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In vitro evaluation of complement deposition and opsonophagocytic killing of *Rhodococcus equi* mediated by poly-*N*-acetyl glucosamine hyperimmune plasma compared to commercial plasma products

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Abstract

Background: The bacterium *Rhodococcus equi* can cause severe pneumonia in foals. The absence of a licensed vaccine and limited effectiveness of commercial *R. equi* hyperimmune plasma (RE-HIP) create a great need for improved prevention of this disease.

Hypothesis: Plasma hyperimmune to the capsular polysaccharide poly-*N*-acetyl glucosamine (PNAG) would be significantly more effective than RE-HIP at mediating complement deposition and opsonophagocytic killing (OPK) of *R. equi*.

Animals: Venipuncture was performed on 9 Quarter Horses.

Methods: The ability of the following plasma sources to mediate complement component 1 (C1) deposition onto either PNAG or *R. equi* was determined by ELISA: (1) PNAG hyperimmune plasma (PNAG-HIP), (2) RE-HIP, and (3) standard non-hyperimmune commercial plasma (SP). For OPK, each plasma type was combined with *R. equi*, equine complement, and neutrophils isolated from horses (n = 9); after 4 hours, the number of *R. equi* in each well was determined by quantitative culture. Data were analyzed using linear mixed-effects regression with significance set at $P < .05$.

Results: The PNAG-HIP and RE-HIP were able to deposit significantly ($P < .05$) more complement onto their respective targets than the other plasmas. The mean proportional survival of *R. equi* opsonized with PNAG-HIP was significantly ($P < .05$) less (14.7%) than that for SP (51.1%) or RE-HIP (42.2%).

Abbreviations: AP, alkaline phosphatase; BHI, brain heart infusion; C1, complement component 1; C1q, complement component 1q epitope; CFU, colony forming unit; HIP, hyperimmune plasma; OD, optical density; OPK, opsonophagocytic killing; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; PMNC, PMN plus complement; PNAG, β-(1→6)-linked poly-*N*-acetyl-glucosamine; PNAG-HIP, PNAG hyperimmune plasma; RE, *Rhodococcus equi* (*R. equi*); RE-HIP, *R. equi* hyperimmune plasma; RPMI, Roswell Park Memorial Institute; SP, standard (non-hyperimmune) plasma.

Conclusions and Clinical Importance: Plasma hyperimmune to PNAG is superior to RE-HIP for opsonizing and killing *R. equi* in vitro. Comparison of these 2 plasmas in field trials is warranted because of the reported incomplete effectiveness of RE-HIP.

KEYWORDS

complement, horse, neutrophils, opsonophagocytic killing, plasma, *Rhodococcus equi*

1 | INTRODUCTION

Rhodococcus equi is a gram-positive bacterium that causes granulomatous pneumonia in foals, a widespread and costly disease for the equine industry. Treatment of *R. equi* pneumonia can be prolonged, have adverse effects for treated foals and their dams,[1] and contribute to development of antimicrobial resistance when antibiotics are used on a widespread basis.[2,3] Thus, there is a great need for prevention of *R. equi* pneumonia. No commercially available vaccines against *R. equi* exist,[1,4] such that prevention of pneumonia is based primarily on transfusion of plasma that is hyperimmune to *R. equi* (RE-HIP).[1,5,6] Transfusion of RE-HIP, however, is not completely effective in decreasing the incidence of *R. equi* pneumonia at horse breeding farms.[1,7-10] Clinical and randomized controlled trials have resulted in variable outcomes for the prophylactic efficacy of RE-HIP.[9-13] Efforts to identify more effective antigenic targets for preparing HIP are warranted.

The capsule-like polysaccharide, β-(1→6)-linked poly-*N*-acetylglucosamine (PNAG), is a highly conserved sugar molecule present on the surface of many pathogenic microbes including *R. equi*.[14] Recently, our laboratory has demonstrated that vaccinating pregnant mares protected their foals against experimental infection with *R. equi* when challenged at approximately 4 weeks of age.[15] Moreover, transfusion of plasma hyperimmune for PNAG (PNAG-HIP) protected additional foals against a similar experimental infection.[15] Although these findings indicate that PNAG is a valid target for immunizing donors to prepare PNAG-HIP, this study only compared foals transfused with standard non-HIP (SP) to those treated with PNAG-HIP[15]; comparison of PNAG-HIP with RE-HIP was lacking. Protection against *R. equi* either by PNAG vaccination of mares or by transfusion of PNAG-HIP to foals was mediated by anti-PNAG antibodies that fix complement and mediate opsonophagocytic killing (OPK) by neutrophils in conjunction with complement.[15] Our objective was to better characterize the protection provided by PNAG-HIP relative to RE-HIP by comparing complement deposition and OPK of PNAG-HIP, RE-HIP, and SP in vitro.

2 | MATERIALS AND METHODS

2.1 | Plasmas and complement source

The following plasma products were used in this study: non-HIP (SP; High-Glo Equine IgG, Mg Biologics, Inc, Ames, Iowa), plasma hyperimmune to *R. equi* (RE-HIP; ReSolution Rhodococcus Equi Antibody, Mg Biologics, Inc), and plasma hyperimmune to PNAG (PNAG-HIP; Mg Biologics, Inc[15]). Concentrations of IgG for each plasma were measured by ELISA performed in duplicate using a commercial kit (Horse IgG ELISA Kit, Abcam, Cambridge, Massachusetts). The complement source was prepared from commercial equine serum (Sigma-Aldrich, St. Louis, Missouri) that was adsorbed twice with *R. equi* (EIDL 5-331) at 4°C for 30 minutes to remove *R. equi*-specific antibodies, as previously described.[15] After adsorption, the complement solution was centrifuged, filter sterilized, diluted to a 40% concentration in phosphate-buffered saline (PBS), and frozen until use.

2.2 | Complement component 1q epitope (C1q) deposition assay

Complement component 1 (C1) deposition assays were conducted to analyze the ability to opsonize *R. equi* by the test plasmas SP, RE-HIP, and PNAG-HIP. Immulon 4 HBX 96-well immunoassay plates (VWR International, Radnor, Pennsylvania) were coated with either the purified PNAG molecule[16] or whole *R. equi*.[4] For PNAG coating, plates were treated with 100 μL of PNAG, purified as described previously,[16] at 0.6 μg/mL and then incubated for a minimum of 3 hours. The strain of virulent *R. equi* used to coat plates (EIDL 5-331; confirmed virulent by polymerase chain reaction and in vivo infection[4,15]) was grown overnight in brain heart infusion broth (BHIB; Becton, Dickinson, and Co, Sparks, Maryland) at 37°C; 100 μL of a bacterial suspension prepared at an optical density (OD) of 1 (corresponding to a concentration of 1.0×10^8 *R. equi*/mL or 2.5 mg/mL of *R. equi* antigen)[4] at a 650-nm wavelength was placed in sensitization buffer (0.04 M PO_4 [pH 7.2]). Plates were incubated for a minimum of 3 hours at 37°C and then stored overnight at 4°C. The plates then were treated with 100 μL of methanol (−20°C) for 5 minutes before blocking for 60 minutes at 37°C with 120 μL of PBS + 1% skim milk (1 g/100 mL) that was heated to 65°C for 60 minutes to pasteurize the solution. All C1 deposition assays were performed in quadruplicate for each test plasma; results of replicates were included in mixed-effects regression modeling (described below in the Statistical Methods section). Samples of each plasma were heat inactivated (56°C for 30 minutes), and 50 μL of each plasma was combined with 50 μL of complement source (100 μL/well) for 60 minutes on a rocker at 37°C. The purpose of heat treating the plasma was to inactivate complement in each plasma product such that, by using a common source of complement, innate differences among plasmas to deposit complement could not be attributed to innate differences in complement activity in the plasma products. After washing 3 times with PBS + 0.05% Tween, affinity-purified goat

anti-complement C1q primary antibody (Cedarlane, Inc, Burlington, North Carolina) was placed in each well (100 μL/ well) at a dilution of 1:1000, and incubated for 60 minutes at room temperature (approximately 22°C). This was followed by 3 wash cycles with PBS + 0.05% Tween, after which an anti-goat IgG-alkaline phosphatase (AP) secondary antibody produced in rabbits (anti-goat IgG-AP, Sigma-Aldrich) was placed in each well at a dilution of 1:2000. Plates were incubated for 60 minutes at room temperature. After another 3 wash cycles, the AP-enzymatic production of color was elicited using a 1 mg/mL of 4-nitrophenyl phosphate disodium salt hexahydrate in substrate buffer for 30 minutes at 37° C. The ODs for each plate were recorded at a wavelength of 450 nm.

2.3 | Opsonophagocytic killing assay

Polymorphonuclear cells (PMNs) for the OPK assays were prepared from fresh equine blood collected from horses of the Department of Large Animal Clinical Sciences (VLCS) research and teaching herd using a double layer Histopaque (Sigma-Aldrich), isolation (densities = 1.077 and 1.191). The blood collection procedure was approved by the Texas A&M University Animal Care and Use Committee (AUP# 2017-0440). For OPK assays, PMNs were collected from 8 adult mares (5-15 years of age) and 1 yearling gelding from the VLCS herd. All of the test subjects were Quarter Horses. Each isolation was confirmed to be composed of >95% neutrophils using microscopy. The sample size was calculated on the basis of previous results of OPK assays,[15] and unpublished data from our laboratory indicating that PMNs from healthy yearlings are similar to horses >1 year of age. The concentration of PMNs was adjusted to 1×10^7 cells/mL in modified Roswell Park Memorial Institute (RPMI) medium 1640 free of phenol red (Lonza Bioscience, Walkersville, Maryland) +5% heat-inactivated (ie, 56°C for 30 minutes) fetal bovine serum (Bovine Serum Gibco, ThermoFisher, Waltham, Massachusetts). The test plasmas for the OPK assay (SP, RE-HIP, and PNAG-HIP [Mg Biologics, Inc]) were diluted 1:10 in RPMI. Overnight cultures of the bacterial strains to be evaluated in the OPK assay were grown in brain-heart infusion (BHI) broth at 37°C. Bacterial suspensions were made at an OD at 650 nm of approximately 0.95 (corresponding to approximately 1×10^8 colony forming units [CFU]/mL) and the final concentration adjusted to 1×10^7 CFU/mL in RPMI for use in the OPK assay. The assay was performed by mixing 100 μL (each) of each of the test plasmas (non-heat-inactivated), the PMN suspension, and the *R. equi* solution. The reference negative control was composed of 100 μL of bacterial solution added to 300 μL of RPMI to maintain a consistent volume among wells. A negative control with only PMNs plus complement (PMNC) was included, to account for the effects of PMNs and complement in the absence of plasma. The PMNC was made by combining 100 μL (each) of PMN suspension, the aforementioned complement source, *R. equi* solution, and RPMI. The assay tubes were incubated on a rotator rack at 37°C for 4 hours; samples were taken at time 0 and after 4 hours. Samples were plated onto BHI plates in either triplicate or duplicate, 50 μL/plate. The CFU were quantified after a 24-hour incubation at 37°C. The proportional survival for a given treatment (PMNC, SP, RE-HIP, and PNAG-HIP) relative to the reference negative control was calculated and used for the outcome measures of OPK. Values of proportional survival >100% reflect bacterial growth and proportions <100% indicate bacterial death.

2.4 | Statistical methods

Data were analyzed using mixed-effects linear regression to evaluate effects of plasma treatment on C1 deposition onto plates coated with PNAG or *R. equi* by ELISA. The absorbance was modeled as the outcome variable. Dependent variables were the dilution, treatment, and their interaction which were modeled as fixed effects, and horse identification modeled as a random effect. Multiple comparisons of pairwise differences among treatments within dilution and dilution within treatment were made using the method of Sidak.[17] To evaluate effects of plasma treatment on opsonic killing of *R. equi* when incubated with neutrophils (and complement), data were analyzed using mixed-effects linear regression. The dependent variable was the proportion of bacteria surviving; treatment was modeled as a fixed effect and horse was modeled as a random effect. The proportional survival (relative to bacteria in media only) was transformed using the $\log_{10}$ function to meet distributional assumptions of the model. Multiple comparisons of pair-wise differences either among treatments within dilution or dilution within treatment were made using the method of Sidak.[17] Significance level for analysis was set at $P < .05$.

3 | RESULTS

The concentrations of IgG determined by ELISA in the SP, RE-HIP, and PNAG were 1462, 2333, and 2116 mg/dL, respectively. The OD at 450 nm was measured for C1 deposition on to PNAG-coated plates at dilutions ranging from 1:100 to 1:6400 for each plasma (Figure 1). The PNAG-HIP deposited significantly more C1 onto PNAG compared to RE-HIP and SP at dilutions of 1:100, 1:200, and 1:400 ($P < .05$). There were no statistically significant differences at any of the dilutions measured between RE-HIP and SP for deposition of C1 onto PNAG. Because of limitations in the amount of binding of the *R. equi* bacteria to the wells, the OD for the C1 deposition on to *R. equi*-coated plates was measured at dilutions ranging from 1:10 to 1:640 for each plasma (Figure 2). The RE-HIP deposited significantly ($P < .05$) more C1 onto *R. equi* compared to PNAG-HIP and SP at dilutions of 1:10, 1:20, and 1:40; there were no significant differences at any of the dilutions evaluated between PNAG-HIP and SP for C1 deposition onto *R. equi*.

Using linear mixed-effects modeling, proportional survival of *R. equi* for the PMNs and complement alone (PMNC, 114.3%; 95% confidence interval [CI], 92.5%-136.2%) did not differ significantly from that of bacteria in media only. All 3 plasma treatments, however, yielded bacterial counts that were significantly ($P < .05$) lower than the media only or PMNC controls (Figure 3). Moreover, the proportional survival by PNAG-HIP (mean, 14.7%; 95% CI, 0%-33.8%) was significantly ($P < .05$) less than that for either the RE-HIP (mean, 41.2%; 95% CI, 22.1%-60.3%) or the SP (mean proportional killing, 51.1%; 95% CI, 32.1%-70.2%), but the

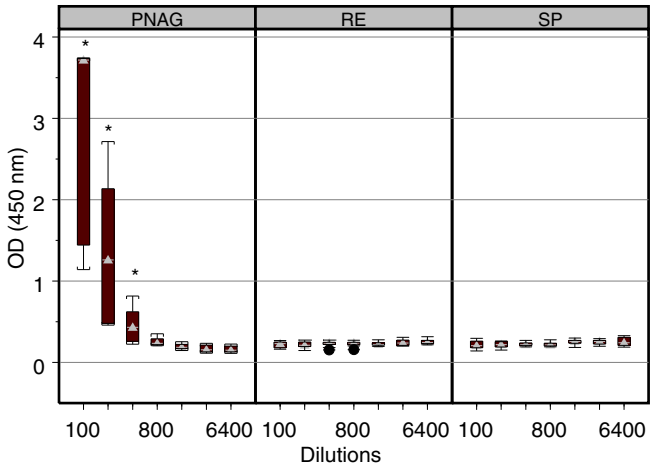

FIGURE 1 Boxplot of optical densities at 450 nm from complement component 1q epitope (C1q) ELISAs measuring C1q deposition onto β-(1→6)-linked poly-*N*-acetyl-glucosamine (PNAG) mediated by 3 different sources of plasma: PNAG hyperimmune plasma (PNAG-HIP), *R. equi* hyperimmune plasma (RE-HIP), and standard (non-HIP) plasma (SP). The PNAG-HIP deposited significantly greater complement onto PNAG at dilutions of 100, 200, and 400. The other plasmas had no statistical difference in deposition. Gray triangles represent median values and black circles represent outliers. Statistical significance ($P < .05$) among treatments indicated by asterisks

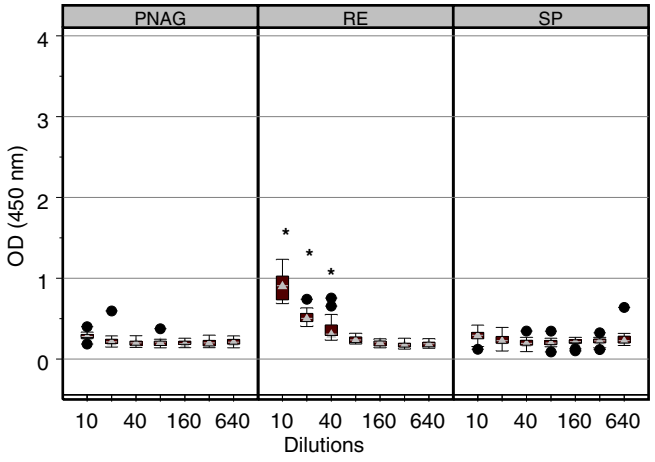

FIGURE 2 Boxplot of optical densities at 450 nm from complement component 1q epitope (C1q) ELISAs measuring C1q deposition onto whole *R. equi* mediated by 3 different sources of plasma. The *R. equi* hyperimmune plasma (RE-HIP) deposited significantly greater C1 onto *R. equi* at dilutions of 100, 200, and 400, whereas there was no significant difference among the other plasmas. Gray triangles represent median values and black circles represent outliers. Statistical significance ($P < .05$) among treatments indicated by asterisks. PNAG, β-(1→6)-linked poly-*N*-acetyl-glucosamine; SP, standard (non-HIP) plasma

proportional survival did not differ significantly between the RE-HIP and SP (Figure 3). The complement of proportional survival is proportional killing. In this context, the proportional killing by PNAG-HIP (mean, 85.3%; 95% CI, 66.2%-100%) was significantly ($P < .05$) higher than the proportional killing of either RE-HIP (mean, 59.8%; 95% CI, 39.7%-87.9%) or SP (mean, 48.9%; 95%, CI, 29.8%-67.9%).

4 | DISCUSSION

Our results indicate that PNAG-HIP was superior to either RE-HIP or SP at depositing complement onto the PNAG antigen and mediating killing of *R. equi* by PMNs. The results from the C1q ELISA indicate that both PNAG-HIP and RE-HIP can activate and deposit complement on to PNAG and *R. equi*, respectively, indicating that they both function to supply complement opsonins essential for bacterial killing. The finding that PNAG-HIP did not deposit significantly more C1 onto whole *R. equi* was attributed to limitations in the amount of antigen that can be bound to each well, the relative amount of PNAG on PNAG-coated plates versus on bacterial surfaces, and the sensitivity of the methods being used. Nevertheless, both HIPs (RE-HIP and PNAG-HIP) were superior to SP at depositing C1 onto their respective targets (HIP and PNAG, respectively), and the difference in OPK between PNAG-HIP and RE-HIP could not be explained by differences in C1q deposition. Because PNAG is a surface polysaccharide, making it functionally equivalent to a bacterial capsule, it is likely that antibody to PNAG deposits the most effective opsonins such as IgG, C3b, iC3b, and other complement factors onto the outer bacterial surface. This is well correlated with enhanced OPK-mediated killing, because IgG and complement receptors on the surface of phagocytes can bind and ingest microbes most efficiently under these conditions, and therefore likely accounts for the differences in OPK between PNAG-HIP and RE-HIP.[18,19] Of note, however, is that in OPK assays previously conducted with *R. equi*, as well as with other microbial targets,[14-16] opsonic killing mediated by antibody to PNAG is completely dependent on the presence of functional complement, suggesting little to no role for the receptors of the constant region of immunoglobulins (ie, FcRs) in this system. Nevertheless, further evaluation of the ability of PNAG-HIP to deposit complement onto *R. equi* is warranted by our results.

In the OPK assay, there was no significant difference between the controls with no plasma (control and PMNC), indicating that neutrophils and complement alone mediated negligible killing in the absence of antibodies or other opsonins that bind to *R. equi* to mediate bacterial killing. Interestingly, for some horse sera the bacteria actually appeared to survive better than in media only (Figure 3; proportional survival >1). The reasons for this finding are unknown, but it is likely that the complement source provided nutrients for bacterial growth.

Each of the plasmas mediated significantly higher OPK (ie, significantly smaller proportion of surviving bacteria) than the PMNC controls in any of the assays, indicating that opsonization with plasma improves the killing capacity of PMNs. Nonetheless, the PNAG-HIP mediated significantly higher OPK (significantly lower proportion of bacteria surviving) than did either the SP or the RE-HIP. As noted above, the difference between RE-HIP and PNAG-HIP was not attributable to the ability of the plasmas to fix complement onto their targets but might be because of the location of the opsonins deposited onto the bacterial cells. Because the concentrations of total IgG were similar in RE-HIP and PNAG-HIP, the observed difference could not be attributed to a difference in IgG content. We conclude that PNAG-HIP was superior to RE-HIP at killing of *R. equi* in vitro. Our finding that PNAG-HIP

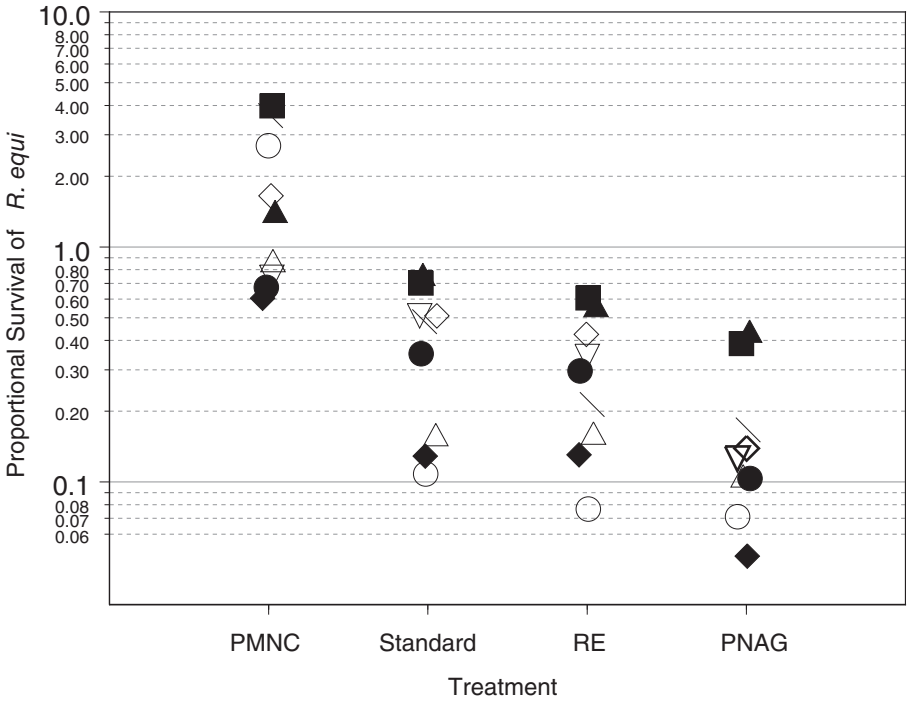

FIGURE 3 Proportion of viable *R. equi* co-cultured with polymorphonuclear leukocytes (PMNs) and complement only (PMNC), standard plasma (SP), *R. equi* hyperimmune plasma (RE-HIP), or β-(1→6)-linked poly-*N*-acetyl-glucosamine-hyperimmune plasma (PNAG-HIP), relative to bacteria in media only. The proportional survival of *R. equi* treated with PMNC did not differ significantly from bacteria grown in media only. The proportional survival of *R. equi* in the presence of PNAG-HIP (14.7%) was significantly ($P < .05$) less than that for RE-HIP (41.2%) or SP (51.2%), but there was no significant difference in *R. equi* survival between RE-HIP and SP

mediated significantly more killing than an equivalent volume of SP in vitro is consistent with the observation that PNAG-HIP mediated significantly more protection than SP against intrabronchial infection of foals.[15]

Although the RE-HIP mediated somewhat more killing than SP, the difference was not significant. The IgG concentration of SP was approximately 70% of the IgG concentration in RE-HIP (and PNAG-HIP), but its concentration was similar to the reported ranges for commercially available plasmas.[20,21] Although data are lacking from either observational studies or randomized controlled field trials, our findings are consistent with prior experimental evidence of similar survival among foals infected with *R. equi* that were transfused with RE-HIP or SP before infection.[22] Further evaluation of the comparison of SP and RE-HIP is warranted inasmuch as our study was limited to in vitro data and was underpowered to detect the observed magnitude of difference between these 2 plasma products.

Our study had a number of limitations. Results of this in vitro study do not provide evidence of superior protection of PNAG-HIP in vivo. Nonetheless, we considered this in vitro study essential preliminary evidence to justify field-based comparisons among plasma products. The complement deposition assays indicated that both PNAG-HIP and RE-HIP deposited complement onto their targets, but PNAG-HIP was superior to RE-HIP in bacterial killing. This discrepancy suggests that the difference in OPK function is not the result of superior complement activation by anti-PNAG antibodies but rather might be because of the location of the opsonins on the surface of the bacteria. The mechanism of action of antibody to PNAG is an ongoing activity in our laboratory. We did not evaluate the ability of PMNs from foals to mediate killing, and evidence exists that there are functional deficits in opsonic capacity and functional responses of neonatal foal PMNs.[23-31] We do note, however, that active vaccination of pregnant mares with a PNAG conjugate vaccine as well as passive administration of PNAG-HIP to newborn foals resulted in an aggregate protection from *R. equi* intrabronchial challenge of 94%, whereas 91% of foals born to control mares in the active vaccine trial or foals receiving SP in the passive protection trial developed *R. equi* pneumonia.[15] The foals were 4 weeks of age at challenge,[15] and likely had functional phagocytes and complement at this point. At the time the study reported here was conducted, we did not have an opportunity to obtain PMNs from young foals. The sex and age distribution of our population was not ideal (8 mares and 1 gelding), but the similar functional responses of all horses suggest that this did not confound our results. Finally, our sample size was modest, and having more horses would have enhanced the precision and power of the study. Only a single strain of *R. equi* (1 for which we have in vitro and in vivo evidence of virulence) was considered in our study. Thus, our results cannot necessarily be extrapolated to other strains, but we have reported that 100% of *R. equi* clinical isolates tested produce surface PNAG antigen.[15] Although considerable genetic heterogeneity of *R. equi* strains has been reported,[32] there is no evidence of variation in virulence or response to plasma treatment among isolates of *R. equi*. It is generally considered that any isolate bearing the virulence-associated plasmid can cause disease in susceptible foals.[1] Despite these limitations, we found convincing evidence that PNAG-HIP mediated significantly more killing than did RE-HIP. The relevance of this finding merits further investigation in vivo.

5 | CONCLUSIONS

In conclusion, PNAG-HIP and RE-HIP are capable of activating complement. Furthermore, PNAG-HIP was functionally superior at mediating killing of *R. equi* by PMNs in vitro than either RE-HIP or SP. Comparison of PNAG-HIP and RE-HIP (or SP) in vivo is warranted.

Further investigations of the mechanism of anti-PNAG antibodies to mediate protection against *R. equi* are warranted.

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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 Vaccine, LLC, and Alopexx Pharmaceuticals, LLC, entities 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 Pharmaceuticals, LLC, and Alopexx Vaccine, LLC. Gerald B. Pier's interests were reviewed and are managed by the Brigham and Women's Hospital and Partners Healthcare 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 Pharmaceuticals, LLC. 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 Pharmaceuticals, LLC. Noah D. Cohen has received an unrestricted gift to the Equine Infectious Disease Laboratory at Texas A&M University to support research from Alopexx Vaccines, LLC.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

The blood collection procedure was approved by the Texas A&M University Animal Care and Use Committee (AUP# 2017-0440).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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