

Hyperimmunized Chickens Produce Neutralizing Antibodies Against SARS-CoV-2

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Abstract

The novel severe acute respiratory syndrome (SARS) coronavirus, SARS-CoV-2, is responsible for the global COVID-19 pandemic. Effective interventions are urgently needed to mitigate the effects of COVID-19 and likely require multiple strategies. Egg-extracted antibody therapies are a low-cost and scalable strategy to protect at-risk individuals from SARS-CoV-2 infection. Commercial laying hens were hyperimmunized against the SARS-CoV-2 S1 protein using three different S1 recombinant proteins and three different doses. Sera and egg yolk were collected at three and six weeks after the second immunization for enzyme-linked immunosorbent assay and plaque reduction neutralization assay to determine antigen-specific antibody titers and neutralizing antibody titers, respectively. In this study we demonstrate that hens hyperimmunized against the SARS-CoV-2 recombinant S1 and receptor binding domain (RBD) proteins produced neutralizing antibodies against SARS-CoV-2. We further demonstrate that antibody production was dependent on the dose and type of antigen administered. Our data suggest that antibodies purified from the egg yolk of hyperimmunized hens can be used as immunoprophylaxis in humans at risk of exposure to SARS-CoV-2.

Introduction

The novel severe acute respiratory syndrome (SARS) coronavirus, SARS-CoV-2, is the etiologic agent of COVID-19, a newly emerged viral respiratory disease in humans. First identified in late 2019, the disease reached pandemic status and has caused over 158 million cases and 3.3 million deaths worldwide by the time of this publication. The COVID-19 landscape is rapidly evolving, and effective strategies to mitigate its spread are urgently needed. Notably, the first emergency use authorizations for a vaccine for the prevention of COVID-19 was issued on December 11, 2020¹. Multiple vaccines and treatments have since been developed and approved for emergency use. Although vaccines appear reasonably effective at reducing the severity of illness associated with COVID-19, the recent emergence of new variants threatens the efficacy of these vaccines. Therefore, ongoing research to develop new vaccines, antiviral drugs, and antibody therapies is critical to reducing the devastating impact of the pandemic. Successful COVID-19 intervention likely requires multiple approaches, and antibody therapies are an attractive strategy to protect at-risk individuals from SARS-CoV-2 infection.

The genetic material of coronaviruses is composed of a single-stranded, positive sense RNA genome. This genome is the largest of the RNA viruses and ranges from 27 to 30 kilobase pairs. SARS-CoV-2 is a betacoronavirus belonging to the family *Coronaviridae*. Coronaviruses cause respiratory and gastrointestinal disease in a wide range of animal species, including mammals and birds. Alphacoronaviruses and betacoronaviruses are known to infect humans and other mammals, and gammacoronaviruses and deltacoronaviruses infect birds, although some can infect mammals. The most notable and extensively studied gammacoronavirus in chickens is infectious bronchitis virus (IBV), against which most commercial hens are immunized.

Coronavirus entry into cells is mediated by the spike protein, most specifically its S1 portion, a peplomer-like structure anchored to the virus membrane in the form of a trimer. In addition, the S1 subunit of this protein determines virus variability and elicits neutralizing antibodies^{2,3,4}. On the globular head of each SARS-CoV-2 S1 protein is a receptor-binding domain (RBD), which specifically recognizes the human angiotensin-converting enzyme 2 (ACE2)⁵. The RBDs switch between one of two conformations. In the standing-up position, one of the RBDs is accessible and available to bind ACE2; in the lying-down position, all three RBDs are inaccessible, which may help evade immune surveillance in infected individuals^{6,7}. The RBD is the most antigenic region of the spike protein in coronaviruses and is thus an attractive site for therapies that target cell entry of the virus.

On August 23, 2020, the U.S. Food and Drug Administration issued an emergency use authorization for investigational convalescent plasma to treat hospitalized COVID-19 patients⁸. The principle behind this therapy is that recovering COVID-19-infected individuals develop SARS-CoV-2-specific antibodies that can be recovered from plasma and administered to ill patients to neutralize the virus if applied systemically. This passive transfer of antibodies is not limited only to SARS-CoV-2-infected patients. Researchers are also applying this concept of virus neutralization to generate SARS-CoV-2-specific antibodies in animals to be used for passive immunization against the virus in humans. Harvesting antibodies from eggs laid by hens that have been immunized against the spike protein of SARS-CoV-2 is an attractive model to produce protective antibodies due to the scalability, convenience, and low cost⁹.

Chickens produce immunoglobulin Y (IgY), which is a homologue of mammalian IgG. An average egg yolk yields 50–100 mg of IgY, of which 2–10% comprise specific antibodies^{10,11}. When hyperimmunizing hens, the amount of antigen-specific IgY produced will depend on the age of the hen, adjuvant, route of application, as well as the dose, antigenicity, and molecular weight of antigen administered to each hen^{12,13,14,15}. A laying hen lays an average of 300 eggs per year, which corresponds to approximately 15–30 g of IgY. Thus, a considerable amount of polyclonal antibody can be non-invasively recovered from the eggs laid by immunized chickens. Other advantages of using IgY in human applications are that it is well tolerated and can be administered orally^{16,17}. Furthermore, IgY neither binds to human rheumatoid factors nor activates the human complement system¹⁶, therefore reducing the risk of inflammatory reactions as a secondary effect of using antibodies produced in different species. These characteristics make chicken IgY a promising source of new therapies for human viral diseases such as COVID-19 in addition to vaccination strategies¹⁸.

Here we demonstrate that hens hyperimmunized against the SARS-CoV-2 recombinant receptor binding domain and/or S1 protein produced neutralizing antibodies against SARS-CoV-2. We further demonstrate that antibody production was dependent on the dose and type of antigen administered. Our data suggest that antibodies purified from the egg yolk of hyperimmunized chickens can be used as immunoprophylaxis in humans at risk of exposure to SARS-CoV-2.

Results

Antigen-specific IgY antibodies were detected in the serum of hyperimmunized hens

Commercial laying hens were immunized against the SARS-CoV-2 S1 protein, and blood was collected 21 days following the second vaccination for antigen-specific ELISAs. Birds receiving 50- μ g doses from all vaccines demonstrated strong antibody responses, which were higher ($p < 0.05$) than negative control titers (Fig. 1). At the lower doses (2.5 and 5 μ g), only birds receiving vaccine C demonstrated positive antibody responses, which were significantly higher than non-immunized control titers at 2.5 μ g. Notably, antibody titers among birds administered vaccine C were not significantly different between doses, whereas antibody titers among birds administered vaccines A and B were significantly higher in the 50- μ g groups compared with their respective 2.5- and 5- μ g groups (Fig. 1A, B, C).

To test the immunogenicity of combining two vaccines, one group of birds received primary vaccine C followed by a boost with vaccine B. ELISAs were performed 21 days following the second immunization to measure both antigen C- and B-specific antibody responses. The birds receiving 50 μ g of vaccine C/B developed strong antigen B- and C-specific IgY titers (Fig. 1D, E), which were significantly greater than their respective negative control titers and titers among birds receiving 2.5- and 5- μ g doses.

Antigen A-, B-, and C-specific IgY antibodies were detected in the yolk of hyperimmunized hens

Beginning one month following the second immunization, eggs were collected weekly for ELISAs to detect antigen A-, B-, and C-specific IgY titers extracted from egg yolks. Yolk extracts were diluted 1:400, and specific IgY for each antigen was measured by ELISA using antigen A-, B-, or C-covered plates (Fig. 2). Antigen-specific IgY titers extracted from yolks collected 6 weeks after the second immunization were significant for all tested antigens in birds receiving 50- μ g doses, regardless of which vaccine they were administered. Among the birds administered vaccine A/A, egg yolk-derived antibodies were detectable at the lower immunization doses, but the titers were only significant for the 2.5- μ g dose. Notably, the vaccine A/A 50- μ g titers were significantly greater than the 2.5- and 5- μ g titers. Egg yolk-derived IgY antibodies from birds receiving the lower doses for vaccine B/B were not significantly different from negative control titers. In contrast, egg yolk-derived IgY of hens from the vaccine C/C groups showed significant antibody titers at all doses (Fig. 2A, B, C).

Egg yolk extractions were also analyzed for C- and B-specific antibody responses in hens receiving a combination of vaccine C and B as first and second dose, respectively. B-specific antibody titers in hens administered vaccine C/B were significant ($p < 0.05$) only in hens receiving the 50- μ g doses (Fig. 2D), which was consistent with the data reported for the hens receiving vaccine B/B. In contrast, C-specific antibody titers in hens receiving vaccine C/B were significant at 2.5-, 5-, and 50- μ g doses, which corresponded to observations noted in hens in the vaccine C/C group (Fig. 2E).

Egg yolk extracts showed a mean protein concentration of 2.42 mg/ml with a standard deviation of 0.48, whereas serum protein concentration had a mean of 56.71 mg/ml with a 2.2 standard deviation.

Hyperimmunized hens produced neutralizing antibodies against SARS-CoV-2

Hens immunized against the SARS-CoV-2 S1 protein were tested for neutralizing antibodies in sera at three weeks after the second immunization and in egg yolk extractions at six weeks following the second immunization. Negative controls included IgY from serum and egg yolk of hens that were either infectious bronchitis virus (IBV)-vaccinated or IBV-naïve. The IBV-naïve birds are tested regularly to confirm the absence of IBV antibodies by ELISA.

Sera and egg yolk IgY extractions from both IBV-vaccinated and IBV-naïve controls did not neutralize virus. Sera from hens receiving vaccine A/A and B/B neutralized virus up to 1:20, whereas sera from hens receiving vaccines C/C and C/B neutralized virus up to 1:40 and 1:80, respectively (Fig. 3A). IgY extractions from egg yolk neutralized virus up to 1:10 among only the hens in the vaccine C/C and C/B groups, and no neutralization was observed among egg yolk samples from hens receiving vaccines A/A and B/B (Fig. 3B).

Discussion

In this study we demonstrate that laying hens hyperimmunized against three different SARS-CoV-2 recombinant spike proteins produced antibodies in sera at good levels. We further confirmed that the dose and type of antigen administered influenced the levels of antibody titers. In addition, we showed that all vaccines induced neutralizing antibodies against SARS-CoV-2, but that vaccine against the glycosylated S1 protein (C/C) and combination of glycosylated S1 and non-glycosylated RBD of S1 (C/B) led to better neutralization. Specifically, neutralization was observed in all groups, but it was at lower serum concentrations among the hens receiving the full-length glycosylated S1 (C) protein at least once, whereas virus neutralization by IgY extracted from egg yolk occurred only in the vaccine C/C and C/B groups.

Compared with the negative control group, the full length and tagged S1 protein elicited a higher level ($p < 0.05$) of IgY titers in serum and egg yolk at all doses, whereas immunization with the RBD fragments (A or B; non-tagged) induced antibody titers higher than the negative control ($p < 0.05$) only when immunized at the highest dose of 50 μg . This observation could be explained by the structural difference between antigens. In contrast to vaccines A and B, in which the S1 fragments contained only the receptor-binding domain, vaccine C comprised the entire extracellular domain of the S1 protein. The increased size and number of potential epitopes may be responsible for the enhanced antigenicity of vaccine C. Another possible explanation could be that vaccine C was produced using a eukaryotic HEK293 expression system. In contrast to the prokaryotic expression system used for manufacturing vaccines A and B, HEK293 cells are capable of the post-translational modifications necessary for the correct folding of the protein¹⁹. Thus, modifications such as phosphorylation or glycosylation of the recombinant protein could

potentially enhance immunogenicity¹⁹. Our data suggest that antibodies generated from vaccine C could therefore be administered at a fraction of the dose necessary for seroconversion to occur. This is crucial when considering potential applications of IgY in prevention strategies against COVID-19.

We also demonstrated that a combination approach using vaccine C followed by vaccine B generated good IgY titers specific for antigen C, which were comparable to the titers observed with vaccine C/C. Similarly, vaccine C/B IgY titers specific for antigen B corresponded to vaccine B/B titers. Importantly, the vaccine C/B combination resulted in neutralizing antibody titers that were at least as good as the titers induced by vaccine C/C. Since the production of proteins using the eukaryotic HEK293 expression system is more costly than using a prokaryotic system, a vaccine combination approach may prove to be a more economical, but equally effective, immunization strategy.

Since the hens had been vaccinated for IBV, a chicken gammacoronavirus, it was important to determine whether cross-neutralization could be observed between samples from IBV-vaccinated hens and SARS-CoV-2. Virus neutralization was not observed by IBV-vaccinated hens, which suggests that IBV antibodies do not cross-react with SARS-CoV-2. Although unsurprising given the enormous genetic variability of coronaviruses, this observation underscores the importance of ensuring that the spike protein used for vaccination is genetically similar to the circulating virus.

As expected, SARS-CoV-2-specific IgY titers were also detected in egg yolk. As shown in our results, IgY titers in yolk were lower than in serum. These outcomes might be related with the efficiency of our yolk antibody extraction method, which is based only on ultrapure water and centrifugation. The egg yolk is a complex structure composed of proteins, lipids, vitamins and minerals. The main component of the egg yolk are lipids in ~ 65% of dry matter, and the lipid-to-protein ratio is about 2:1²⁰. In addition, lipids in the yolk are exclusively associated with lipoprotein assemblies, i.e. triglycerides, phospholipids and cholesterol²⁰. This intricate lipid association with proteins can complicate protein extraction from the yolk if the lipids are not diluted appropriately, which might have been the case with our IgY extraction method. Anecdotally, we have worked with a private company upscaling the hyperimmunization and egg production systems. They have used a polyethylene glycol precipitation to extract IgY from yolks with better results (Michelle Hawkins, CAMAS, personal communication). The polyethylene glycol method has been tested by several researchers^{14,21} and seems to be a better method to address the issue of fats present in the egg yolk. This methodology is crucial for pure antibody extractions and formulation of preventative products, such as local use sprays containing IgY.

A recent publication demonstrated that IgY extracted from egg yolk of hens immunized with the RBD of SARS-CoV-2 could prevent entry and replication of SARS-CoV-2 *in vitro*²². These results strengthen the case for administering egg yolk-derived IgY to reduce SARS-CoV-2 infection.

The IgY neutralizing effects measured in this study were performed using the USA-WA1/2020. Like many other RNA viruses including the coronaviruses, SARS-CoV-2 is prone to frequent mutations^{23,24}. Thus far, thousands of mutations have been observed at the nucleotide level in the SARS-CoV-2 genome, indicating

that SARS-CoV-2 has already become heterogeneous²³. The recent emergence of more prevalent new variants has already resulted in reduced efficacy of current vaccines, so it is critical to closely monitor the evolution of SARS-CoV-2 to ensure that prophylactic antibody administration targeted toward the SARS-CoV-2 RBD retains sufficient neutralizing activity.

Methods

Constructs and Virus

The constructs used for vaccines A and B included the SARS-CoV-2 S1-derived RBD protein #0570, which included amino acids 328–533 and a His tag (vaccine A) and SARS-CoV S1-derived RBD amino acids 319–542 and a His tag (vaccine B), using a bacterial based *in vitro* expression system (Sutro Biopharma, South San Francisco, CA, USA). The reference virus used to obtain these constructs was Wuhan strain. The construct used for vaccine C consisted of the SARS-CoV-2 S1 protein, including amino acids 16–685 and an Fc-tag, which was expressed in HEK293 cells (Cat # S1N-C5255, AcroBiosystems, Newark, DE, USA); these proteins were glycosylated.

Virus neutralization experiments were performed using the 2019-nCoV/USA-WA1/2020 strain sourced from an infected patient in Washington state.

Experimental Design

Two hundred 85-week-old laying hens were transported from a table egg layer farm in the California Central Valley to the UC Davis Teaching and Research Animal Care Services facility, where they were placed on pine shavings in climate-controlled BSL-2 rooms. After one week of acclimatization, the hens received two immunizations administered twelve days apart. Vaccines were prepared as an oil-water emulsion with an equal volume of Freund's incomplete adjuvant (Thermo Scientific, IL, USA) using an Ultra-Turrax T25 High-Speed Homogenizer (IKA, Staufen, Germany) at 25,000 rpm for 10 min. The experimental groups were as follows: vaccine A twice (A/A), vaccine B twice (B/B), vaccine C twice (C/C), vaccine C followed by vaccine B (C/B), and adjuvant only (negative control). Each treatment group was divided into the following subgroups receiving a 2.5-, 5-, or 50- μ g dose. All vaccines were administered by intramuscular route in the pectoral muscle in 0.5-mL volumes. Blood was collected from the ulnar vein upon arrival, 21 days following the second immunization, and at the end of the experiment (6 weeks post second dose). Blood was centrifuged and serum was stored at 4 °C for ELISA. One month following the second immunization, eggs were collected every week and stored at 4 °C for IgY extraction. In the sixth week after the last immunization, the hens were humanely euthanized by CO₂ inhalation, immediately followed by cervical dislocation.

Animal care and experimental procedures were performed in compliance with all federal and institutional animal use guidelines. The University of California is an Association for Assessment and Accreditation of Laboratory Animal Care accredited institution. UC Davis institutional animal care and use committee

(IACUC) approved the experimental design and testing protocols performed in the hens. Protocol #20851. In addition, our study is reported in accordance to ARRIVE guidelines.

IgY Extraction from Egg Yolk

IgY extraction was performed as described previously²⁵, with minor modifications. Nine eggs per group were cracked open. The egg white was discarded, and the egg yolk was washed with phosphate-buffered saline (PBS). The egg yolk was punctured, and the vitelline membrane was discarded. The yolk was transferred to a 50-mL sterile centrifuge tube and Milli-Q® water (Millipore purification system, Bedford, MA, USA) was added for a yolk dilution of 1:5. The tube was vortexed vigorously and stored overnight at 4 °C. The following day, the tubes were centrifuged at 1,000 x *g* for 30 min at room temperature. Since the supernatant was still yellow, a second dilution/centrifugation step was performed. Ten mL of the supernatant was collected and mixed with 40 mL of Milli-Q® water for a final yolk dilution of 1:10, and centrifugation was performed at 2,000 x *g* at 15 °C for 30 min. The supernatant, now clear, was obtained and filtered through a Millex -HV 0.45 µm filter (Millipore, Bedford, MA, USA) and stored at 4 °C. To measure the IgY extraction efficiency, protein concentration of the yolk extract from 9 egg yolks was individually measured and compared with protein concentration from 10 individual serum samples obtained at necropsy from vaccine C group at 50 µg using the Take 3 plate on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

IgY Antibody Titers

Serum and yolk IgY titers were measured by enzyme-linked immunosorbent assay (ELISA). Wells were coated with 0.5 µg of each of the antigens in coating buffer and incubated at 4 °C overnight. All wash steps were performed with PBS-Tween 20 (0.05% Tween 20). Plates were blocked with 5% milk in PBS-Tween 20 for 1 hour at room temperature. Serum samples were diluted 1:3200 in Sample Diluent, provided by the manufacturer, and purified yolk samples were diluted 1:400 in Milli-Q® water. The rest of the ELISA was performed using the reagents provided by the commercial IDEXX infectious bronchitis Ab Test (IDEXX, Westbrook, ME, USA), according to the manufacturer's instructions. Data were presented as sample to positive (S/P) ratio, which was calculated from the optical density (O/D) values using the following equation: (mean of test sample – mean of negative control) / (mean of positive control – mean of negative control). One positive control was carefully standardized for each antigen, and one negative control was used for all antigens. An S/P ratio allows titers to be compared across multiple plates, but S/P ratios should not be compared between antigens or between serum and egg yolk-derived IgY because a different positive control was used for each antigen and sample type.

Plaque Reduction Neutralization Assay

Antibodies derived from pooled sera and egg yolk from hens receiving vaccine A/A, B/B, C/C and B/C at 50-µg dose were assessed for their ability to neutralize SARS-CoV-2 using a plaque reduction neutralization assay (PRNA). Briefly, the antibodies were diluted 1:10 in culture medium and incubated in two-fold serial dilutions with 100 plaque forming units (PFUs) of the Washington Strain of SARS-CoV-2 (USA-WA1/2020). SARS-CoV-2 was diluted in supplemented DMEM at a 1:1 ratio. Virus was then added

to the antibody samples and allowed to incubate for 1 h at 37°C and 5% CO₂. After incubation, viral plaque assay was conducted to quantify viral titers. Twelve-well plates were previously seeded with Vero cells (ATCC CCL-81) at a density of 2 X 10⁵ cells per well. Media was aspirated from plates and virus-antibody samples were transferred to wells, one sample per well. Plates were incubated for 1 h at 37°C and 5% CO₂. After infection, a 1:1 overlay consisting of 0.6% agarose and 2X Eagle's Minimum Essential Medium without phenol red, supplemented with 10% fetal bovine serum (FBS), non-essential amino acids (Gibco, 11140-050), 1 mM sodium pyruvate, 2 mM L-glutamine, 1% P/S was added to each well. Plates were incubated at 37°C for 48 h. Cells were fixed with 10% formaldehyde for 1 h at room temperature. Formaldehyde was aspirated and the agarose overlay was removed. Cells were stained with crystal violet (1% CV w/v in a 20% ethanol solution). Viral titer of SARS-CoV-2 was determined by counting the number of plaques and represented as plaque-forming units (PFU) and as relative percentage of neutralization with respect to the negative control.

Statistical Analysis

The data were analyzed using GraphPad Prism v.8.4.3 software (GraphPad Software, Inc., La Jolla, CA, USA; www.graphpad.com; RRID:SCR_002798). A Kruskal-Wallis test with Dunn's posttest was performed to compare treatment groups for each vaccine combination. Virus neutralization data by groups was compared by unpaired t tests. Significant differences were determined at $p < 0.05$.

Declarations

CONTRIBUTION TO THE FIELD

This study contributes to the continuous search for effective ideas to find preventative approaches against pandemic viruses such as SARS-CoV-2. The SARS-CoV-2-specific IgY generation in yolk provides an inexpensive, reliable and scalable way of providing protection to billions of people at risk of SARS-CoV-2 infection.

Author Contributions

Conceptualization: M.W. and D.M-R. Study design: R.G. Execution of experiment and sample collection and processing: E.A., S.E. and R.G. Design and performance of virus neutralization assay: A.N. Data analysis: E.A. and R.G. Writing: E.A. All authors have read and agreed to the final version of the manuscript.

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Competing Interests

All authors declare no competing interests.

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Figures

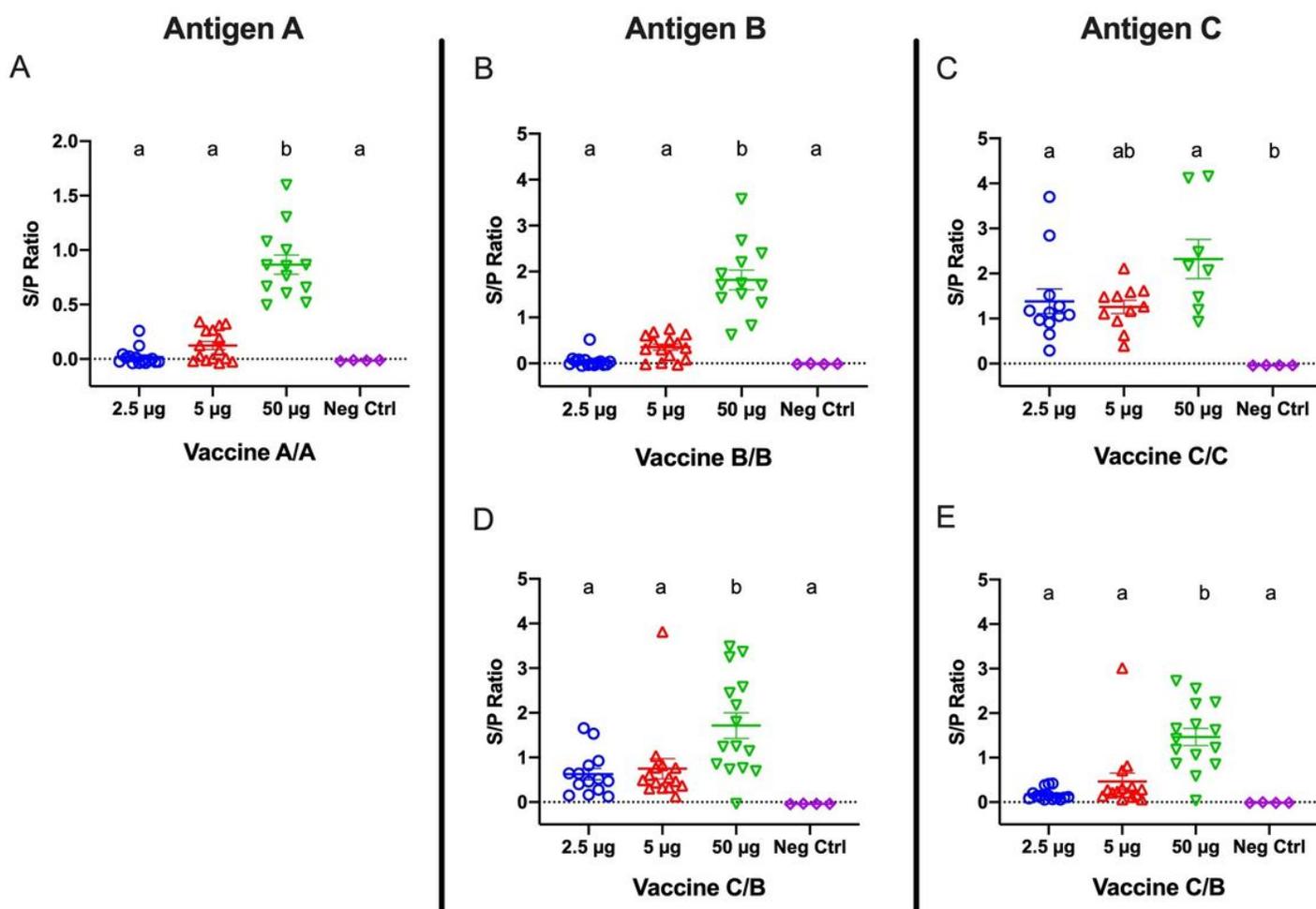


Figure 1

IgY titers in serum collected 21 days following the second vaccination for groups receiving (A) vaccine A/A, (B) vaccine B/B, (C) vaccine C/C, and (D, E) vaccine C/B combinations. Hens were inoculated twelve days apart. Antigen A-, B-, and C-coated plates were used for panels (A), (B, D) and (C, E), respectively.

Error bars indicate the mean and standard error. Significant differences are indicated by letters (a, b) above the data points ($p < 0.05$).

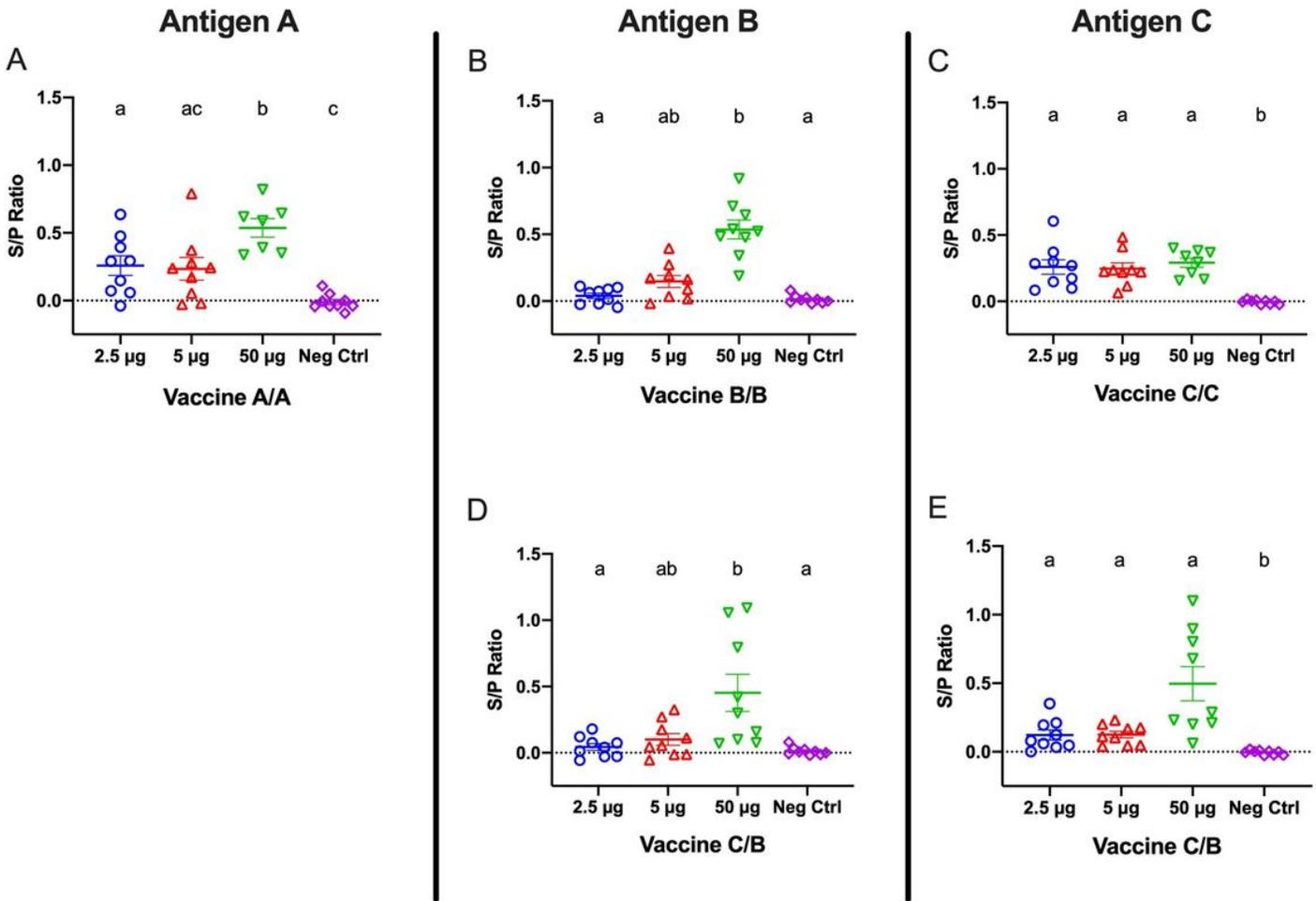


Figure 2

IgY titers from egg yolk collected six weeks following the second vaccination for groups receiving (A) vaccine A/A, (B) vaccine B/B, (C) vaccine C/C, and (D, E) vaccine C/B combinations. Hens were inoculated twelve days apart. Antigen A-, B-, and C-coated plates were used for panels (A), (B, D) and (C, E), respectively. Error bars indicate the mean and standard error. Significant differences are indicated by letters (a-c) above the data points ($p < 0.05$).

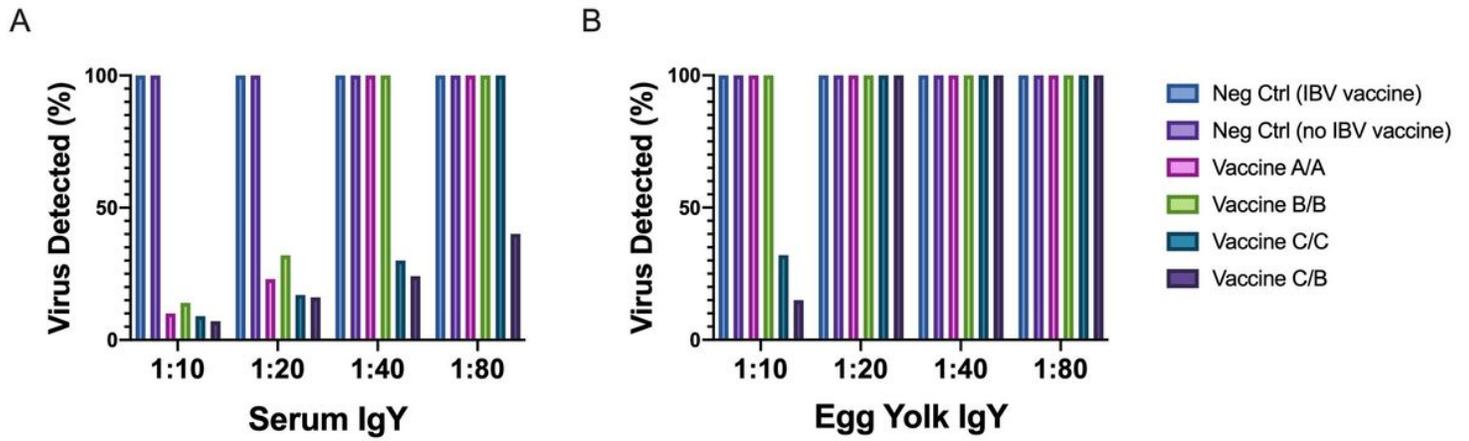


Figure 3

A plaque reduction neutralization assay (PRNA) was performed to detect SARS-CoV-2-specific neutralizing antibodies in (A) sera and (B) egg yolk IgY extractions from hens receiving 50 µg of vaccine A/A, B/B, C/C, and C/B. Controls included negative control hens that were either vaccinated or not vaccinated for IBV. Sera was collected at three weeks after the second vaccination, and egg yolk was harvested beginning at six weeks after the second vaccination. Viral titer of SARS-CoV-2 was determined by counting the number of plaques and represented as relative percentage of neutralization with respect to the negative control.