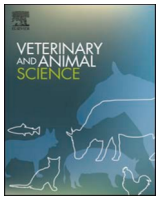
Veterinary and Animal Science 23 (2024) 100326

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas



Development of egg yolk-based polyclonal antibodies and immunoprophylactic potential of antigen-antibody complex against infectious bursal disease

Sanaullah Sajid [a,*], Sajjad ur Rahman [a], Mashkoor Mohin [a], Zia ud Din Sindhu [b]

[a] Institute of Microbiology, University of Agriculture Faisalabad, Pakistan
[b] Department of Parasitology, University of Agriculture Faisalabad, Pakistan

ARTICLE INFO

Keywords:
Immune complex
Infectious bursal disease virus
Immunoglobulin-Y
Polyclonal antibodies
Bursa of Fabricius

ABSTRACT

A study conducted in the Faisalabad district sampled 50 cases across five IBD outbreaks, revealing an alarming 80 % infection rate among poultry. Our research focused on developing an immune complex (Antigen-antibody complex) with potential immunoprophylactic benefits to counter this formidable threat. Our study was based on producing egg yolk-derived polyclonal antibodies (IgY) targeting IBDV. Commercial layer birds were immunized with inactivated IBDV, yielding IgY antibodies extracted from their eggs exhibited substantially higher and more enduring antibody titers, with a geometric mean titer of 104. Further research involved the creation of an immune complex (ICx) where antigen was extracted from infected bursae tissues. The immunogenic response of ICx was assessed in poultry birds after a challenge with a virulent strain of IBD virus and compared to a conventional IBDV vaccine in poultry. Results revealed significantly higher and more enduring antibody titers induced by the ICx, offering enhanced protective immunity against the IBDV challenge, as evidenced by lower Bursa to body-weight ratios in vaccinated birds.

Introduction

A balanced diet is essential for maintaining good health, with protein being a crucial component that plays a vital role in structural development. The recommended daily protein intake is approximately 102.7 gs per person, yet approximately 66 % of Pakistan's population suffers from protein deficiency. To meet this demand for protein, the poultry industry plays a significant role in livestock production (Abedullah et al., 2007; Wei et al., 2006). The poultry sector is advantageous due to its short productive span, reproductive characteristics, global distribution, and favorable egg production.

Moreover, the poultry industry is a key driver of Pakistan's economic development and poverty alleviation. Pakistan is home to 33,146 officially registered poultry farms, collectively providing shelter and care to a population of 89.4 million poultry birds. This diverse avian community comprises 16.9 million layers, 70.7 million broilers, and 17.5 million breeder flocks. The poultry sector contributes approximately 1.3 % to Pakistan's national GDP (Hussain et al., 2015). Despite its importance, the poultry industry faces the persistent challenge of immunosuppression, which escalates with intensive commercial farming. Viruses are the primary reason, as they infect and damage immune cells, leading to immunosuppression. Several factors contribute to decreased production and hindered growth in these immunosuppressed flocks, including chronic diseases, and suboptimal vaccine responses. Furthermore, antibiotic resistance and drug residues in poultry meat substantially threaten human health (Bughio et al., 2017).

Infectious bursal disease also known as Gumboro, a significant immunosuppressive disease affecting poultry, is primarily induced by the infectious bursal disease virus (IBDV). This virus belongs to the Avibirnaviridae group, a member of the Birnaviridae family. Infectious bursal disease virus stands out as a non-enveloped, double-stranded RNA (dsRNA) virus characterized by a genome consisting of two segments, labeled as A and B, and exhibits a size range of about 55–60 nanometers in diameter. While IBDV comprises two serotypes, Serotype-I is notorious for causing avian disease, while Serotype-II predominantly infects various avian species, including turkeys, without resulting in clinical disease (Jackwood et al., 2011; Sajid et al., 2020; Van den Berg et al., 2004).

The economic repercussions of Infectious Bursal Disease (IBD) on the poultry industry extend beyond mere mortality and morbidity; it

* Corresponding author.
E-mail address: sanaullahsajid@gmail.com (S. Sajid).

https://doi.org/10.1016/j.vas.2023.100326

Available online 29 November 2023

significantly impairs overall flock performance. IBDV primarily targets the Bursa of Fabricius (BF), where it replicates within B-lymphocytes, leading to humoral suppression due to B cell depletion. This makes infected birds highly susceptible to opportunistic pathogens, resulting in substantial economic losses. Within Pakistan's poultry sector, these losses accumulate to an annual deficit of approximately 3.9 million kilograms of poultry meat with a market price of approximately $14 million (Jackwood et al., 2008). Unfortunately, no specific treatment for IBD makes vaccination and biosecurity crucial for disease management (Vukea et al., 2014). IBDV's Serotype-I, the primary cause of avian IBD, exhibits two major antigenic groups known as classical and variant. The virus's antigenic characteristics are predominantly determined by the hypervariable region of VP2 (hvVP2), specifically the amino acids located at the apex of loop structures designated PBC, PDE, PFG, and PHI. Even single-point mutations in these regions can drive antigenic drift, rendering existing IBD vaccines less effective (Lupini et al., 2020; Zachar et al., 2016).

IBDV's resilience and stability against chemical and physical agents allow it to persist at farms for several weeks. This robustness, coupled with the lack of specific treatments, necessitates the development of novel strategies for managing and treating respiratory diseases in poultry (Shabbir et al., 2016). One alternative approach to treating infections in immunocompromised individuals is passive immunization, which avoids the potential side effects of vaccination. Polyclonal antibodies derived from immunized animal sera have traditionally been used for passive immunization, but face challenges related to safety and standardization. Monoclonal antibodies (mAbs) offer promise but are limited by their high production costs (Keller & Stiehm, 2000).

Egg yolk immunoglobulins (IgYs) have emerged as a potential solution. These IgYs, produced by various avian species, including chickens, share functional similarities with mammalian IgG. IgYs in chicken sera are transferred to embryos through egg yolks. This suggests that IgY-based therapy could have a longer circulating half-life, enhancing its efficacy against infections. IBD remains a leading cause of mortality in the poultry industry, with significant global impacts. Using IgY antibodies for passive immunity presents a promising option for treating such diseases. Producing IgY is a non-invasive, cost-effective alternative to traditional methods that require repeated bleeding of laboratory animals. IgY demonstrates high stability across a wide range of pH levels and temperatures, making it a suitable candidate for immunotherapy (Hussain et al., 2004; Khan et al., 2017).

A modified immune complex vaccine was developed to advance therapeutic possibilities, combining yolk antibodies (IgY) with a viral antigen. The objective of this study was to investigate the efficacy of the modified ICx in controlling IBD among poultry population.

Materials and methods

Statement of ethics

This animal study was conducted with the approval of the Institutional Biosafety and Bioethics Committee (IBC), University of Agriculture Faisalabad. Permission to conduct the research was granted with reference number 588/ORIC, ensuring that all necessary safety and ethical considerations were met following the guidelines of National Biosafety Rules 2005 and Punjab Animal Health Act 2019.

Bursae retrieval and histopathological analysis

A total of 50 Bursa of Fabricius samples were collected from five suspected Infectious Bursal Disease (IBD) outbreaks (birds of age from 22 to 49 Days) across various commercial farms in Faisalabad district. These outbreaks were characterized by observable symptoms in the affected birds, including atrophy, and Bursae inflammation. Histopathological examinations of the collected tissue samples were conducted following the procedure described by Bancroft and Stevens (1996). In brief, the BF tissues were initially fixed in a 10 % formalin solution for 7 days. Subsequently, the samples underwent a series of procedures, including washing to remove the fixative, dehydration using a graded ethanol series, and clearance in xylene. The tissues were then embedded in paraffin and sliced into sections with a thickness of 3 μm utilizing a microtome. These tissue sections were affixed to glass slides, for a 24 h drying period at 37 °C. Following this, the tissues were subjected to a 30 min incubation at 45 °C and subsequently stained with Hematoxylin and Eosin stains, following the method outlined by (Jones et al., 2008).

Molecular identification of IBDV-VP2

The process began with isolating RNA from the bursa sample, followed by synthesizing complementary DNA (cDNA) using the ABM EasyScript™ Reverse Transcriptase cDNA Synthesis kit, adhering to the manufacturer's guidelines. The PCR was conducted using an ABM PCR kit, with a designed primer set (Previously designed by Sajid et al., 2020) tailored to partially amplify the VP2 sequence from base 751 to base 1449 of genome segment A of IBDV. The PCR product, explicitly targeting the VP2 gene, was subsequently purified using a PCR purification kit from QIAGEN, USA, following the manufacturer's protocol. To determine the nucleotide sequences of the virus, the purified VP2 DNA underwent DNA sequencing.

The acquired nucleotide sequence data were subjected to analysis using various software tools, including MegaAlign, BioEdit, and Lasergene DNASTAR. The primary focus of the phylogenetic analysis centered on VP2 nucleotide sequences, which extended from nucleotide 751 to 1449 (Sajid et al., 2020).

IBD virus propagation

To cultivate the IBDV, a specific procedure was followed. Firstly, 10-day-old embryonated specific pathogen-free (SPF) eggs, were obtained from the poultry farm at the University of Agriculture in Faisalabad, Pakistan. These eggs were infected with 10^3 embryo infective dose (EID_{50}) in the Chorioallantoic membrane (CAM). After infection, the eggs were placed in an incubator at 37 °C for 96 h. Following this incubation period, they were subsequently chilled for 4 h. After 96 h, observations were made for CAM lesions and embryo mortality and the CAM was harvested from these eggs containing the propagated virus (Malik et al., 2006).

Layer bird immunization and IgY purification

In the animal housing facility, White Leghorn hens, specifically layers, were utilized for an immunization study. These layers were injected with infectious bursal disease virus antigen, and each of the ten layer birds received a subcutaneous injection (0.5 ml). For four weeks, the birds were provided with bi-weekly booster injections of the preparation, which included the Montanide adjuvant. Egg collection commenced one week after the initial injection, and the collected eggs were stored at a temperature of 4 °C until further processing.

The egg yolk was separated from the egg white and then washed twice with distilled water at a temperature of 4 °C. To adjust the pH to 5.0, 1 N HCl was added, followed by the introduction of charcoal (0.01 %). The pH was subsequently re-adjusted to 4.0, and the diluted yolk underwent freezing at −20 °C overnight. Afterward, the frozen yolk was thawed at 4 °C and then centrifuged at 10,000 × g for 10 min at the same temperature. The resulting supernatant was collected and supplemented with ammonium sulfate at 0.24 g/ml concentration. This mixture was incubated at 25 °C for 30 min with continuous stirring. Subsequently, the samples underwent centrifugation at 10,000 × g and 4 °C for 12 min, with the supernatant being discarded. The resulting pellet was reconstituted in a small volume of 2 M ammonium sulfate solution and re-incubated at 25 °C for another 30 min. After a subsequent centrifugation step at 10,000 × g and 4 °C for 12 min, the supernatant was again

discarded, and the pellet was re-suspended in a fraction of phosphate buffer saline (PBS) before being stored at -20 °C for further processing (Mahmood & Siddique, 2006).

Quantifying antigen and antibodies: RPHA & IHA

IgY antibodies were sensitized using washed sheep RBCs. This sensitization process involved mixing an equal volume of sheep RBCs with diluted IgY (2 ml) in the presence of Chromium Chloride as a coupling agent (at a ratio of 1:400) in a test tube. The test tube was then incubated at 37 °C for 20 min, preparing a 1 % sensitized RBC suspension. Subsequently, the Reverse Passive Hemagglutination (RPHA) test was conducted in a 96-well microtitration plate with a U-bottomed design. The virus (antigen) was subjected to a two-fold dilution in 50µl of normal saline, followed by adding 1 % sensitized RBCs (50µl) to all wells, while the last well served as a control. The microtitration plate was incubated for 30 min at 37 °C, and the degree of agglutination was observed.

Similarly, for the Indirect Hemagglutination Test (IHA), a two-fold serial dilution of IgY antibodies was prepared, and 1 % sensitized human O-negative RBCs with antigen were added to each well of the microtitration plate. The plate was gently tapped to ensure uniform dispersion of RBCs and then incubated at 37 °C for 30 min. The degree of agglutination in each well was compared to the negative control, which contained normal saline and sensitized RBCs (Jayasundara et al., 2017).

Immune complex vaccine: preparation, sterility, safety, and stability studies

The antigen-antibody complex vaccine developed by the unification of a known concentration (2HA titer) of IBDV with (2HA titer) chicken antibodies (IgY) obtained from layer eggs. Equal amounts of the antigen and antibodies were thoroughly mixed in a 1:1 ratio to form an antigen-antibody complex, which was then kept at temperatures below 10 °C for future use. Sterility testing involved aseptically inoculating the prepared vaccine (0.5 ml) into fluid thioglycolate media and blood agar plates. These inoculated tubes and plates were incubated for 72 h at 37 °C to check for growth. In safety studies, six SPF rabbits were divided into three groups, with one group serving as a negative control. The second group received the immune complex vaccine subcutaneously, and the rabbits were closely monitored for any signs of adverse effects for seven days post-injection. Stability studies involved storing ICx at two different temperature conditions, below 10 °C and 37 °C, while recording suspension and color parameters for up to eight months (Lee et al., 2011).

ICx vaccine: immune response and protective efficacy

The birds were raised on the ground, where wood shavings were utilized as the bedding material. Each group was accommodated in its dedicated enclosure. Prior to introducing the chickens, a hygiene protocol was observed. All enclosures were cleaned and disinfected using gaseous disinfectants to ensure a sanitized environment for the birds. Group 1 served as the control group and received no vaccinations. In Group II, a single subcutaneous dose of 0.2 ml of the immune complex vaccine was administered. Group III received an initial 0.2 ml dose of the immune complex vaccine on day one, followed by a booster dose of 0.2 ml after one week. Group IV was inoculated with 0.2 ml of a commercially available IBDV live vaccine (Himmvac). To assess antibody titers and evaluate the vaccine's performance compared to the commercially available vaccine, serum samples were collected from all groups on days 0, 7, 14, 21, 28, 35, and 42.

The viral challenge was conducted on the 21st day of age by administering EID50 /1 ml of IBDV orally and ocularly to poultry birds. Following the challenge, the birds were closely monitored for disease symptoms, bursa to body weight ratio, and underwent postmortem examination to assess bursal pathology. Chicken sera collected from all groups were tested for IBD-specific antibodies using an I-ELISA kit with a single dilution (1:500) of serum. Serum samples with an S/P ratio less than or equal to 0.2 were considered negative, while those with an S/P ratio greater than 0.2 were considered positive (Kalenik et al., 2014).

Results

Insights of bursa histopathology and pathogenesis

Infected birds exhibited a range of clinical signs, including ruffled feathers, loss of appetite, diarrhea, depression, and even death. Morbidity was widespread, affecting all infected flocks, with mortality rates ranging from 70 % to 80 %. Postmortem examinations revealed significant signs such as bursal atrophy and lesions, hemorrhaging in the thigh and pectoral muscles, darkened discoloration of the pectoral muscles, and a yellowish gelatinous material covering the external surface of the bursa. These findings were often accompanied by multiple hemorrhages and areas of necrosis. Histopathological analysis of the affected bursae provided further insights into the impact of IBDV infection. The examination revealed a spectrum of acute and chronic lesions in the examined bursae. These lesions included lymphoid follicle depletion, severe atrophy with infiltration of inflammatory cells (predominantly heterophils), and necrosis. Over the course of the infection, the necrosis of lymphocytes led to the formation of karyorrhectic debris. This phase was accompanied by edema, hyperemia, and reticuloendothelial cell hyperplasia (Fig. 1).

Molecular characterization of IBDV

A partial region of 699 nucleotides in segment A, referred to as VP2, was amplified and sequenced to examine the genetic characteristics of Infectious Bursal Disease Virus (IBDV) strains in the study area. These sequences were subsequently aligned, and phylogenetic analysis was conducted to understand the evolutionary relationships among the strains. As previously characterized in our earlier study (Sajid et al., 2021), the IBDV strains in this study were grouped into seven distinct genogroups, reflecting the virus's genetic diversity. This genetic diversity was confirmed by the PCR results. The phylogenetic analysis provided further insights into the genetic relationships among these genogroups, emphasizing the complex evolutionary landscape of IBDV. This information is essential for understanding the genetic dynamics of the virus and its implications for disease management and vaccine development.

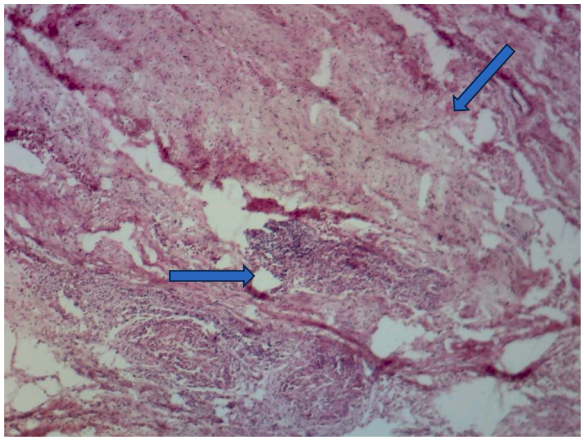


Fig. 1. Histopathological examination at 40X revealed lymphoid depletion of the follicles, necrosis of lymphocytes with pyknotic or karyorrhectic cells, and fibrosis of the bursal stroma.

Propagation of pathogens in avian eggs

Embryonated chicken eggs were closely monitored daily, with any deceased embryos occurring within the first 24 h post-inoculation. After 96 h, the eggs were cooled to 4°C, and the Chorioallantoic membrane (CAM) was carefully harvested into a sterilized petri dish. In the initial and second passages, a 90 to 100 % mortality rate was observed among the embryos, which gradually decreased to 70 % in the third passage. Grossly, the chicken embryos displayed abdominal distention with edema and noticeable hemorrhages on various body parts such as the cerebrum, skin, toe joints, and CAM. However, these lesions were less severe in the third passage compared to the first and second passages. The harvested CAM was subsequently separated and subjected to centrifugation at 1500 rpm for 20 min to isolate the virus in the supernatant. The presence of the infectious bursal disease virus (IBD virus) in the supernatant was verified using the agar gel precipitation test (AGPT), employing known antiserum that had been raised in rabbits. The results of the agar gel precipitation test provided confirmation of the presence of the IBD virus in the embryonated eggs that had been inoculated. Within 24 h of incubation, a distinct line of precipitation became evident. In comparison, there was an absence of any precipitation line in the negative control well (Fig. 2).

Birds' immunization status evaluated through IgY in eggs and virus titration

Egg yolk antibodies, known as IgY, were assessed quantitatively and qualitatively using the Indirect Hemagglutination test (IHA). In the first week, the IgY titer in IHA displayed a geometric mean titer (GMT) of 3.7. During the second week, the titer varied with a GMT of 17.1. In the third week, the IgY titer showed a notable increase and was recorded with a GMT of 39.4, and the fourth week exhibited the highest IgY titer with a GMT value of 104. Subsequently, purified IgY was obtained through ammonium sulfate precipitation. The IBD virus was quantified using a reverse passive hemagglutination test, which utilized a 4HA titer of IgY determined through the IHA test. Sample well displayed hemagglutination with a 4HA titer, while the negative control well served as a critical test component. No hemagglutination was observed in the negative control well, and its titer was recorded as 0HA. This result served as a baseline, indicating that hemagglutination only occurred when the IBD virus was present and active (Fig. 3).

Sterility, safety, and stability assessment of the vaccine

The sterility assessment of the immune complex was conducted after seven days of incubation, revealing no observed turbidity or signs of bacterial growth. Similarly, the inoculation of the immune complex on blood agar medium plates yielded negative results, indicating the absence of bacterial contamination. A one-week post-immunization observation period was implemented for the safety studies of the immune complex vaccine in rabbits. During this time, the body temperatures of the rabbits remained within the normal range (101 ± 0.3 ℉ to 103 ± 0.3 ℉), with no recorded mortality. Mild inflammation at the injection site was observed, but other parameters remained within the expected normal range. Following the immune complex vaccine injection, the rabbits initially exhibited reduced feed intake. However, by day five post-immunization, feed intake increased to normal. A similar pattern was observed for water intake, with a decrease during the first two days post-immunization followed by a return to normal levels by day six (Table 1).

The stability of the immune complex was assessed under two temperature conditions: storage below 10 °C and at 37 °C for a duration of up to eight months. When stored below 10 °C, the immune complex suspension maintained its color for the first month, but the titer gradually decreased, reaching 70 % in the eighth month. Conversely, when stored at 37 °C, the antigen titer decreased significantly, reaching 60 % within the first two months, and immiscibility and color changes were observed after one month (Table 2).

Assessing immune responses in chickens

The immune response study explored the effectiveness of different vaccines on chickens and investigated how immune responses varied across groups receiving distinct types of vaccinations. The groups in focus were those administered with an immune complex vaccine and those given a commercially available live vaccine for Infectious Bursal Disease (IBD). Antibody response revealed a dynamic pattern in antibody levels across different time intervals. Initially, the group receiving the immune complex vaccine showed a strong antibody response (1st & 2nd week) compared to the commercial vaccine group. By the 4th week, this distinction in antibody levels between the two groups became less pronounced. However, a notable shift was observed later in the study (5th week), with the commercial vaccine group experiencing a decrease in antibody levels while the immune complex vaccine group maintained a relatively stronger immune response (Fig. 4).

To evaluate the level of protection provided by different vaccines,

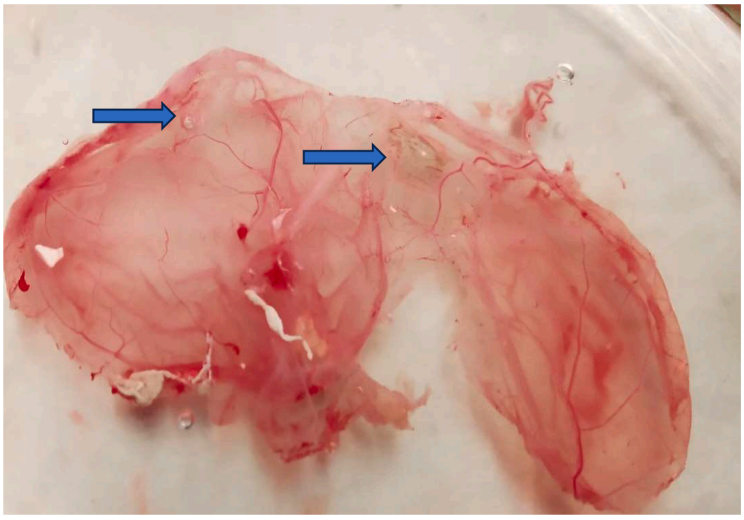


Fig. 2. Visualizing gross lesions elicited on the Chorioallantoic membrane by infectious bursal disease virus in chicken embryonated eggs.

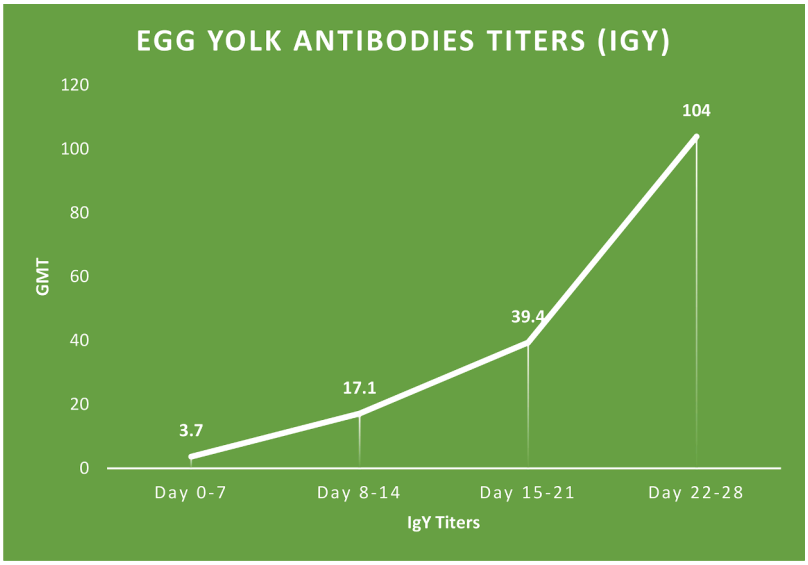


Fig. 3. Investigating the geometric mean titers of egg yolk antibodies in response to Montanide-adjuvanted antigen over a 28-day post-injection period.

Table 1
Investigation of safety test parameters in rabbits inoculated with an immune complex vaccine.

Post immunization (Hours)	Observations				
	Local inflammation	Body temperature	Food Intake (g/Kg)	Water Intake (mL/Kg)	Change in Body Weight (Grams)
24	Present	103.7 ± 0.85	25 ± 5	25 ± 5	1950 ± 10
48	Present	102.20 ± 3.0	35 ± 5	40 ± 5	1955 ± 10
72	Resolved	102.50 ± 0.35	50 ± 5	55 ± 5	1970 ± 10
96	-	103.15 ± 0.85	85 ± 5	70 ± 5	1980 ± 10
120	-	101.45 ± 0.40	100 ± 5	100 ± 5	1995 ± 10
144	-	101.78 ± 0.35	100 ± 5	100 ± 5	2000 ± 10
168	-	102.40 ± 0.75	100 ± 5	100 ± 5	2000 ± 10

the Bursa to body weight ratio (B.B. ratio) was calculated. Among all the vaccinated groups, it was evident that they exhibited a higher B.B. ratio when compared to the negative control group. However, an interesting observation was made at 35 days of age; specifically, the B.B. ratio in G-III (ICx) was significantly lower than in the other vaccinated groups. Moving to day 21, both the ICx groups (G-II, G-III) and the commercial vaccination group (G-IV) did not show significant alterations in the Bursa, except for minor B-cell proliferation in G-IV. Similarly, there were no substantial changes observed in G-II, G-III, and G-IV following virus challenge. However, the control group (G-I) presented severe degenerative changes, apoptosis, inflammatory cell infiltration, and central necrosis. By day 28, G-II and G-III exhibited healthy epithelial linings of the Bursa and highly activated cortical lymphocytes. In contrast, G-IV showed immune-reactive lymphoid follicles and mild interstitial connective tissue thickening due to fibroblastic proliferation. In comparison, G-IV (commercial vaccine) displayed only mild bursa enlargement, while both G-II and G-III (ICx) exhibited normal bursa conditions. Importantly, all vaccinated groups demonstrated no significant differences in body weights throughout the study period.

Discussion

In poultry, infectious bursal disease (IBD) is the second most significant viral disease, following Newcastle disease. IBD's immunosuppressive nature leads to economic losses by compromising the birds' immune responses, resulting in poor feed conversion ratios and increased susceptibility to secondary infections. This disease's economic impact is twofold: high mortality rates, especially in young chicks, and prolonged immunosuppression that hinders vaccine effectiveness (Dey et al., 2019). The infectious bursal disease virus (IBDV) is of particular interest because it primarily targets the Bursa of Fabricius while sparing other organs (Zachar et al., 2016). The Bursa, which reaches its peak development at six weeks, becomes highly susceptible to rapid viral replication, making IBDV particularly lethal at this stage. IBDV's main characteristic is the depletion of lymphoid cells, and the Bursa's atrophy suggests that apoptosis plays a role in the disease's progression (Kalenik et al., 2014).

In this study, IBD's gross lesions included petechial hemorrhages in

Table 2
Monitoring the integrity and longevity of immune complex IBDV antigen for an eight-month duration.

Month of storage	Temperature below 10 °C			Temperature below 37 °C		
	Color	Suspension	Immunization	Color	Suspension	Immunization
1st	Opaque white	Homogenous	90 %	Opaque White	Homogenous	75 %
2nd	Opaque white	Homogenous	81 %	Yellowish	Immiscible	60 %
3rd	Opaque white	Homogenous	78 %	Yellowish	Immiscible	42 %
4th	Opaque white	Homogenous	75 %	Yellowish	Immiscible	37 %
5th	Opaque white	Immiscible	75 %	Yellowish	Immiscible	25 %
6th	Opaque white	Immiscible	73 %	Yellowish	Immiscible	17 %
7th	Opaque white	Immiscible	72 %	Yellowish	Immiscible	11 %
8th	Opaque white	Immiscible	70 %	Yellowish	Immiscible	08 %

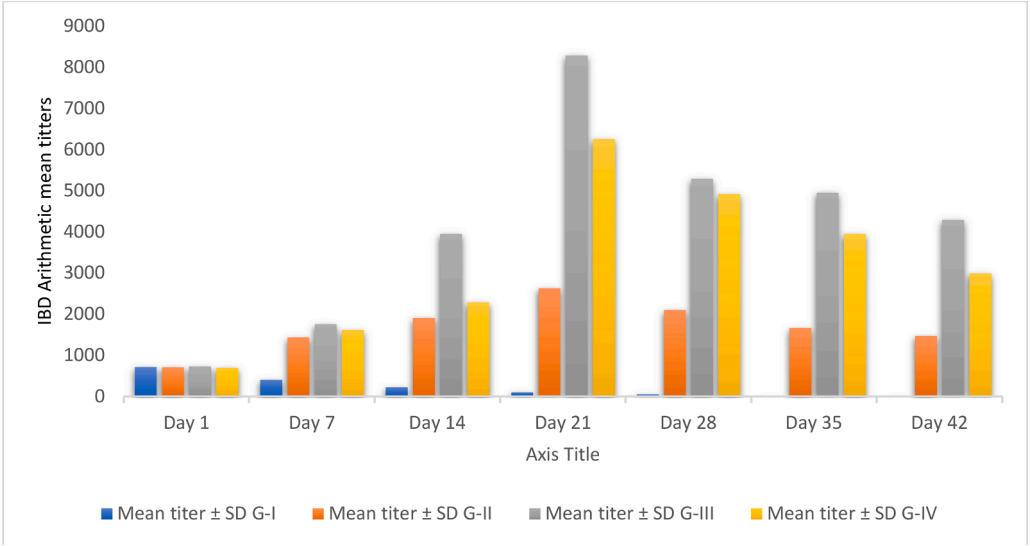


Fig. 4. Examination of immune response patterns induced by immune complex antigen compared to a conventional commercial IBDV vaccine.

the Bursa of Fabricius, muscle discoloration, and increased intestinal mucus. The Bursa initially swelled due to hyperemia and edema, doubling in size by the 4th day post-infection. Additional findings included necrotic foci with petechial hemorrhages on the Bursa's mucosal surface, an enlarged spleen with gray foci, and hemorrhages at the gizzard-proventriculus junction. Microscopic examination revealed lymphocyte degeneration in the Bursa's medullary region, followed by the replacement of hyperplastic reticuloendothelial cells and heterophils. Histologically, the Bursa exhibited fibroblast and reticuloendothelial cell proliferation, necrosis, and phagocytosis of plasma cells and heterophils. Bursal follicles eventually appeared as empty spaces, along with fatty degeneration called microcyst. Virus replication and apoptosis were predominantly observed in the Bursa of Fabricius. The study also highlighted the importance of histopathological analysis in understanding the vaccine's protective mechanism. The absence of severe degenerative changes and the presence of healthy epithelial linings and activated lymphocytes in the ICx-vaccinated groups by day 28 post-vaccination provide histological evidence of the vaccine's efficacy in protecting the Bursa from IBDV-induced damage (Jackwood et al., 2011).

This research has significant implications for poultry health management, particularly in regions where IBDV is prevalent. The development of a stable, effective, and safe vaccine like the ICx could revolutionize the approach to controlling IBD in poultry. The study underscores the potential of using egg yolk-derived antibodies for passive immunization strategies, offering an innovative avenue for vaccine development in avian diseases (Jackwood et al., 2008; Shabbir et al., 2016)

Global trade and the use of IBD vaccine strains have contributed to the emergence of new, pathogenic IBD virus strains. Pakistan has seen the dominance of classical IBD virus strains until the 1980s. A key mutation at position 222, from proline (P) to threonine (T), has been associated with the shift from classical to variant Genogroups, possibly due to widespread vaccine usage in rearing flocks. Adapting field strains of IBDV from Bursa to cell culture is challenging and often requires extensive passaging in Chorioallantoic membrane or embryonated eggs. The virus was inoculated into embryonated eggs and monitored daily in this study. After 96 h, the Chorioallantoic membrane (CAM) was harvested, resulting in 100 % embryo mortality during the 1st and 2nd passages, reduced to 70 % in the 3rd passage (Jayasundara et al., 2017).

This study highlights the effectiveness of the ICx vaccine in controlling IBDV in poultry. Traditional vaccines and biosecurity measures were successful until highly virulent IBDV strains emerged, complicating vaccination efforts, particularly in the presence of maternally derived antibodies. To address this challenge and improve early vaccination, the study employed an immune complex (ICx) vaccine strategy. This approach involves combining the IBDV antigen with homologous antibodies to create an antigen-antibody complex. This complex is administered to poultry birds, effectively jumpstarting their immune response. The ICx vaccine utilized chicken IgY antibodies, which offer several advantages, including reduced animal handling and increased IgY production from egg yolks. The IgY antibodies were purified from egg yolks using ammonium sulfate precipitation. The study successfully demonstrated the early development of IgY antibodies in layer bird egg yolks, which can be used for protective effects and experimentation. The study demonstrated the potential of egg yolk derived IgY antibodies in providing an effective immune response against the Infectious Bursal Disease Virus (IBDV). The increasing trend in IgY titers observed over the initial weeks post-vaccination underscores the efficacy of this approach in stimulating the immune system. This is consistent with previous research highlighting the advantages of IgY-based immunization, particularly its specificity and relatively long half-life in circulation, which enhances its effectiveness against infections (Lee et al., 2011).

This study assessed the effectiveness of an immune complex (ICx) vaccine against infectious bursal disease virus (IBDV) in poultry. The ICx vaccine was produced by injecting IBDV into layer birds and extracting IgY antibodies from their egg yolks. This vaccine was compared with a commercial live IBD vaccine. The study evaluated various parameters, including performance, bursa-to-body weight ratio, bursa histopathology, and antibody response in vaccinated birds. Results showed that all vaccinated groups were protected against IBDV, with the ICx group demonstrating promising results. The ICx vaccine's advantage lies in its ability to bypass maternal antibodies and provide protection even in the presence of high maternal antibody levels. The Bursa to body weight ratio (B.B. ratio) served as a critical measure of the vaccines' protective capabilities. The elevated B.B. ratios in vaccinated groups compared to the control group highlight the notion that vaccination plays a significant role in preserving the health of the Bursa of Fabricius, a crucial organ for avian immunity.

The immune complex vaccine's stability under different temperature conditions and its safety profile in rabbits further add to its viability as a practical and safe option for large-scale use in poultry. The minimal side effects observed in the safety studies align with the requirements for a viable poultry vaccine, especially in a field setting. Further research could focus on the large-scale application of the ICx vaccine in diverse

poultry settings to assess its efficacy across different environmental conditions and bird populations. Additionally, exploring the mechanistic aspects of how the ICx vaccine stimulates and sustains the immune response at the molecular level would provide deeper insights into its mode of action.

Conclusion

Infectious bursal disease (IBD) poses significant economic challenges in poultry due to its immunosuppressive nature and impact on bird health. The infectious bursal disease virus (IBDV) primarily targets the Bursa of Fabricius, resulting in lymphoid cell depletion and apoptosis. This study detailed the gross and histopathological characteristics of IBD and highlighted the importance of vaccination in disease control. The emergence of new IBDV strains, global trade, and vaccine use have influenced virus evolution. Mutations, particularly the Proline (P) to Threonine (T) change at position 222, have led to shifts in IBDV genogroups, impacting vaccine efficacy. The study introduced an immune complex (ICx) vaccine strategy using IgY antibodies from chicken egg yolks. This approach demonstrated early IgY development in layer bird egg yolks, showcasing its potential for protective effects and experimentation. Overall, the ICx vaccine showed promise in controlling IBDV in poultry, offering an alternative to traditional vaccines. This research contributes to understanding IBDV dynamics and presents a valuable vaccination strategy for enhanced disease management in poultry.

Statement of ethics

This animal study was conducted with the approval from Institutional Biosafety and Bioethics Committee (IBC), University of Agriculture Faisalabad. Permission to conduct the research was granted with reference number 588/ORIC, ensuring that all necessary safety and ethical considerations were met following the guidelines of National Biosafety Rules 2005 and Punjab Animal Health Act 2019.

CRediT authorship contribution statement

Sanaullah Sajid: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Sajjad ur Rahman:** . **Mashkoor Mohin:** . **Zia ud Din Sindhu:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Acknowledgments

We acknowledge Dr. Rai Shafqat, Deputy Director, CVH Faisalabad, for his kind cooperation in procuring the filed samples and Dr. Mudasser Habib, Principal Scientific Officer, NIAB, Dr. Salahuddin Shah, Principal Scientific Officer, NIAB for their support and cooperation during the research.

References

Abedullah, A., Maqbool, A., & Bukhsh, K. (2007). Issues and economics of poultry production: A case study of Faisalabad, Pakistan. *Pakistan Veterinary Journal, 27*(1), 25–28.

Bancroft, J., & Stevens, A. (1996). *The haematoxylin and eosin: Theory and practice of histological techniques,; ch 6* (pp. 99–112). Churchill Livingstone.

Bughio, E., Jatoi, A. S., Memon, M., Bughio, R., Khoso, P. A., Khoso, Z. A., & Baloch, A. A. (2017). Effect of age and route of administration on the efficacy of live infectious bursal disease vaccines in broiler. *Sarhad Journal of Agriculture, 33*(2).

Dey, S., Pathak, D. C., Ramamurthy, N., Maity, H. K., & Chellappa, M. M. (2019). Infectious bursal disease virus in chickens: prevalence, impact, and management strategies. *Veterinary Medicine: Research and Reports*, 85–97.

Hussain, I., Rasool, M. H., & Mahmood, M. S. (2004). Production of hyperimmune serum against infectious bursal disease virus in rabbits. *Pakistan Veterinary Journal, 24*, 179–183.

Hussain, J., Rabbani, I., Aslam, S., & Ahmad, H. A. (2015). An overview of poultry industry in Pakistan. *World's poultry science journal, 71*(4), 689–700.

Jackwood, D. J., Sommer-Wagner, S. E., Crossley, B. M., Stoute, S. T., Woolcock, P. R., & Charlton, B. R. (2011). Identification and pathogenicity of a natural reassortant between a very virulent serotype 1 infectious bursal disease virus (IBDV) and a serotype 2 IBDV. *Virology, 420*(2), 98–105.

Jackwood, D. J., Sreedevi, B., LeFever, L. J., & Sommer-Wagner, S. E. (2008). Studies on naturally occurring infectious bursal disease viruses suggest that a single amino acid substitution at position 253 in VP2 increases pathogenicity. *Virology, 377*(1), 110–116.

Jayasundara, J. M. K. G. K., Walkden-Brown, S. W., Katz, M. E., Islam, A. F. M. F., Renz, K. G., McNally, J., & Hunt, P. W. (2017). Pathogenicity, tissue distribution, shedding and environmental detection of two strains of IBDV following infection of chickens at 0 and 14 days of age. *Avian Pathology, 46*(3), 242–255.

Jones, M. L., Bancroft, J. D., & Gamble, M. (2008). Connective tissues and stains. *Theory and Practice of Histological Techniques, 6*, 135–160.

Kalenik, B., Sawicka, R., Góra-Sochacka, A., & Sirko, A. (2014). Influenza prevention and treatment by passive immunization. *Acta Biochimica Polonica, 61*(3).

Keller, M. A., & Stiehm, E. R. (2000). Passive immunity in prevention and treatment of infectious diseases. *Clinical Microbiology Reviews, 13*(4), 602–614.

Khan, R. S. A., Sajid, S., Habib, M., Ali, W., Shah, M. S. U. D., & Sarfraz, M. (2017). History of Gumboro (infectious bursal disease) in Pakistan. *Saudi Pharmaceutical Journal, 25*(4), 453–459.

Lee, M. S., Lin, Y. C., Lai, G. H., Lai, S. Y., Chen, H. J., & Wang, M. Y. (2011). One-step reverse-transcription loop-mediated isothermal amplification for detection of infectious bursal disease virus. *Canadian Journal of Veterinary Research, 75*(2), 122–127.

Lupini, C., Quaglia, G., Mescolini, G., Russo, E., Salaroli, R., Forni, M., … Catelli, E. (2020). Alteration of immunological parameters in infectious bronchitis vaccinated–specific pathogen-free broilers after the use of different infectious bursal disease vaccines. *Poultry science, 99*(9), 4351–4359.

Mahmood, M. S., & Siddique, M. (2006). Comparative efficacy of RT-PCR, AGPT and reverse passive haemagglutination assay for the detection of infectious bursal disease virus in broilers. *Pakistan Veterinary Journal, 26*(4), 167.

Malik, M. W., Ayub, N., & Qureshi, I. Z. (2006). Passive immunization using purified IgYs against infectious bursal disease of chickens in Pakistan. *Journal of Veterinary Science, 7*(1), 43–46.

Sajid, S., Rahman, S. U., Mohsin Gilani, M., Sindhu, Z. U. D., Ali, M. B., Hedfi, A., … Mahmood, S. (2021). Molecular characterization and demographic study on infectious bursal disease virus in faisalabad district. *Plos one, 16*(8), Article e0254605.

Sajid, S., ur Rahman, S., Nayab, S., Khan, I. U., & Javeed, A. (2020). 71. Infectious bursal disease virus cloning and structural protein (VP2) expression in Escherichia coli. *Pure and Applied Biology (PAB), 9*(1), 743–749.

Shabbir, M. Z., Ali, M., Abbas, M., Chaudhry, U. N., & Munir, M. (2016). Molecular characterization of infectious bursal disease viruses from Pakistan. *Archives of Virology, 161*, 2001–2006.

Van den Berg, T. P., Morales, D., Eterradossi, N., Rivallan, G., Toquin, D., Raue, R., Zierenberg, K., Zhang, M. F., Zhu, Y. P., Wang, C. Q., & Zheng, H. J. (2004). Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. *Avian Pathology, 33*(5), 470–476.

Vukea, P. R., Willows-Munro, S., Horner, R. F., & Coetzer, T. H. (2014). Phylogenetic analysis of the polyprotein coding region of an infectious South African bursal disease virus (IBDV) strain. *Infection. Genetics and Evolution, 21*, 279–286.

Wei, Y., Li, J., Zheng, J., Xu, H., Li, L., & Yu, L. (2006). Genetic reassortment of infectious bursal disease virus in nature. *Biochemical and Biophysical Research Communications, 350*(2), 277–287.

Zachar, T., Popowich, S., Goodhope, B., Knezacek, T., Ojkic, D., Willson, P., Ahmed, K. A., & Gomis, S. (2016). A 5-year study of the incidence and economic impact of variant infectious bursal disease viruses on broiler production in Saskatchewan, Canada. *Canadian Journal of Veterinary Research, 80*(4), 255–261.