Impact of prepartum administration of a vaccine against infectious calf diarrhea on nonspecific colostral immunoglobulin concentrations of dairy cows[1]

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LAY SUMMARY

Unlike human babies, calves do not receive protective immune proteins (immunoglobulins) from the mother before birth, so a sufficient volume of immunoglobulin-rich colostrum of adequate quality must be consumed within hours of birth. It can be a challenge to meet this requirement for all dairy calves. Prior to calving, cows can be vaccinated with a vaccine against specific infectious causes of calf diarrhea to stimulate elevated concentrations of specific immunoglobulins in their colostrum, which is consumed by their calves to protect them until their own immune systems develop. We enrolled cows that were either vaccinated or not with a calf diarrhea vaccine and, using novel laboratory techniques, measured concentrations of immunoglobulin classes A, G and M in their colostrum. As expected, vaccinated cows had elevated concentrations of vaccine-specific immunoglobulins in their colostrum. However, they also had elevated non-vaccine-specific concentrations of immunoglobulin M. The vaccine may therefore have stimulated a nonspecific increase in colostral immunoglobulin M concentrations. Further research is necessary to confirm the preliminary findings of the present study and determine the mechanism for this apparent nonspecific increase in colostral immunoglobulin M concentrations, whether it may occur in other immunoglobulin classes, and whether it may benefit calf health and growth.

TEASER TEXT

We used novel techniques to show that prepartum vaccination of dairy cows with a calf diarrhea vaccine increased colostral concentrations of not only vaccine-specific immunoglobulins of but also non-vaccine-specific immunoglobulin M. More work is required to confirm these preliminary findings, whether the effect may occur in other immunoglobulin classes, and whether this may be a new tool for improving calf health and productivity through enhanced colostrum quality.

ABSTRACT

Passive transfer of colostral immunoglobulins from the cow to the calf is essential for calf health. The objective of this study was to determine if prepartum administration of a vaccine stimulates increased concentrations of colostral immunoglobulins of dairy cows beyond what is explained by vaccine-specific immunoglobulins. A prospective cohort study was conducted on a spring-calving commercial dairy farm that had a policy of only vaccinating cows with even ear tag numbers with a calf diarrhea vaccine, while cows with odd ear tag numbers were left unvaccinated. Cows in the vaccinated group (even ear tag numbers, n=204) received a sensitizer and booster vaccination with a vaccine against bovine rotavirus (serotypes G6 and G10), bovine coronavirus and *E. coli* having the K99 pili adherence factor. A sensitizer was given because the study vaccine was different to the vaccine previously used. Cows in the control group (odd ear tag numbers, n=194) received a 2 mL subcutaneous sterile saline solution. Both groups received two treatments at a three-week interval, completing the treatments approximately two weeks prior to the planned start of calving. During the calving period, technicians separated calves from cows immediately after parturition and prior to suckling, and cows were completely milked out within six hours of parturition. Vaccine-specific, total, and nonvaccine-specific (total minus vaccine-specific) concentrations of immunoglobulin classes A, G1, G2a and M (IgA, IgG1, IgG2a and IgM respectively) were quantified by mass spectrometry for 20 colostrum samples from each treatment group. Predicted mean non-vaccine-specific colostral IgM concentrations were 8.76 (95% CI =7.18-10.67) and 5.78 (95% CI =4.74-7.05) mg/ml for vaccinated and control cows respectively (p =0.005). Predicted mean non-vaccine-specific colostral IgG1 concentrations were 106.08 (95% CI =92.07-120.08) and 95.30 (95% CI =81.30-109.31) mg/ml among vaccinated and control cows respectively, however these means were not significantly different (p=0.278). It is thus possible that the vaccine, in addition to specifically managing infectious calf diarrhea,

may also have non-specific benefits by improving colostrum quality through increased non-vaccine-specific colostrum IgM concentrations. Further research is necessary to determine the mechanism for these preliminary findings, whether the effect may occur in other immunoglobulin classes, and what impacts it may have on calf health outcomes.

Keywords: vaccination, colostrum, immunoglobulin, dairy cow

ABBREVIATIONS

AQUA	Absolute quantification
BCS	Body condition score
ELISA	Enzyme-linked immunosorbent assay
FcRn	Neonatal fragment crystallizable receptor
HPLC	High performance liquid chromatography
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LC-MS	Liquid chromatography – mass spectrometry
MS	Mass spectrometry
PBS	Phosphate-buffered saline
PSC	Planned start of calving
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SRM	Selected reaction monitoring

INTRODUCTION

Cattle have synepitheliochorial placentas that are impermeable to maternal antibodies (Borghesi et al., 2014), so the passive transfer of immunoglobulins from the cow to the calf after birth via colostrum is essential for the health, growth and survival of calves. Female dairy calves with serum immunoglobulin concentrations <12 mg/mL at 24-48 hours of age were shown to have a 6.78% risk of mortality in the first 180 days of life, compared to a 3.33% risk among calves with concentrations >12 mg/mL (Robison et al., 1988). Neonatal serum immunoglobulin concentration was positively associated with milk production in the first lactation (DeNise et al., 1989). Calves fed 4 L of high-grade colostrum within one hour of birth had approximately half the veterinary costs, grew significantly faster and produced more milk in their first lactation than calves fed 2 L of the same colostrum within one hour of birth (Faber et al., 2005).

Successful acquisition of immunoglobulins relies on the consumption of colostrum of sufficient quantity and quality within 12 hours of birth (Osaka et al., 2014), leading to the term “the three Q’s” of colostrum (quickly, quantity and quality). Several management interventions have been recommended to maximize colostrum quality, such as optimising cow nutrition and timing of vaccination, separate harvesting and storage of first milking colostrum and preferential feeding thereof to calves at their first feed after birth, transfer to the calf rearing facility and feeding of newborn calves twice-daily and improving the preservation of stored colostrum (Denholm et al., 2017b; Cuttance et al., 2018; Denholm et al., 2018; Menichetti et al., 2021). However, no intervention should be expected to completely remove the risk of suboptimal colostrum quality.

Vaccination of the dam has been shown to improve the protective value of colostrum by increasing colostrum titres of specific immunoglobulins, particularly immunoglobulin G (Crouch et al., 2001; Recca et al., 2003; Žuffa et al., 2019), but it is possible that it may also

improve colostrum quality by increasing overall immunoglobulin concentrations. Denholm et al. (2017a) assessed the quality of pooled colostrum fed to newborn calves by Brix refractometry, which has been shown to provide a useful indication of colostrum quality (Bielmann et al., 2010). The mean Brix % was 18.1% on farms that vaccinated all cows with a commercial multivalent vaccine for the prevention of infectious diarrhea in their calves (ScourGuard 4(K), Zoetis, Auckland, New Zealand), which was higher than that for farms that vaccinated no cows (16.3%), or where only partial herd vaccination was used (15.6%) (p=0.033). Subsequently, (Immler et al., 2021) found a 1.8% increase in the colostrum Brix % among cows given a prepartum rotavirus/coronavirus vaccine. However, further work is necessary because, when vaccination is applied at the herd level, it is not possible to isolate the effect of the vaccine from other farm-level effects on colostrum quality and, it is unclear whether the increased Brix % was caused by increased concentrations of specific vaccine-induced immunoglobulins or an overall increase in immunoglobulin concentration caused by vaccine-stimulated immunoglobulin secretion.

Previous research into the effects of vaccination on bovine colostral immunoglobulin concentrations has focused on pathogen-specific immunoglobulins (Saif et al., 1984; Snodgrass et al., 1991; Žuffa et al., 2019), or total immunoglobulins measured directly by radial immunodiffusion (Wilson et al., 1972) or indirectly by Brix refractometry (Denholm et al., 2017a; Immler et al., 2021). To the authors' knowledge, only a single study has measured both total and vaccine-specific immunoglobulin concentrations in cattle (Fleenor and Stott, 1983), but non-vaccine-specific concentrations were not reported. There is evidence that vaccines can provide benefits beyond their effects on target pathogens due to heterologous effects (Cortese et al., 2020), and administration of an adjuvant alone has been shown to increase colostral anti-rotaviral antibody concentrations (Van Opdenbosch et al., 1981). Given the possibility that vaccines may provide benefits through nonspecific stimulation of

the immune system, and the lack of studies reporting non-vaccine-specific immunoglobulin concentrations in bovine colostrum, we conducted a pilot study to explore our hypothesis that prepartum vaccination of pregnant dairy cows with ScourGuard 4(K) stimulates an increase in the total concentration of colostral immunoglobulins beyond what is explained by vaccine-specific immunoglobulins.

To determine whether vaccination increases immunoglobulin concentrations in colostrum beyond the specific immune response, we needed to accurately measure the total and vaccine-specific concentrations of the principal immunoglobulin isotypes (i.e., IgA, IgG1, IgG2a, and IgM) in colostrum. Antigen-specific immunoglobulin concentrations in colostrum following vaccination are typically reported as titers rather than absolute measurements and largely follow the magnitude of the response observed in serum. However, titers do not indicate precise immunoglobulin concentrations, making it difficult to draw comparisons between studies due to diagnostic kit and laboratory variation. Mass spectrometry provides a multiplexed method to quantify each of the principal immunoglobulin isotypes within complex samples and has been explored in clinical settings to assess human immune responses (Remily-Wood et al., 2014). As no bovine-specific version of this mass spectrometry approach has been described to date, we developed new methods to provide high-throughput immunoglobulin concentration analysis in colostrum.

Our objective was to determine whether prepartum vaccination of pregnant dairy cows with a multivalent vaccine for the prevention of infectious diarrhea in their calves stimulated an increase in the concentration of colostral immunoglobulins beyond what was explained by vaccine-specific immunoglobulins.

MATERIALS AND METHODS

All procedures in the study were approved by the Kaiawhina Animal Ethics Committee (protocol number AEC 004/20).

Field procedures

A prospective cohort study was conducted in the winter and spring of 2020 on a single, commercial, seasonally-spring calving dairy farm in the Waikato region of New Zealand. The cattle were Jersey/Friesian cross, grazing a predominantly ryegrass and white clover pasture and supplemented with hay, maize silage, and palm kernel extract during the winter and spring without anionic adjustment, and they produce approximately 4,100 liters of milk per cow annually. The dietary nutrient composition was not available. The farm was purposely selected due to its history of partial vaccination to control calf diarrhea, having consistently vaccinated all cows with even ear tag numbers for the past five years (Rotavec Corona, MSD Animal Health, Wellington, New Zealand).

The cow was the experimental unit. The sample size was calculated to compare differences in colostral IgG concentration between vaccinated and unvaccinated cows. Assuming that IgG concentration was normally distributed and had a standard deviation of 25 mg/mL (Quigley et al., 2013), a difference in IgG concentration in the colostrum of vaccinated and unvaccinated cows of 23 mg/mL could be detected with a sample size of 20 cows in each group with 80% power and 95% confidence.

All cows in the herd with parity of ≥1 (n=398) were eligible for enrolment. Heifers that were due to calve for the first time during the calving season were excluded.

On 11 June 2020 (study day 0), 31 days prior to the herd's planned start of calving (PSC, = 12 July 2020) cows were allocated to either a treatment ("vaccinated") or control ("control") group depending on their tag number (even tag = vaccinated, odd tag = control).

Cows in the vaccinated group (n=204) received a 2 mL subcutaneous sensitizer and booster vaccination with a multivalent vaccine registered to aid in the prevention of neonatal diarrhea caused by bovine rotavirus (serotypes G6 and G10), bovine coronavirus and *E. coli* having the K99 pili adherence factor (ScourGuard 4(K), Zoetis, Auckland, New Zealand), and then again 21 days later. The New Zealand approved label directions for ScourGuard 4(K) state that previously unvaccinated heifers or cows should receive two intramuscular doses at least three weeks apart, with the second dose given 2-12 weeks prior to calving. Because prior vaccination status was based on vaccinations administered by farm staff, and because a different vaccine had been used previously, the cows in this group received two vaccinations (as would be given for a full primary course) to ensure that all cows were fully vaccinated. Cows in the control group (n=194) received a 2 mL subcutaneous sterile saline solution at both time points. Cows were excluded on day 21 if they were diagnosed non-pregnant, had a body condition score (BCS) <4.5 when measured on a 10-point scale (Roche et al., 2004), had been treated for illness within the previous 30 days or were diagnosed with an illness by a veterinarian.

All cattle were under typical farm management and were grazed together from drying off to parturition. On the 20th of July 2020 (39 days following the first vaccination), veterinary technicians commenced observation of the "springer" mob (cows approaching parturition) between 8am and 5pm. The observers were unaware of which cows were in the vaccinated or control groups as they were different technicians to those who administered the treatment and were unaware of the treatment allocation system. Observation was carried out at a distance from the calving paddock (~50 m) to avoid interrupting the cows' natural calving process. A cow was only enrolled if the calving was observed. Any cows that calved out of observation hours may have fed their calves and were therefore excluded.

Following a successful observed calving, calves were separated from the cow prior to suckling and taken to the calf rearing facility. Recently calved eligible cows were milked in the afternoon of the same day of observation, within six hours of parturition. The cows were milked into an individual volumetric milking bucket to allow the volume of the first milking colostrum to be measured and an approximately 50ml four-quarter sample taken for analysis by the veterinary technician. This process of observation, calf removal and colostrum sampling continued until a minimum of 20 cows in each group had been sampled, ending on 2 August 2020 (day 52).

At the time of first milking, the cow's BCS was recorded by a technician. Times of calving and first milking were recorded, and we extracted cow breed, parity, calving date (and hence vaccination to calving interval (days)) from herd records using Infovet Software (Zoetis, Auckland, New Zealand). Cows were excluded on the day of colostrum sampling if parturition occurred >10 days earlier than expected, if they had a BCS <4.0, if they had been diagnosed with an illness by a veterinarian and/or treated for illness within the previous 30 days, or had less than four functioning and non-mastitic quarters.

The four-quarter samples were transported back to the veterinary clinic laboratory, pooled, and measured for Brix percentage using a portable, optical, temperature compensating Brix refractometer (Brand LH-T32, Shoof International Ltd), and recorded. We calibrated the Brix refractometers daily using two drops of distilled water. The samples were frozen and stored at -20 °C at the veterinary laboratory for approximately two months.

To select the colostrum samples to be sent for immunoglobulin analysis, colostrum samples were blocked by colostrum volume to control for this potential confounder variable, and samples were selected using systematic random sampling. After excluding one cow due to low BCS (BCS = 3.0) and one cow with a spurious colostrum volume (18L), the remaining 45 samples were ranked on colostrum volume from smallest to greatest volume. A single

random number was generated in Microsoft Excel (Microsoft Corporation, Redmond, WA) of either 1 (treatment) or 2 (control). First, the sample with the smallest volume from the treatment group corresponding to the random number was selected. Then the sample with the smallest volume from the other treatment group was selected. Then the sample with the second smallest volume from the first treatment group was selected. This alternating process continued until 20 samples were selected from each treatment group. The balance of the treatment groups was then checked for the remaining confounding variables (age and BCS). The groups were balanced, so no further matching was undertaken. This subset of unthawed samples was personally delivered to the University of Waikato's School of Biological Sciences within 40 minutes of being removed from the freezer. Treatment groups were not identified on the samples and the laboratory staff was blinded to treatment group allocation.

Laboratory procedures

High-throughput analysis of colostral immunoglobulin concentrations was undertaken by quantitative mass spectrometry. Total immunoglobulin concentrations were measured directly from colostrum samples following fragmentation by tryptic digest. In contrast, ScourGuard 4(K) specific antibodies were first isolated by immunoglobulin pulldown before fragmentation. A chromatography resin was prepared for this purpose by conjugating antigens extracted from the vaccine formulation to agarose beads. The general mass spectrometry workflow is summarized visually in Figure 1. The assay was calibrated using commercial enzyme-linked immunosorbent assays for each of the bovine immunoglobulin isotypes.

Isotype quantification by mass spectrometry

Colostrum samples were thawed, mixed well, and subsamples of 2 mL taken for analysis. These samples were spun at 17,000 g for 10 mins at 4 °C to separate fat layers from the protein-rich supernatant. Immunoglobulin concentrations were determined using a targeted liquid chromatography – mass spectrometry (LC-MS) /mass spectrometry (MS) quantification assay using heavily labeled internal standards. This method provides absolute quantification (AQUA) of proteins of interest using a selected reaction monitoring (SRM) strategy where multiple ions of interest are quantified using mass spectrometry. The specificity of the assay is derived from accurate precursor mass, retention time, collision energy optimization, and selected fragment-ion masses. In brief, proteins of interest (immunoglobulin classes A, G1, G2a and M [IgA, IgG1, IgG2a and IgM respectively]) were digested with trypsin in a solution containing heavily labeled internal peptide standards. The digest was separated by reverse-phase high performance liquid chromatography (HPLC), and the tryptic peptides were detected using a triple-quadrupole mass spectrometer. The signal intensity from select fragment ions of the endogenous target peptides was then compared to the corresponding signal intensity of synthetic tryptic peptides of known abundance (AQUA peptide) from a 9-point calibration curve. A correction factor was applied to each isotype to control for incomplete tryptic digestion effects by calibration against colostral immunoglobulin concentrations obtained from commercial enzyme-linked immunosorbent assay (ELISA) kits for bovine IgG, IgA and IgM (Bethyl Laboratories, TX, USA).

Creation and characterization of vaccine-specific agarose beads

A volume of 50 mL of ScourGuard 4(K) was dialyzed overnight at 4 °C into 2 L of 0.05 M sodium carbonate pH 10 using a 6-8 kDa cut-off Spectrapor tubing (Thermo Fisher Scientific, MA, USA) to remove non-antigenic components of the formulation. The dialyzed fraction was spun at 5000 g for 1 h at 4 °C. The supernatant fraction was concentrated in 3

kDa Amicon spin columns (Merck, NJ, USA) according to the manufacturer's instructions and stored. Insoluble components were resuspended in 10 mL of 0.05 M sodium carbonate pH 10 and sonicated on ice using a microtip for 3 mins with an amplitude of 4 and pulses of 1 s on followed by 1 s off (Q700 QSonica, CT, USA). Sonicated material was centrifuged at 5000 g for 30 mins at 4 °C and filtered through a 0.22 µm filter. This material was mixed with the concentrated supernatant fraction to complete the antigen extraction.

We added 10 mL of extracted antigen components (~50 mg) to 10 mL of washed and equilibrated (0.05 M sodium carbonate pH 10) AminoLink Plus Coupling Resin (Thermo Fisher Scientific) in a 50 mL tube. The slurry was incubated at 4°C overnight with gentle rotation. The next day, 5 mL of pH 7.2 phosphate buffered saline (PBS) and 100 µL of cyanoborohydride solution (5.0 M in 1 M NaOH) were added and rotated for 4 h at room temperature. The slurry was gradually added to a 10 mL chromatography column (Thermo Fisher Scientific) and then washed with 20 mL of 1 M Tris-HCl, pH 7.4. The slurry was resuspended and rotated with 5 mL of pH 7.2 PBS and 100 µL of cyanoborohydride solution for 30 mins at room temperature. The resin was finally washed with 50 mL of 1 M NaCl and stored in PBS at 4 °C. Control resin was prepared by the same method, but PBS was substituted for the extracted antigen components.

To confirm the binding specificity and capacity of the created resin, three colostrum samples were randomly selected from the vaccinated group of animals for immunoglobulin pulldown with both the antigen-specific and negative control resins. Briefly, a five-fold dilution series was created in pH 7.2 PBS and 160 µL of each dilution was added to 20 µL of antigen conjugated resin or control resin in 1.5 mL tubes. Samples were incubated at room temperature with mixing for 1 h and washed four times using PBS with centrifugation of 2000 g for 1 min between washes. Samples and controls were analyzed by gel electrophoresis on 12 % sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) Tris-

glycine gels with Precision Plus Unstained ladder (Bio-Rad, CA, USA). A GS-900 calibrated densitometer was used to obtain gel images and Image Lab software used to determine the background binding of the chromatographic approach.

Vaccine-specific immunoglobulin pull down for mass spectrometry

Colostrum samples were diluted 25-fold in 50 mM di-sodium phosphate pH 7.4 to ensure underloading of the ScourGuard 4(K) chromatography resin. Diluted colostrum (160 µL) was added to 40 µL of ScourGuard 4(K) resin in chromatography filter spin columns (Bio-Rad) and incubated with rotation for 1 h at room temperature. Samples were washed three times with 0.5 mL of 50 mM di-sodium phosphate pH 7.4 by centrifuging at 100 g. To elute bound antibodies, the resin was incubated with 200 µL of denaturation buffer (9 M Urea, 3 M Thiourea, 50 mM Ammonium bicarbonate, 12.5 mM dithiothreitol) for 1 h at room temperature with rotation before centrifuging at 300 g. Mass spectrometry was performed as described above.

Statistical analysis

The null hypothesis was that, within each immunoglobulin class, any difference in mean colostral immunoglobulin concentration between the vaccinated and control groups was limited to differences in vaccine-specific immunoglobulin concentration. Statistical analysis was performed in R version 4.0.2 (R Core Team, 2021).

The outcome variables comprised the concentrations of the four immunoglobulin classes of interest (IgG1, IgG2, IgA and IgM) and total immunoglobulin concentration at the cow level. The single primary exposure variable was treatment group. Because of their possible association with colostrum quality and hence their potential to be confounders of treatment effect, the balance of treatment groups was assessed for a set of cow-level secondary exposure variables: age (yr), BCS, colostrum volume (L), vaccination to calving

interval (d) and calving to sampling interval (h). Breed was not included as all cows were ≥ 8/16 Jersey and there was thus little variation in breed.

Descriptive analyses, including distributional plots and tables, were conducted on all variables to visualize their distributions and check for missing data and outliers or spurious observations. Relationships between pairs of variables were visualized with frequency tables and plots. Pairwise associations between categorical and continuous variables were tested by ANOVA or the Kruskal-Wallis rank-sum test according to their distributions. Balance of treatment groups for secondary exposure variables was tested by ANOVA for normally distributed variables and Kruskal-Wallis rank-sum test for nonparametric variables.

For each immunoglobulin class, we compared the distributions of vaccine-specific, total, and nonvaccine-specific colostral immunoglobulins for control and vaccinated groups. Concentrations of nonvaccine-specific immunoglobulins were defined as the difference between total and vaccine-specific immunoglobulin concentrations for each cow. We present the distributions of each immunoglobulin class by treatment group graphically as box plots because some immunoglobulin classes were not normally distributed and hence the median and interquartile ratio are more meaningful.

To test for treatment group differences in mean vaccine-specific, total, and nonvaccine-specific immunoglobulin concentrations, we constructed simple linear regression models. The models were tested for the assumptions of independence, linearity, homoscedasticity, and normally distributed residuals by evaluation of diagnostic plots. Homogeneity of variance was also checked by the Levene test. If the residuals were not normally distributed, immunoglobulin concentrations were natural log-transformed and then back transformed to present the results. If the residuals were normally distributed but the variance was not homogenous, we used the Welch ANOVA. We could not present the

between-group differences and their confidence intervals jointly because the coefficients of log-transformed dependent variables are on a ratio scale instead of an absolute difference, so the scales differ between immunoglobulin classes. We therefore calculated predicted mean immunoglobulin concentrations for each treatment group and each immunoglobulin class. Values from models of log-transformed immunoglobulin concentrations were back-transformed to the original scale and adjusted for bias (Zeng and Tang, 2011).

RESULTS

Technicians harvested colostrum from a total of 47 cows (n=24 control cows and n=23 vaccinated cows). Two cows were excluded due to low BCS (n=1) and a spurious colostrum volume (n=1), leaving 45 eligible samples. After matching, 20 samples from each treatment group were randomly selected for analysis (Figure 2). The cows were dried off between 2 April and 30 May 2020, a mean of 66 (min = 51, max = 114) days prior to calving. The age of three cows was unknown (n=1 vaccinated and n=2 control group) and they were excluded from analyses of age but retained for the remaining analysis. Secondary exposure variables were evenly balanced across the two treatment groups (Table 1, Figure 3). Mean colostrum Brix values were 26.20% and 24.65% for vaccinated and control cows respectively but the difference was not statistically significant (Table 1).

Vaccine specific immunoglobulin isolation

We isolated the vaccine antigen fraction and covalently linked it to agarose beads with high efficiency as determined by SDS-PAGE gel (Supplementary Figure 1). Strong binding of ScourGuard 4(K) specific antibodies was observed over a range of colostrum dilutions with only minor levels of nonspecific binding to the negative control resin (Figure 4). Under reducing conditions, distinctive bands were observed at ~50 kDa and ~25 kDa for immunoglobulin heavy and light chains respectively. Background proteins comprised 8.4 ± 4.1 % of the total proteins bound across the replicates.

Vaccine specific immunoglobulin concentrations

We found 2.2- to 3.0-fold higher concentrations of vaccine specific IgG1, IgG2a and total immunoglobulins in the colostrum of vaccinated cows compared to control cows, and no difference in the concentration of IgA (Table 2, Figure 5A, Supplementary Tables 1-2). The difference in predicted mean vaccine specific colostral IgG1 concentration between vaccinated and control cows was 0.98 (95% CI = 0.61-1.36) mg/ml. We also found a 1.6-fold increase in the concentration of IgM. IgG2a concentrations were lower but still within the detection range of our assay. Three animals in the control group also showed significantly raised immunoglobulin concentrations above the quantified non-specific binding rate: two cows had elevated IgM and one cow had elevated IgA (Figure 5A, Supplementary Table 1).

Total and non-vaccine specific immunoglobulin concentrations

While no significant differences were observed for the other immunoglobulin isotypes, we found a 1.5-fold increase in total IgM concentrations associated with vaccination (Table 3, Figure 5B, Supplementary Tables 3-4).

After subtracting vaccine-specific from total immunoglobulin concentrations, we again found a 1.5-fold increase in total IgM concentrations associated with vaccination but no significant differences for the other immunoglobulin isotypes (Table 4, Figure 5C). Furthermore, the difference in mean non-vaccine-specific colostral IgM concentration between vaccinated and control cows (2.72 mg/ml) was larger in magnitude than the difference in vaccine-specific IgM concentration (0.15 mg/ml).

DISCUSSION

Quantifying the concentrations of representative peptide fragments for each of the principal immunoglobulin isotypes in colostrum (IgA, IgG1, IgG2a, IgM) provides a comprehensive method to assess the immune response. Using this method, we determined the

concentrations of vaccine-specific and total immunoglobulin concentrations in colostrum purified by affinity chromatography, and thus inferred the concentrations of non-vaccine-specific immunoglobulins. In contrast to titers generated by serial dilution, we present absolute concentrations that future researchers may use for comparison.

The preparation of pooled antigens from the ScourGuard 4(K) vaccine means immunoglobulin responses to each of the individual antigenic components were not resolved. As there is no guarantee that each antigen will have similar immunogenicity, we cannot exclude the possibility that some antigens will induce higher immunoglobulin concentrations for specific classes than others. If an immunoglobulin concentration overloads the chromatography resin binding capacity for a select antigen our method will underreport the total antigen-specific immunoglobulin concentration. To minimize the chance of overloading for any given antigen and to enable quantitation by capturing all binding immunoglobulins, a conservative dilution of 1:25 was selected for subsequent experiments.

Previously reported colostral immunoglobulin concentrations have significant variation and are heavily influenced by variables such as cow parity or the calving to sampling interval (Denholm et al., 2018). A study of 507 Holstein dairy cows from commercial herds found total IgG concentration increased from 83.5 mg/mL to 113.3 mg/mL with increased lactation number (1 > 4+) as measured by radial diffusion (Kehoe et al., 2011). The total average IgG concentration of 100.53 mg/mL reported here for a mixed age herd falls with the ranges identified by this study. Mean colostrum concentrations after birth in 20 Swedish Friesian cows were 90, 2.8, 1.6, and 4.5 mg/mL for IgG1, IgG2a, IgA, IgM respectively, and those concentrations rapidly declined over the first 48 hours after parturition (Elfstrand et al., 2002). Samples collected from 88 Jersey cows as soon as possible after parturition were 65.8, 2.4, and 1.7 mg/mL for IgG, IgM, and IgA respectively (Quigley et al., 1994). We observed slightly higher mean concentrations of IgM (6.22 mg/mL) and IgA (9.61

mg/mL) among the unvaccinated cows in our study. It is unclear why the cows in our study had slightly elevated colostral IgA and IgM concentrations, but we believe the most likely explanation is the immediacy of sampling after parturition, as other studies have also reported elevated mean colostral IgA (>10 mg/mL) immediately after birth (Zhao et al., 2010). However, there may be other factors that elevated colostral immunoglobulin concentrations in the present study, such as genetics, nutrition, and energy balance.

Vaccine-specific immunoglobulins

Calf diarrhea vaccines have been developed to increase the concentrations of specific vaccine immunoglobulins in the colostrum, particularly IgG1 (Saif et al., 1984; Gonzalez et al., 2021), so the elevated IgG1 and IgG2a concentrations we observed among vaccinated cows were expected. The much more elevated concentrations of IgG relative to IgM in our study indicate a class-switched immunoglobulin response as expected following a primary vaccine dose accompanied by second boost after 21 days (Roco et al., 2019). Despite similar serum concentrations, IgG1 is preferentially trafficked over IgG2a across the mammary epithelium, presumably by a neonatal Fc receptor (FcRn) mediated mechanism (Hurley and Theil, 2011).

We did not expect to see a significant increase in vaccine-specific IgM concentrations *a priori* because rotaviral vaccines are formulated to increase IgG concentrations. Serum IgM concentrations have been shown to rise early but transiently in the immune response to vaccination as part of the primary immune response, followed by a more specific and sustained elevation in IgG concentrations (Eschbaumer et al., 2016). Our finding is consistent with the work of Saif et al. (1983), who also found that vaccination of pregnant cows with a rotaviral vaccine induced higher colostral concentrations of IgM than unvaccinated cows, though whether the elevation was due to vaccine-specific or non-vaccine-specific IgM was not determined. However, this elevation does not appear to be a consistent response to

vaccination. Administering a *Clostridium perfringens* B-toxin vaccine to pregnant cows in the prepartum period induced elevated colostral IgG but not IgM concentrations (Fleenor and Stott, 1983). Given the narrow window of 17-30 d between administration of the booster vaccination and parturition and the limited sample size of the present study, further research is necessary to explore the relative increases in concentrations of each immunoglobulin isotype across time after vaccination.

There was a marked absence of a vaccine-specific IgA response in the present study, which is typically produced locally in the mammary gland (Stelwagen et al., 2009), and is a likely consequence of the route and site of vaccine administration (Boerhout et al., 2018; Nagasawa et al., 2019).

Elevated colostral vaccine-specific immunoglobulin concentrations in some control cows suggests they may have had prior exposure to either a calf diarrhea vaccine or to circulating strains of rotavirus, coronavirus, and/or *E. coli*. This may have masked a larger difference between vaccinated and control groups than we detected. Future trials should enroll immunologically naïve animals, ideally nulliparous heifers, by screening serum concentrations of vaccine-specific immunoglobulins before immunization.

Non-vaccine-specific immunoglobulins

In addition to an unexpectedly large increase in vaccine-specific colostral IgM concentrations, we also observed a substantial increase in non-vaccine-specific colostral IgM concentrations associated with vaccination. As the first immunoglobulins secreted to a significant level during an immune response, IgM is frequently of low affinity and relies on multimerization to improve binding (Eisen, 2014). Low binding affinity could prevent IgM capture by the chromatography capture method we used, which would result in the underrepresentation of this population in the antigen-specific fraction analyzed. However,

such low binding would result in antibodies that are largely non-functional against the target vaccine antigens. We cannot draw any conclusions about the IgM's functionality from the present study, but it is possible that ScourGuard 4(K) immunization non-specifically raised colostrum IgM concentrations.

The present study did not detect a significant effect of vaccination on non-vaccine specific colostral IgG1 concentrations. However, we made two observations about IgG1 that, while not statistically significant, should be explored further: 1) most of the 12.9 mg/mL numerical group difference in predicted mean non-vaccine-specific total immunoglobulin concentration was explained by the 10.78 mg/ml numerical difference in IgG1, and 2) the numerical difference in group mean non-vaccine specific IgG1 concentration was larger in magnitude than the 0.98 mg/ml significant difference in vaccine specific IgG1. It is possible that a lack of statistical power due to a small sample size impeded our ability to detect an effect of vaccination on non-vaccine-specific colostral IgG concentrations in the present study. Larger sample sizes are recommended for future work to verify whether those observations were true effects or chance findings.

Potential implications for calf and cow health, and future work

The non-targeted but beneficial effects of vaccination on immune protection are gaining recognition (Goodridge et al., 2016). For example, vaccination of cows in the prepartum period or on the day of calving with a modified live bovine respiratory syncytial virus, modified live bovine herpesvirus-1 and modified live parainfluenza virus type 3 vaccine was associated with a lower risk of removal from the herd and a reduction in the incidence of clinical mastitis in the following lactation (Cortese et al., 2020). This pilot study revealed a potential nonspecific immune response to prepartum vaccination of dairy cows, including IgG, the immunoglobulin class of most interest, but several aspects should be explored further to confirm these preliminary findings.

Further work is required to determine the specificity of the IgM pool in the present study and establish whether the observed increase is linked to individual vaccine antigens, or the adjuvant used. ScourGuard 4(K) contains a Quil A adjuvant, a known immunomodulator that has been shown to trigger both humoral and cell-mediated responses, and is a member of the *Quillaja* saponins, which have demonstrated mitogenic activity and the ability to induce B and T cell proliferation (Rajput et al., 2007). The nonvaccine-specific increase in colostral IgM concentrations we observed may therefore be explained by an adjuvant-antigen immune response activation of innate and adaptive immunity.

The vaccinated cows received a full primary course in the present study despite having been vaccinated in previous years. A full primary course was administered in case there were cows that had been inadvertently unvaccinated. It is unclear whether a repeat primary course would have altered the immune response to vaccination compared to a single booster. We ran the study in a herd whose manager was prepared to leave 50% of the cows unvaccinated and where there was a history of only vaccinating cows with even ear tag numbers. This meant that, with the exception of the technicians and the laboratory analysis, blinding was not possible. While the cows in each treatment group were grazed and managed together and there was no difference in the interval from second vaccination to parturition, we recommend blinding the farm staff in future studies.

Our study did not quantify serum immunoglobulin concentrations in the cows. We suggest another opportunity for future work lies in determining if there is a nonspecific serological immune response to vaccination, and whether that may benefit the cow.

Considering the limitations of this pilot study, future investigations of the preliminary findings of the present study should (1) have a larger sample size to address the possible lack of power of the present study, (2) mask treatment groups to achieve blinding, (3) examine the role of the adjuvant by repeating the study with an adjuvant-only group, (4) enroll naïve

heifers to control for prior exposure to the vaccinal pathogens or vaccination itself and to measure the response to a standard primary course in previously unvaccinated animals, (5) measure serum immunoglobulin responses in the cows to explore the immunoglobulin dynamics and the potential for a benefit to vaccinated cows, (6) explore the immunoglobulin response for each vaccine antigen to determine if one or more antigens drive the nonspecific response, and (7) measure clinical outcomes among calves fed colostrum with higher nonspecific IgM and IgG concentrations.

CONCLUSIONS

We have rejected the null hypothesis that any differences in colostral immunoglobulin concentrations between the vaccinated and control groups were limited to differences in vaccine-specific immunoglobulin concentration. Concentrations of non-vaccine-specific IgM were significantly elevated among vaccinated cows, but we did not detect elevations in the other immunoglobulin classes. We recommend further research informed by the limitations of this study to explore the effect of vaccination on non-vaccine specific colostral IgG concentrations and identify the mechanism for vaccine-driven nonspecific increases in colostral IgM concentrations.

DISCLOSURES

We wish to disclose that G Chambers was employed by Zoetis New Zealand at the time of the study, and that Zoetis New Zealand sponsored the study. The vaccine (ScourGuard 4(K)) used in the trial is distributed by Zoetis.

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TABLES

Table 1. Distribution of exposure variables for cows in each treatment group in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations

	Median (IQR)[1]				Mean (SD)	
Treatment group	Age (yr)	BCS	Treatment-calving interval (d)[2]	Calving-sample interval (h)[3]	Colostrum volume (L)	Colostrum Brix (%)
Control (n=20)	6 (4.5, 8)	4.5 (4.5, 4.6)	21.5 (20.8, 23)	3.1 (1.4, 3.9)	4.59 (1.73)	24.65 (3.12)
Vaccinated (n=20)	6 (4, 6)	4.5 (4.5, 5)	20 (18.8, 23)	4.3 (1.9, 4.7)	4.38 (2.04)	26.20 (3.89)
All cows	6 (4, 7)	4.5 (4.5, 4.8)	21 (19, 23)	3.5 (1.7, 4.5)	4.48 (1.87)	24.43 (3.57)
P-value	0.21	0.7	0.14	0.24	0.73	0.17

1. IQR = interquartile range
2. Interval between second vaccination (vaccinated group) or saline injection (control group) and calving
3. Interval between calving and colostrum harvesting

Table 2. Predicted mean concentrations of vaccine-specific colostral immunoglobulins for control (n=20) and vaccinated (n=20) cows in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations

	Mean (95% CI) concentration (mg/ml)		
Immunoglobulin class	**Control group**	**Vaccinated group**	**P-value**
IgG1	0.489 (0.288, 0.690)	1.472 (1.271, 1.674)	<0.001
IgG2a[1]	0.011 (0.008, 0.015)	0.023 (0.017, 0.031)	0.001
IgA[1]	0.086 (0.061, 0.120)	0.081 (0.058, 0.113)	0.798
IgM[1]	0.322 (0.243, 0.426)	0.505 (0.382, 0.668)	0.027
IgG	0.499 (0.293, 0.705)	1.496 (1.290, 1.703)	<0.001
Total Ig	0.895 (0.577, 1.214)	2.096 (1.778, 2.414)	<0.001

1. Values were log transformed and then transformed back to the original scale due to non-normality.

Table 3. Predicted mean concentrations of total colostral immunoglobulins for control (n=20) and vaccinated (n=20) cows in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations

	Mean (95% CI) concentration (mg/ml)		
Immunoglobulin class	**Control group**	**Vaccinated group**	**P-value**
IgG1	95.79 (81.69, 109.89)	107.55 (93.45, 121.65)	0.240
IgG2a	4.74 (3.97, 5.51)	4.74 (3.97, 5.50)	0.993
IgA	9.61 (7.50, 11.72)	9.05 (6.94, 11.16)	0.707
IgM[1]	6.10 (5.00, 7.44)	9.27 (7.60, 11.30)	0.005
IgG	100.53 (85.97, 115.09)	112.29 (97.73, 126.84)	0.255
Total Ig	116.36 (99.82, 132.90)	130.46 (113.93, 147.00)	0.220

1. Values were log transformed and then transformed back to the original scale due to non-normality.

Table 4. Predicted mean concentrations of non-vaccine-specific colostral immunoglobulins for control (n=20) and vaccinated (n=20) cows in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations

	Mean (95% CI) concentration (mg/ml)		
Immunoglobulin class	**Control group**	**Vaccinated group**	**P-value**
IgG1	95.30 (81.30, 109.31)	106.08 (92.07, 120.08)	0.278
IgG2a	4.73 (3.96, 5.50)	4.71 (3.95, 5.48)	0.972
IgA[1]	9.87 (7.79, 12.50)	8.73 (6.88, 11.10)	0.462
IgM[1]	5.78 (4.74, 7.05)	8.76 (7.18, 10.67)	0.005
IgG	100.03 (85.57, 114.49)	110.79 (96.33, 125.24)	0.294
Total Ig	115.46 (99.08, 131.84)	128.37 (111.99, 144.75)	0.267

1. Values were log transformed and then transformed back to the original scale due to non-normality.

FIGURE CAPTIONS

Figure 1. Workflow for analysis of colostrum samples by antigen-specific pull-down and mass spectrometry in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations. Colostrum samples were directly digested for quantification of total immunoglobulins by isotype or passed through a chromatographic step to isolate antigen-specific immunoglobulins.

Figure 2. Inclusion and exclusion of cows in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations.

Figure 3. Frequency histograms of the interval from second vaccination to parturition for cows in each treatment group in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations.

Figure 4. Analysis of background binding of colostral proteins to ScourGuard 4(K) vaccine conjugated chromatography resin in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations. A selected reaction monitoring strategy was used for the quantification of isotype abundance. Colostrum samples were sequentially diluted and mixed with either unconjugated or conjugated resin created with isolated ScourGuard 4(K) antigens. All samples were run on a 12 % SDS-PAGE gel under reducing conditions (representative gel shown).

Figure 5. Vaccine-specific (A) and total (B) colostral immunoglobulin concentrations by immunoglobulin class, and nonvaccine-specific colostral IgM concentrations (C), in a cohort study evaluating the effect of prepartum vaccination of pregnant dairy cows with a calf diarrhea vaccine on colostrum immunoglobulin concentrations. Nonvaccine-specific colostral IgM concentrations were calculated as total minus vaccine specific immunoglobulin concentrations. Boxes extend from the 25th to the 75th percentiles, with a line at the median. Whiskers extend from the box to values no more than 1.5 times the interquartile range. Data beyond the end of the whiskers are deemed outliers and are plotted individually. P values for tests of differences between groups are indicated on the plots. Refer to the Methods section for an explanation of the statistical testing.

Figure 1

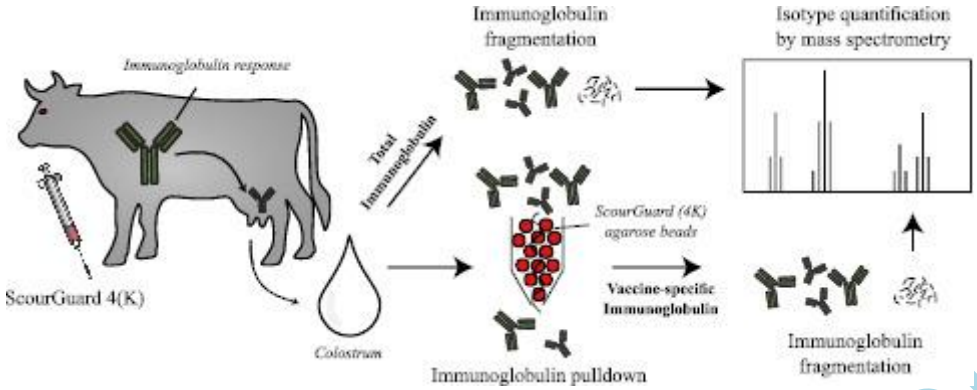

Figure 2

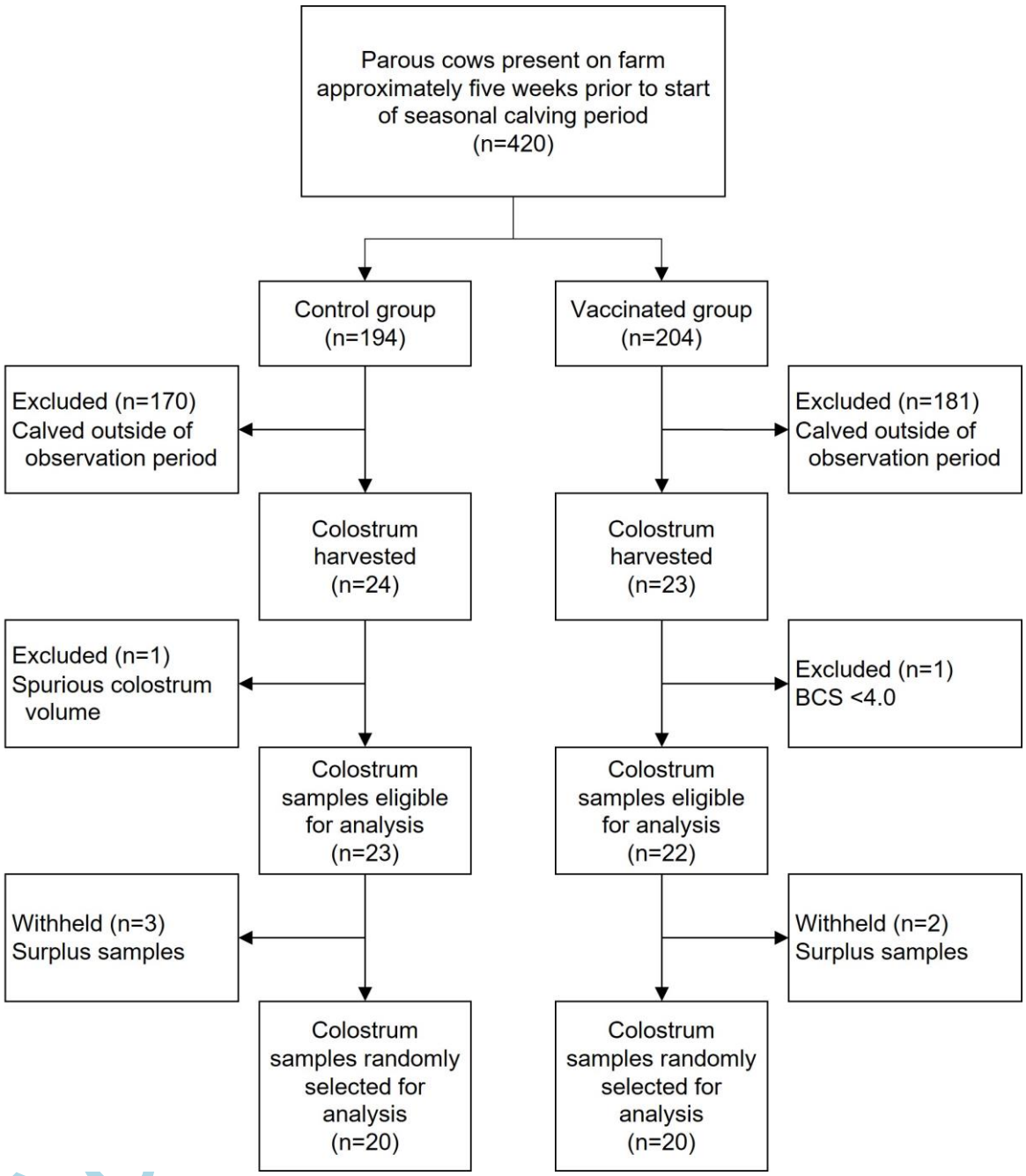

Figure 3

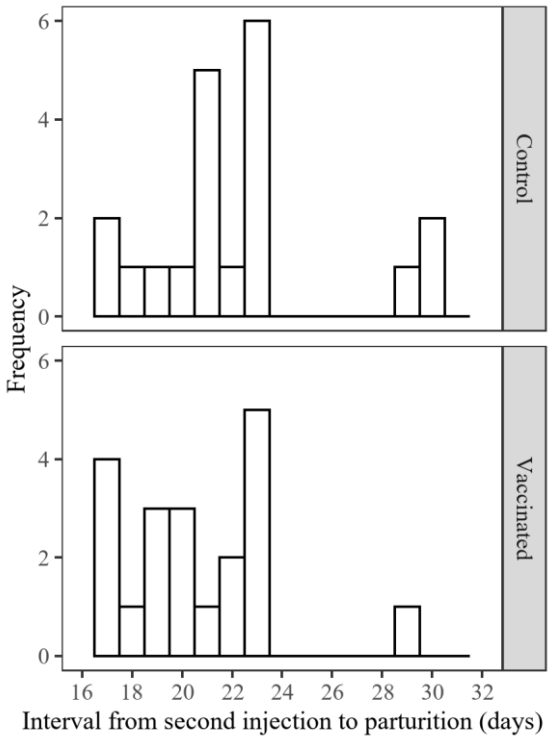

Figure 4

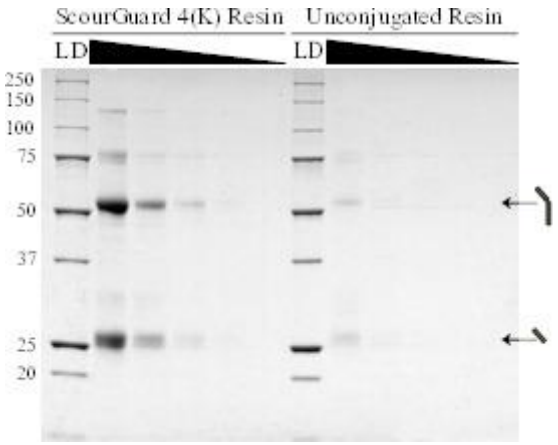

Figure 5