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Experimental Study on the Effect of Different Routes of Administration on the Immunogenicity of Live Infectious Bursal Disease Vaccine

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Abstract

Background: Infectious bursal disease is a contagious viral infection that primarily affects young chickens and can cause significant morbidity and mortality. Vaccination with live attenuated infectious bursal disease viruses has been used to prevent the disease. The primary mechanism of the protective immune response in infectious bursal disease infection is humoral immunity. The route of live infectious bursal disease vaccine administration can affect its immunogenicity. This paper aims to determine the immunogenicity of a live infectious bursal disease vaccine administered *via* different routes of administration.

Methods: The complete random block design method was used to divide 60 mixed sexes, 7-day-old Bovan brown chickens breeds into six experimental groups, each with ten chickens: Five treatments and one control group. Each experimental group received two doses of the live Infectious bursal disease vaccine at 7 and 21 days old *via* drinking water, intra ocular, intranasal, subcutaneous routes and intramuscular. As a challenge control, the non-immunized control group was used. The indirect enzyme linked immunosorbent assay was used to test serum samples for antibody titers.

Results: There is no significant difference between the mean of the sample positivity ratio of group one through three at 95% confidence interval whereas the statistical result revealed that the presence of significant antibody titer difference was seen in comparison of control group with subcutaneous and intramuscular. The mean antibody SP ratio of group four and five were found to be superior to other groups.

Conclusion: This finding suggested that for better antibody response, the subcutaneous and intramuscular routes of administration are recommended whereas drinking water, intraocular and intranasal routes are not recommended. Further research is also required to better understand why one administration route induces more effective immunity than the others.

Keywords: Antibody; Chicken; IBD; Immunogenicity; Route of administration; Vaccine

Introduction

Infectious bursal disease, also known as gumboro disease, is an economically important acute, highly contagious viral infection of young chickens. Despite numerous advances in the management of this disease, losses in commercial and indigenous poultry continue [1]. The virus that causes infectious bursal disease is termed Infectious Bursal Disease Virus (IBDV) and it belongs to the avibirnavirus family. Infectious bursal disease infection activates all immune system branches in chickens. Infection with IBDV weakens both the humoral and cellular arms of the immune system. The virus can cause B cell lysis and alter antigen presenting cells because it prefers actively dividing B cells and mature B cells. Vaccination of chickens with high quality vaccines is the primary method of controlling IBD (gumboro disease). Live attenuated and inactivated vaccines are commonly used for IBD prevention. Since it provides the neutralizing Abs target the conformation dependent Viral Protein two (VP2) epitopes, humoral immunity is essential for protecting against IBDV infection [2].

Poultry vaccines including Infectious bursal disease can be given orally, subcutaneously, intramuscularly, *via* wing web, drinking water, eye drops or spray. The relative immunogenicity of vaccines delivered *via* these routes varies depending on the vaccine [3]. If a vaccine is not administered *via* the recommended immunization site or route, it may result in poor vaccine protection in poultry flocks. Drinking water vaccination is a common method of administering a live IBD vaccine. It can also be administered as eye drops, intra nasal sprays, and parenteral injections.

The primary method for assessing the immune response to vaccination is serological testing. Enzyme Linked Immuno Sorbent Assay (ELISA) is the most commonly used serological test for detecting IBDV antibodies. If the level of antibodies (or titer) in the serum is high and the variation in titer responses between individuals is low, vaccination is considered successful.

Despite the regular use of live attenuated IBD vaccines and different vaccination practices, IBD have remained as the most important infectious diseases threatening the village chicken and

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commercial poultry production in most parts of the world [4]. It has great economic importance because of the mortality and morbidity it causes. At present, the majority of vaccines used in Ethiopia are being produced by the National Veterinary Institute (NVI), Bishoftu, Ethiopia. Although the NVI recommended that the vaccine could be administered through the drinking water and ocular routes, some other manufacturers also recommend the intramuscular route. However, the effects of these routes of administration on the immunogenicity of the IBD vaccine were not well studied, and the effective route of administration is yet to be identified. As previously reported by Pozo, et al., following vaccination, the route of administration could have an impact on immune response profiles. As a result, the current study was designed to investigate the role of the live IBD vaccine administration route on chicken immunization using antibody titer (ELISA) and protection against IBDV (efficacy) as a tool. Therefore the objectives of this study are to determine the most effective route of administration of live infectious bursal disease vaccine and to compare the major route of administration recommended by different manufacturers including national veterinary institute of Ethiopia.

Materials and Methods

Experimental site

The experimental study was carried out at Ethiopia's National Veterinary Institute (NVI) in Bishoftu, Oromia regional state starting from November, 2021 to May, 2022 (Figure 1). The institute is located 45 km southeast of Addis Ababa, at 9ºN latitude and 40ºE longitude and 1900 meters above sea level. The annual rainfall average is 851 mm, with the long rainy season accounting for 84% of the total (June to September). With minimum and maximum temperatures of 8.9°C and 26.2°C, respectively, the average humidity level is 58.6 percent (NMSA, 2010) [5].

The national veterinary institute is a government organization that was established in 1964 under the ministry of agriculture to manufacture various veterinary vaccines. This company has received several international accreditation certificates for vaccine production and disease diagnosis. Among these certificates are the following: It has been certified with the International Standard Organization/ Quality Management System (ISO/QMS 9001: 2008) by an international accrediting company ISOQAR and its primary mandate is the development, manufacturing, sales and distribution of veterinary vaccines. Its research and development laboratory for serological tests has also been ISO/IEC 17025:2005 certified (NVI public communication office).

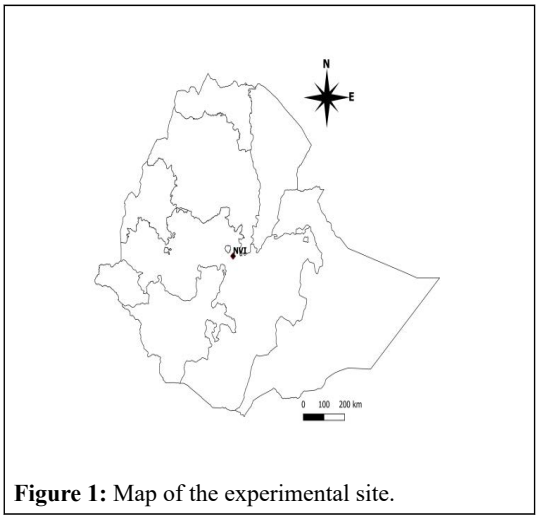

Figure 1: Map of the experimental site.

Experimental animals

Specific pathogen free eggs of a Bovan brown breed hen from the NVI's animal breeding and quarantine department were hatched in a hatchery incubator at the NVI's research and development directorate. A total of 60, healthy chicks of both sexes were chosen and reared in a pre-prepared room. These chicks were randomly and equally assigned to various experimental groups and were kept in separate isolation units for the duration of the experiments under suitable conditions (Figure 2).

Figure 2: Chicks reared in a prepared room after hatching before grouping.

Experimental animal husbandry and management

The ceiling, walls and floor of the experimental house were all disinfected with 1% formalin. Drinkers, feeders and buckets, among other things, have been cleaned and disinfected before being introduced into the homes. The house was kept closed before the chickens were introduced. All groups were housed in one experimental room until the experiment began and then they were randomly assigned to six groups coded as G1, G2, G3, G4, G5 and G6

each with ten birds. According to the schedule below, each group was inoculated with live IBD vaccines *via* a different route. The chickens were fed a commercial starter ration that was purchased throughout the study. Grower rations and water were provided on an as needed basis. Antibiotics (oxytetracycline), minerals and vitamins were combined in a sachet (Vytlet) and administered seven days after each episode of bleeding. Chickens were kept in a separate sterile room under strict hygienic and standard management conditions until the end of the post-challenge experiment. Throughout the experiment, the birds were monitored twice a day for any clinical signs. Every day, the number of chickens that died was recorded [6].

Experimental design and methodology

The experimental study design was used to investigate the effects of five different routes of administration on the immunogenicity of the IBD vaccine. Live attenuated intermediate standard strain IBDV vaccine was prepared by following the NVI, standard operating procedure of IBD vaccine production. Specific pathogen free eggs originated from NVI were used for dual purpose one part was for CEF preparation after 11 day incubation and the other part was hatched using hatchery incubator. Antibody titers against IBDV were determined in serum samples collected at 5, 21, 28, 35 and 42 days age of chicken. Depending on the date of vaccination, each blood collection day was labeled as day zero, day 14, day 21, day 28 and day 35, respectively. The hatched chicks were tested for the presence of maternal antibodies using an AC-ELISA (ID-Vet product) before the experiment began. In the experiment, all chicks with low maternal antibody (sample positivity ratio <0.3) were used. Using the Complete Random block Design (CRD) method, 67 day old mixed sex (Bovine brown breed) chickens were divided into 6 experimental groups of 10 chicks each.

Source of working seed of IBDV vaccine and challenge virus: Attenuated working seed of IBDV vaccine intermediate standard strain, lot number 01/22 with a titer of log 106.4 titer/ml, was obtained from NVI quality assurance department, vaccine seed preparation section and used for vaccine production (Figure 3).

The NVI research and development department's virology laboratory provided a highly virulent IBD virus strain with a known identity that had previously been stored for experimental purposes. Prior to use, both the challenge virus and the vaccine were tested for its titer. They were stored at -20°C under strict conditions after their titers were determined.

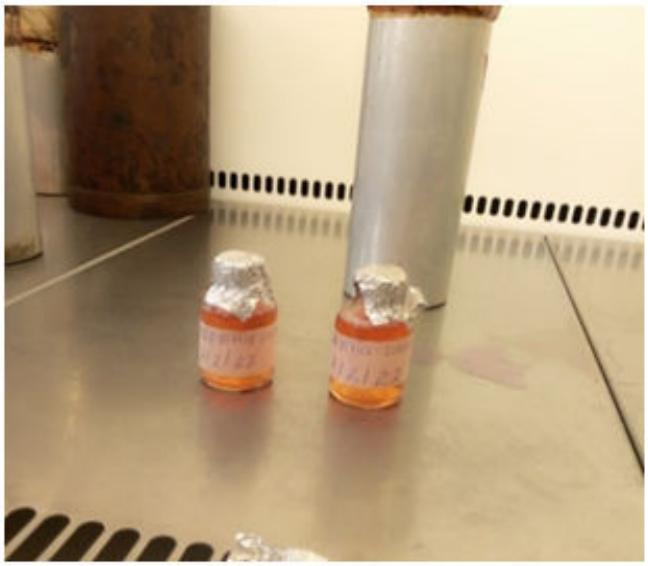

Figure 3: Working seed of IBDV vaccine, intermediate standard strain (LC-75) and challenge virus (VVIBDV).

Titration of IBD vaccines and challenge virus: The OIE method was used to titre IBDV experimental vaccines, IBD challenge viruses and Reed and Muench method was used to calculate Tissue Culture Infective Dose (TCID50). Both IBDV vaccine strains and field isolate challenge viruses were titrated tenfold, beginning with 10^{-1} by mixing 1 ml of virus in 9 ml of diluents (GMEM base medium) and then transferring 1 ml of the previous virus dilution to the next using a sterile pipette. Fifty microliters (100 µl/well) of each virus dilution (10^{-1} to 10^{-10}) were distributed into the wells of their respective rows of flat-bottom microtiter plates containing established Chicken Fibroblast Cell lines (CFC).

Then all the wells were covered over flown by dispensing hundred µl/well GMEM medium with 2% calf serum and incubate at 37°C in a CO_2 incubator for seven days. The titer for each vaccine and challenge virus was determined by using Reed and Muench formula.

$$\log 10 = X_0 - \left(\frac{d}{2}\right) + \frac{d(\varepsilon ri)}{ni}$$

Where;

x_o: Log 10 of reciprocal of the lowest dilution at which all set monolayer's are positive.

d: Log 10 of the dilution factor that is the difference between the log dilution intervals.

ni: Number of positive test monolayer's out of ni.

Ɛ (ri/ni): Ɛ(P) sum proportion of the tests beginning at the lowest dilution showing 100% positive result.

The summation is started at dilution X_o.

Route of vaccine administration and grouping of birds: At seven days, chickens were randomly assigned to six experimental groups, five treatment groups and one control group based on the route of

vaccine administration. Each group was labeled G1, G2, G3, G4, G5 and G6 sequentially. The first five groups in the treatment group were immunized through drinking water, intra ocular, intranasal, subcutaneous and intramuscular respectively. The sixth group (G6) was not vaccinated and serves as a challenge control.

Reconstitution of the vaccines and vaccinations of each group: Four vials containing 100 doses of IBD vaccine were used for each group. Group one (vaccination *via* drinking water) birds were suspended from water for one hour in the morning to ensure that all birds were exposed to water containing an IBD vaccine. A freeze dried vial of 100 doses was reconstituted in a 6000 ml of chlorine free water and 300 ml was given to a group of ten chicks assigned to group one. To keep the temperature stable, an ice pack was used. Vaccines for group 2 birds (vaccinated intraocularly) were dissolved in 10 mL of saline water. Each bird was restrained with its head to one side and a 100 microliter drop of the vaccine was placed in the uppermost eye and held in place for a few seconds after administration until the bird blinks to ensure the full dosage remained in the eye. Group three (intranasal vaccination) bird vaccines were reconstituted in the same manner as group two and 100 microliters of the vaccine solution were placed in the uppermost nasal opening [7].

A 100 dose vaccine vial was dissolved in 10 ml of saline water and 0.1 ml of the vaccine solution was injected into the loose skin at the back of the neck using a 1 ml syringe in group four (vaccinated subcutaneously) birds. The same solution and volume of vaccine used in group four were injected into the thigh muscle using a 1 ml syringe for group five (intramuscular vaccination). 14 days after primary vaccination (at 21 days) by following the same procedure, secondary vaccination was delivered to each group (Table 1).

Classification	Route of administration	No. chicks	Age of Vaccination	Blood collection days (pi)	Challenge days (pi)
Group 1	D/W	10	7 and 21	0,14,21,28,35	35
Group 2	I/O	10	7 and 21	0,14,21,28,35	35
Group 3	I/N	10	7 and 21	0,14,21,28,35	35
Group 4	S/C	10	7 and 21	0,14,21,28,35	35
Group 5	I/M	10	7 and 21	0,14,21,28,35	35
Group 6	Control group	10	Unvaccinated	0,14,21,28,35	35

Table 1: Experimental design summary.

Collection of blood

After disinfecting with 70% alcohol, two milliliter blood samples were collected from each bird's wing vein on different days using a 23 gauges and 3 ml sterile disposable plastic syringe. The blood samples were left at room temperature in a slant position for 10 hours, and sera samples were harvested into cryo-vials and labeled. The harvested serum samples were kept at -20°C until the laboratory analysis.

Antibody detection

Indirect ELISA: The indirect ELISA test was carried out in a 96 well microplate coated with purified IBDV antigen, a positive control, a negative control, concentrated conjugate (10x), dilution buffer 3 and dilution buffer 14, wash concentrate (20x), substrate solution, and stop solution (0.5 M). The entire ELISA test was carried out at the NVI research and development directorate.

Before being dispensed into the appropriate number of wells, the test sera were diluted according to the established protocol or kit instructions. After incubation under the appropriate conditions, the sera are removed from the plates and the wells are thoroughly washed. Anti-chicken immunoglobulin conjugated to enzyme was dispensed into the wells, and the plates were re-incubated as needed. Before adding a substrate containing a chromogen that causes a color change in the presence of the enzyme used, the plates are emptied and rewashed [8]. After a final incubation step, the substrate/chromogen reaction was stopped, and the color reactions were quantified by measuring the optical density of each well. The Sample to Positive (S/P) ratio was calculated for each test sample as follows.

$$\text{SP Value} = \frac{\text{OD Sample} - \text{OD NC}}{\text{OD PC} - \text{OD NC}}$$

Where;

SP: Samples to positive ratio.

OD sample: Optical Density of the test sera.

ODNC: Optical Density of Negative Control.

ODPC: Optical Density of Positive Control.

ELISA validity test

SP value >0.3 and OD of negative control <0.25 is considered as valid. But, if S/P value is less than 0.3 and the mean OD values of the positive control is <0.250 the entire result of the kit is considered as invalid according to manufacturer instruction.

ELISA test result interpretation

- S/P ratio <0.3 or antibody titer <875 is negative.
- S/P ratio >0.3 or antibody titer >875 is positive.

Data management and statistical analysis

All data was collected and entered into the Microsoft (MS) excel spreadsheet programme to create a database, which was then analyzed using graph pad prism version 5 (GraphPad software, Inc., San Diego, CA) and subjected to a one-way Analysis of Variance (ANOVA). Each

treatment group was compared to the control group using the t test. Differences between groups were considered significant at $p<0.05$.

Results

Immunogenicity test results

The results of ELISA tests for MDA performed on serum collected from day 5 old chicks prior to vaccination (day zero) revealed that the antibody SP ratio was on average 0.026378, indicating that there was no maternal antibody at all because the SP ratio positivity cut off point is far from the above number, which is SP>0.3. Throughout the study period, the mean SP ratio for the control group was 0.037018, confirming that it is a true negative control in this experiment. On each day of the antibody test, no significant change in the SP ratio was observed in the control group.

There is no significant change in antibody titer 14 days after primary vaccination in groups one through three, *i.e.* drinking water, intraocular, and intranasal. A significant change in antibody titer is observed in a vaccine administered *via* both parental routes. The chicken in group four (subcutaneous) has the greatest change in antibody titer, which is 0.508, compared to 0.019 before vaccination on day zero. Fourteen days after primary vaccination, group five (intramuscular) also shows a change in antibody titer. It means SP ratio increased from 0.04 before vaccination to 0.363 after vaccination, a significant number.

The chickens were boosted on day 14 post inoculation and 7 days later, the S/P ratio was significantly increased in groups four and five, while a minor change was observed in the other group, though not statistically significant. The S/P ratio or antibody titer begins to decline 28 days after primary immunization (14 days after boosting). On day 35, all groups' show a decrease in antibody titer except intranasal, which shows a slight increase (Figure 4).

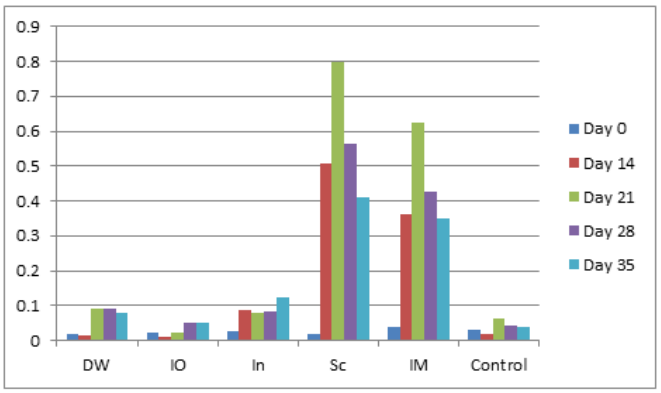

Figure 4: Mean SP ratio of each group at different time by chart.

Note: DW: Drinking Water; IO: Intra Ocular, IN; Intranasal, SC: Subcutaneous, IM: Intramuscular.

Comparison of immunogenicity test result: As shown on below tables, when comparing each treatment group to the control group on day seven after vaccination, only groups four (subcutaneous) and four (intramuscular) react to the vaccination. As it's seen on the below ANOVA table drinking water vaccination, intra ocular route and intranasal route were not significantly different from non-vaccinated group throughout the study period at 95% confidence interval. Even though there is some positive sample in group one yet statistically it's not significant as indicated in Table 2. It's expected to be by chance. In intra nasal also there is positive sample at day 21 after vaccination but statistically insignificant as shown below. In subcutaneous and intramuscular group after vaccination starting from day 14 significant difference in SP ratio was seen in compared with control group (Figure 5).

Days (PI)	Mean SP ratio of control group versus treatment group				
	Drinking water	Intra ocular	Intra nasal	Subcutaneous	Intramuscular
	T test	T test	T test	T test	T test
Day 0	0.1388	0.06489	0.01562	0.1265	0.1322
Day 14	0.03491	0.1211	0.852	6.026	4.24
Day 21	0.3333	0.492	0.1732	9.055	6.911
Day 28	0.6028	0.08541	0.5042	6.38	4.729
Day 35	0.5174	0.1232	1.035	4.582	3.831
P value	P>0.05 at all	P>0.05 at all	P>0.05 at all	P<0.001 except at day 0	P<0.001 except at day 0
Summary	Non-significant	Non-significant	Non-significant	Significant	Significant
Note: The mean difference is significant at the (p<0.05). Pi-Post inoculation.					

Table 2: Mean SP ratio of control group versus treatment group.

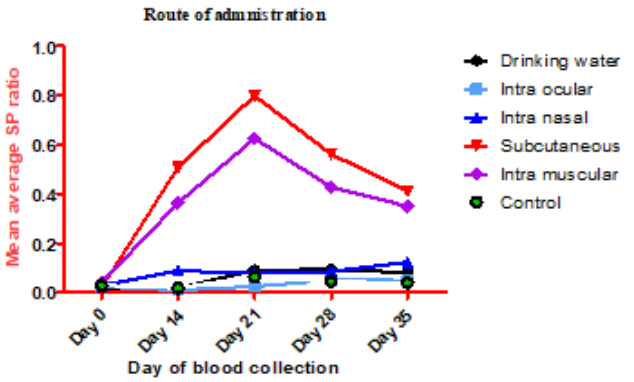

Figure 5: Graph showing route of administration and antibody titer (Sp ratio) at different day after vaccination.

Discussion

One of the key questions addressed in this study was the role of the route of administration of the live IBD vaccine in immunizing chickens against infectious bursal disease. It was investigated using antibody titer (ELISA) and protection against IBDV (efficacy) as parameters. A serology test using indirect ELISA was performed 5 days before inoculation to check for maternal antibodies that could jeopardize the experiment. Because they were derived from pathogen free eggs, all groups of chickens had antibody titers that were lower than the positive range, as expected.

According to this finding, drinking water, intra nasal and eye drops, which are commonly used, were not the best options because the number of birds that seroconverted when they were susceptible to infection was low when compared to the other routes. On day 14 post-inoculation, all groups of chickens were boosted, which increased antibody production as well as the mean S/P ratio or antibody titer. This observation is consistent with Bitew, et al., previous reports, which demonstrates the importance of the booster dose. The intermediate plus vaccine induced antibody levels at 14 days after vaccination, according to Rautenschlein, et al.

Following the primary vaccination, 14 days later their mean SP ratios were 0.016, 0.09 and 0.080 for drinking water, intraocular and intranasal, respectively, which is less than the sample positivity cut off point? Throughout the study, they exhibit a low antibody response. These three routes do not have a clear advantage over one another. Although the national veterinary institute of Ethiopia (the largest veterinary vaccine manufacturing company) recommends drinking water, this route results in a very low immune response in this experiment. Oyekunle reported similar findings that drinking water is not suitable for IBD vaccination. Although it is suitable for mass vaccination, relying on vaccine transmission from bird to bird is risky, as it can result in excessive rolling type reactions of prolonged duration and delayed immunity in the flock. On the other hand chick's vaccinated intra ocularly and intranasally, had low immune responses and protection rates. This result was contrasting with report by Abdel-Alim and Kawkab who found that live intermediate IBDV vaccines were immunogenic with better immune response in eye drop vaccinated groups.

Intramuscular and subcutaneous routes could be beneficial. According to Wang, et al., they showed very similar responses in clinical studies. This argument is consistent with a notion of IM vaccination, results in significant recruitment of immune cells, resulting in local inflammation. The high level of protection provided by subcutaneous vaccination may be due to significant differences in the cellular composition of muscle dermal tissues, which may affect these vaccination outcomes. As previous studies by Wiendl, et al., in which it was discovered that the DC populations in lymph nodes draining the intramuscular and subcutaneous injection sites were different, this could lead to different antigenic specific immune responses.

Conclusion

This finding suggested that vaccination *via* subcutaneous route is superior and preferable to other vaccine administration routes, with intramuscular coming in second. When chicks were vaccinated subcutaneously, they produced at higher levels of antibodies and protection. The antibody response to drinking water, intra ocular and intra nasal injections is low. An increase in antibody titer following a booster dose demonstrates the importance of the booster dose. It suggested that during this time period, chicks could be susceptible to field virus. Contrary to many manufacturers' recommendations, it was discovered that drinking water and intraocular routes is not protective. Although the drinking water method is simple to use for mass vaccination, it is not the preferred route for protective antibody response. Individual vaccinations such as subcutaneous and intramuscular are laborious and require skilled personnel to perform, but they are effective in terms of antibody production.

Ethics Approval and Consent to Participate

All animal experiments, including the collection of chicken sera were carried out in accordance with protocols approved by the institutional review board of the national veterinary institute's Animal Research Ethics and Review Committee (ARERC) and in accordance with approved animal care guidelines and protocols. Throughout the experiment, animals were handled in accordance with the ARERC's standard guidelines, with adequate feed, water and health care. Animal ethical clearance certificate was issued after the implementation of the guideline subsequently to end of the experiment.

Consent for Publication

Not applicable.

Availability of Data and Materials

All data and materials are within this published paper.

Competing Interests

The authors declare that there is no competing interest.

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