


Randomized, controlled trial comparing *Rhodococcus equi* and poly-*N*-acetyl glucosamine hyperimmune plasma to prevent *R equi* pneumonia in foals

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Abstract

Background: Hyperimmune plasma raised against β -1 \rightarrow 6-poly-*N*-acetyl glucosamine (PNAG HIP) mediates more opsonophagocytic killing of *Rhodococcus equi* (*R equi*) than does *R equi* hyperimmune plasma (RE HIP) in vitro. The relative efficacy of PNAG HIP and RE HIP to protect foals against *R equi* pneumonia, however, has not been evaluated.

Hypothesis: Transfusion with PNAG HIP will be superior to RE HIP in foals for protection against *R equi* pneumonia in a randomized, controlled, blinded clinical trial.

Animals: Four hundred sixty Quarter Horse and Thoroughbred foals at 5 large breeding farms in the United States.

Methods: A randomized, controlled, blinded clinical trial was conducted in which foals were transfused within 24 hours after birth with 2 L of either RE HIP or PNAG HIP. Study foals were monitored through weaning for clinical signs of pneumonia by farm veterinarians. The primary outcome was the proportion of foals that developed pneumonia after receiving each type of plasma.

Results: The proportion of foals that developed pneumonia was the same between foals transfused with RE HIP (14%; 32/228) and PNAG HIP (14%; 30/215).

Conclusions and Clinical Importance: Results indicate that PNAG HIP was not superior to a commercially available, United States Department of Agriculture-licensed RE HIP product for protecting foals against *R equi* pneumonia under field conditions.

KEYWORDS

bacterial, clinical trials, humoral immunity, neonatology, *Rhodococcus*, transfusion

Abbreviations: 95% CI, 95% confidence interval; C1q, complement component 1q; glm, generalized linear model; HIP, hyperimmune plasma; OD, optical density; OR, odds ratio; PBS, phosphate-buffered saline; PNAG, β -1 \rightarrow 6-poly-*N*-acetyl glucosamine; PNAG HIP, β -1 \rightarrow 6-poly-*N*-acetyl glucosamine hyperimmune plasma; RE HIP, *Rhodococcus equi* hyperimmune plasma.

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1 | INTRODUCTION

Although many organisms cause respiratory disease in foals, *Rhodococcus 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 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, 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.

2 | MATERIALS AND METHODS

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 $P < .05$; (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 (ie, 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 (eg, 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 (eg, perinatal asphyxia/hypoxic-ischemic encephalopathy) were excluded.

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) (Figure S1). 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 (eg, 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°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 (eg, stillbirth, neonatal sepsis). These forms were stored in binders that were returned to investigators at Texas A&M University when the study was completed.

2.3 | Foal health monitoring

All study foals were monitored at least twice daily through weaning (ie, age ≥ 5 months) by farm veterinary medical and technical staff for signs of pneumonia including lethargy, coughing, depressed attitude, fever (rectal temperature $> 39.4^{\circ}\text{C}$), increased respiratory rate (≥ 60 breaths/min) or effort (abdominal lift, flaring nostrils), and extrapulmonary manifestations of *R equi* infection such as poly-synovitis 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 $> 39.4^{\circ}\text{C}$); (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 (Figure S2) 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.

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 (ie, 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°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 $0.6\ \mu\text{g}/\text{mL}$ of purified PNAG diluted in sensitization buffer ($0.04\ \text{M PO}_4$, pH 7.2) overnight at 4°C . Plates were washed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, blocked with $120\ \mu\text{L}$ of PBS containing 1% skim milk for 1 hour at 37°C , and washed again. Dilutions of different foal sera were added in $50\ \mu\text{L}$ volumes, after which $50\ \mu\text{L}$ 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 $100\ \mu\text{L}$ 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, $100\ \mu\text{L}$ 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 (ie, blinded to both the disease status of the foal and with which plasma the foal was transfused).

TABLE 1 Distribution of foals by farm and type of plasma transfused

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%)

Note: Distribution of foals by farm and type of plasma transfused in a randomized, controlled trial comparing the relative efficacy of RE HIP and PNAG HIP to protect foals against *Rhodococcus equi* pneumonia.

Abbreviations: PNAG HIP, β -1 \rightarrow 6-poly-N-acetyl glucosamine hyperimmune plasma; RE HIP, *Rhodococcus equi* hyperimmune plasma.

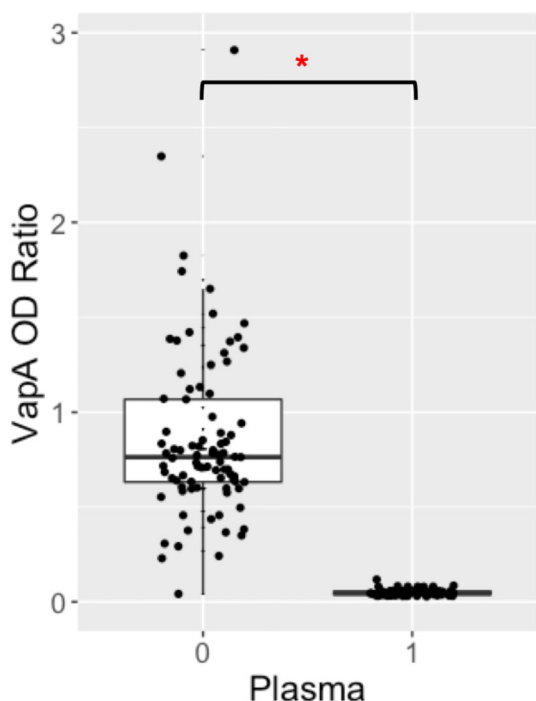


FIGURE 1 Comparison of VapA optical density ratios between *Rhodococcus equi* hyperimmune plasma and PNAG hyperimmune plasma. Boxplot of optical density (OD) ratios from ELISAs measuring antibody activity against purified VapA protein in 153 plasma samples, stratified by plasma type. Of those samples, 85 (42 healthy foals, 43 pneumonia foals) were RE HIP samples and 68 (32 healthy foals and 36 pneumonia foals) were PNAG HIP samples. Samples of RE HIP had significantly higher (asterisks represent $P < .05$) VapA OD ratios than samples of PNAG HIP. PNAG HIP, β -1 \rightarrow 6-poly-N-acetyl glucosamine hyperimmune plasma; RE HIP, *Rhodococcus equi* hyperimmune plasma

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

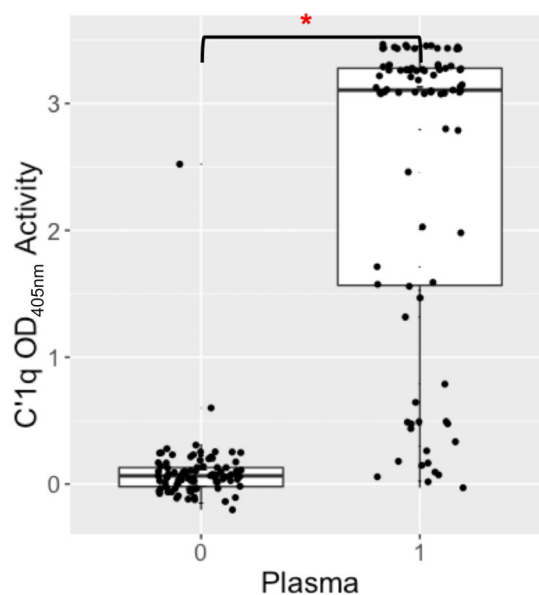


FIGURE 2 Comparison of C'1q relative optical density between *Rhodococcus equi* hyperimmune plasma and PNAG hyperimmune plasma. Boxplot of relative optical density (OD_{405nm}) antibody activities for deposition of C'1q onto PNAG by plasma samples from 171 plasma samples, stratified by plasma type. Of these samples 92 (49 healthy foals, 43 pneumonia foals) were from RE HIP, and 79 (43 healthy foals, 36 pneumonia foals) from PNAG HIP. Samples from PNAG HIP had significantly higher (asterisks represent $P < .05$) C'1q OD_{405nm} activities than RE HIP. PNAG HIP, β -1 \rightarrow 6-poly-N-acetyl glucosamine hyperimmune plasma; RE HIP, *Rhodococcus equi* hyperimmune plasma

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 2×2 table, using the fisher.test command in R. Significance was set at $P < .05$ for all analyses.

TABLE 2 Odds ratios of developing pneumonia for type of plasma transfused to foal and farm of origin

Variable	Odds ratio (95% CI)	P value
Plasma type		
PNAG HIP	1 (Not applicable)	Not applicable
RE HIP	0.74 (0.44-1.23)	.24
Farm		
Texas 1	1 (NA)	NA
Texas 2	1.33 (0.43-4.08)	.62
Kentucky	0.86 (0.31-2.41)	.77
Oklahoma	1.25 (0.50-3.13)	.63
New York	5.99 (2.63-13.65)	<.001

Note: Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *Rhodococcus equi* among 443 foals from 5 farms transfused with either RE HIP or PNAG HIP.

Abbreviations: CI, confidence interval; PNAG HIP, β -1 \rightarrow 6-poly-*N*-acetyl glucosamine hyperimmune plasma; RE HIP, *Rhodococcus equi* hyperimmune plasma.

3 | RESULTS

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 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 ($P < .05$) higher among RE HIP compared to PNAG HIP (Figure 1). The relative OD values for C'1q deposition were significantly ($P < .05$) higher among foals that were transfused with PNAG HIP compared to RE HIP (Figure 2). Among foals transfused with RE HIP, there was no significant difference ($P = .68$) of OD ratios for VapA of plasma samples between those that developed pneumonia and those that did not develop pneumonia (Figure S3). Among foals transfused with PNAG HIP, there was no significant difference ($P = .6$) in the relative OD values for C'1q of samples from foals that developed pneumonia and foals that did not develop pneumonia (Figure S4).

Farm	PNAG HIP		RE HIP		OR (95% CI) ^a	P value ^b
	Pneumonia	Healthy	Pneumonia	Healthy		
Kentucky	1 (2%)	46 (98%)	6 (13%)	42(87%)	0.30 (0.04-1.40)	.27
Oklahoma	6 (10%)	52 (90%)	6 (10%)	54(90%)	1.39 (0.45-4.47)	.57
New York	17 (38%)	28 (62%)	12 (23%)	40 (77%)	2.67 (1.22-5.99)	.02
Texas 1	6 (15%)	35 (85%)	2 (5%)	41 (95%)	3.51 (0.75-25.04)	.15
Texas 2	0 (0%)	24 (100%)	6 (24%)	19 (76%)	ND ^c	.02 ^c

Note: Odds ratios (and 95% CIs) estimated using logistic regression for the outcome of pneumonia attributed to *Rhodococcus equi* for each of 5 farms where foals were transfused with either RE HIP or PNAG HIP.

Abbreviations: 95% CI, 95% confidence interval; PNAG HIP, β -1 \rightarrow 6-poly-*N*-acetyl glucosamine hyperimmune plasma; RE HIP, *Rhodococcus equi* hyperimmune plasma.

^aOR = odds of pneumonia among foals transfused with PNAG HIP relative to RE HIP.

^bP values derived from logistic regression unless indicated by footnote "c."

^cND = not determined because incalculable due to complete separation; P value derived using Fisher's exact test.

TABLE 3 Individual farms distribution of foals within plasma type and odds ratios (OR) of pneumonia based on plasma type

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; $P = .24$), adjusted for the effect of farm. The odds of pneumonia were approximately 6-fold higher ($P < .001$) for foals at the New York farm compared to the reference farm Texas 1 (Table 2). The cumulative incidence and effect of plasma type varied among farms (Table 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.

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 antibodies 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 misclassification 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 hyperimmunized 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.

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CONFLICT OF INTEREST DECLARATION

Gerald B. Pier is an inventor of intellectual properties (human monoclonal antibody to PNAG and PNAG vaccines) that are licensed by Brigham and Women's Hospital to Alopexx Inc, an entity in which Gerald

B. Pier also holds equity. As an inventor of intellectual properties, Gerald B. Pier also has the right to receive a share of licensing-related income (royalties, fees) through Brigham and Women's Hospital from Alopexx Inc. Gerald B. Pier's interests were reviewed and are managed by the Brigham and Women's Hospital and Partners Health care in accordance with their conflict of interest policies. Colette Cywes-Bentley is an inventor of intellectual properties (use of human monoclonal antibody to PNAG and use of PNAG vaccines) that are licensed by Brigham and Women's Hospital to Alopexx Inc. As an inventor of intellectual properties, Colette Cywes-Bentley also has the right to receive a share of licensing-related income (royalties, fees) through Brigham and Women's Hospital from Alopexx Inc. Sarah C. Meyer and Patrick J. Sutter work for MG Biologics that produced the plasma used for this project and thus might have potential earnings from plasma sales, but they had no part in the study design other than masking plasma and did not participate in data analysis. No other authors have a conflict of interest.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

This study was approved by the Texas A&M University IACUC and the Clinical Research Review Committee of the Texas A&M College of Veterinary Medicine & Biomedical Sciences (Protocol #2018-0429), and included informed owner consent for participation.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

HUMAN ETHICS APPROVAL DECLARATION

The authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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