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Technical note

Optimizing selection of large animals for antibody production by screening immune response to standard vaccines

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ABSTRACT

Antibodies made in large animals are integral to many biomedical research endeavors. Domesticated herd animals like goats, sheep, donkeys, horses and camelids all offer distinct advantages in antibody production. However, their cost of use is often prohibitive, especially where poor antigen response is commonplace; choosing a non-responsive animal can set a research program back or even prevent experiments from moving forward entirely. Over the course of production of antibodies from llamas, we found that some animals consistently produced a higher humoral antibody response than others, even to highly divergent antigens, as well as to their standard vaccines. Based on our initial data, we propose that these “high level responders” could be pre-selected by checking antibody titers against common vaccines given to domestic farm animals. Thus, time and money can be saved by reducing the chances of getting poor responding animals and minimizing the use of superfluous animals.

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1. Introduction

Large animals have long held important roles in diverse areas of biomedical research. Small animals are much more common, due to their ease of use and the availability of genetic manipulation systems; but large animals including goats, sheep, horses, donkeys and camelids all have distinct advantages. Large animals tend to be better than murine models for diseases such as tuberculosis, influenza, asthma and Crohn's disease, among others (Conti et al., 2014). Traditionally, horses have been used in the production of antisera for the treatment of various diseases. For example, antisera against diphtheria, tetanus and snake venom are in high demand, particularly in places where vaccination programs are not available (Coghill et al., 1940; Hanly et al., 1995; Wilde et al., 1996; Wagner et al., 2009). In antibody production, domestic farm animals are used when large volumes of antisera and antibody are needed, or when smaller animals are not phylogenetically different enough from the antigen of interest (Coghill et al., 1940; Wagner et al., 2009). Additionally, with the advent of recombinant single-domain antibodies (termed nanobodies), which are now routinely used in both research and clinical applications, interest in using camelid species for antibody production has climbed sharply (Hamers-Casterman et al., 1993; Harmsen and De Haard, 2007; Muyldermans, 2013; Fridy et al., 2014; Klarenbeek et al., 2015). While large domestic animals are crucial for these reasons, their use is often prohibitive due to the high cost

associated with using them, the facilities necessary to house them, and the ethical issues associated with experimentation on large mammals (Hanly et al., 1995; Wilde et al., 1996; Hein and Griebel, 2003; Conti et al., 2014).

Animals that fail to respond to antigen are a well-known problem during polyclonal antibody production (Garvey et al., 1977; Hanly et al., 1995). This is so common an occurrence that investigators will usually inject more animals than necessary to ensure an immune response. In smaller animals such as rabbits and mice this may not pose many issues, as the cost of upkeep for these animals and the amount of antigen required to inoculate them is low. However, large animals have high husbandry costs, and can sometimes demand large amounts of antigen to produce a strong immune response, so a large animal non-responder represents a substantial monetary setback that many researchers cannot afford. A method that can predict which animals will be likely non-responders to an antigen before it is purchased and set aside for biomedical research would thus be of significant utility. By reducing the chances of getting poor responding animals, time and money can be saved, and superfluous animal use can be avoided.

In humans, most studies of immune response have focused on person-to-person variation in response to single vaccines (Ovsyannikova et al., 2006; Poland et al., 2013; Tsang et al., 2014). However, it would be more beneficial if it were possible to predict what the response to one antigen in a single animal would be by looking at a previous response to a different antigen entirely. In one study on pigs, animals were bred to have either a high immune response or low immune response. One metric used to determine the strength of immune response was serum

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concentration and avidity of anti-hen egg-white lysozyme antibodies (Appleyard et al., 1992; Mallard et al., 1992). When vaccinated against *Actinobacillus pleuropneumoniae*, pigs bred to have high immune responses had higher antibody titers and produced antibodies with higher avidity than those bred for low immune responses (Magnusson et al., 1997). This correlation implies that it may be possible to predict how well an animal will produce antibodies to one antigen based on their reactions to other antigens.

A convenient method we found to test a large animal's immune response is based on common vaccinations. The CDC suggests that all herd animals, especially animals that come in contact with humans and those that are of high value, be vaccinated against rabies (National Association of State Public Health Veterinarians, 2011). Additionally, *Clostridium perfringens* toxoids type B and type D and *Clostridium tetani* toxin are a cause of high morbidity and mortality in domestic farm animals, and prophylaxis via vaccination is a common herd management practice (Stiles et al., 2013). Because these immunizations are so prevalent, most large animals that will be used for antibody production will be vaccinated against these, or similar, antigens. Within a single farm, it is typical for a herd to be managed in a uniform fashion, removing many variables such as last vaccination date, vaccine brand, dose, and route of vaccination. Farms also tend to have standard adjuvants and injection protocols for experimental antibody production. All of these factors make it possible to readily analyze the response against vaccine antigens, and use those responses to predict how an animal will respond to future antigens.

During the course of previous work we observed that certain animals consistently produced high concentrations of antigen-specific serum antibodies to several highly divergent antigens. We hypothesized that if an animal is consistent with its level of antibody production, then future antigen response can be predicted by previous outcomes within a single animal. For this study we developed an ELISA method to analyze an animal's antibody titers against two common vaccinations, CDT and rabies, as well as mCherry and GFP antigens. Using this assay, we identified a correlation between antibody titers to common vaccines and titers to experimental antigens injected for the purpose of antibody production.

2. Materials and methods

The ELISA assay was designed based on standard published protocols (Bishop et al., 1984; Miura et al., 2008). Briefly, llama serum was saved from previous studies (Fridy et al., 2014); upon delivery, all serum was stored in 0.02% sodium azide to prevent microbial growth. Biographical and handling information for llamas and alpacas can be found in Tables 1 and 2. Llamas (*Lama glama*) and alpacas (*Vicugna pacos*) were used in previous and ongoing nanobody production efforts (Fridy et al., 2014). All animals were obtained from Capralogics, Inc., and all animal procedures were performed by Capralogics according to their Institutional Animal Care and Use Committee. Nunc-Immuno 96 well plates (Sigma) were coated with antigen. Vaccines were prepared for use as coating antigen as followed: Nobivac 3-Rabies Rabies vaccine

Table 1
Biographical information for immunized llamas and alpacas.

ID Number	Species	Name	Sex	Age	Last rabies	Last CDT
3484	Llama	Barbie	Female	~8 years	N/A	N/A
3485	Llama	Pacino	Male	~7 years	N/A	N/A
4761 ^a	Llama	Marley	Male	3 years	6/26/2013	3/26/2014
4762	Llama	Big Papa	Male	7 years	6/26/2013	3/4/2014
5094 ^a	Llama	Marley	Male	4 years	6/26/2013	3/26/2014
5095	Llama	Briffitt	Male	10 years	3/21/2014	3/21/2014
5096	Llama	Makeba	Female	8 years	3/21/2014	3/21/2014
5097	Alpaca	Ziti	Female	4 years	8/9/2013	3/26/2014
5098	Alpaca	Paulie	Male	2 years	1/31/2014	3/26/2014

^a These numbers represent the same animal used in two different experiments.

Table 2

Animal immunizations. All initial immunizations were performed subcutaneously with complete Freund's adjuvant (CFA). All boosters were performed subcutaneously 21 days apart with incomplete Freund's adjuvant (IFA), except where otherwise noted. Preimmune bleeds were taken before initial injection. Test bleeds were taken 10 days after boosters; production bleeds were taken 10 days after the final booster. Animals in this chart are separated by species, antigens injected and project start date. Antigens in bold are those whose response was measured with ELISAs in this paper.

ID number	Antigen	First injection	Boosters	Project start date
3484	GFP -His ₆	5 mg	5 mg × 5 ^b	2/26/2010
3485	GFP -His ₆	5 mg	5 mg × 5 ^b	2/26/2010
4761 ^a	FLAG ₃ - mCherry -FLAG ₃	5 mg	5 mg × 3	5/17/2013
4762	FLAG ₃ - mCherry -FLAG ₃	5 mg	5 mg × 3	5/17/2013
5094 ^a	GFP	1 mg	1 mg × 3	4/25/2014
5095	GFP	1 mg	1 mg × 3	4/25/2014
5096	GFP	1 mg	1 mg × 3	4/25/2014
5097	GFP	1 mg	1 mg × 3	4/25/2014
5098	GFP	1 mg	1 mg × 3	4/25/2014

^a These numbers represent the same animal used in two different experiments.

^b The first two boosters were given 21 days apart, the following two were given 5 months later, 21 days apart from one another. A final booster was given 6 months after this, using complete Freund's adjuvant.

(Merck) was diluted 1:16 in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃ pH 9.6). Vision CDT vaccine (Merck) was diluted 1:256 in coating buffer. To improve sensitivity and reliability of the assay, GFP and mCherry were crosslinked before coating. Proteins were prepared for use as coating antigen as follows: 50 µL of 4.3 mg/ml GFP or mCherry in PBS was mixed with 50 µL of 0.43 mg/mL BSA in PBS. 40 µL of ice cold 72% TCA was added and the mixture was incubated on ice for 10 min, then harvested by centrifugation at 14,000 rpm at 4 °C for 10 min. The pellet was gently washed with 500 µL of PBS and centrifuged again for 5 min. It was then resuspended in 200 µL 1% formaldehyde in PBS and sonicated, then incubated at room temperature for 1 h. GFP was then diluted 1:25 in coating buffer, and mCherry was diluted 1:500 in coating buffer. 100 µL/well for each antigen was plated (for final antigen amounts of 4 µg or 0.2 µg per well respectively) and plates were incubated overnight at 4 °C.

The next day coating solution was removed and plates were blocked for two hours at room temperature with 300 µL/well of 5% fat free skim milk in TBS. Plates were then washed three times with 300 µL/well wash buffer (0.1% Tween-20 in TBS). Serum bleeds diluted in dilution buffer (0.1% BSA, 0.05% Tween-20 in TBS) were used as primary antibody. In the case of animals 3484, 3485, 4761, and 4762, serum used for ELISAs was obtained after all boosters were given. For animals 5094–5098, serum for all antigens was obtained after two boosters were given. Serum was serially diluted 1:3 from 1:5000 to 1:10,935,000 for all antigens. 100 µL/well of primary antibody was pipetted onto plates, and plates were incubated for 2 h at room temperature. After the incubation, plates were washed three times with 300 µL/well wash buffer. Next, 100 µL/well 1:25,000 HRP-conjugated Goat α-Llama antibody (Bethyl) diluted in dilution buffer was used as secondary. Plates were incubated two hours at room temperature, then washed three times with 300 µL/well wash buffer. 100 µL per well of 3,3',5,5'-Tetramethylbenzidine (TMB) (Thermo) was added and allowed to develop for 5 min, at which time the reaction was quenched with 100 µL 2 M H₂SO₄. Absorbance at 450 nm was measured with a BioTek Synergy NEO plate reader. Each experiment was performed in triplicate.

The absorbance at 450 nm was plotted against the log(concentration) of the reciprocal dilution of the serum used as the primary antibody. Titer cutoffs corresponding to a range of ODs were calculated in the following manner: A minimum OD and a maximum OD were chosen for each group of animals, and the titer values for five evenly spaced ODs in this range were interpolated from fitting a sigmoidal 4 Parameter Logistic curve to the data. The extreme values were selected by taking, e.g., the maximum OD for which a value was obtained for each animal and then finding the minimum of these values, ensuring that the selected cutoff would be in

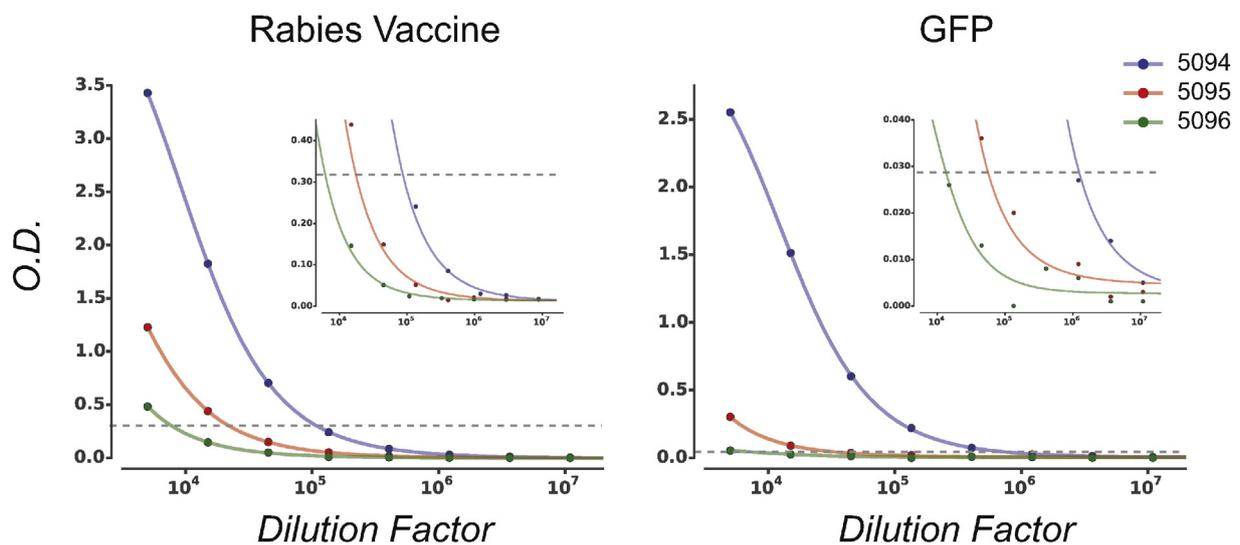


Fig. 1. Rabies vaccine or GFP responses by ELISA. Representative ELISA curves from three llamas concurrently immunized with GFP (5094, 5095, and 5096) are shown for serum activity against rabies vaccine (left) or GFP (right). A sigmoidal 4-parameter logistic curve was fit to each serum titration. An intermediate cutoff (fourth out of five tested) was selected to calculate titers (gray dashed line). A zoomed-in view of the low-response range for each plot is shown inset.

the measured OD range for every animal in the group. Animals were grouped based upon species (Llama or Alpaca), type of Antigen (GFP or mCherry), and date administered.

For each selected titer cutoff, the correlation between vaccine and antigen response was measured by fitting a linear regression model and calculating Pearson and Spearman correlation coefficients (*r*-values) for the data. The *p*-values for these measures were obtained by permutation tests: Vaccine response levels (*x*-values) were kept constant while shuffling the Antigen response levels (*y*-values) over the entire range of possible permutations. This was manageable due to the relatively small size of the data set (9 animals). The number of possible permutations for a data set of size *n* is *n!* – in this case 362,880 (9!) permutations. The *r*-values were calculated for each permutation producing distributions for the null hypothesis, namely that the Vaccine and Antigen responses are

not correlated. The proportion of the *r*-values from the permutations that are greater than the value calculated from the original data is the reported *p*-value.

The Python programming language (package “SciPy”) was used to automate the process of fitting curves to the data, calculating the correlation statistics for the range of titer cutoffs and running the permutations. Scripts used can be provided upon request.

To determine serum concentrations of IgG, preimmune or test bleed serum was incubated with Protein A sepharose (Life Technologies). Serum was diluted 10-fold in 20 mM Na-phosphate, pH 7.0 and incubated with an excess of Protein A sepharose for 30 min. at room temperature. Resin was washed 3 times with 20 mM Na-phosphate, pH 7.0, and bound protein was eluted by heating for 10 min. at 72 °C in LDS. Elutions were separated by SDS-PAGE and visualized by Coomassie Blue staining.

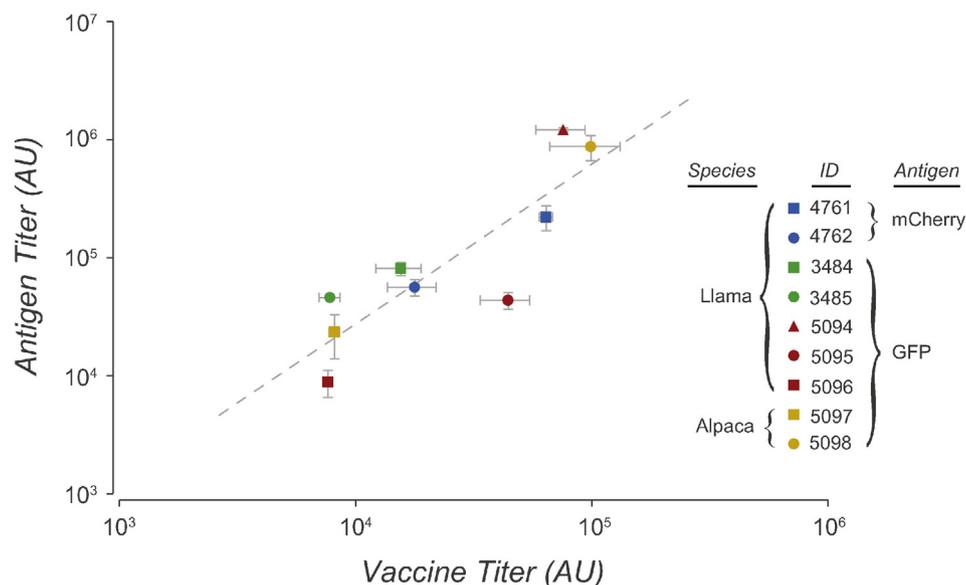


Fig. 2. Correlation of llama or alpaca vaccine and antigen titers. Those animals of the same species and injected with identical antigens are shown in the same color (see Table 2). Species and antigen types are indicated. All animals' antigen titers were measured with a GFP ELISA, except for 4761 and 4762, which were titrated with an mCherry ELISA. The combined vaccine titer was obtained by averaging each animal's response to the rabies and CDT vaccines. All titers were calculated based on an intermediate cutoff (fourth highest out of five calculated). Experiments were performed in triplicate, and error bars reflect s.e.m. values. A linear regression was performed (dashed gray line), which had an R^2 value of 0.75 ($p = 0.003$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Bovine gamma globulin (BgG) standards were run concurrently to allow absolute quantification. Densities of heavy chain V_{H1} (~43–46 kDa) and V_{H2} (~50 kDa) fragments were quantified using ImageJ2 and calibrated using the BgG standards. Pearson and Spearman correlation tests with antigen titers were performed as above.

3. Results

Over the course of several studies using camelid antibodies, we immunized a total of 6 llamas and 2 alpacas with either GFP or mCherry, or both in the case of one animal. Given the structural similarity of these two fluorescent proteins, and the otherwise identical immunization conditions, we reasoned that these animals' antigen and vaccine immune responses could be compared, and used to determine any potential correlation. All animals were maintained at the same facility, and were inoculated with both rabies and CDT vaccines, allowing us to do an analysis with these two vaccines. These vaccines are formulated with proprietary adjuvants, distinct from the CFA used in the antigen immunizations. It was reasoned that differences in adjuvants would not impact relative trends of immune response, and our results should therefore also extend to other classes of adjuvants when CFA is not an option (Eckersley et al., 2011).

Probing with dilutions of serum bleeds from all animals, we performed ELISA assays against immobilized antigen (GFP or mCherry) and CDT and rabies vaccine mixtures. In calculating serum titers from these ELISAs, we were unable to define a baseline cutoff using negative control sera, as unvaccinated animals were unavailable; all llamas and alpacas are routinely vaccinated around 6–8 weeks old, and maternally transferred antibodies could be present even before this time. To overcome this, we calculated titers using five different cutoff values across the observed data range. As only relative titers are necessary for this analysis, this was chosen as the most comprehensive approach allowing comparison between animals injected with the same antigen and vaccine.

Serum titers against each antigen or vaccine were calculated using different cutoffs and plotted against each other (Fig. 1 and Supplementary Fig. 1). The middle range cutoffs all showed consistent results, and a representative result was selected (Fig. 2). Responses to CDT and rabies vaccines were averaged, but similar trends were observed for each independently. For this cutoff, linear regression showed significant positive correlation, with Pearson and Spearman correlation tests giving coefficients of 0.86 and 0.83 respectively, with two-tailed p-values of 0.002 and 0.006. Aside from the lowest cutoff tested (nearing the assay baseline, thus much more sensitive to noise), regressions using all cutoffs gave Pearson and Spearman coefficients ranging from 0.79 to 0.87 and 0.77 to 0.9, respectively. Thus a statistically significant correlation is consistently observed between an animal's titer against CDT and rabies vaccines, and its response to a subsequent GFP or mCherry immunogen.

To determine whether total IgG levels contribute to observed differences in antigen titers, we quantified the levels of both V_{H1} and V_{H2} heavy chain in serum samples (Supplementary Fig. 2). When compared to the measured antigen responses, no statistically significant correlation is observed with either V_{H1} , V_{H2} or total IgG (Pearson and Spearman p values >0.1), indicating that the immune response generated is not predicated on total IgG levels. Given the particular interest in llama and alpaca V_{H1} IgG variants, it is also notable that the overall V_{H1} to V_{H2} ratios are relatively consistent from animal to animal, and do not significantly change upon immunization.

4. Discussion

While our data are necessarily preliminary, given the difficulties in performing a sufficiently large-scale animal study for unambiguous statistical strength, we have identified a significant pattern whose implications could prove to save both time and money. More work needs to be done on this topic to fully characterize how consistently the humoral immune response to one antigen correlates to others within a single

animal, and we encourage others to analyze new or existing sera stocks to further test the trends presented here. As it stands, our method of screening animal vaccine response by ELISA is a low cost, simple way to increase the odds of choosing an animal likely to give a high humoral immune response to a chosen antigen; moreover, pre-screening animals for high vaccine is unlikely to have any significant disadvantages as a strategy over omitting the prescreen, and is a strategy we now follow. Additionally, we found that once we had one animal that we knew was a high responder, we could continue using that animal for other antigens. After injecting eight different antigens over the course of two years into one llama, this animal still consistently gives a high serum titer response to new antigens. Overall, therefore, vaccine prescreening offers multiple ways to save money at little upfront cost. By predicting which animals will be more likely to respond, and reusing those that do respond, fewer animals need to be used for antibody production experiments. Using fewer animals means less animal distress and sacrifice, and less money wasted by laboratories that cannot afford to deal with response failures.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2016.01.006>.

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