. An aliquot of the transfused plasma (2 ml) was collected immediately post-transfusion from the residual plasma in the infusion set for ELISA testing as described below. Plasma aliquots were stored frozen at -20$^{o}$C at the farm until shipped frozen overnight to the Equine Infectious Disease Laboratory at Texas A&M University. Investigators at each farm recorded on a preprinted roster the name, birth date, type of plasma transfused (0 or 1), and whether a plasma sample for ELISA testing for the study was collected from the foal. The roster also was used to indicate foals that were excluded from the study (*e.g*., stillbirth, neonatal sepsis). These forms were stored in binders that were returned to investigators at Texas A&M University when the study was completed.

**4.2.3. Foal Health Monitoring**

All study foals were monitored at least twice daily through weaning (*i.e*., age ≥ 5 months) by farm veterinary medical and technical staff for signs of

pneumonia including lethargy, coughing, depressed attitude, fever (rectal temperature > 39.4°C), increased respiratory rate (≥ 60 breaths/min) or effort (abdominal lift, flaring nostrils), and extrapulmonary manifestations of *R. equi* infection such as polysynovitis and uveitis. Foals with clinical signs had a CBC and thoracic ultrasonography performed. Foals were diagnosed with presumed *R. equi* pneumonia by the farm veterinarian(s) if they had ultrasonographic evidence of pulmonary abscess(es) or consolidation > 2 cm in maximal diameter and any 2 of the following clinical findings: (a) cough; (b) fever (rectal temperature > 39.4°C); (c) lethargic attitude; (d) increased respiratory rate and effort; and (e) leukocytosis (white blood cell count > 13,000 cells/μl or fibrinogen > 400 mg/dl). No farms in the study-based diagnosis of *R. equi* only on findings of thoracic ultrasonography for screening. Those making the diagnosis of *R. equi* pneumonia were masked to the type of plasma transfused to the foals. A study data form (Appendix C) was completed by a farm veterinarian for each eligible foal when it was weaned. Diagnosis of *R. equi* pneumonia was determined before data analysis and before unmasking of plasma type. All foals that developed pneumonia were treated according to the high standard of care of the participating farm.

### 4.2.4. ELISA Testing

A subset of plasma samples from both transfusion groups was tested to verify foals received the assigned plasma type. For ELISA testing, plasma samples were selected from all foals that developed *R. equi* pneumonia and 1 to

3 healthy foals that received the same plasma type that were closest matched to the foal by birthdate and same lot number of plasma. Our goal was to identify 2 healthy foals for each pneumonic foal but this was not always possible, but on average we had approximately 2 healthy foals for each pneumonic foal. For VapA, 153 samples were tested of which 85 (55 healthy foals, 30 pneumonia foals) were RE HIP samples and 68 (42 healthy foals and 26 pneumonia foals) were PNAG HIP samples. One hundred seventy-one samples were tested for C´1q deposition. Of those samples, 92 (62 healthy foals, 30 pneumonia foals) were from RE HIP samples, and 79 (53 healthy foals, 26 pneumonia foals) were from PNAG HIP samples. The purpose of this testing was to assess the internal validity of the study (*i.e*., extent to which foals had been correctly assigned to their respective plasma). The rationale for this sampling strategy is that it was unbiased by plasma type and ensured representation of all foals that developed pneumonia.

As noted above, an aliquot of the transfused plasma (2 ml) was collected immediately post-transfusion from the residual plasma in the infusion set and shipped to either Mg Biologics for VapA ELISA or to the Department of Medicine in the Brigham & Women's Hospital at Harvard Medical School for deposition of C´1q onto PNAG. Plasma samples were stored at -80$^{o}$C until thawed for testing. Samples from foals transfused with RE HIP were expected to have high levels of activity against VapA and foals transfused with PNAG HIP were expected to have high levels of activity for deposition of C´1q onto PNAG. Samples were tested for activity against VapA at Mg Biologics using the approved assay for assessing

potency of their United States Department of Agriculture-licensed RE HIP product; details of this standardized and regulated assay are proprietary. The ratio of the optical density (OD) of the sample to the OD of the positive control was used as the outcome for the VapA ELISA. Testing for serum endpoint activities for deposition of C′1q onto purified PNAG was performed as previously described.[28] Briefly, ELISA plates (Maxisorp, Thermo Scientific, Rochester, New York) were coated with 0.6 µg/ml of purified PNAG diluted in sensitization buffer (0.04 M PO4, pH 7.2) overnight at 4°C. Plates were washed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, blocked with 120 µl of PBS containing 1% skim milk for 1 hour at 37°C, and washed again. Dilutions of different foal sera were added in 50 µl volumes, after which 50 µl of 10% intact, normal horse serum was added as a source of C′1q. After 60 minutes of incubation at 37°C, plates were washed and 100 µl of goat anti-human C′1q, which also binds to equine C′1q, diluted 1 : 1000 in incubation buffer was added and plates were incubated at room temperature for 60 minutes. After washing, 100 µl of rabbit anti-goat IgG whole molecule conjugated to alkaline phosphatase and diluted 1:1000 in incubation buffer was added, and then incubated for 1 hour at room temperature. Washing and developing of the color indicator then was performed by adding p-nitrophenyl phosphate substrate, and color development determined after 60 minutes at room temperature. Optical density 405 nm values of this highest serum dilution tested were used to determine relative activity. Negative OD values after background subtraction (no primary antibody added) indicated sera with less

activity than this control.[28] Those testing the samples for VapA and C′1q deposition were blinded to the status of the samples (*i.e*., blinded to both the disease status of the foal and with which plasma the foal was transfused).

### 4.2.5. Data Analysis

The primary study outcome was the proportion of foals that developed pneumonia attributed to *R. equi* as defined above. Data were analyzed using descriptive and inferential methods. For descriptive purposes, data were summarized as proportions using figures or text. For inferential analysis, we compared the OD ratio of VapA and relative OD activities for C′1q deposition onto PNAG between foals transfused with RE HIP and those transfused with PNAG HIP using the generalized linear model (glm) function with an identity link using R statistical software (Version 3.3.3, R Core Team, Vienna, Austria), with OD ratios (for VapA) or relative OD (for C′1q) as the dependent (outcome) variable and plasma group as the independent variable. The Wilcoxon rank-sum test was performed using the wilcox.test function in R statistical software to compare OD ratios of VapA among foals transfused with RE HIP and relative OD for C′1q among foals transfused with PNAG HIP between foals that developed pneumonia and foals that did not develop pneumonia. Logistic regression was performed for the binary outcome of pneumonia (yes or no) with farm and plasma type included as independent effects. The effect of plasma type (adjusted for effects of individual farm) was reported as the odds ratio (OR) and the 95% confidence interval (95% CI) for the OR, estimated using maximum likelihood methods. Logistic regression

also was performed for data from each individual farm for the binary outcome of pneumonia with plasma type as an independent effect. The effect of plasma type for each farm was reported as the OR and 95% CI for the OR, estimated using maximum likelihood methods. Logistic regression was performed using the glm function with a binomial link in R statistical software. Fisher's exact test was used to compare proportions of foals with pneumonia within farms when cells had values of 0 in the referent 2 x 2 table, using the fisher.test command in R. Significance was set at $P < 0.05$ for all analyses.

### 4.3. Results

#### 4.3.1. Study Population

A total of 460 foals were transfused, of which 231 received RE HIP (plasma 0) and 229 received PNAG HIP (plasma 1). No reactions to plasma were reported during or after transfusion. Seventeen foals were lost to follow-up. Fourteen foals from the New York farm were lost to follow-up: 11 unexpectedly left the farm before weaning such that their *R. equi* pneumonia status could not be monitored in accordance with study protocols, and 3 foals died from causes unrelated to *R. equi* infection. By chance, all of the foals that left the farm before weaning were assigned to the PNAG HIP group. Two foals at the Oklahoma farm and 1 foal from Kentucky were lost to follow-up because of death to causes unrelated to *R. equi* infection. The distribution of the type of each plasma by farm was tabulated for the 443 foals included in the study (Table 4.1). Three of the 443 foals (0.7%) died from *R. equi* pneumonia. All 3 foals that died received plasma 0 (RE HIP); 2 foals

that died were from the Texas 2 ranch, and the other foal was from the New York farm.

A subset of plasma samples from both the transfusion groups were tested to verify foals received the assigned plasma type, as described above. The OD ratios for VapA were significantly ($P < 0.05$) higher among RE HIP compared to PNAG HIP (Figure 4.1). The relative OD values for C´1q deposition were significantly ($P < 0.05$) higher among foals that were transfused with PNAG HIP compared to RE HIP (Figure 4.2). Among foals transfused with RE HIP, there was no significant difference ($P = 0.68$) of OD ratios for VapA of plasma samples between those that developed pneumonia and those that did not develop pneumonia (Appendix D). Among foals transfused with PNAG HIP, there was no significant difference ($P =0.6$) in the relative OD values for C´1q of samples from foals that developed pneumonia and foals that did not develop pneumonia (Appendix E).

**Table 4.1. Distribution of foals by farm and type of plasma transfused**

Distribution of foals by farm and type of plasma transfused in a randomized, controlled trial comparing the relative efficacy of *R. equi* hyperimmune plasma (RE HIP) and β-1→6-poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP) to protect foals against *R. equi* pneumonia.

| Farm | Foals Transfused with RE HIP | Foals Transfused with PNAG HIP | Total |
|---|---|---|---|
| New York | 52 (54%) | 45 (46%) | 97 (100%) |
| Kentucky | 48 (51%) | 47 (49%) | 95 (100%) |
| Texas 1 | 43 (51%) | 41 (49%) | 84 (100%) |
| Texas 2 | 25 (51%) | 24 (49%) | 49 (100%) |
| Oklahoma | 60 (51%) | 58 (49%) | 118 (100%) |
| **Total** | 228 (51%) | 215 (49%) | 443 (100%) |

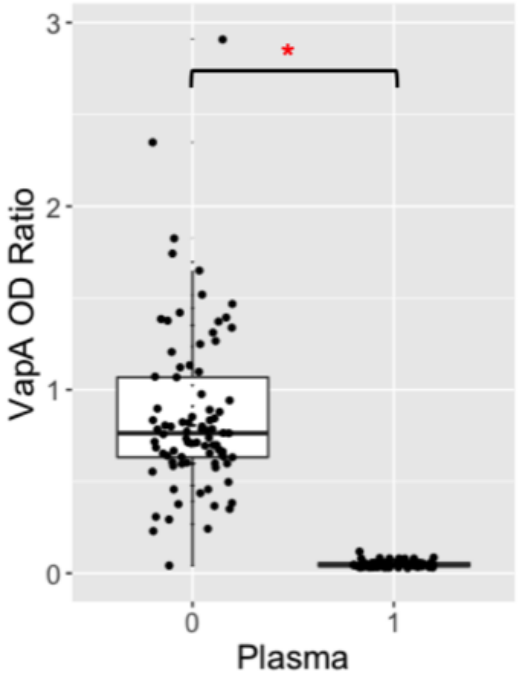


**Figure 4.1. Comparison of VapA optical density ratios between *R. equi* hyperimmune plasma and PNAG hyperimmune plasma.**

Boxplot of optical density (OD) ratios from ELISAs measuring antibody activity against purified VapA protein in 153 plasma samples, stratified by plasma type. Of those samples, 85 (42 healthy foals, 43 pneumonia foals) were *R. equi* hyperimmune plasma (RE HIP) samples and 68 (32 healthy foals and 36 pneumonia foals) were β-1→6-poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP) samples. Samples of RE HIP had significantly higher (asterisks represent $P < 0.05$) VapA OD ratios than samples of PNAG HIP.

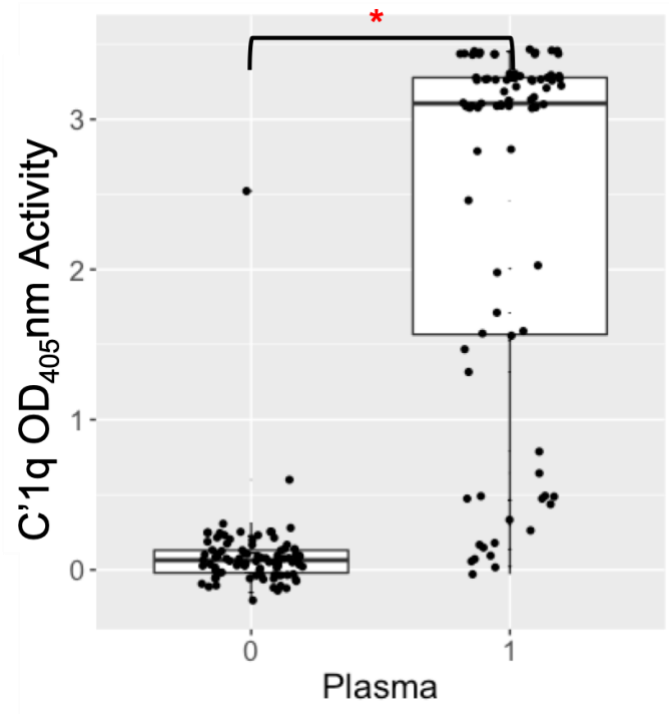


**Figure 4.2. Comparison of C´1q relative optical density between *R. equi* hyperimmune plasma and PNAG hyperimmune plasma**

Boxplot of relative optical density ($OD_{405}$nm) antibody activities for deposition of C´1q onto β-1→6-poly-*N*-acetyl glucosamine (PNAG) by plasma samples from 171 plasma samples, stratified by plasma type. Of these samples 92 (49 healthy foals, 43 pneumonia foals) were from *R. equi* hyperimmune plasma (RE HIP), and 79 (43 healthy foals, 36 pneumonia foals) PNAG hyperimmune plasma (PNAG HIP). Samples from PNAG HIP had significantly higher (asterisks represent $P < 0.05$) C´1q $OD_{405}$nm activities than RE HIP.

### 4.3.2. Association of Plasma Type with Pneumonia

The proportion of foals that developed pneumonia was the same among foals transfused with RE HIP (14%; 32/228) and PNAG HIP (14%; 30/215). Using multivariable logistic regression with pneumonia as the binary outcome and plasma type and farm as dependent variables, the odds of pneumonia in foals transfused with RE HIP were not significantly higher than among foals transfused with PNAG HIP (OR, 0.74; 95% CI, 0.44-1.23; P = 0.24), adjusted for the effect of farm. The odds of pneumonia were approximately 6-fold higher (P < 0.001) for foals at the New York farm compared to the reference farm Texas 1 (Table 4.2). The cumulative incidence and effect of plasma type varied among farms (Table 4.3): PNAG HIP was significantly inferior to RE HIP at the New York farm, whereas PNAG HIP was significantly more effective than RE HIP at a Texas farm.

**Table 4.2. Odds ratios of developing pneumonia for type of plasma transfused to foal and farm of origin**

Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *R. equi* among 443 foals from 5 farms transfused with either *R. equi* hyperimmune plasma (RE HIP) or β-1→6-poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP).

| Variable | Odds Ratio (95% CI) | P Value |
|---|---|---|
| Plasma Type | | |
| PNAG HIP | 1 (NA) | NA |
| RE HIP | 0.74 (0.44 – 1.23) | 0.24 |
| Farm | | |
| Texas 1 | 1 (NA) | NA |
| Texas 2 | 1.33 (0.43 – 4.08) | 0.623 |
| Kentucky | 0.86 (0.31 - 2.41) | 0.774 |
| Oklahoma | 1.25 (0.50 – 3.13) | 0.633 |
| New York | 5.99 (2.63 – 13.65) | 0.00002 |

**Table 4.3. Individual farms distribution of foals within plasma type and odds ratios (OR) of pneumonia based on plasma type**

Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *R. equi* for each of 5 farms where foals were transfused with either *R. equi* hyperimmune plasma (RE HIP) or β-1→6-poly-*N*-acetyl glucosamine hyperimmune plasma (PNAG HIP).

| | PNAG HIP | | RE HIP | | | |
|---|---|---|---|---|---|---|
| **Farm** | **Pneumonia** | **Healthy** | **Pneumonia** | **Healthy** | **OR (95% CI)*** | **P value#** |
| Kentucky | 1 (2%) | 46 (98%) | 6 (13%) | 42 (87%) | 0.30 (0.04 – 1.40) | 0.27 |
| Oklahoma | 6 (10%) | 52 (90%) | 6 (10%) | 54 (90%) | 1.39 (0.45 – 4.47) | 0.57 |
| New York | 17 (38%) | 28 (62%) | 12 (23%) | 40 (77%) | 2.67 (1.22 - 5.99) | 0.02 |
| Texas 1 | 6 (15%) | 35 (85%) | 2 (5%) | 41 (95%) | 3.51 (0.75- 25.04) | 0.15 |
| Texas 2 | 0 (0%) | 24 (100%) | 6 (24%) | 19 (76%) | ND** | 0.02** |

*OR = odds of pneumonia among foals transfused with PNAG HIP relative to RE HIP;

95% CI = 95% confidence interval

#P values derived from logistic regression unless indicated by **

**ND = Not determined because incalculable due to complete separation; P value derived using Fisher's exact test.

## 4.4. Discussion

Cumulatively, the odds of developing *R. equi* pneumonia were not significantly different between foals that were transfused with commercially available RE HIP and those transfused with PNAG HIP. The finding that effects of

plasma type varied among farms and that within-farm effects were occasionally significant underscores the importance of conducting a large-scale, multisite study for assessing clinical efficacy. Results from single farms, particularly with small sample size, might not reflect effects at other farms. The cumulative incidence of *R. equi* pneumonia at most of the farms was markedly lower than those reported at these farms for recent preceding years. This relatively low incidence decreased the statistical power of the study. The reason for this lower incidence is unclear, but some of the farms historically transfused foals with 1 L (rather than 2 L) of RE HIP to prevent *R. equi* pneumonia, and observational epidemiological studies indicate that transfusing 2 L of RE HIP is superior to 1 L for decreasing the incidence of *R. equi* pneumonia. [29, 30]

Results of ELISA testing of plasma samples for VapA and C´1q deposition onto PNAG indicated that misclassification of the type of plasma administered to study foals was unlikely. Some low ELISA results for both VapA and C´1q were observed among foals transfused with RE HIP and PNAG HIP, respectively. These results could have been attributable to mishandling of either the plasma product or the sample aliquot at the farm or in the laboratory, or to variation of antibody activity among batches or lots.[32] For example, thawing plasma at too high of a temperature can result in denaturing of immunoglobulins, and delays in sample freezing, exposure to high ambient temperatures, or improper thawing in the laboratory could have impacted quality of antibodies in the aliquots submitted for testing. These low results were not associated with *R. equi* pneumonia, a

specific farm, plasma lot, or month of transfusion (data not shown). Because RE HIP and PNAG HIP were tested for adequate antibody activities against VapA and C´1q deposition, respectively, before shipping, we believe that degradation of antibodies in the plasma sample collected immediately after transfusion is the most likely explanation of the unexpectedly low ELISA results for some samples. Interestingly, 2 RE HIP samples had high relative OD values for C´1q deposition onto PNAG. These findings might be attributable to samples classified as RE HIP that were actually PNAG HIP. However, these samples also had high VapA titers, indicating that these donors might have had background antibody activity to PNAG despite being hyperimmunized against *R. equi*. Because PNAG is found on the surface of many different bacteria,[35] a plasma donor could have produced functional anti- bodies against PNAG as a result of infection or natural exposure.

Despite using a randomized, controlled, and blinded study design, our study had some limitations. The principal limitation is that we did not perform tracheobronchial aspiration to collect fluid to submit for microbiologic culture of *R. equi*, PCR to confirm virulence of any *R. equi* isolated, and cytologic evaluation of the tracheobronchial aspirate fluid to substantiate the diagnosis of *R. equi* pneumonia. This limitation was unavoidable because large horse breeding farms neither routinely perform nor would consent to tracheobronchial aspiration of all foals suspected to have *R. equi* pneumonia. Thus, we cannot exclude the possibility that mis- classification of the primary outcome masked true effects of either of the plasma types. However, the randomized design made it improbable

that this misclassification would have differed between the 2 plasma treatments. We believe the diagnostic criteria used for our study reflect standard practices at horse breeding farms in the United States. We did not include a negative control group of foals that were not transfused with plasma or transfused with plasma from donors that were not hyper- immunized against *R. equi* or PNAG. Thus, we could not assess whether either plasma decreased the incidence of *R. equi* pneumonia at the participating farms. The participating farms were not willing to consent to have a group of foals forego plasma transfusion. We also cannot draw any conclusions about the efficacy of transfusing with 2 or 1 L of either HIP, nor can we draw any conclusions about the efficacy of PNAG HIP relative to RE HIP when foals are transfused with only 1 L of plasma. We did not have post-transfusion serum samples from foals to determine antibody activity against VapA or PNAG to better understand the association of antibody activities in the individual foals with odds of developing pneumonia.

Despite these limitations, we believe that our results provide evidence that PNAG HIP was not superior to RE HIP for preventing *R. equi* pneumonia. Well-designed, large-scale clinical trials are needed to characterize the efficacy of transfusing foals with RE HIP (or PNAG HIP) and the efficacy of transfusing 2 L vs 1 L.

## 4.5. References


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# 5. CONCLUSIONS AND FUTURE DIRECTIONS

The use of hyperimmune plasma (HIP) as a prophylactic treatment for *Rhodococcus equi* (*R. equi*) pneumonia in foals is widespread, but questions and controversy exist regarding the efficacy and use of this product. As the only product licensed by the United States Department of Agriculture (USDA) to protect foals from *R. equi* pneumonia and in the absence of a licensed vaccine against *R. equi*, it is critically important to improve evidence of the effectiveness of this product. This dissertation was the result of our efforts to better understand the impact on the cumulative incidence of *R. equi* pneumonia of the volume transfused to foals and the anti-*R. equi* antibody activity either in the serum of HIP-transfused foals or in HIP transfused to foals. We also wanted to test the superiority of a novel HIP from donor horses hyperimmunized against β-1→6 poly-*N*-acetyl glucosamine (PNAG HIP) compared to *R. equi* HIP (RE HIP). In this chapter, I summarize the impact of my contributions to the field and discuss my ideas for future directions to better understand the role of HIP and antibodies in preventing *R. equi* foal pneumonia.

## 5.1. Impact of Our Work

Although more work remains to be done, we have further demonstrated efficacy of HIP transfusion for preventing *R. equi* pneumonia and provided evidence that transfusion of a dose of HIP of approximately 40 ml/kg (*i.e.*, 2 L to an average-sized Thoroughbred or Quarter Horse foal) is superior to transfusion

of 20 ml/kg of HIP (*i.e.*, 1 L to an average-sized Thoroughbred or Quarter Horse foal, a volume which is still used to transfuse foals at many horse-breeding farms). We conducted a retrospective cohort study comparing transfusion of 2 L of RE HIP with transfusion of ≤1 L to protect foals against subclinical pneumonia, and demonstrated that transfusion of 2 L of RE HIP was superior to transfusion of ≤1 L.[1] This finding is important for farm managers and veterinarians when considering costs for materials and labor and patient safety of their program for controlling *R. equi* pneumonia. Although the results were compelling, the observational design of the study has inherent limitations. Specifically, management practices on the farm differed between foals transfused with 2 L and foals transfused with ≤1 L such that these practices could have confounded the association between the volume/dose of HIP and (subclinical) pneumonia.[1] Although not included in this dissertation, we subsequently conducted a retrospective cohort study at 2 farms in New York in which we observed that transfusion of 2 L of RE HIP was superior to transfusion of 1 L for preventing clinical pneumonia attributed to *R. equi*.[2] This observational study was not confounded by difference in management between the 2 groups of study foals, but it had the important limitation of lacking random assignment of the volume of HIP transfused to individual foals which introduces the potential for selection bias. Nonetheless, the consistency of the findings from these 2 retrospective studies is encouraging.

Another important finding reported from these observational studies is that no foals transfused with 2 L of HIP at age ≤24 hours had an adverse reaction to the HIP. This is an important finding because of concern for transfusion-associated circulatory overload (TACO);[3] however, results from >200 foals in these 2 studies provides evidence to veterinarians of the safety of transfusion with 2 L at age ≤24 hours. This evidence is supported by another retrospective study of >400 foals that were transfused with 2 L of HIP within 24 hours of birth with no adverse reactions.[4] This safety information helped us in our research when we recruited farms to participate in our clinical trial study comparing RE HIP and PNAG HIP because managers at some farms had concerns about the safety of transfusing foals with 2 L of HIP at age ≤24 hours. Anecdotally, after the clinical trial managers and veterinarians from those farms were convinced that transfusing 2 L of HIP by age 24 hours provides better protection than either transfusing foals with 1 L at age ≤24 hours or transfusing 1 L to foals at age ≤24 hours and then again 3 to 4 weeks later. Nevertheless, great need exists for a well-designed, randomized, controlled, blinded, multi-farm clinical trial comparing transfusion of 2 L of RE HIP with transfusion of 1 L. Ideally, design of trials evaluating the volume of plasma transfused to foals should include an untreated (*i.e.*, no HIP) control group to demonstrate efficacy of HIP; however, our experience has been that horse-breeding farms are unwilling to withhold plasma from a group of foals because they consider it to provide some degree of protection to foals. While we agree that it would be unethical to withhold a

treatment from foals that has been demonstrated to have protective effects, Chapter 1 illustrated the limitations in our understanding of the effectiveness of RE HIP for preventing pneumonia in foals. Answering the question of the optimal dose of HIP is of substantial importance to the equine industry.

Given the evidence from our studies that a higher dose/volume of RE HIP is more protective against *R. equi* foal pneumonia, we investigated whether the level of antibody activity against *R. equi* in foals transfused with HIP was responsible for the greater protection observed (*i.e.*, greater volume of HIP = larger amount of *R. equi*-specific antibodies in transfused foals). We demonstrated a significant association between reduced odds of *R. equi* pneumonia with higher antibody activities against the virulence-associated protein A (VapA) of *R. equi* in serum samples of foals transfused with RE HIP, and with higher antibody activities against PNAG and greater deposition of complement component 1q (C'1q) onto PNAG in serum samples from foals transfused with PNAG HIP.[5] Although our evidence is indirect, these results suggest that the protective effect of transfusion with a larger volume of HIP is likely attributable to transfer of a larger amount of antibodies targeting *R. equi*, consistent with experimental results reported by Hooper-McGrevy.[6] This information is important for both plasma manufacturers and plasma consumers to ensure they are producing plasma products that ***consistently*** have high levels of antibody activity against *R. equi*. It is also important for consumers of HIP to be aware that varying concentrations between manufacturers and among lots[7] are likely to influence

effectiveness of HIP, and that products with higher levels of antibody activity against *R. equi* are likely to provide superior protection. Consumers also need to be diligent in following the instructions from the manufacturers on how to thaw the plasma correctly, as thawing the plasma incorrectly can potentially denature the antibodies, inactivate complement, and possibly damage other as yet unidentified protective components of HIP.

We also compared the effectiveness of PNAG HIP with RE HIP in a randomized, controlled, blinded clinical trial at breeding farms in multiple U.S. states. The rationale for this comparison was our evidence that immunization of dams against PNAG or transfusion of foals with PNAG HIP provided protection against experimental challenge,[8] and results of a small-scale *in vitro* study that indicated that PNAG HIP was superior to RE HIP.[9] Results of our clinical trial, however, demonstrated that the cumulative effectiveness of PNAG HIP was identical to that of RE HIP. Interestingly, at some farms PNAG HIP was superior to RE HIP for preventing *R. equi* pneumonia and at others the RE HIP was superior to PNAG HIP.[10] The differences in effectiveness observed among farms in this study illustrates the importance of large-scale, randomized, controlled, blinded clinical trials, because smaller studies can yield unstable estimates of effectiveness that are not reproducible. Conversely, effectiveness might truly vary among farms for reasons not yet understood, including confounding factors such as management or methods for diagnosing pneumonia.

## 5.2. Future Directions

Our retrospective cohort studies indicate that transfusing foals with 2 L of HIP is superior to transfusing 1 L to prevent clinical and subclinical pneumonia attributed to *R. equi*. But, as noted above, great need exists for well-designed randomized, controlled trials to provide strong clinical evidence for the superior efficacy of transfusing foals with 2 L of HIP because observational studies are prone to selection bias, information bias, collider bias, and confounder bias. Sample size requirements and logistics for conducting these studies will make them expensive and difficult to conduct. It is not clear that these studies will be conducted in the foreseeable future given the limitations of funding for equine research.

Although several studies[1,2,4,5,10] indicate that transfusion of 2 L of HIP to foals at age $\leq$24 hours is safe, there are inherent risks associated with plasma transfusion including hypersensitivity reactions,[11] transfusion associated lung injury (TRALI),[12] and TACO.[3] The latter is of particular concern for transfusing 50 kg foals with 2 L of HIP. Experimental and observational studies should be conducted to better characterize the hemodynamic effects and frequency of adverse events associated with transfusing 2 L of HIP to foals.

Some farms continue to transfuse foals with 1 L of HIP soon after birth and a second liter at age 3 to 4 weeks. We do not recommend this practice because a second transfusion later increases the likelihood of immune reactions to plasma and requires a second procedure where restraint for intravenous catheterization

and transfusion puts foals at risk of injury (self-induced or iatrogenic). Foals are also believed to be infected with *R. equi* soon after birth,[13,14] and it has been shown that plasma is more effective as a prophylaxis treatment than as a therapeutic treatment.[15,16] However, randomized, controlled trials are needed to provide farm managers, equine veterinarians, and foal owners with strong evidence about whether it is better to transfuse foals with 1 or 2 L of HIP soon after birth or 1 L soon after birth followed by 1 L at age 3 or 4 weeks. A trial to answer this question will require a large sample size to obtain adequate statistical power because of the expected magnitudes of effects and because of variability in diagnostic practices and cumulative incidences of pneumonia among farms. A non-inferiority study design would allow us to answer the question of whether 2 L is within a pre-defined limit deemed non-inferior to transfusing 1 L shortly after birth followed by 1 L at age 3 or 4 weeks of age. My belief is that veterinarians and farm mangers would choose to transfuse foals with 2 L of HIP once shortly after birth rather than twice if it were known to be non-inferior to transfusing foals a second time at age 3 or 4 weeks because it would require less labor, be safer for the foal, and be more cost-effective by eliminating a second catheterization and veterinarian farm-call fee.

Our work and that of others indicates that greater antibody activity against *R. equi* is associated with protection. This work is largely based on activity of total IgG. Evidence exists that the IgGa ($IgG_1$) sub-isotype mediates protection against *R. equi*,[17,18] but conflicting evidence also exists.[19] A logical next step would be to

evaluate the association between development of pneumonia attributed to *R. equi* and activities of IgGa ($IgG_1$) and IgGb ($IgG_{4/7}$) recognizing VapA by enzyme-linked immunosorbent assay (ELISA) either in serum of foals or units of HIP transfused. In addition to investigating the protective effects of the sub-isotypes of IgG, the association of activities/concentrations of other components (*e.g.* complement, cytokines, fibronectin, etc.) in HIP with risk of pneumonia also need to be investigated.

Due to the patient safety and cost concerns associated with the administration of HIP, it is important to continue to research novel treatments to improve convenience and safety. In the last few years, production of messenger RNA (mRNA) has become more accessible.[20,21] Our laboratory is using this technology to produce mRNA encoding a monoclonal antibody (mAb) targeting VapA and then delivering it to the foals via nebulization. By nebulizing the mRNA, the foal's own cells should produce high doses of antibodies in the lungs within hours of transfection. This is important because the lungs are considered the site of infection with *R. equi* via inhalation. This novel approach to passive immunization will hopefully be more effective at protecting and will be safer for the foals. Importantly, this approach is distinct from a mRNA vaccine in which an antigen is being delivered to the foal's immune system in order to provoke an active immune response in several weeks' time. Another novel treatment that should be explored for prophylaxis of *R. equi* is the use of hyper immunoglobulins (HIG) containing anti-VapA immunoglobulins from plasma collected from

hyperimmunized horses to VapA. HIGs are made from concentrating HIP from multiple donors to achieve a consistent, high level of specific antibodies per dose.[22,23] HIGs are similar to HIP in that they provide immediate immunity. If a cost-effective way to produce HIG could be established, it would allow for a safer option than HIP because the volume administered intravenously is smaller and the risk of TACO would be reduced. A potential down-side to HIG is that the foal would receive less complement and other opsonins such as cytokines that is contained in HIP and we still don't know how much of a role they play in the protective effect of HIP. It is likely that other novel technologies (*e.g.*, monoclonal antibody products) exist that can and will be explored for improving passive immunization to protect foals against *R. equi* pneumonia.

Our studies have made an impact on the current practice of veterinarians and farm managers for transfusing RE HIP by demonstrating that transfusing 2 L of RE HIP with high activity to VapA shortly after birth is both safe and effective as a prophylactic treatment of *R. equi* pneumonia. These findings, however, are far from conclusive and – as discussed above – further research is needed.

**5.3. References**

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APPENDIX A:
SUMMARY OF AGE OF TRANSFUSION FOR MULLER *ET AL.,* 5 YEAR FIELD STUDY

| Year | Age of transfusion | Incidence of *R. equi* pneumonia in foals transfused with RE HIP | Incidence of *R. equi* pneumonia in controls |
|---|---|---|---|
| 1988 | Foals born in January or February were transfused in March foals born in March were transfused by age 30 days | 0% (0/68) | Not applicable |
| 1989 | Foals born prior to April 1 were transfused in early April foals born after April 1 were transfused between ages 30 to 60 days | 3% (3/101) | 43% (6/14) |
| 1990 | Foals born prior to April 1 were transfused in early April, foals born after April 1 were transfused during first 2 weeks after birth | 8% (10/120) | 50% (4/8) |
| 1991 | The majority of foals were transfused in April and May at an average age of 6 weeks | 3% (4/126) | 60% (3/5) |
| 1992 | The majority of foals were transfused in April and May at an average age of 6 weeks | 5% (5/109) | No controls |

Summary of the ages when foals were transfused during each year and the incidence of *R. equi* pneumonia in foals transfused with *R. equi* hyperimmune plasma (RE HIP) and control foals that were not transfused by group from the study by Muller and Madigan.[38]

APPENDIX B:

PICTURE OF PLASMA USED IN STUDY


Plasma units were masked for this study: Research 0 (Plasma 0) was *Rhodococcus equi* hyperimmune plasma and Research 1 (Plasma 1) was β-1→6-poly-*N*-acetyl glucosamine hyperimmune plasma.

# APPENDIX C:

## STUDY DATA FORM

Data Collection Form: Plasma Study Foal ID __ __ __

**FOALING DATA**

1. Foal study identification number: _____
2. Name or identification of foal's dam: _____
3. Date of foal's birth (Month/Day/Year): ___/___/_____
4. Sex of foal (M = MALE or F = FEMALE): _____
5. Plasma TYPE given: _____
6. Plasma volume given: __ (Liters)
7. Date plasma given: (Month/Day/Year): ___/___/_____
8. 6 ml of foal serum collected in tiger top tube (circle one): YES or NO
9. Reaction to plasma (circle one): YES or NO
    a. If yes, please describe:

**DIAGNOSTIC DATA**

10. Was the foal treated for *R. equi* pneumonia? YES or NO
11. Which clinical or laboratory findings did it have (**circle all that apply**)
    a. Fever (rectal temperature > 103.0ºF)
    b. Increased respiratory rate (> 60 breaths/minute)
    c. Increased respiratory effort (flaring nostrils, abdominal effort to breath)
    d. Coughing
    e. Depressed/lethargic attitude (less active/vigorous, less suckling)
    f. Abnormal sounds in lung or trachea
    g. Increased white blood cell (WBC) concentration (> 13,000 cells/µl)
    h. Increased fibrinogen concentration (> 400 mg/dl) or SAA
12. Ultrasound findings:
    a. Maximum sum of diameter of lesions at any exam: _____(cm)
13. WBC at time of diagnosis: __ __ __ . __ __ (x $10^3$ cells per µL)

Data Collection Form: Plasma Study Foal ID __ __ __

14. Tracheal wash done (circle one): YES or NO ___

**FOLLOW-UP DATA**

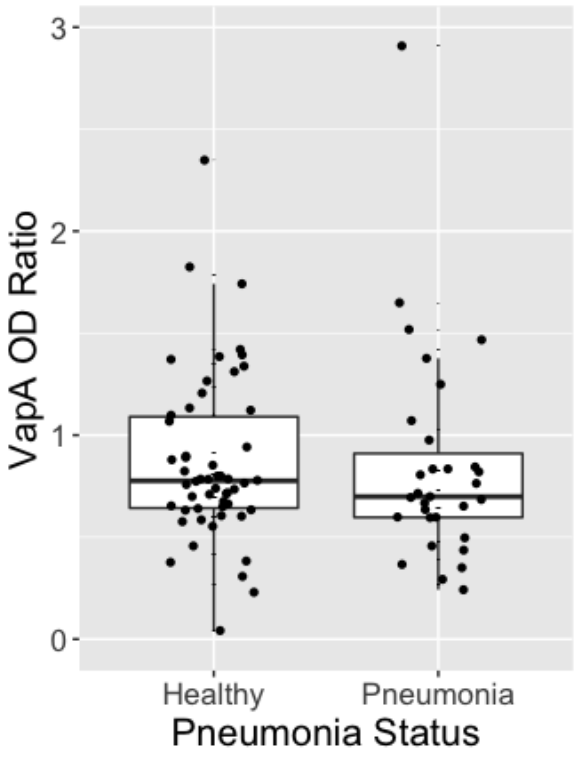
15. Date first clinical signs detected (Month/Day/Year): ___ /___ /____
16. Last day of treatment(s) (Month/Day/Year): ___ /___ /____
17. Duration of ultrasound lesions: __ __ (Weeks)
18. Survived: YES or NO _____
19. Any extra-pulmonary* disorders? YES or NO ___

* Signs other than the lungs like swollen joints, ocular problems, etc.

Study data form was completed for each eligible foal by farm personnel

APPENDIX D:

VapA OPTICAL DENSITY (OD) RATIOS OF SAMPLES OF RE HIP

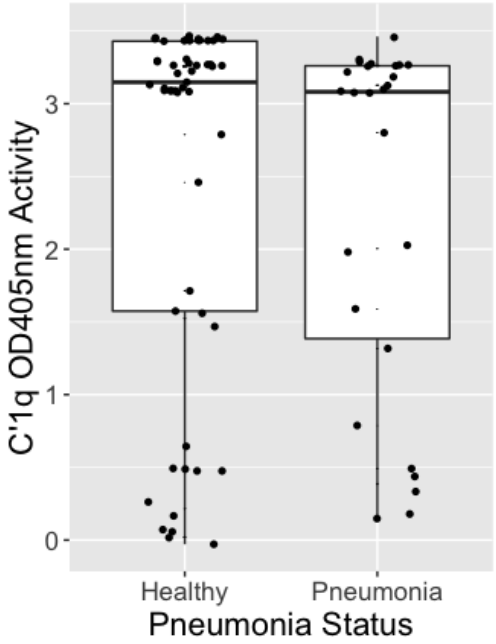



**VapA optical density (OD) ratios of samples of RE HIP transfused to foals that developed pneumonia and foals that did not develop pneumonia.**

Boxplot of OD ratios from ELISAs measuring antibody activity against purified VapA protein from 85 RE HIP samples (n = 42 samples from foals that did not develop pneumonia [Pneumonia Status = Healthy] and n = 43 samples from pneumonia foals [Pneumonia Status = Pneumonia]). There was no significant (P = 0.68) difference in VapA OD ratios for samples from foals that developed pneumonia and those that did not develop pneumonia.

APPENDIX E:

C´1q RELATIVE OPTICAL DENSITY OF SAMPLES OF PNAG HIP




**C´1q relative optical density ($OD_{405}$nm) of samples of PNAG HIP transfused to foals that developed pneumonia and foals that did not develop pneumonia.**

Boxplot of relative optical density ($OD_{405}$nm) antibody activities for deposition of C´1q onto PNAG from 79 PNAG HIP samples (n = 43 samples from foals that did not develop pneumonia [Pneumonia Status = Healthy] and n = 36 samples from pneumonia foals [Pneumonia Status = Pneumonia]). There was no significant (P = 0.60) difference in the relative $OD_{405}$nm values for C´1q for samples from foals that developed pneumonia and those that did not develop pneumonia.