Development of IGY-based immunoprophylaxis in poultry: a novel approach for epidemic prevention

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Abstract

The poultry industry is increasingly challenged by infectious diseases caused by pathogens such as Escherichia coli, Salmonella spp., Enterococcus spp. and Clostridium perfringens. The use of antibiotics to control these infections has led to the emergence of antibiotic-resistant strains, necessitating alternative approaches. This study aimed to develop an efficient method for producing high-quality IgY antibodies from egg yolks of hens immunized with a bacterial consortium of these pathogens. Two antigen concentrations, 1:1:1:1 and 3:3:1:1, were used to immunize laying hens and eggs were collected post-25 days for IgY extraction and purification.

Bacterial antigens were prepared and characterized before immunization. Egg yolks were isolated and IgY antibodies were extracted using a series of steps involving polyethylene glycol (PEG) precipitation and dialysis. The protein concentration of the purified IgY was measured photometrically at 280 nm, yielding a consistent concentration of 9.3268 mg/ml for both antigen concentrations. SDS-PAGE analysis confirmed the presence of IgY with the expected molecular weight. The results demonstrate that IgY antibodies can be efficiently produced against a consortium of common poultry pathogens using the outlined methodology. The consistent protein concentration and successful characterization by SDS-PAGE highlight the reproducibility and reliability of the extraction process.

Keywords: *Escherichia coli, Salmonella spp., Enterococcus spp., Clostridium perfringens,* SDS-PAGE, IgY antibodies.

Introduction

The global poultry industry faces numerous challenges, primarily associated with the prevalence of infectious diseases, which can cause significant economic losses and impact food security. Traditionally, antibiotics have been used extensively to prevent and treat bacterial infections in poultry. However, the overuse of antibiotics has led to the emergence of antibiotic-resistant bacteria, posing a serious threat to both animal and human health. This growing concern has necessitated the search for alternative methods to control infections in poultry and one promising solution is the use of immunoglobulin Y (IgY) antibodies derived from egg yolks.[7] IgY antibodies are the functional equivalent of mammalian IgG antibodies but are naturally produced by birds, reptiles and amphibians. They are transferred from the hen to the offspring via the egg yolk, providing passive immunity to the chick. The use of IgY antibodies in immunoprophylaxis has several advantages over conventional antibiotics. First, IgY does not induce resistance in bacteria, making it a safer alternative for long-term use. Second, IgY antibodies are specific to their target pathogens, reducing the risk of disrupting the natural gut flora. Third, the production of IgY is non-invasive and can be scaled up relatively easily by collecting eggs from immunized hens.[8]

Bacterial pathogens in poultry

Escherichia coli: Avian pathogenic *E.coli* (APEC) is a significant cause of colibacillosis in poultry, leading to respiratory infections, septicemia and mortality. APEC infections can result in substantial economic losses due to decreased growth rates, increased mortality and the cost of treatments.[1,2,6]

Salmonella spp.: *Salmonella enteritidis* and *Salmonella typhimurium* are among the most common serovars causing salmonellosis in poultry. These infections can be asymptomatic or cause gastrointestinal diseases in birds. Importantly, *Salmonella* can be transmitted to humans through contaminated poultry products, posing a public health risk.[9,12]

Enterococcus spp.: *Enterococcus faecalis* and *Enterococcus faecium* are part of the normal gut flora but can become opportunistic pathogens, leading to infections such as septicemia and endocarditis in poultry. Enterococci are known for their ability to acquire antibiotic resistance, complicating treatment options.[10,13]

Clostridium perfringens: This bacterium is the causative agent of necrotic enteritis in poultry, a disease characterized by severe intestinal damage, leading to high morbidity and mortality rates. Clostridium perfringens infections are often associated with changes in diet or other stress factors in poultry production.[4]

Potential benefits of IgY Technology: IgY antibodies have been recognized for their potential in passive immunization strategies against various pathogens. Unlike mammalian IgG, IgY does not bind to Fc receptors or activate the complement system in mammals, reducing the risk of inflammation and other side effects. The production of IgY involves immunizing hens with the target antigen, leading to the incorporation of IgY antibodies into the egg yolk. These

antibodies can then be harvested non-invasively from the eggs.[3,11]

The use of IgY in poultry has been explored for several applications, including the prevention and treatment of infectious diseases, enhancing gut health and improving overall performance. Studies have demonstrated the efficacy of IgY against a range of pathogens including bacteria, viruses and parasites. IgY antibodies have also been used to neutralize toxins and promote gut health by modulating the gut microbiota.[3,11] This study addresses the critical need for alternative strategies to control bacterial infections in poultry. By developing a reliable method for producing high-quality IgY antibodies, this research contributes to the broader goal of reducing antibiotic use in poultry farming. The findings from this study have the potential to enhance poultry health and productivity, reduce the risk of antibiotic resistance and improve food safety for consumers. [3,11]

This study represents a significant step forward in the application of IgY technology for poultry health management. The development of IgY-based immunoprophylaxis offers a promising alternative to traditional antibiotics, aligning with global efforts to promote sustainable and responsible use of antimicrobials in animal agriculture. The primary aim of this study is to develop a reliable and efficient method for producing high-quality IgY antibodies against a consortium of common bacterial pathogens in poultry: *Escherichia coli, Salmonella spp.*, *Enterococcus spp.* and *Clostridium perfringens*. These pathogens were selected due to their significant impact on poultry health and productivity.[5]

Material and Methods

Antigen preparation: For the preparation of antigens, we obtained bacterial cultures from the American Type Culture Collection (ATCC). The details of the bacterial strains used are summarised in the table 1. Mini cultures were grown from these bacterial strains to prepare the antigens. Fig. 1 depicts the steps involved in the preparation of bacterial cultures and the resulting antigen samples.

Culturing bacteria

1. **Mini Cultures:**
   - The bacterial strains were reconstituted from ATCC-provided frozen cultures and inoculated into respective growth media.
   - Each bacterial species was cultured separately under optimal growth conditions (e.g. temperature, oxygen levels) to ensure robust growth.
   - Cultures were incubated until they reached the mid-logarithmic phase as indicated by OD600 measurements.

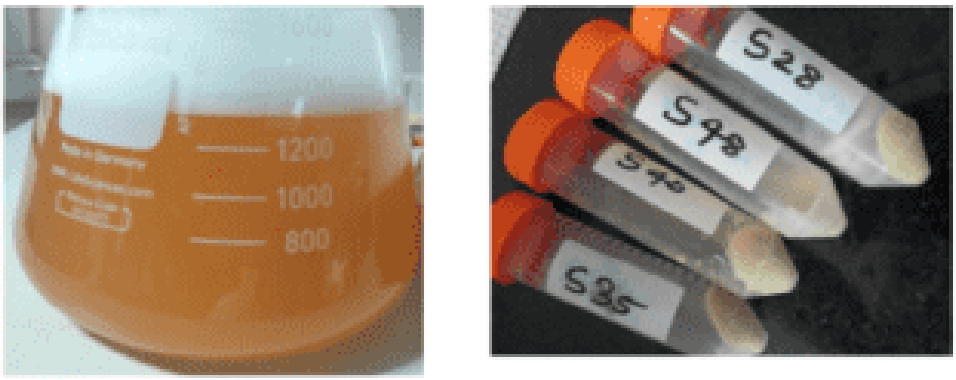

Figure 1: Preparation of Crude Soluble Extract as Antigen (CSEA)

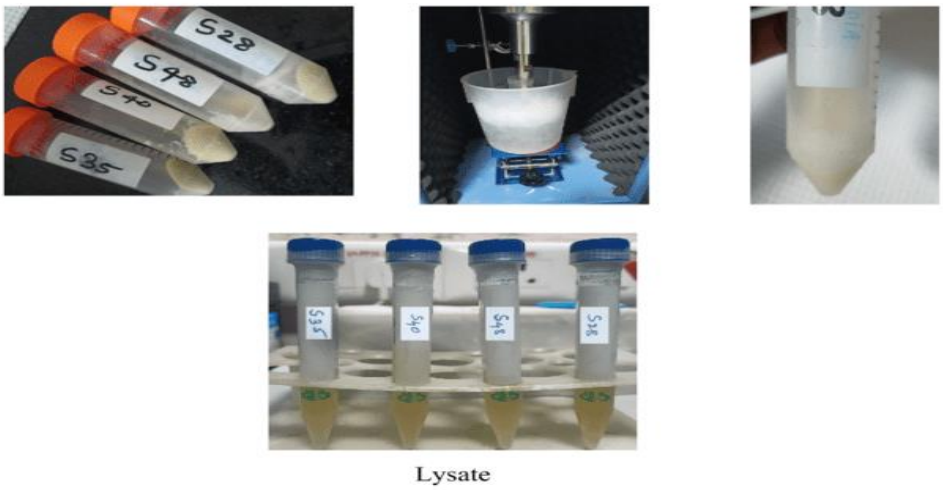

Figure 2: Mini culture and antigen preparation

Table 1
Bacteria used for antigen preparation and its respective catalogue numbers from ATCC

S.N.	Bacteria used as antigen	Mostly found Bacterial Sps. in poultry	ATCC Catalogue Number
1.	*Escherichia coli*	Avian pathogenic *E.coli*	25922
2.	*Salmonella species*	*Salmonella Enteritidis*	35664
3.	*Clostridium perfringens*	Type A *clostridium*	13124
4.	*Enterobacteria*	ESBL-producing Enterobacter (*Enterobacter cloacae*)	13047

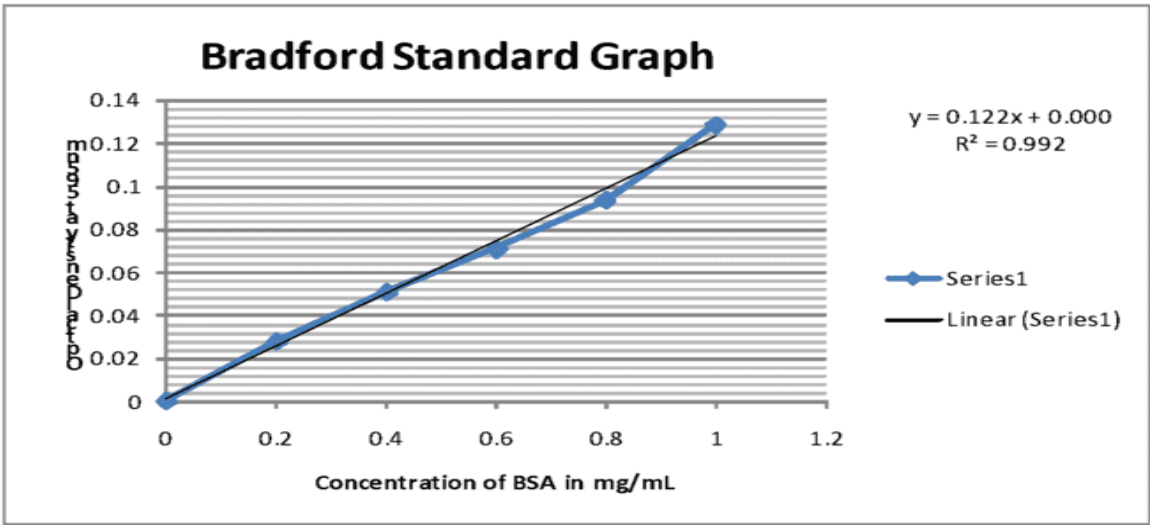

Figure 3: Bradford Standard Graph

2. **Harvesting Cells:**
   - Once optimal growth was achieved, the cultures were harvested by centrifugation at 5,000 x g for 10 minutes to pellet the bacterial cells.
   - Supernatants were discarded and pellets were washed twice with sterile phosphate-buffered saline (PBS) to remove residual media components.

Preparation of crude soluble extract as antigen (CSEA)

1. **Cell Lysis:**
   - The bacterial pellets were re-suspended in a minimal volume of PBS and subjected to mechanical disruption using a sonicator. The sonication process involved applying pulses of ultrasonic energy to break open the bacterial cells and release intracellular contents.
   - The sonicated samples were kept on ice to prevent overheating and protein denaturation during the process.

2. **Lysate Processing:**
   - The sonicated bacterial suspensions were centrifuged at 10,000 x g for 20 minutes to separate cell debris from the soluble proteins.
   - The supernatants, containing the soluble protein fractions, were carefully collected and stored at -20°C until further use.

3. **Protein Quantification:**
   - The protein concentration of each antigen preparation was determined using the Bradford protein assay. This colorimetric assay involves the binding of Coomassie brilliant blue dye to proteins, resulting in a measurable color change.
   - A standard curve was generated using known concentrations of bovine serum albumin (BSA) and the protein concentrations of the bacterial lysates were calculated based on the absorbance readings at 595 nm.

The protein concentrations of the prepared antigens were essential to ensure consistent and effective immunization doses for the poultry hens. The cultures were then concentrated to prepare the antigens which involved the creation of two different antigen concentrations: 1:1:1:1 and 3:3:1:1 ratios of *E. coli*, *Salmonella*, *Enterococcus* and *Clostridium perfringens* respectively. The prepared antigens were then used for the immunization of poultry hens.

Immunization of poultry hens: Two groups of hens were immunized with the prepared antigen concentrations. Group A was injected with the 1:1:1:1 antigen concentration and group B was injected with the 3:3:1:1 concentration. The immunizations were administered via intramuscular injection. Following the immunization, eggs were collected from these hens 25 days post-injection.

Egg Yolk Preparation: The eggshells were carefully cracked and the yolks were transferred to a yolk spoon to remove as much egg white as possible. The yolks were then rolled on filter paper to remove remaining egg white and the yolk membrane was pierced with a lancet. The yolk content was poured into a 50 ml tube and the volume was recorded.

Initial Extraction: Twice the yolk volume of PBS was added to the yolk. Then, 3.5% PEG 6000 of the total volume was added and the mixture was vortexed and rolled on a mixer for 10 minutes. This step separated the suspension into two phases: a phase with yolk solids and fatty substances and a watery phase containing IgY and other proteins. The tubes were centrifuged at 4°C for 20 minutes at 10,000 rpm. The supernatant was filtered and transferred to a new 50 ml tube.

Secondary Extraction: 8.5% PEG 6000 (w/v) was added to the supernatant, vortexed and mixed on a roller for 10 minutes. After centrifugation at 4°C for 20 minutes at 10,000 rpm, the supernatant was discarded. The pellet was dissolved in PBS to a final volume of 10 ml, mixed with 12% PEG 6000 (w/v) and 0.02% sodium azide and rolled for 10 minutes. After a final centrifugation, the pellet was dissolved in 800 µl PBS.

Dialysis: The solution was transferred to a dialysis bag and dialyzed overnight in 0.1% saline, then in PBS for three hours the next morning. After dialysis, using 14 kda bag, sample was transferred to centricon of 100 kda to concentrate protein and final sample was stored at -20°C for further use.

Protein content measurement: The protein content of the IgY preparations was measured using a spectrophotometer at 280 nm. The IgY samples were diluted 1:50 with PBS and the protein concentration was calculated using the Lambert-Beer law with an extinction coefficient of 1.33 for IgY.

SDS-PAGE Analysis: The quality and purity of the isolated IgY antibodies were assessed using SDS-PAGE. Samples from both antigen concentrations (1:1:1:1 and 3:3:1:1) were analyzed to determine the presence of IgY.

Table 2
Prepared antigens and its respective concentration known from Bradford

S.N.	Antigen	Concentration (mg/ml)
1.	Ag1(*E.Coli*)	0.670
2.	Ag2 (*Salmonella*)	0.384
3.	Ag3 (*Enterobacter*)	0.321
4.	Ag4 (*Clostridium perfringens*)	0.047

Figure 4: Immunization and Egg collection process

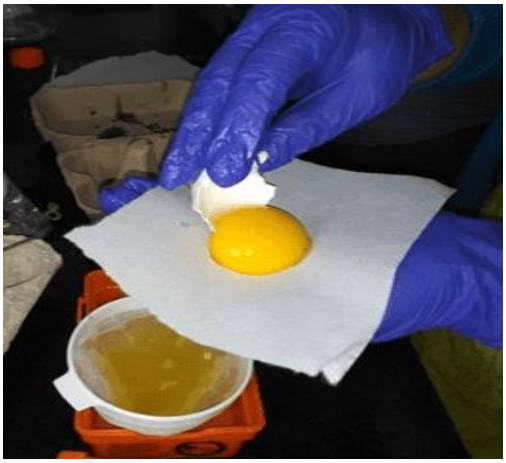

Figure 5: Egg yolk extraction

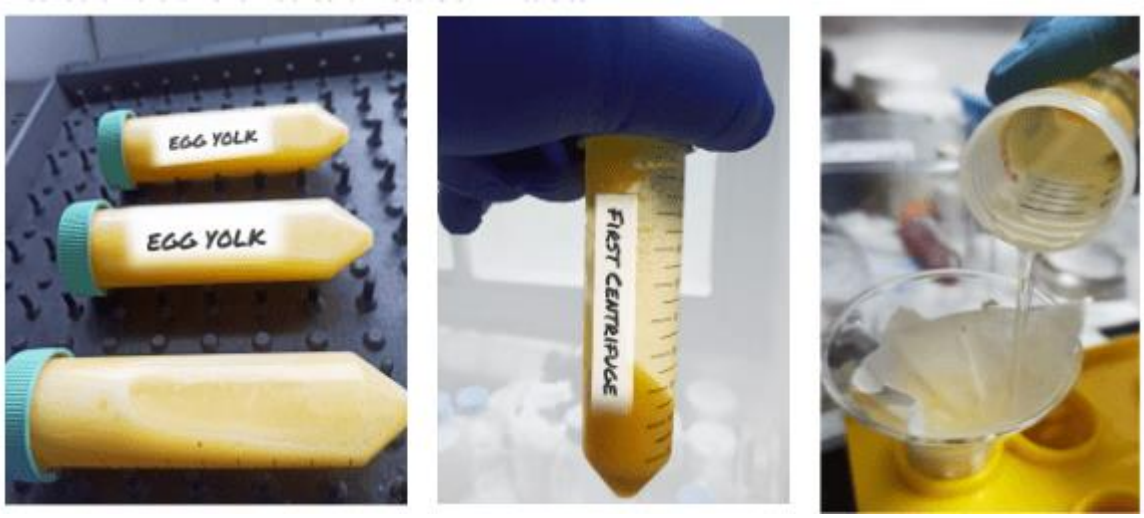

Figure 6: Egg yolk centrifugation and supernatant collection

Figure 7: Dialysis

Results

The immunization of poultry hens with the bacterial consortium led to the successful production of IgY antibodies, which were subsequently isolated from the egg yolks. The isolation and purification process yielded high-quality IgY, as confirmed by SDS-PAGE and protein concentration measurements.

Protein concentration: The protein concentration of the IgY preparations from both antigen concentration groups (1:1:1:1 and 3:3:1:1) was measured using a spectrophotometer at 280 nm. The IgY samples were diluted 1:50 with PBS and the protein concentration was calculated using the Lambert-Beer law with an extinction coefficient of 1.33 for IgY. Surprisingly, the results indicated that both groups had the same protein concentration:

UV-VIS Spectrophotometry: The UV-Vis spectrophotometry graph for the 1:50 diluted IgY sample is shown. The graph displays the absorbance spectrum of the IgY antibodies across a wavelength range of 200-400 nm. The graph shows a peak absorbance at around 280 nm, which is characteristic of presence of protein concentration. The absorbance at this wavelength was used to determine the protein concentration using the spectrophotometer. The presence of distinct peaks indicates that the IgY antibodies were successfully isolated and purified from the egg yolk, with minimal contamination from other proteins or substances. The clear peak at 280 nm further confirms the purity and concentration of the IgY samples.

SDS-PAGE Analysis: The SDS-PAGE analysis showed distinct bands corresponding to the heavy and light chains of IgY, confirming the successful isolation and purification of the antibodies. The molecular weights of the IgY heavy and light chains matched the expected values, indicating the purity of the samples. The SDS-PAGE analysis demonstrated that both antigen concentrations produced IgY antibodies with similar molecular weights. The intensity of the bands was comparable between the two groups, indicating similar yields of IgY despite the different antigen concentrations used for immunization.

Discussion

The results of this study demonstrate that immunization with a bacterial consortium can effectively induce the production

of IgY antibodies in poultry. Interestingly, both antigen concentration groups (1:1:1:1 and 3:3:1:1) yielded the same IgY protein concentration of 9.3268 mg/mL. This finding suggests that a higher concentration of antigens does not necessarily correlate with an increased yield of IgY in the egg yolk. The isolation and purification protocol used in this study proved to be effective in obtaining high-quality IgY antibodies. The use of PEG for precipitation, followed by dialysis, successfully removed unwanted proteins and lipids, resulting in a pure IgY preparation. The purity of the IgY was confirmed by SDS-PAGE, which showed distinct bands corresponding to the heavy and light chains of IgY with no significant contamination. The comparable IgY yields from both antigen concentration groups could imply that the hens reached a saturation point in their IgY production capability.

Table 3
IgY Protein Concentration in Poultry Samples

S.N.	Sample	Protein Concentration (mg/ml)
1.	Blank	0.0000
2.	1:1:1:1 Concentration	9.3268
3.	3:3:1:1 Concentration	9.3268

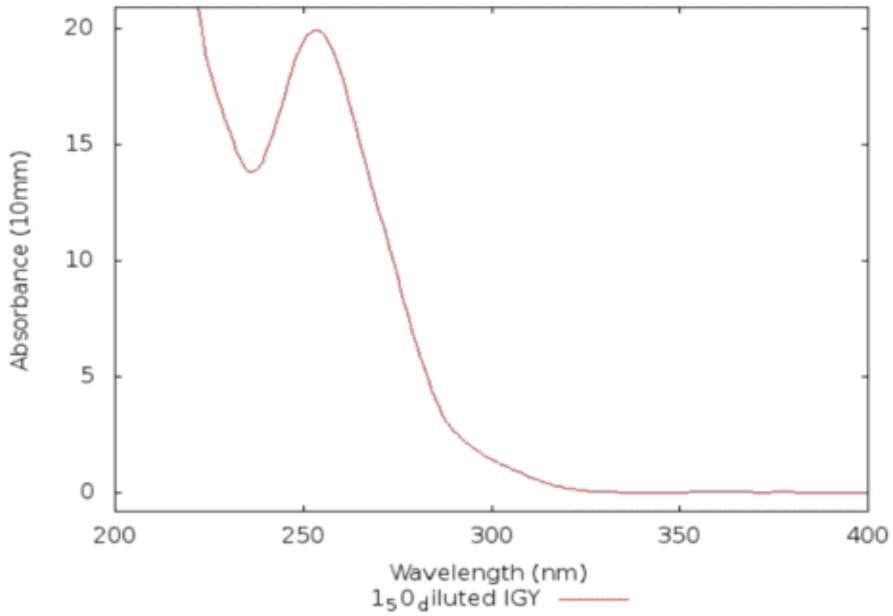

Figure 8: Absorbance spectrum at 280nm with Wavelength on X-axis and Absorbance on Y-axis

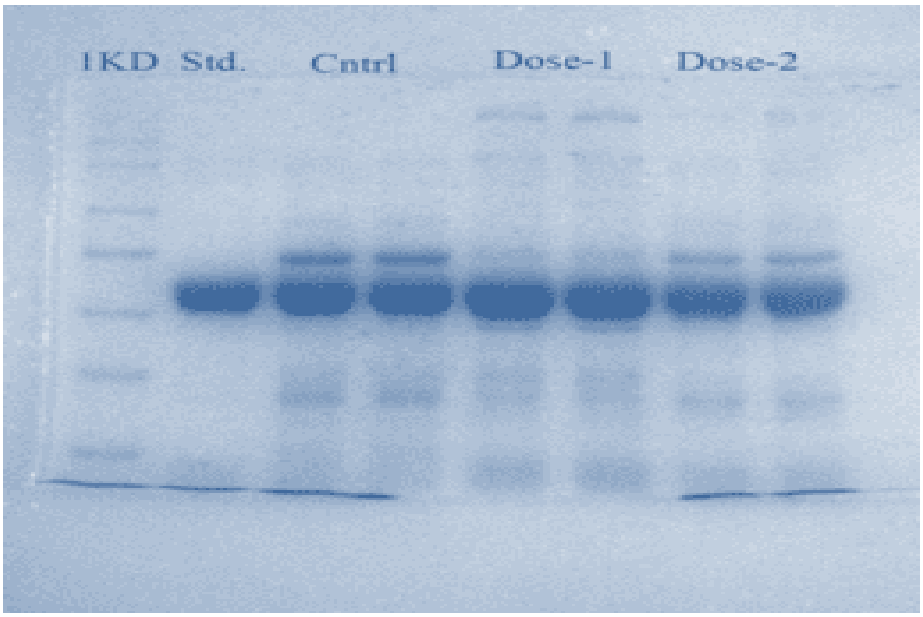

Figure 9: SDS-PAGE to determine the isolation of IgY from 1:1:1:1 concentration of antigen consortium

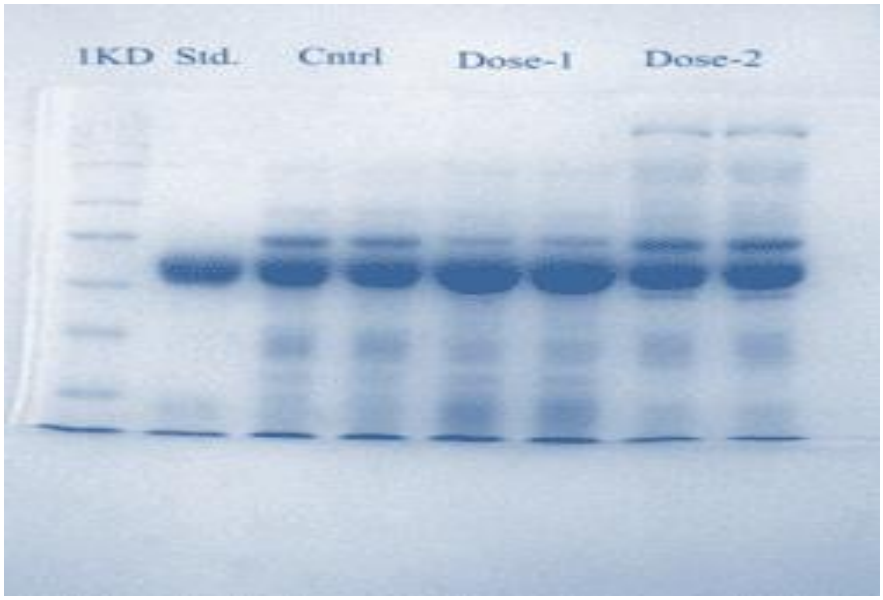

Figure 10: SDS-PAGE to determine the isolation of IgY from 3:3:1:1 concentration of antigen consortium

This finding is crucial for optimizing immunization protocols, as it suggests that lower antigen concentrations may be sufficient to induce maximum IgY production, potentially reducing the cost and stress on the animals.

Overall, this study provides a promising approach for producing IgY antibodies for use in immunoprophylaxis in poultry. The findings suggest that optimizing antigen concentrations can significantly influence IgY yield, which could have important implications for the development of IgY-based treatments and preventive measures in the poultry industry. Further research is needed to explore the mechanisms behind the observed saturation effect and to optimize immunization strategies for different bacterial consortia.

Conclusion

This study successfully demonstrated the production of IgY antibodies in poultry through immunization with a bacterial consortium containing *E. coli*, *Salmonella*, *Enterococcus* and *Clostridium perfringens*. The key findings include:

1. **Effective IgY Production:** Both antigen concentration groups (1:1:1:1 and 3:3:1:1) yielded the same IgY protein concentration of 9.3268 mg/mL, indicating that varying the antigen concentration within these ranges does not significantly affect IgY yield.
2. **High Purity IgY Isolation:** The isolation and purification protocol, which included PEG precipitation and dialysis, effectively produced high-purity IgY antibodies, as confirmed by SDS-PAGE analysis.
3. **Optimal Antigen Concentration:** The results suggest that lower antigen concentrations can be as effective as higher concentrations for IgY production, potentially reducing costs and stress on the hens.

These findings are significant for the development of IgY-based immunoprophylaxis in poultry, as they provide a cost-effective and efficient method for producing high-quality IgY antibodies. Further research could explore the saturation point of IgY production and optimize immunization strategies for different bacterial antigens, enhancing the practical applications of IgY antibodies in preventing and controlling poultry diseases.

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