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PNAG-specific equine IgG_1 mediates significantly greater opsonization and killing of *Prescottella equi* (formerly *Rhodococcus equi*) than does $IgG_{4/7}$

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ABSTRACT

Prescottella equi (formerly *Rhodococcus equi*) is a facultative intracellular bacterial pathogen that causes severe pneumonia in foals 1–6 months of age, whereas adult horses are highly resistant to infection. We have shown that vaccinating pregnant mares against the conserved surface polysaccharide capsule, β-1 → 6-linked poly-*N*-acetyl glucosamine (PNAG), elicits opsonic killing antibody that transfers via colostrum to foals and protects them against experimental infection with virulent.

R. equi. We hypothesized that equine IgG_1 might be more important than $IgG_{4/7}$ for mediating protection against *R. equi* infection in foals. To test this hypothesis, we compared complement component 1 (C1) deposition and polymorphonuclear cell-mediated opsonophagocytic killing (OPK) mediated by IgG_1 or $IgG_{4/7}$ enriched from either PNAG hyperimmune plasma (HIP) or standard plasma. Subclasses IgG_1 and $IgG_{4/7}$ from PNAG HIP and standard plasma were precipitated onto a diethylaminoethyl ion exchange column, then further enriched using a protein G Sepharose column. We determined C1 deposition by enzyme-linked immunosorbent assay (ELISA) and estimated OPK by quantitative microbiologic culture. Anti-PNAG IgG_1 deposited significantly ($P < 0.05$) more C1 onto PNAG than did $IgG_{4/7}$ from PNAG HIP or subclasses IgG_1 and $IgG_{4/7}$ from standard plasma. In addition, IgG_1 from PNAG HIP mediated significantly ($P < 0.05$) greater OPK than $IgG_{4/7}$ from PNAG HIP or IgG_1 and $IgG_{4/7}$ from standard plasma. Our findings indicate that anti-PNAG IgG_1 is a correlate of protection against *R. equi* in foals, which has important implications for understanding the immunopathogenesis of *R. equi* pneumonia, and as a tool for assessing vaccine efficacy and effectiveness when challenge is not feasible.

1. Introduction

Prescottella equi (*P. equi*, formerly *Rhodococcus equi*) is a ubiquitous facultative intracellular pathogen that causes severe pyogranulomatous pneumonia in foals 3 weeks to 6 months of age, and is among the leading causes of morbidity and mortality for foals [1,2]. Although effective treatments exist [3–6], no vaccine to prevent *P. equi* pneumonia in foals is commercially available. Recently, our laboratory has demonstrated that vaccination of pregnant mares targeting the highly conserved microbial surface

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Abbreviations: PNAG, β-1→6-linked poly-*N*-acetyl glucosamine; CFU, colony forming unit; C1, complement component 1; OPK, opsonophagocytic killing; PMN, polymorphonuclear cells; HIP, hyperimmune plasma; DEAE, diethylaminoethyl; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; OD, optical density; ELISA, enzyme-linked immunosorbent assay.

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polysaccharide, β-1 → 6-linked poly-*N*-acetyl glucosamine (PNAG) protects their foals against subsequent intrabronchial infection with virulent *P. equi* [7]. Antibodies do not cross the equine epitheliochorial placenta during gestation [8] and thus foals are born agammaglobulinemic. Consequently, vaccination of mares during late gestation was presumed to protect foals by antibodies transferred postnatally from mares to foals via colostrum. Foals born to PNAG-vaccinated mares acquired antibody to PNAG from colostrum of the immunoglobulin (IgG) subclass 1 (IgG_1; a.k.a., IgGa) and subclass $IgG_{4/7}$ (a.k.a., IgGb), with differences in titers between vaccinates and controls of the former being generally higher than those of the latter. This finding was considered potentially important clinically because different equine IgG subclasses are known to mediate different effector functions in immune responses [9–13]. Direct comparison of the function of these subclasses, however, was not determined in our prior study [7].

Conflicting data exist regarding the role of IgG subclasses in immunity to *P. equi* [7–10] and other equine pathogens [11,12]. It has been proposed that the predominant subclass response against *P. equi* reflects a protective Th1-type response (mediated by IgG_1) or a non-protective Th2-type response (mediated by $IgG_{3/5}$ and $IgG_{4/7}$) [8]. Other studies, however, indicate that the $IgG_{4/7}$ subclass provides protective immunity against *P. equi* [9,10] and other intracellular pathogens [11,12]. Immunoglobulin G can opsonize *P. equi* for efficient phagocytosis by equine polymorphonuclear cells [13,14], and both IgG_1 and $IgG_{4/7}$ contribute to protection against bacterial infection by their ability to: (1) elicit a strong respiratory burst from equine peripheral blood mononuclear cells [9]; (2) bind to Fc receptors (FcRs) on effector cells [9,15]; and, (3) bind complement (C′) component 1 (C1) and activate C′ via the classical pathway [9]. Although $IgG_{3/5}$ also elicits a respiratory burst and interacts with FcRs [9], there is contradictory evidence as to whether it fixes C′, or inhibits C′ fixation by IgG_1 or $IgG_{4/7}$ [8,15,16]. The presence of $IgG_{3/5}$ antibody to *P. equi* is indicative of exposure to infection, but not of a protective immune response [17]. The finding that anti-PNAG IgG_1 titers were generally higher relative to foals of unvaccinated mares than those of $IgG_{4/7}$ in vaccinated and protected foals suggested that anti-PNAG IgG_1 could represent a correlate of protective immunity in foals. Thus, we sought to directly compare functional responses (*viz.*, deposition of C1 onto PNAG and opsonophagocytic killing [OPK] of virulent *P. equi* by polymorphonuclear cells [PMNs]) of IgG_1 isolated from plasma hyperimmune to PNAG (PNAG HIP) with those of $IgG_{4/7}$ from PNAG HIP, and with those of IgG_1 or $IgG_{4/7}$ from standard plasma.

2. Materials and methods

2.1. Enrichment and isolation of IgG subclasses IgG_1 and $IgG_{4/7}$

We performed the IgG subclass isolation from plasma at the Texas A&M University Protein and Chemistry Laboratory, based on the method of Sugiura et al. [18]. The plasma source used for the isolation of IgG subclasses was standard, non-immune, horse plasma with low titers to *P. equi* (Immunoglo Serial 1700, Mg Biologics, Ames, IA, USA) and PNAG HIP produced using previously described methods by Mg Biologics [7]. We removed the contaminants from 50 ml of plasma (either PNAG HIP or non-immune) using dextran sulphate to precipitate lipoproteins in the presence of cations such as Ca^{2+}, and dialysis with a 5 M Tris pH 8.0 buffer for desalination of the resulting protein solution (supernatant). Then we passed the desalted protein solution through a diethylaminoethyl (DEAE) Sepharose ion exchange column (GE Healthcare, Chicago, IL, USA) to which IgG_1 and $IgG_{4/7}$ do not bind. We collected the flow-through material into different fractions and detected presence of IgG_1 and $IgG_{4/7}$ by standard sandwich ELISA. The flow-through fractions positive for IgG_1 and $IgG_{4/7}$ were bound to a Protein G Sepharose column (GE Healthcare) and eluted using a pH gradient ranging from 8.0 to 2.0 to separate IgG_1 from $IgG_{4/7}$ on an AKTA pure chromatography system controlled by Unicorn (v7.0.3, GE Healthcare). We collected the fractions in Tris buffer (1 M, pH 7.5), combined, and concentrated using spin columns with molecular weight cutoff (MWCO) of 10,000 (VIVASPIN 15R, Sartorius Stedim Biotech, Goettingen, Germany). Isolation of IgG steps were monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [60].

2.2. ELISA fordetection of IgG subclasses

We detected the concentration (in ng/ml) of IgG_1 and $IgG_{4/7}$ subclasses collected from the chromatography fractions by standard sandwich ELISA using duplicates in a 96-well plate coated with horse IgG_1 or $IgG_{4/7}$. After 1 h of incubation at room temperature (approximately 22 °C), we washed the plate 5 times and blocked for 30 min at room temperature. We washed the plate again before adding diluted standard and IgG subclass samples from chromatography fractions and incubated at room temperature for 1 h. Afterwards we added diluted horseradish peroxidase conjugated antibodies against IgG_1 and $IgG_{4/7}$ to the corresponding wells, and incubated for another hour at room temperature. After a final wash, SureBlue Reserve One Component TMB Microwell Peroxidase Substrate (SeraCare, Gaithersburg, MD, USA) we added to the wells and incubated for 15 min at room temperature in the dark. We stopped the reaction before determining the optical densities at 450 nm by using microplate reader Synergy 2 (Biotek, Winooski, VT, USA).

2.3. Polymorphonuclear (PMN) cell isolation

For all procedures requiring blood in this study, blood was collected from university-owned donor horses using a protocol approved by the Texas A&M University IACUC (AUP# 2017-0440). To isolate equine PMNs, we collected whole blood in plastic sodium heparin vacutainer tubes (ThermoFisher Scientific, Grand Island, NY, USA) by layering over Histopaque 1191 (Sigma-Aldrich Co, St. Louis, MO, USA) and Histopaque 1077 (Sigma-Aldrich Co), and centrifuging at 700*g* for 30 min at 22 °C yielded 2 opaque rings or layers. We collected PMNs from the middle layer, washed once in 1X Hanks' Balanced Salt Solution (ThermoFisher Scientific), and resuspended in 1X RPMI medium.

2.4. Complement acquisition from depleting *P. equi*-specific antibodies from serum

We used the C′ source from commercial equine serum (Sigma-Aldrich Co) diluted to a 40% solution containing *P. equi* which was grown overnight in brain-heart infusion broth (Sigma-Aldrich Co) and suspended to a concentration of an optical density at 600 nm (OD_{600} nm) of 1. To allow time for *P. equi*-specific antibodies in the commercial serum to bind to the *P. equi* cells, we incubated this serum/*P. equi* solution on ice for 30 min. After this incubation time, we centrifuged the serum solution for 3 min at 6000*g* at 4 °C, discarded the pellet, incubated the supernatant again with *P. equi* at an OD_{600} nm of 1 for 30 min on ice, and centrifuged for 3 min at 6000*g* at 4 °C. To remove residual bacterial cells we filter-sterilized the supernatant through a 0.2-μm filter, and then we aliquoted, and stored at −80 °C.

2.5. Complement protein C1 deposition

This assay was performed as previously described [7]. Briefly, we sensitized ELISA plates with PNAG and added dilutions of equine standard plasma, PNAG HIP, or the IgG subclasses in 50-μl volumes with 50 μl of 10% intact horse serum as the source of C1 (Sigma-Aldrich Co). After 60 min incubation at 37 °C, we washed the plates and added 100 μl of goat anti-human C1 (Cedarlane, Burlington, NC, USA), which also binds to equine C1, diluted 1:1000, and incubated the plates at room temperature for 60 min. After washing, we added 100 μl of rabbit anti-goat IgG whole molecule (Sigma-Aldrich Co) conjugated to alkaline phosphatase diluted to 1:2000 and incubated for 1 h at room temperature. We finished by washing the plate and developing the color indicator before reading the optical densities.

2.6. Opsonophagocytic killing (OPK) assay

We determined the OPK of *P. equi* in the presence of PMNs, C′, and antibodies in 2-ml round-bottom tubes. The total volume of each tube was 400 μl and comprised of 4×10^6 equine PMN (1×10^7 cells/ml), 40% *P. equi*-absorbed horse serum as a C′ source, 4×10^6 cfu of *P. equi* (1×10^7 cfu/ml), and 10 μg/ml antibodies of either subclass (IgG_1 or $IgG_{4/7}$) isolated from standard plasma or PNAG HIP. We used two negative controls: a bacterial control (Contrl Bact), comprised only of media and *P. equi*; and a control omitting antibodies (Contrl C′PMN; presence of media, *P. equi*, equine C′ and PMNs). We incubated the tubes with end-over-end rotation for 90 min then serially diluted and plated for bacterial enumeration.

2.7. Statistical analysis

We analyzed the C1 deposition data (OD_{405} nm) using linear mixed-effects regression. The outcome variable was the OD value and the dependent fixed variables were source of antibody (standard plasma, PNAG HIP, and IgG_1 and $IgG_{4/7}$ from either PNAG HIP or standard plasmas a categorical variable), concentration (as an ordered categorical variable), and their bivariate interaction terms, with individual experiment as a random effect. The 95% confidence intervals were estimated using maximum likelihood methods. For post hoc comparisons among dilutions and treatments, we used the method of Sidak [19], a significance level of $P < 0.05$.

We analyzed the OPK data from 16 experiments conducted using PMNs from 16 horses to compare the killing capacity of PMNs infected with either IgG_1 or $IgG_{4/7}$ derived from PNAG HIP or standard plasma. We determined the proportional killing by dividing the number of bacteria recovered from PMNs by the number from control samples. We analyzed data using linear mixed-effects regression with the proportional killing as the dependent variable (standard plasma, PNAG HIP, IgG_1P, IgG_1S, $IgG_{4/7}P$, and $IgG_{4/7}S$), and estimated the 95% confidence intervals using maximum likelihood methods. For post hoc comparisons among dilutions and treatments we used the method of Sidak [19], a significance level of $P < 0.05$. For all linear mixed-effects regression models, the goodness of model fit was assessed by inspecting diagnostic residual plots and examining AIC and BIC values.

3. Results

3.1. Enrichment of IgG subclasses from equine plasma

To compare functional responses of IgG subclasses IgG_1 and $IgG_{4/7}$ from PNAG HIP and standard plasma, we first needed to enrich these subclasses from plasma. We used a DEAE ion exchange column to enrich whole IgG molecules from standard commercial equine plasma and PNAG HIP, provided by the same manufacturer (Mg Biologics, Ames, IA) (Fig. 1). This resulted in the release of IgG into chromatography fractions collected from the flow-through represented in the first peak in Fig. 1. We confirmed the presence of IgG_1 and $IgG_{4/7}$ in this flow-through by ELISA (Table 1). We stripped the remaining proteins bound to the DEAE column from the column with a salt wash-out (second peak in Fig. 1). We monitored the quality of the IgG enrichment from equine plasma by using SDS-PAGE on saved samples of the enrichement process: the original whole plasma, the supernatant from dextran sulphate precipitation, the dialysate from desalination, chromatography fractions with whole IgG (first peak of DEAE ion exchange column in Fig. 1), and chromatography fractions from the column salt wash-out (second peak of DEAE ion exchange column in Fig. 2).

3.2. Verification of IgG enrichment using SDS-PAGE

To detect potential protein loss during the enrichment process, we performed SDS-PAGE on samples of the different fractions obtained from the plasma enrichment of IgG (Fig. 2).

We found substantial amounts of albumin present in the whole (untouched) plasma, supernatant (after precipitation), and dialysate (after desalination), suggesting that there was no major protein loss. We showed fractions from the first peak in Fig. 1 to be depleted of albumin, and to instead have strongly enriched bands that corresponded to heavy and light IgG chains, confirming presence of IgG (see Fig. 2). The strong band of albumin reappeared in the wash-out (second peak in Fig. 1), that had only trace bands of heavy and light chains of IgG. It should be noted that this gel was heavily loaded with proteins such that small amounts of protein can be detected. This figure demonstrates the successful enrichment of the fractions containing predominately IgG that was substantiated by the results of ELISA testing (Table 1).

3.3. Isolation of IgG_1 and $IgG_{4/7}$ using protein G Sepharose columns

For the isolation of IgG_1 and $IgG_{4/7}$ from the flow-through in the DEAE ion exchange columns (first peak to the left, Fig. 1) we used a Protein G Sepharose column (Fig. 3). A continual decrease in pH (represented by the green line in Fig. 3) allowed for the capture of IgG_1 in the fractions represented in the smaller peak beneath the pH gradient in Fig. 3. Further decrease in the pH allowed for the dissociation of $IgG_{4/7}$ from the Protein G Sepharose column and its collection into the fractions represented by the taller peak beneath the pH gradient in Fig. 3. We confirmed the presence or absence of IgG_1 and $IgG_{4/7}$ in the eluted fractions by ELISA (Table 2).

3.4. Complement component C1 deposition of standard plasma, PNAG HIP and their IgG subclasses

To test for differences in classical C′ pathway activation between IgG_1 and $IgG_{4/7}$ from PNAG HIP and standard plasma, we compared their ability to deposit C1 onto PNAG. We found that antibodies from whole PNAG HIP (positive control) deposited significantly ($P < 0.05$; linear mixed-effects modeling, using the method of Sidak for pair-wise comparisons) more C1 onto PNAG than did those from whole standard plasma (Fig. 4), subclasses IgG_1 or $IgG_{4/7}$ isolated from PNAG HIP, and subclasses IgG_1 or $IgG_{4/7}$ isolated from standard plasma. IgG_1 isolated from PNAG HIP deposited significantly ($P < 0.05$) more C1 than did whole standard plasma, $IgG_{4/7}$ derived from PNAG HIP, and IgG_1 or $IgG_{4/7}$ from standard plasma. No other differences were significant. For the PNAG HIP and subclasses IgG_1 and $IgG_{4/7}$ derived from it, however,

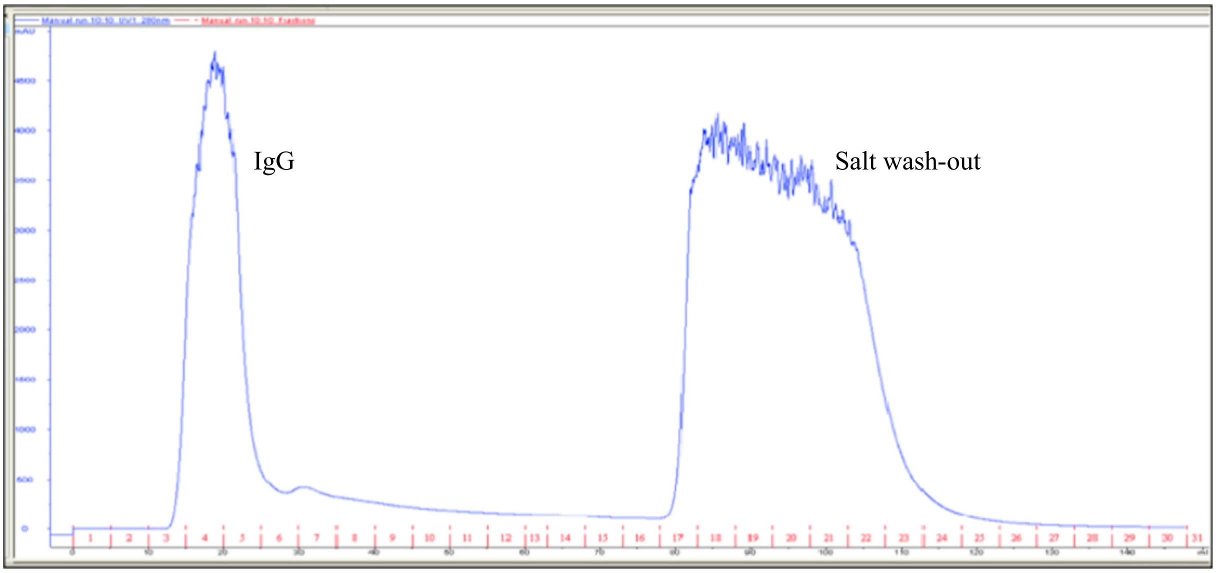

Fig. 1. Protein profile of DEAE ion exchange column. First peak to the left depicts flow-through of IgG (includes IgG subclasses IgG_1 and $IgG_{4/7}$), which does not bind to the DEAE ion exchange column. The second peak to the right represents other proteins which were retained in the DEAE ion exchange column and were removed with a salt wash-out.

Table 1
Concentrations (ng/ml) of IgG_1 and $IgG_{4/7}$ from the combined fractions of the flow-through of the DEAE ion exchange column (first peak in Fig. 1). An ELISA plate coated with antibodies specific for either equine IgG_1 or $IgG_{4/7}$ immunoglobulins detected these IgG subclasses in the flow-through from PNAG hyper immune plasma (PNAG HIP) and standard plasma (Standard) (shown in two-fold dilutions). Dilutions of flow-through highlighted in orange were tested in wells coated with IgG_1 and dilutions of flow-through highlighted in blue were tested in wells coated with $IgG_{4/7}$. The concentration of IgG_1 in the PNAG HIP and standard plasmas was detected within the range of concentrations set by the standard IgG1 (approx. 150–7 ng/ml). The subclass $IgG_{4/7}$ was highly concentrated in the flow-through of PNAG HIP and standard plasma, as every dilution surpassed the concentration intervals set by the $IgG_{4/7}$ standard (>209 ng/ml). Concentrations of each IgG subclass dilution represent the mean of the dilution in duplicate.

ELISA of DEAE flow-through					
		Plates coated with			
		Anti-IgG_1 (ng/ml)		Anti-$IgG_{4/7}$ (ng/ml)	
Standard IgG_1 & $IgG_{4/7}$	**Flow-through dilution**	**PNAG HIP**	**Standard**	**PNAG HIP**	**Standard**
200	Neat	139.35	149.05	>209	>209
100	1:2	108.05	74.73	>209	>209
50	1:4	67.22	50.38	>209	>209
25	1:8	48.65	32.10	>209	>209
12.5	1:16	30.98	20.11	>209	>209
6.25	1:32	21.73	12.31	>209	>209
3.125	1:64	14.32	7.49	>209	>209
Blank	-	3.24	4.47	9.517	6.11

there were significant ($P < 0.05$; linear mixed-effects modeling using the method of Sidak for pair-wise comparisons) decreases of ODs with dilution. Collectively, these results indicate that IgG_1 derived from PNAG HIP deposited significantly ($P < 0.05$) more C1 onto PNAG than did $IgG_{4/7}$ from PNAG HIP, or IgG_1 or $IgG_{4/7}$ isolated from standard plasma.

3.5. Determination of OPK activity using IgG subclasses

To further assess the functional capacity of each subclass, we determined opsonic killing of *P. equi* by PMNs in the presence of C′. We used 2 negative controls: a bacterial control (Contrl Bact, Fig. 5) comprised of only media and *P. equi*, and a second control omitting antibodies (Contrl C′PMN, Fig. 5; presence of media, *P. equi*, equine C′, and equine PMNs). We incubated tubes with end-over-end rotation for 90 min, and then serially diluted and plated for bacterial enumeration. Each of the IgG subclasses resulted in significant ($P < 0.05$; linear mixed-effects modeling) killing relative to the Contrl Bact, but the Contrl C′PMN did not differ significantly from the Contrl Bact ($P = 0.2106$; linear mixed-effects modeling). The proportion of bacteria surviving that were opsonized by IgG_1 from PNAG HIP (mean proportion surviving, 69%; 95% CI 61–78%) was significantly ($P < 0.05$; linear mixed-effects modeling using the method of Sidak for pair-wise comparisons) less than those opsonized with IgG_1 from standard plasma (mean proportion surviving 79%; 95% CI, 70–87%), $IgG_{4/7}$ from PNAG HIP (mean proportion surviving, 81%; 95% CI, 73–89%), and $IgG_{4/7}$ from standard plasma (mean proportion surviving, 80%; 95% CI, 72–88%) (Fig. 5). The proportion of bacteria surviving that were opsonized by IgG_1 from PNAG HIP was also significantly ($P < 0.05$; linear mixed effects modeling using the method of Sidak for pair-wise comparisons) less than that of bacteria treated without antibodies (Cntrl C′PMNs = 90%; [95% CI, 79–100%]). No other pairwise comparisons among treatments differed significantly (Fig. 5).

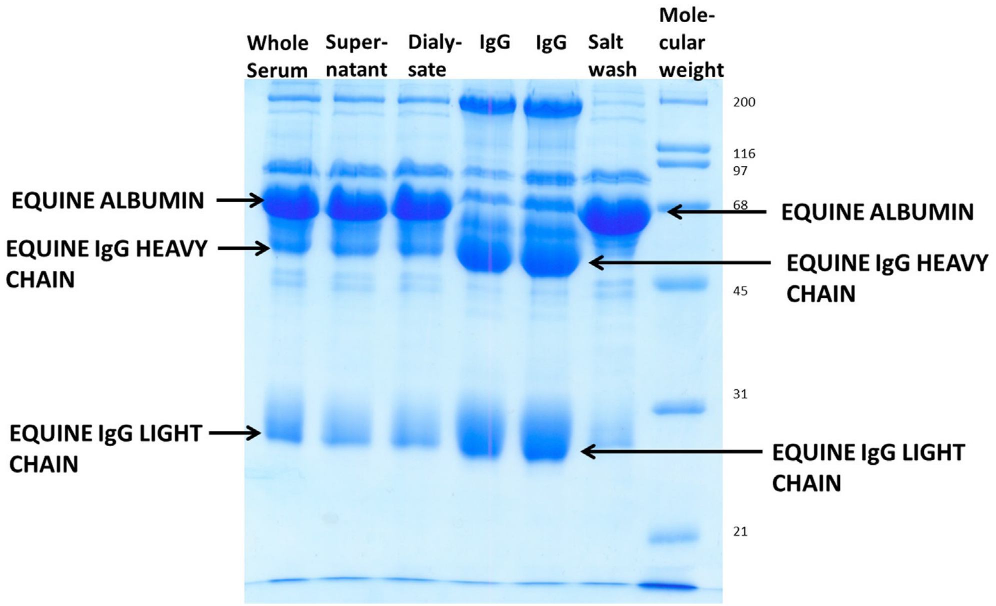

Fig. 2. SDS PAGE of samples from different phases of plasma enrichment. Columns left to right: whole serum; supernatant from dextran sulphate; dialysate after desalination; IgG from flow-through (from first peak to the left of DEAE in Fig. 1); salt wash-out used to clean column of other retained proteins (from second peak of DEAE in Fig. 1); and, molecular weight standard. Whole serum, supernatant, dialysate, and the wash-out all had robust bands that correspond to albumin protein (see arrows). The flow-through, containing IgG molecules, was clear of the albumin band and showed prominent bands that correspond to equine IgG heavy and light chains (see arrows).

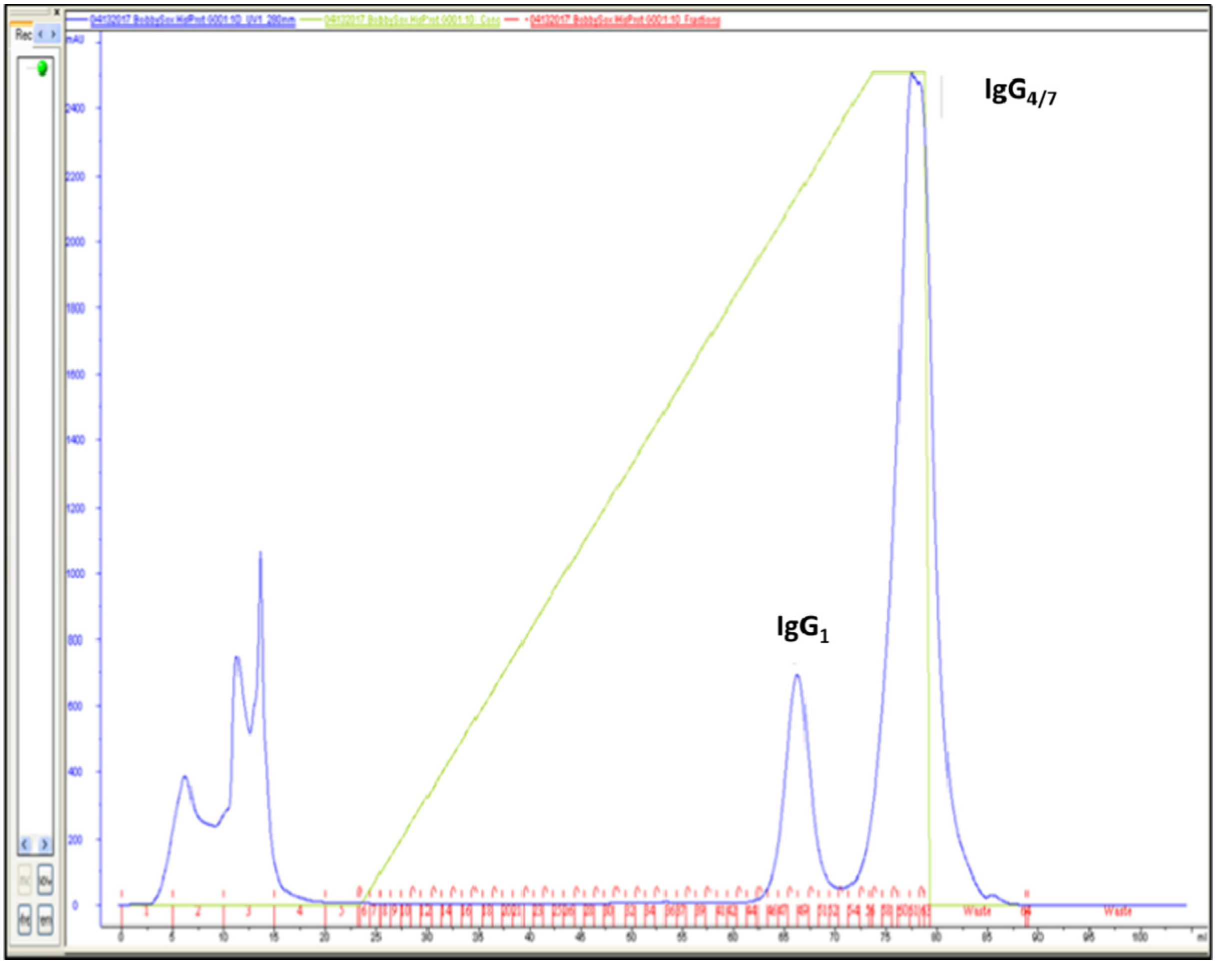

Fig. 3. Protein profile of the isolation of IgG subisotypes using a pH gradient of 8.0–2.0 in a protein G sepharose column. The subisotype IgG$_1$ was eluted into fractions (comprising of the smaller peak to the left) by a pH gradient (green line) while other proteins remained bound to the protein G column. Further decrease in pH (rise in the green line) eluted IgG$_{4/7}$ into another set of fractions (represented by the taller peak to the right). The elution of the 2 IgG subclasses at different pH values allowed for their isolation. Presence of these subclasses in these fractions was confirmed by ELISA (see Table 2).

Table 2

Concentrations (ng/ml) of IgG_1 and $IgG_{4/7}$ subclasses were simultaneously determined by ELISA from the chromatography fractions collected from the protein G column from Fig. 3. Fractions (Fx) 47–51 (highlighted in orange) corresponded to the smaller peak in Fig. 3. In these fractions, the presence of IgG_1 was detected at a peak concentration of 63.25 ng/ml. There was no $IgG_{4/7}$ detected in these fractions. Fractions 57–65 (highlighted in blue) corresponded to the second (taller) peak in Fig. 3 where the presence of $IgG_{4/7}$ was detected by ELISA at a peak concentration of 93.9 ng/ml. There was no IgG_1 detected in these fractions. Neither IgG subclasses were detected by ELISA in the remaining fractions before and after the pH gradient.

Fx	IgG_1 ELISA Conc (ng/ml)	$IgG_{4/7}$ ELISA Conc (ng/ml)	Dilution factor	ng/ml	ug/ml
1	0	0	5000	0	0
2	0	0	5000	0	0
3	0	0	5000	0	0
4	0	0	5000	0	0
45	0	0	5000	0	0
47	4.041	0	5000	20205	20.205
49	12.650	0	5000	63250	63.250
51	6.985	0	5000	34925	34.925
55	0	0	5000	0	0
57	0	2.893	5000	14465	14.465
59	0	14.430	5000	72150	72.150
61	0	18.790	5000	93950	93.950
63	0	11.750	5000	58750	58.750
65	0	2.850	5000	14250	14.250
67	0	0	5000	0	0

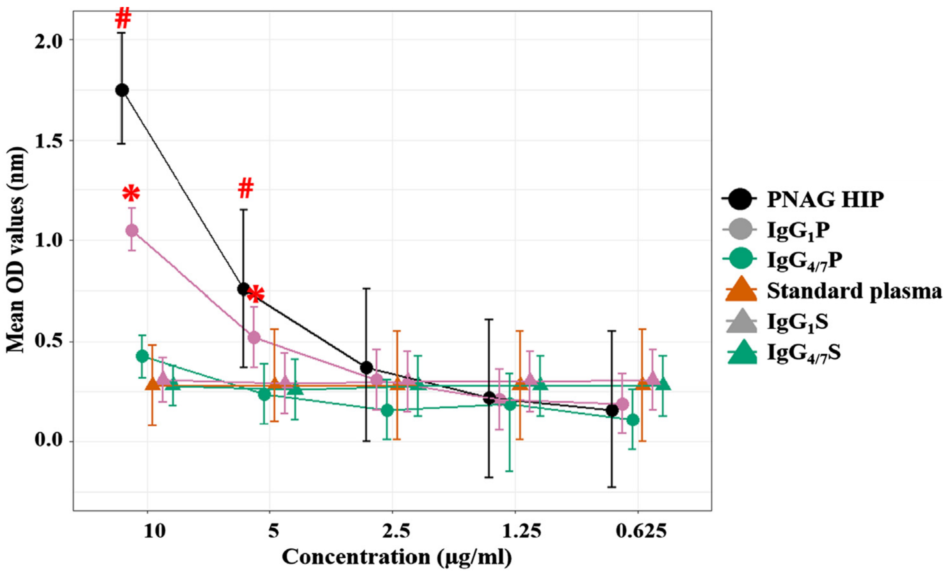

Fig. 4. Impact of IgG-subisotype and source on PNAG C1q deposition. Model-estimated mean OD values for deposition of C1q onto PNAG for standard plasma, PNAG HIP, IgG_1 from PNAG HIP (IgG_1P), $IgG_{4/7}$ from PNAG HIP ($IgG_{4/7}P$), IgG_1 from standard plasma (IgG_1S), and $IgG_{4/7}$ from standard plasma ($IgG_{4/7}S$). Plasmas and IgG subclasses were serially diluted by 1:1 from 10 to 0.625 µg/ml. It should be noted that the concentration for plasma is reflective of IgG_1 in plasma (but not $IgG_{4/7}$). The OD values are derived from the amount of complement deposited onto plates coated with PNAG as determined by ELISA. Sidak method for mean comparison for concentrations with different symbols differed significantly ($P < 0.05$) from the other conditions (*PNAG HIP; $^{\#}$IgG1P).

4. Discussion

We have previously shown that maternal vaccination with PNAG protects foals against experimental infection with live, virulent *P. equi*, and that this protection is likely mediated following colostral transfer to foals of maternal antibodies raised to PNAG [7]. Moreover, relative differences between titers of vaccinated foals and control foals of anti-PNAG IgG_1 were greater than those for $IgG_{4/7}$, suggesting anti-PNAG IgG_1 was more important for immunity to *P. equi*. To further investigate this possibility, we compared the *in vitro* capacity of these 2 IgG subclasses enriched from standard plasma and PNAG HIP to deposit C1 on to PNAG which leads to efficient OPK. Our results indicate a functional basis for the observed association between relatively greater concentrations of IgG_1 against PNAG than $IgG_{4/7}$ in protected foals [7,20–22] supporting the importance of anti-PNAG IgG_1 as a correlate of immunity against *P. equi* infection in foals.

There is compelling evidence that antibodies can protect against intracellular infections, although conflicting results exist [20–22]. Toxin neutralizing antibodies can contribute to protection against progression of *P. equi* pneumonia [23], though the protection they mediate is incomplete [23]. Beyond neutralizing activity, antigen-specific antibodies can alter inflammatory responses against certain intracellular pathogens through FcR-mediated sig-

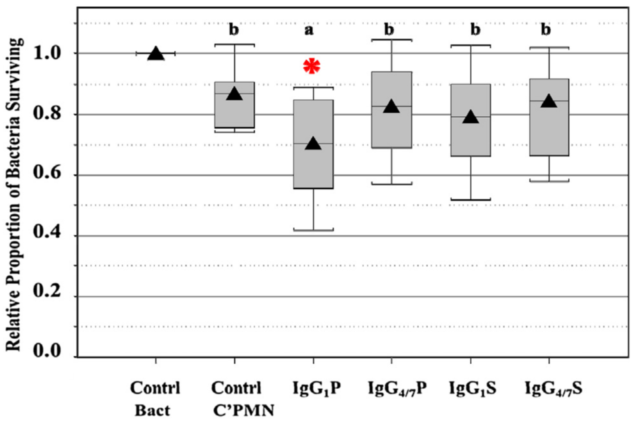

Fig. 5. Effect of IgG-subclass and source on PMN killing of opsonized *P. equi*. Boxplot of proportional equine PMN killing of virulent *P. equi* when opsonized with IgG_1 derived from either PNAG HIP or standard plasma (IgG_1P and IgG_1S, respectively) or $IgG_{4/7}$ derived from PNAG HIP or standard plasma ($IgG_{4/7}P$ and $IgG_{4/7}S$, respectively). Controls had bacteria and media only (Contrl Bact; reference category) or bacteria, media, complement (C′), and polymorphonuclear (PMN) cells (no antibodies; Contrl C′PMN). Triangles represent median values; bottoms and tops of boxes represent the 25th and 75th percentiles, respectively. Whiskers extend to a multiple (1.75) of the inter-quartile distance. Sidak method for mean comparison: boxes labelled with different letters differ significantly ($P < 0.05$) relative to control.

naling [24], altering microbial physiology [25,26], stimulating respiratory burst [27], opsonizing and activating C′ [28], enhancing phagocytosis [16], and via antibody-dependent cellular cytotoxicity of OPK [35,37]. Activation of FcR could play an important role in *P. equi* protection, as the interaction of antibodies with FcεRII-CD23 or FcγIIIa leads to control of proliferation of ingested of *Mycobacterium tuberculosis* (Mtb) [29], an organism similar to *P. equi*. Antibody binding to the surface of the facultative intracellular pathogens *C. neoformans* [26] or Mtb [25] triggers transcriptional responses intracellularly that can interfere with microbial physiology. Antibodies may enter cells via pinocytosis [30,31] and mediate protection against intracellular pathogens by activity within infected cells. For example, antibodies mediate protection against *Listeria monocytogenes*, by neutralizing the toxin listeriolysin *within* infected phagocytic cells; the toxin is delivered to the bacterial phagosome by intracellular transport [32].

The ability of antibodies to mediate killing of intracellular pathogens can vary among IgG subclasses. In this study, we report that anti-PNAG IgG_1 – but not anti-PNAG $IgG_{4/7}$ – mediated protective responses against *P. equi*. In other studies of *P. equi* infections, foals that remained healthy after experimental infection [10,42,43] with *P. equi* or during natural exposure [9,44] to *P. equi* had high IgG_1 titers against *P. equi*, which were indicative of either immunity or humoral response to *P. equi.* There is also a link between IgG subclasses and Th1 and Th2 cytokine responses [42,45,46]. It is possible that IgG_1 directs a Th1-type response resulting in enhanced cell-mediated immunity (CMI) against *P. equi* [33–35], whereas $IgG_{4/7}$ and $IgG_{3/5}$ are associated with greater Th2 responses that do not enhance CMI [9]. A Th1 response has been linked to protection against *P. equi* in mice [42–47]. Varying protection among different subclasses also has been observed for other intracellular pathogens such as Mtb, in which murine IgG subclasses IgG_1 and IgG_3 prolonged survival of mice infected with Mtb that were associated with CMI [20,27]. Arabinomannan-specific IgG_3 elicited by a polysaccharide conjugate vaccine targeting Mtb conferred protection in infected mice [20], possibly by altering expression of genes regulating bacterial metabolism [25]. Human IgG_2 is responsible for immunity to certain bacterial infections by binding to bacterial capsular polysaccharide antigens on which it forms hexamers that increase its avidity for C1 [36]. Studies with *C. neoformans* show IgG_1 – but not IgG_3 – against a capsular polysaccharide protects against infection in mice [37]. Opsonization of *C. neoformans* with IgG_1 increases phagocytosis by macrophages and arrests intracellular fungal growth [38].

The mechanisms by which anti-PNAG IgG_1 mediates protection against *P. equi* are not fully explored, but a number of mechanistic insights have been experimentally derived. It has recently been shown that PNAG derived from the surface of intracellular *P. equi* appears to be transported to the cytoplasmic membrane of infected macrophages [7]. The PNAG on the surface of the infected macrophages was detected by an anti-PNAG monoclonal antibody, which could also facilitate deposition of C1 and chemotaxis of neutrophils to the infected cells, with subsequent lysis of the macrophages and release of the intracellular microbes for further opsonic killing. Furthermore, antibodies to PNAG increased the release of IFN-γ from PBMCs isolated from foals born to vaccinated mares in response to PNAG [7]. Equine subclass IgG_1 could orchestrate interactions with other cell types by activating FcR on effector cells, and consequently mediating phago-lysosomal fusion within alveolar macrophages infected with virulent *P. equi* [32,57]. The enhanced ability of equine IgG_1 from PNAG HIP to mediate killing of *P. equi* could be due to higher specificity (epitope location and accessibility) than that of $IgG_{4/7}$ to *P. equi*, as occurs for specific subclasses in mediating protection against other intracellular pathogens [25,57,58]. These mechanisms likely change as the foal's immune system matures and requirements change, but more studies are needed.

The impetus for this study was our previous finding that foals born to PNAG-vaccinated mares had higher serum titers of anti-PNAG IgG_1 than $IgG_{4/7}$, and PNAG antibodies were significantly associated with protection [7]. The present study provides evidence that anti-PNAG IgG_1 is functionally superior to $IgG_{4/7}$ and thus represents a correlate of protective immunity. Definitively establishing the relative contributions of IgG_1 and $IgG_{4/7}$ for protecting against *P. equi* pneumonia would require passive transfer of anti-PNAG IgG of each subclass to foals followed by experimental challenge. Isolating each individual subclass from at least 20 L of plasma (assuming a minimum of 10 foals, each requiring the antibody in 2 L of PNAG hyperimmune plasma) would be extremely difficult even with industrial-sized chromatography columns for which our laboratory is not equipped. Using rodent models for these experiments isn't feasible for a number of reasons. Experimental *P. equi* infections in mice result in increased tissue burdens of bacteria in the liver and spleen (and sometimes the lungs) [41–43], reflecting systemic infection and bacteremia. When infections occur in the lungs of mice, the resulting inflammatory response is acute and does not result in pyogranulomas as occurs in foals [1,41,44–48]. Moreover, the murine and equine IgG subclasses do not correspond [9,39,40]. Guinea pigs also are not suitable models of *P. equi* pneumonia [49].

Although it remains to be demonstrated whether anti-PNAG IgG_1 administered to foals specifically mediates protection *in vivo* against either experimental or natural infection with *P. equi*, our previous evidence that foals protected against *P. equi* had higher concentrations of IgG_1 than $IgG_{4/7}$ [7] and results of this study provide strong evidence that equine IgG_1 antibody to PNAG is a mediator of protective immunity to *P. equi* in foals from pregnant mares vaccinated against PNAG. Intracellular clearance of *P. equi* likely relies on the cooperation between innate, humoral, and CMI [14] factors. The possibility to protect against intracellular pathogens such as *P. equi* by vaccines that elicit humoral immunity requires further understanding of how antibodies interact with leukocytes and alter host cell-pathogen interactions to control and eliminate intracellular infections. In horses, differences in functional activities of IgG subclasses appear to impact such protective immunity, indicating that better understanding of protective immunity against *P. equi* and other intracellular pathogens can be garnered from further characterizing the function of IgG subclasses in host-agent interactions.

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Author contributions

(1) Joana Rocha – Performed OPK and C1 assays, assisted with isolation of subclasses, and took primary responsibility for writing the manuscript; (2) Lawrence J. Dangott – Oversaw isolation and enrichment of IgG subclasses, participated in designing the project and interpreting and reporting results; (3) Waithaka Mwangi and Robert C. Alaniz – Conceived the project and participated in interpreting and reporting results; (4) Angela I. Bordin – Assisted in designing the study, and interpreting and reporting results; (5) Colette Cywes-Bentley – Assisted with assay development, study design, and interpreting and reporting study results; (6) Sara D. Lawhon and Suresh D. Pillai – Contributed to conceiving the study and interpreting and reporting results; (7) Jocelyne M. Bray – Assisted with performing laboratory assays and participated in interpreting and and reporting results; (8) Gerald B. Pier – Co-senior author, provided oversight for design, conduct, interpretation, and reporting of results; (9) Noah D. Cohen – Senior author, provided oversight for design, conduct, interpretation, and reporting of results, and performed statistical analyses.

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Conflict of interest

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 GBP also holds equity. As an inventor of intellectual properties, GBP 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. GBP'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, CC-B 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.

All authors attest they meet the ICMJE criteria for authorship.

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